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Biodegradation of bioaccessible textile azo dyes by *Phanerochaete chrysosporium*

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Abstract

Azo dyes are important chemical pollutants of industrial origin. Textile azo dyes with bioaccessible groups for lignin degrading fungi, such as 2-methoxyphenol (guaiacol) and 2,6-dimethoxyphenol (syringol), were synthesised using different aminobenzoic and aminosulphonic acids as diazo components. The inocula of the best biodegradation assays were obtained from a pre-growth medium (PAM), containing one of the synthesised dyes. The results of the dye biodegradation assays were evaluated every 7 days, by the decrease of the absorbance at the maximum wavelength of the dye, by the decrease of the sucrose concentration in the culture medium and by the increase of the biomass during the 28 days of assay. It was observed that the extent of dye biodegradation depended on the sucrose concentration, on the degraded dye structure and, on the dye present in the PAM medium.

Keywords: Azo dyes; Chemical structure/extent of biodegradation; Bioaccessibility; White rot fungus; *Phanerochaete chrysosporium*

1. Introduction

Synthetic azo dyes are extensively used in the textile and dyestuff industries. Effluents from these industrial processes are usually resistant to biological treatment and the azo dyes are considered recalcitrant xenobiotic compounds due to the presence of a N=N bond and other possible groups that are not easily biodegraded, as for instance the sulphonic group (SO_3H). The azo dyes are toxic, and also become harmful to the environment by the formation of aromatic amines (anilines), which are carcinogenic and/or mutagenic. The anilines are formed by the reductive cleavage of the azo bond that, usually, occurs in anaerobiose conditions by many strains of bacteria (Chung and Stevens, 1993; Gottschalk and Knackmuss, 1993). In addition, under aerobic or anaerobic–aerobic conditions using a bacterial consortium, mineralisation of dyes can occur (Haug et al., 1991; Dubrow et al., 1996; Malpei, 1996). More recently, dye biodegradation by yeasts (Luo and Liu, 1996) in particular biodegradation of textile azo dyes in batch-aerated cultures (Martins et al., 1999) have been investigated.

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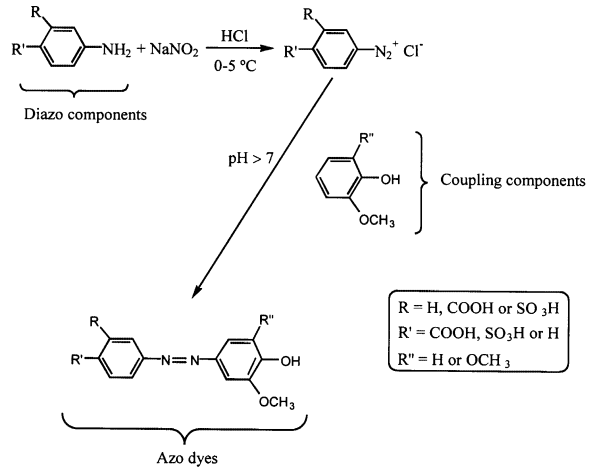


Fig. 1. Scheme of the azo dyes synthesis.

Ligninolytic fungi have shown the ability to degrade numerous aromatic organopollutants including polycyclic aromatic hydrocarbons via oxidative mechanisms (Bogan and Lamar, 1995; Hammel, 1995). Some of these filamentous fungi, that are able to degrade cellulose, hemicellulose and lignin, as the white rot fungus *Phanerochaete chrysosporium*, have also shown the capacity of biodegrading azo dyes till their complete mineralisation by oxidative mechanisms, avoiding the formation of anilines as intermediates (Paszczynski et al., 1991; Spadaro et al., 1992; Zhou and Zimmermann, 1993). The azo dyes biodegradation is enhanced under specific conditions of the culture medium, particularly under nitrogen-limiting conditions (Spadaro et al., 1992) and it was shown that the biodegradation also depends on the chemical structure of the azo dye, nature of the substituents and their relative position (Pasti-Grigsby et al., 1992; Paszczynski et al., 1992).

In this work, eight textile azo dyes were synthesised using aminobenzoic and aminosulphonic acids as diazo components and bioaccessible groups such as 2-methoxyphenol (guaiacol) and 2,6-dimethoxyphenol (syringol) as coupling components. The bioaccessible groups are present in the lignin structure and seem to be access points to the fungus ligninolytic enzymes produced by *P. chrysosporium* (Paszczynski et al., 1991; Spadaro et al., 1992). The biodegradation of the eight synthesised azo dyes by *P. chrysosporium* was studied in order to establish the relationship between the chemical structure of the dye and the extent of biodegradation, after defining the optimal conditions for the assays. The biodegradation assays were performed in liquid medium, under nitrogen-limiting conditions, since ligninolytic activity of *P. chrysosporium* is induced in these conditions (Keyser et al., 1978; Kirk et al., 1978; Jeffries et al., 1981; Glenn and Gold, 1983; Spadaro et al., 1992; Ollikka et al., 1993).

2. Material and methods

2.1. Azo dyes synthesis

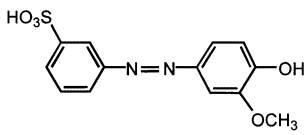
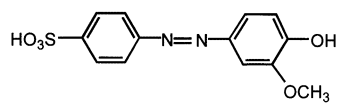
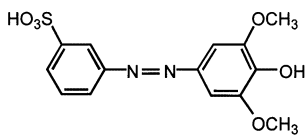
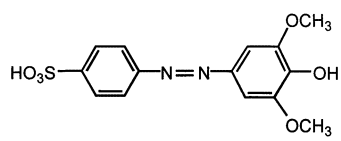
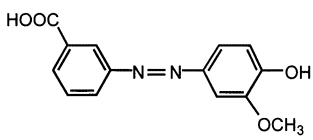
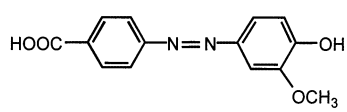
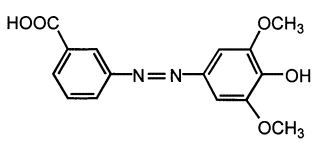
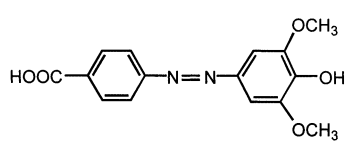
Eight azo dyes were synthesised (Fig. 1) by diazotation of *meta* or *para* aminobenzoic or aminosulphonic acids (diazo components) and coupling with guaiacol or syringol (coupling components).

To a solution of the diazo component (25 mmol) in NaOH 5% (16 ml) and water (46 ml), a solution of $NaNO_2$ (20 mmol) in water (4 ml) was added and stirred. The resulting solution was added slowly with continuous stirring to a mixture of HCl_{conc} (4 ml) and ice (50 g) maintaining the temperature between 0 and 5 °C. A precipitate was formed. Then the diazotised acid was added portionwise to the cooled coupling component solution (20 mmol) in NaOH 5% (40 ml) with stirring. Finally, NaCl (40 g) was added and the mixture was stirred continuously during 30 min at room temperature. The precipitated azo dye was filtered and washed with ethanol and ether.

The synthesised dyes (Table 1) were characterised using UV-visible, IR and ^{1}H-NMR spectroscopy and mass spectrometry.

The nomenclature presented in this paper, expressing the diazo component → coupling component, is used in textile chemistry to suggest the synthetic process. The siglas used refer the kind of

Table 1
Structure of the eight synthesised azo dyes

•S*m*-g dye 3-aminobenzenesulphonic acid→guaiacol 	•S*p*-g dye 4-aminobenzenesulphonic acid→guaiacol 
•S*m*-s dye 3-aminobenzenesulphonic acid→syringol 	•S*p*-s dye 4-aminobenzenesulphonic acid→syringol 
•C*m*-g dye 3-aminobenzoic acid→guaiacol 	•C*p*-g dye 4-aminobenzoic acid→guaiacol 
•C*m*-s dye 3-aminobenzoic acid→syringol 	•C*p*-s dye 4-aminobenzoic acid→syringol 

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).

2.2. Microorganism

The *Phanerochaete chrysosporium* Burdsall and Eslyn, 1974 white rot fungus strain MUM 95.01

(= ATCC 24725), used in this study was obtained from the culture collection of Micoteca da Universidade do Minho (MUM). Stock cultures were maintained on TWAcellulose medium at 4 °C, with periodic transfer.

3. Media

The liquid culture medium, LCM (sucrose 5 g l^{-1}, ammonium sulphate 0.5 g l^{-1}, yeast nitrogen base, YNB without amino acids and ammonium sulphate 1.7 g l^{-1}, L-asparagine 1 g l^{-1} and one of the synthesised azo dyes 50 mg l^{-1}) was used in all biodegradation assays. The final pH was 4.5. The stock solutions of the dyes (100 ×), YNB (10 ×) and L-asparagine (10 ×) were filter sterilised using 0.45 μm membranes. The solid culture media tested to grow the inocula for the experiments, the pre-growth media, were: tap water agar, TWA (agar 15 g l^{-1} in tap water); malt extract agar, MEA (malt extract 30 g l^{-1}, mycological peptone 5 g l^{-1} and agar 15 g l^{-1}); and pre adaptation medium, PAM (LCM with S*m*-g 50 mg l^{-1} and solidified with agar 15 g l^{-1}). TWAcellulose (TWA with a strip of cellulose paper) was used as an inducer medium of the enzymatic system of the fungus.

3.1. 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 TWAcellulose culture plate, was inoculated at the centre of a pre-growth culture plate: TWA, MEA or PAM. Incubation was carried out for 7 days at 30 °C. For the biodegradation assays, 100 ml of LCM medium in 250 ml flasks were inoculated with five plugs of 8 mm from the periphery of a pre-growth 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. Results presented are the mean of at least two independent assays and in all situations, standard deviations were less than 5%.

3.2. Biomass concentration

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

3.3. Sucrose concentration

The sucrose concentration was determined by a colorimetric method, using the Sucrose/D-glucose kit/test from Boehringer Mannheim (cat.No.139041), reading the absorbance values at 340 nm. This kit was used as recommended by the manufacturer with the following modifications—(1) the blank sucrose sample was done with the sample solution but without the kit enzyme suspension; (2) a miniaturised procedure was set up using a 300 μl microwell plate in an ELISA spectrophotometer SLT Spectra; (3) to obtain sucrose concentration, a standard curve was earlier constructed using the same sugar as standard. It was established that the initial sucrose concentration present in LCM medium corresponded to 100%.

3.4. Dye concentration

The concentration of the dye in the samples was determined in a spectrophotometer UV/VIS Jasco 7850 ranging from 250 to 500 nm, by the decrease of the intensity of the absorption at the maximum wavelength (λ_{max}) of the dye. It was established that the absorbance value of the LCM medium containing the initial concentration of the dye corresponded to 100% of dye. For these dyes, the absorption spectra are pH value dependent. Therefore, buffer solutions of citric acid/sodium hydrogenphosphate (Dawson et al., 1986) of different pH values (3.0–6.4) were prepared to select the optimum pH value in which the intensity of absorbance at λ_{max} was higher (pH 4.4). The spectra of the biodegradation assays were obtained from 1 ml of samples diluted with 2.5 ml of the appropriate buffer solution.

4. Results and discussion

In order to select the dye to be used in the pre-growth medium, PAM, the eight synthesised dyes were screened using a concentration of 50 mg l^{-1}. S*m*-g was chosen to be one of the dyes where a clearly fungal decolourisation of the medium was observed.

To investigate the optimal conditions for the biodegradation assays in LCM medium, pre-growth media were tested as source of inoculum: MEA, TWA and PAM. The results (Fig. 2) show that little biodegradation of S*m*-g occurred in the flasks inoculated using MEA (20% at day 14) whereas in the flasks inoculated using TWA a significant biodegradation was observed (64% at day 14). In contrast, the flasks inoculated using PAM exhibited a strong biodegradation of 75% at day 7, which at day 14 had increased up to 83%. Since the best result, in the biodegradation assay using LCM with S*m*-g dye, was obtained with the fungus pre adapted to PAM, this medium was used as pre-growth medium in further biodegradation assays. From these results, it can be concluded that a nutrient rich medium, like MEA, is not a suitable medium to obtain the inoculum of the fungus to biodegrade the azo dyes, even when it has been earlier maintained and grown in an enzymatic inducer and nutrient poor medium such as TWAcellulose. It is very well established that the fungus *P. chrysosporium* has the best enzymatic activity performance under nutrient-limiting conditions, which were the cases of TWA and PAM pre-growth media, as well as LCM used

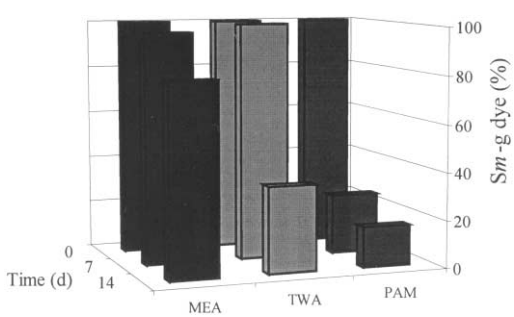


Fig. 2. Effect of different pre-growth media on S*m*-g biodegradation.

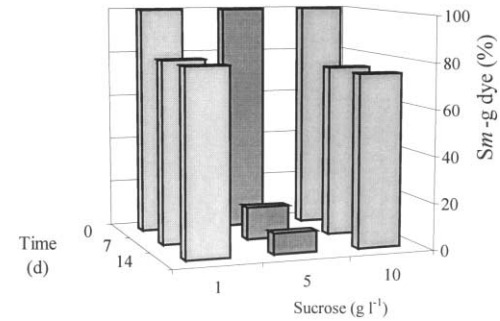


Fig. 3. Effect of different sucrose concentrations on S*m*-g biodegradation.

in biodegradation assays. We presume that the *P. chrysosporium* ligninolytic system is involved in azo dye biodegradation, and that the S*m*-g dye present in PAM would constitute a good adaptation medium for the biodegradation of the other seven dyes.

In preliminary studies, it was observed that for the initial biodegradation of the synthesised azo dyes, an additional carbon source, in our case sucrose, must be present in the culture medium confirming the results obtained by other authors (Blondeau, 1989; Morgan et al., 1993). Therefore, three sucrose concentrations (1, 5 and 10 g l^{-1}) in LCM were tested to find out the best dye biodegradation. The results presented in Fig. 3 clearly show that 5 g l^{-1} of sucrose yielded the highest S*m*-g biodegradation. Consequently, this sucrose concentration in the LCM medium was used in all further experiments.

In the biodegradation assays, a rapid consumption of sucrose with a concomitant increase of biomass and decolourisation of the LCM medium was generally observed after 7 days of incubation. It was also observed in all experiments that no significant variation of dye concentration was caused either by photo-degradation or by dye adsorption to the biomass since it remained colourless during the time of assay. Fig. 4 shows an example of the evolution of the three parameters in study during the time course of the C*m*-s dye biodegradation assay. During the first 7 days of incubation, an increase of biomass was observed, sucrose was totally consumed and the concentration of the dye decreased to 20%. These

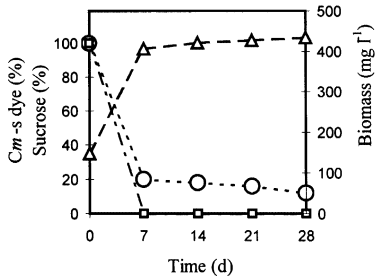


Fig. 4. C*m*-s biodegradation by *P. chrysosporium* pre-grown in PAM with S*m*-g dye. C*m*-s percentage (○), sucrose percentage (□) and biomass concentration (△).

data indicate a cometabolic situation followed by a slight fungal growth at the expense of the dye only. Other authors also found cometabolic pathways in the biodegradation of aromatic compounds by fungi isolated from contaminated soils (Bollag, 1972; Hofrichter et al., 1993), observing also the decrease of these compounds after the exhaustion of the cosubstrate used in the culture medium.

The extent of biodegradation of the eight synthesised dyes using PAM as pre-growth medium is presented in Fig. 5. High percentages of dye biodegradation were obtained (80% on average) using dyes either with guaiacol or syringol as coupling components (Fig. 5). This means that the improvement of the bioaccessibility of the dyes, through the design of their chemical structures to incorporate groups present in lignin, was achieved.

The best results of dye biodegradation were obtained using S*p*-g (89%), C*m*-s and C*m*-g (88%), S*m*-s (85%) and, finally, S*m*-g (83%). The complete sequence obtained from Fig. 5 is: S*p*-g > C*m*-s and C*m*-g > S*m*-s > S*m*-g > C*p*-s > C*p*-g > S*p*-s. Considering the relationship between the dye chemical structure and its biodegradability we suggest these assumptions—(1) comparing C*m*-s versus C*m*-g, C*p*-s versus C*p*-g and, S*m*-s versus S*m*-g, syringol is a better coupling component than guaiacol; (2) comparing C*m*-s versus S*m*-s, C*p*-s versus S*p*-s and, C*m*-g versus S*m*-g the carboxylic group is more adequate than the sulphonic acid group; (3) comparing C*m*-s versus C*p*-s, C*m*-g versus C*p*-g and, S*m*-s versus S*p*-s *meta*-position is better than *para*-position. Taking these assumptions into consideration, the model chemical structure for an azo dye to be easily degraded will be C*m*-s and, on the other hand, the chemical structure not recommended will be S*p*-g. Surprisingly, the latter was the dye that showed better biodegradation performance in our assays, followed by the former one.

Since S*p*-g showed the best biodegradation performance it was decided to study the possible influence of the dye present in the pre-growth medium PAM on the biodegradation results, comparing the initially selected S*m*-g dye with a dye differing in the coupling component, S*m*-s, and a dye differing in the acid position, S*p*-g.

The results presented on Fig. 6 clearly show that the best S*p*-g biodegradation was achieved when *P. chrysosporium* was pre adapted to a dye having a sulphonic group in *meta*-position (S*m*-g or S*m*-s) independently of the coupling component nature. Comparing these two dyes it is observed that, at day 7, the best results for the

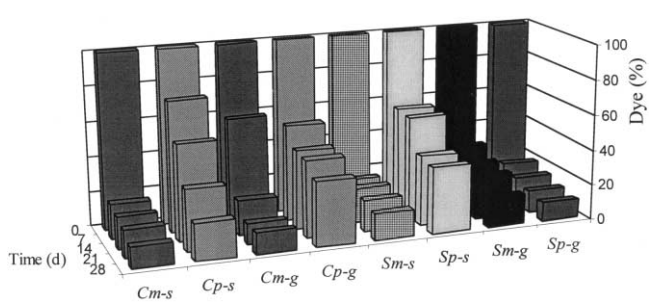


Fig. 5. Biodegredation of the azo dyes by *P. chrysosporium* pre-grown in PAM with *Sm*-g dye.

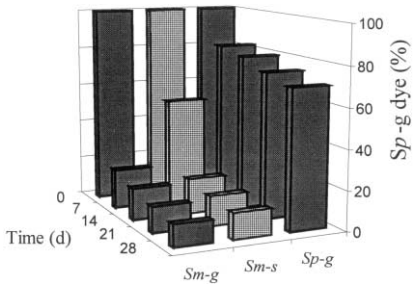


Fig. 6. Effect of S*m*-g, S*m*-s or S*p*-g in PAM on biodegradation of S*p*-g by *P. chrysosporium*.

biodegradation of S*p*-g are obtained when S*m*-g is used in PAM medium. This is exclusively due to the fact that both S*m*-g in the PAM, and S*p*-g in the LCM, have the same guaiacol coupling component. Finally, when the fungus was pre adapted to S*p*-g itself, little biodegradation (30% at day 28 only) was observed (Fig. 6). This means that it is not strictly necessary that the dye present in the PAM should be the same for a successful biodegradation, confirming that S*m*-g is an appropriate dye to be used in the pre adaptation of the fungus, even for dyes having the sulphonic acid group in *para*-position. From these results, we can conclude that biodegradation of these dyes is also dependent on the dye structure present in the pre-growth medium.

As a final conclusion, the results show a relationship between the extent of biodegradation and the chemical structure of the dyes either to be degraded or to be used in the pre-growth medium. To improve the knowledge about this relationship, additional work has been done in our laboratory (data to be published).

Acknowledgements

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