Comparative study of toxicity of azo dye Procion Red MX-5B following biosorption and biodegradation treatments with the fungi Aspergillus niger and Aspergillus terreus

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HIGHLIGHTS

- We show that incomplete biodegradation of dyes led to formation of toxic metabolites.
- In the biosorption process not occurred formation of toxic metabolites.
- The fungi A. niger and A. terreus showed good ability to decolorization of solutions.
- The organisms L. sativa and A. salina proved to be good indicator of acute toxicity.

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ABSTRACT

Azo dyes are an important class of environmental contaminants and are characterized by the presence of one or more azo bonds (−N=N−) in their molecular structure. Effluents containing these compounds resist many types of treatments due to their molecular complexity. Therefore, alternative treatments, such as biosorption and biodegradation, have been widely studied to solve the problems caused by these substances, such as their harmful effects on the environment and organisms. The aim of the present study was to evaluate biosorption and biodegradation of the azo dye Procion Red MX-5B in solutions with the filamentous fungi Aspergillus niger and Aspergillus terreus. Decolorization tests were performed, followed by acute toxicity tests using Lactuca sativa seeds and Artemia salina larvae. Thirty percent dye removal of the solutions was achieved after 3 h of biosorption. UV–Vis spectroscopy revealed that removal of the dye molecules occurred without major molecular changes. The acute toxicity tests confirmed lack of molecular degradation following biosorption with A. niger, as toxicity to L. sativa seed reduced from 5% to 0%. For A. salina larvae, the solutions were nontoxic before and after treatment. In the biodegradation study with the fungus A. terreus, UV–Vis and FTIR spectroscopy revealed molecular degradation and the formation of secondary metabolites, such as primary and secondary amines. The biodegradation of the dye molecules was evaluated after 24, 240 and 336 h of treatment. The fungal biomass demonstrated considerable affinity for Procion Red MX-5B, achieving approximately 100% decolorization of the solutions by the end of treatment. However, the solutions resulting from this treatment exhibited a significant increase in toxicity, inhibiting the growth of L. sativa seeds by 43% and leading to a 100% mortality rate among the A. salina larvae. Based on the present findings, biodegradation was effective in the decolorization of the samples, but generated toxic metabolites, while biosorption was effective in both decolorization and reducing the toxicity of the solutions.

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1. Introduction

Synthetic dyes are widely used as raw materials in the production of inks, foods, cosmetics, paper and textiles. The azo class of synthetic dyes has the largest and most diverse number of representatives listed in the Colour Index (Pandey et al., 2007). These dyes are characterized by the presence of one or more azo bonds (−N=N−) as well as aromatic rings and sulfonic groups and are widely used in textile processes. It is estimated that approximately 10000 colors are produced on an industrial scale, 20% of which are employed in the textile industry and 15% are released into the environment during synthesis, processing or application (Guaratini and Zanoni, 2000; Murugesan et al., 2007; Liao et al., 2013).
Textile effluents are highly colored and discarding these effluents into water bodies without prior treatment inevitably affects the balance of aquatic ecosystems. Azo dyes are generally recalcitrant due to their xenobiotic nature and exhibit considerable resistance to decolorization treatments (Novotny et al., 2006; Khalid et al., 2009; Hua et al., 2013). Anaerobic azo dye reduction, the reductive cleavage of azo linkage, is the first stage in the complete anaerobic–aerobic degradation, resulting in aromatic amine as metabolites. Aromatic amines, which are formed during anaerobic treatment, are generally colorless and hazardous. Though mineralization of the aromatic amines under aerobic conditions is more common, it was reported that a few aromatic amines that are characterized by the presence of hydroxyl and carboxyl groups can be mineralized under anaerobic conditions. As a result, combined anaerobic and aerobic conditions are essential for the complete biodegradation of colored wastewaters (Çinar and Demiröz, 2010).

The discussion on the potable water supply has gained prominence throughout the world due to the importance of preserving this precious resource. In recent decades, there has been increasing concern regarding water pollution by colored effluents, which contribute greatly to the deterioration of aquatic environments. Thus, treatments aimed at the decolorization and reduction in the toxicity of these effluents have been the object of discussion in many countries (Prigione et al., 2008).

Currently, there is no general treatment for the decolorization of textile effluents. Among the many existing forms of treatment, none is completely effective and some require a combination of techniques to be efficient, the high costs of which render such methods unfeasible. Thus, effective alternative treatments are needed. The greater strictness of environmental legislation has contributed to the development of new technologies for the treatment of textile effluents, such as biosorption and biodegradation.

The biosorption process constitutes the adhesion of a chemical species to the surface or pores of a substrate of biological origin (Chiou, 2002), such as chitin, yeasts, filamentous fungi, algae and bacteria. These biosorbents have a large variety of functional groups that form complexes with dye molecules to allow their subsequent removal from a system (Crini, 2006). Adsorption techniques have been widely used for the treatment of colored effluents and have industrial applications due to the advantage of using low-cost adsorbents with high rates of decolorization.

Biodegradation is a process by which different types of microorganisms are capable of converting a complex chemical molecule into a simpler molecule. Such microorganisms use the molecules as a carbon source to obtain the energy necessary for growth and the maintenance of their metabolism (Solis et al., 2012). Filamentous fungi have been widely studied for use in the biodegradation of dyes. These microorganisms are abundant in the environment and are able to quickly adapt their metabolism to different sources of carbon and nitrogen in the quest for survival (Glenn and Gold, 1983; Enayatzamir et al., 2010; Sivasamy and Sundarabal, 2010; Gomi et al., 2011).

Another issue involving the effects of azo dyes on ecosystems regards the fact that chemical tests alone do not adequately portray the impact of these pollutants. Thus, biological systems (organisms) are required for the determination of the toxic effects. Toxicity testing has become increasingly important due to the growing complexity of chemicals discharged into the environment on a daily basis. Moreover, the harmful effects of azo dyes in water bodies extend far beyond visual pollution, as these dyes contain amines and benzidines, which have a high potential for toxicity in the form of metabolites following molecular degradation (Al-Sabti, 2000; Gottlieb et al., 2003; Wang et al., 2005; Rizzo, 2011).

Toxicity tests can be conducted with different organisms, such as larvae of the microcrustacean Artemia salina (brine shrimp). The A. salina test is fast, inexpensive and does not require special equipment or large samples. The aim is to determine the larval survival rate following exposure to the substance being tested (Meyer et al., 1982). The natural tolerance of A. salina is as an advantage over to other bioindicators that are less adapted to different variations in the abiotic environment (Nunes et al., 2006). Plants also form an important group of organisms used in toxicity tests and are excellent indicators of the mutagenic effects of environments polluted by toxic substances (Fiskesjö, 1985; Yi and Meng, 2003). Acute toxicity studies are carried out with seeds for the evaluation of the phytotoxic effects of different compounds. In such tests, sublethal effects are generally evaluated, such as the inhibition of germination and root growth (Sobrero and Ronco, 2008; Rizzo, 2011).

The toxicity of the effluents should be analyzed both before and after decolorization treatments, as a requirement for the recycling of wastewater is that the end toxicity must be lower than the initial toxicity. Moreover, the formation of toxic metabolites during treatment is undesirable. Therefore, it is important to obtain information on the intermediate structures formed during the degradation processes of dyes (Constapel et al., 2009).

In line with the search for novel techniques for the treatment of textile wastewater, the aim of the present study was to evaluate the biosorption and biodegradation of the azo dye Procion Red MX-5B in solutions with the filamentous fungi Aspergillus niger and Aspergillus terreus. The acute toxicity of these solutions was evaluated before and after microbiological treatment using seeds from Lactuca sativa and A. salina larvae.

2. Materials and methods

The fungi A. niger and A. terreus in preliminary microbiological tests indicated higher capacity of decolorization of the solutions under pre-determined condition for each treatment, so were used in studies of biosorption and biodegradation of azo dye Procion Red MX-5B respectively. The concentration of the dye equal to 200 µg/mL solutions were determined after preliminary toxicity tests with A. salina and L. sativa, this being a concentration that before the treatments do not cause significant harm to such organisms.

2.1. Azo dye

The azo dye Procion Red MX-5B (CAS 17804-49-8) was obtained from I.C.I. Brazil S.A. The chemical used was FW 615.34, with λmax = 537 nm. Dye purity of 40% (see Fig. 1).

2.2. Microorganisms

The biosorption and biodegradation tests were respectively performed with the filamentous fungi A. niger (CCT 1435) and
A. terreus (CCT 2679) obtained from the culture collection of André Tosello Foundation for Research and Technology. The fungi were used in their morphogenetic physical form, using the pelletizing method proposed by Marcanti-Contato et al. (1997). The fungal biomass was determined by dry weight of the pellets suspensions.

2.3. Biosorption

The biosorption study (adapted Corso and Almeida, 2009) of Procion Red MX-5B was performed in aqueous solution in 100 mL Erlenmeyer flasks containing 25 mL of dye solution at a concentration of 200 µg/mL, pH 4.0, and 3 mg/mL of fungal biomass (A. niger pellets). The test was carried out in triplicate. After preparation, the flasks were incubated at 30 ± 1 °C for 3 h. Decolorization of the solutions was monitored by UV–Vis spectrophotometry (Shimadzu 2401PC). Following the period of contact between the adsorbent biomass and dye, the solutions were centrifuged at 5000 rpm for 10 min. UV–Vis analyses were performed in the 720–240 nm region using a quartz cuvette with an optical path of 5 mm. The degree of decolorization was calculated from the results of absorbance at \( \lambda_{\text{max}} = 537 \) nm.

2.4. Biodegradation

The biodegradation test (adapted Vitor and Corso, 2008) of Procion Red MX-5B was performed in aqueous solution with the fungus A. terreus. In 100 mL Erlenmeyer flasks containing 25 mL of dye solution at a concentration of 200 µg/mL, pH 4.0, and 3 mg/mL of fungal biomass pellets, the flasks were incubated at 30 ± 1 °C. Samples were analyzed after 24, 240 and 336 h of treatment. For the UV–Vis and Fourier transform infrared (FTIR) analyses, the solutions were centrifuged at 5000 rpm for 10 min. UV–Vis analyses were performed in the 720–240 nm region using a quartz cuvette with an optical path of 5 mm.

2.5. FTIR analysis of metabolites formed in biodegradation treatment

The decolorization of the solutions in the biosorption and biodegradation treatments was monitored by UV–Vis spectrophotometry. FTIR analysis (Shimadzu FTIR-8300) was also performed to obtain more detailed information on the transformation of the dye after the biodegradation treatment. FTIR spectra provide information on molecular structure and this method is a useful tool in the analysis of metabolites formed after the biotransformation of dye molecules. For the FTIR analysis, samples were dried at 105 °C. KBr discs were then prepared at a ratio of 1 mg of sample: 149 mg of KBr. The discs were placed in suitable holders and readings were performed in the mid-infrared region (400–4000 cm\(^{-1}\)) with 16 scans at a resolution of 4 cm\(^{-1}\).

2.6. Toxicity assessment with seeds from L. sativa

Considering the high degree of sensitivity of plants to toxic substances, the aim of the first toxicity experiment was to determine the inhibition of root growth in seeds from L. sativa (TopSeed Garden) before and after the biosorption and biodegradation treatments. For such, Petri dishes were lined with filter paper, to which 20 seeds and 3 mL of the test solution were added. The plates were individually wrapped in plastic film to avoid the evaporation of moisture and placed in a climatic chamber at 21 ± 1 °C in the absence light for 72 h. The positive control was ZnSO\(_4\) 0.05 N and the negative control was distilled water. Untreated dye solutions at a concentration of 200 µg/mL were also tested. At the

Table 1

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Decolorization (%)</th>
<th>Root growth replica 1 (cm)</th>
<th>Root growth replica 2 (cm)</th>
<th>Mean root growth (cm)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (distilled water)</td>
<td>–</td>
<td>0.77</td>
<td>0.74</td>
<td>0.76</td>
<td>–</td>
</tr>
<tr>
<td>Dye solution before treatment</td>
<td>–</td>
<td>0.70</td>
<td>0.74</td>
<td>0.72</td>
<td>5.26</td>
</tr>
<tr>
<td>Dye solution after treatment</td>
<td>30</td>
<td>0.85</td>
<td>0.88</td>
<td>0.87</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The experimental results of the biosorption and biodegradation tests were expressed as the percentage of decolorization, which was obtained from Eq. (1).

\[
\text{Decolorization (\%) = } \frac{\lambda_{\text{max}}537 \text{ initial} - \lambda_{\text{max}}537 \text{ final}}{\lambda_{\text{max}}537 \text{ initial}} \times 100
\]
end of the exposure period, measurements of the roots were taken and growth inhibition was calculated using Eq. (2).

\[
\text{Inhibition (\%)} = \frac{\text{root growth negative control} - \text{root growth dye solution}}{\text{root growth negative control}} \times 100
\]  

(2)

2.7. Toxicity assessment with A. salina larvae

A. salina has been used as a bioindicator of the toxicity of textile effluents, since the high degrees of salinity and conductivity in these effluents are critical parameters for freshwater species. A. salina larvae were obtained after hatching from dry eggs in artificial sea water with constant aeration. Recently hatched (24 h) larvae were used for the tests, which were performed in test tubes containing 2 mL of artificial sea water and 3 mL of dye solution, totaling 5 mL of test solution. Artificial sea water was used as the control. After preparation of the tubes, 10 larvae were added and were left exposed to the solutions (untreated Procion Red MX-5B and solutions following the microbiological treatments) for 48 h, after which the mortality rate was calculated to determine toxicity.

3. Results and discussion

3.1. Biosorption

In the biosorption interaction test between the fungus A. niger and solutions of Procion Red MX-5B, the percentage of decolorization of the samples was approximately 30%. No change occurred in the UV–Vis spectrum of the dye following biosorption, indicating no significant structural changes in the dye molecules following treatment (Fig. 2).

The acute toxicity test performed with seeds of L. sativa revealed a reduction in the inhibition of root growth from approximately 5% (control solution 200 µg/mL) to 0% following the biosorption interaction. With the A. salina larvae, no toxicity occurred either before or after decolorization treatment with the
fungal biomass. Table 1 displays the data on root growth of the L. sativa seeds.

The results of the toxicity test confirm the absence of breakdown of the dye molecules during biosorption treatment, indicating the lack of formation of potentially toxic metabolites. These findings demonstrate that the biosorption process was effective at both decolorizing and reducing the toxicity of the solutions.

3.2. Biodegradation

In the biodegradation process with the fungus A. terreus, decolorization of the solutions was 98% after 336 h of treatment. However, significant spectral changes in the dye molecules were found following microbiological treatment (Fig. 3).

After 24 h of treatment it is possible to identify the biodegradation process of the molecules, because occurred changes the UV–Vis spectrum in the regions of maximum absorbance of the dye. Changes remained after 240 h and were more pronounced after 336 h indicating the slow biodegradation of molecules. The long period of biodegradation can be explained by the fact the use of only one species of microorganism in the study and high molecular complexity of the dye.

The changes indicate that enzymes azo reductase produced by A. terreus was able to degrade the dye molecules. This finding is important, since the improper disposal of these substances can exert a negative impact on aquatic ecosystems. Despite the high decolorization rate, the toxicity test with L. sativa seeds revealed increased root growth inhibition from approximately 5–50%, denoting a 10-fold increase in the degree of toxicity. Table 2 displays the data on root growth.

The original dye solution was non-toxic to A. salina larvae. However, toxicity following the biodegradation treatment was 100%, with the death of all larvae exposed to the treated solution. Fig. 4 displays the data on larvae mortality following microbiological treatment.

These results demonstrate that the biodegradation process of the dye was incomplete, as there was no degradation of the secondary metabolites formed during treatment. The formation of these metabolites during microbiological treatments is highly undesirable due to the increase in toxicity, which can exert a severe impact on the environment. These findings underscore the importance of determining the toxicity of effluents following decolorization processes before discharging wastewater into the environment. Thus, the absence of color does not necessarily translate to an absence of toxicity, as incomplete degradation can lead to greater adverse effects in comparison to effluents discarded in their crude form due to the formation of highly toxic metabolites during the biodegradation of dye molecules.

3.3. FTIR analysis of metabolites formed during biodegradation

The FTIR spectra show the structures of dyes in greater detail. In the comparison of the control Procion Red MX-5B solution and those subjected to the biodegradation treatment, sharp spectral changes were found after contact with the biomass pellets of the fungus A. terreus. The analysis of these spectra allows estimating what compounds were likely formed after the degradation of the dye molecules. Fig. 5 displays the spectra before and after microbiological treatment.

The main changes occurred in the region spanning from 2000 to 400 cm\(^{-1}\). Moreover, the increase in the intensity of the bands from 1650 to 1500 cm\(^{-1}\) was proportional to the increase in treatment time (24, 240 and 336 h). This region is indicative of the angular deformations of the NH\(_2\) group of primary amines, azo groups and vibration of the C–N bond in amine groups (Gup et al., 2007; Pachhade et al., 2009; Wharfe et al., 2010).

In all treatments, an increase occurred in intensity of the band at 1340 cm\(^{-1}\), which corresponds to the stretching of the C–N bond of aromatic primary amines (Kalme et al., 2007). After 240 and 336 h of treatment, two bands emerged – one at 1080 and another at 987 cm\(^{-1}\), which respectively correspond to the C–N bond of primary and secondary amines (Silverstein et al., 1994). The changes from 850 to 800 cm\(^{-1}\) in all treatments correspond to the presence of chlorine and aromatic rings (Polunin et al., 2008). Increased intensity was also found at 545 cm\(^{-1}\), corresponding to the presence of sulfonic groups and aromatic rings (Telke et al., 2010).

The bands at 1141 and 1049 cm\(^{-1}\) are characteristic of sulfonated groups and vibrations of the C–OH bonds in naphthol. The reduction in the intensity of these bands in the FTIR spectra Fig. 6. Proposed degradation pathways for azo dye Procion Red MX-5B by A. terreus.
indicates a possible breakage of these bonds during the biodegradation of the dye molecules (Jadhav et al., 2008; Dhanve et al., 2009; Deveoglu et al., 2012).

All structural changes evident in the FTIR spectra indicate that the Procion Red MX-5B molecules were degraded by the enzymatic action of the fungus A. terreus and this degradation led to the formation of different secondary metabolites, such as primary and secondary amines. Fig. 6 displays a hypothetical degradation scheme for the dye molecules and their possible metabolites. The use these dyes has been drastically reduced in Europe due to national regulations and textile quality labels, but may still be a problem in non-European countries. It is a high risk of the use of azo dyes that give rise to toxic and carcinogenic amines (Schneider et al., 2004).

4. Conclusions

Based on the present findings, seeds from L. sativa and the larvae of A. salina proved to be good indicators of acute toxicity and were sensitive to changes in the toxicity of the solutions after the biosorption and biodegradation treatments. From the toxicological standpoint, the removal of dye molecules by means of biosorption offers the advantages of a shorter period of contact with the microorganism, the absence of major changes in the dye molecules and the formation of metabolites with potential toxicity. Moreover, biodegradation under the conditions proposed in the present study should be carried out for a much longer period of time in an attempt to degrade the metabolites formed during microbiological treatment.

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