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ENVIRONMENTAL BIOTECHNOLOGY

AZO DYE DECOLORIZATION BY *BACILLUS* spp.

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ABSTRACT

Azo dyes are of great importance in the textile industry. These compounds are xenobiotics, and recalcitrant, and promote color in water courses; if not treated properly, they cause considerable environmental impact. Seeking economically viable and environmentally friendly solutions, strains of microorganisms isolated from soil were used in decolorization tests for 4 azo dyes, demonstrating a high potential for decolorizing these compounds. The strains that performed above 80% in most of the dyes tested were identified as belonging to the *Bacillus* genus.

Keywords: Azo dye. *Bacillus* sp. Dye degradation. Textile effluent. Biological water treatment.

1 INTRODUCTION

Dyes are molecules responsible for providing color to the object to which they are fixed and are used in the most diverse industrial sectors. The textile industry is one of the most dependent on these compounds, consuming around two-thirds of all global dye production[1]. After dyeing the fabric, some of the dyes applied do not attach to the fiber, being released into waterways. These compounds are xenobiotic and recalcitrant and promote color in the aquatic ecosystem[2,3], interfering with the penetration of sunlight. Furthermore, azo dyes and their intermediates have toxic, carcinogenic, and mutagenic potential[1]. Several alternatives have been studied to find an ecologically friendly way to degrade these compounds, and biological treatments have demonstrated promising results. The use of microorganisms and their free and immobilized enzymes are possible and economically viable alternatives for treating this waste[4]. In the present work, isolation was carried out, and the discoloration potential of different strains of *Bacillus* spp. was investigated.

2 MATERIAL & METHODS

Microorganisms were isolated from soil microcosms constructed using humus and gardening soil in a 1:1 ratio. Three different types of microcosms were constructed: C (control), which was watered with a dye-free solution; L (Methyl Orange), which was watered with a solution containing 0.1 g/L of the azo dye methyl orange; and A (Remazol Blue), moistened with a solution containing 0.1 g/L of the anthraquinone dye Remazol Blue. A 1 g aliquot of soil was transferred to 100 mL saline solution (NaCl 0.85%), and 1 ml of suspension was diluted by serial dilution and transferred to Petri dishes containing solid Luria Bertani (LB) medium. After the colonies grew, they were isolated in solid LB based on their morphology and stored for decolorization assays.

In the first stage of decolorization tests, each isolated strain was pre-inoculated separately in LB broth to increase cell mass, centrifuged, and resuspended in saline. A 1 ml aliquot of the microbial pellet resuspended in saline was transferred to a decolorization broth containing Methyl Orange dye at a concentration of 15 mg/L under static conditions (at room temperature without aeration) and under agitation (approximately 150 rpm) and maintained under these conditions for 7 days. All experiments were performed in duplicate. On the 2nd, 5th, and 7th day, aliquots were removed to evaluate dye degradation. The loss of color was evaluated through the difference between the absorbance of the control group (without microorganisms) and the inoculated samples at the wavelength corresponding to the dye peak (455 nm), as follows:

$$Decolorization\ (\%) = \frac{\Delta\ Absorbance}{Inicial\ absorbance}\ x\ 100$$

The strains that showed a decolorization rate above 70% were selected for the second stage of decolorization tests, which involved the degradation of 3 different azo dyes: Reactive Intense Black N, Reactive Red CA, and Brilliant Violet 5R. The test parameters remained the same as those used in the first test stage, changing only the final concentration of the dyes, viz. 25 mg/L of black dye and 50 mg/L of red and violet dyes. The strains defined as those that showed satisfactory performance in decolorizing all dyes had their genome sequenced by Illumina. Genome sequences were assembly using SPAdes and annotated using RASTtk pipeline on RAST server, and BLAST was performed on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the 16S rRNA coding region.

For cytotoxicity assay, hepatocellular carcinoma cell line (HepG2) and kidney cell line (VERO) were maintained in Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with 10% fetal bovine serum, and 0.1% gentamicin (10 mg/mL) at 37°C and 5% CO_2. Cell viability with different proportions of Methyl Orange decolorization medium, before and after degradation by strain C7.1, was evaluated using tetrazolium salt (MTT).

3 RESULTS & DISCUSSION

All 21 strains of microorganisms isolated showed decolorizing potential of the Methyl Orange dye in both static (without aeration) and agitation conditions, although the best results were obtained in conditions without aeration (Figure 1). Of these, 15 strains had a discoloration rate greater than 70%.

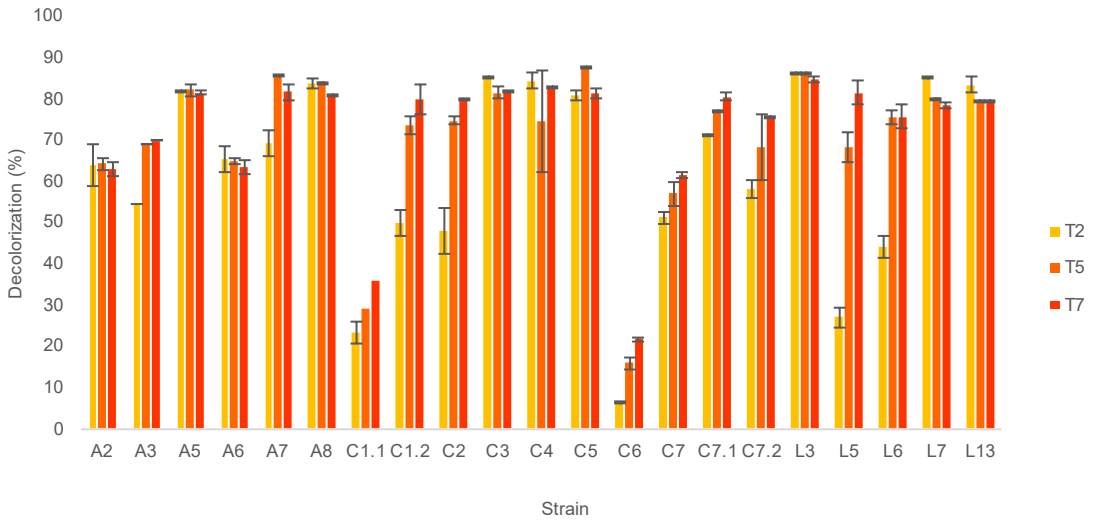

Figure 1 Decolorization of methyl orange in static condition over 7 days by the isolated strains. T2 represents 2nd day, T5 the 5th day and T7 7th day.

As the best results were obtained in static conditions, the strain that degraded more than 70% of methyl orange was cultivated using other dyes (Figure 2) in static conditions. The 3 strains (C7.1, L6, and L7) that showed discoloration greater than 80% in all azo dyes used in the test had their genome sequenced.

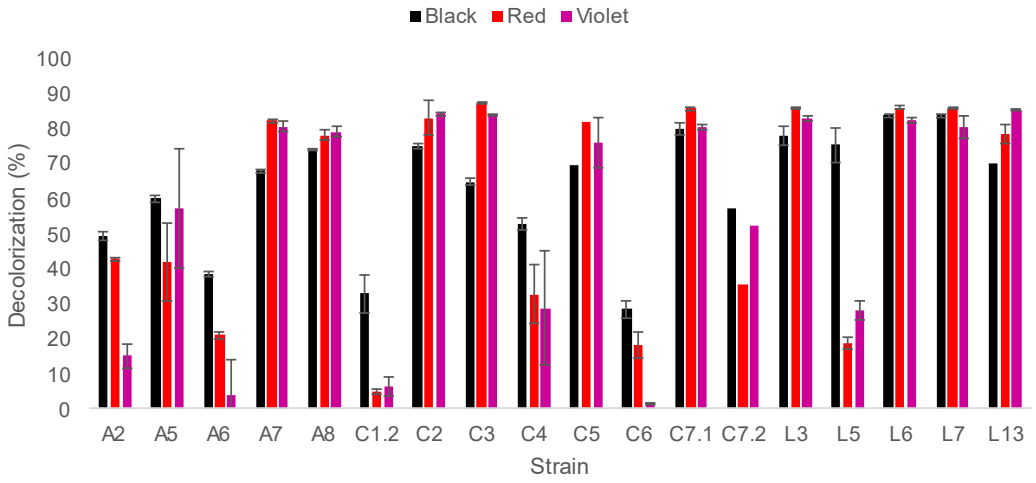

Figure 2 Decolorization of the azo dyes Reactive Intense Black N (Black), Reactive Red CA (Red), and Brilliant Violet 5R (Violet) after 7 days of cultivation in static conditions at room temperature (approximately 28ºC).

Genome sequencing data from strains C7.1, L6, and L7 suggest that all the strains belong to the genus *Bacillus* spp. There are reports in the literature of the use of these microorganisms in the degradation of azo dyes by enzymatic mechanisms through enzymes of the oxidoreductase class, such as azoreductases, laccases, and lignin and manganese peroxidases. In the present work, the mostly static discoloration of azo dyes provides evidence that the main enzyme related to the breakdown of the dye molecule is azoreductases since these enzymes, for the most part, are sensitive to oxygen and are the main ones involved in the

cleavage of the azo bond, resulting in the breakdown of the chromophore group and loss of color, producing aromatic amines. Cytotoxicity assays carried out using the HepG2 strain demonstrated a reduction in cell viability after treatment with the C7.1 strain; however, in preliminary assays using the decolorization medium without the addition of dye (data not shown), there was a reduction in cell viability, which may have occurred due to the release of some virulence factor expressed by the microorganism that may have interfered with cell viability. In the VERO line, there was a slight variation in viability before and after treatment, suggesting that the metabolites produced during degradation were not toxic to this cell line (Figure 3).

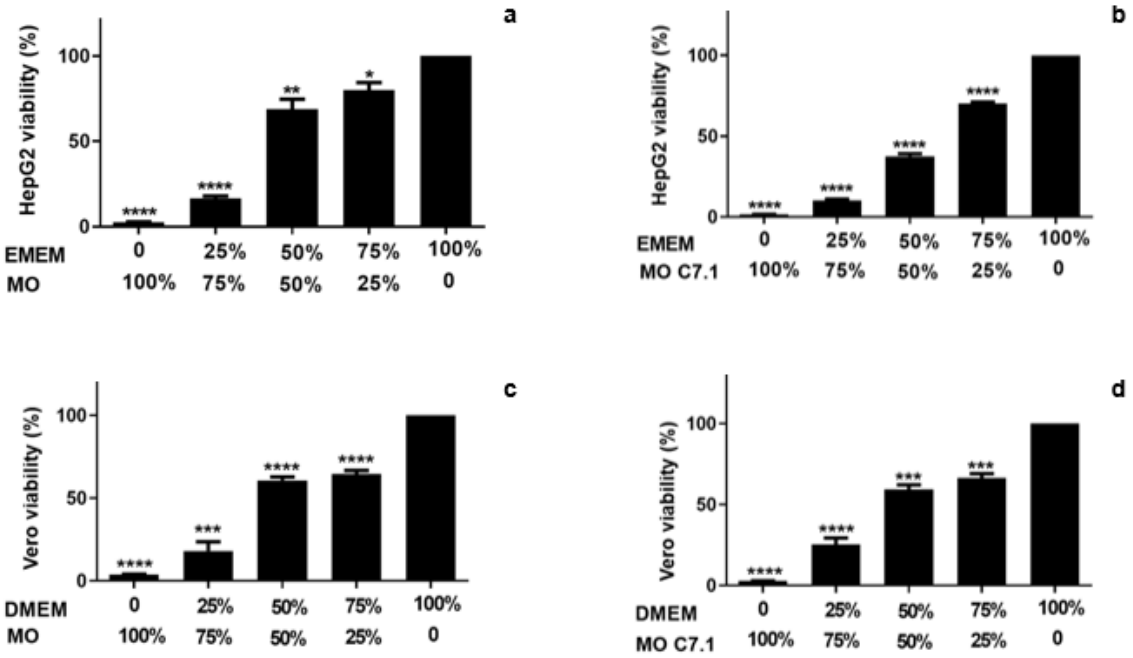

Figure 3 Viability of the hepatocellular carcinoma cell line (HepG2) in decolorization medium artificially contaminated with the dye methyl orange (MO) in static condition before (a) and after treatment with the C7.1 strain (b). Viability of cell line obtained from green monkey kidney (VERO) in decolorization medium artificially contaminated with methyl orange dye (MO) in static condition before (c) and after treatment with strain C7.1 (d).

4 CONCLUSION

The results demonstrate the biotechnological potential of species of the genus *Bacillus* to degrade recalcitrant compounds, including different dyes of the azo class. The biotechnological potential of *Bacillus* spp. strains, as well as their enzymes, demonstrate that they can be used to treat this type of effluent.

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