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Decolorization and Detoxification of Industrial Wastewater Containing Indigo Carmine by *Aspergillus niger* AN400 in Sequential Reactors

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Abstract: Effluents from the textile industry are an active problem in the sector and one of the world's main environmental problems. The conventional treatments applied are not always efficient in terms of compliance with legislation, and, in many cases, the efficiency of treatment is guaranteed by the enormous energy expenditure involved, camouflaging the momentary problem and not effectively treating it. In this work, batch reactors with immobilized biomass of *Aspergillus niger* AN400 were arranged in series for the treatment of real textile wastewater containing approximately 20 mg/L of indigo carmine. Sucrose was added as a co-substrate in concentrations of 1 g/L and 0.5 g/L, in the first and second reactors, respectively, over 19 cycles of 48 h. The highest decolorization rate in the system was (93 ± 4) %, with the largest amount removed in the first reactor (90 ± 6) %, occurring mainly by biological means. The production of aromatic by-products from the initial degradation of the dye molecule was reflected in the lower removal efficiency of dissolved organic matter: 52% in the first reactor, and 25% in the second reactor. The number of colonies of fungi was higher than that of bacteria, 2.24:1 and 2.44:1 in the first and second reactors, respectively. The treated effluent in the system showed less toxicity than the raw effluent, and this demonstrates the potential of this technology in the treatment of textile effluents containing indigo carmine.

Keywords: bioremediation; filamentous fungi; sequencing batch reactors; sucrose; textile effluent

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1. Introduction

The textile industry is a very important economic sector, whose use of colorants stands out in comparison with other industrial sectors [1]. The denim textile industry is one of the branches of textile production that is highly represented in the sector and the main dye used is indigo carmine, with between 3 and 12 g of dye needed per pair of jeans [2]. Indigo carmine is also used in the food, paper, pharmaceutical, and cosmetic industries, and in medicine, it is used in diagnostic methods and surgical procedures [2,3]. In recent decades, its production has increased, reaching up to 80,000 tonnes/year and its usage is steadily increasing [2–6]. The problem with using colorants is that part of the dye is not retained by the fabric (20–30%), so a large proportion ends up in textile effluents [1,7]. About 280 tons of dye are released into water bodies every year, globally [8,9].

The discharge of textile effluents into water bodies, in addition to the visual impact due to the intense color, reduces the penetration of light into the aquatic environment, hindering photochemical and biological activities that are essential for aquatic life [10]. Dyes are

relevant sources of pollution to the hydrosphere due to their complex organic molecular structure and recalcitrant behavior in conventional wastewater treatment methods [10]. They also exhibit mutagenic, carcinogenic, and toxic characteristics [11]. For instance, indigo carmine is a highly persistent, chemically and photolytically stable recalcitrant dye, with high solubility (10 g/L) and whose removal from wastewater through conventional processes has proven inefficient [3]. This is not only an environmental problem but also a public health concern, since exposure to this dye can cause skin irritation, dermatitis, corneal and conjunctival damage, and cancer [3,8]. Moreover, the global indigo carmine market consumes over 4.5 billion liters of water annually [12]. Accordingly, the textile industry in general, and the denim textile industry in particular, seeks innovative and sustainable processes to treat end-of-line effluents to ensure compliance with discharge limit values, as well as minimize negative impacts on the surrounding environment and address water recycling [13].

Conventional wastewater treatment consists of a combination of physical, chemical, and biological processes and operations to remove solids, organic matter, and, sometimes, nutrients from wastewater. However, conventional treatment options are frequently ineffective [14], while secondary pollution and inefficient removal of organic load upon discoloration demand the use of advanced approaches [15], so there is an urgent need to develop cost-effective and environmentally friendly treatment approaches for adequately treating dye-containing wastewater prior to its final disposal into the environment.

Biological treatments of colored wastewater have advantages over physical and chemical approaches because they can effectively remove dyes from large volumes of wastewater, also removing COD and BOD, are more easily applicable, generate less sludge, require fewer chemical reagents, are less expensive, have energy-saving characteristics, and are safer for the environment [16–19]. Biological processes that apply fungi can be effective solutions for the treatment of textile wastewater because they are able to remove color and mineralize these complex and recalcitrant compounds through oxidative enzymes involved in breaking down the molecular structure, generating smaller compounds that can be used as a carbon source [20]. The best results of mycoremediation occur through the immobilization of microbial cells inside reactors [17,21]. This favors contact between the mycelium and liquid medium, which allows for access to the substrate and protection against shear forces, overloads, and unfavorable environmental conditions [17,21]. These systems provide the maintenance of active enzymes for a longer time, allowing for greater production of secondary metabolites [21]. In the mycoremediation processes, the existence of an easily assimilable carbon source is a crucial factor to improve the uptake of pollutants by the fungi [22]. Therefore, the microorganisms are able to have the carbon available in the medium, in the appropriate concentration to induce enzyme production without repressing the oxidation of the pollutant ad maintain the metabolic activities that contribute to the complete mineralization of the pollutant molecule [23].

Most of the research on treatment technologies developed at the laboratory scale have been carried out with simulated wastewaters, prepared under optimum conditions for the microorganisms and enzymes, with either carried out at a low scale, which represents an obstacle when the developed treatments have to be applied in real scenarios [24]. In this work, the ability of *Aspergillus niger* AN 400 to biodegrade indigo carmine was studied by feeding two sequential batch reactors, arranged in series, with an industrial textile effluent and sucrose as a co-substrate. *Aspergillus niger* is a member of the Ascomycetes group, which produces various types of enzymes, such as laccases. Laccases are used in the mineralization of dyes, catalyzing demethylation reactions with the subsequent rupture of aromatic rings [25]. *Aspergillus niger* is readily available and widely studied in the field of bioremediation. Its well-characterized genome and established cultivation methods make it a practical choice for research and industrial applications. It has been extensively studied for its biotechnological applications, including enzyme production and bioremediation not only for the biodegradation of textile dyes [26,27] but also for various other pollutants, such as pesticides [28], surfactants [29], microplastics [30], and aromatic

hydrocarbons [31,32]. It is a species of great versatility, with the ability to adapt to various environmental conditions and substrates. It is a robust organism and can tolerate a wide range of pH, temperature, and salinity conditions. This resilience and adaptability allows it to thrive in different wastewater environments, an advantage in wastewater treatment applications (including recalcitrant dyes) where conditions may fluctuate. It is also easy to manipulate and colonize, with it found in various terrestrial environments due to its ability to degrade diverse substrates and adapt to the most adverse conditions.

2. Materials and Methods

2.1. Cultivation, Production, and Counting of Fungal Spores

Aspergillus niger AN 400 was inoculated in sterile Petri dishes containing Sabouraud agar media, which were previously sterilized under the pressure of 1 to 1.5 Kgf·cm^2 at 121 °C for 20 min. Fungi were cultivated for 5 days at ±28 °C. Subsequently, the fungal spores were removed with saline solution of sodium chloride (NaCl) at 0.9%, with Tween 80 added, transferred to a sterile flask, and stored at 0 °C. For the spore count, 50 µL of the suspension was withdrawn and stirred in a vortex shaker and 950 µL of Tween 80 solution was added, resulting in a 1:20 dilution. Then, 20 mL of this suspension was transferred to a 0.1 mm deep Neubauer chamber with a minimum area of 1/400 mm^2. The spore count was carried out using an optical microscope (Bioval, L-1000 series, São Paulo, Brazil) with 400× magnification. Equation (1) was used to calculate the number of spores.

$$\text{Spores (mL)} = \text{spores counted} \times \text{dilution} \times 2.5 \times 10^5 \quad (1)$$

2.2. Immobilization of the Biomass

Polyurethane foam was cut into cubes of 1 inch of edge (30 g), washed with soap and tap water, and dried at 50 °C. Subsequently, polyurethane cubes were packed into polyethylene networks which were placed into two reactors that were fed with 4 L of basal medium containing (mg/L): glucose (5000); $MgSO_4{\cdot}7H_2O$ (1000); $NaNO_3$ (4000); K_2HPO_4 (800); $CaCl_2{\cdot}2H_2O$ (40); $CuSO_4{\cdot}5H_2O$ (320); H_2MoO_4 (200); $MnSO_4{\cdot}H_2O$ (200); $Fe_2(SO_4)_3{\cdot}H_2O$ (200); $ZnSO_4{\cdot}7H_2O$ (160). After that, 0.1 g/L of streptomycin antibiotic and 1 mL/L of Vishniac solution (mg/L) were added: H_3BO_3 (50) $FeCl_2{\cdot}4H_2O$ (2000); $ZnCl_2$ (50) $MnCl_2{\cdot}4H_2O$ (500); $CuCl_2{\cdot}2H_2O$ (38); $AlCl_3{\cdot}H_2O$ (90); $CoCl_2{\cdot}6H_2O$ (2000). Then, 2×10^6 spores/mL were inoculated in each reactor and kept with natural aeration for the initial fixation of spores in the cubes supports within the first 24 h. After this time, air was supplied by mini aerators. Biofilm growth lasted 10 days, and the medium was replaced with a new one on the fifth day.

2.3. Industrial Effluent

This study was carried out with a real effluent from a textile facility located in the metropolitan region of Fortaleza, state of Ceará, Brazil. The textile industrial wastewater has an average dye concentration of (18 ± 6) mg/L. Characterization of the real effluent is presented in Table 1.

Table 1. Characterization of the textile effluent used in this study.

Effluent Characterization	Concentration (mg/L)
COD total	7046 ± 1586
COD dissolved	6220 ± 1475
Dye	100 ± 4
Ammonia	45.87 ± 12
Nitrite	9.73 ± 3
Nitrate	2 ± 1
pH	10 ± 1

COD, chemical oxygen demand.

2.4. Experimental Setup

Two glass bottle reactors with a capacity of 5 L and working volumes of 4 L (R1) and 3.6 L (R2) were sealed with a plastic cover and used in the experiments (Figure 1). The first reactor was fed with real effluent diluted to 20% (*v*/*v*)—20 mL of effluent to 80 mL of tap water, sucrose (1 g/L) as a co-substrate, and streptomycin antibiotic (0.1 g/L) to minimize medium contamination by opportunistic bacteria. After 48 h of reaction, the effluent of R1 was introduced into the second reactor, 0.5 g/L sucrose was added, and the reaction time set was to 48 h.

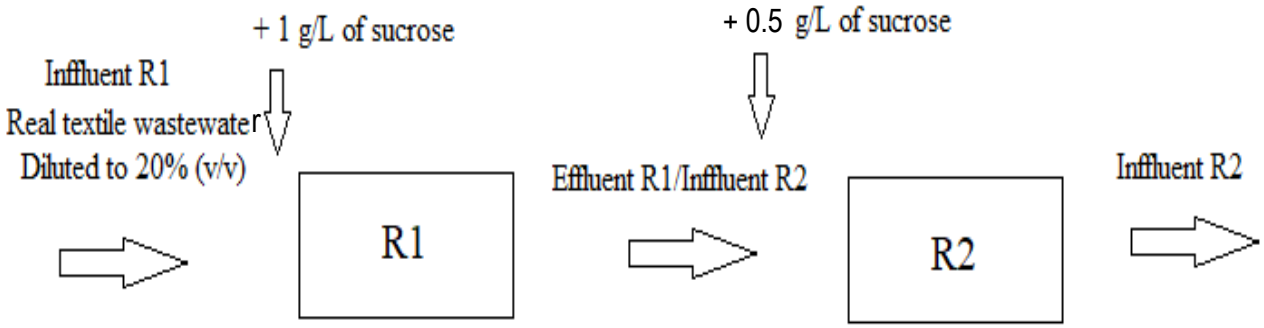

Figure 1. Schematic representation of the operational strategy of sequential batch reactors R1 and R2.

The pH of the medium was (5 ± 0.2) in both reactors to provide better conditions for fungal growth. The reactors were operated in 19 cycles (under the same conditions), over 133 days, and their performance was monitored by determining COD and pH (according to APHA [33]) and dye removal. For dye removal analysis, the influent and effluent samples from both reactors were analyzed through UV-Vis spectrophotometry between 200 and 1000 nm using a UV-vis spectrophotometer (Genesys 150, Thermo Scientific™, Waltham, MA, USA). The maximum wavelength of the dye was identified at 610 nm.

2.5. Determination of A. niger Mycelium-Adsorbed Dye

For evaluating the dye adsorption onto the mycelium, a foam cube with attached biomass was withdrawn from each reactor and transferred to a 250 mL Erlenmeyer flask containing 100 mL of saline solution and glass beads. The cube was vigorously stirred in a vortex to facilitate the removal of the biomass adhering to the polyurethane cubes. The medium was centrifuged at 3500 rpm and the released biomass was transferred to test tubes with 10 mL of methanol for dye extraction. After 20 min, a further 5 mL of methanol was added to the tubes and centrifuged again at 3500 rpm, and the supernatant was analyzed via spectrophotometry. The determination of dye adsorbed onto the polyurethane foam cubes was evaluated by placing the polyurethane foam cubes (15 g) in a beaker (5 L), which were previously washed with soap and dried in an oven at 50 °C. Then, 4 L of diluted wastewater at 20% (*v*/*v*) was added and the beaker was shaken for 1 h. Aliquots were withdrawn at 10 min intervals.

2.6. Toxicity Bioassay

The bioassay to assess the detoxification of the effluent during the indigo carmine removal process was carried out in accordance with Romero and Cantú [34], utilizing common onion, *Allium cepa* L. (2n = 16). This choice was based on its excellent chromosomal condition, making it widely employed for examining the cytogenetic effects caused by various chemical compounds in biological material [35]. Onion bulbs (*Allium cepa*) were peeled, and the old roots were carefully removed. The base bulbs were placed in plastic cups containing tap water and kept for three days at 25 °C in a humidity-free place protected from light. After this period, growth roots were removed. The aim of this procedure was to obtain bulbs with the same growth pattern by removing the roots grown during the three days and then using the bulbs.

To assess the roots growth inhibition, the base of the bulbs was placed in plastic cups containing the samples: IR1 (influent of R1), ER1 (effluent of R1), IR2 (influent of R2), and ER2 (effluent of R2), and after 72 h, the roots were measured. As a negative control, onions exposed only to tap water were used. The assay was carried out in triplicate, The inhibition percentage was determined in accordance with Equation (2).

$$I = 100 \times (Lc - Ln)/Ln \quad (2)$$

where "I" corresponds to the inhibition percentage (%); "Lc" is the length of the roots of the onion bulb negative control (cm); and "Ln" is the length of the roots of onion bulbs in wastewater (cm).

2.7. Colony-Forming Unit Count

At the end of reactor operation, the biofilm samples were subjected to the colony-counting procedure for fungi and bacteria to determine their predominance. Colony counting was performed using the spread-plate technique with selective medium for fungi, Martin, and half-nutrient agar for bacteria (23 g/L). The Martin medium has the following composition (mg/L): K_2HPO_4 (1000), peptone (5000), KH_2PO_4 (500), $MgSO_4 \cdot 7H_2O$ (500), dextrose (10,000), yeast extract (500), rose Bengal (33), and agar (18,000). For counting the fungal microorganisms, the antibiotic streptomycin was added to the plates at the concentration of 3 µg/mL in order to avoid bacterial growth. For bacterial colony counting, the antibiotic was omitted.

Polyurethane cubes were removed from each reactor at the end of the experiments and introduced into vials containing 60 mL of saline solution (0.9% NaCl) and glass beads in order to detach the biomass. Aliquots of 1 mL were transferred successively to 9 mL of saline to yield concentrations of 10^{-2}, 10^{-3}, and 10^{-4}. Then, 0.1 mL of sample dilution was added to the solid culture media. The inoculated plates were incubated at 28 °C, for 120 h. The colonies were counted, and the number of CFUs/mL were calculated (Equation (3)).

$$CFU = \frac{\text{Number of colonies on the Petri dish} \times \text{Dilution factor}}{\text{Inoculum volume}} \quad (3)$$

3. Results and Discussion

3.1. Decolorization of Textile Industrial Wastewater

Although textile industrial wastewater has an average dye concentration of (18 ± 6) mg/L, in the sixth cycle of operation, the concentration in R1 was higher than this initial value (36.2 mg/L), resulting in an indigo carmine concentration of 11.1 mg/L in the final effluent of this reactor (Figure 2). This can possibly be explained by desorption of the dye adsorbed in the previous cycles. In the first cycle, the fungus could still be adapting to the effluent and synthesizing the enzymes to degrade the dye, and the main removal mechanism may have been adsorption. The higher dye removal in the reactor R1 occurred in the tenth cycle, were 96% of the dye was removed, and the average dye concentration decreased to 2 mg/L, with it remaining constant throughout the 19 cycles.

Analyzing the performance of R2, the highest efficiency, 88%, was obtained in the fifth cycle, when the dye concentration in the final effluent was 0.7 mg/L. Between cycles 10 and 19, there was no dye removal. Indeed, the performance of R1 was more stable, with little oscillation in efficiency over the operational cycles, so that the standard deviation (StD) in R1 (StD ± 8) was nine times lower than that presented in R2 (StD ± 59). During the sixteenth cycle of reactor R2, the final effluent displayed a dye concentration of 3.99 mg/L, slightly higher than the influent concentration of 2.75 mg/L. This increase was attributed to desorption of dye from the biofilm within this reactor. Observation during the exit of the final effluent in this cycle revealed fragments of detached biomass. However, for the majority of the operational period, the final effluent from the series reactor system appeared clarified, as illustrated in Figure 3.

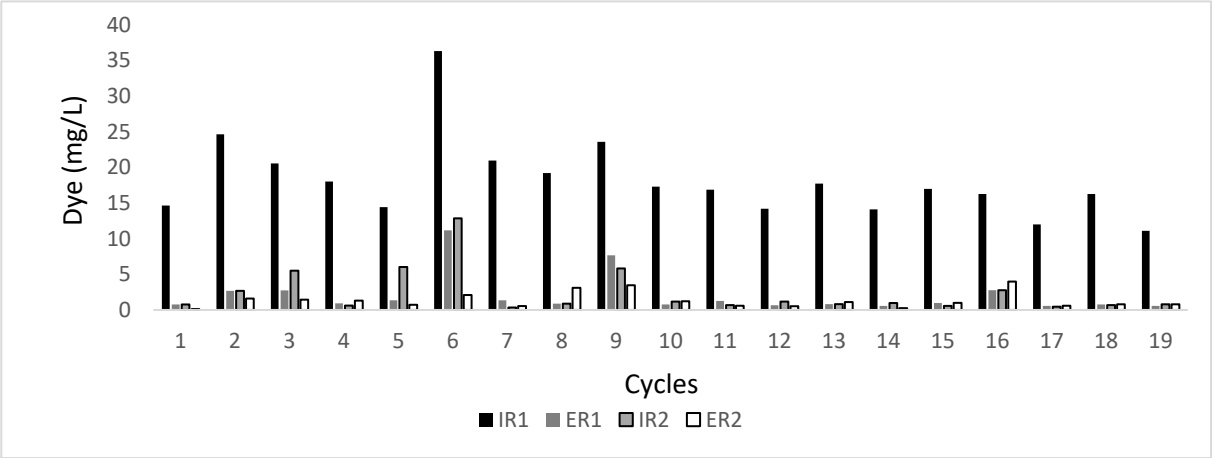

Figure 2. Dye concentration in influent (IR) and effluent (ER) of R1 and R2 reactors over the course of operating cycles.

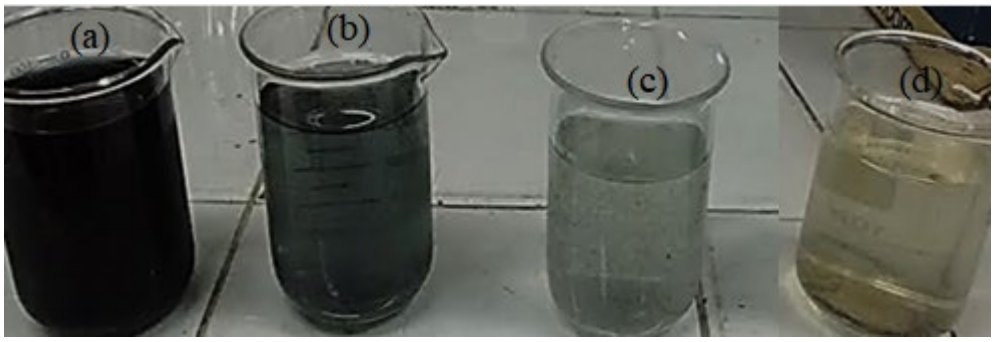

Figure 3. Samples from the 8th cycle of operation of sequential batch reactors (SBR) in series: (**a**) untreated wastewater; (**b**) influent of R1; (**c**) effluent of R1/influent of R2; (**d**) effluent of R2.

It should be noted that the amount of dye entering R2 was always very low, which may have contributed to the low removal speed, since it was at a limited concentration. Indeed, the lower efficiency in R2 may be attributed to the low concentration of indigo (2.4 ± 2) mg/L compared to sucrose (0.5 g/L) in the influent. The sucrose:dye ratio used was 250:1, which may have inhibited indigo carmine consumption in reactor R2, since sucrose is a preferential substrate and was available in high concentrations. According to Pizato et al. [22], the growth of fungi and color removal occurs only when a primary source of carbon and nitrogen are available in the medium. The presence of a primary carbon source in a sufficient concentration is essential for the growth of biomass and the production of specific enzymes that assist in pollutant degradation; however, the sucrose:dye ratio can influence the dye mineralization process; in excess, it can suppress the utilization of other more complex carbon sources [22,23]. Sugars such as sucrose, glucose, fructose, or yeast extracts, among others, are widely used as the primary source of carbon, with them playing a crucial role in the biodegradation process [36]. They can serve as electron donors to break chemical bonds (such as azo and anthraquinone) in dye molecules during mineralization. Additionally, they provide energy to the organism to respond to toxicity stresses. The proper selection and optimization of the input quantity of these carbon sources into the reactor are essential to achieve effective performance in dye degradation by microorganisms.

In general, the removal efficiencies in the R1–R2 system were above 75% during the operational period. The average dye removal in the system (R1–R2) was (93 ± 4) %, and the maximum dye removal was 99% during the first cycle, resulting in the lowest dye concentration (0.1 mg/L) at the system outlet. The minimum removal occurred after 16 cycles (75.2%) when the maximum dye concentration in the R2 reactor effluent was 4.0 mg/L. This

corresponds to an average dye concentration in the effluent of (1.3 ± 0.81) mg/L. Color was visually absent in the final effluent of the R1–R2 system, complying with the provisions of Brazilian legislation [36,37].

COD and pH variations in the influent and effluent of reactors R1 and R2 are shown in Figure 4. The average COD removal efficiency was (53 ± 19) % in R1, while R2 only showed efficiency of (33 ± 21) %, with dissolved organic matter concentrations in the reactors' effluent of (1376.8 ± 340) mg/L and (880 ± 183) mg/L, respectively. The high COD values in the R2 influent are due the presence of sucrose, micronutrients, and antibiotic, which were added to the effluent of the first reactor. Nevertheless, COD removal in R2 was higher than that of the dye, which once again suggests that sucrose was used more than the dye in this reactor.

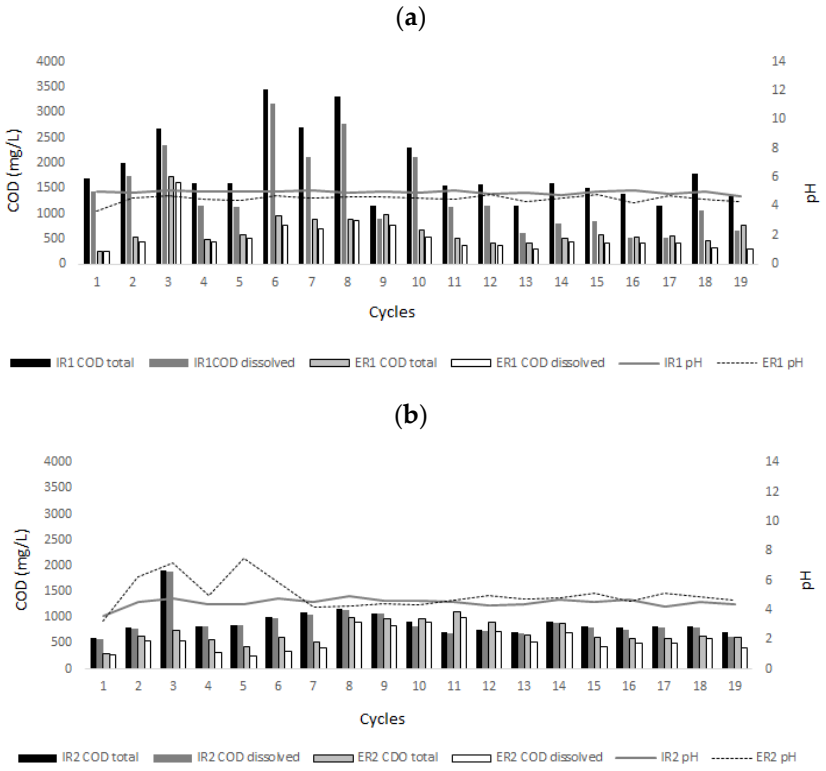

Figure 4. Variations in COD and pH in the influent and effluent of reactors R1 (**a**) and R2 (**b**).

Dye and organic matter removal from various textile effluents in reactors with the immobilized biomass of nine different white rot fungi species was studied by Anastasi et al. [38]. High color removal (80%) was achieved with *Trametes pubescens* MUT2400 in 48 h. However, there was evidence of the formation of metabolites derived from the breakdown of the dye molecule, which were not assimilated and, therefore, caused an increase in COD in the effluent from 485 mg/L to 645 mg/L.

The pH of the R1 and R2 influents remained within the optimum range for the fungus (pH ≈ 5). The average pH of the effluent was 4.7 ± 0.8 in R1, and 5.6 ± 1.5 in R2, ranging from 3.2 (cycle 1) to 7.8 (cycle 19); thus, it may be necessary to correct the pH before discharge into the water body receptor.

3.2. Dye Removal through Adsorption

The results of the adsorption test revealed that polyurethane foam has the capacity to adsorb 4.3 mg of dye per gram of polyurethane. During system operation, 1395.9 mg and 182.7 mg of dye mass were applied to R1 and R2, respectively (i.e., 93.1 mg per gram of support in R1 and 12.2 mg per gram of support in R2), concentrations well above the adsorption capacity of polyurethane foam. These results indicate that indigo carmine removal was not due to absorption on polyurethane foam. On the other hand, there is a

strong affinity of fungal mycelia for dyes [39]. Yet, the amount of indigo carmine adsorbed on the mycelium of *Aspergillus niger* AN 400 was lower than the mass of dye that was removed in the reactors, 0.55 mg/g of mycelium in reactor R1 and 1.05 mg/g in reactor R2. These results show that the microbial cells were able to metabolize about 1141.1 mg of dye, reaching a removal percentage of 86% of the initial mass of dye, and that indigo carmine was removed mainly via the biological pathway. As analyzed via spectroscopy, the greatest decrease occurred in the wavelength of the chromophore (62% in R1 and 76% in R2), and at 238 nm, this percentage was 50% in R1 and 31% in R2, indicating the presence of functional groups from the benzene ring during the breakdown of the dye molecule.

3.3. Toxicity Assessment

The partial mineralization of indigo carmine was reflected in the toxicity of the final effluent. Although the toxicity analysis demonstrated decreased toxicity in the effluent in relation to the influent, it is still considerable. Indeed, when the bulbs were placed in contact with the textile influent, there was no growth of their roots, but when in contact with the treated effluent in reactors arranged in a series system, there was a bulb growth corresponding to a decrease in toxic effects, and inhibition decreased from 89.2% to 79.1% (Table 2, Figure 5). The still high toxicity observed after fungal treatment may be related to the formation of more toxic by-products, such as isatin, an intermediate metabolite of the biological degradation of indigo carmine, previously identified, whose final product is 2-aminobenzoic acid, an aromatic amine that under the action of the laccase enzyme produced by fungi can result in free radicals that can be further degraded or polymerized [40,41]. According to Wang et al. [42], the action of laccase enzymes facilitates the cleavage of the C=C bond of indigo carmine, leading to the formation of the intermediate product isatin 5-sulfonic acid. The subsequent biodegradation of this intermediate yields additional by-products, including isatin, methanol, and 2-aminobenzaldehyde, which may possess higher toxicity levels compared to the original molecule. Campos et al. [40] also documented the generation of anthranilic acid as a by-product during the degradation process of isatin. However, besides the small decrease in toxicity after treatment, it had a significant effect on the growth of onion bulb (*Allium cepa*) roots when exposed to the R2 effluent, which was almost double that of the growth with the R1 influent.

Table 2. Onion root growth in tap water, and growth inhibition (*Allium cepa*) in the presence of the influent wastewater of industrial textile wastewater (IR1) and in the R1 and R2 effluents (ER1 and ER2).

Sample	Root Length (cm)	Growth Inhibition (%)
Tap water	4.9 ± 1.70	0
IR1	0.53 ± 0.68	89.2 ± 15
ER1	0.76 ± 0.75	84.5 ± 7
ER2	1.03 ± 0.65	79.1 ± 10

The toxicity of the treated effluent containing indigo carmine seems to be higher than that other classes of dyes reported in the literature (Table 3). However, the comparison is not straightforward as different toxicity assessment methods have been used and for different dyes. In addition, model waters, with much lower complexity than industrial waters, such as the one used in this study, were used, with the exception of Pizato et al. [22], who also used a real effluent (diluted 25 (v/v)) and for which the inhibition using *Artemia salina* was 100%, even though they obtained a color removal efficiency of up to 92% (Table 3). This increase in toxicity was associated with the formation of by-products that were more toxic than the parent compound. In the present work, although the growth of onion roots in the final treated effluent was lower in relation to the control, they still grew, resulting in a decrease in toxicity compared to the influent.

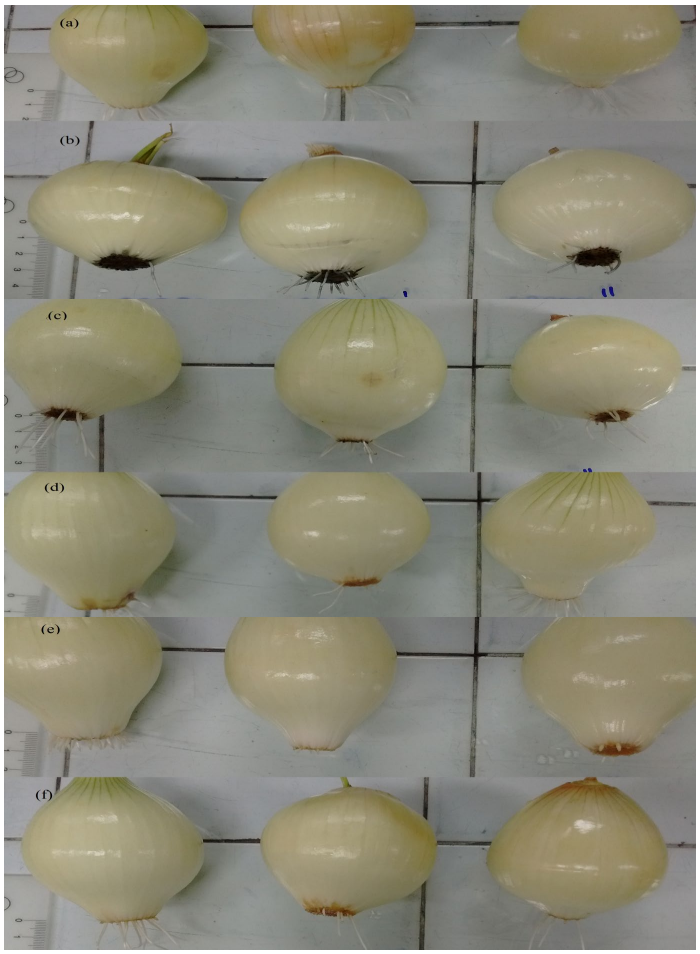

Figure 5. Onion roots growing whilst immersed in (**a**) tap water/the control; (**b**) untreated wastewater; (**c**) the influent of R1; (**d**) the effluent of R1; (**e**) the influent of R2; and (**f**) the effluent of R2 during the 8th cycle.

Table 3. Removal of dyes by fungi and toxicity assessed after treatment—comparison of this work with other published works.

Reference	Fungal Species	C_0 * (mg/L)	Dye	Dye Class	Toxicity Test	Inhibition (%)
This work	*Aspergillus niger* AN400	18 ± 6	Indigo Carmine	Indigoid	Onion bulbs (*Allium cepa*)	79.1 ± 10
Pizato et al. [22]	*Lasiodiplodia theobromae* MMPI	NM	NM	NM	*Artemia salina*	100
Rybczyńska-Tkaczyk et al. [43]	*Bjerkandera adusta CCBAS 930*	10	Alizarin Blue Black B	Anthraquinone	*Lepidium sativum* L.	39
Chen et al. [44]	*Penicillium simplicissimum*	50	Violet Metil	Triphenylmethane	*Vigna radiata*	21.06
		50	Malachite Green	Triphenylmethane	*Vigna radiata*	29.05
		50	Cotton Blue	Triphenylmethane	*Vigna radiata*	0
Sosa-Martínez et al. [45]	*Phanerochaete chrysosporium*	50	Malachite Green	Triphenylmethane	*Vibrio fischeri*	0
		50	Congo Red	Azo	*Vibrio fischeri*	18.31

* C_0: initial dye concentration; NM: not mentioned.

The formation of more toxic by-products from the degradation of the original dye has been reported in the literature. Rybczyńska-Tkaczyk et al. [43] associated the increased toxicity with the formation of phenolic compounds from the rupture of Alizarin Blue Black and Acid Blue 129 molecule bonds by *Bjerkandera adusta*, in the presence of 0.25% glucose. There was inhibition of *Lepidium sativum* growth with treated Alizarin Blue Black (39%) and Acid Blue 129 (40%) model effluents, which resulted from the formation of phenolic by-products. It is important to note that for these authors, besides those using model wastewaters, the dye concentration was lower than that of the indigo carmine in the present study, and even so, increased toxicity after treatment was obtained. Contrarily, although Chen et al. [44] obtained and effluent of lower toxicity than that of the present research (Table 3), these authors reported that the effluent presented higher levels of toxicity than the influent when the medium was prepared with Methyl Green and then subjected to the action of *Penicillium simplicissimum* in reaction time of 24 h. Thus, even with high dye removal rates (97.5% for Methyl Green and 97.1% Malachite Green), a final effluent with more toxic by-products than the dye was produced, but for Cotton Blue (96.1%), no inhibition was observed with the treated effluent via the same process. On the other hand, Malachite Green and Congo Red dyes, when submitted to the action of *Phanerochaete chrysosporium* in a synthetic medium with corn cob as a co-substrate, did not result in a final effluent with greater toxicity [45]. Malachite Green (41.84%) and Congo Red (69.70%) were not completely removed from the medium; removal percentages of 41.84% and 69.70%, respectively, were obtained, but the final effluent from Malachite Green treatment did not cause toxicity to *Vibrio fischeri*. Even for the medium containing Congo Red, the effluent toxicity level after fungal treatment was considered low (18.31%) [45]. Based on this, the molecular structure of the dye may be determinant of its greater or lesser mineralization and, consequently, detoxification [43].

3.4. Microorganisms' Viability after Treatment

At the end of the reactors' operation, the number of fungal colonies and bacteria was recorded. In R1, 9.30×10^6 CFUs/mL of fungi and 4.15×10^6 CFUs/mL of bacteria were observed, while in R2, 9.93×10^6 CFUs/mL and 4.07×10^6 CFUs/mL of fungi and bacteria were obtained, respectively. In both reactors, the participation of bacteria in the process can be justified by the use of industrial wastewater and the fact that the reactors did not operate under aseptic conditions. Fungi and bacteria have diverse metabolic capabilities, allowing them to degrade a wide range of pollutants [17,45]. Fungi, for instance, produce extracellular enzymes capable of breaking down complex organic molecules, while bacteria may specialize in the degradation of specific compounds or metabolites. By monitoring both groups, we could gain insight into which organisms were driving the degradation of the dye in the real wastewater used, for optimize treatment strategies accordingly, when applying this at the industry level. Moreover, one has to have in mind that fungi and bacteria may compete for resources or engage in symbiotic relationships within the bioremediation system [17,45]. Therefore, understanding the relative abundance of each group can provide insight into potential competitive interactions or beneficial partnerships that influence overall treatment efficiency. For instance, certain fungi may produce compounds that inhibit bacterial growth, while others may enhance bacterial activity through nutrient cycling or other mechanisms [45]. However, the fungal community was predominant, approximately double the number of bacterial colonies in both reactors. Fungi are often more resilient to harsh environmental conditions, such as extremes of pH or temperature, compared to bacteria. In environments where traditional bacterial bioremediation may be limited, fungi can play a crucial role in degrading pollutants. Monitoring both fungi and bacteria allows for a comprehensive assessment of microbial community resilience and adaptability in response to changing environmental conditions during bioremediation [46]. The existence of biofilm consisting of both bacteria and fungi did not demonstrate a detrimental impact on treatment efficiency, particularly given the predominant growth of fungi in the environment.

4. Conclusions

The sequencing of batch reactions with immobilized *Aspergillus niger* AN 400 biomass showed good performance for color removal of industrial wastewater containing indigo carmine, with an overall efficiency of 90%. However, the degradation of the dye was partial and resulted in the formation of by-products during the breakdown of the dye molecule. The high sucrose:dye ratio (250:1) in the influent of R2 caused inhibition of the consumption of indigo carmine by the excess sucrose, which is the easiest substrate to be metabolized. The adsorption tests on the mycelium and on the support material showed that indigo carmine removal occurred mainly via biological action.

In addition to great color removal, a reduction in toxicity was obtained in the treated effluent as compared to the influent, thus demonstrating the potential of the proposed system for textile wastewater treatment. It is also important to note that in natura wastewater such as the one used in the present work is much more complex than model waters usually used, because it has dye and other compounds of a toxic nature, but the wastewater after treatment with *Aspergillus niger* showed lower toxicity, which was advantageous since it is an industrial effluent.

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