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Textile dyeing wastewater treatment by *Penicillium chrysogenum*: design of a sustainable process

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ABSTRACT

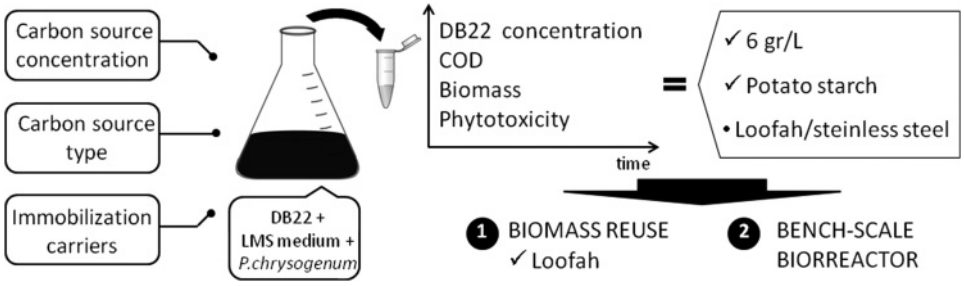
In this work a parametric study and a bench bioreactor degradation test of Direct Black 22 (DB22) by *Penicillium chrysogenum* was performed as a first approach to an industrial application, framed within a policy of sustainable processes development. Three ancillary carbon sources and their optimum initial concentrations were studied. These were: glucose, potato starch and potato industry wastewater. Their optimum initial concentration was 6 g/L. The use of potato starch as co-substrate showed the highest decolorization rate and COD removal. Degradation of DB22 using different immobilization supports (stainless steel sponge, loofah sponge and polyethylene strips) was studied and the results showed that the time needed for the treatment decreased from 6 to 4 d. Phytotoxicity was evaluated in the final products of the immobilized cells assays, using *Lactuca sativa* seeds. For all treatments phytoxicity was reduced with respect to the untreated wastewater, except for the assays using polyethylene strips. Finally, the reuse of the biomass attached to different carriers and the performance of the treatment of DB22 in.a 1 L bench scale bioreactor were tested. *P. chrysogenum* decolorized at least four sucesives reuses. The reactor assays showed a better performance of the treatment.

Key words: bioreactor, fungus, sustainable bioprocess, textile wastewater

HIGHLIGHTS

- *Penicillium chrysogenum* shows good potential to decolorize the DB22 dye.
- A parametric study of the biodegradation of an azo dye was performed to design a sustainable bioprocess.
- Phytotoxicity of the dye was reduced after the treatment.
- Biomass reuse and a bench bioreactor assay were successfully performed.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Textile wastewater discharges usually have adverse impacts on the environment due to its organic load, in terms of : Total Organic Carbon (TOC), Biological Oxygen Demand (BOD) (100–3,000 mgO_2 L^{-1}), Chemical Oxygen Demand (COD)

(10–2,250 $mgO_2\ L^{-1}$) dye concentration (10–250 mg L^{-1}), a wide range of pH (5–12), suspended solids, salinity and the presence of recalcitrant organic compounds (Ceretta *et al.* 2021). Color interferes with penetration of sunlight into waters, inhibits photosynthesis and the growth of aquatic biota, and interferes with gas solubility in water bodies (Banat *et al.* 1996). Dyes have been extensively used in textile, food, plastic, printing, leather, cosmetics and pharmaceutical industries, with an annual worldwide production of synthetic dyes of 7.10^5 tons (Katheresan *et al.* 2018), of which almost 30,000–150,000 tons are annually discharged into aquatic environments (Anjaneya *et al.* 2013). Specially, the textile industry is a substantial consumer of water and produces high volumes of contaminated water; the azo dyes are among the most important contaminants (Solís *et al.* 2012). Azo dyes are aromatic compounds with one or more azo groups (–N = N–) and represent the most important and largest class of synthetic dyes used in commercial applications (Elisangela *et al.* 2009). Many dyes are suspected to be toxic and carcinogenic, and some are synthesized using known carcinogens such as benzidine (Kariminiaae-Hamedaani *et al.* 2007).

Different methods for removing dyes have been proposed including physical, chemical, advanced oxidation and electrochemical processes. However, disadvantages such as high operating cost, incomplete azo dye degradation, possible secondary pollution, and high energy consumption, hinder the practical applications of these methods in full-scale wastewater treatment facilities (Cui *et al.* 2021). Comparatively, biological degradation methods have proven to be more cost-efficient, environmentally friendly and reliable than physicochemical methods (He *et al.* 2018), since they stimulate the biotransformation of toxic contaminants to sustainable and nontoxic end products such as carbon dioxide, minerals and inorganic substances, which require less water and energy compared to physical and chemical methods (Abd Ellatif *et al.* 2020). Extensive research has been done to examine the biodegradation of azo-dyes by either bacteria or fungi, and the results have shown that bacterial degradation of azo dyes can result in aromatic amines, compounds that can inhibit the activity of microorganisms, and may produce aryl amines that are more toxic, carcinogenic and mutagenic than the parent compound (Shabbir *et al.* 2017). On the other hand, a textile wastewater treatment with fungi avoids the problems of sludge disposal and secondary pollution (He *et al.* 2018). Furthermore, fungal cells can be easily immobilized, which allows the design of semi continuous wastewater treatment for an industrial application. Immobilized fungal wastewater treatment has several advantages, such as the reuse of the biomass and an easy liquid–solid separation (Rodríguez Couto 2009). Also, immobilized cultures tend to improve the effectiveness in wastewater treatment (Przystaś *et al.* 2017). Many types of carriers have been studied to immobilize fungi: the use of a natural support instead of an artificial inert one has the advantage that the material mimics the natural habitat of the fungi and it would be an environmentally friendly way for waste management (Rodríguez Couto *et al.* 2004). Additionally, some synthetic material has also been described as very suitable for fungi immobilization, as one of the advantages found on some of these materials is that dyes do not adsorb onto it (Rodríguez Couto *et al.* 2004).

One of the most important factors involved in wastewater treatment with fungi is the ancillary carbon source type and concentration (Przystaś *et al.* 2017), which have a great influence not only on decolorization effectiveness but also in the process cost and their viability in full-scale application (Sumathi & Manju 2000).

Penicillium chrysogenum, an ascomycota fungus, shows a good potential for decolorization applications, decolorizing and degrading not only the three different azo dyes independently but also the mixture of them (Durruty *et al.* 2015). With the aim of designing a sustainable process, some variables involved in the textile wastewater treatment with fungi were studied. Alternative carbon sources and their initial concentration were evaluated during the Direct Black 22 (DB22) degradation by *P. chrysogenum*. Several biomass carriers and phytotoxicity of the degradation products were tested. Finally, as a first approach to an industrial application, the biomass reuse capability and the degradation of DB22 in.a bench-scale reactor were studied.

2. MATERIAL AND METHODS

2.1. Chemicals

The textile azo-dye Direct Black 22 (DB22) was used in this work and it was kindly provided by Gama SA, a local textile industry. Potato starch was kindly provided by McCain SA (Balcarce, Argentina). All the other chemicals were analytical grade and obtained from Biopack unless stated otherwise.

2.2. Microorganism

P. chrysogenum ERK 1 (GenBank, accession numbers HQ336382 and HQ336383) was maintained in potato dextrose agar (PDA, Gibco) at room temperature for 14 days. This fungus was isolated from commercial crop soils from Balcarce, Buenos Aires province, Argentina, as described by Wolski *et al.* (2012).

2.3. Inoculum

A conidia solution was used as inoculum for decolorization assays. To obtain this solution 6 PDA agar discs of 4 mm diameter containing the fungal mycelium were inoculated into 150 mL of liquid mineral salt medium (LMS) supplemented with 9 g/L of glucose and 0.2 g/L of DB22. LMS was composed by $MgSO_4 \cdot 7H_2O$ 0.1 g/L, K_2HPO_4 1 g/L, NH_4NO_3 1 g/L, KCl 0.1 g/L and 25 μL of trace elements solution (in $mg \cdot L^{-1}$: $MnSO_4$ 15.4, $FeCl_3$ 40, $ZnSO_4 \cdot 7H_2O$ 6.3, $CuSO_4 \cdot 5H_2O$ 2.5, $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$ 0.5) and deionized water. The initial pH was 7.0 and the media was sterilized. The cultures were incubated at room temperature in the dark, with shaking at 120 rpm for 5 days. Then the solution was filtered through a muslin.

2.4. Decolorization assays

For decolorization assays, 250 mL Erlenmeyer flasks were used with a final working volume of 150 mL, of which 135 mL were composed by LMS medium with a DB22 concentration fixed to 0.2 g/L and 15 mL of the conidia solution described above. The concentration of the DB22 azo dye was chosen based on the concentration found in the real textile wastewater that has been previously reported by our research group. The characterization of the wastewater showed a DB22 concentration of 205.15 ± 2.21 mg/L (Ceretta *et al.* 2017). Non-inoculated flasks were used as controls.

All the assays were incubated at room temperature in the dark, with orbital shaking at 120 rpm for 14 days. Different settings were performed to evaluate several parameters.

2.4.1. Initial carbon source concentration and type

The capability to simultaneous degrade and decolorize different azo-dyes by *P. chrysogenum* supplemented with 9 g/L of glucose has been previously reported (Durruty *et al.* 2015). To study the effect of the initial carbon source concentration in the process and find the optimum value for this variable, four initial glucose concentrations were tested: 2, 4, 6 and 8 g/L.

With the aim of studying the influence of alternative, and cheaper, ancillary carbon sources, glucose, potato starch and potato wastewater were tested. Potato starch is a by-product of the potato industry and potato wastewater is a residue from the same industry. Glucose and starch assays were performed with the optimum value for the initial carbon source concentration. Potato wastewater was prepared by liquefying 1 kg of potato per liter of deionized water for 2 minutes. Solids were removed and potato starch was added until the initial COD value was reached. Each flask was inoculated and incubated as described in section 2.4.

2.4.2. Immobilization carriers

Three different fungal biomass carriers were studied: stainless steel sponge (3 g per flask), because it is inert and reusable; polyethylene strips (2 g per flask), for being an industrial waste (discarding of a bag factory); and loofah (1 g per flask), as a model material of natural supports. Flasks without carriers were used as control. Each flask was inoculated and incubated as described in section 2.4.

2.4.3. Biomass reuse

Biomass reuse was studied using stainless steel and loofah as carriers. After decolorization was reached the treated solution was discharged and 150 mL of simulated textile wastewater with the optimum initial carbon source concentration were added to refill each flask. Three extra loads were used to test the biomass attached reuse. Each reuse assay was inoculated and incubated as described in section 2.4.

2.4.4. Bench-scale bioreactor

Decolorization of DB22 was studied in a stirred tank bioreactor of 1.7 L capacity with 1 L of working volume, batch operated with aeration and stirring control. The stirring speed was set at 150 rpm; air was supplied at 0.8 VVM and temperature fixed at 20 °C (room temperature). The initial pH was 7.0 and the run was carried out without pH control. The selected immobilization carrier was used. Microorganisms were inoculated from 100 mL of the conidia solution, DB22 concentration was fixed to 0.2 g/L and the optimum initial carbon source concentration was added to the LMS solution.

2.5. Analytical methods

Sampling was done at regular intervals to determine COD, color and pH; 1.5 mL were collected and clarified (centrifuged at 7,500 g for 5 min) to separate cellular or other suspended debris. COD close reflux method (5220D) was used (APHA, AWWA, WEF 1997). The measured soluble COD represented growth substrate since the contribution of the dye to the

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COD was found negligible. A solution with 0.2 g/L of DB22 was tested for COD with the same protocol. Color was measured as absorbance (UV VIS spectrophotometer Pg instruments T80) at a wavelength of 480 nm, which was the wavelength of the maximum absorbance in the visible region for DB22. Then the concentration of DB22 (mg/L) was calculated using a calibration curve, in agreement with Lambert–Beer's law. pH was measured in a HANNA HI 2,211 pH Meter. Fungal growth was determined by measuring the mycelium dry weight. The fungal mycelium was filtered and dried at 100 °C until constant weight.

2.6. Phytotoxicity assays

Phytotoxicity was evaluated for the final products of the immobilized fungal assays, Percentage of germination and root elongation were measured in *Lactuca sativa* seeds according to the Environmental Protection Agency's methodology (United States Environmental Protection Agency (EPA) 1996). First, the Lethal Concentration 50 (LC_{50}) of untreated wastewater was calculated using TRAP software, version 1.30a (EPA 1999). Then, from the endpoint of each treatment aliquots were taken and diluted until the corresponding LC_{50} was obtained. Ten seeds plus 3 mL of the corresponding solution were placed in Petri dishes. Distilled water and untreated simulated wastewater were used as negative and positive controls, respectively. Then, the seeds were incubated in the dark at 25 °C until the radicle length was 1.5 cm in negative control dishes. Finally, germination percentage and root elongation (measured with Image J software) were calculated. Three replicates were done for each treatment.

2.7. Statical analysis

The statistical analysis and data management were performed by using Origing 8.0. Factorial ANOVA was applied for statistical analysis of the data. The means were compared by using LSD test.

3. RESULTS AND DISCUSSION

3.1. Initial carbon source concentration

With the aim of optimizing the amount of additional carbon source needed for the wastewater treatment, different initial glucose concentrations were studied. Figure 1(a) shows a good decolorization for all the concentrations tested. This ability of the fungus to decolorize DB22 agrees with previously reported data (Durruty *et al.* 2015). Figure 1 also shows how decolorization took place (Figure 1(a)) while the fungus grew (Figure 1(c)) and the COD decreased (Figure 1(b)) as reported by Durruty *et al.* (2015). It is known that fungus has a requirement for a primary substrate such as glucose to achieve color reduction (Sumathi & Manju 2000). When the growth substrate was depleted, the fungus stopped growing and some dye desorption was observed, suggesting that microbial decolorization was carried out by biosorption and degradation simultaneously, as previously reported (Solís *et al.* 2012). Results show how dye desorption is faster when the final biomass growth is lower, because a higher biomass growth avoids this phenomenon (Sen *et al.* 2016). Although the analytical method to measure biomass makes it difficult to see the growth trend, the Figure 1(c) clearly shows a growth delay and a lower decolorization rate at high initial glucose concentrations. Solís *et al.* (2012) reported that high carbon concentrations lead to low decolorization because the microorganisms use the carbon source preferentially to the dye. However, Wuhrmann *et al.* (1980) have postulated that in the presence of certain co-substrates the degradation of azo compounds produces toxic metabolites that inhibit growth. These phenomena were evident at high initial carbon source concentration in this work. Thus, an initial glucose concentration of 6 g/L, which presented the highest decolorization rate and a longer retention time of DB22, was selected for following tests.

3.2. Carbon source type

The use of additional carbon sources may involve a significant cost in wastewater treatment. Therefore, the replacement of glucose by cheaper carbon sources, waste or byproducts of local industries was studied within in a policy of sustainable process development. Table 1 shows the ratio between each parameter and the same parameter to glucose. It can be observed that tests with potato starch as a carbon source resulted in a significant improvement of the treatment with respect to glucose. A decolorization efficiency 1.1 times higher than glucose assay was achieved. Furthermore, assays with potato starch as growth substrate showed a maximum decolorization rate 1.73 times faster than glucose assays. The maximum growth rate was 1.5 times faster than glucose assay. Durruty *et al.* (2015) postulate that decolorization is associated to growth, so a higher growth rate leads to a higher decolorization rate It has been reported that many microorganisms that can use

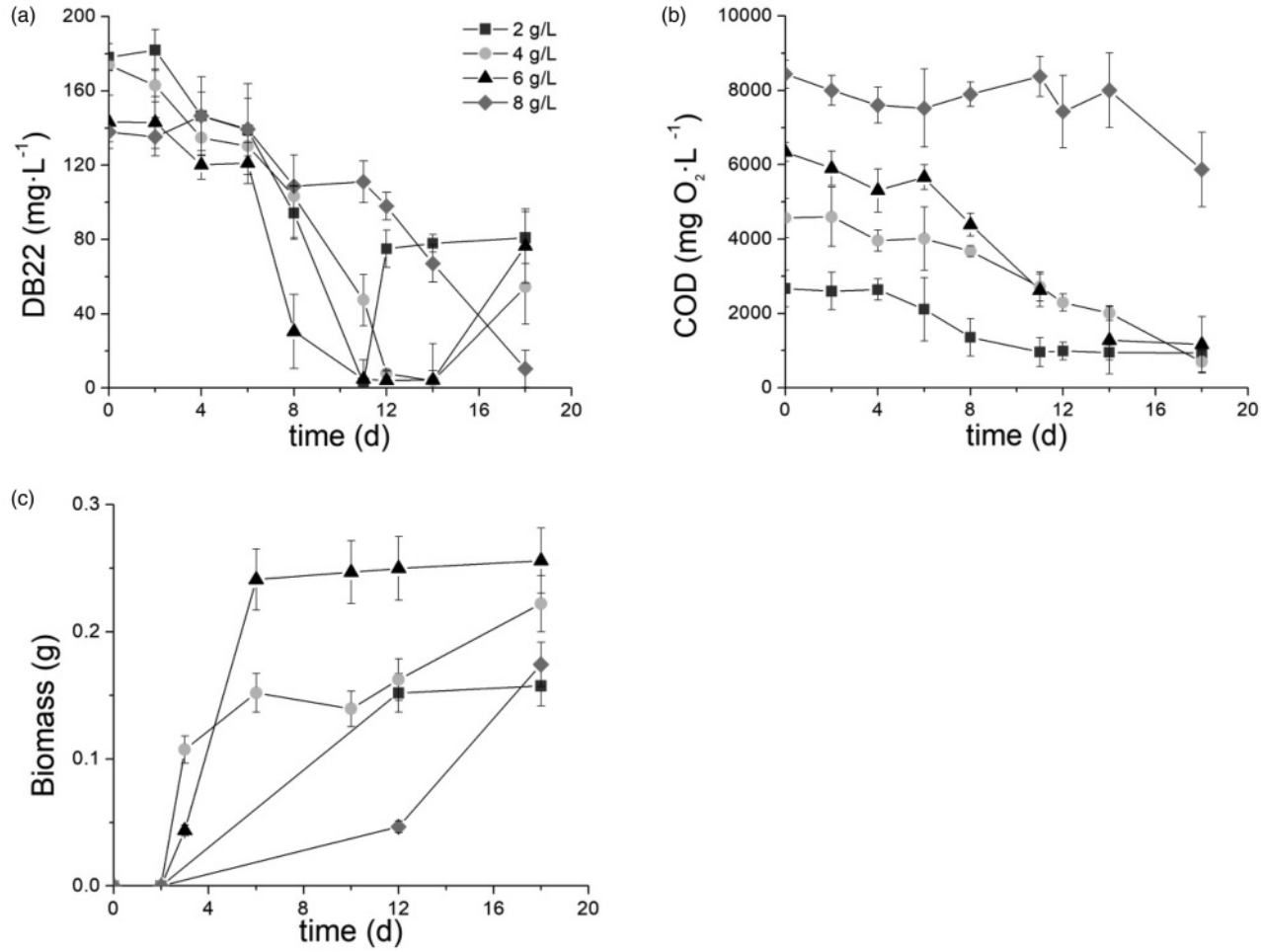

Figure 1 | DB22 decolorization (a), COD depletion (b) and Biomass growth (c) by *P. chrysogenum* for different initial glucose concentrations.

Table 1 | Carbon source type assays for DB22 treatment by *P. chrysogenum*

	Glucose	Potato starch	Potato wastewater
Decolorization efficiency	1.0	1.10	0.38
Maximum decolorization rate	1.0	1.73	0.80
Maximum biomass growth rate	1.0	1.50	0.50

Parameters ratio regarding to glucose for the alternative carbon sources studied.

starch as a growth substrate improved the dye removal (Ruiz-Arias *et al.* 2010). On the other hand, the use of potato wastewater as growth substrate did not show a good performance. The degree of decolorization achieved was 0.38 with respect to glucose assays. Moreover, both the degradation rate and the growth rate were slower, proving that the carbon source type can affect not only the process cost, but also the effectiveness (Katheresan *et al.* 2018). This is due to the role as electron donor of the carbon source, which are necessary for the breakage of the azo bond (Yang *et al.* 2009). Differences on the molecular weight and chemical structure may significantly affect the utilization mechanism of these types of carbon sources. Thus, potato starch was selected as growth substrate for DB22 treatment.

3.3. Immobilization carriers

Figure 2(a) shows that the use of immobilized cells improved the decolorization rate and the retention time of DB22, compared to free cells tests. Total decolorization was achieved in 4 days, whereas the tests with free cells needed 6 days. This behavior was previously reported by Krastanov *et al.* (2013), who suggested that using immobilization carriers improved the fungus growth speed. Moreover, immobilized cells have less dense fiberpacking in comparison with free fungal biomass, so microorganisms have a larger surface area available for dye adsorption. This reduced the mass transfer limitations, which in turn increased access to pollutant degradation (Przystaś *et al.* 2017). Furthermore, Thongchul & Yang (2003) suggested that the rheological features of the medium turned to be more favorable for oxygen supply and mass transfer using immobilization carriers improving the process performance. No significant differences were observed in the use of different carriers tested over decolorization performance (ANOVA TEST $p < 0.05$). Adsorption of dyes was also observed in every carrier type. Results showed an adsorption performance on polyethylene strips of $15 \pm 1.3\%$, whereas the adsorption on stainless steel and loofah was observed negligible. Then, the improvement in the decolorization process using immobilized cells cannot be attributed solely to the carriers' adsorption.

Azo dye degradation can lead to the formation of aromatic amines, compounds reported as carcinogenic and muthagenic (Kariminiaae-Hamedaani *et al.* 2007), so toxicity evaluation is important to evaluate the degradation products after a wastewater treatment. In this study, phytotoxicity test showed that both the percentage of germination and the root elongation were greater for the treated wastewater than without treatment, except in assays using polyethylene strips as carrier (Figure 3). In this case, percentage of germination and the root elongation were inhibited significantly. In order to locate the source of this phenomenon, a control test was carried out, where 150 mL of distilled water in an Erlenmeyer flask with the same amount of polyethylene carrier were sterilized. Results showed that the germination percentage and root elongation where null. Therefore, it can be deduced that the particles that come off the polyethylene support are highly phytotoxic. This demonstrated that for any bioremediation technology it is very important to assess the toxicity of the pollutants and metabolites formed after dye degradation in order to study the feasibility of the method (Jadhav *et al.* 2011). This result suggested that the decolorization treatment by *P. chrysogenum* was accompanied by reduction in phytotoxicity only in the cases that biomass was supported in loofah or stainless steel. Therefore, these two alternatives turned out to be the best treatment options.

3.4. Biomass reuse

The performance of fungi biomass reuse over decolorization of DB22 was tested. Results showed that at least four loads of the liquid media with azo dye were decolorizated by the supported fungus (Figure 4(a)). From the first reuse the decolorization speed increased significantly, decreasing the time needed for the treatment from 4 to 2 days. Figure 4(c) shows how the fungus grows between cycles. Solís *et al.* (2012) suggest that microbial decolorization was carried out by biosorption and degradation simultaneously and Durruty *et al.* (2015) showed that the fungus degrades the dye while growing. Although in this work it is

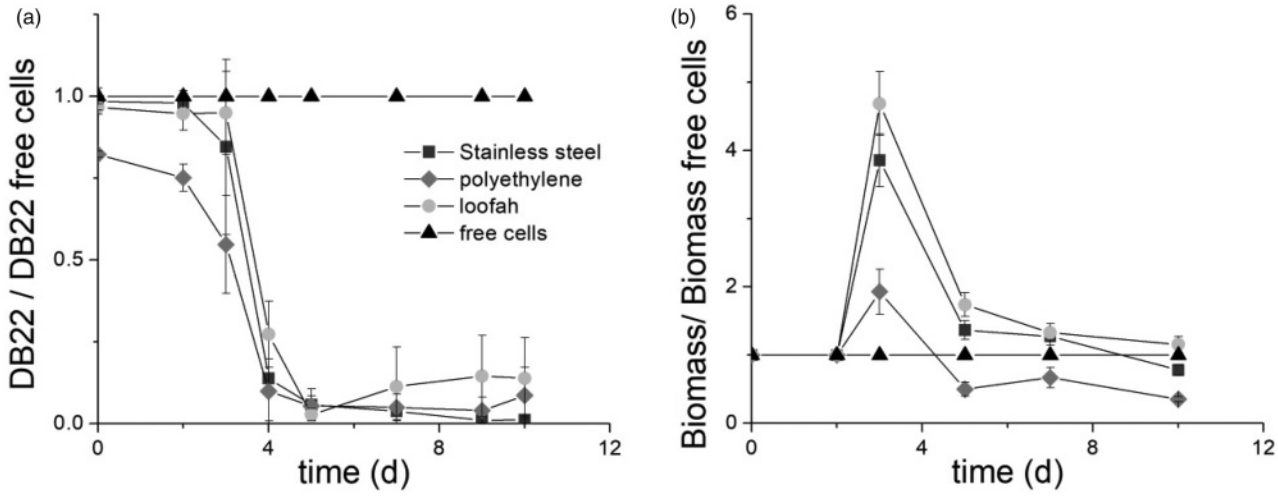

Figure 2 | DB22 decolorization (a) and Biomass growth (b) by *P. chrysogenum* for different immobilization carriers, with respect to free cells tests.

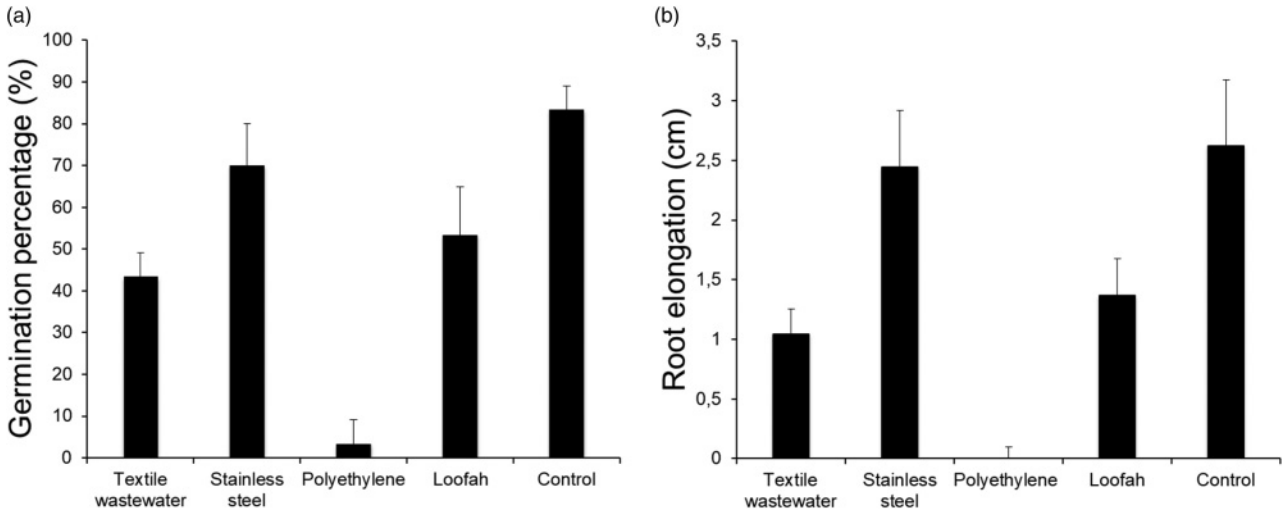

Figure 3 | Germination percentage (a) and root elongation (b) for treated wastewater using different biomass carriers, untreated simulated wastewater (Textile wastewater) and distilled water (Control).

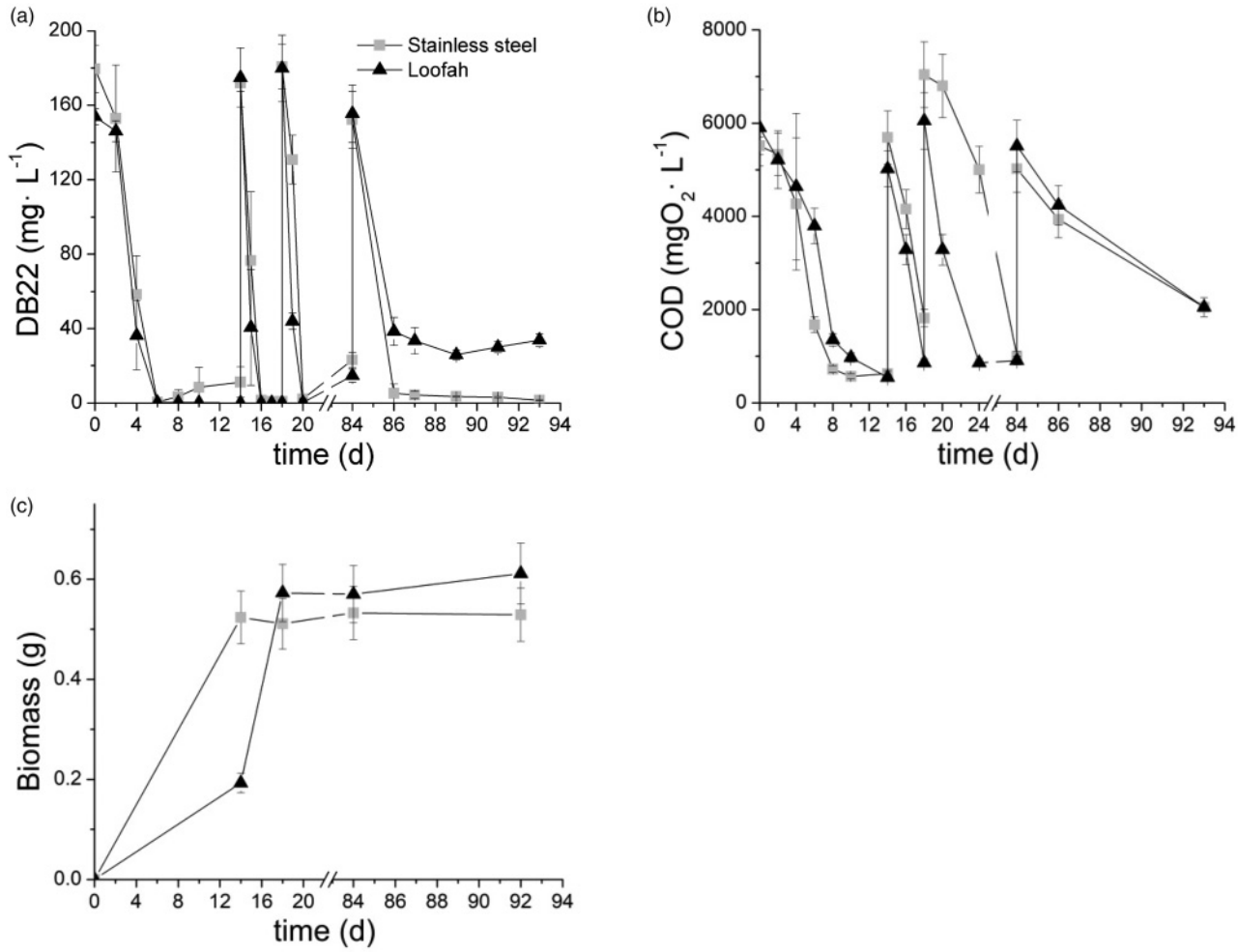

Figure 4 | DB22 decolorization (a), COD depletion (b) and Biomass growth (c) by *P. chrysogenum* during biomass reuse for stainless steel and loofah carriers.

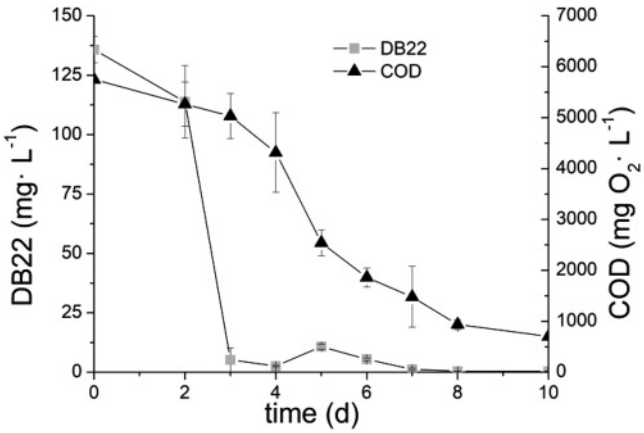

Figure 5 | DB22 decolorization and COD depletion by *P. chrysogenum* in a bench-scale bioreactor.

shown that the dye is removed during the reuse, the ratio of sorption/degradation cannot be quantified due to the nature of the system. The decrease in the time needed for the treatment is due to the higher amount of the starting biomass, grown in the previous cycle (Figure 4(c)). In addition, good decolorization efficiency and COD removal was observed in the assays using stainless steel sponge and loofah, reaching DB22 removal efficiencies of 99.7 $\pm$ 0.01% and 99.9 $\pm$ 0.5%, respectively.

The beginning of the fourth cycle was delayed 64 days after the end of the third cycle with the aim of evaluating the stability and the performance of the attached fungus after a dead time. Figure 4(a) showed that high decolorization efficiency and speed were achieved, which proved the capability to restart after a period of time. Regarding the affinity for the support, no biomass loss was recorded between the third and fourth cycle for both carriers (Figure 4(c)). This proved that filamentous fungi have a natural tendency to adhere to surfaces and become immobilized, as reported by Rodríguez Couto (2009).

Results showed no difference in the performance between the two different supports tested. In addition, due to its robustness and regenerating ability, this strain was considered reusable, which is essential for its long-term applicability to bioremediation of dye-polluted wastewater (Rodríguez Couto 2009). The use of a natural support instead of an artificial, inert one for the fungi is advantageous because immobilization material mimics the natural habitat of the fungi (Rodríguez Couto 2009). Furthermore the loofah was selected as the biomass carrier for this process because (1) the use of natural supports prioritize the design of a sustainable process, and (2) loofah is used as a model of other natural supports like residues of the agricultural industry.

3.5. Bench-scale bioreactor

Testing in laboratory-scale reactor systems is essential to scale the model from the laboratory to the pilot plant (Zhang & He 2015). Figure 5 shows the DB22 decolorization and the COD depletion in a 1 L bioreactor by *P. chrysogenum*. High levels of decolorization were achieved in three days, with a DB22 concentration below 5 mg/L. These results demonstrate that the reactor assays did not show the same behavior as the laboratory scale assays. An ideal scale-up experiment establishes an environment in which the production organism displays at least the same productivity and physiology as in the laboratory process (Tajsoleiman *et al.* 2019). In this case the scaling-up improved the process performance. A decisive change when assays passed from the orbital-shaker Erlenmeyer flask to the bioreactor, was the aeration rate, which is one of the process operating conditions that affects the dissolved oxygen available for the aerobic process (Crater & Lievense 2018). Therefore, the degradation performance observed in the bench-scale bioreactor encourages the industrial-scale application.

4. CONCLUSIONS

In this work a parametric study of the decolorization of DB22 by *P. chrysogenum* on a laboratory scale allowed us to design a sustainable process for textile wastewater treatment. These fungal cells demonstrated to be easily immobilized, which allowed the biomass reuse. Finally, the ability of the strain to be reused in successive treatments and the reproducibility of the tests in a laboratory-scale reactor are promising for the applicability of the process a larger scale.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICTS OF INTEREST

The authors declare there is no conflict.

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