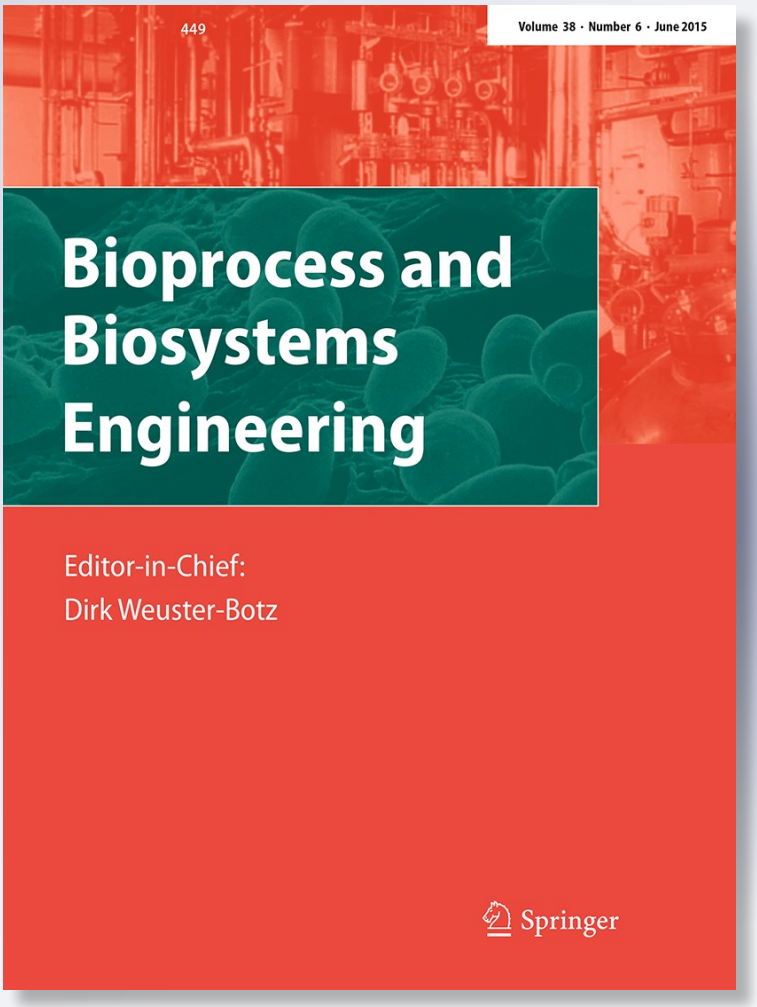
A kinetic study of textile dyeing wastewater degradation by Penicillium chrysogenum

Ignacio Durruty, Diana Fasce, Jorge Froilán González & Erika Alejandra Wolski

Bioprocess and Biosystems Engineering

ISSN 1615-7591
Volume 38
Number 6

Bioprocess Biosyst Eng (2015)
38:1019-1031
DOI 10.1007/s00449-014-1344-9



Springer

Bioprocess Biosyst Eng (2015) 38:1019–1031
DOI 10.1007/s00449-014-1344-9

ORIGINAL PAPER

A kinetic study of textile dyeing wastewater degradation by *Penicillium chrysogenum*

Ignacio Durruty · Diana Fasce · Jorge Froilán González · Erika Alejandra Wolski

Received: 21 August 2014 / Accepted: 18 December 2014 / Published online: 3 January 2015

Abstract The potential of *Penicillium chrysogenum* to decolorize azo dyes and a real industrial textile wastewater was studied. *P. chrysogenum* was able to decolorize and degrade three azo dyes (200 mg L^{-1}), either independently or in a mixture of them, using glucose as a carbon source. A kinetic model for degradation was developed and it allowed predicting the degradation kinetics of the mixture of the three azo dyes. In addition, *P. chrysogenum* was able to decolorize real industrial wastewater. The kinetic model proposed was also able to predict the decolorization of the real wastewater. The calibration of the proposed model makes it a useful tool for future wastewater facilities' design and for practical applications.

Keywords Fungal biodegradation · Azo dyes · Wastewater · Degradation kinetics · Phytotoxicity · FTIR

Introduction

The environmental problems created by the textile industry have received increased attention for several decades because this industry is one of the largest generators of contaminated effluents [1], due to the discharge of untreated or poorly treated effluents. Wastewater resulting from these processes has adverse impacts in terms of total organic carbon (TOC), biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, salinity, color, a wide range of pH (5–12) and the recalcitrance of organic compounds, such as azo dyes [2, 3].

Azo dyes are aromatic compounds with one or more azo groups (–N=N–) and are the most important and largest class of synthetic dyes used in commercial applications, mainly in the textile industry [4, 5]. During the dyeing process, approximately 10–15 % of the dyes used are released into the wastewater in the form of colored wastewater [4, 6]. This can lead to acute effects on exposed organisms due to the toxicity of the dyes, for example: the abnormal coloration of phytoplankton and the reduction in photosynthesis because of the absorbance of the light that enters the receiving water bodies [7, 8]. The release of such dyes also alters the pH, increases the BOD and COD, and imparts intense colorations to the receiving water bodies, giving rise to public concern about water quality. The presence of unnatural colors is esthetically unpleasant and tends to be associated with contamination.

Several physicochemical and biological methods were adapted for the reduction of azo dyes to achieve decolorization. In recent years, new biological processes for dye degradation and wastewater reutilization have been developed including aerobic and anaerobic bacteria and fungi. Among fungi, the most widely researched are the ligninolytic fungi or white rot basidiomycete fungi. However, white rot fungi had two majors limiting factors to be used in the industry namely: lignocellulosic substrates requirement and many species have slow growth kinetics [9].

Many non-basidiomycetes fungi are also able to degrade aromatic compounds and other complex structures [10, 11]. Nevertheless, studies about degradation of complex aromatic compounds and particularly decolorization of azo dyes using non-basidiomycetes fungi are scarce [9, 12–15].

I. Durruty · J. F. González · E. A. Wolski (✉)
Grupo de Ingeniería Bioquímica, Fac. Ingeniería, Universidad Nacional de Mar del Plata, J.B. Justo 4302, 7600 Mar Del Plata, Buenos Aires, Argentina
e-mail: ewolski@mdp.edu.ar

D. Fasce
INTEMA, CONICET, UNMDP, J.B. Justo 4302, 7600 Mar Del Plata, Buenos Aires, Argentina

For example, an *Aspergillus flavus* isolate was able to decolorize the azo dye Reactive red 198 to an extent of 85 % approximately [6] and *Penicillium* sp. QQ decolorized the azo dye Reactive Brillant Red X-3B by bioadsorption [14], as well as *Penicillium* spp. (ATCC 74414) decolorized two polymeric non-azo dyes [12]; however, it is not clear whether if decolorization occurs by bioadsorption, biodegradation or both mechanisms. Recently, Saroj et al. [9] reported the biodegradation of three azo dyes independently by an isolate of *Penicillium oxalicum* SAR-3, which showed a significant level of degradation at 100 and 300 mg L^{-1} of the azo dyes. All these studies tested the decolorization or degradation of the azo dyes independently and without carrying out a kinetic study of the process, which is also an important aspect to consider for wastewater treatment, since the development of a model allows simulating and predicting the behavior of the other azo compounds and its mixtures for industrial applications. In addition, the study of the degradation of different azo dyes and its mixtures is very important, since dyeing is usually done in batch processes, using different dyes, and the discharged wastewaters contain a mixture of them.

In this work, we report on the decolorization by biodegradation process of three azo dyes, the mixture of them and the real wastewater by a *Penicillium chrysogenum* isolate. Additional information on the products of the biodegradation is presented via FTIR analysis and phytotoxicity assays. A kinetic model was developed based on experimental observations and fitted to the results of experiments with individual azo dyes. The development of kinetic models that represent the microbial behavior is of paramount importance since they are valuable for industrial biotechnology. Mathematical models should be able to assist in the rational design of microbial processes in which they are utilized [16]. Finally, the kinetic model was evaluated on the degradation of a real wastewater.

Materials and methods

Azo dyes and wastewater

The azo dyes Direct Yellow 86, Direct Black 22 and Direct Blue 200 were selected because they are among the dyes mostly used in local textile industry. Real wastewater was collected from a local industry (Gama S. A., Mar del Plata, Argentina). On the sampling day, the factory was using Direct Black 22 on dying process step. Therefore, the wastewater contained Direct Black 22 and the pH was about 11–12. Both wastewater and azo dyes were kindly provided by Gabriela Fioramonti, manager of Gama S. A.

Microorganism

A *Penicillium chrysogenum* ERK 1 isolate (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 [11].

Decolorization

For decolorization assays, the fungus was inoculated directly from the PDA plate into 150 mL of liquid mineral salt (LMS) medium, which was previously sterilized. LMS contained: deionized water 1,000 mL, glucose 9 g, $MgSO_4{\cdot}7H_2O$ 0.1 g, K_2HPO_4 1 g, NH_4NO_3 1 g, KCl 0.1 g and 25 μL of trace elements solution (in mg 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). For the azo dye assays, 200 mg L^{-1} of the dyes mentioned above or a mixture of them in equimolar quantities was added and pH was adjusted to 6.0. For wastewater assays, the wastewater was mixed with LMS in a 10 % (v/v) and 20 % (v/v), ever with a final volume of 150 mL. Each flask was inoculated with six PDA agar disks of 4 mm diameter containing the fungal mycelium. The cultures were incubated at room temperature, with shaking at 120 rpm and in the dark. Non-inoculated flasks and inoculated flasks without the dyes were used as controls.

Every day the mycelium from each sample flask was filtered and azo dyes' content of the liquid medium was measured after removing the mycelia. In addition, chemical oxygen demand (COD) of the supernatant and biomass were measured [17].

In additional experiments, PDA plates with and without dyes (controls) were prepared and inoculated with agar disks (4 mm) containing the fungal mycelium. Plates were incubated at 25 °C in the dark. The extent of the mycelia was measured daily in two perpendicular directions to evaluate growth inhibition in the presence of the different dyes tested. After 15 days, the mycelia growth speed index (MGSI) was calculated [18, 19].

All experiments were carried out in triplicate, and the results show the mean value of three independent experiments.

To measure decolorization, sampling was done every other day from the decolorizing media. One milliliter of each flask was collected and clarified (centrifuged at 7500*g* for 4 min) to prevent absorbance interference from cellular or other suspended debris, and was monitored spectrophotometrically. The wavelengths for Direct Yellow 86 (380 nm), Direct Black 22 (480 nm), Direct Blue 200 (550 nm) and the mixture of them (585 nm) were

determined experimentally as the wavelength with maximal absorbance at the visible region. The concentration of azo dyes (mg L^{-1}) was calculated using a calibration curves, in agreement with Lambert–Beer's law.

The decolorization efficiency of different isolates was expressed as described by Jasińska et al. [15]:

$$\text{Decolorization } (\%) = 100\frac{(A_0 - A)}{A_0} \quad (1)$$

where A_0 is the initial absorbance and A is the absorbance of medium after decolorization at the λ max (nm) of each dye.

Kinetic model

Since no inhibition by azo dyes was observed on the experiments, the specific growth rate (μ) can be fitted to a monod kinetic model and can be considered to be the same in every assay:

$$\frac{dX}{dt} = \mu \cdot X = \frac{\mu_{max} \cdot G}{K_m + G} \cdot X \quad (2)$$

where X is the dry weight of fungi per unit of liquid volume and G is the concentration of growth substrate (glucose). The kinetics parameters μ_{max} and K_m are the maximum specific growth rate and the Monod constant, respectively. In a first approach, the parameters μ_{max} and X_0 were obtained by linear fitting at early times (until day 7) where the specific growth rate could be considered constant and equal to μ_{max}. At these conditions, Eq. 2 can be integrated and linearized as: $\ln(X) = \ln(X_0) + \mu_{max}\, t$. This equation was fitted between day 0 and day 7, because it resulted linear until this day ($R^2 = 0.9192$). The K_m parameter was estimated using data from the later stages of the runs, where the substrate concentration is very low and the specific growth rate approaches $\mu_{max}\ G/K_m$.

The growth substrate is considered as measured COD since the contribution of the dyes to the total COD was find negligible. Solutions with 200 mgL^{-1} of each dye were tested for COD with the same protocol and the measurements fall within the error of the method.

Also, the degradation of growth substrate (G) is related to the biomass growth by the growth substrate to biomass yield coefficient $Y_{X/G}$:

$$\frac{dG}{dt} = -\frac{1}{Y_{X/G}}\frac{\mu_{max} \cdot G}{K_m + G} \cdot X \quad (3)$$

where the growth substrate to biomass yield coefficient ($Y_{X/G}$) was obtained by linear fitting from X vs G plot, using the data from all the assays together.

Due to the fact that all data are not used to fit all parameters and the use of "early" and "late" concepts is subjective, a further step of model optimization was performed using a general non-linear regression framework. The values from the first approach were used as initial condition to obtain optimized values of μ_{max} and K_m.

The degradation/decolorization of azo dyes must also be evaluated and modeled. Preliminary results have shown that decolorization did not proceed in the absence of growth substrate. Furthermore, the decolorization rate is associated with biomass growth.

$$\frac{dS_i}{dt} = (-r_i) \propto (r_X) \quad (4)$$

where S is the concentration of azo dye and i represents every azo dye. Since the dyes used are a non-growth substrate, the definition of a yield coefficient is not appropriate. The decolorization ratio (b) is defined as described on Eq. 5. Since the concentration of the non-growth substrate (dye) influences its own degradation, b also must be a function of it. The effect of the azo compound concentration on the b parameter can be modeled with a Michaellis Menten type equation. This kinetic equation assumes that there is a saturating concentration of the dye, above which the rate reaches a maximum value due to metabolic limitations.

$$\frac{(-r_i)}{r_X} = b_i = \frac{b_{max,i} \cdot S_i}{Ks_i + S_i} \quad (5)$$

where b_{max} is the maximum decolorization ratio, K_s is the half saturation constant. These kinetic parameters b_{max} and K_s were obtained by non-linear fitting of Eq. 5.

Later, the decolorization of every azo dye can be modeled solving simultaneously the Eq. 6 to every dye with Eqs. 2 and 3 using a 4th-order Runge–Kutta method.

$$\frac{dS_i}{dt} = b \cdot \mu \cdot X = \frac{b_{max,i} \cdot S_i}{Ks_i + S_i}\frac{\mu_{max} \cdot G}{Ks + G} \cdot X \quad (6)$$

Thus, the decolorization kinetic rate is a linear function of biomass and it depends on growth substrate (co-metabolic pathway).

Therefore, with the aim of evaluating the predicting capabilities of the model, the decolorization of the mixture was modeled using the parameters fitted for each substrate.

The saturation of all the compounds must be considered to predict the behavior of a mixture. Based on the premise that all the dyes are degraded by the same enzymatic pool, the saturating effect of the other compounds ($j \neq i$) is taking into account by a competitive inhibition using the K_s of the compound as inhibition constant. This methodology has been widely used in the bibliography since the K_s represents the relative enzyme affinity [20–22]. Thus, the decolorization ratio (b) for a compound i on the mixture is:

$$b_i = \frac{b_{\max,i} \cdot S_i}{Ks_i\left(1 + \sum_{j \neq i}^{j} \frac{S_j}{Ks_j}\right) + S_i} \quad (7)$$

In turn:

$$S_{\text{mixture}} = \sum S_i \quad (8)$$

and, furthermore, in batch cultures the degradation of the total mixture can be predicted as:

$$\frac{dS_{\text{mixture}}}{dt} = \frac{d\sum S_i}{dt} = \sum \frac{dS_i}{dt} = \sum (r_i) \quad (9)$$

Enzyme assays and cytochrome P450 inhibition experiments

The azo reductase activity in liquid cultures and in the fungal mycelium was measured after 4, 6 and 8 days of cultivation, when decolorization was noticeable. Cultures were harvested and the mycelium was filtered onto a Whatman GF/A filter using a Büchner funnel. The liquid culture was used as the enzyme source. The mycelia separated from the liquid culture were washed three times with 20 mM sodium phosphate buffer (pH 7), resuspended in the same buffer and homogenized using a Wheaton glass homogenizer. The extract collected was centrifuged at 12,000*g* during 15 min and the supernatant recovered was used to measure the enzymatic activity.

Azo reductase was estimated according to Jasińska et al. [15]. The assay mixture contained 200 µL of a 20 mM sodium phosphate buffer (pH 7), 250 µL of 0.1 mM NADH, 250 µL of 20 mM azo dyes and 300 µL of the enzyme extract. The blank contained all reagents except the fungal sample. Absorbance of the remaining azo dyes was monitored at $\lambda_{\max}$ (nm) of each dye (described above), for 1 min and enzyme activity was expressed in mM min^{-1} mL^{-1} of the enzyme.

Batch experiments in Erlenmeyer flasks were also performed to study the effect of a cytochrome P450 inhibitor. The inhibitor, 1-aminobenzotriazole (ABT), was added to a final concentration of 1 mM to 150 mL of defined medium. The experiment was carried out as described by Baccar et al. [23]. The same repeated batch experiments were performed with two types of controls: one without the inhibitor [control (1)] and another one using killed fungus [control (2)]. Killed controls received 4 mL of a sodium azide solution (5 g L^{-1}) to 100 mL of the medium containing the fungus.

All the experiments were performed in triplicate.

Fungal growth and biomass

Fungal growth was determined by measuring the mycelium dry weight. The fungal mycelium was filtered onto a Whatman GF/A filter, rinsed twice with distilled water, and dried at 100 °C until constant weight. Biomass was calculated as milligrams of dry weight per volume of reactor (L).

FTIR analysis

The metabolites produced after degradation of the azo dyes were extracted with equal volumes of ethyl acetate.

FTIR spectra were obtained in transmission mode using a Nicolet 6700 device, from Thermo Scientific, in the mid-infrared region of 400–4,000 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. Original dye samples were ground with KBr and pressed into transparent disks, and thin films from ethyl acetate extracts were obtained by casting the solutions onto NaCl windows.

Phytotoxicity studies

The toxicity of the original and decolorized azo dyes was assessed by measuring the phytotoxicity effect on seed germination of wheat (*Triticum aestivum*), according to Osma et al. [24] with some modifications. LMS and distilled sterile water were used as control. Five replicates of ten seeds were used for each treatment. The seeds were submerged in LMS supplemented with the different dyes independently (200 mg L^{-1}) (a non-inoculated control sample was also cultivated) and the decolorized azo dyes after fungal treatment. Decolorized azo dyes showed a low pH (between 3 and 4), since the acidity of these residues is toxic by itself; before beginning the phytotoxicity studies, the decolorized azo dyes were adjusted to pH 6.

After 5 days of incubation in the dark at 25 °C, the seed germination (percentage) and plumule and radical length of seeds immersed in the solutions mentioned before were measured.

Results and discussion

Decolorization and degradation analysis

For the decolorization kinetic studies, the fungus was grown in batch cultures in LMS medium, with glucose as a carbon source, supplemented with 200 mg L^{-1} of the azo dyes as well as the mixture of these. The concentration of the azo dyes used during the experiment is approximately the concentration present in the real textile wastewater. The study of different azo dyes and its mixtures is very important because different dyes are used simultaneously or independently in different dyeing process. Decolorization was measured spectrophotometrically using the peak of absorbance for each azo dye at the visible region and expressed as mg L^{-1} (Fig. 1). *P. chrysogenum* decolorizes

the three azo dyes tested and the mixture of them, at room temperature and with shaking at 120 rpm. Decolorization of the azo dye Direct Blue 200 reaches 87.5 % in 12 days, while decolorization of Direct Yellow 86, Direct Black 22 and the mixture of three dyes was between 98 and 100 % in 12 days. At day 8, the Direct Yellow 86, Direct Black 22 and the mixture already reaches 94, 96 and 99 % of decolorization, respectively. During the decolorization process, a fall in pH was observed, from pH 6 at time zero to pH between 3.5 and 4.5 at the end of the assay, for all the dyes tested.

Controls without inoculation did not show dye decolorization. Controls without dyes and inoculation with the fungus showed a good growth. A list of decolorization parameters for white rot fungi has been reported by Levin et al. [25], showing that decolorization percentages by *Penicillium chrysogenum* are within the range of some very well-known white rot fungi like *Phanerochaete chrysosporium*, *Trametes versicolor* and *T. trogii*. Therefore, *P. chrysogenum* shows a good potential for further decolorization applications. Figure 2 shows the biomass in the presence of different azo dyes and a control in the absence of dyes together with the depletion of total COD, which represent the growth substrate since the contribution of the dyes to the total COD was found negligible. The ANOVAs analysis showed that there are no significant differences in the biomass growth or in COD depletion among treatments. In addition, the MGSI was similar between the fungus growing in plates with and without azo dyes, showing that the azo dyes did not inhibit the fungal growth.

Since Direct Blue 200 did not inhibit the mycelia growth rate, it can be assumed that the reduction in the decolorization rate with respect to the other azo dyes could be attributed to the minor differences in the structure of the azo dye (Fig. 3). Elisangela et al. [4] reported on the degradation of mono, diazo and triazo dyes by *Staphylococcus arlettae* and found that the decolorization time is related to the chemical structure of the dyes, showing that

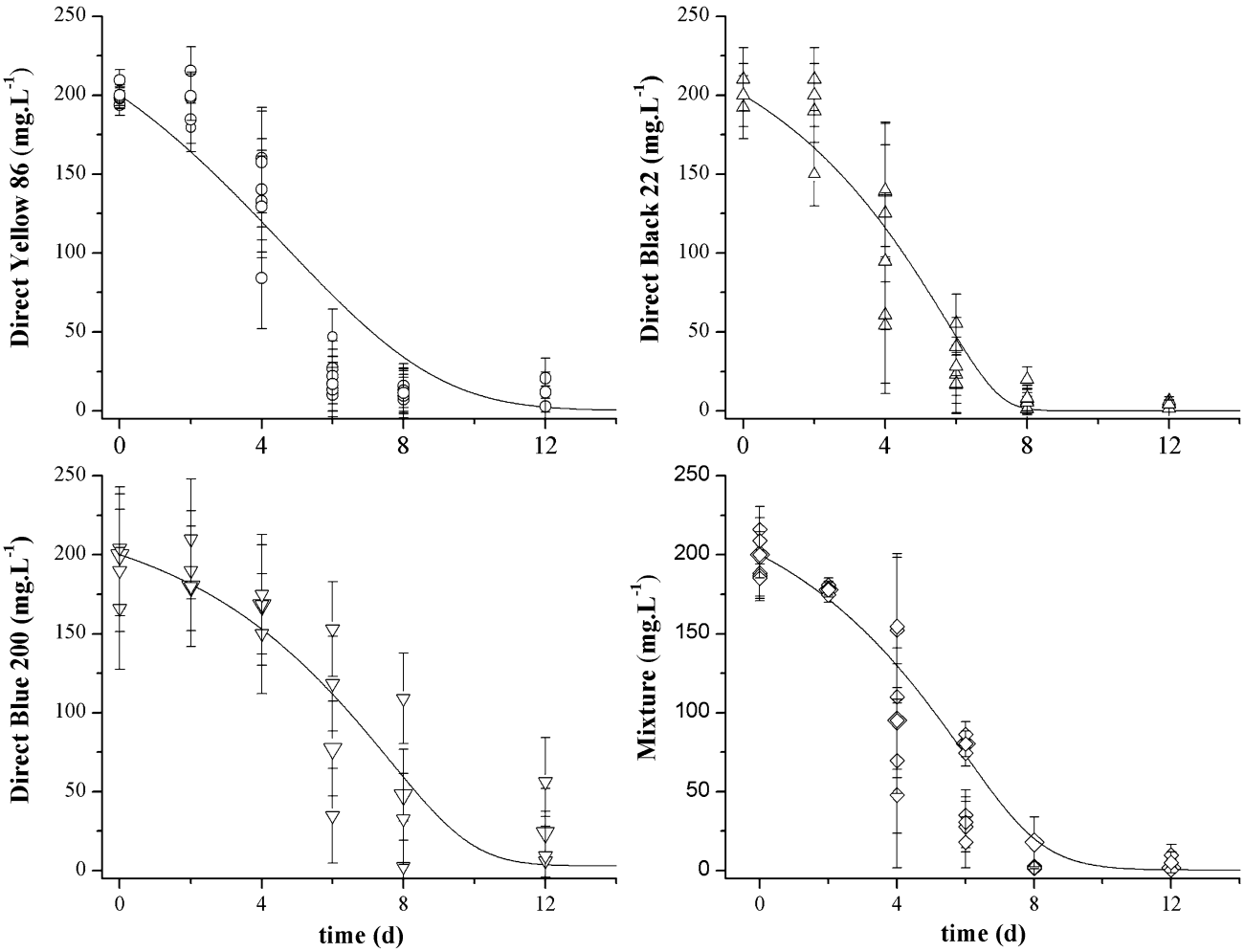


Fig. 1 Kinetics of azo dyes' decolorization by *P. chrysogenum*. *Empty symbols* represent experimental values. *Solid lines* represent kinetic model fitting for Direct Yellow 86, Direct Black 22 and Direct Blue 200 and the values predicted by the model for the mixture

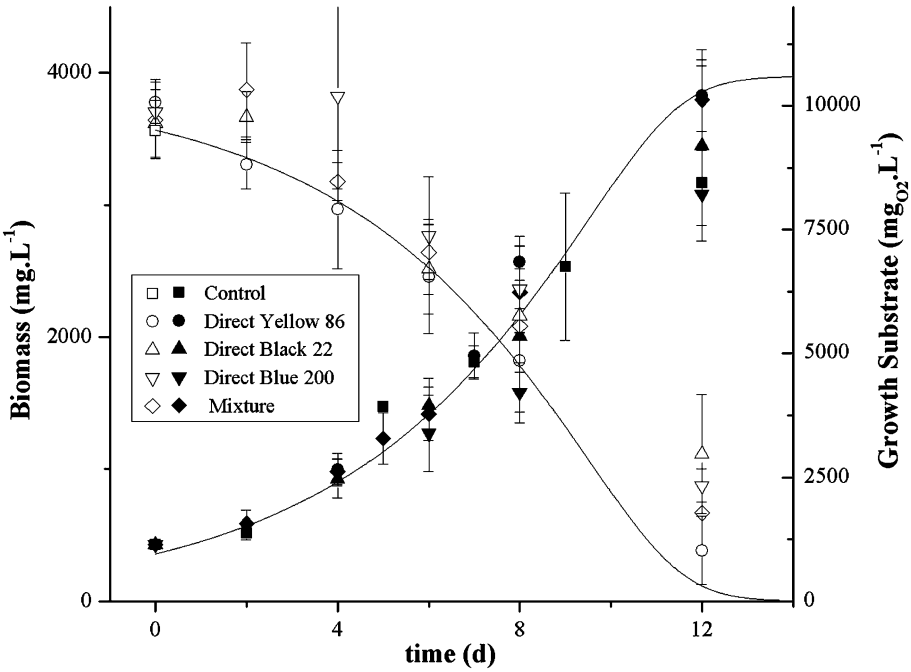
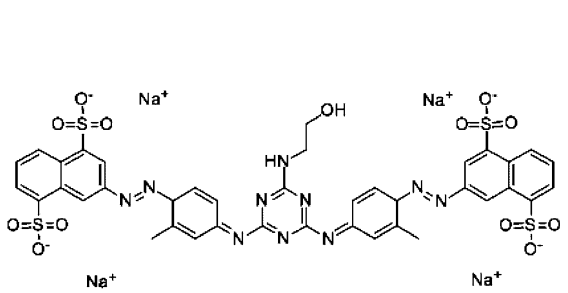
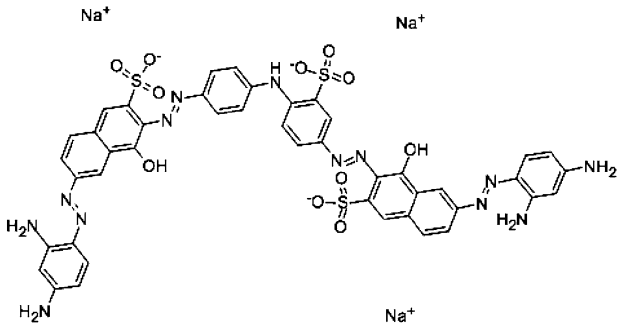


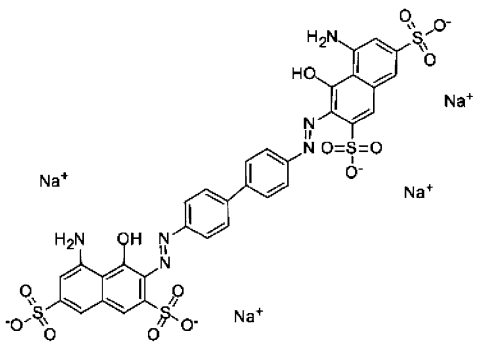
Fig. 2 Biomass growth and COD depletion. *Fill symbols* express biomass (dry weight per unit of reactor volume), and *empty symbols*, express growth substrate (COD). The *solid lines* represent the model fitting



Direct Yellow 86



Direct Black 22



Direct Blue 200

Fig. 3 Chemical azo dyes structures

the presence of more azo bonds in the structure implied longer decolorization times. However, our results showed that Direct Black 22, which has four azo bonds, is decolorized faster than Direct Blue 200. On the other hand, methyl orange was more easily decolorized than xylidine, being both mono azo dyes but with some differences in the terminal or substituent groups of the aromatic structure [25]. It has been reported that the chemical structures of the dyes largely influence their decolorization rate and a linear relationship exists between the redox potential of the azo dyes and the decolorization efficiency when laccases are involved [26]. Although no laccase activity was detected in *P. chrysogenum* [11], differences in decolorization rate were also correlated with chemical structure of the dyes, since Direct Blue 200 shows more steric hindrance to access to the azo bonds and has two hydroxyl groups near of them, which can form hydrogen bond and stabilize the molecule.

As mentioned above, studies about decolorization of azo dyes which also show degradation of them using non-basidiomycete filamentous fungi are scarce. For example, Bergsten-Torralba et al. [27] describe the decolorization of 200 mgL^{-1} of the monoazo RR198 and diazo RB214 dyes in 7 days by *Penicillium simplicissimum*, assuming that decolorization involved adsorption followed by degradation. Also, another isolate of *Penicillium* sp. which decolorizes 100 mgL^{-1} of an azo dye (Reactive Brillant Red X-3B) by bioadsorption in aerobic conditions with sucrose as a carbon source was reported [13]. However, in both studies they did not carry out any assay to differentiate between adsorption and degradation process or to analyze the degradation of azo dyes mixtures. Recently, Saroj et al. [9] have reported the decolorization of three azo dyes independently by an isolate of *Penicillium oxalicum* SAR-3, showing that biodegradation occurs; nevertheless, they did not analyze the azo dyes as a mixture.

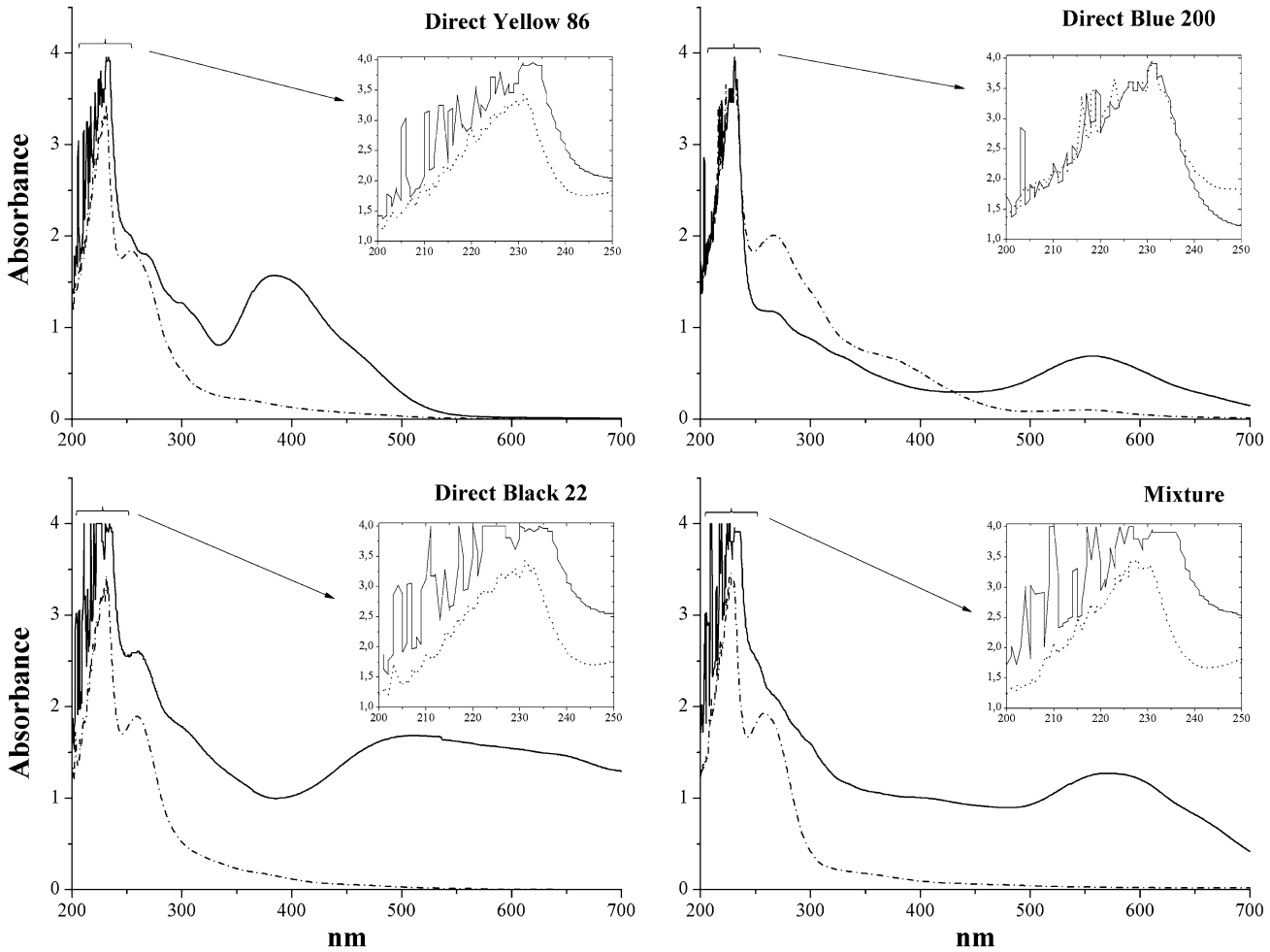


Fig. 4 UV–visible spectral analysis of LMS medium with azo dyes before and after fungal treatment. *Continuous lines* LMS supplemented with 200 mg $L^{-1}$of the azo dyes and inoculated with *P. chrysogenum* at cero time. *Dash-dot lines* LMS plus 200 mg L^{-1} of azo dyes after the end of the treatment

It is very important for the treatment of wastewater from the textile industry to elucidate if decolorization is followed by degradation. The analysis of the resulting products is also considered relevant. For this reason, biodegradation of the azo dyes and the mixture was monitored by UV–visible spectra and FTIR analysis (Figs. 4 and 5, respectively).

The spectra of the azo dyes treated with the fungus showed that the absorbance peaks in the visible region disappeared indicating complete decolorization for all the dyes tested (Fig. 4). The typical absorption peak of the hydrogenated azo bond structure is around 245 nm [4, 28], and all azo dyes tested showed a reduction in the absorbance at this wavelength suggesting the partial disruption of the azo bond.

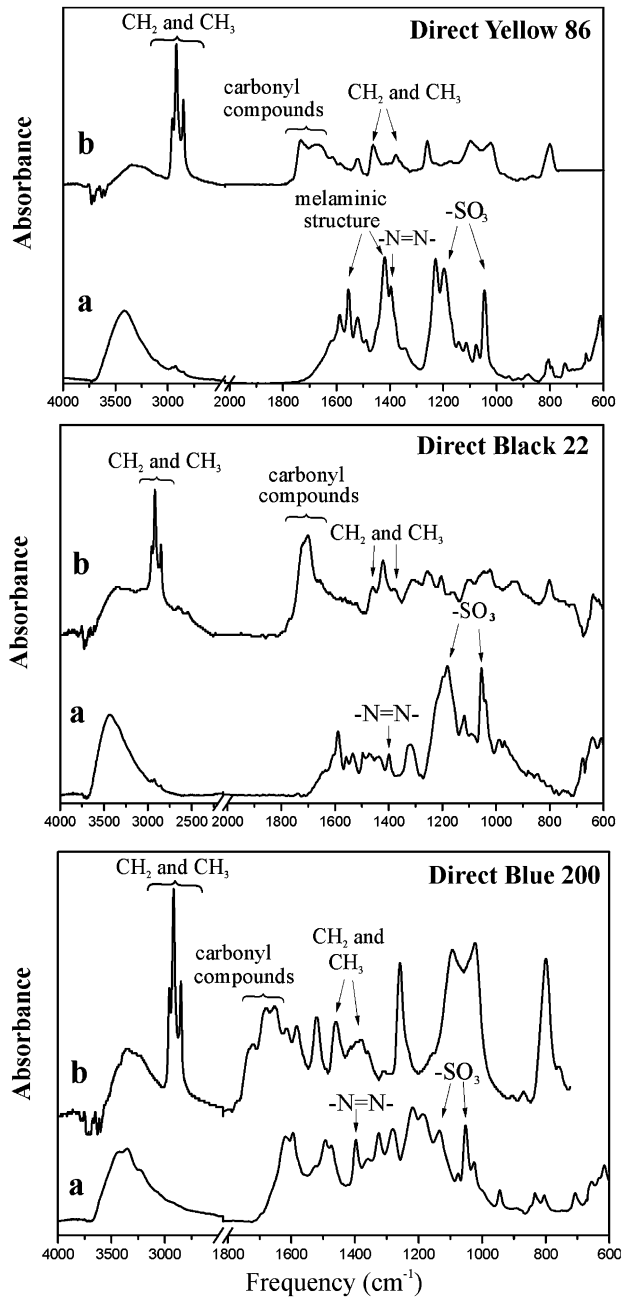


Fig. 5 FTIR spectrum of azo dyes before (**a**) and after (**b**) fungal treatment

Direct Black 22 showed a peak at 260 nm, which decreased after fungal treatment, indicating the partial degradation of aromatic rings [4, 29]. It can also be observed another peak at 220 nm corresponding to the benzene ring [4], which disappeared after fungal treatments (Fig. 4), and a peak at 230 nm can be seen in the decolorization residue.

The spectra for Direct Yellow 86 showed two major peaks at 380 and 230 nm, the last one may be corresponding to benzene rings which diminished in the decolorization residue, while the first peak disappears totally indicating complete decolorization. After fungal treatment, the peak at 260 nm is more noticeable suggesting the decrease of aromatic rings (Fig. 4).

Figure 4 shows that even though for Direct Blue 200 there is a decrease in the intensity of the UV-spectra and a peak can now be clearly observed at 260 nm, suggesting that the destruction of the azo-conjugated group released aromatic rings. For the mixture of the three azo dyes, similar spectra were observed.

Analysis of decolorization products by FTIR

Fourier transform infrared spectroscopy (FTIR) analysis of decolorization products showed that there are significant differences between the spectra of pure azo dyes and those after fungal treatment (Fig. 5). After treatment, a pronounced decrease in the intensity of the peaks at 1,187, 1,050 ± 5 and 1,400 cm^{-1} was observed for all the azo dyes tested. The first ones are characteristics of asymmetric and symmetric stretching of the $–SO_3$ groups from sulfonic acid salts [30] and the last one of the non-protonated aromatic azo groups [31]. Thus, it may be inferred that cleavage of the azo linkage took place and fragments containing sulfonic groups, soluble in aqueous media, were generated (Fig. 5). These results agree with the spectra analysis where the absorbance of the characteristic peaks of azo bonds decreases (Fig. 4). For Direct Blue 200 and Direct Yellow 86, an increase in the peaks at 2,956, 2,922 and 2,852 cm^{-1}, 1,460 and 1,380 cm^{-1}, related to CH_2 and CH_3 groups was observed after fungal treatment, indicating that degradation led to the formation of aliphatic compounds. The same peaks appeared in the Direct Black 22 residues [32] have already reported the formation of aliphatic ethers during degradation of a textile effluent by a bacterial consortium, suggesting the formation of aliphatic compounds. All decolorization residues also showed the

appearance of several overlapped peaks in the 1,730–1,650 cm^{-1} region, which can be associated with acids, amides, salts, and quinone structures. The same results were observed by Elisangela et al. [4] with the bacteria *S. arlettae*. The presence of organic acids is coincident with a significant fall in the pH during degradation process. In addition, the spectrum of the treated Direct Yellow 86 shows a marked decrease of peaks at 1,555 and 1,419 cm^{-1} associated with a melaminic structure present in this azo dye (Fig. 3), which shows that it is has been degraded.

A large fraction of the aromatic amines from azo dyes is susceptible to autoxidation, producing water-soluble and highly colored dimmers, oligomers and eventually dark-colored polymers with low solubility [33]. However, in our experiments, no increase in color in the visible region was observed, suggesting that the aromatic amines were degraded.

Finally, FTIR analysis confirmed that *Penicillium chrysogenum* not only decolorizes the azo dyes but also degrades.

Enzyme assays and cytochrome P450 inhibition experiment

The transformation processes of synthetic dyes are often mediated by several oxidoreductive enzymes such as laccase, peroxidase and reductase. In a previous research it was described that *P. chrysogenum* ERK1 did not show laccase, manganese or lignin peroxidase activities [11]. However, only catechol dioxygenase activity was observed. Therefore, azo reductase activities were tested during the experiments reported here. In liquid cultures, the supernatants as well as the whole homogenized cultures were used to examine the reductase activity. The results revealed that *P. chrysogenum* did not show reductase activity. In addition, intracellular systems, which generally are present in most fungi, such as cytochrome P450 monooxygenase, may also be involved in dye decolorization. Experiments were conducted to determine if cytochrome P450 was involved in the decolorization of the azo dyes. The results showed that the cytochrome P450 inhibitor (ABT) did not affect the decolorization process. In the assays carried out in the presence of the cytochrome P450 inhibitor, the fungus decolorizes the 89 ± 0.2 %, while controls without inhibitor decolorize 90 ± 1 %. This result suggests that the enzyme cytochrome P450 is not involved in the degradation of these azo dyes.

On the other hand, the killed fungus experiments (done in the presence of sodium azide) decolorized only 18 ± 1 % of the azo dye, showing that adsorption is not the main mechanism for azo dyes' decolorization by *P. chrysogenum*.

Phytotoxicity

The phytotoxicity of the original and decolorized azo dyes was assessed by observing the germination percentage and measuring the length of the plumule and radicle of wheat seeds. This is one of the most common phytotoxicity assays used in the literature, which measures the relative seed germination and relative plumule and radicle elongation that are sensitive to the presence of phytotoxic compounds [24]. Several species have been traditionally used for evaluating phytotoxicity. Since there are no standardized seed species in use worldwide [24], wheat (*Triticum aestivum*) was used for this assay, because it is a common crop in Argentinean fields.

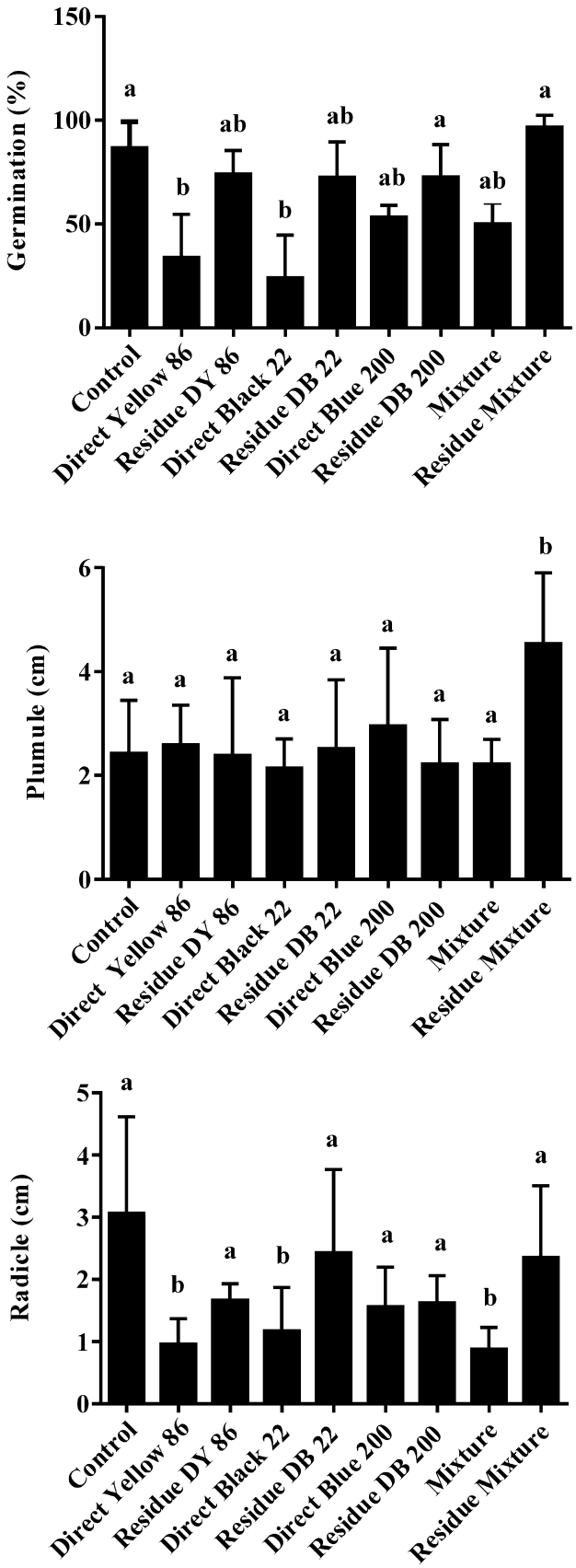
Figure 6 shows that Direct Yellow 86 and Direct Black 22 were the most toxic azo dyes tested in this work, considering both seed germination and the length of the radicle. While, Direct Blue 200 did not show significant differences with respect to the control. The mixture of the three azo compounds did not affect the seed germination, but reduced the length of the radicle significantly. On the other hand, the dyes tested individually did not affect the length of the plumule, except for the residue of the mixture of the dyes, which showed an increase in the mean length of the plumule.

After fungal treatment, all the decolorized azo dyes reduced its toxicity on seed germination. Direct Yellow 86, Direct Black 22, Direct Blue 200 and the mixture of the azo dyes after degradation reduced the phytotoxicity in a 40, 48, 20 and 47 %, respectively (Fig. 6). Although the differences are not significant for Direct Blue 200 and the mixture of the azo dyes, the degradation products of Direct Yellow 86 and Direct Black 22 show significant differences with respect to the non-decolorized azo dye. In addition, no differences were observed on seed germination among the decolorized Direct Yellow 86 and Direct Black 22 and control (water), demonstrating that the reduction in the toxicity on seed germination for both dyes was very important. When the effect on the radicle length was tested, degradation product of Direct Yellow 86, Direct Black 22, and the mixture of the dyes showed a significant reduction in toxicity, while the residue of Direct Blue 200 did not show differences (Fig. 6).

Finally, the decolorization and degradation by *P. chrysogenum* reduced the toxicity of the azo dyes on wheat seed, mainly in the case of Direct Yellow 86 and Direct Black 22.

Kinetic model fitting

Since the contribution of azo dyes to COD is negligible, it is assumed that the total COD represents the concentration of growth substrate (G). The values obtained for



◀**Fig. 6** Phytotoxicity analysis of the azo dyes degradation products. The toxicity of the original and decolorized azo dyes (residue) was assessed by measuring seed germination of wheat and the length of plumule and radicle. Mixture is the combination of the three azo dyes in equimolar quantities. The phytotoxicity assay was carried out at 25 °C, during 7 days with photoperiod (12 h). Control was carried out using water. *Bars* represents the mean ± SD of three independent experiments with two replicates per each. *Different letters* mean significant differences at $P < 0.05$ (Kruskal–Wallis ANOVA test)

parameters μ_{max}, K_m and X_0 after fitting and optimization were: 0.273 ± 0.023 day^{-1}, 163 ± 112 mg$_{O_2}$ L^{-1} and 359 ± 1.12 mg L^{-1}, respectively. The value found for $Y_{X/G}$ was 0.380 ± 0.023 mg$_X$/mg$_{O_2}$. The specific growth rate [$\mu = \mu_{max}\, G/(K_s + G)$] values observed in this work are similar to those reported for *P. chrysogenum* using glucose as a carbon source in the absence of pollutants [34, 35] and are in the range of previously found for other pollutants [10, 36]. It also seems to indicate that the dyes evaluated do not inhibit the mycelia growth in the range of concentration study.

The values of decolorization kinetic parameters $b_{max,i}$ and K_{s_i} obtained for every dye are depicted in Table 1. The values for b_{max} obtained clearly showed that Direct Blue 200 presented the slowest decolorization rate. Furthermore, Direct Blue 200 presents a higher half saturation constant (K_s), which indicate a lower affinity substrate–biomass than the other ones. Since this work is the first which reports kinetic parameters for fungal dye degradation, a direct comparison with literature is not available. However, the instant velocities observed here (as r_i) fall into the range reported in the literature for global degradation rates [25]. The development of mathematical kinetic models not only allows to carry out a deeper kinetic study but also presents a useful tool for field and industrial applications, in situ bioremediation or wastewater facilities' design. This work is the first to develop a mathematic kinetic model for azo dyes' decolorization and degradation by fungal cultures.

The values fitted by the model are also shown in Fig. 1 as solid lines. It is clearly shown how the model fits properly to every dye alone (Fig. 1a–c). Furthermore, the model showed its capability to predict successfully the decolorization of the mixture. In this way, the parameters from Table 1 were used in Eq. 7. The values predicted

Table 1 Kinetic parameters for dye decolorization

Dye	b_{max} (mg$_S$/mg$_X$)	K_s (mg$_S$/L)
Direct Yellow 86	0.159 ± 0.019	25.658 ± 5.958
Direct Blue 200	0.109 ± 0.010	46.055 ± 4.780
Direct Black 22	0.179 ± 0.011	25.162 ± 6.118

by the model for mixture degradation are also shown in Fig. 1.

Real wastewater

Several assays were performed to evaluate the capability of the fungus to treat the real industrial wastewater. Different volumes of the real industrial wastewater were mixed with LMS media (expressed as % v/v), and decolorization was evaluated. *P. chrysogenum* was able to decolorize the real industrial wastewater up to 20 % (v/v) of wastewater in LMS. Figure 7 shows the experimental values obtained from real wastewater decolorization.

The methodology, when parameters obtained from laboratory data are used to predict fresh data, is called validation [16, 37]. This step is very important to evaluate the kinetic model to be used directly or the need for modifing it [37, 38]. The fully prediction of biomass by the model is also shown in Fig. 7 (solid lines). In this work, the proposed model predicts very well the fungal growth in the real wastewater with original values of parameters μ_{max}, K_m and $Y_{X/G}$. This validates the growth model for industrial applications and also indicates that dye concentration or the complex matrix of wastewater did not affect the biomass growth.

In turn, when kinetic parameters obtained for Direct Black 22 were used to predict the decolorization, the rates predicted by the model were higher than those observed experimentally. This phenomenon could be attributed to the complex matrix of the textile wastewater which contains high concentration of other compounds needed for industrial process, such as Na_2CO_3, NaOH among others, used as mordants. These chemicals can affect the metabolism of the microorganism. In these cases, it is necessary to modify the kinetic parameters to fit the model to real conditions [38, 39]; this methodology is called calibration [37, 38]. Kinetic parameters for Direct black 22 must be refitted for real wastewater decolorization. The value of b_{max} was recalibrated to 0.081 ± 0.015 and K_s was not modified. The values of Direct black 22 concentration predicted by the model after calibration are shown as solid lines in Fig. 7. It shows that the model was able to predict successfully the decolorization of the real wastewater.

The good predictions of the developed model for mixture degradations and its capability to predict real wastewater decolorization encourage its use to simulate/predict the behavior of the other azo compounds and its mixtures for industrial applications, facilities' design and optimization process. Furthermore, it is a stepping stone for future applied research on dye wastewater treatment.

Conclusions

In view of the obtained results, it can be concluded that *Penicillium chrysogenum*, a non-basidiomycete fungus, has a great potential to decolorize and degrade not only the three azo dyes independently but also the mixture of them even in a complex wastewater matrix in the presence of glucose as a carbon source. Decolorization rates showed some differences depending on the dye structure. In this work, based on the UV–visible spectra and FTIR analysis, it was demonstrated that decolorization is

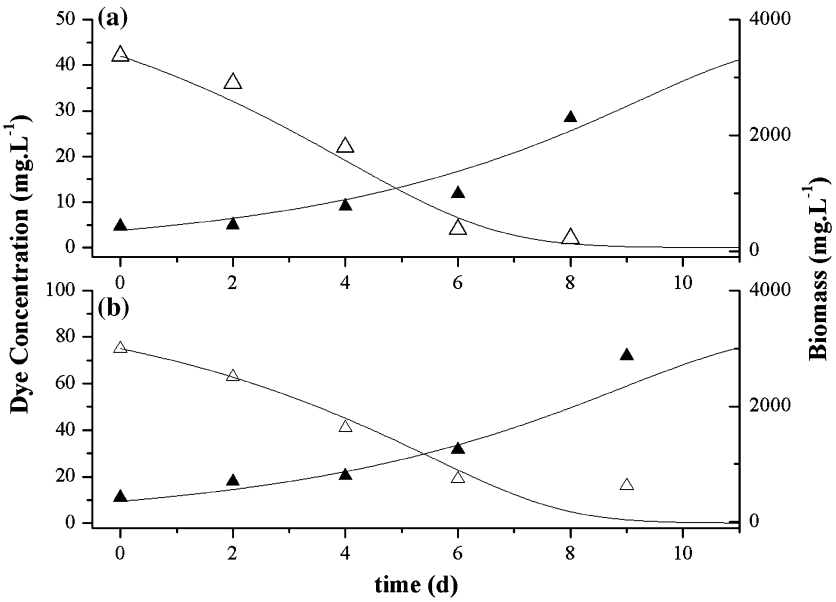


Fig. 7 Real wastewater assay. *Empty symbols* represent Direct Black 22 concentration in real wastewater and *filled symbols* represent biomass. *Symbols* represent experimental values and *solid lines* represent calibrated model prediction. **a** Wastewater-LMS 10 % (v/v), **b** wastewater-LMS 20 % (v/v)

followed by degradation. This work is the first which reports kinetic parameters for fungal azo dyes degradation and evaluates them in real wastewater degradation. The use of mathematical kinetic models allowed performing a deeper kinetic study. Furthermore, the kinetic model development represents a useful tool in field and industrial applications.

Acknowledgments This research was supported by the Universidad Nacional de Mar del Plata, the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT). The authors would like to thank Gabriela Fioramonti from Gama S. A. for kindly providing the azo dyes and wastewater samples.

References

1. Robinson T, McMullan G, Marchant R, Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour Technol 77(3):247–255. doi:10.1016/S0960-8524(00)00080-8
2. Solís M, Solís A, Pérez HI, Manjarrez N, Flores M (2012) Microbial decolouration of azo dyes: a review. Process Biochem 47(12):1723–1748. doi:10.1016/j.procbio.2012.08.014
3. Kuberan T, Anburaj J, Sundaravadivelan C, Kumar P (2011) Biodegradation of azo dye by *Listeria* sp. Int J Environ Sci 1(7):1760–1770
4. Elisangela F, Andrea Z, Fabio DG, de Menezes Cristiano R, Regina DL, Artur C-P (2009) Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process. Int Biodeterior Biodegrad 63(3):280–288. doi:10.1016/j.ibiod.2008.10.003
5. Saratale RG, Saratale GD, Chang JS, Govindwar SP (2011) Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem Eng 42(1):138–157. doi:10.1016/j.jtice.2010.06.006
6. Esmaeili A, Kalantari M (2012) Bioremoval of an azo textile dye, Reactive Red 198, by *Aspergillus flavus*. World J Microbiol Biotechnol 28(3):1125–1131. doi:10.1007/s11274-011-0913-1
7. Durán N, Esposito E (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl Catal B Environ 28(2):83–99. doi:10.1016/S0926-3373(00)00168-5
8. Mester TN, Tien M (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. Int Biodeterior Biodegrad 46(1):51–59. doi:10.1016/S0964-8305(00)00071-8
9. Saroj S, Kumar K, Pareek N, Prasad R, Singh RP (2014) Biodegradation of azo dyes Acid Red 183, Direct Blue 15 and Direct Red 75 by the isolate *Penicillium oxalicum* SAR-3. Chemosphere. doi:10.1016/j.chemosphere.2013.12.049
10. Aranciaga N, Durruty I, González JF, Wolski EA (2012) Aerobic biotransformation of 2, 4, 6-trichlorophenol by *Penicillium chrysogenum* in aqueous batch culture: degradation and residual phytotoxicity. Water SA 38(5):683–688
11. Wolski EA, Barrera V, Castellari C, González JF (2012) Biodegradation of phenol in static cultures by *Penicillium chrysogenum*: catalytic abilities and residual phytotoxicity. Rev Argentina Microbiol 44:113–121
12. Zheng Z, Levin RE, Pinkham JL, Shetty K (1999) Decolorization of polymeric dyes by a novel Penicillium isolate. Process Biochem 34(1):31–37. doi:10.1016/S0032-9592(98)00061-2
13. Gou M, Qu Y, Zhou J, Ma F, Tan L (2009) Azo dye decolorization by a new fungal isolate, *Penicillium* sp. QQ and fungal-bacterial cocultures. J Hazard Mater 170(1):314–319. doi:10.1016/j.jhazmat.2009.04.094
14. Ramalingam SN, Shanmugapriya S, Shakthipriyadarshini S, Sadasivam S, Shanmugapraksh M (2010) Decolorization of textile dyes by *Aspergillus tamarii*, mixed fungal culture and *Penicillium purpurogenum*. J Sci Ind Res India 69:151–153
15. Jasińska A, Różalska S, Bernat P, Paraszkiewicz K, Długoński J (2012) Malachite green decolorization by non-basidiomycete filamentous fungi of *Penicillium pinophilum* and *Myrothecium roridum*. Int Biodeterior Biodegrad 73:33–40. doi:10.1016/j.ibiod.2012.06.025
16. Almquist J, Cvijovic M, Hatzimanikatis V, Nielsen J, Jirstrand M (2014) Kinetic models in industrial biotechnology—improving cell factory performance. Metab Eng. doi:10.1016/j.ymben.2014.03.007
17. APHA (1998) Standard methods for the examination of water and wastewater, 20th edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC
18. Borges Pereira R, Ciavareli Lucas G, Perina FJ, Vilela deResende ML, Alves E (2011) Potential of essential oils for the control of brown eye spot in coffee plants. Ciéncia e Agrotecnologia 35:115–123
19. Perlatti B, da Silva MF, Fernandes JB, Forim MR (2012) Validation and application of HPLC–ESI–MS/MS method for the quantification of RBBR decolorization, a model for highly toxic molecules, using several fungi strains. Bioresour Technol 124:37–44. doi:10.1016/j.biortech.2012.08.032
20. Alvarez-Cohen L, McCarty PL (1991) Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl Environ Microbiol 57(4):1031–1037
21. Bielefeldt AR, Stensel HD (1999) Modeling competitive inhibition effects during biodegradation of BTEX mixtures. Water Res 33(3):707–714. doi:10.1016/S0043-1354(98)00256-5
22. Gérard C, Tyson John J, Novák B (2013) Minimal models for cell-cycle control based on competitive inhibition and multisite phosphorylations of Cdk substrates. Biophys J 104(6):1367–1379. doi:10.1016/j.bpj.2013.02.012
23. Baccar R, Blánquez P, Bouzid J, Feki M, Attiya H, Sarrà M (2011) Decolorization of a tannery dye: from fungal screening to bioreactor application. Biochem Eng J 56(3):184–189. doi:10.1016/j.bej.2011.06.006
24. Osma JF, Toca-Herrera JL, Rodríguez-Couto S (2010) Transformation pathway of Remazol Brilliant Blue R by immobilised laccase. Bioresour Technol 101(22):8509–8514
25. Levin L, Grassi E, Carballo R (2012) Efficient azoic dye degradation by *Trametes trogii* and a novel strategy to evaluate products released. Int Biodeterior Biodegrad 75:214–222. doi:10.1016/j.ibiod.2012.10.005
26. Zille A, Ramalho P, Tzanov T, Millward R, Aires V, Cardoso MH, Ramalho MT, Gübitz GM, Cavaco-Paulo A (2004) Predicting dye biodegradation from redox potentials. Biotechnol Prog 20(5):1588–1592. doi:10.1021/bp049963i
27. Bergsten-Torralba LR, Nishikawa MM, Baptista DF, Magalhães DP, Silva Md (2009) Decolorization of different textile dyes by *Penicillium simplicissimum* and toxicity evaluation after fungal treatment. Br J Microbiol 40:808–817
28. Qing C (1989) Chemistry of dye intermediates. Press Beijing, China
29. Feng W, Nansheng D, Helin H (2000) Degradation mechanism of azo dye C. I. reactive red 2 by iron powder reduction and photooxidation in aqueous solutions. Chemosphere 41(8):1233–1238. doi:10.1016/S0045-6535(99)00538-X

30. Socrates G (1994) Infrared characteristic group frequencies. Tables and charts. Wiley, UK
31. Wojciechowska M, Wojciechowski G, Wasiak W (2003) Spectroscopic and semiempirical studies of the phototropic species of 8-amino-5,8′-azo-bis-naphthalene-2-sulphonic acid. J Mol Struct 658:125–133
32. Phugare SS, Kalyani DC, Surwase SN, Jadhav JV (2011) Eco-friendly degradation, decolorization and detoxification of textile effluent by a developed bacterial consortium. Ecotox Environ Safe 74:1288–1296
33. Kudlich M, Hetheridge MJ, Knackmuss HJ, Stolz A (1999) Autoxidation reactions of different aromatic *o*-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes. Environ Sci Technol 33:896–901
34. Yuan J, Liu Y, Geng J (2010) Stoichiometric balance based macrokinetic model for *Penicillium chrysogenum* in fed-batch fermentation. Process Biochem 45(4):542–548. doi:10.1016/j.procbio.2009.11.015
35. Paul GC, Syddall MT, Kent CA, Thomas CR (1998) A structured model for penicillin production on mixed substrates. Biochem Eng J 2(1):11–21. doi:10.1016/S1369-703X(98)00012-6
36. Wolski E, Durruty I, Haure P, González J (2012) *Penicillium chrysogenum*;: phenol degradation abilities and kinetic model. Water Air Soil Pollut 223(5):2323–2332. doi:10.1007/s11270-011-1026-z
37. Donoso-Bravo A, Mailier J, Martin C, Rodríguez J, Aceves-Lara CA, Wouwer AV (2011) Model selection, identification and validation in anaerobic digestion: a review. Water Res 45(17):5347–5364
38. Béline F, Boursier H, Daumer ML, Guiziou F, Paul E (2007) Modelling of biological processes during aerobic treatment of piggery wastewater aiming at process optimisation. Bioresour Technol 98(17):3298–3308. doi:10.1016/j.biortech.2006.07.004
39. Durruty I, Ayude MA (2014) Modeling of an industrial anaerobic digester: a case study for undergraduate students. Chem Eng Educ Spring, 71–78