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ORIGINAL PAPER

Biological decolorization of xanthene dyes by anaerobic granular biomass

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Abstract Biodegradation of a xanthene dyes was investigated for the first time using anaerobic granular sludge. On a first screening, biomass was able to decolorize, at different extents, six azo dye solutions: acid orange 7, direct black 19, direct blue 71, mordant yellow 10, reactive red 2 and reactive red 120 and two xanthene dyes—Erythrosine B and Eosin Y. Biomass concentration, type of electron donor, induction of biomass with dye and mediation with activated carbon (AC) were variables studied for Erythrosine B (Ery) as model dye. Maximum color removal efficiency was achieved with 4.71 g VSS L^{-1}, while the process rates were independent of the biomass concentration above 1.89 g VSS L^{-1}. No considerable effects were observed when different substrates were used as electron donors (VFA, glucose or lactose). Addition of Ery in the incubation period of biomass led to a fivefold increase of the decolorization rate. The rate of Ery decolorization almost duplicated in the presence of commercial AC (0.1 g L^{-1} AC_0). Using different modified AC samples (from the treatment of AC_0), a threefold higher rate was obtained with the most basic one, AC_{H_2}, as compared with non-mediated reaction. Higher rates were obtained at pH 6.0. Chemical reduction using Na_2S confirmed the recalcitrant nature of this dye. The results attest that decolorization of Ery is essentially due to enzymatic and adsorption phenomena.

Keywords Anaerobic process · Biological decolorization · Eosin · Erythrosine · Xanthene dyes

Abbreviations

AC	Activated carbon
AC_0	NoritROX0.8 activated carbon
AC_{O_2}	Surface modified activated carbon, prepared by chemical oxidation O_2 and thermal treatments under H_2 (AC_{H_2}) or N_2 (AC_{N_2}) flow
AC_{HNO_3}	Surface modified activated carbon, prepared by chemical oxidation with HNO_3 (AC_{HNO_3})
AC_{H_2}	Activated carbon treatments under H_2 (AC_{H_2}) or N_2 (AC_{N_2}) flow
AC_{N_2}	Activated carbon treatments under N_2 (AC_{N_2} flow
COD	Chemical oxygen demand (mg O_2 L^{-1})
C.I.	Colour index
DAD	Diode array detector
Ery	Erythrosine B
Eos Y	Eosin Y

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VSS Volatile suspended solids
AO7 Acid orange 7
DB19 Direct black 19
DB71 Direct blue 71
MY10 Mordant yellow 10
RR2 Reactive red 2
RR120 Reactive red 120

Introduction

A variety of synthetic dye stuffs are released by industry during manufacturing processes. Their presence in the environment is of greater concern, since they may generate serious public health and environmental problems. Some of these compounds have mutagenic or carcinogenic activity (Caliman and Gavrilescu 2009; Gavrilescu 2009). Several techniques (chemical, physical, and biological) may be applied for the decolorization of wastewater containing dyes, but each method has technical and economic limitations (Apostol and Gavrilescu 2009; Cailean et al. 2009; Caliman and Gavrilescu 2009; Momenzadeh et al. 2011; SenthilKumar et al. 2010).

Biological treatment methods may be preferred for decolorization as environmentally friendly and relatively inexpensive ones (Costa et al., 2009; Van der Zee and Villaverde, 2005; Gavrilescu and Chisti, 2005). Anaerobic treatment generally gives good color removal efficiencies in opposition with aerobic conditions where low color removal efficiencies are achieved, because oxygen is a more efficient electron acceptor, therefore having more preference for electrons than azo dyes (Dos Santos et al. 2004). Treatment systems composed of mixed microbial populations achieve a higher degree of biodegradation and mineralization due to the synergistic metabolic activities of the microbial community and have considerable advantages over the use of pure cultures (Saratale et al. 2011; Venkata Mohan et al. 2011). In a microbial consortium, the individual strains may attack the dye molecule at different positions or utilize metabolites produced by the co-existing strains for further decomposition (Saratale et al. 2011). Most of the research focuses on the anaerobic decolorization of the most representative class of dyes, azo compounds (Firmino et al. 2010; Saratale et al. 2011).

Xanthene represent a distinct class of dyes that are use in food, cosmetics, paper, and ink, due to superior dyeing and coloring properties, but have poor biodegradability and some of them are toxic (Itoh and Yatome 2004). Only six reports on enzymatic oxidation of xanthene dyes are available (Itoh and Yatome 2004; Jesus et al. 2010) and any about enzymatic reduction.

The aim of this study was to test and investigate the effect of non-acclimated anaerobic granular sludge on a xanthene dye, Erythrosine B (Ery B), under batch anaerobic conditions; this being a first report on the decolorization of xanthene dyes containing solutions by a mixed anaerobic culture.

The results in batch and in continuous assays, can give different conclusions, especially in the case of biological degradation with mixed cultures. However, as a starting approach for this type of studies, batch assays are useful and indicative of expected kinetics. From the indication of the batch assays we anticipate a difficult anaerobic biodegradability of this dye, even in continuous assays. Different approaches, combining oxidative and biological processes or redox mediators among others, are being tested.

A first screen of dye biodegradation by the biomass was done for six model azo dyes—acid orange 7, direct black 19, direct blue 71, mordant yellow 10, reactive red 2 and reactive red 120 and two xanthene dyes—Ery B and Eosin. Ery B was selected for further studies on the biological degradation of xanthene dyes. The effect of various parameters on the Ery biodegradation was then, for the first time, studied: (i) biomass concentration; (ii) electron donor (VFA, glucose, and lactose); (iii) biomass induction with dye; (iv) pH; and (v) dye concentration. The transfer of reducing equivalents from a primary electron donor (co-substrate) to a terminal electron acceptor (azo dye) generally acts as the process rate limiting step in anaerobic azo dye reduction (Van der Zee et al. 2003) and activated carbon, as an insoluble catalyst, has been shown as a good alternative to the soluble ones, with the advantage of being recovered and reused with facility. For the first time, the effect of a redox mediator on xanthene dyes biodegradation was assessed by adding a commercial (AC_0) and surface modified activated carbon samples, prepared by chemical oxidation with HNO_3 (AC_{HNO_3}) and O_2 (AC_{O_2}) and thermal treatments under H_2 (AC_{H_2}) or N_2 (AC_{N_2}) flow (Pereira et al., 2010). Besides in a

biological system, Ery reduction was assessed with different concentrations of a known chemical reducing agent, Na_2S (Cervantes et al. 2007; Pereira et al. 2010; Van der Zee et al. 2001). Work in order to obtain the biodegradation products is ongoing and we aim to provide the information in near future publications.

Experimental

Chemicals

The xanthene dyes used were the Erythrosine B (Ery B, C.I. 45430, dye content 95 %) and Eosin Y (Eos Y, C.I. 45380, dye content 85 %). The azo dyes acid orange 7 (AO7, C.I. 15510, dye content 85 %), direct black 19 (DB19, C.I. 35255, dye content 50 %), direct blue 71 (DB71, C.I. 34140, dye content 50 %), mordant yellow 10 (MY10, C.I. 14010, dye content 85 %); reactive red 2 (RR2, dye content 40 %) and reactive red 120 (RR120, dye content 50 %), were selected as azo dye model compounds. All the dyes were purchased from Sigma-Aldrich and used without additional purification. The chemical structures of the dyes are illustrated in Fig. 1. Stock solutions of 14 mM were prepared in deionized water.

NoritROX0.8 commercial activated carbon type (pellets of 0.8 mm diameter and 5 mm length) was

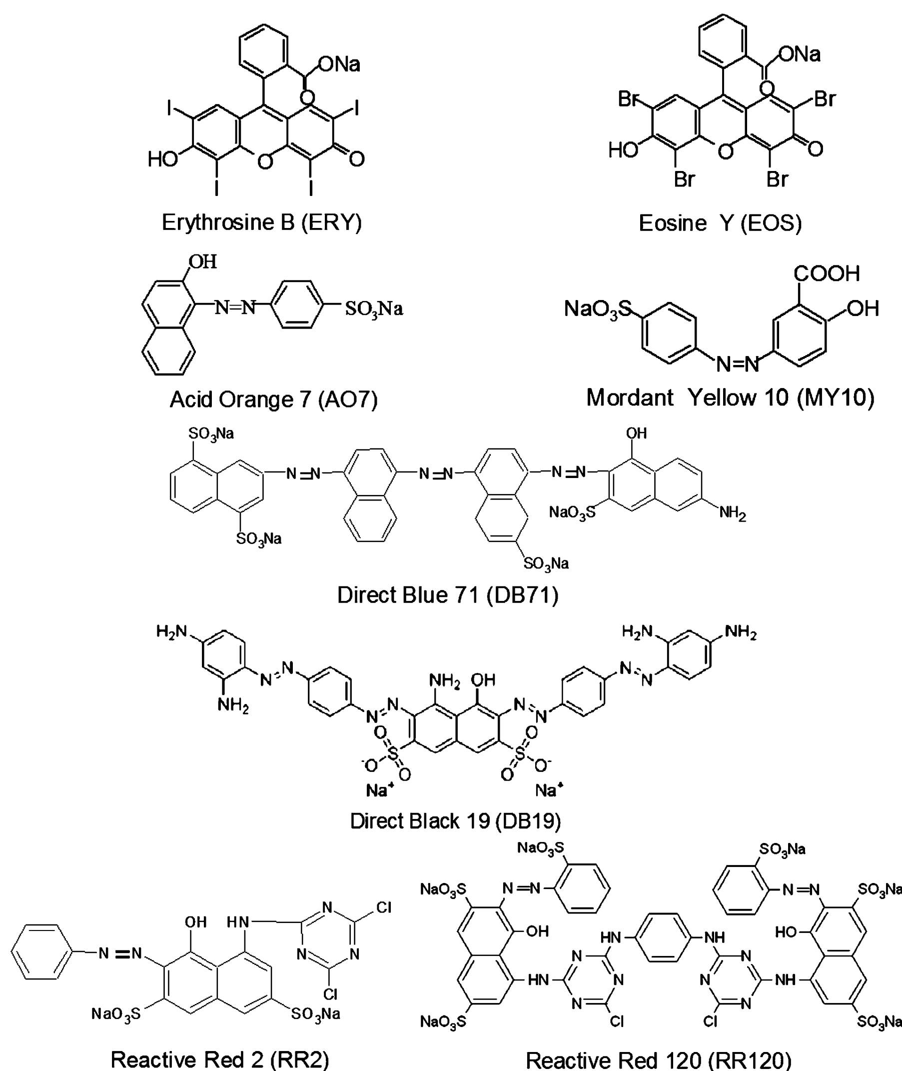

Fig. 1 Molecular structure of the azo and xanthene dyes

used, as supplied by Norit (the registered mark of Noric Americas Inc.), as a starting material (sample AC_0). AC samples with different chemical composition on the surface, maintaining the original textural properties as much as possible, were prepared by chemical oxidation (AC_{HNO_3} and AC_{O_2}) or thermal treatment (AC_{N_2} and AC_{H_2}) and characterized in Engineering Faculty of Porto University as described by Pereira et al. (2010).

The substrates, macronutrients and sodium sulphide (Na_2S) were all from Sigma or Fluka, at the highest analytic grade purity commercially available.

Granular anaerobic biomass

Non-acclimated anaerobic granular sludge was collected from wastewater treatment plant of "Central de Cervejas", Vialonga, Portugal. The volatile suspended solids content of the biomass was determined as 0.0943 g VSS g^{-1}.

Dye degradation by anaerobic granular biomass

Eight model dyes were screened for biodegradation using an anaerobic granular biomass: three azo (AO7, MY10 and RR2), a disazo (RR120), a trisazo (DB71), a poliazo (DB19) and two xanthene dyes (Ery and Eos). Batch assays were conducted in 120 mL serum bottles with butyl rubber stopper, containing the biomass, 0.94 g VSS L^{-1}, the substrate and macronutrients in a total volume of 50 mL of medium, that was buffered at a pH of 7 ± 0.2 with $NaHCO_3$ (2.5 g L^{-1}). The headspace of the serum bottles was flushed with the N_2:CO_2 (80/20 v/v) and pre-incubation of the sludge was done overnight at 37 °C, in a rotary shaker at 120 rpm. As macronutrients, 2.8 g L^{-1} NH_4Cl, 2.5 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.06 g L^{-1} $CaCl_2$ were added. Volatile fatty acids (VFAs: acetic, propionic, and butyric acid, 1:10:10) were supplemented as electron source for the reduction (2 g COD L^{-1}). After the pre-incubation period, the dye was added with a syringe from the stock solution to a final concentration of 0.3 mM. The serum bottles were further incubated at 37 °C in a rotary shaker at 120 rpm, for 1 day. All the experiments were prepared in triplicate.

Biological Ery B degradation

For the anaerobic biological dye degradation, batch assays were performed in the same conditions as described before. Various parameters that could affect the reaction were evaluated: biomass concentration (0.94, 1.89, 3.77, and 4.71 g VSS L^{-1}); dye concentration (0.1–0.6 mM); substrate (VFAs, glucose, lactose, 2 g COD L^{-1}) and biomass induction with dye (0.03 mM: 10 % of the use in decolourisation assay). The effect of activated carbon (AC) as redox mediator was also studied by incubation with 0.1 g L^{-1} and 0.5 g L^{-1} of commercial and surface modified AC. In these assays, the amount of biomass used was 0.94 or 3.77 g VSS L^{-1}. In order to evaluate the dye adsorption on AC, assays in the same conditions but without biomass were conducted in parallel. Isotherms of adsorption on AC were also determined according to Potgieter (1991), with AC_0 concentrations between 0.1 and 0.5 g L^{-1} and dye concentration of 0.3 mM. Batch experiments using 0.3 mM of Ery in the presence or absence of 0.1 g L^{-1} of AC_0, in the same conditions but at pH 6, were also run with the aim of evaluating how the pH decrease to six could affect the reactions.

Adsorption of Ery B on biomass

With the purpose of estimating the dye adsorption on biomass, batch assays were performed in the same conditions as described previously, using 0.3 mM of Ery B, with inactive (autoclaved at 120 °C, for 1 h) or inhibited biomass (3.77 g VSS L^{-1}). Inhibition was done by immersing the biomass (previous washed with PBS—phosphate buffered saline) in 2.5 % (v/v) of a glutaraldehyde solution and 37 % of formaldehyde. These two aldehyde fixatives are frequently used in light and electron microscopy (Artvinli 1975).

Fixation stabilizes and cross-links organic molecules within the cellular material, thus preserving the morphology and making the cells more resistant to subsequent treatments.

Glutaraldehyde significantly changes the physical, chemical, and biological characteristics of proteins, although its penetration is slow and heterogeneous.

It decreases the affinity of cell wall proteins for acid dyes, probably as a result of the rapid blocking of amino groups of the proteins. This effect may also occur with tissue proteins. Contrary, formaldehyde penetrates the tissues homogeneously, rapidly terminates the catabolic reactions, and only slightly denatures the proteins (Artvinli 1975). Thus, glutaraldehyde–formaldehyde

solution can be suitable for tissue preparative techniques when the fixation time is reduced.

VFAs were used as substrate. Biological solutions decolorization with active biomass (3.77 g VSS L^{-1}) but without nutrients and carbon source was also assayed.

Chemical Ery B reductions

Batch assay were performed for chemical reduction of Ery using Na_2S. The effect of pH was evaluated in the range of 4–10. Batch assays were performed in 120 mL serum bottles closed with butyl rubber stopper, containing 50 mL of 100 mM Britton Robinson (BR) buffer (100 mM phosphoric acid, 100 mM boric acid and 100 mM acetic acid titrated to the desired pH with 0.5 M NaOH). The bottles were flushed with the N_2:CO_2 (80/20 v/v) and a volume to give the Na_2S desired concentration (in the range 1–6 mM) was added with a syringe. The bottles were pre-incubated overnight at 37 °C, in a rotary shaker at 120 rpm. After the pre-incubation period, 0.3 mM of dye was added with a syringe from the stock solution. The serum bottles were further incubated at 37 °C in a rotary shaker at 120 rpm. The process reaction was monitored visually. In order to study the effect of sodium sulphide concentration, an assay with higher amount, 100 mM, was also conducted, at pH 7, and the reaction was monitored spectrophotometricaly.

Analytical techniques

Color decrease was monitored spectrophotometricaly in a 96-well plate reader (ELISA BIO-TEK, Izasa). At select intervals, samples were withdrawn (300 μL), centrifuged at 5000 rpm for 10 min to remove the biomass and/or AC and diluted, with the same buffer as of the reaction, to obtain less than one absorbance unit (AU), due to the high absorbance of the dye, even at low concentrations. The visible spectra (300–900 nm) were recorded and dye concentration calculated at λ_{max}. Molar extinction coefficients were determined for each dye at λ_{max}: $\varepsilon_{524nm} = 67{,}282\ M^{-1}\ cm^{-1}$ for Ery; $\varepsilon_{510nm} = 60{,}826\ M^{-1}\ cm^{-1}$ for Eos; $\varepsilon_{480nm} = 9{,}600\ M^{-1}\ cm^{-1}$ for AO7; $\varepsilon_{540nm} = 28{,}637\ M^{-1}\ cm^{-1}$ for RR2; $\varepsilon_{350nm} = 15{,}519\ M^{-1}\ cm^{-1}$ for MY10; $\varepsilon_{590nm} = 76{,}716\ M^{-1}\ cm^{-1}$ for DB71; $\varepsilon_{660nm} = 12{,}813\ M^{-1}\ cm^{-1}$ for DB19 and $\varepsilon_{510nm} = 4{,}934\ M^{-1}\ cm^{-1}$ for RR120. All the experiments were prepared in triplicate.

Color removal (R) was calculated according to Eq. 1:

$$R(\%) = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

where A_0, is the absorbance for λ_{max} at the beginning of incubation and A_t, the absorbance at λ_{max} at a selected time (t).

First-order reduction rate constants were calculated in OriginPro 6.1 software, applying the equation $C_t = C_o + C_i e^{-kt}$, where C_t is the concentration at time t; C_o, the offset; C_i, the concentration at time initial time; k, the first-order rate constant (h^{-1}) and t is the accumulated time of the experiment.

HPLC analyses were performed in a HPLC (JASCO AS-2057 Plus) equipped with a DAD (Diode Array Detector) detector. A C18 reverse phase Nucleodur MNC18 (250 × 4.0 mm, 5 μM particle size and pore of 100 Å from Machenerey-Nagel, Switzerland) column was used. The following solvent systems were used as mobile phase: solvent A (ACN) and solvent B (Sodium acetate buffer, pH 5.3). Compounds were eluted at a flow rate of 0.7 mL min^{-1} and at room temperature, with a linear gradient of mobile phase from 10 to 100 % of solvent A, over 15 min, followed by isocratic condition with 100 % of solvent A over 10 min. Compounds elution was monitored at λ_{max} of each dye and 230 nm.

Results and discussion

Dye biodegradation

The studies regarding azo dye anaerobic reduction indicated that the process is a non-specific and presumably extracellular process, in which reducing equivalents from either biological or chemical source are transferred to the dye (van der Zee et al. 2000, 2003; Ong et al. 2005; Karatas et al. 2009). All azo dyes tested in our experiments were, at a different extent, reductively degraded by the granular biomass (Table 1).

Previous studies carried out by van der Zee et al. (2000, 2003) on azo dyes chemical decolorization using sulphide pointed out the presence of a lag phase: the reaction rates were initially slow, but accelerated

Table 1 Screening for the biological decolorization of different dye solutions (0.3 mM of dye). Batch assays were conducted at 37 °C and 120 rpm (The amount of anaerobic granular biomass was 0.94 g VSS L^{-1}; as electron donor was used VFAs)

Dye	Class	Decolorization (%)
Acid orange 7	Azo	73 ± 3
Direct black 19	Azo	26 ± 6
Direct blue 71	Azo	69 ± 2
Mordant yellow 10	Azo	87 ± 1
Reactive red 2	Azo	55 ± 1
Reactive red 120	Azo	87 ± 4
Erythrosine B	Xanthene	20 ± 4
Eosin Y	Xanthene	7 ± 1

in time according to the extent to which the dye was reduced. Based on these observations, it was hypothesized that products of azo dye reduction may increase the rate of the reduction process, i.e., the reaction has an autocatalytic nature.

In our study, biodegradation of all dyes proceeded without lag periods and followed the first-order kinetics, since decolorization in anaerobic sludge environments is a combined process of biotic and abiotic reactions. Ong et al. (2005) shows that azo dyes can be reduced in a direct chemical reaction with bulk biogenic reducing agents, but they can also be reduced by biological reactions, either directly as an enzymatically catalyzed reaction or indirectly via reduced enzyme cofactors. The indirectly biological reduction (redox mediator catalyzed) of the dye, which acts as a redox mediator, may determine the increment of the biodegradation rate. A similar situation was discussed by van der Zee et al. (2003).

Better results were obtained for MY10 and RR120, ~90 %. High degradation degrees were also obtained for AO7 and DB71, ~70 %, and, at less extent, for RR2, 55 %. The poliazo DB19 was the azo dye degraded at lower extent, 26 %.

The xanthene dyes tested were more recalcitrant, with color decrease of merely 20 % and 7 %, for Ery and Eos, respectively. Most of the decolorization studies using anaerobic sludge bacteria concentrate on azo dye containing solutions. Due to the lack of information available on xanthene dyes biodegradation, Ery B, though the low degradation obtained in the conditions tested, was chosen as a substrate for further studies. Only one work on Ery B biodegradation is available (Pereira et al. 2009). Jesus et al. (2010) results on dye removal by the fungus *Neurospora crassa* 74A suggested the possibility of biodegradation in proportion to the contact time between the dye and the fungal biomass. Itoh and Yatome (2004) have reported the aerobic biodegradation of six other xanthene dyes also by a white rot fungus, *Coriolus versicolor*, but as compared to our results with anaerobic bacterial biomass, where color removal occurred in few hours, their process took some days. Indeed, the decolorization of fluorescein, 4-aminofluorescein, and 5-aminofluorescein, all containing –OH groups, with growing cells was 79, 51, and 52 % after 7 days and 85, 95, and 92 % after 14 days incubation, respectively. With the other three xanthene dyes that they have tested, Rhodamine B, Rhodamine 123 hydrate, and Rhodamine 6G, not containing OH groups, color removal was not observed. With cell free extracts of *Coriolous versicolor* only the first three dye solutions were decolorized as well. Oxidation of the dyes was attributed to the enzymatic action of a laccase. Enzymatic oxidation of Rhodamine B by a *Trametis versicolor* laccase was reported by Khammuang and Sarnthima (2009). Xanthene dyes oxidation by a horseradish peroxidase (HRP) was found to occur only in the presence of the natural compound indole-3-acetic acid (IAA) in O_2-containing solutions (Krilov and Chebotareva 1993). More recently, Lan et al. (2006) have reported on the degradation of Rhodamine B with a lignin peroxidase coupled with glucose oxidase.

Biological degradation of Ery B

Effect of biomass amount

Degradation of Ery B was studied at different initial sludge concentrations. The UV–visible spectra with 1.89 g VSS L^{-1} of biomass show a decrease of the maximum band, corresponding to 46 % of color removal. No new peaks corresponding to products formation were observed in the spectrum recorded after the treatment, 24 h (Fig. 2a). In the HPLC chromatogram, a decrease of the peak at the retention time (R_t) of 10.1 min, corresponding to Ery, was also obtained and 70 % of decolorization calculated (the decolorization calculated value were not presented in Fig. 2a). Lower degrees of decolorization by spectrophotometry are expected since the color of substrate

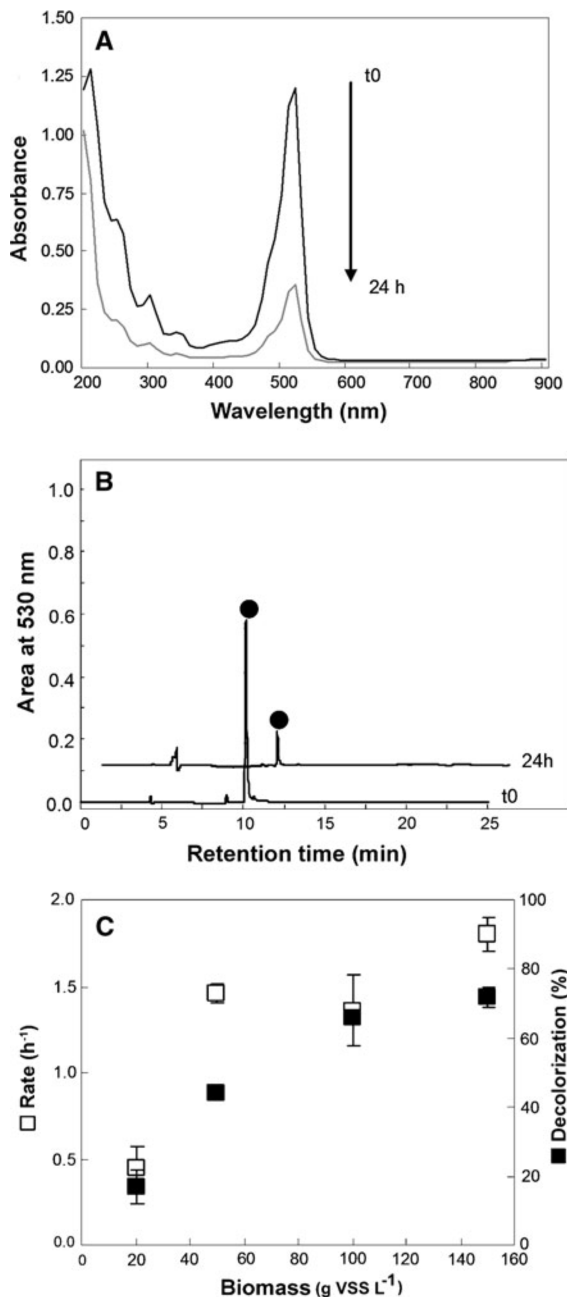

Fig. 2 UV–visible spectra of the of 0.3 mM Erythrosine solution biological decolorization, with 1.89 g VSS L^{-1} of biomass (**a**); degree (*filled square*) and rates (*open square*) of Erythrosine B (0.3 mM) solution biological decolorization at increasing biomass concentration **(b)** and at increasing dye concentrations (biomass concentration of 3.77 g VSS L^{-1}) (**c**)

and products can contribute to the absorbance. On the other hand, by HPLC, the dye is chromatographically separated from products of the enzymatic reaction (Pereira et al. 2009). The relationship between biomass amount, dye degradation rate and percentage is presented in Fig. 2b. As can be observed from Fig. 2c, color decrease displayed a saturation type of dependency with regard to sludge concentration, the removal percentage of dye increased with the increased of biomass amount and then leveled at ~70 % for higher than 3.77 g VSS L^{-1}. The first order degradation rates have also increased with increase of the initial biomass concentration from 0.94 g VSS L^{-1} to 1.89 g VSS L^{-1}, but for higher amounts it was maintained constant (1.5 h^{-1}), even though the higher color removal degree with 3.77 g VSS L^{-1} and 4.71 g VSS L^{-1} of biomass. Those results suggest the occurrence of two phenomena, dye enzymatic degradation, and adsorption. Indeed, after centrifugation, the granules were slightly colored. Several researchers have pointed out the capacity of various microbial biomasses (bacteria, yeasts, fungi, and algae) to absorb or accumulate dyes (Pearce et al. 2003; Crini 2006), which seems to take place essentially at the cell wall level. The main advantages of biosorption are its high selectivity and efficiency, good removal from large volumes and the potential cost effectiveness. Moreover, both living and dead biomasses can be used to remove hazardous organics. However, with living organisms higher decolorization of dyes aqueous solutions may be achieved due to the combination of both phenomena: adsorption and enzymatic degradation (Bakshi et al. 1999; Aretxaga et al. 2001). A saturation type curve of azo dye removal with increasing biomass concentration was also observed by Kalyuzhnyi et al. (2006). They have explained that, taking into account a regenerating function of sludge for reducing agents participating in azo dye splitting. Under increasing sludge concentration, hence, increasing metabolic activity per volume unit, the concentration and transport of reducing agents became less and less rate limiting for extracellular azo dye reduction.

Effect of electron donor

The primary electron donor may play a role in the biodegradation reactions in individual cultures by changing and stimulating enzyme activities and, in mixed cultures, by stimulating specific trophic groups of microorganisms (Van der Zee and Villaverde 2005). Some primary substrates may be better suitable

for delivering reducing equivalents to the dyes during degradation reactions, either because of the substrate itself or because of the microorganisms involved. Ery B biodegradation was tested with three different substrates (mixture VFA, glucose, and lactose) in order to study the effect of electron donor. The biomass concentration was 0.94 g VSS L^{-1}, to diminish the dye removal by adsorption. Color removal from the solution occurred with all the tested substrates, which suggests that the process is relatively non-specific with respect to its electron donor. Comparing the three substrates, better results were obtained with glucose and lactose. For those two substrates, similar degrees of decolorization (~35 %) and rates (~1.0 h^{-1}) were obtained (Table 2). With VFAs as substrate the degree and rate of decolorization was half of the obtained with glucose and lactose. This finding is in agreement with previous studies that investigated the role of various electron donors on the reduction of dyes, concluding that the rates vary with the type of substrate by stimulating specific microorganisms in a mixed culture (Van der Zee et al. 2001; Dos Santos et al. 2003). Li et al. (1999) have also concluded that the carbon sources could affect the biodegradation rates of the xanthene dye rose bengal. The use of different substrates may also affect the enzymatic reaction that take place for dye degradation, once also different enzymes may be involved in the reaction.

Effect of biomass induction with dye

The pre-incubation with lower amounts of dye (or other compounds) may activate (constitutive enzymatic system) or induce (inducible or adaptive enzymes) the genes for the expression of the enzymes involved on its degradation. Incubation is also important for living cells adaptation and sometimes can even decrease the toxicity exerted by the compound. We have pre-incubated biomass with 0.03 mM of Ery (10 % of the concentration tested in the further biodegradation assays). All the three co-substrates were again tested. Although the induction decreased the process efficiency with the substrates glucose and lactose (only ~25 % color removal), the rate was similar as with non induced biomass (~1 h^{-1}) with glucose as substrate and slightly higher (~1.5 h^{-1}) with lactose. The extent of solution decolorization with VFAs was maintained as in non-induced system (~20 %), but biomass pre-incubation led to a ~threefold rate increase (1.4 ± 0.2 h^{-1}) (Table 2). With VFA, decolorization is almost the same (17–20 %) though that the rates almost triplicate. This may be due to the fact that the equilibrium is reached faster, but at the final of the experiment, the extent of decolorization is the same. With glucose and lactose, the extent of decolorization decreases. This may be due to experimental results: if we look to all results (Tables 1, 2, 3, 4, and 5), the extent of decolorization with biomass (without AC), independent of the conditions, is always ~20–30 %.

Since the differences between induced and non-induced biomass capacity for Ery B removal are very low, with the exception of reaction rate increase with VFAs as substrate, constitutive enzymes may be involved in the biodegradation. The rate increase may be due to the adaptation of the biomass to the dye, prior to the decolorization process. Constitutive fungal enzymes, involved in the degradation of the xanthene dye Rose Bengal, could also be stimulated by the pre-incubation with the dye (Li et al. 1999).

Table 2 Effect of different co-substrates on biological Erythrosine B (0.3 mM) solution decolorization with non-induced and induced biomass (0.03 mM of dye) (the amount of biomass was 0.94 g VSS L^{-1})

Substrate	Decolorization (%)		Rate (h^{-1})	
	No induction	Induction	No induction	Induction
VFA	17 ± 5	20 ± 5	0.5 ± 0.1	1.4 ± 0.2
Glucose	36 ± 3	25 ± 1	1.2 ± 0.1	1.1 ± 0.2
Lactose	38 ± 2	23 ± 1	0.8 ± 0.2	1.5 ± 0.3

Activated carbon as redox mediator

The transfer of reducing equivalents from a primary electron donor (co-substrate) to a terminal electron acceptor (azo dye) generally acts as the process rate limiting step in anaerobic azo dye reduction (Van der Zee et al. 2003). The large majority of the studies indicated the use of water-soluble compounds as redox mediators for azo dye reduction. Some investigations demonstrated redox-mediating properties of non-soluble materials.

The addition of redox mediators like quinones and flavine-based compounds has been shown to accelerate this electron transfer, and higher degradation rates can be achieved in bioreactors (Dos Santos et al. 2003;

Cervantes et al. 2001). In recent studies it is specified that activated carbon, graphite, and alginate accelerate azo dye reduction in biological systems (Mezohegyi et al. (2007); Van der Zee et al. (2003); Van der Zee et al. 2003; Pereira et al. 2010; Guo et al. (2007); Van der Zee and Cervantes (2009)). Van der Zee and Cervantes (2009) indicated that activated carbon or other insoluble materials with surface-associated or entrapped redox-active functional groups can be considered valuable insoluble redox mediators.

The biological degradation results of Ery B in the presence of 0.1 g L^{-1} and 0.5 g L^{-1} of a commercial (AC_0) and structurally modified AC are presented in Tables 3 and 4. The first two lines from Table 3 presented the data obtained for Ery adsorption using two AC dosages (in the absence of biomass), line 3 and 4 presented the values obtained for Ery decolorization in the presence of two biomass concentration, but in the absence of AC, the last two lines include the data obtained in the experiments conducted both in the presence of biomass and AC.

Table 3 Degrees and rates of Erythrosine B (0.3 mM) solution decolorization with different AC_0 concentration (0.1 and 0.5 g L^{-1}) and biomass (0.94 and 3.77 g VSS L^{-1})

Conditions	Decolorization (%)	Rate (h^{-1})
AC (0.1 g L^{-1}) no biomass	10 ± 3	0.34 ± 0.18
AC (0.5 g L^{-1}) no biomass	46 ± 1	0.59 ± 0.04
Biomass (0.94 g VSS L^{-1}) no AC	20 ± 4	0.26 ± 0.05
Biomass (3.77 g VSS L^{-1}) no AC	66 ± 1	1.36 ± 0.20
AC (0.1 g L^{-1}) + biomass (0.94 g VSS L^{-1})	26 ± 3	0.61 ± 0.01
AC (0.5 g L^{-1}) + biomass (0.94 g VSS L^{-1})	56 ± 3	0.63 ± 0.15
AC (0.1 g L^{-1}) + biomass (3.77 g VSS L^{-1})	72 ± 3	1.44 ± 0.32
AC (0.5 g L^{-1}) + biomass (3.77 g VSS L^{-1})	80 ± 2	1.30 ± 0.21

Although the similar degree of Ery B solution decolorization with 0.94 g VSS L^{-1} of biomass either in the non-mediated or mediated (0.1 g L^{-1} AC_0) assays, the rate almost duplicated which suggests that enzymatic degradation occurs. With higher biomass concentration, 3.77 g VSS L^{-1}, degrees and rates of solution decolorization were independent of the presence of AC_0. It is worth to mention that the use of five times more AC does not led to an increase of the rates, but lead to an increase of color removal from the medium, specially with 0.94 g VSS L^{-1} of biomass (almost the double), probably due to adsorption phenomena. Indeed, in the adsorption assays only with the commercial AC_0, a ~fivefold increase of dye adsorption on the material was obtained by increasing the AC concentration from 0.1 to 0.5 g L^{-1}. The use of small amounts is of utmost importance due to the excessive costs of AC (Pereira et al., 2010). In the biological assays with different treated AC samples, the degree of solution color decrease is very similar for all the conditions (25–30 %), except with AC_{HNO_3} with lower extent, only 15 %, but the rates increased with the increase of sample basicity, i.e., with increase of pH_{pzc} (Table 4). As can be seen in Table 4, the presence of AC accelerates the reaction. With AC_{HNO_3} and AC_{O_2}, there is no adsorption (only 1–3 % decolorization). If the decolorization extent in the presence of the different AC samples is always in the same order and the rate increases (two to threefold), that means that a reaction occurs.

In the presence of AC_{H_2}, the rate is almost the triple as in the absence of AC and the double as compared with other treated samples. The pH_{pzc} (pH of the point of zero charge) is a critical value for determining quantitatively the net charge (positive or negative) carried on the AC surface as a function of the solution pH. The assay was performed at pH 7 and

Table 4 Biological decolorization of Erythrosine B solution (0.3 mM) with different AC samples (0.1 g L^{1}). Biomass concentration of 0.94 g VSS L^{-1}

AC sample	No biomass	Biomass	
	% Decolorization	% Decolorization	Rate (h^{-1})
No AC	–	20 ± 4	0.26 ± 0.05
AC_{HNO_3} (pH_{pzc} = 2.7)	3 ± 3	15 ± 1	0.44 ± 0.00
AC_{O_2} (pH_{pzc} = 4.5)	1 ± 1	24 ± 1	0.62 ± 0.23
AC_0 (pH_{pzc} = 8.4)	10 ± 1	23 ± 3	0.61 ± 0.01
AC_{H_2} (pH_{pzc} = 9.2)	12 ± 4	29 ± 1	0.90 ± 0.03

for pH > pH_{pzc}, AC becomes negatively charged, what is the case of AC_{HNO_3} and AC_{O_2} (Pereira et al. 2010). Once the dye is anionic, the repulsion between AC and dye will increase. These phenomena impede the electron changes and, consequently, the adsorption of the dye on AC. On the other hand, when pH < pH_{pzc}, AC becomes positively charged (as the case of AC_0 and AC_{H_2}) and attraction between AC and anionic dye occurs, resulting an increase in dye adsorption. Indeed, in the controls without biomass, decolorization was obtained, though at low degree, only in the solutions containing AC_0 and AC_{H_2} (~10 %).

The isotherms of Ery adsorption on AC_0 were also determined. The Langmuir (K_L) and Freundlich constants (K_F) and the correlation coefficients are presented in Table 5. The values of the correlation coefficients, ~0.99, indicated that data fit well to both isotherms model. The essential feature of the Langmuir isotherm can be expressed by means of R_L, a dimensionless constant referred to as separation factor or equilibrium parameter. The values of R_L indicate the shape of isotherms to be either favorable ($0 < R_L < 1$), unfavorable ($R_L > 1$), linear ($R_L = 1$) or irreversible ($R_L = 0$). The R_L values obtained in our study, 0.148, indicated that Ery adsorption on AC_0 was a favorable process. The Freundlich constant K_F and n are the indicative of the extent of the adsorption and the degree of nonlinearity between solution concentration and adsorption. The slope range between 0 and 1 is a measure of surface heterogeneity, becoming more heterogeneous as its value gets closer to zero. The sorption intensity is given by n. The value of n above one, 3.44, means that the adsorption took place by chemical interactions between dye and AC.

Table 5 Adsorption of Erythrosine B on Activated Carbon (AC_0): Langmuir (K_L) and Freundlich (K_F) isotherm constants. *Xm* is the limiting amount of adsorbate that can be taken up per mass of adsorbent (mg Ery g^{-1} AC) and n is a constant of the Freundlich isotherm

Isotherm type	Parameter			
	Xm	K_L	R_L	R^2
Langmuir	416.66	0.023	0.148	0.9809
		K_F	*n*	R^2
Freundlich		75.24	3.44	0.9824

Effect of pH

The effect of pH on Ery B degradation was tested at pH values of 6.0 and 7.0, in the presence and absence of activated carbon (AC_0). The ideal pH range for anaerobic digestion is very narrow, between 6.8 and 7.2. The growth rate of methanogens is greatly reduced below pH 6.6 (Mosey and Fernandes 1989). Though the optimal pH of methanogenesis being around 7.0, the optimum pH of hydrolysis and acidogenesis has been reported in the range between pH 5.5 and 6.5 (Adebiyi et al. 2011; Sohrabnezhad et al. 2010). By those reasons, we have only tested this two pH values, 6.0 and 7.0. In the dye solutions without AC, the decolorization percentage was three-fold higher at pH 6.0, 36 ± 3 %, as compared with the decolorization at pH 7.0, 13 ± 4 %, (data not shown). The decrease of the pH has also duplicated the rate of the reaction: 0.85 ± 0.11 h^{-1} and 0.42 ± 0.05 h^{-1} at pH 6.0 and pH 7.0, respectively. The presence of AC in the reaction solution did not affect significantly either the decolorization percentage or reaction rates at pH 6.0 (37 ± 4 % and 0.88 ± 0.14 h^{-1}). Contrarily, at pH 7.0, an increase of both was obtained when the mediator was present (26 ± 3 % and 0.61 ± 0.01 h^{-1}). Though, the results were worse than the obtained with lower pH.

Adsorption of Ery B on biomass

In biotic assay without carbon source and nutrients, the removal percentage of Ery B from the medium was lower than in the presence of nutrients, 47 ± 5 and 68 ± 1 %, respectively (data not shown). The result suggests the occurrence of both phenomena on the dye decrease, adsorption, and enzymatic degradation. Even without the addition of exogenous sources of carbon and electrons, sludge can use endogenous energy reserves for dye removal, justifying the 47 %. Additionally, biodegradable organics may be introduced within the sludge. When dye removal is only due to the physical process of adsorption, the color is eliminated, but the dye remains in the solid phase. With both phenomena, first the dye is adsorbed and then enzymatically degraded by the cells. In the abiotic experiments, with autoclaved biomass, the degree of dye removal is the same as with active biomass, 69 ± 1 %. This result may lead to the opposite conclusion that dye removal derives only

from the adsorption on cells. Although, the process of autoclaving may change the cell membranes of the microorganisms and also the granular sludge morphology facilitating the adsorption of dye, as compared with active and in perfect conditions cells. Interestingly, when the biomass was inhibited with gluteraldehyde and formaldehyde, the color removal from the solution was much higher, 90 ± 1 %. The inhibition process may have caused changes in cell membrane, such as zeta potential (i.e., the surface charge), which have affected the dye adsorption and also degradation. To explain this result, further studies of cell microbiology should be made.

Effect of dye concentration

The kinetic of Ery degradation by 3.77 g VSS L^{-1} of biomass was studied with different initial dye concentrations (Fig. 3a). The other xanthene dye with similar structure, Eosin Y, was also tested for comparison (Fig. 3b). The difference between the molecules is the substitution of the four iodine atoms in Ery to four bromide atoms in Eosin. It is merit to make a note that, contrarily to the observed with Ery, the centrifugation of the reaction medium containing Eos, the granules were colorless, suggesting that Eos did not adsorbed on cells during the process. In terms of kinetics, similar behavior was observed for both dyes, first-order rates increased with increasing the dye concentration followed by a decrease at higher dye amounts, 0.4 mM of Ery and 0.9 mM of Eos. The maximal rates were 1.08 ± 0.09 h^{-1} for Ery and 2.97 ± 0.22 h^{-1} for Eos. Inhibitory effect at high dye concentration occurred with both dyes, being higher for Ery. In anaerobic processes, dyes are used as final electron compounds and the co-substrate as electron donors, at higher dye concentrations probably the bacteria can use also dyes as co-substrate and a competition between both substrates may result in kinetics inhibition. Other possibility may be the toxicity exerted by the dyes when used at high levels. Inhibition kinetics was also reported by other authors for the degradation of azo (Pereira et al. 2009) and anthraquinone dyes (Adebiyi et al. 2011). In this study, a complex system is present, where more than one microorganism from the sludge and many enzymes may be involved, instead of a single enzymatic reaction.

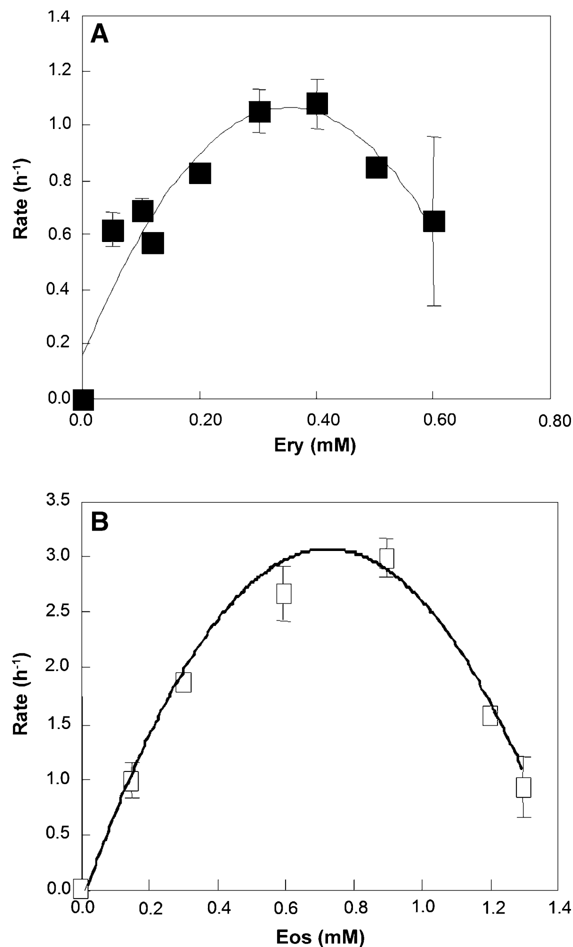

Fig. 3 Rates of Erythrosine B (**a**) and Eosine (**b**) decolorization at increasing dye concentrations. Biomass concentration of 3.77 g VSS L^{-1}

Chemical reduction of Ery B

Chemical Ery B reduction was tested with sulphide at different pH values. By a visual screening of the dyed solution, no significant decolorization was observed at concentrations of 1 mM to 6 mM of Na_2S. Increasing the Na_2S to 100 mM, the color changed from pink to purple. In the visible spectra, a decrease of the absorbance of the dye was followed by an increase of absorbance at around 600 nm reaching the equilibrium after 20 h, due to the formed products of dye reduction (data not shown). The percentage of color removal was 43 ± 6 %. The results confirm that the xanthene dye Ery B is a high recalcitrant dye towards anaerobic reduction, once a very high amount of sulphide is necessary to cause its degradation. As

postulated by other authors, for the reduction of azo dyes by sulphide, according to the stoichiometry of dye reduction by sulphide, 2 mol of sulphide are required per mole of azo dye when sulphide is oxidized to elemental sulphur (Van der Zee et al. 2003; Pereira et al. 2010; Sohrabnezhad et al. 2010). For xanthene dye reduction, any previous work is available.

Conclusions

A screening of dye biodegradation with anaerobic granular biomass showed its capacity to degrade all the azo dyes tested. In the similar reaction conditions, the two xanthene dyes, Ery and Eos, were degraded at lower extent. Many factors were studied, for the first time, on the biological Ery dye reduction. With increasing biomass amounts, maximum color removal efficiency of 72 % was achieved with 4.71 g VSS L^{-1} biomass. Inhibition at high dye concentration was observed with a maximum rate at 0.4 mM. Substrate inhibition was also obtained with Eos Y, for concentrations higher than 0.9 mM. No considerable effects using different substrates as electron donors (VFA, glucose or lactose) were obtained. Induction of biomass with little amounts of Ery B decreased the percentage dye removal from the reaction solution, but accelerated the reaction. Although the similar degree of Ery B degradation with 0.94 g VSS L^{-1} of biomass either in the non-mediated or mediated (0.1 g L^{-1} AC_0) assays, the rate almost duplicated in the presence of the catalyst, which suggests that enzymatic degradation occurs. That phenomenon was not observed for higher biomass concentration. The use of five times more AC does not led to an increase of the rates, but an increase of color removal was achieved. Using different modified AC samples, better results occurred with the most basic one, 1.5-fold and threefold higher rates with AC_{H_2}, as compared with non-treated AC (AC_0) and non-mediated reaction, respectively. Decreasing the pH from 7.0 to 6.0 resulted in a threefold improvement of Ery degradation rate. Chemically Ery B reduction occurred only with high Na_2S concentration, 100 mM. Our results led to the conclusion that decolorization of Ery containing solutions may be due to enzymatic and adsorption phenomena. Biodegradation products will be provided in future publications.

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