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Application of cellulose-immobilized riboflavin as a redox mediator for anaerobic degradation of a model azo dye Remazol Golden Yellow RNL

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ABSTRACT

This study described the anaerobic degradation of the azo dye Remazol Golden Yellow RNL (RGY-RNL) using cellulose-immobilized riboflavin (MC 3) as the redox mediator. This new solid support containing immobilized riboflavin was synthesized from succinylated mercerized cellulose, and was characterized by elemental analysis, FTIR, and solid-state ^{13}C NMR. MC 3 was resistant to pH 2–9, and anaerobic degradation of RGY-RNL using MC 3 in the presence of anaerobic sludge yielded a zero order degradation rate constant ($k_{0,obs}$) equal to 0.189 mg/L h, which was 56% better than experiments carried out without a redox mediator. Color removal efficiency after 48 h of degradation averaged 89.4% in experiments with MC 3 and 72% without the addition of a redox mediator. These results showed that MC 3 can be used to immobilize redox mediators, allowing reduction of wastewater treatment costs.

1. Introduction

Highly-colored effluents generated by different types of industries, especially the textile sector, are a matter of great environmental interest (Field and Brady, 2003; Méndez-Paz et al., 2005). The textile industry employs several steps to give the fabrics the properties and characteristics desired in the final product, of which dyeing is one of the most important, and it is estimated that 10–15% of dyes used in the dyeing process do not adhere to the fibers and end up in the textile effluent (Corrêa et al., 2009). Apart from their potential toxicity, dyes and pigments are recalcitrant (resistant to degradation) and may remain in the environment for long periods, accumulating in water and soil if not suitably treated before being discharged (dos Santos et al., 2007).

Most dyes used for textile industries (60–70%) are aromatic azo compounds, which bear the functional group R—N=N—R′, in which R and R′ are usually substituted aromatic compounds (van der Zee et al., 2003). Azo dyes are removed by a non-specific reduction mechanism under anaerobic conditions, but the low rate of degradation is the primary issue when adopting anaerobic reactors to remove these compounds from industrial wastewater (van der Zee et al., 2001a).

Studies have indicated the main limitation for azo dye reduction under anaerobic conditions is transferring the reducing equivalents generated by the cells during the metabolic process to the azo dye (Field and Brady, 2003). Redox mediators (RM) such as riboflavin and quinone groups, can improve this electron transfer, and studies have been performed to find cheap sources of such catalysts or for immobilizing them onto solid supports (Alvarez et al., 2010; Cervantes et al., 2011; dos Santos et al., 2004; Field and Brady, 2003; Martínez et al., 2013). Riboflavin is soluble in water and normally lost in the treated wastewater, while immobilization onto an insoluble solid support would reduce costs by allowing the recovery and reuse of riboflavin.

Cellulose is the most abundant renewable biopolymer in nature. This homopolymer of β-D-glucopiranose units can undergo chemical modifications through the reaction of its primary and secondary hydroxyl functional groups, and such chemical modifications have yielded new materials with specific physicochemical properties (Alvarez et al., 2010). Gurgel et al. (2008) and Gurgel and Gil (2009) prepared modified cellulose containing internal carboxylic acid anhydride functional groups through the reaction of succinylated cellulose (containing carboxylic acid groups) with acetic anhydride. Such functional groups are excellent electrophiles that can react with nucleophilic compounds containing amine or hydroxyl

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functional groups, resulting in the formation of amide or ester linkages on the modified cellulose.

In this study, succinylated mercerized cellulose was reacted with acetic anhydride to yield internal carboxylic acid anhydride functional groups used to incorporate riboflavin (Rib) onto the solid support through the formation of ester linkages. The efficiency of this new solid support as a redox mediator was evaluated during anaerobic degradation of the model azo dye Remazol Golden Yellow RNL (RGY-RNL).

2. Materials and methods

2.1. Materials

Microcrystalline cellulose and sulfanilic acid (≥99.0%) were purchased from Sigma-Aldrich (cat. No. 31,069-7 and S5263, respectively, Brazil). Riboflavin (Fig. 1a), succinic anhydride, acetic anhydride, acetic acid, pyridine, sodium hydroxide, ethanol, acetone, and methylene chloride were purchased

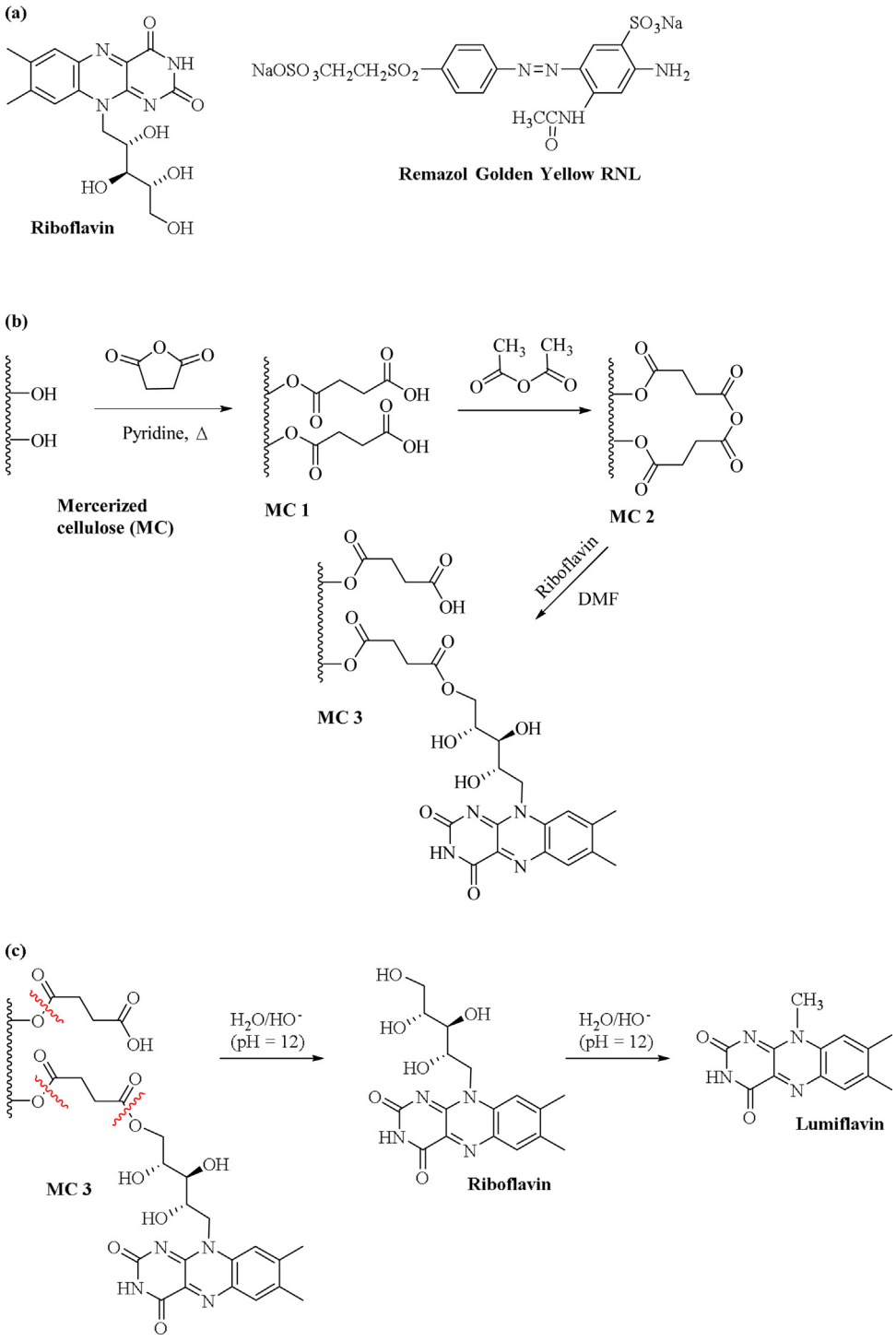

Fig. 1. (a) chemical structures of riboflavin (Rib) and Remazol Golden Yellow RNL (RGY-RNL) azo dye, (b) synthetic route used to incorporate riboflavin onto mercerized cellulose and (c) treatment used to hydrolyze (at pH 12) riboflavin attached onto MC 3 yielding lumiflavin.

from Vetec (Brazil). Hydrochloric acid, diethyl ether, *N*,*N*′-dimethylformamide (DMF), and dimethylsulfoxide (DMSO) were purchased from Synth (Brazil). Remazol Golden Yellow RNL (RGY-RNL, $C_{16}H_{18}N_4O_{10}S_3Na_2$, Fig. 1a) azo dye was kindly provided by a local textile industry and used without further purification. Yeast extract containing 50 μg/g of riboflavin was purchased from Himedia.

2.2. Apparatus and operational conditions

Flat-bottom amber glass bottles (250 mL) with polypropylene caps were used as reactors and inoculated with anaerobic sludge from a small-scale UASB reactor at the Center of Research and Training on Sanitation (CePTS) UFMG/COPASA, located at the Arrudas Wastewater Treatment Plant (WWTP) in Belo Horizonte, Brazil. The nutrient solution contained glucose (except in the experiments 5, 10, and 11), RGY-RNL (except in the experiments 1–3), yeast extract (except in the experiments 1–4 and 6–11), riboflavin (except in the experiments 1, 2, 4, and 8–11) and a macronutrient solution (composition is described in Supplementary Table 1). Redox mediators tested were MC 3 (cellulose-immobilized riboflavin), riboflavin and yeast extract (containing ~50 μg riboflavin per g of yeast extract). The efficiency of color removal following Remazol Golden Yellow RNL degradation was assessed by measuring the absorbance (λ_{max} = 410 nm) of the centrifuged suspension with a UV–vis spectrophotometer (model HP 8453).

2.2.1. Methods

The experiments (except the modification of microcrystalline cellulose) were performed in triplicate. Pyridine was refluxed with NaOH pellets overnight and distilled. *N*,*N*′-Dimethylformamide (DMF) was treated with 4 Å molecular sieves (Merck) overnight and distilled under reduced pressure. Succinic anhydride, acetic anhydride, and dimethyl sulfoxide (DMSO) were used without further purification.

2.2.2. Chemical modification of microcrystalline cellulose

2.2.2.1. Mercerization. Microcrystalline cellulose (15 g) was added to a polyethylene beaker containing 400 mL of 20% (w/v) NaOH and the suspension stirred for 18 h at 25 °C. The suspension was filtered (sintered disk filter funnel, porosity 3) and the retentate washed with distilled water until pH 7 was reached in the wash. The mercerized microcrystalline cellulose (MC) was washed with 95% ethanol and dried at 85 °C for 90 min.

2.2.2.2. Preparation of MC 1. MC (11.66 g) and succinic anhydride (34.8 g) were placed in a round-bottom flask, anhydrous pyridine (233 mL) added and the suspension heated at 120 °C with constant stirring for 6 h. The succinylated mercerized cellulose (MC 1) was separated by filtration (sintered glass funnel, porosity 3) and washed successively with a solution of 1 mol/L acetic acid in methylene chloride, 95% ethanol, distilled water, 0.01 mol/L HCl, distilled water, and acetone. The MC 1 was dried at 80 °C for 1 h.

2.2.2.3. Preparation of MC 2. MC 1 (9.4 g) and acetic anhydride (265 mL) were added to a round-bottom flask and heated at 100 °C for 24 h with constant stirring. The suspension was separated by filtration (sintered glass funnel, porosity 3), washed with diethyl ether (previously treated with a 4 Å molecular sieve) and dried at 100 °C for 20 min.

2.2.2.4. Preparation of MC 3. MC 2 (produced from 9.4 g of MC 1) and riboflavin (2.35 g) were placed in a round-bottom flask, anhydrous DMF (140 mL) added and the suspension heated at 75 °C for 24 h under constant magnetic stirring. MC 3 was separated by filtration (sintered glass funnel, porosity 3), washed with DMF, DMSO, an excess of distilled water, 95% ethanol, and acetone, and dried at 80 °C for 30 min. The water wash was monitored at 267 nm using an UV–vis spectrophotometer, which corresponded to the maximum absorption of riboflavin in aqueous medium. The synthetic route used to prepare MC 3 is shown in Fig. 1.

2.2.3. Characterization of the materials

The synthetized materials (previously dried in an oven at 90 °C) were characterized by weight gain, elemental analysis, FTIR and ^{13}C NMR. For FTIR characterization, 1 mg of the material was mixed with 100 mg of KBr (spectroscopy grade) and spectra recorded from 400 to 4000 cm^{-1} with 32 scans at a resolution of 4 cm^{-1}. Elemental analysis was performed on a CHNS/O Perkin Elmer Series II, model 2400 analyzer. ^{13}C NMR (solid state) of MC 3 was recorded on a Bruker Avance III-400 spectrometer at room temperature and measurements obtained at frequencies of 100 MHz with magic angle spinning of 5 kHz using the CPTOSS technique.

2.2.4. Evaluation of chemical stability of MC 3 as a function of pH

As ester functional groups (used to incorporate riboflavin onto MC 3) can be hydrolyzed at pH values below 2 and greater than 9, the chemical stability of MC 3 was assessed in aqueous solutions as a function of pH. For this, 20.0 mg portions of MC 3 were added to 250 mL Erlenmeyer flasks, 100.0 mL of HCl or NaOH aqueous solutions at pH 2, 9, 12, or 14 were added to individual flasks and stirred (100 rpm) for 24 h at 25 °C. The suspensions were separated by filtration (sintered glass funnel, porosity 3) and thoroughly washed with distilled water. The absorbance of the

Table 1
Experimental conditions used to evaluate the stability of MC 3, the adsorption of RGY-RNL on anaerobic sludge, the influence of the nutritional medium and the use of different redox mediators on color removal.

Experiment	MC 3 concentration (mg/L)	Riboflavin concentration (mg/L)	Yeast extract (mg/L)	Glucose concentration (mg/L)	Biomass concentration (mg/L)	Nutrient solution (mL)
1 (Control)[a]	–	–	–	250	650	10
2[a]	50	–	–	250	650	10
3[a]	–	3.56	–	250	650	10
4 (Control)[b]	–	–	–	250	650	10
5[b]	0.245	–	–	250	–	10
6[b]	0.245	–	–	–	–	–
7[a,b]	0.245	–	–	–	650[c]	10
8 (Control)[b]	–	–	–	250	650	10
9[b]	–	–	350	–	650	10
10[b]	0.245	–	–	250	650	10
11[b]	–	0.0175	–	250	650	10

[a] Concentration of biomass (expressed as volatile suspended solids—VSS) in the stock anaerobic sludge = 12.66 g/L.
[b] Remazol Golden Yellow RNL concentration was 50 mg/L in all reaction flasks.
[c] The biomass was autoclaved.

washes was measured on a UV–vis spectrophotometer (model HP 8453) at 267 nm and 354 nm, which corresponded to the maximum absorbance wavelengths of riboflavin in acidic and basic aqueous media, respectively.

2.2.5. Determination of riboflavin concentration on MC 3

Riboflavin in aqueous solutions pH 9 and above, is predominantly in the form of lumiflavin (Fig. 1c) (Penzkofer, 2012). The amount of riboflavin incorporated on MC 3 was assessed by hydrolyzing the ester bond used to attach riboflavin onto the succinylated mercerized cellulose at basic pH. MC 3 (31.0 mg) was placed in a 250 mL Erlenmeyer flask, 50.0 mL of a 0.1 mol/L NaOH solution (pH 12) added and shaken (100 rpm) for 24 h at 25 °C. The suspension was centrifuged and the absorbance of the yellowish supernatant was measured on a UV–vis spectrophotometer at 354 nm, the maximum absorption wavelength (λ_{max}) for lumiflavin at pH 12, and the concentration of lumiflavin estimated by comparison to a calibration curve.

2.2.6. Evaluation of the stability of MC 3 in the reaction flaks

To verify the chemical stability of MC 3 under the experimental conditions used, experiments were carried out in the presence of all components of the reaction medium (with exception of RGY-RNL), and Table 1 shows the experimental conditions used. After the incubation period (24 or 48 h), samples were removed, centrifuged and the absorbance measured on a UV–vis spectrophotometer at 267 and 354 nm, the maximum absorption wavelengths (λ_{max}) for riboflavin in acidic and basic aqueous media, respectively.

2.2.7. Influence of the reaction medium on the degradation of RGY-RNL dye

Experiments were performed to determine whether color removal occurred by adsorption of RGY-RNL onto the anaerobic sludge by inoculating flasks with autoclaved (121 °C for 20 min) anaerobic sludge. The influence of reaction medium components (e.g. nutrient solution) and whether their adsorption onto MC 3 affected color removal efficiency were also evaluated. Experimental conditions are described in Table 1.

2.2.8. Anaerobic degradation of Remazol Golden Yellow RNL (RGY-RNL)

Experiments were performed to (1) evaluate the influence of MC 3 on anaerobic degradation of Remazol Golden Yellow RNL, (2) compare the performance of MC 3 in relation to well-known redox mediators (riboflavin and yeast extract), and (3) investigate the contribution of RGY-RNL adsorption onto MC 3 and/or anaerobic sludge. All experiments were carried out in triplicate and the degradation of Remazol Golden Yellow RNL was monitored by measuring the absorbance of the supernatants at 410 nm after centrifugation at 3600 rpm for 20 min.

Batch experiments were performed in inoculated reaction flasks with a total volume of 200 mL (nutritional conditions shown in Supplementary Table 1, Chernicharo (2007)) and experimental conditions set so the COD:N:P ratio was ~350:5:1 (concentration of macro- and micronutrients in the solution according to Aquino et al., 2007). The concentration of anaerobic biomass (collected from a small-scale UASB reactor fed with sewage) was estimated by analysis of volatile suspended solids (VSS) according to APHA (Clesceri et al., 1998). The initial concentration of biomass, RGY-RNL and yeast extract in the reaction flasks were fixed at 650 mg/L, 50 mg/L, and 350 mg/L, respectively. Concentrations of glucose, RGY-RNL, yeast extract, nutrient solution, and microorganisms in the reaction flasks were determined from optimization studies realized in our research group. In experiments performed with riboflavin or MC 3 as the redox mediators, the amount of riboflavin was proportional to that contained in reaction flasks in which yeast extract was used as the redox mediator. Control flasks were inoculated with active biomass without the redox mediator (RM).

All reaction flasks were purged with nitrogen (White Martins, 99.999% purity), sealed, incubated at 25 °C with mechanic stirring (150 rpm) and monitored for 48 h by sampling (sampling frequency varied with the type of experiment) 3 mL of the content by using plastic syringes. Table 1 shows the conditions used in batch experiments for the anaerobic degradation of Remazol Golden Yellow RNL assisted by redox mediators (riboflavin, yeast extract, and MC 3).

2.2.9. Aromatic amines analysis

Aromatic amines generated in the anaerobic degradation of Remazol Yellow Gold RNL were analyzed by high performance liquid chromatography (HPLC) using Shimadzu chromatography equipped with a diode array UV–vis detector set at 191 nm and an ion exchange column Aminex HPX-87H (300 × 7.8 mm, Bio-Rad) at 55 °C with 0.01 mol/L H_2SO_4 as eluent and a flow rate of 0.6 mL/min according to Baêta et al. (2013). Samples were centrifuged before injection (30 μL). The separated aromatic amines generated as byproducts of sulfonated azo dye degradation (e.g. sulfanilic acid derivatives) can be detected at 191 nm according to Pinheiro et al. (2004).

3. Results and discussion

3.1. Synthesis and characterization of solid supports

3.1.1. Synthesis and characterization of MC 1

Microcrystalline cellulose was mercerized to convert cellulose I into cellulose II. This rearrangement of crystal packing increases the separation of chains, providing easier access to the hydroxyl groups of cellulose, reducing packing efficiency, facilitating penetration of succinic anhydride, promoting a higher degree of substitution and more uniform succinylation due to a greater number of available hydroxyl groups (Gurgel et al., 2008). Mercerized microcrystalline cellulose was reacted with succinic anhydride using pyridine as the solvent and catalyst, which yielded succinylated mercerized cellulose (MC 1) (Gurgel et al., 2008). The weight gain percentage after succinylation was calculated as follows:

$$\text{wgp}\ \ (\%) = \left(\frac{m_f - m_i}{m_i}\right) \times 100 \quad (1)$$

where m_f (g) is the weight of modified mercerized cellulose and m_i is the weight of mercerized cellulose.

The weight gain percentage (wgp) obtained after succinylation was 105.2%, due to the incorporation of succinyl functional groups through the formation of ester linkages with the primary and secondary hydroxyl groups of cellulose, which released carboxylic acid groups (Fig. 1b). The number of carboxylic groups (n_{COOH}) released after succinylation reaction was estimated at 7.1 mmol/g by back-titration (Karnitz et al., 2007).

MC 1 was characterized by FTIR, and the spectra of MC and MC 1 are shown in Fig. 2a. When comparing the FTIR spectrum of MC 1 with the spectrum of unmodified mercerized cellulose (MC), the appearance of bands at 2964 and 2924 cm^{-1} can be attributed to asymmetric and symmetric stretching of methylene groups (—CH_2—), while the bands at 1425 and 1160 cm^{-1} were attributed to deformation vibration of hydroxyl groups and the stretching of C—O groups from the dimer in carboxylic acid. A strong band at 1739 cm^{-1} was attributed to asymmetric and symmetric stretching of the ester bond carbonyl group (—O—C═O) introduced by succinylation (Gurgel et al., 2008). These bands confirmed the incorporation of succinic anhydride through ester linkages with a consequent release of carboxylic acid groups.

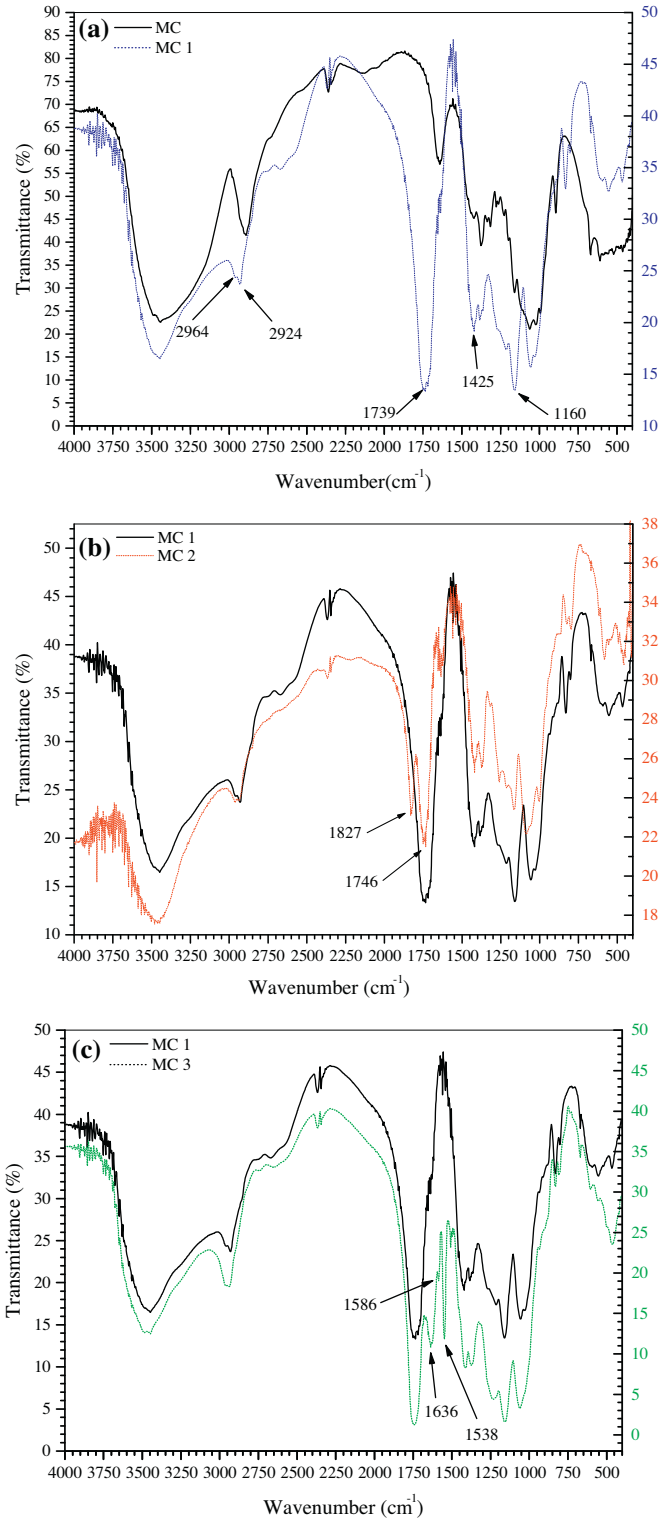

Fig. 2. (a) FTIR spectra of MC and MC 1, (b) MC 1 and MC 2, and (c) MC 1 and MC 3.

3.1.2. Synthesis and characterization of MC 2

Succinylated mercerized cellulose (MC 1) was reacted with acetic anhydride (solvent and dehydrating agent) to promote the formation of internal anhydride functional groups from carboxylic acid groups, yielding MC 2. These internal anhydride groups were excellent electrophiles capable of reacting with nucleophilic groups such as the primary hydroxyl groups of riboflavin, which were better nucleophiles than secondary hydroxyl groups (Fig. 1b). In this study, the nucleophile was the primary hydroxyl group riboflavin and the reaction of riboflavin with MC 2 allowed incorporation of riboflavin onto the solid support through formation of an ester bond.

The synthesis of MC 2 was confirmed through FTIR analysis and the spectra of MC 2 and MC 1 are shown in Fig. 2b. When comparing the spectrum of MC 2 to the spectrum of MC 1, the appearance of bands at 1827 and 1746 cm^{-1} were attributed to the formation of carboxylic acid anhydride functional groups. In general, these two bands were separated by a maximum wavelength range of 81 cm^{-1} which is characteristic of the presence of a functional anhydride group (Nakanishi and Solomon, 1977). The changes seen in the spectrum of MC 2 (with respect to MC 1) confirmed the formation of internal anhydride functional groups on the solid support.

3.1.3. Synthesis and characterization of MC 3

MC 3 was obtained by the reaction of MC 2 with riboflavin using DMF as the solvent. The concentration of riboflavin chemically bonded to MC 3 was assessed by hydrolyzing the ester bond in an aqueous solution at pH 12 (Fig. 1c) and quantifying lumiflavin (a riboflavin byproduct at pH 12, Fig. 1c). Analysis indicated a lumiflavin concentration in the supernatant of 44.19 mg/L, and considering all lumiflavin in the supernatant was released by hydrolysis of MC 3, riboflavin content was 71.3 mg/g.

MC 3 was characterized by infrared spectroscopy with FTIR, ^{13}C NMR and elemental analysis. The FTIR spectra of MC 1 and MC 3 are shown in Fig. 2c, where the three bands at 1548; 1586 and 1636 cm^{-1} were due to the incorporation of riboflavin. Abe et al. (1986) studied the infrared spectra of riboflavin and its derivatives, indicating the bands at 1552, 1580 and 1583 cm^{-1} could be attributed to stretching vibrations of $C_{24}{=}N_{25}$ and $C_{19}{=}N_{20}$ bonds in the isoalloxazin unit rings (see Fig. 3), while the band at 1636 cm^{-1} was due to vibrations in the frequency of the isoalloxazin ring carbonyl group ($C_{23}{=}O$).

The elemental analysis for MC 1 and MC 3 revealed carbon, hydrogen, and nitrogen contents of 43.71 and 35.04%, 5.20 and 3.99%, and 0.27 and 2.25%, respectively. These results showed an increase in nitrogen content after modification of MC 1 with riboflavin to produce MC 3, which confirmed the incorporation of riboflavin into cellulosic matrix. The solid-state ^{13}C NMR (CP/MAS) spectrum of MC 3 is shown in Fig. 3. The ^{13}C NMR spectra of MC 3 exhibited chemical shifts attributed to the presence of cellulose and riboflavin in the chemical structure of this material (Keller et al., 1983; Melo et al., 2011). The signals at 105 and 90 ppm were related to the involvement of the C-1 and C-4 carbons (respectively) in the acetal bond of the $\beta(1 \rightarrow 4)$-D-glucopiranose units of cellulose. The signal at 75 ppm was attributed to secondary carbons bonded to the hydroxyl groups of riboflavin (C-14, C-15, and C-16), cellulose (C-2 and C-3) and to the tertiary carbon of the cellulose structure (C-5). As a consequence of succinylation, the modified cellulose contained ester bonds formed after esterification of the primary hydroxyl groups in the glucopiranose units of cellulose. Thus, the signal at 66 ppm was related to the C-6 of cellulose, whereas the signal at 175 ppm was attributed to carbonyl groups at C-1′, C-4′, C-8, and C-11. Additionally, the signal at 66 ppm indicated the C-13 from riboflavin, which was involved in the ester bond linking riboflavin to the succinylated cellulose through the primary hydroxyl groups of riboflavin at C-13. The carbons in the riboflavin aromatic rings appeared as broad signals from 110 to 150 ppm, while the chemical shifts at 125, 137, 155, and 160 ppm were related to the presence of aromatic rings in the chemical structure of MC 3, which indicated the chemical modification of MC 2 to bind riboflavin. These data and published studies evaluating succinylated cellulose (Gurgel and Gil, 2009; Melo et al., 2011) clearly demonstrated the incorporation of riboflavin onto cellulose through the formation of ester bonds.

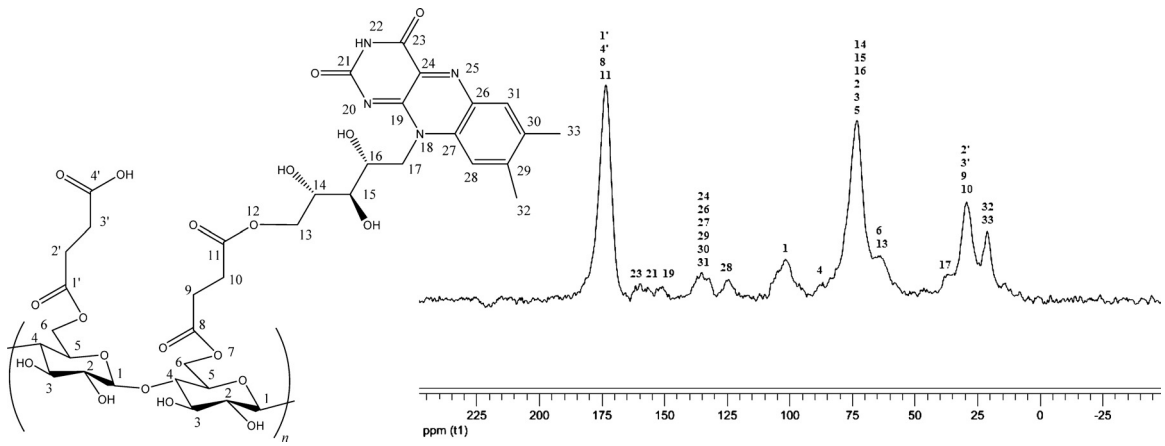

Fig. 3. Solid-state ^{13}C CP/MAS NMR spectrum of MC 3.

3.2. Evaluation of the stability of MC 3 as a function of pH

MC 3 was treated with an aqueous HCl solution (pH = 2) for 24 h, and no signal was seen at 267 nm for the washes, which confirmed the ester bond used to attach riboflavin onto MC 3 was not cleaved at pH 2 (MC 3 was stable at pH ≥ 2). Treatment of MC 3 with an aqueous NaOH solution (pH = 9) for 24 h also showed no signal at 354 nm in the washes. However, at pH 11 and 12, the ester bond was hydrolyzed, which could be verified by the appearance of peaks at 354 nm in the washings. These observations indicated the ester bond used to attach riboflavin onto MC 3 was stable at pH 2–9.

3.3. Influence of the reaction medium on the stability of MC 3

Experiments were carried out to evaluate the influence of the reaction medium on MC 3 stability, and were performed in the presence of all components of the reaction medium with exception of RGY-RNL. No riboflavin was released from MC 3 (by hydrolysis of ester bond) under the experimental conditions used (Table 2). If a release had occurred, there would have been an increase in absorbance over time for the supernatants of experiment 2 (MC 3 was added). The absorbance of supernatants from experiment 3 was twice as high as those observed in experiment 2, which indicated that if riboflavin were released, it would have been detected.

3.4. Influence of the reaction medium on degradation of RGY-RNL dye

These experiments were carried out to evaluate RGY-RNL removal by adsorption onto anaerobic sludge and/or reaction with medium components. Adsorption of the RGY-RNL dye onto anaerobic sludge (experiment 7), MC 3 (experiment 6) or medium components (experiment 5) did not result in significant decolorization. Reasonable dye (color) removal only occurred in experiment 4 due to RGY-RNL reduction by anaerobic microorganisms in the absence of a redox mediator.

Table 2
Influence of reaction medium on the stability of MC 3.

Experiment[a]	Absorbance (267 nm)		
	t = 0 h	t = 24 h	t = 48 h
1[b]	0.224	0.217	0.215
2[c]	0.252	0.216	0.238
3[d]	0.504	0.470	0.492

[a] All experiments were carried out with anaerobic sludge and without RGY-RNL.
[b] Control flasks, incubated without MC 3.
[c] Experiments carried out with MC 3.
[d] Experiments carried out with riboflavin instead of MC 3.

3.5. Anaerobic degradation of RGY-RNL dye using MC 3 as the redox mediator

In addition to the experiments evaluating anaerobic degradation of RGY-RNL dye using MC 3 as redox mediator (experiment 10), experiments using redox mediators such as soluble riboflavin (experiment 11) and yeast extract (experiment 9) were performed. The effect of each redox mediator on the decolorization of RGY-RNL in an anaerobic system is shown in Fig. 4.

According to dos Santos (2005), color removal of azo dyes in anaerobic systems without the addition of redox mediators varies from 60 to 80%. Corrêa et al. (2009) reported that color removal efficiencies of the azo dye blue Drimarem HF-RL in anaerobic systems varies 39–45% within the first 24 h, with a final degradation efficiency of 91% after 150 h. van der Zee et al. (2001a) reported a 30% color removal efficiency in a bench-scale UASB reactor kept at 30 °C without redox mediators and fed with the azo dye reactive red (RR2), while a 95% color removal efficiency was seen for different azo dyes in anaerobic systems in less than 6 days (van der Zee et al., 2001b).

In this study, removal efficiency of RGY-RNL by anaerobic biomass in the absence of RM was 30.7% within the first 24 h and

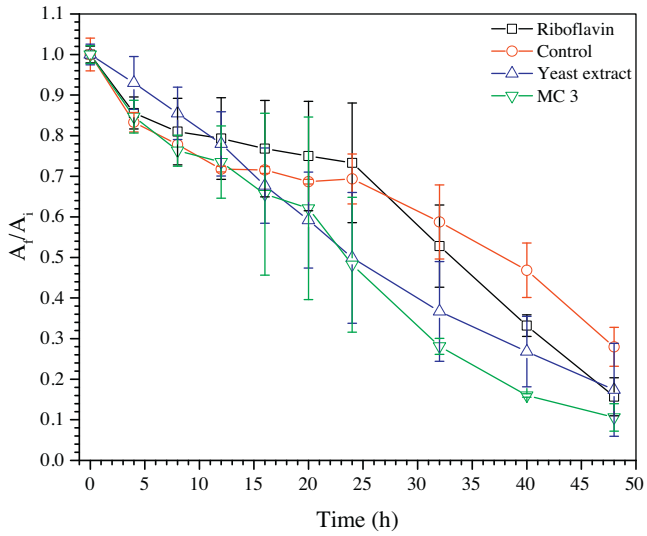

Fig. 4. Anaerobic degradation of azo dye RGY-RNL using different redox mediators.

72.1% after 48 h in batch mode at 25 °C, which were in agreement with results reported by dos Santos (2005). It should be noted that the maximum removal efficiency in this study was seen after 48 h, while over 144 h of degradation time was required in the study of Corrêa et al. (2009).

The use of redox mediators such as anthraquinone sulfonate (AQS), disulfonated anthraquinone (AQDS), yeast extract, and riboflavin (among others) have led to significant improvements in azo dye removal kinetics under anaerobic conditions (Baêta et al., 2012; Corrêa et al., 2009; Costa et al., 2010; Field and Brady, 2003; dos Santos, 2005). Costa et al. (2010) reported a 2.27-fold increase in the degradation kinetic rate constant for reactive red 2 (RR2) azo dye in anaerobic reactors fed with AQDS. van der Zee et al. (2001a) demonstrated an increase in color removal from 30% to 88% by adding AQDS as the catalyst in an anaerobic system used for the degradation of RR2 dye. dos Santos (2005) found a 2.9-fold increase in the RR2 color removal rate in reaction flasks that received AQS when compared to flasks without. When yeast extract was used as the redox mediator (riboflavin) in the degradation of blue azo dye Drimaren HF-RL, Corrêa et al. (2009) and Baêta et al. (2012) report color removal efficiencies up to 87% within the first 24 h of degradation, and a final removal efficiency higher than 90% after 150 h. Field and Brady (2003) found the degradation rate of Yellow mordant 10 dye increases 61% with the addition of riboflavin in anaerobic systems operating in batch mode.

RGY-RNL color removal efficiency increased from 30.7 to 50.1% within the first 24 h after addition of yeast extract (source of the redox mediator riboflavin), which increased to 82.6% after 48 h. When riboflavin was added, degradation of RGY-RNL dye was 26.7% within the first 24 h, which increased to 84.3% after 48 h. These results were similar to those reported by Corrêa et al. (2009) for the same times of dye degradation. Possible explanations for the differences between the results of this study and those reported in literature may due to factors such as the chemical structure of the dyes used in the experiments, the ratio of dye to redox mediator (can cause differences in the transfer rate of electrons between RM and dye) and even the electron donors used.

The degradation rate constant for RGY-RNL dye using MC3 was improved by 1.56-fold when compared with experiments without addition of redox mediators and followed the zero-order kinetic model. This result is similar to those reported in the literature. Several authors evaluated the use of immobilized redox mediators to improve the kinetics of azo dye decolorization in anaerobic treatment systems. Three factors are important to compare various reducing systems using immobilized redox mediators such as type of solid support, redox mediator for immobilization, and the structure of dyes. Cervantes et al. (2010) has improved the dye removal rate constant of methyl orange by 8.8-fold using NQS immobilized onto ion exchange R1 type resin and by 1.9-fold using NQS immobilized onto ion exchange R2 type resin. AQDS immobilized to nanoparticles of $Al(OH)_3$ also improved the dye degradation rate constant for reactive red 2 (RR2) by 7.5-fold, while AQDS immobilized onto ion exchange resin improved the dye degradation constant 1.9-fold (Alvarez et al., 2010). Humic substances (HS) attached to ion exchange resin (AER) increase degradation rates for RR2 by 2-fold (Cervantes et al., 2011). Martínez et al. (2013) reported the degradation of RR2 using immobilized HS supported on an AER in an upflow anaerobic sludge reactor (UASB). The decolorization efficiency of RR2 increased ~90% in comparison with the control UASB reactor operated without immobilized HS. Amezquita-Garcia et al. (2014) investigated the anchorage of two anthraquinones (AAQ and AQDS) on surface of polyacrilonitrile activated carbon fibers (ACFs) to obtain materials with redox functional groups on their surface to be applied for the treatment of contaminants with electron withdrawing groups. These authors reported that only AQDS anchored on ACFs was an effective redox mediator improving the reductive conversion of 4-nitrophenol (4NP) to 4-aminophenol. The catalytic properties of AQDS-ACFs improved the reduction of 4NP by 49% in comparison with ACFs alone. Many studies have shown the influence of the structure of dyes in the anaerobic reduction process using immobilized redox mediators. Yuan et al. (2012) have immobilized AQS on ceramsites and found an increase of degradation rate constant for Acid dye yellow 36 (AY36) by 8.0-fold, while for RR2, Acid red 27 (AR27), and Acid orange 7 (AO7), the increases in degradation rate were 2.3, 2.7, and 2.8-fold, respectively, when compared to experiments carried out without redox mediator. Guo et al. (2007) reported that anthraquinone immobilized on calcium alginate beads had an increase in decolorization rates for reactive brilliant red K-2BP, reactive brilliant red X-3B, acid black 10B, acid scarlet GR, acid red B and Acid red G of 1.5–2, 1.57, 1.88, 2.1, 1.65, and 1.48-fold, respectively. Lu et al. (2010) also reported AQS immobilization on polyurethane foam (PUF) and found an increase in degradation rates for RR2 by 4-fold, while Zhou et al. (2014) investigated decolorization of Reactive red K-2G using AQS immobilized on PUF in an upflow anaerobic bioreactor. These authors reported improvements in decolorization efficiencies by 1.47-fold in comparison with the control.

The results obtained in this study indicated a removal efficiency of 51.8% for anaerobic degradation of RGY-RNL dye in the presence of riboflavin immobilized onto MC 3 after 24 h of incubation. This result was similar to those experiments using yeast extract as the source of redox mediators (50.1%), twice as high as when soluble riboflavin was used (26.7%), and higher than those which did not receive any redox mediator (control flasks, 30.7%). After 48 h, the color removal efficiency in the flasks without redox mediator was 72.1 ± 0.1 and 89.4 ± 0.0% for those incubated with MC 3, which was higher than when yeast extract (82.6%) and riboflavin (84.3%) were used as redox mediators.

In comparison to other studies on azo dyes such as RR2 (van der Zee et al., 2001a) and Blue Drimaren HF-RL (Corrêa et al., 2009) which were degraded anaerobically for 24 h without redox mediators, the percentages of color removal obtained in this study in the presence of MC 3 were significantly higher. This study indicated MC 3 was as efficient as riboflavin and yeast extract in the anaerobic decolorization of RGY-RNL, and could be employed for the anaerobic degradation of other azo dyes.

In order to provide evidence of the reduction process of the RGY-RNL dye mediated by immobilized riboflavin derivative (MC 3) through the azo cleavage, the released aromatic amines were analyzed by HPLC. According to Pinheiro et al. (2004), the degradation of azo dyes containing sulfonic acid groups such as the model dye used in this study generates as byproducts aromatic amines with sulfonic groups such as analogous of sulfanilic acid. Baêta et al. (2015, 2013) have studied the anaerobic degradation of RGY-RNL dye with powdered activated carbon and evaluated the byproducts released. The possible degradation byproducts of RGY-RNL dye were evaluated as sulfanilic acid derivatives (Baêta et al., 2013). Fig. 5 shows the chromatograms of samples collected from anaerobic degradation experiments, which were carried out in the same conditions described in Section 2.2.8. These experiments were accomplished with and without (control) addition of MC 3. Standards solutions of sulfanilic acid and volatile fatty acids (VFA) (intermediates in anaerobic digestion) (Baêta et al., 2013) were also analyzed by HPLC. The nature of byproducts generated in the experiments of anaerobic degradation of RGY-RNL dye was suggested by comparing retention times of the standard solution of sulfanilic acid and samples subjected to anaerobic degradation using MC 3 as RM (Fig. 5). The retention time of sulfanilic acid was very close to those aromatic amines released in the anaerobic degradation of RGY-RNL dye in the presence of MC 3. Similar observations were reported by Baêta et al. (2013) for the anaerobic degradation of RGY-RNL in an

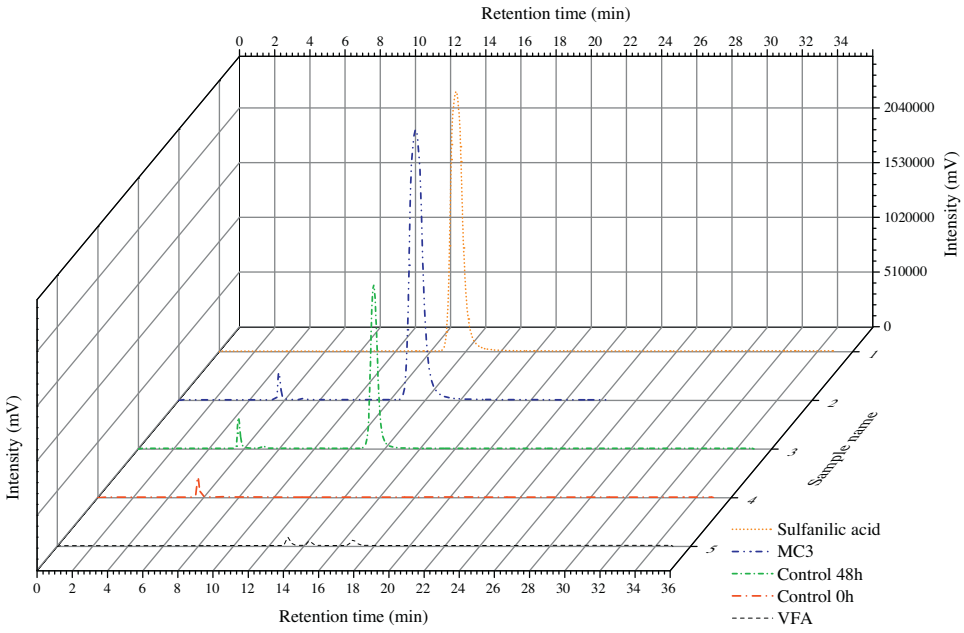

Fig. 5. Chromatogram (signal at λ = 191 nm) of standard solutions and supernatant of anaerobic degradation of RGY-RNL dye with MC 3 in batch mode. Standard VFA solution (5); control 0 h (4); control 48 h (3); MC 3 (2), and standard sulfanilic acid solution (1).

Table 3
Parameters obtained from the linear fit for zero-order and first-order kinetic models for anaerobic degradation of RGY-RNL.

Experiment	Kinetic model	R^2	$k_{0,obs}$ (mg/L h)	$k_{1,obs}$ (h^{-1})	$t_{1/2}$ (h)
Control	Zero-order	0.9291	0.0121	–	41.3
MC 3	Zero-order	0.9826	0.0189	–	26.4
Riboflavin	Zero-order	0.9369	0.0158	–	31.6
Yeast extract	First-order	0.9822	–	0.0364	19.0

upflow UASB reactor using yeast extract and powdered activated carbon as sources of redox mediators. The azo cleavage of RGY-RNL using MC 3 resulted in the formation of a large amount of sulfanilic acid derivatives than those experiments performed without MC 3 (Fig. 5). This observation proves the efficiency of MC 3 to enhance the electron transfer rate in the reduction of azo bond.

The chemical stability is an important characteristic of an immobilized redox mediator. Most of the studies reported in the literature are related to the immobilization of quinone-based redox mediators on solid supports such as ion exchange resins, calcium alginate, and nanoparticles of metal oxides through adsorption, electrostatic interaction and entrapment techniques and yielded good mechanical strengths during the removal process (Alvarez et al., 2010; Cervantes et al., 2010, 2011; Guo et al., 2007; Martínez et al., 2013). In this study, the mediator redox riboflavin was immobilized on cellulose by a covalent bond. A chemical bond is considered a stronger bond for incorporation of a redox mediator on solid support than immobilization by adsorption, for example. Therefore, the attachment of riboflavin on cellulose can provide good mechanical and chemical properties to this solid support to resist for the anaerobic degradation process. Lu et al. (2010) and Yuan et al. (2012) adopted a similar strategy for the immobilization of redox mediators on polyurethane foam and ceramsite, respectively.

Anaerobic degradation of RGY-RNL dye as a function of time was evaluated using three kinetic models: zero-order, first-order and second-order. Experiments performed without redox mediators (control) and with MC 3 as the redox mediator followed the zero-order kinetic model. Similar results were reported by Field and Brady (2003), where riboflavin was used as the redox mediator in the anaerobic reduction of azo dye mordant yellow 10, where the zero-order kinetic model explained the experimental data. Table 3 summarizes the kinetic results.

The use of MC 3 as the redox mediator increased the zero-order degradation rate constant ($k_{0,obs}$) of RGY-RNL 1.56-fold compared to control (Table 3), which was similar to the $k_{0,obs}$ reported by Field and Brady (2003) for anaerobic degradation of Mordant yellow 10 in the presence of riboflavin. Despite this increase, the color removal rate was lower than those reported in the literature for immobilized redox mediators (Alvarez et al., 2010; Cervantes et al., 2010; Lu et al., 2010). However, the comparison was not straightforward, since Cervantes et al. (2010) and Lu et al. (2010) immobilized different redox mediators (AQDS, humic substances and NQS) on different supports to degrade a different azo dye (RR2).

Summarizing the results obtained in this study, MC 3 was as efficient as riboflavin and yeast extract in the anaerobic decolorization of RGY-RNL dye, and therefore, it could be employed for the anaerobic degradation of other azo dyes. An advantage of MC 3 in relation to riboflavin and yeast extract is that MC 3 can be recovered and reused in the anaerobic degradation process.

4. Conclusions

The results have shown that riboflavin was covalently bound to cellulose and the resulted material (MC3) was chemically stable at pH 2 to 9, covering the pH range for anaerobic microorganisms. It was demonstrated that degradation of RGY-RNL was not due to adsorption or chemical reduction. The use of MC 3 increased the RGY-RNL degradation rate constant by 56%, which was similar when riboflavin or yeast extract were used as redox mediators. The zero-order kinetic model best described the decolorization process

in the presence of MC 3. The use of immobilized redox mediators as MC 3 could allow reduction of wastewater treatment costs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2014.10.059.

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