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Decolorization kinetics of the azo dye Reactive Red 2 under methanogenic conditions: effect of long-term culture acclimation

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Abstract

The biological decolorization of the textile azo dye Reactive Red 2 was investigated using a mixed, mesophilic methanogenic culture, which was developed with mixed liquor obtained from a mesophilic, municipal anaerobic digester and enriched by feeding a mixture of dextrin/peptone as well as media containing salts, trace metals and vitamins. Batch decolorization assays were conducted with the unacclimated methanogenic culture and dye decolorization kinetics were determined as a function of initial dye, biomass, and carbon source concentrations. Dye decolorization was inhibited at initial dye concentrations higher than 100 mg l^{-1} and decolorization kinetics were described based on the Haldane model. The effect of long-term culture exposure to the reactive dye on decolorization kinetics, culture acclimation, as well as possible dye mineralization was tested using two reactors fed weekly for two years with an initial dye concentration of 300 mg l^{-1} and a mixture of dextrin/peptone. The maximum dye decolorization rate after a 2-year acclimation at an initial dye concentration of 300 mg l^{-1} was more than 10-fold higher as compared to that obtained with the unacclimated culture. Aniline and the *o*-aminohydroxynaphthalene derivative resulting from the reductive azo bond cleavage of the dye were detected, but further transformation(s) leading to dye mineralization were not observed. Reactive Red 2 did not serve as the carbon and energy source for the mixed culture, and dye decolorization was sustained by the continuous addition of dextrin and peptone. Thus, biological decolorization of reactive azo dyes is feasible under conditions of low redox potential created and maintained in overall methanogenic systems, but supply of a biodegradable carbon source is necessary.

Introduction

Azo dyes are the most common synthetic dyes used in textile, printing, pharmaceutical, cosmetic and food industries. Reactive dyes, the only textile colorants designed to bond covalently with cellulosic fibers (i.e. cotton), are extensively used in the textile industry because of the variety of their color shades, high wet fastness profiles, ease of application, brilliant colors, and minimal energy consumption (Aspland 1997). Due to competition between the cellulosate ($CellO^-$) and OH^- ions in reactive dyebaths, under typical reactive dyeing conditions (pH $\geqslant$10, temperature $\geqslant$ 60 °C, salt 60–100 g l^{-1}), 20–50% of the dye remains in the spent dyebath in its unfixed, hydrolyzed form, which has no affinity for the fabric, resulting in colored effluent (Pearce et al. 2003; Ryes & Zollinger 1989). Thus, the management of spent reactive dyebaths is a pressing environmental problem for the textile industry.

Reactive dyes are both highly water soluble due to a high degree of sulfonation, and non-degradable under the typical aerobic conditions found in conventional, biological treatment systems (Beydilli et al. 2000; Hao et al. 2000; Kalyuzhnyi

& Sklyar 2000; Pagga & Brown 1986; Pagga & Taeger 1994; Pearce et al. 2003; Vandevivere et al. 1998). Additionally, reactive dyes adsorb very poorly to biological solids, resulting in residual color in discharged effluents, and creating major aesthetic problems (Pagga & Taeger 1994; Pierce 1994). In contrast, reductive cleavage of the azo bond results in the decolorization of azo dyes and formation of aromatic amines under anaerobic conditions (Beydilli et al. 2000; McMullan et al. 2001; Nigam et al. 1996; Stolz 2001; Tan et al. 1999; van der Zee et al. 2001a, b). The resulting aromatic amines, which are generally considered to be both toxic and resistant to further degradation under anaerobic conditions, can be degraded under aerobic conditions (FitzGerald & Bishop 1995; Kalyuzhnyi & Sklyar 2000; Rajaguru et al. 2000; Seshadri et al. 1994; Tan et al. 1999). However, the aromatic amines resulting from the reductive decolorization of the azo dye Remazol Brilliant Violet 5R were not degraded in the aerobic phase of an anaerobic-aerobic sequencing batch reactor in spite of a number of operational changes made (such as increased duration of aerobic phase, hydraulic retention time and dye/carbon source concentration ratio) (Lourenco et al. 2003). On the other hand, although limited, the complete degradation of aromatic amines under anaerobic conditions has been reported (Donlon et al. 1997; Razo-Flores et al. 1997). In contrast to the wealth of information regarding the transformation of azo compounds, relatively less is known about the transformation of complex reactive textile azo dyes despite the fact that their use has increased due to the worldwide increase in cotton use. Furthermore, most reported research relates to either simple, model azo compounds or azo dyes with relatively simple molecular structures. Information on the biotransformation of commercially significant reactive azo dyes with complex molecular structures, especially on the long-term effect of such dyes on biological processes, is therefore limited.

The objective of the work presented here was to investigate the biological decolorization of the azo dye Reactive Red 2 (RR2) – a commercially important, monoazo, dichlorotriazinyl reactive dye – under methanogenic conditions. The effect of initial dye, biomass, and carbon source concentration on azo dye decolorization and methanogenic culture activity were evaluated. In addition, the effect of long-term culture exposure to RR2 on dye decolorization kinetics, and possible culture acclimation and dye mineralization were tested. The work is part of a broader investigation into the biological decolorization of spent reactive textile dyebaths and their reuse as process water in closed-loop dyeing operations.

Materials and methods

Dye

Commercial quality RR2 was obtained from BASF Corp., Charlotte, NC and used without any further purification. The dye structure, as well as the results of dye characterization have been reported previously (Beydilli et al. 2000). Previously conducted RR2 decolorization tests under methanogenic conditions using both unreacted and reacted (i.e. hydrolyzed) RR2 resulted in similar rate and extent of decolorization (Beydilli et al. 1998; Beydilli et al. 2000). However, in order to be consistent with the broader investigation regarding the decolorization of spent reactive textile dyebaths, reacted RR2 was used in the present study. Preparation of reacted dye solutions was based on the simulation of typical textile dyebath conditions as follows. Aliquots of 5.0 g dye and 25.0 g Na_2CO_3 were dissolved in deionized water (resulting in pH 11), heated to 80 °C for 1 h and then diluted to 1 l after cooling while adjusting to pH 7 with 0.1 N HCl.

Culture

A methanogenic culture developed with mixed liquor from a mesophilic, municipal anaerobic digester and fed with a mixture containing 4 g l^{-1} dextrin, 2 g l^{-1} peptone and culture media was used in this study. The media contained (in g l^{-1}): K_2HPO_4, 0.9; KH_2PO_4, 0.5; NH_4Cl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.10; $MgCl_2 \cdot 6H_2O$, 0.20; $FeCl_2 \cdot 4H_2O$, 0.10; $NaHCO_3$, 6.7. Also, 10 ml l^{-1} each of vitamin and trace metal stock solutions were added to the media (Beydilli et al. 2000). To avoid any abiotic dye reduction and color removal, the culture medium did not include any chemical reductant. The methanogenic culture was maintained at 35 °C with continuous mixing using a magnetic stirrer, and batch-fed once every 7 days resulting in a retention

time of 35 days. The ultimate digestibility of the dextrin/peptone mixture by the mixed, methanogenic culture measured over a 54-days incubation was 90.4 ± 1.3% (mean ± standard deviation). The specific methane production value was 357 ± 5 ml CH_4 per g COD destroyed at 1 atm and 35 °C. The steady-state gas-phase CH_4 and CO_2 concentration of the methanogenic culture was 58.4 ± 0.6 and 41.6 ± 0.5% (mean ± standard deviation), respectively, and H_2 was not detected.

Decolorization assays

Most of the batch decolorization assays were performed in duplicate, unless otherwise mentioned, following a similar procedure. Biomass taken from the methanogenic stock culture, which was not previously exposed to any dye, was anaerobically transferred into a 9-l glass carboy and starved for 3 days before using it as an inoculum for the decolorization assays. Except for the assays testing the long-term exposure of the methanogenic culture to RR2 using semicontinuously-fed reactors, all assays were performed in 500-ml serum bottles sealed with rubber stoppers and aluminum crimps, pre-flushed with helium gas. Aliquots of pre-starved methanogenic culture were anaerobically transferred to the serum bottles along with a dye stock solution, a bicarbonate solution, and a dextrin/peptone stock solution (used as the carbon source), to arrive at a 400 ml total liquid volume and 585 mg l^{-1} bicarbonate. The initial dye, biomass and dextrin/peptone concentration varied, depending on the type of batch decolorization assay (see below).

A batch assay was conducted to test the effect of initial RR2 concentration. The initial biomass and dextrin/peptone concentration was 2000 mg volatile suspended solids (VSS) l^{-1} and 750/375 mg l^{-1}, respectively, and the RR2 concentration was 50, 300, 500, 1000, and 2000 mg l^{-1}. A control culture (i.e., a culture without dye) was also prepared. An abiotic control was also prepared with only media and RR2. Previously conducted assays with an inactivated methanogenic culture resulted in negligible RR2 adsorption (Beydilli 2001). Single dye addition was used in this assay. In another batch decolorization assay, the effect of initial biomass concentration on the color removal kinetics was tested at three initial concentrations – 2000, 200, and 20 mg VSS l^{-1} – using 300 mg l^{-1} RR2 and 750/375 mg l^{-1} dextrin/peptone along with an abiotic control with only media and RR2. A third bioassay was conducted with an initial biomass and RR2 concentration of 2000 and 300 mg l^{-1}, respectively, and dextrin/peptone was tested at three levels: 100, 10, and 0% (750/375, 75/37.5, and 0/0 mg l^{-1}, respectively). For all batch assays, the culture serum bottles were incubated in the dark at 35 °C, static with only periodic mixing by hand.

In order to test the effect of long-term exposure of the methanogenic culture to RR2, two methanogenic cultures were set up in 2-l glass reactors pre-flushed with helium. Aliquots of 1400 ml methanogenic stock culture along with 235 ml media, reacted RR2 stock solution and dextrin/peptone stock solution were transferred to the two reactors to arrive at a liquid volume of 1750 ml in a total reactor volume of 2260 ml. The initial RR2, dextrin and peptone concentration in these reactors was 300, 686 and 343 mg l^{-1}, respectively. The reactors (designated as R1 and R2) were batch fed every 7 days as follows: 350 ml of mixed liquor was removed and replaced with 105 ml of 5,000 mg l^{-1} reacted RR2 stock solution, 235 ml media and 10 ml of stock dextrin/peptone solution. This feeding schedule resulted in a hydraulic (and solids) retention time of 35 days. Both cultures were maintained at 35 °C and mixed periodically using magnetic stirrers. After more than 2-years of feeding as described above, one of the reactors (R2) was fed only with RR2 and anaerobic media without any dextrin and peptone addition.

In all decolorization assays, the dye concentration was monitored over the incubation period using a spectrophotometer and/or an HPLC unit. Total gas, methane, pH, and oxidation-reduction potential (ORP) were monitored. Volatile fatty acids (VFAs), dissolved organic carbon (DOC) and biomass were also measured over the incubation period in selected bioassays.

Analytical methods

VSS, ORP and pH were measured by following procedures outlined in *Standard Methods* (APHA 1998). Total gas production, methane, VFAs, DOC, and particulate organic carbon (POC) analyses were performed as previously described (Beydilli et al. 1998, 2000). Biomass concentrations were expressed as VSS based on a POC versus VSS calibration curve.

All spectrophotometric analyses were carried out using a UV/Vis Hewlett Packard 8453 spectrophotometer equipped with a diode array detector. Samples analyzed with the spectrophotometer were first centrifuged for 15 min at 14,000 rpm in 1.5 ml polypropylene microcentrifuge tubes and the supernatants were diluted with deionized water. Absorbance spectra between 200 and 700 nm were recorded. Absorbance measurements at the first RR2 maximum absorbance wavelength of 512 nm were also recorded. Spectrophotometric absorbance measurements of decolorized RR2 solutions at 512 nm resulted in residual color attributed to dye decolorization products and/or autoxidation products. However, HPLC analyses of the same samples revealed the complete disappearance of RR2. In addition to monitoring the RR2 concentration, HPLC analyses were carried out to monitor the formation of aromatic amines (aniline and the *o*-aminohydroxynaphthalene derivative) resulting from the reductive azo bond cleavage of the dye.

HPLC analyses were performed using a Hewlett Packard Series 1100 HPLC unit equipped with a diode array detector, a Waters Spherisorb ODS-2 column (25 cm × 4.6 mm ID, 5 μm thickness) and a Waters Spherisorb ODS guard column (1 cm × 4.6 mm ID, 5 μm thickness). The ion-pairing chromatography technique was used and the mobile phase consisted of different ratios of methanol/water solutions with 0.005 M tetrabutylammonium hydroxide (ion-pairing reagent). The following eluent composition (methanol/water, % v/v) was used: 30/70 for the first 5 min, then the ratio of methanol increased and reached 50/50 in 40 min, and was held constant until the end of the run (70 min). The eluent flow rate was 0.8 ml min^{-1} and the sample injection volume was 25 μl. Samples used for HPLC analyses were prepared by centrifugation as described above and the resulting sample supernatants were further filtered through 0.22 μm nylon syringe filters.

Results and discussion

Reactive Red 2 decolorization kinetics

Effect of initial dye concentration

The decolorization of RR2 was tested at an initial concentration range from 50 to 2000 mg l^{-1}. The cultures were monitored for dye concentration for 41 days, and for total gas and methane production for a longer period of 250 days. Within the first 41-day incubation, RR2 at all initial concentrations decolorized completely. The primary reasons for further incubation and monitoring for total gas and methane production were to determine whether further mineralization of the dyes would be observed as well as to determine if recovery of the inhibited methanogenic culture activity after the complete dye decolorization would take place. The pH values for all cultures were within the range of 7.3–7.4 and the ORP values varied between −195 and −251 mV. The initial, volumetric rates of decolorization as a function of initial RR2 concentration are presented in Table 1. As the initial RR2 concentration increased, the initial decolorization rate increased, but decreased at and beyond an RR2 concentration of 300 mg l^{-1}.

After 250 days of incubation, the ratio of total gas (and methane) produced by the RR2-amended cultures to that by the control culture was 1.04(1.02), 1.18(1.15), 0.65(0.60), 0.61(0.52), and 0.63(0.53) for an initial RR2 concentration of 50, 300, 500, 1000, and 2000 mg l^{-1}, respectively. Therefore, a decline in both total gas and methane production was observed at and above an initial RR2 concentration of 500 mg l^{-1}. Methane production was depressed more than total gas production. At higher dye concentrations, decolorization continued in spite of the observed decline in gas and methane production rate. The observed culture inhibition is probably due to the accumulation of dye breakdown products (e.g., aromatic amines which are known to be toxic to methano-

Table 1. Initial volumetric decolorization rate of RR2 in mixed, methanogenic cultures as a function of initial dye concentration[a]

Initial RR2 Concentration (mg l^{-1})	Initial decolorization rate (mg l^{-1} d^{-1})[b]	r^2
50	92.0 ± 3.0	0.998
300	105.6 ± 8.4	0.991
500	99.2 ± 15.2	0.999
1000	90.3 ± 27.0	0.923
2000	87.7 ± 9.4	0.985

[a]Initial biomass and dextrin/peptone concentration 2000 mg VSS l^{-1} and 750/375 mg l^{-1}, respectively.
[b]Mean ± standard error.

gens) but not necessarily to the dyes, because after almost complete color removal, the inhibited cultures showed no signs of recovery. However, continued decolorization, in spite of the observed culture inhibition, further supports the conclusion that maintenance of reduced conditions is sufficient for the cleavage of the azo bond to occur resulting in dye decolorization.

Effect of initial biomass concentration

The effect of initial biomass concentration on RR2 decolorization was tested at 1, 10, and 100% biomass levels. This assay lasted for 11.7 days. The pH in the control and the dye-amended cultures was within 7.0 and 7.2. The ORP values in the control culture and the dye-amended 1, 10, and 100% biomass level cultures were, −204, −203, −150, and −145 mV, respectively. Therefore, the ORP values in the 10% and 1% biomass cultures were significantly lower than in the 100% biomass culture. The effect of initial biomass concentration on color removal kinetics of RR2-amended cultures is shown in Figure 1a. The initial rate of decolorization in the 100, 10, and 1% biomass cultures was 89.7, 32.1, and 17.7 mg l^{-1} per day, respectively. Therefore, the initial rate of decolorization decreased significantly in the cultures with dilute initial biomass concentration, however, the decrease was not directly proportional to the initial biomass concentration. On the other hand, after 1.5 day of incubation the rate of decolorization in the 100, 10, and 1% biomass cultures was 28.6, 24.7, and 20.7 mg l^{-1} per day, respectively. Therefore, despite the slower rate of decolorization at the beginning of the incubation period, the 10 and 1% biomass cultures subsequently decolorized RR2 at rates comparable to that of the culture initially containing 100% biomass. The extent of decolorization at the end of 11.7-day incubation was 86.7, 65.1, and 54%, for the cultures with 100, 10, and 1% biomass, respectively. No loss of RR2 was observed in the abiotic control containing only media and RR2. Therefore, all of the color removal observed in the cultures was due to the presence of the methanogenic culture. The methane production in the RR2-amended cultures with varying initial biomass concentrations as well as the control culture is shown in Figure 1b. The RR2-amended culture with 100% initial biomass exhibited a methane production profile almost identical to that of the control culture. On the other hand, the RR2-amended cultures with 10% and 1% initial biomass concentration showed slower rates of methane production. At the end of 11.7 days incubation the methane production by the RR2-amended cultures was as follows compared to the RR2-unamended control culture: 94.3, 76.2, and 20.4% for the cultures containing 100, 10, and 1% biomass, respectively. Therefore, during the incubation period only a fraction of the carbon source was processed and converted to methane in the cultures with 10% and 1% biomass. Similarly, RR2 decolorization kinetics were also slower. The slower rate of RR2 decolorization does not necessarily indicate that the methanogenic culture activity level was important, because ORP values in the dilute biomass culture set were also lower. In addition, because of the initial dilution of the methanogenic stock culture,

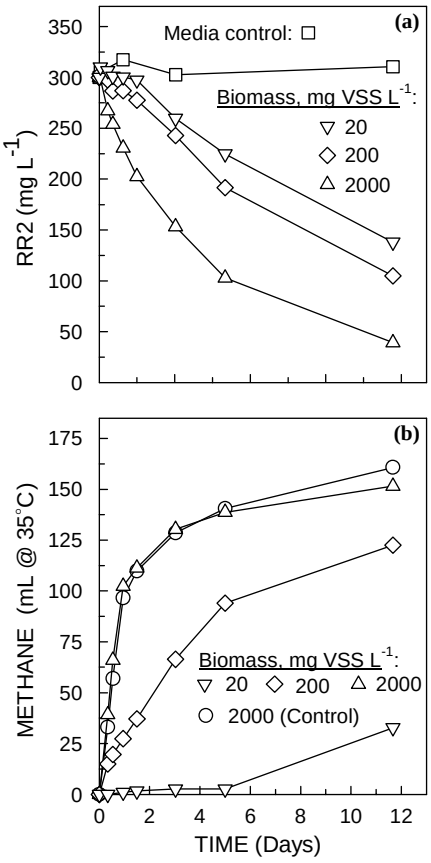

Figure 1. Effect of initial biomass concentration on the decolorization kinetics of Reactive Red 2 (a) and methane production (b) by the methanogenic culture (Initial RR2 300 mg l^{-1}; initial dextrin/peptone 750/375 mg l^{-1}).

extracellular co-factors, which may act as electron carriers and mediate azo reduction, were also diluted. Hence, the observed difference in the rate of RR2 decolorization was more likely the result of the combined effect of different factors.

Effect of carbon source concentration on decolorization kinetics

The effect of initial carbon source concentration on RR2 decolorization was tested at 0, 10, and 100% dextrin/peptone levels. The incubation for this assay lasted 11.7 days The pH in the control and the dye-amended cultures was within 7.0 and 7.3. The ORP values in the control culture and the dye-amended 0, 10, and 100% carbon source amended cultures were, −204, −190, −193, and −230 mV, respectively. Therefore, unlike the different initial biomass concentration series, the ORP values in this assay were relatively similar. The effect of different initial carbon source concentrations on RR2 decolorization kinetics is shown in Figure 2a. The initial rates of decolorization were 89.7, 54.0, and 46.0 mg l^{-1} per day, for the 100%-, 10%-, and no-carbon source amended cultures, respectively. Increased initial carbon source concentration resulted in faster kinetics of RR2 decolorization. The extent of RR2 decolorization at the end of 11.7 days incubation was 86.7, 78.0, and 75.0%, for the 100%-, 10%-, and no-carbon source amended cultures, respectively. The methane production in the cultures with varying initial carbon source concentrations as well as in the control culture is shown in Figure 2b. At the end of 11.7 days incubation the methane production by the RR2-amended cultures was as follows compared to the RR2-unamended control culture (corrected for the methane produced by the dextrin/peptone-unamended control): 93.3, 16.2, and 8.7% for the cultures containing 100%, 10%, and no initial carbon source, respectively. The decreased level of methane production is due to carbon source limitations. It is noteworthy that even the RR2-amended culture without any carbon source added initially decolorized RR2 at a rate and to an extent similar to the 100% carbon source amended culture. Therefore, reducing power present in the culture background was sufficient for RR2 decolorization. Donlon et al. (1997) reported similar results, indicating that azo dye decolorization took place initially in a reactor fed only with dye. However, dye decolorization diminished over time as the endogenous substrates were gradually depleted.

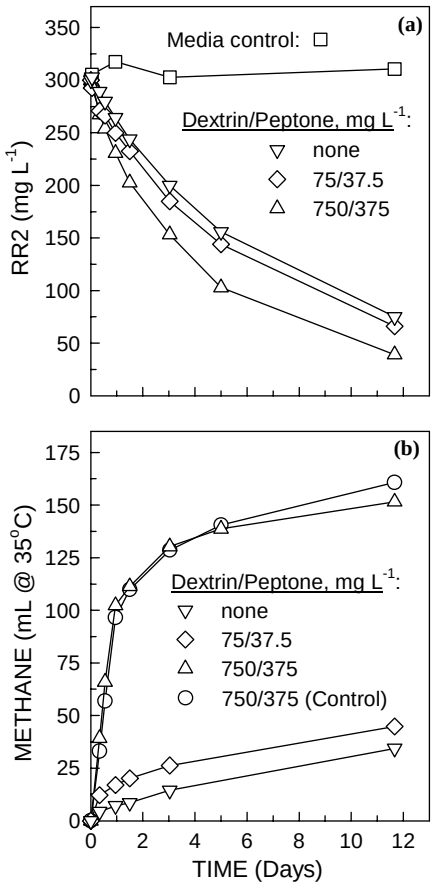

Figure 2. Effect of initial carbon source concentration on the decolorization kinetics of Reactive Red 2 (a) and methane production (b) by the methanogenic culture (Initial RR2 300 mg l^{-1}; initial biomass concentration 2000 mg VSS l^{-1}).

Kinetic modeling of Reactive Red 2 decolorization

Kinetic modeling of azo dye decolorization was performed using the data set obtained from the assay testing the effect of different initial dye concentration on dye decolorization kinetics. The effects of different initial biomass and carbon source concentrations were not included in the analysis because neither the biomass concentration nor the carbon source concentration as tested in this research directly affected the dye decolorization kinetics. As discussed above, co-factors present in the culture background and endogenous substrate contribute to dye decolorization and

could not be isolated from the direct effects of biomass and external carbon source concentration. Assuming saturation kinetics with respect to both biomass and carbon source, a Michaelis–Menten type expression with a substrate inhibition term, i.e., the Haldane model was used:

$$V = \frac{V_{max} C}{K_m + C + \frac{C^2}{K_i}} \quad (1)$$

where, V is the initial volumetric decolorization rate (mg dye l^{-1} per day), V_{max} is the apparent, maximum initial volumetric decolorization rate (mg dye l^{-1} per day), C is the initial dye concentration (mg l^{-1}), K_m is the half velocity coefficient (mg l^{-1}), and K_i is the inhibition coefficient (mg l^{-1}). When referring to batch kinetics, the notation V_{max} (here and elsewhere in this paper) refers to the apparent V_{max} value, i.e. the maximum initial volumetric decolorization rate under the experimental conditions of the assay (e.g., initial dye, electron donor, and biomass concentration, etc.). The inhibition term (K_i) was included to account for a possible inhibitory effect of high dye concentrations on the dye decolorization rates. Accordingly, a small K_i value (K_i comparable to that of the K_m value) indicates inhibition of the dye decolorization due to the presence of high concentrations of dye, while a large K_i value (K_i much larger than the K_m value) indicates that the presence of high concentrations of dye did not have a significant inhibitory effect on dye decolorization. Although, dye decolorization may not necessarily be directly related to the culture enzymatic activity, this model was considered as adequate to describe the rate of dye decolorization as a function of dye concentration. Azo reduction may not necessarily be directly due to the azoreductase activity of the culture. However, the reducing equivalents used for azo reduction are the result of the culture metabolic activity.

RR2 decolorization at different initial concentrations (50, 300, 500, 1000, and 2000) was modeled as follows. Initial volumetric decolorization rates (V, mg dye l^{-1} per day) were determined using linear regression at each different initial RR2 concentration series (see above). Subsequently, the initial volumetric decolorization rates were correlated with the corresponding initial dye concentration according to Equation (1) by using the statistical package in SigmaPlot 6.0 (SPSS Inc., Chicago, IL) and performing a least-squares, non-linear regression. Initial RR2 decolorization rate data were fitted to the Haldane model (Equation 1) and results are presented in Figure 3a. A depression in the rate of decolorization was observed at an initial RR2 concentration of 100 mg l^{-1} and higher. From the non-linear regression, the values for V_{max}, K_m, and K_i were determined as 109 ± 5 mg l^{-1} d^{-1}, 8.6 ± 3.9 mg l^{-1}, and 7285 ± 2517 mg l^{-1} (mean ± standard error; $r^2 = 0.994$), respectively. The model predicted initial RR2 decolorization rate without any inhibition (i.e. $K_i = \infty$) at an initial RR2 concentration of 2000 mg l^{-1} is 108.4 mg l^{-1} per-day as opposed to the experimentally observed (i.e., inhibited) RR2 decolorization rate of 87.7 mg l^{-1} per day (Figure 3a). Therefore, a relatively high

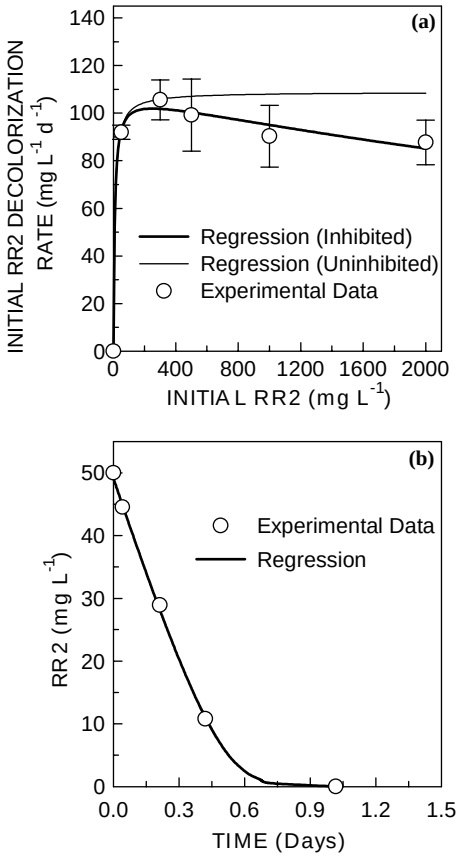

Figure 3. Reactive Red 2 decolorization kinetics by the methanogenic culture: (a) Non-linear fit of initial decolorization rate data to the Haldane model as a function of initial Reactive Red 2 concentration (Error bars represent standard errors); (b) Non-linear fit of Reactive Red 2 concentration data to the integrated Michaelis–Menten model at an initial Reactive Red 2 concentration of 50 mg l^{-1}.

initial RR2 concentration of 2000 mg l^{-1} resulted in a moderate degree of inhibition of the dye decolorization process as is also shown by the relatively high K_i value as compared to the K_m value.

RR2 decolorization kinetics at sub-inhibitory initial RR2 concentrations were described using the Michaelis-Menten model:

$$V = -\frac{dC}{dt} = \frac{V_{max}C}{K_m + C} \quad (2)$$

Integration of equation 2 results in the following Equation:

$$C_t = C_0 - K_m \ln(C_t/C_0) - V_{max}t \quad (3)$$

Use of Equation (3) implies that the biomass concentration remains constant throughout the batch decolorization assay. As a result of a relatively high initial biomass concentration used, the biomass concentration varied by less than 10% during the relatively short incubation period in these assays, thus justifying the use of Equation (3). The experimental data obtained for RR2 decolorization at an initial concentration of 50 mg l^{-1} were used to illustrate the Michaelis-Menten model fit (Figure 3b). From the non-linear regression, the values for V_{max}, and K_m were determined as 122 ± 1 mg l^{-1} per day, and 8.8 ± 0.7 mg l^{-1}, respectively (mean ± standard error; $r^2 = 0.999$). Note that, the V_{max} value obtained through regression of experimental data from the batch RR2 decolorization experiment at an initial RR2 of 50 mg l^{-1} is specific to the particular system (i.e. initial RR2 concentration of 50 mg l^{-1} and initial biomass concentration of 2000 mg l^{-1}).

A similar analysis was performed with initial volumetric decolorization rate data obtained with the same mixed, methanogenic culture and two other reactive, but unreacted azo dyes: Reactive Black 5 (RB5; a diazo, di-vinyl sulfone dye) and Reactive Yellow 17 (RY17; a monoazo, vinyl sulfone dye) at a concentration range of 50–2000 mg l^{-1}. The V_{max} and K_m values were as follows for the RB5 and RY17, respectively (mean ± standard error): 3797 ± 920 mg l^{-1} per day, 1510 ± 667 mg l^{-1} ($r^2 = 0.977$; $n = 6$) and 805 ± 108 mg l^{-1} per day, 1018 ± 285 mg l^{-1} ($r^2 = 0.988$; $n = 6$) (Beydilli 2001). It is noteworthy that the initial decolorization rates for both RB5 and RY17, at the highest dye concentration tested (i.e. 2000 mg l^{-1}), were much higher than that for the RR2. In addition, decolorization was not inhibited at this high dye concentration. Overall, RB5 had the fastest decolorization kinetics among the three reactive dyes tested, as indicated by the highest V_{max} value. Based on molecular weight values of 615.35, 991.85, and 683.81 g mol^{-1} for RR2, RB5, and RY17, respectively, and assuming equal dye purity, the above measured V_{max} values correspond to 0.18 ± 0.01, 3.83 ± 0.93, and 1.18 ± 0.16 mM per day, respectively. Hence, the initial volumetric decolorization rate was actually higher for the dyes with higher molecular weight. This observation further supports a widely accepted mechanism in which azo dye decolorization takes place extracellularly, via electron transfer mediation (Stolz 2001; Pearce et al. 2003). Similarly, van der Zee et al. (2000) investigated the decolorization of twenty azo dyes by granular sludge from an upflow anaerobic sludge blanket (UASB) reactor. Among the azo dyes tested, six were reactive dyes, including RR2 and RB5. The initial dye concentration tested varied between 100 and 300 mg l^{-1} and first-order decolorization kinetics were reported for all dyes (half-life of these dyes varied between 1 and 100 h). Although the first-order rate constant values varied greatly among the different dyes, a correlation between dye molecular weight and first-order decolorization rate constants was not found. The first-order decolorization rate constant for RR2 at an initial dye concentration of 370 mg l^{-1} was reported as 0.31 per day, indicating that 50% decolorization occurred at 2.35 days incubation. In the present study, at an initial RR2 concentration of 300 mg l^{-1}, 50% decolorization occurred at 1.63 d incubation. Similarly, the first-order decolorization rate constant for RB5 at an initial dye concentration of 540 mg l^{-1} was reported as 5.0 per day, indicating that 50% decolorization occurred at 0.14 day incubation (van der Zee et al. 2000). In the present study, at an initial RB5 concentration of 500 mg l^{-1}, 50% decolorization of RB5 took place at 0.17 d incubation, indicating comparable RB5 decolorization kinetics in both cultures.

Effect of long-term culture acclimation on decolorization kinetics

The two batch-fed, semicontinuous reactors (R1 and R2) were maintained according to the previously mentioned schedule for over a period of two

years under identical feeding conditions. A detailed characterization of a 7-day feeding cycle (cycle-0) showed that both reactors performed similarly in terms of RR2 and DOC removal, as well as aniline, total gas and methane, and VFA profiles over the incubation period (Figure 4). Acetic and propionic acids were the only VFAs detected. It is noteworthy that residual acetic acid was left at the end of each feeding cycle, whereas propionic acid was produced and totally consumed within each feeding cycle. The aniline concentration at the beginning of the feeding cycle was 11.3 mg l^{-1}. As RR2 decolorized rapidly within the first 6 h of incubation, the aniline concentration rapidly increased to 19 mg l^{-1}. Based on RR2 purity determined via aniline recovery upon RR2 reduction using sodium dithionite

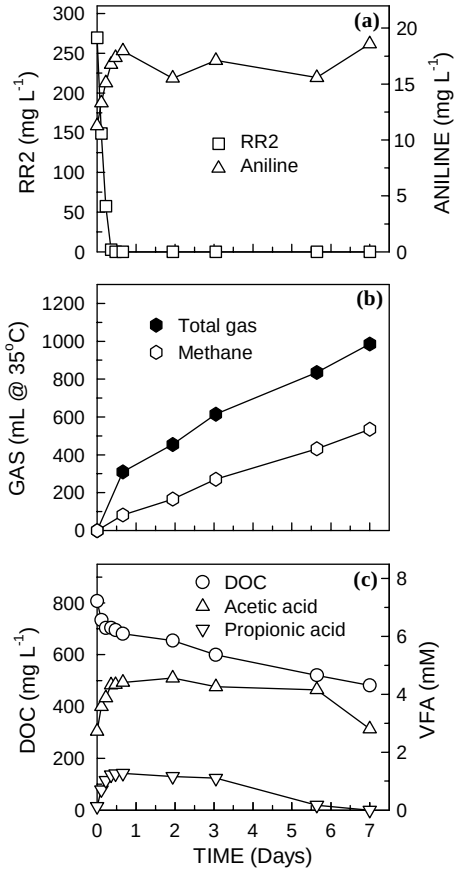

Figure 4. Parameter variation during a typical feeding cycle of the semicontinuously-fed reactors amended with Reactive Red 2 (Initial RR2 300 mg l^{-1}; both reactors performed identically during cycle-0, where RR2 and dextrin/peptone were added to both reactors).

(Beydilli 2001), the recovery of aniline was about 74%. The initial rates of RR2 decolorization in R1 and R2 were 1021 and 922 mg l^{-1} per day after two years of weekly addition of RR2 and dextrin/peptone. The initial rate of decolorization by the unacclimated methanogenic culture during the first RR2 addition at the same level (i.e. 300 mg l^{-1}) was 95.3 mg l^{-1} per day. Therefore, long-term exposure of the methanogenic culture to RR2 resulted in about a 10-fold enhancement of the rate of RR2 decolorization. The increased RR2 decolorization rate upon long-term exposure indicates that both the reduced environment in the culture and culture acclimation play a significant role in the decolorization kinetics of RR2. Electron equivalent balance calculations for R1 and R2 showed that during each feeding cycle, the electron equivalents used for RR2 decolorization represented only 1.5% of the electron equivalents used for methane production. Thus, only a very small fraction of the electron equivalents available in the culture medium was utilized for RR2 decolorization.

After the characterization of cycle-0, weekly dextrin/peptone addition continued for R1 but not for R2, while RR2 was added to both reactors. R1 and R2 were monitored for another 10 feeding cycles (i.e., 10 weeks). The pH in both reactors was always 7.0 ± 0.1. The variation of most of the parameters monitored in both R1 and R2 during the last 10 feeding cycles is shown in Figure 5. The biomass concentration in R1 remained relatively constant over the 10 feeding cycles, and the concentration at the end of the 10th feeding cycle was 1475 mg VSS l^{-1}. In contrast, the biomass concentration in R2 decreased steadily over the 10 feeding cycles, and reached 130 mg VSS l^{-1} at the end of the 10th feeding cycle. The continuous solid line in Figure 5b represents the simulated dilution effect due to culture wastage and fresh media addition on biomass concentration in the R2 reactor assuming zero net biomass growth, and completely mixed conditions. As shown in Figure 5b, the biomass concentration in R2 closely followed the simulated dilution effect. These results indicate that, in the absence of an external carbon source (dextrin/peptone), culture growth stopped in R2 and its biomass concentration decreased over time as a result of wasting and media plus RR2 feeding at the beginning of each feeding cycle. The decrease of biomass in R2 also indicates

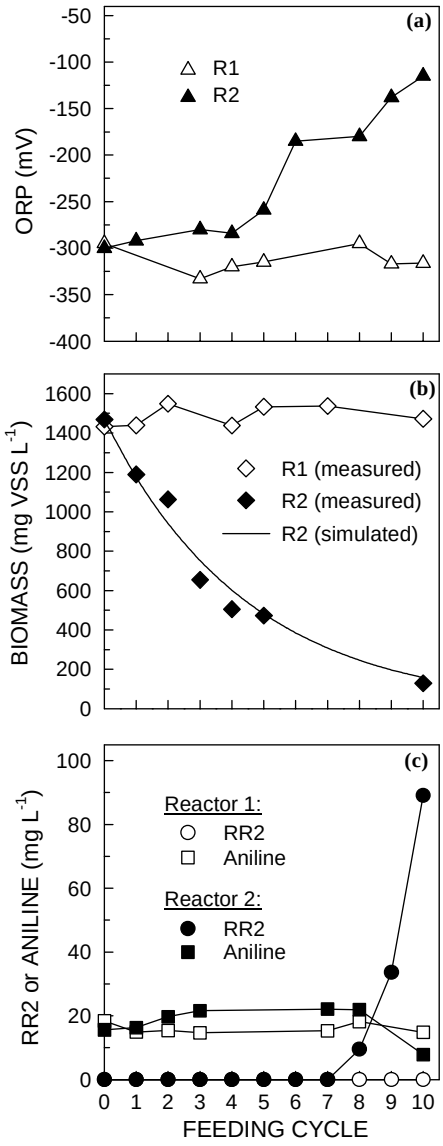

Figure 5. Parameter variation in the two methanogenic reactors (R1 and R2) at the end of each feeding cycle for the last, consecutive ten cycles.

that RR2 was not utilized as a carbon and energy source by the mixed culture. The RR2 was not detected in R1 at the end of each feeding cycle. On the other hand, RR2 was detected in R2 at the end of the 8th and subsequent feeding cycles and reached 89 mg l^{-1} at the end of the 10th feeding cycle. Concurrent with the detection of RR2 in the R2 reactor at the end of the 8th and subsequent feeding cycles, the concentration of aniline sharply decreased as a result of incomplete RR2 decolorization, as well as the dilution effect due to reactor wasting and feeding, whereas the aniline concentration in R1 remained relatively constant over the 10 feeding cycles (Figure 5c). Methane production in the R2 reactor ceased by the end of the second cycle, and the low methane production recorded during the first feeding cycle was attributed to the residual carbon source (e.g., acetic acid) at the end of the previous feeding cycle during which dextrin/peptone was supplied to R2 for the last time.

The initial RR2 decolorization rate in R2 during cycle-0, cycle-5, and cycle-10 was 922, 419, and 233 mg l^{-1} per day, respectively. The initial RR2 decolorization rate in R2 during cycle-10 represents a 75% decrease compared to the initial decolorization rate during cycle-0, when R2 was fed with dextrin/peptone for the last time. On the other hand, the initial rate of decolorization in the unacclimated methanogenic culture was 95.3 mg l^{-1} per day and decolorization was almost complete at the end of 7-day incubation, while after 10 feeding cycles without dextrin/peptone amendment, R2 achieved only 70% RR2 decolorization within 7 days (Figure 6). The observed decline in RR2 decolorization in R2 over the last ten cycles, when dextrin and peptone were not added to R2, is attributed to both a decline in biomass concentration and the availability of reducing equivalents.

Similar to the results presented here, others have reported that, for the decolorization of azo dyes, addition of a co-substrate was necessary to provide the reducing equivalents for azo reduction and dye decolorization (Carliell et al. 1995; Donlon et al. 1997; Lourenco et al. 2003). The only complete biodegradation of an azo dye under methanogenic conditions was reported by Razo-Flores et al. (1997). These researchers used azodisalicylate, which has a relatively simple molecular structure. Upon azo cleavage of azodisalicylate in the adapted methanogenic culture, 5-aminosalicylic acid was produced and subsequently mineralized to methane and ammonia. It is important to note that 5-aminosalicylic acid contains a carboxylic group, which facilitates further degradation under methanogenic conditions. In addition, the observed biodegradation was culture- and dye-specific. In the research presented here, it was found that even after two years of exposure to RR2, the mixed, methanogenic culture did not achieve biodegradation beyond azo bond

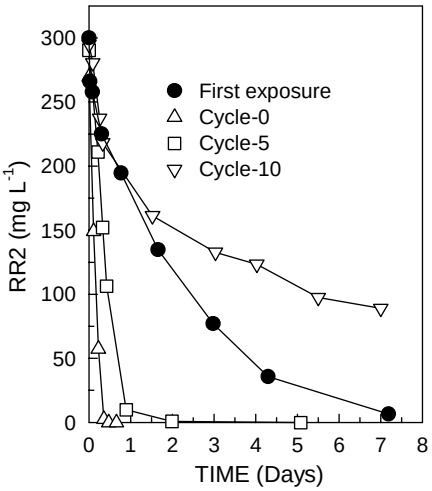

Figure 6. Effect of the long-term acclimation of the methanogenic culture in Reactor 2 to Reactive Red 2 as well as carbon source availability on the decolorization kinetics of Reactive Red 2 (Initial RR2 and biomass concentration 300 mg l^{-1} and 1450 mg VSS l^{-1}, respectively; dextrin/peptone was supplied in cycle-0, but not in cycle-1 through cycle-10; the biomass concentration in cycle-5 and cycle-10 was 475 and 130 mg VSS l^{-1}, respectively).

cleavage. Similar results relative to the recalcitrance of aromatic amines resulting from azo bond reduction of an azo dye were reported by Lourenco et al. (2003) based on a study which used an anaerobic-aerobic sequencing batch reactor for over 180 days.

Conclusions

Overall, the results of this study indicate that a biological component was responsible for RR2 decolorization under methanogenic conditions as evidenced by the increased rates of RR2 decolorization upon long-term culture acclimation. However, RR2 was not utilized as a source of carbon and energy by the mixed methanogenic culture used in this study, even after two years of weekly RR2 additions. Further transformation and mineralization of aniline and the *o*-aminohydroxynaphthalene derivative resulting from the reductive azo bond cleavage of RR2 were not observed. These observations further prove that complete biodegradation of complex azo dyes does not generally take place under strictly anaerobic, methanogenic conditions. Thus, addition of an external carbon and energy source is necessary to achieve and maintain a high decolorization rate.

Although biological decolorization of reactive dyes, especially azo dyes, under methanogenic conditions has been proven to be feasible, reactive dyeing processes use salt at relatively high concentrations (typically over 60 g NaCl or Na_2SO_4 l^{-1}), which undoubtedly will have a detrimental effect on the microbial activity of non-halotolerant microorganisms, especially if undiluted textile wastewater (i.e., dyebaths) are to be biologically decolorized before their final disposal or with the intent of reusing such streams as process water in the textile plant. Biological decolorization of reactive dyes at relatively high dye and salt concentrations, typically encountered in textile reactive dyebaths, under anaerobic conditions using halophilic microorganisms has been a major research effort in this laboratory for the renovation of reactive textile dyebaths and their reuse in the dyeing process. In-plant application of a closed-loop dyeing and decolorization system has the potential to not only decrease wastewater volume but also minimize fresh water consumption and the discharge of textile pollutants, such as salt and dyes, thereby leading to an overall improvement of water quality through the effective management of spent dyebaths at their source.

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