Degradation of azo dye Mordant Yellow 10 in a sequential anaerobic and bioaugmented aerobic bioreactor

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Abstract: Complete biodegradation of azo dyes requires an anaerobic and aerobic step, in the anaerobic step sulfonated azo dyes (SADs) are reduced, yielding (sulfonated) aromatic amines ((S)AAs) which can be degraded aerobically. The complete biodegradation of the SAD Mordant Yellow 10 (MY10) was studied in a sequential anaerobic and aerobic bioreactor. Anaerobically, MY10 was reductively cleaved and the resulting aromatic amines, 5-aminosalicylic acid (5-ASA) and sulfanilic acid (SA), were both recovered in high stoichiometric yields. One of the AAs, 5-ASA, was readily degraded under aerobic conditions. However, SA was not degraded aerobically in the continuous experiment because no SA-degrading bacterial activity was present in the system. Therefore, a SA-degrading enrichment culture derived from Rhine sediment was used as an inoculum source. This enrichment culture was bioaugmented into the aerobic reactor by increasing the hydraulic retention time (HRT), thus enabling SA-degrading activity to develop and maintain in the aerobic reactor. After decreasing the HRT, the SA-degrading activity remained in the bioreactor and the stoichiometric recovery of sulfate (a SA biodegradation product) indicated the mineralization of SA after bioaugmentation. Batch experiments with aerobic reactor sludge confirmed the biodegradation of SA and 5-ASA. The sequential anaerobic and aerobic bioreactor was able to completely remove the sulfonated azo dye MY10 at a maximum loading rate of 210 mg MY10 (lreactor d)–1 after the appropriate microorganisms for aerobic degradation of SA were bioaugmented into the aerobic bioreactor.

Keywords 5-aminosalicylic acid; azo dye reduction; Mordant Yellow 10; sequential anaerobic aerobic; sulfanilic acid; bioaugmentation

Introduction
The production of dyes is extensive: up to 10^8 kg were manufactured in 1987 in the United States (Zollinger, 1987). During production and usage of dyes and pigments, an estimated amount of 10–15% is released into the environment mainly via wastewater (Vaidya and Datye, 1982). If dyes are divided in different groups by their chemical structure then azo dyes are the largest (60–70%) group (Carliell et al., 1995). These compounds are mainly used in textile, printing, pharmaceutical and food industry for colouration. Many azo dyes are substituted with a sulfonate group. SADs absorb visible light owing to the conjugated system of one or more azo groups (R1–N=N–R2) substituted with aromatic groups. Owing to the toxic and carcinogenic character of some azo dyes (Chung and Cerniglia, 1992) as well as their breakdown products, the emission of these compounds should be minimized. A good way to minimize the emission of azo dyes is via biological degradation.

The initial step in the biodegradation of azo dyes is the reduction of the azo linkage, which generally results in the decolouration of the compound. Azo dye reduction is a ubiquitous process under anaerobic conditions if co-substrate is present to donate electrons (Walker, 1970; Carliell et al., 1995; Razo-Flores et al., 1996). The azo dye reduction products, the (S)AAs, are not very likely to be degraded under anaerobic conditions (Kuhn and Sufliita, 1989). Therefore, these compounds are excreted after the anaerobic treatment step.

Under aerobic conditions many AAs are readily degraded (Brown and Laboureur, 1983). In contrast, SAAs are not very likely to be degraded under aerobic conditions. Only some reports describe complete degradation of SAAs when used as carbon and energy
source (Nortemann et al., 1986; Thurnheer et al., 1986; Feigel and Knackmuss, 1988; Hooper, 1991). One advantage of SAAs compared with unsulfonated analogues is that the SAAs are less toxic than their unsulfonated counterpart (Jung et al., 1992). Experiments at our laboratory revealed that only a two of the seven SAAs tested were degraded in aerobic batch experiments using activated sludge or Rhine sediment (Tan et al., in preparation). Thus, a combination of anaerobic and aerobic treatment for the degradation of SADs is only possible if the microorganisms are present to degrade the SAAs. A way to stimulate the biodegradation of these SAAs is to bioaugment the aerobic reactor with strains or enrichments able to degrade these compounds.

The aim of this study is to assess if MY10 can be completely mineralized under continuous conditions in a sequential anaerobic and aerobic bioreactor. The major problem with regard to this aim is the aerobic biodegradation of the reduction product SA. Therefore, special attention will be given to the optimization of biodegradation efficiency of the aerobic reactor.

Methods
Sequential anaerobic/aerobic reactor set-up

In this study, MY10 was continuously treated in a sequence of an anaerobic and an aerobic lab-scale bioreactor (Figure 1). The anaerobic reactor, a 2.5 l expanded granular sludge blanket (EGSB, diameter 0.05 m and height 1.0 m), was connected to a 1.0 l aerobic activated sludge reactor (diameter 0.06 m and height 0.30 m). The upflow velocity of the anaerobic EGSB reactor was kept 2.0 m h⁻¹ by recycling the EGSB effluent with a recycle factor of 40. The flow rate of the compressed air to aerate the bioreactor was 75 l h⁻¹. The washed-out aerobic activated sludge could settle in a conical settler (0.3 l) from which a sludge recycle stream was led back into the aerobic reactor.

The influent consisted of a synthetic wastewater containing MY10 (100–200 mg l⁻¹), NaHCO₃ (1.0 g l⁻¹), and mineral medium (Tan et al., 1999c). Ethanol (EtOH, 1.0–2.0 g chemical oxygen demand (COD) l⁻¹) was added as the cosubstrate for the reduction of the azo dye.

Biomass
Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating effluent of an alcohol distillery of Nedalco (Bergen op Zoom, The Netherlands), was used for the experiments. The granular sludge was washed and sieved to

Figure 1 Schematic drawing of the sequential anaerobic EGSB and aerobic reactor. (1. anaerobic EGSB reactor; 2. aerobic reactor; 3. settler; 4. influent; 5. effluent; 6. EGSB recycle stream; 7. air supply; 8. gas liquid separator)
remove the fine particles before use in the reactor and batch tests. Both the aerobic and anaerobic reactors were seeded with the Nedalco biomass with an amount of 20.0 g volatile suspended sludge (VSS; (APHA, 1985) per litre reactor volume.

Aerobic Rhine sediment was collected along side the banks of the river near Lexkesveer in Wageningen (The Netherlands). The sediment (100 g) was suspended in 500 ml water and sieved (pore size 1.5 mm). This diluted sediment was used for the aerobic enrichment experiment. All biomass sources were stored at 4°C before usage.

Batch assays

**Anaerobic activity, toxicity and biodegradation experiments.** The anaerobic activity, toxicity and biodegradation assays experiments were conducted via the method described before (Donlon *et al.*, 1995; Razo-Flores *et al.*, 1996).

**Aerobic biodegradation experiments.** The aerobic biodegradation assays of 5-ASA and SA were performed as described before by Tan *et al.* (1999a). Enrichments were obtained the same way as the biodegradation assays. After degradation occurred, the batches were repetitively fed with compound and supplied with sufficient oxygen. The degrading biomass was frequently transferred in fresh and sterilized mineral medium.

Analyses

**UV method for SA and 5-ASA and High Pressure Liquid Chromatography (HPLC) for MY10, SA and 5-ASA.** The UV and HPLC method for the determination of SA, 5-ASA and MY10 were described before (Tan *et al.*, 1999c).

**Gas chromatography (GC) for EtOH and GC method for methane.** Gas composition (O₂, N₂, CO₂ and CH₄) and ethanol concentrations were measured via the GC methods described before (Tan *et al.*, 1999a).

**HPLC for Sulfate.** The mobile-phase (10.8 mM, potassium biphthalate) for the sulfate HPLC method was pumped with a high-pressure precision pump model 480 (Separations, Hendrik Ido Ambacht, The Netherlands) and a flow rate of 1.2 ml min⁻¹ through the HPLC system. A 20 µl sample was injected with a Midas autosampler (Separation, Hendrik Ido Ambacht, The Netherlands) and anions were separated on a reverse-phase Vydx 302 IC405 anion column (Vydac, Hesperia CA, USA; length 250 mm × ID 4.6 mm). The anions were detected with a Waters 431 conductivity detector (Millipore, Etten-Leur, The Netherlands).

**Chemical Oxygen Demand (COD).** The soluble COD was measured colourimetrically. First, the samples were centrifuged 5.0 min at 10,000 rpm. Secondly, 5.0 ml volume samples were treated with 3.0 ml 0.083 N K₂Cr₂O₇ and 7.0 ml 18M H₂SO₄ which contains 0.034 M Ag⁺. Thirdly, the closed sample tubes were stored in a 150°C oven for two hours. Finally, after cooling the samples were measured at 600 nm with a Cecil CE 1011 spectrophotometer (Cecil Instruments Limited Technical Center, Cambridge, United Kingdom).

Results and discussion

The results of the experiments will be presented and discussed separately for both reactors. First, the results of the anaerobic reactor will be presented. Secondly, the results of the aerobic reactor will be shown and discussed. At the end, the complete sequential anaerobic and aerobic reactor will be evaluated.
The main results of the anaerobic EGSB bioreactor are summarized in Table 1. These results clearly show that the anaerobic EGSB bioreactor performed well, as it achieved complete decolouration of MY10 with increasing loads of MY10. The cosubstrate ethanol was completely degraded in the anaerobic reactor and converted into methane and carbon dioxide. No inhibitory effects of the applied azo dye MY10 and its biodegradation products SA and 5-ASA to the anaerobic granular sludge were observed. The acetoclastic methanogenic activity remained high and even increased at the end of the experiment (Table 1) where azo dye concentrations up to 159 mg MY10 l–1 were applied. No inhibition was expected since the IC50 values for these compounds were 952 and 444 mg l–1 for MY10 and 5-ASA, respectively (Donlon et al., 1997). Batch experiments with SA even did not show any inhibition of the acetoclastic methanogenic activity up to concentration of 1.0 g SA l–1 (results not shown).

Both aromatic amines were recovered in high yields after the anaerobic reduction of MY10. SA was recovered in high stoichiometric percentages (Table 1). Anaerobic degradation of SA is not likely since this compound was not degraded in anaerobic biodegradation experiments after long test periods (Kuhn and Suflita, 1989; Razo-Flores et al., 1996). The other aromatic amine, 5-ASA, was only recovered for 60–80% which was probably due to autoxidation of this compound before measurements or to partial anaerobic degradation of 5-ASA. Indeed, the presence of bacteria capable of degrading 5-ASA anaerobically was demonstrated in an anaerobic biodegradability experiment in the period An2. The result of the biodegradation experiment clearly shows the anaerobic biodegradation of 5-ASA (Figure 2). The amount of methane produced during biodegradation of 5-ASA was 101% of the theoretical methane production, calculated via the Buswell equation (Tarvin and Buswell, 1934). Biodegradation was also confirmed by stoichiometric recovery of ammonium (results not shown). The fact that 5-ASA was biodegraded by anaerobic granular sludge is not surprising because this was already reported (Razo-Flores et al., 1996). After anaerobic degradation of 5-ASA was proven, the HRT was increased from 0.97 to 1.22 d between days 405 to 423 to stimulate the anaerobic bacteria capable of degrading

### Table 1: Operational parameters and results of the anaerobic EGSB bioreactor (average values)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
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<th>Ar2</th>
<th>Ar3</th>
<th>Ar4</th>
<th>Pe1#</th>
<th>Pe2</th>
<th>Pe3</th>
<th>Pe4</th>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>1.00</td>
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<td>1.59</td>
<td>1.05</td>
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<tr>
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<td>100</td>
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<td>137</td>
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<td>92</td>
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<td>143</td>
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**Efficiencies**

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<th>Ar3</th>
<th>Ar4</th>
<th>Pe1#</th>
<th>Pe2</th>
<th>Pe3</th>
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<tr>
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<td>97</td>
<td>100</td>
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<td>88</td>
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<td>5-ASA</td>
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<td>61</td>
<td>79</td>
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<td>79</td>
<td>57</td>
<td>47</td>
<td>67</td>
<td>67</td>
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</table>

Influent concentrations of the compounds. **rem. = removal and rec. = recovery. ***Removal and recovery based on the influent and effluent concentrations. @Measured at the beginning of the period. #Period Pe1 started at day 125; between days 89 and 124 no measurements were done. Anaerobic EGSB bioreactor

The main results of the anaerobic EGSB bioreactor are summarized in Table 1. These results clearly show that the anaerobic EGSB bioreactor performed well, as it achieved complete decolouration of MY10 with increasing loads of MY10. The cosubstrate ethanol was completely degraded in the anaerobic reactor and converted into methane and carbon dioxide. No inhibitory effects of the applied azo dye MY10 and its biodegradation products SA and 5-ASA to the anaerobic granular sludge were observed. The acetoclastic methanogenic activity remained high and even increased at the end of the experiment (Table 1) where azo dye concentrations up to 159 mg MY10 l⁻¹ were applied. No inhibition was expected since the IC50 values for these compounds were 952 and 444 mg l⁻¹ for MY10 and 5-ASA, respectively (Donlon et al., 1997). Batch experiments with SA even did not show any inhibition of the acetoclastic methanogenic activity up to concentration of 1.0 g SA l⁻¹ (results not shown).
5-ASA. However, no positive effect on the anaerobic degradation of 5-ASA was observed. Probably, this was because of the too slow growth of the anaerobic 5-ASA-degrading biomass.

Aerobic bioreactor
A summary of the operational parameters and the results of the aerobic bioreactor performance as summarized in Table 2. For a period of more than 240 days, no SA degradation took place as it was recovered at nearly stoichiometric amounts in the effluent of the aerobic reactor. Nevertheless, in batch degradation experiments using Rhine sediment as inoculum source SA biodegradation could be obtained and an aerobic enrichment culture was developed. This enrichment was already successfully used to bioaugment batches in which MY10 was degraded under integrated anaerobic and aerobic conditions (Tan et al., 1999c). Therefore, this further enriched culture was also used to bioaugment the aerobic continuous reactor in period Pe3. In order to establish good retention of the SA-degradation culture, the HRT of the aerobic reactor was increased to from 0.37 to 3.3 d on day 261. Afterwards, the HRT was slowly decreased to its original value. The SA-degrading biomass still maintained its ability to degrade SA for more than 160 days. Degradation of SA was also confirmed in the stoichiometric recovery of sulfate during period An2 (Figure 3).

Table 2 Operational parameters and results of the aerobic reactor (average values)

<table>
<thead>
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<th>Parameter</th>
<th>Unit</th>
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</tr>
<tr>
<td>End period day</td>
<td>21 42 68 88 188 245 279 295 335 405 425</td>
<td></td>
</tr>
<tr>
<td>SA* mg l⁻¹</td>
<td>-</td>
<td>25 37 78 57 72 56 70 53 70 65</td>
</tr>
<tr>
<td>5-ASA* mg l⁻¹</td>
<td>-</td>
<td>17 26 48 38 52 39 35 21 31 37</td>
</tr>
<tr>
<td>HRT day</td>
<td>0.54</td>
<td>0.52 0.55 0.49 0.59 0.37 2.10 0.28 0.28 0.38 0.67</td>
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Efficiencies ***

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% rem.**</th>
</tr>
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<td>SA</td>
<td>1 1 1 3 7 93 93 70 97 98</td>
</tr>
<tr>
<td>5-ASA</td>
<td>91 93 100 97 100 88 100 92 100 100</td>
</tr>
</tbody>
</table>

* influent concentrations are due to the anaerobic reduction of MY10. ** rem. = removal. *** Removal efficiencies based on the influent concentrations.

Figure 2 Anaerobic biodegradation of 5-ASA with 8.0 g volatile suspended solids (Nedalco granular sludge) per litre. 5-ASA biodegradation (○); methane (■); methane (×) production control without 5-ASA degradation.
The aerobic reactor was originally inoculated with granular sludge. Granular sludge showed the ability to aerobically degrade aniline (Tan et al., 1999b) the unsulfonated analogue of SA. During the reactor experiment the granular sludge developed more like activated sludge. Since the aerobic bioreactor was only fed with SA and 5-ASA, the biomass, which developed, was specialized to degrade these compounds. No further research was done to locate on a micro scale the fate of the SA-degrading bioaugmented biomass.

Aerobic batch experiments with the aerobic reactor sludge (sample at the end of the reactor experiments) clearly revealed the mineralization of SA and 5-ASA (Figure 4). Oxygen uptake and stoichiometric sulfate production associated with SA degradation also confirmed these results. Figure 4 also shows that during 5-ASA biodegradation, also autoxidation occurs in the sterile control. The formation of all kinds of 5-ASA autoxidation products in aqueous solutions was already reported (Jensen et al., 1992). However, the active batches show that biodegradation is faster than autoxidation and is the dominant route for removal for 5-ASA (Figure 4).

Combined sequential anaerobic and aerobic reactor
In general, the sequential system showed a good removal of the SAD MY10, co-substrate and its constituent products SA and 5-ASA. Both AAs were produced in the anaerobic phase and degraded in the aerobic phase. The aerobic biodegradation of SA had to be stimulated via bioaugmentation of a SA-enrichment culture. Bioaugmentation was successful and retention of the SA-degrading biomass was obtained.
The treatment of industrial wastewater polluted with SADs in a sequential system will be more difficult since many different aromatic intermediates will be formed which also need to be degraded. Of these, the aerobic biodegradation of the SAAs can be considered a major problem. Therefore, further research is needed on how the SAAs’ degradation can be improved and efficiently incorporated in the total reactor system.

Conclusions
The complete degradation of MY10 was clearly demonstrated in a sequential anaerobic (EGSB) and aerobic (activated sludge) continuous bioreactor at a maximum loading rate 210 mg MY10 (l<sub>reactor</sub> d<sup>−1</sup>). The aerobic reactor was successfully bioaugmented with an aerobic SA-degrading enrichment culture. The degradation of SA was also confirmed by stoichiometric production of sulfate in batch as well as in continuous experiments. Furthermore, this research showed that bioaugmentation of xenobiotic degrading microorganisms is a useful tool for the removal of recalcitrant compounds such as SADs and SAAs.

References

