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Decolorization and Partial Degradation of Selected Azo Dyes by Methanogenic Sludge

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Abstract

The toxicity potential and decolorization of three acid azo dyes (Acid Orange 6, Acid Orange 7, and Acid Orange 52) by methanogenic granular sludge from an anaerobic expanded granular sludge bed reactor was assayed. Complete bioreduction was found for all three azo dyes. Sulfanilic acid and 4-aminoresorcinol were detected from the decolorization of Acid Orange 6, sulfanilic acid and 1-amino-2-naphtol were detected from the reduction of Acid Orange 7, and sulfanilic acid and *N*,*N*-dimethyl-1,4-phenylenediamine (DMP) were found to be intermediates of Acid Orange 52 degradation. Sulfanilic acid and 1-amino-2-naphtol were persistent in the anaerobic conditions, whereas 4-aminoresorcinol was completely mineralized by anaerobic sludge and DMP was transformed into 1,4-phenylenediamine. Enrichment cultures obtained via consecutive passages on basal medium with only azo dye as a carbon and an energy source seemed to be morphologically heterogeneous. Baculiform and coccus cells were found when viewed under a light microscope. Cocci were joined in chains. Because anaerobic sludge contains sulfate-reducing bacteria and therefore may generate sulfide, azo dyes were tested for chemical decolorization by sulfide to compare rates of chemical and biologic reduction.

Index Entries: Azo dyes; methanogenic sludge; toxicity; bioreduction; chemical reduction.

Introduction

Azo dyes represent a class of complex aromatics containing one or more azo groups and are widely used in textile and other industries *(1,2)*.

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All the azo dyes currently used are synthetic compounds and, hence, xenobiotics. The presence of these aromatics may create serious environmental problems because the products of their transformation, aromatic amines, possess mutagenic or carcinogenic activities for living cells *(3)*. Research associated with the development of dye treatment technology is currently of great interest *(4–6)*. The majority of azo dyes are readily transformed by anaerobic microorganisms through splitting of the azo bond with formation of aromatic amines. The aromatic amines are colorless, so the azo bond splitting represents a decolorization process *(7)*. The next stage of azo dye degradation is the conversion of aromatic amines. Mineralization of the aromatic amines in aerobic conditions is common. Note that some aromatic amines are readily autooxidized in the presence of oxygen to oligomeric and polymeric substances. On the other hand, anaerobic bacteria are able to degrade some aromatic amines characterized by the presence of hydroxyl and carboxyl groups *(8,9)*.

The goal of the present study was to investigate toxicity for methanogens and anaerobic biodegradability of three acid azo dyes widely used in Russia in the presence of methanogenic granular sludge. The biologic azo dye reduction rate was evaluated and compared with the rate of chemical reduction by sulfide, which might be generated by sulfate-reducing bacteria presented in anaerobic sludge.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO), were of analytical grade, and were not further purified.

Biomass and Basal Medium

Methanogenic granular sludge from an expanded granular sludge bed reactor treating brewery wastewater (Efes-Moscow) was used.

The basal medium in all batch experiments contained 5000 mg/L of $NaHCO_3$, 280 mg/L of NH_4Cl, 10 mg/L of $CaCl_2 \cdot 2H_2O$, 250 mg/L of K_2HPO_4, 100 mg/L of $MgSO_4 \cdot 7H_2O$, 100 mg/L of yeast extract, 0.05 mg/L of H_3BO_3, 2 mg/L of $FeCl_3 \cdot 4H_2O$, 0.05 mg/L of $ZnCl_2$, 0.05 mg/L of $MnCl_2 \cdot 4H_2O$, 0.03 mg/L of $CuCl_2 \cdot 2H_2O$, 0.05 mg/L of $(NH_4)_2SeO_3 \cdot 5H_2O$, 2 mg/L of $AlCl_3 \cdot 6H_2O$, 0.05 mg/L of $NiCl_2 \cdot 6H_2O$, 0.1 mg/L of $Na_2SeO_3 \cdot 5H_2O$, 1 mg/L of EDTA, and 36% HCl; pH 7.0.

The pH of the culture broth was controlled potentiometrically during all experiments.

Assays

The toxicity of the azo dyes at concentrations of 0.5, 1, 2, 3, and 4 m*M* was determined using aceticlastic methanogenic activity assays *(8)*. These assays were performed in 120-mL glass serum vials closed with a butyl

rubber septum and an aluminum cap. The bottles containing 50 mL of basal medium were flushed with argon. The aqueous solution of investigated dyes and granular sludge (1.0 g of volatile suspended solids [VSS]/L) were added. Then 0.5 mL of acetate was added from a solution containing 2.85 *M* sodium acetate. The assay bottles were incubated at 30°C for 10 d. The concentration of CH_4 and pressure in the headspace of the serum flasks were recorded throughout the incubation. Specific aceticlastic activities of the sludge were calculated from a linear segment of kinetic curves of methane production. The aceticlastic activity of the untreated sludge with a toxicant was taken as a control. From the concentration dependence of the specific activities, 50% inhibiting concentration (IC_{50}) values for the azo dyes were estimated. The assays were performed in duplicate.

Azo dye reduction experiments were performed in 120-mL serum bottles. These bottles were filled with 50 mL of basal medium, granular sludge (1.0 g of VSS/L), and 1 m*M* Acid Orange 6 or Acid Orange 7, or 0.2 m*M* Acid Orange 52. Then they were closed with a butyl rubber septum and an aluminum cap. The headspace of the bottles was flushed with argon. The assays were performed in duplicate. An experiment in the presence of ethanol was also carried out at a concentration 1 m*M* as external electron donor. The assay bottles were incubated at 30°C for 40 d. The concentrations of methane and cosubstrate were measured by gas chromatography, and dye concentrations were measured spectrophotometrically. For this purpose, samples were centrifuged and diluted with a phosphate buffer (1 *M*, pH 7.0) in a 1:20 ratio.

To take into account production of methane from the cell autolysis, control experiments were carried out without dyes. Degradation of dyes by reaction with the components of the basal medium was considered in the control experiment in the absence of methanogenic sludge.

The concentration of azo dyes may decrease as a result of adsorption on the sludge particles. Therefore, experiments with autoclaved sludge (killed cells) were used.

The chemical azo dye reduction assays were performed identically to the biologic assays with the exception that sulfide was added to final concentrations of 5, 0.5, or 0.05 m*M* instead of granular sludge. The concentrations of the dyes were 0.5, 0.25, or 0.025 m*M*, respectively.

Analyses

The methane and carbon dioxide contents in the headspace of the serum flasks were determined on a gas chromatograph LHM 8MD (Yagot, Moscow, Russia) equipped with a steel column (2 m) packed with Porapak QS. The temperature of the column, injector port, and conductivity detector was 50°C. Argon was used as the carrier gas with a flow rate of 30 mL/min. The volume of the gas sample was 200 µL.

Volatile fatty acids and alcohols were detected using a gas chromatograph Chrom-5 (Laboratorni Pristroje, Praha, Czechoslovakia) equipped with a glass column (1 m) packed with Chromosorb 101. The temperatures

of the column, injector port, and flame ionization detector were 190, 200, and 200°C, respectively. Argon was used as the carrier gas with a flow rate of 30 mL/min. The volume of the aqueous sample was 1 µL.

Azo dye concentrations were measured spectrophotometrically with a Shimadzu UV-1202 spectrophotometer (Shimada, Kyoto, Japan) at a maximum absorbance wavelength of 428 nm (Acid Orange 6), 490 nm (Acid Orange 7), and 465 nm (Acid Orange 52). Samples were centrifuged, and then aliquots of 100 µL were diluted in a 2-mL 0.1 *M* phosphate buffer (pH 7.0) solution and measured in a 1.0-cm quartz cuvet.

The aromatic amines formed were analyzed using high-performance liquid chromatography (HPLC). The reverse-phase column was packed with Diasorb 130-C_{16}T and had a film thickness of 6 µm. The chromatograph (Gilson, Villiers la Bel, France) was equipped with an ultraviolet detector operated at 225 nm. Methanol with 1% acetic acid was used as the liquid phase with a flow rate of 1 mL/min. The pressure was 14 MPa and the volume of the aqueous sample was 10 µL.

Results and Discussion

Toxicity of Azo Dyes

The literature describing the toxicity of azo dyes for anaerobic microorganisms is substantial *(8,10)*; however, we did not find such data for the three azo dyes used in our study. Generally, sulfonated azo dyes are more toxic than the corresponding unsulfonated compounds for anaerobic microorganisms *(7)*. Note that from all the classes of organisms participating in the process of anaerobic biodegradation, methanogens are the slowest-growing organisms, so toxic compounds may have a strong influence on them. Thus, the toxicity assays of Acid Orange 6, Acid Orange 7, and Acid Orange 52 were undertaken regarding the aceticlastic methanogenic activity of anaerobic sludge. The results are presented in Fig. 1.

It was observed that toxicity increased among Acid Orange 6, Acid Orange 7, and Acid Orange 52 for which IC_{50} values were found to be 2.50, 0.90, and 0.25, respectively. An increase in methanogenic activity was observed up to an Acid Orange 6 concentration of 2 m*M*. This was probably owing to an additional input of acetate from degradation of 4-aminoresorcinol (breakdown product of this dye).

Biodegradation of Azo Dyes

Biodegradation of Acid Orange 6, Acid Orange 7, and Acid Orange 52 under anaerobic conditions has not been sufficiently studied. In the literature, there are some data only on Acid Orange 7 decolorization under methanogenic conditions *(11)*.

It was found that all of the azo dyes tested were completely decolorized (Fig. 2). Color elimination occurred owing to cleavage of the azo bond. In all cases, the reaction products were colorless. The addition of

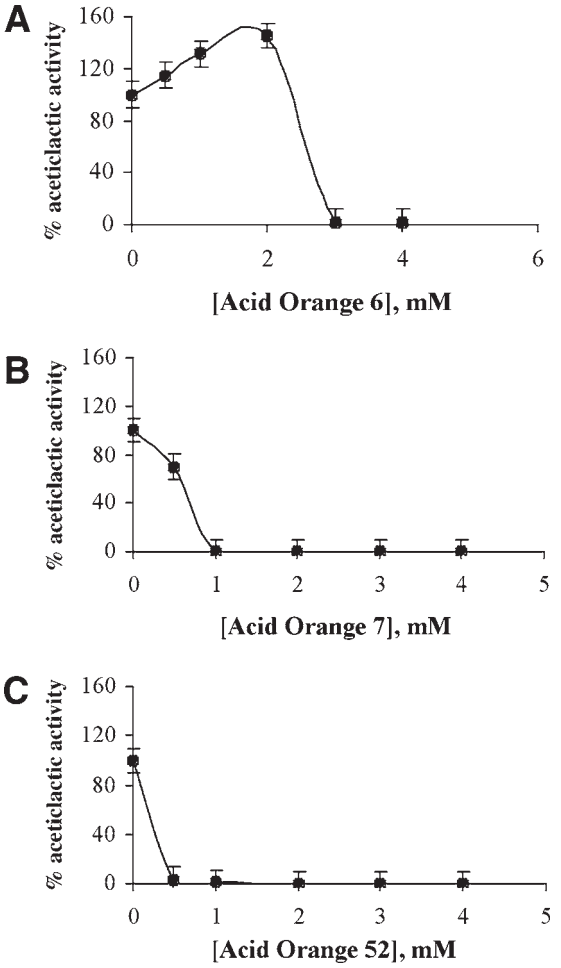


Fig. 1. Influence of concentrations of tested azo dyes on aceticlastic methanogenic activity of anaerobic sludge: **(A)** Acid Orange 6; **(B)** Acid Orange 7; **(C)** Acid Orange 52.

ethanol as a donor of reductive equivalent for splitting of azo bond did not enhance decolorization rates of azo dyes. The fact that anaerobic sludge can easily degrade various azo dyes points to the nonspecific character of this process. The bioreduction of azo bond proceeded without a lag period and followed first-order kinetics with respect to dye concentration:

$$C = C_0 \times e^{-kt}$$

in which C is the current concentration of azo dye at given time (M), C_0 is the initial concentration (M), t is time (d), and k is kinetic (first-order) constant (d^{-1}).

Kinetic constants were calculated from the time course of azo dye concentration. The determined values of these constants were 0.20 ± 0.02, 0.30 ± 0.03, and 0.49 ± 0.03 d^{-1} for Acid Orange 6, Acid Orange 7, and Acid

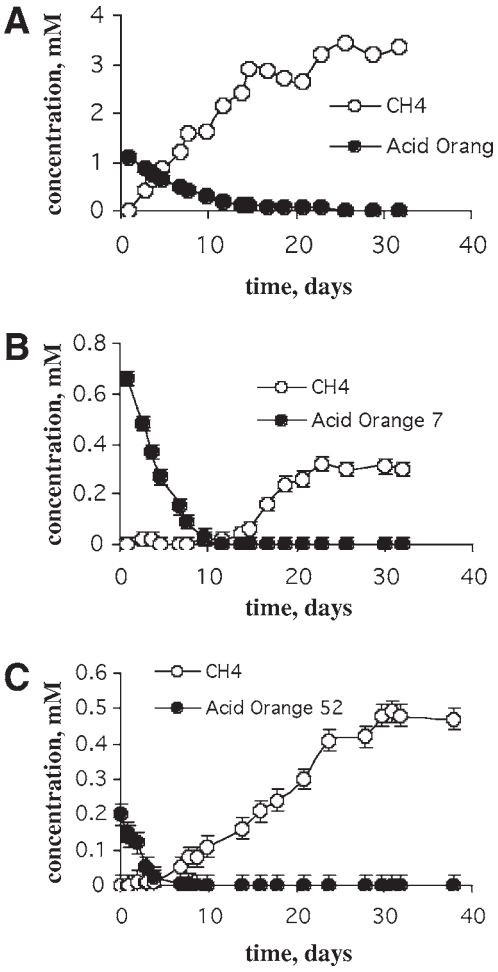
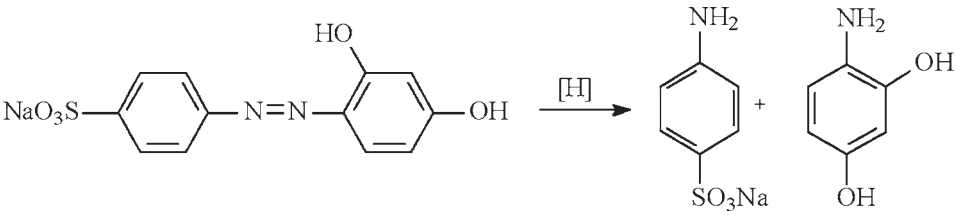


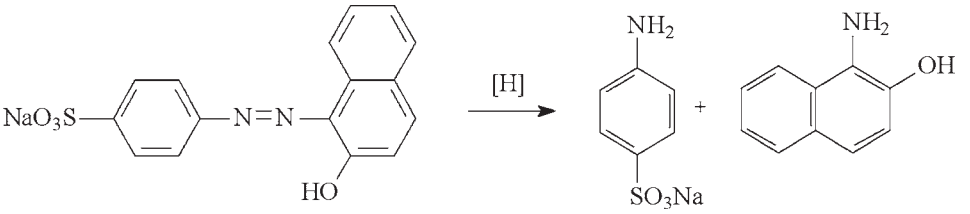
Fig. 2. Bioconversion of azo dyes in anaerobic environments: **(A)** Acid Orange 6; **(B)** Acid Orange 7; **(C)** Acid Orange 52. Net methane production was calculated by subtracting background methane production in the control (without exogenous substrate) from that in the test vials.

Orange 52, respectively. These data are not in accordance with the toxicity of azo dyes. In spite of the high toxicity of Acid Orange 52, the rate of its reduction was highest. Note that methanogens, for which toxicity has been determined, do not take part in azo dye decomposition. In addition, the rate of azo dye reduction is a function of its chemical structure, although toxicity can apparently be related to infringement of cell membrane. The reduction of azo dyes was mediated biologically since assays in the absence of sludge and in the presence of autoclaved sludge (killed cells) did not show any decolorization of azo dyes.

A change in pH may result in color shift or even decolorization of dye solution, and, therefore, the pH of the culture broth was controlled. At all periods of cultivation, the pH was about 7.0.



Scheme 1.

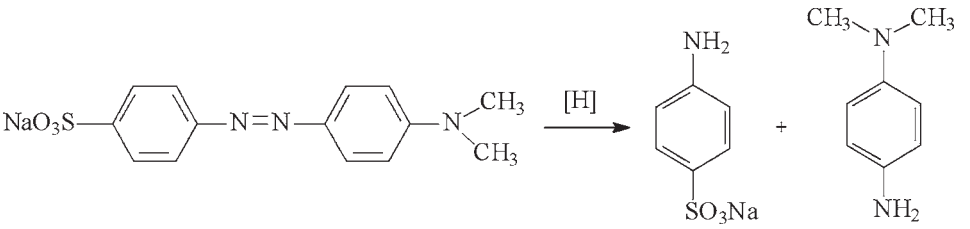


Scheme 2.

During decolorization of Acid Orange 6, gradual methane accumulation was observed. This result manifested owing to mineralization of breakdown products of this azo dye. Two aromatic amines appeared as intermediates of Acid Orange 6 reduction: sulfanilic acid and 4-aminoresorcinol (see Scheme 1). Sulfanilic acid is known to be persistent to biodegradation in anaerobic environments *(7)*. HPLC showed that sulfanilic acid concentration remained fairly constant. The other product of Acid Orange 6 degradation (4-aminoresorcinol) was not found by HPLC, so it seems to have been mineralized by sludge. This supposition was confirmed by methane production. Theoretically, the amount of methane produced as the result of 4-aminoresorcinol mineralization is three times more than the initial concentration of 4-aminoresorcinol (in m*M*). This was proven by experimental data (Fig. 2A).

Sulfanilic acid and 1-amino-2-naphtol were detected from the reduction of Acid Orange 7, and this finding is in accordance with the literature *(11)* (see Scheme 2). Both these substances are persistent in anaerobic conditions *(7,11)*. Low methane production was detected during an incubation period of 1 mo (Fig. 2B). This means that sulfanilic acid and 1-amino-2-naphtol were not mineralized by the sludge.

Acid Orange 52 was also completely decolorized by methanogenic sludge. Two aromatic amines were determined after dye reduction: sulfanilic acid and *N*,*N*-dimethyl-1,4-phenylenediamine (DMP) (see Scheme 3). Immediately after azo dye decolorization, an increase in methane production was observed (Fig. 2C). This can be attributed to demethylation of DMP and formation of 1,4-phenylenediamine, as confirmed by HPLC. Theoretically, two molecules of methane appear from DMP conversion, and this fact was proven by the experimental data. By contrast, 1,4-phenylenediamine was resistant to further degradation.



Scheme 3.

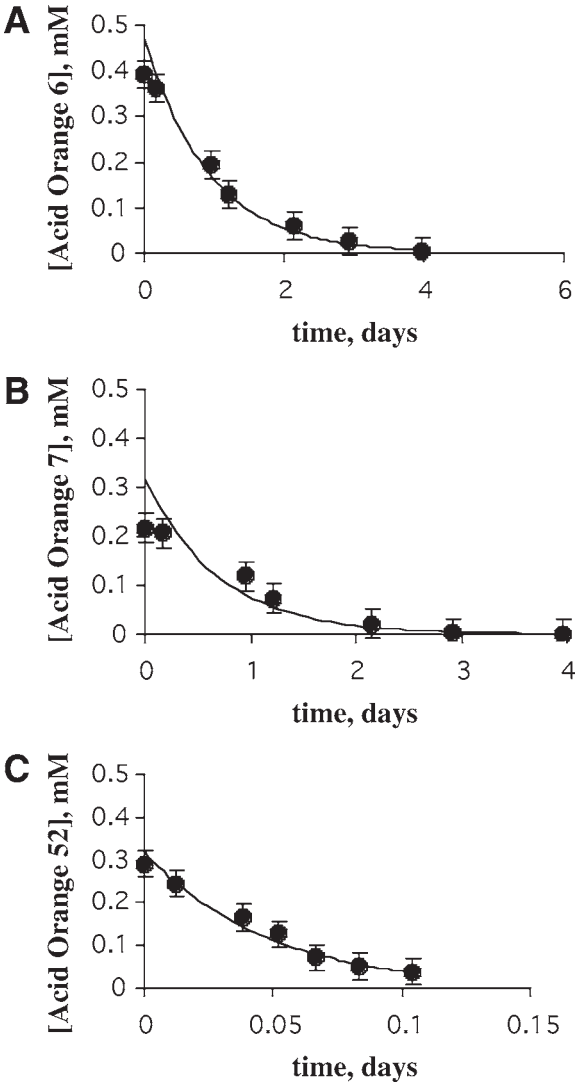


Fig. 3. Decolorization of tested azo dyes by sulfide (initial sulfide concentration of 5 m*M*): **(A)** Acid Orange 6; **(B)** Acid Orange 7; **(C)** Acid Orange 52.

Table 1
First-Order Rate Constant Values for Azo Dye Decolorization by Sulfide

	Sulphide (m*M*)		
	5	0.5	0.05
k (Acid Orange 6), d^{-1}	1.07 ± 0.06	0.10 ± 0.01	0.07 ± 0.01
k (Acid Orange 7), d^{-1}	1.44 ± 0.09	0.40 ± 0.08	0.20 ± 0.04
k (Acid Orange 52), d^{-1}	20 ± 1	0.88 ± 0.04	0.20 ± 0.02

Enrichment cultures obtained via consecutive passages on a basal medium with only azo dye as a carbon and an energy source seemed to be morphologically heterogeneous. Baculiform and coccus cells were found when observed under a light microscope. Cocci were jointed in chains.

Reduction of Azo Dyes by Sulfide

Anaerobic granular sludge usually contains sulfate-reducing bacteria, therefore, sulfide can be present in the sludge and may facilitate azo dye reduction. Thus, azo dye decolorization was studied without sludge but under sulfide concentrations of 0.05, 0.5, and 5 m*M*. From Fig. 3 it can be seen that all dyes under study were reduced by sulfide. The products in all cases were found to be the same as for bioreduction. A linear relationship was found between the rates of azo dye reduction and sulfide concentrations; that is, the rate of reduction increased with an increase in sulfide concentration. Chemical azo dye reduction also followed first-order kinetics with respect to dye concentration. However, in contrast to biologic decolorization, a lag period was observed in the beginning of the reduction by sulfide, especially at low sulfide concentrations. This fact may be related to a reaction of sulfide and the remaining oxygen in the medium; apparently, traces of oxygen remained in the medium in spite of argon flushing and a part of the sulfide reacted with the remaining oxygen. For all sulfide concentrations, Acid Orange 52 had the highest value of kinetic constant and Acid Orange 6 had the lowest, as in the case of biodegradation (Table 1). This confirms the fact that the rate of azo dye reduction, either biologic or chemical, depends on the chemical structure of the substrate.

Determination of the sulfide concentration in the culture broth showed that the sulfide was <0.001 m*M*. It seems that bioreduction of azo dyes is not directly related to sulfide generated by anaerobic sludge.

Conclusion

The toxicity of three azo dyes (Acid Orange 6, Acid Orange 7, and Acid Orange 52) was determined regarding aceticlastic methanogenic activity of anaerobic sludge. Acid Orange 6 was less toxic for methanogens compared with Acid Orange 7 and Acid Orange 52.

All the azo dyes tested were completely decolorized by methanogenic anaerobic sludge. First-order kinetics described azo dye reduction. Sulfanilic acid and 4-aminoresorcinol were detected from the decolorization of Acid Orange 6, sulfanilic acid and 1-amino-2-naphtol were detected from the reduction of Acid Orange 7, and sulfanilic acid and DMP were found to be intermediates of Acid Orange 52 degradation. Sulfanilic acid and 1-amino-2-naphtol were persistent under anaerobic conditions, whereas 4-aminoresorcinol was completely mineralized by anaerobic sludge. Furthermore, DMP was transformed into 1,4-phenylenediamine.

Azo dye reduction by sulfide at concentrations of 5, 0.5, and 0.05 m*M* was investigated. Lag periods were observed in the beginning of the reaction between azo dyes and sulfide, which may be related to the reaction of sulfide and the remaining oxygen in the medium. The highest rate of reduction (both chemical and biologic) was found for Acid Orange 52, and the lowest for Acid Orange 6.

Acknowledgment

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