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P. Rajaguru · K. Kalaiselvi · M. Palanivel · V. Subburam

Biodegradation of azo dyes in a sequential anaerobic–aerobic system

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Abstract A sequential anaerobic–aerobic treatment process based on mixed culture of bacteria isolated from textile dye effluent-contaminated soil was used to degrade sulfonated azo dyes Orange G (OG), Amido black 10B (AB), Direct red 4BS (DR) and Congo red (CR). Under anaerobic conditions in a fixed-bed column using glucose as co-substrate, the azo dyes were reduced and amines were released by the bacterial biomass. The amines were completely mineralized in a subsequent aerobic treatment using the same isolates. The maximum degradation rate observed in the treatment system for OG was 60.9 mg/l per day (16.99 mg/g glucose utilized), for AB 571.3 mg/l per day (14.46 mg/g glucose utilized), for DR 112.5 mg/l per day (32.02 mg/g glucose utilized) and for CR 134.9 mg/l per day (38.9 mg/g glucose utilized).

Introduction

Synthetic dyes are used extensively for textile dyeing, paper printing, colour photography and as additives in petroleum products. Approximately 40,000 different dyes and pigments are used industrially and over 7×10^5 tons of these dyes are produced annually worldwide (Zollinger 1987). It is estimated that 10–15% of the dyes used in textile processing is lost in effluent during the dyeing process (Vaidya and Datye 1982). A necessary criterion for the use of these dyes is that they must be highly stable in light and during washing. They must also be resistant to microbial attack. Therefore, they are not readily degradable under natural conditions and are typically not removed from wastewater by conventional wastewater-treatment systems (Anliker 1979; Pagga and Brown 1986). Azo dyes are the largest group of these dyes used in industry. They also exhibit structural variety and so as a group they are not uniformly susceptible to microbial attack (Meyer 1981). As with other aromatic pollutants, microorganisms have only a limited capacity to degrade azo dyes under natural conditions.

In the microbial degradation of azo dyes, the initial process is their decolorization. The highly electrophilic azo bond must be cleaved for azo decolorization to take place. Under aerobic conditions neither the activated sludge (Shaul et al. 1991) nor aerobic bacterial isolates were able to degrade azo dyes (Wuhrmann et al. 1980; Haug et al. 1991). On the other hand, various azo dyes were shown to be decolorized by anaerobic sludge (Brown and Laboureur 1983; Brown and Hamburger 1987), anaerobic sediments (Weber and Wolfe 1987) and by pure cultures of bacteria incubated anaerobically (Meyer 1981; Haug et al. 1991). Dye decolorization was also reported in continuous anaerobic reactions with immobilized biomass (Zaoyan et al. 1992). The reductive cleavage of the azo bond resulted in the formation of aromatic amines as end-products. However, most such end-products of anaerobic degradation of azo compounds are further degraded by aerobes (Kulla 1981; Thurnheer et al. 1986; Weber and Wolfe 1987; Thurnheer et al. 1988; Dickel et al. 1993).

The degradation of recalcitrant compounds with xenobiotic characteristics requires unusual catabolic activities, which may not be found in a single organism. Characteristically, the microbial degradation of sulfonated aromatics is often accomplished by mixed cultures (Dangmann et al. 1996). The sequential anaerobic–aerobic bacterial degradation system has been shown to be efficient in the degradation of the sulfonated azo dye Mordant Yellow 3 (Haug et al. 1991). On the basis of these observations, the present paper describes a sequential anaerobic–aerobic system for the degradation of the sulfonated azo dyes Orange G (OG), Amido black

P. Rajaguru (✉) · K. Kalaiselvi · M. Palanivel
Department of Environmental Science,
PSG College of Arts and Science,
Coimbatore–641014, India

V. Subburam
Department of Environmental Science, Bharathiar University,
Coimbatore–641048, India

10B (AB), Direct red (DR) and Congo red (CR) using the resistant microorganisms isolated from the dye effluent-contaminated soils (Fig. 1).

Materials and methods

Chemicals

All dyes and chemicals were obtained from either E. Merk (Mumbai, India) or SD-Fine Chemicals (Mumbai, India). The dyes were purified by recrystallization before being used for the biodegradation studies. For recrystallization, the dyes were dissolved in boiling methanol and filtered through Whatman No. 1 filter paper. The filtrate was allowed to crystallize at room temperature. The crystals were collected and used for further studies.

Experimental set-up and design of the sequential anaerobic–aerobic system

The continuous two-stage anaerobic–aerobic bioreactor system used in this study was set-up according to the design described by Dickel et al. (1993), with slight modifications. The treatment system mainly consisted of an up-flow fixed-bed column (anaerobic part) and an agitated tank (aerobic part). The substrate solution was allowed to flow from a storage tank into the up-flow fixed-bed column. For pressure compensation, the storage bottle was connected to a nitrogen-filled balloon. The length of the up-flow fixed-bed column made of polyvinyl chloride was 50 cm and the diameter was 7.5 cm. A perforated plastic plate was inserted at the bottom. In order to allow immobilization of the anaerobic biomass, the column was filled with plastic beads (2-mm diameter) as the bacterial support. The interstitial fluid volume of the column was 1 l. The efflux at the top of the anaerobic column was allowed to drain into an aerated laboratory fermentor vessel. The aerobic culture

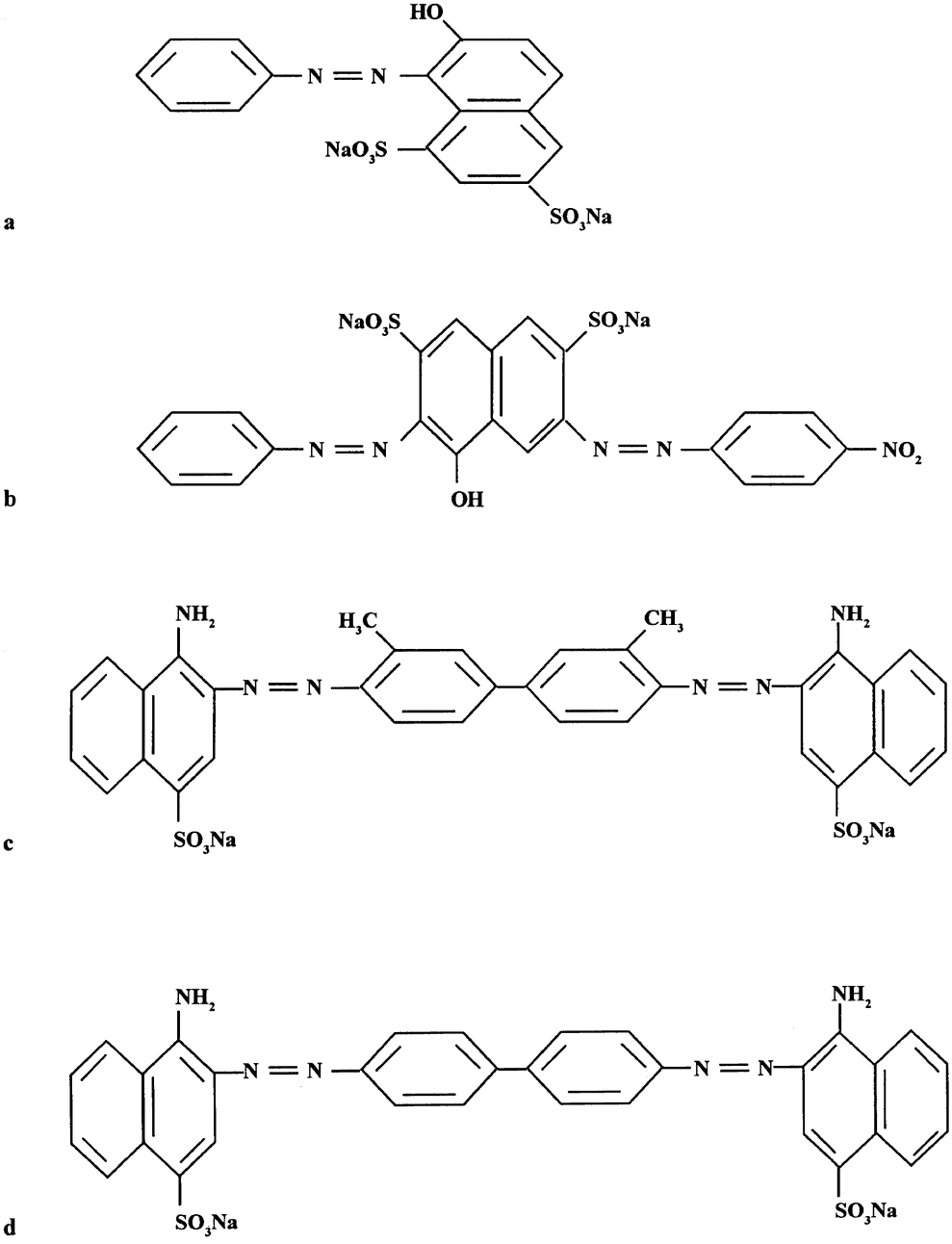

Fig. 1a–d Azo compounds used in this study. **a** Orange G (CI 16239 = Acid orange 10); **b** Amido black 10B (CI 20470 = Napthol blue black); **c** Direct red 4BS (CI 23500 = Direct red 2); **d** Congo red (CI 22120 Direct red C)

volume was also 1 l and the rate of aeration was maintained at 2 l/min. The surplus from the aerobic culture vessel was conveyed into a waste tank by a plastic tube. The flow rate of the whole system was maintained at 30 ml/h resulting in a hydraulic retention time (HRT) of 3 days in the anaerobic–aerobic bioreactor system.

Media and inoculum

Four bacterial strains (pseudomonads) isolated from dyeing effluent-contaminated soils were used as the inoculum for the anaerobic and aerobic parts of the two-stage bioreactor system. For inoculation of the reactor system, 1 l nitrogen-free mineral medium containing (per litre) 2.38 g KH_2PO_4, 5.55 g K_2HPO_4, and 1.0 g $MgSO_4 \cdot 7\ H_2O$, pH 7.0) and supplemented with 5.0 g/l glucose and 0.2 g/l yeast extract was seeded with the bacterial isolates and preincubated for 48 h at 30 °C. The mixed bacterial culture was divided into two equal portions: one was transferred to an anaerobic column packed with the supporting material and the other was transferred to an aerobic vessel. In order to equilibrate the sequential bioreactor, nitrogen-free mineral medium containing glucose (20 mM) and OG (5 mg/l) was allowed to pass from the storage bottle through the system (flow rate, 15 ml/h; HRT, 6 days). During an adaptation period of 4 weeks the OG concentration was increased to 10 mg/l by means of a linear gradient. After a further 2-week period, the flow rate was enhanced to 30 ml/h (HRT, 3 days) and the concentration of OG was raised, at 20-day intervals, to 25, 50 and 100 mg/l. The whole system was run at ambient temperature. The concentrations of the dye, amine, ammonia, glucose and cellular protein in the efflux of the anaerobic fixed-bed column and the aerobic agitation tank were measured spectrophotometrically twice a week.

The same experimental procedure was followed to assess the extent of degradation of AB (10, 25, 50 and 100 mg/l), DR (25, 50, 100 and 200 mg/l) and CR (25, 50, 100, and 200 mg/l). Throughout the experiment the flow rate and the HRT were maintained at 30 ml/h and 3 days, respectively.

Aromatic amines were quantified by diazotization and azo coupling with *N*-(1-naphthyl)-ethylenediamine according to the method of Bratton and Marshall (1939). Amines released from anaerobic reduction of OG and AB were quantified using aniline as the standard and those from DR and CR were measured using benzidine as the standard. Ammonia was determined colorimetrically by the phenol-hypochlorite reaction (Gerhardt et al. 1981). The quantitative determination of glucose was done using the Nelson–Somagyi method (Somagyi 1952).

In order to quantify the biomass in the system, cellular protein was determined in the efflux from the anaerobic fixed-bed column and that from the aerobic agitated tank. For this purpose, approx. 1 ml of the efflux sample was centrifuged (3,000 rpm, 30 min). The resultant cellular pellet was dissolved by boiling in 1-M NaOH for 20 min; an aliquot of this was used for protein determination by Lowry's method (Lowry et al. 1951).

To characterize the composition of the aerobic biomass, after 3 months samples were taken from the aerobic tank and inoculated into mineral medium with or without glucose and supplemented with 100 mM aniline as the sole source of nitrogen and/or carbon and incubated at 30 °C for 3 days.

Results

The results show complete reduction of OG in the feed solution with the concomitant release of amine by the anaerobic biomass up to a dye concentration of 50 mg/l. However, when the inlet concentration of the dye was raised to 100 mg/l, a decrease in the level of reduction of the dye was observed, as evidenced by the appearance of the dye in the efflux from the anaerobic column (Fig. 2). Breakthrough occurred at an inlet concentration of ≥100 mg/l (0.22 mM). At the breakthrough concentration of OG, 40.9% of the dye was recovered as amine in the efflux, which corresponds to a reduction rate of 60.9 mg dye/l per day or 16.99 mg dye/g glucose utilized. The breakthrough concentration of non-reduced OG was 15.4 mg/l (0.034 mM).

When the amine-containing effluent from the anaerobic fixed-bed column was passed into the agitated

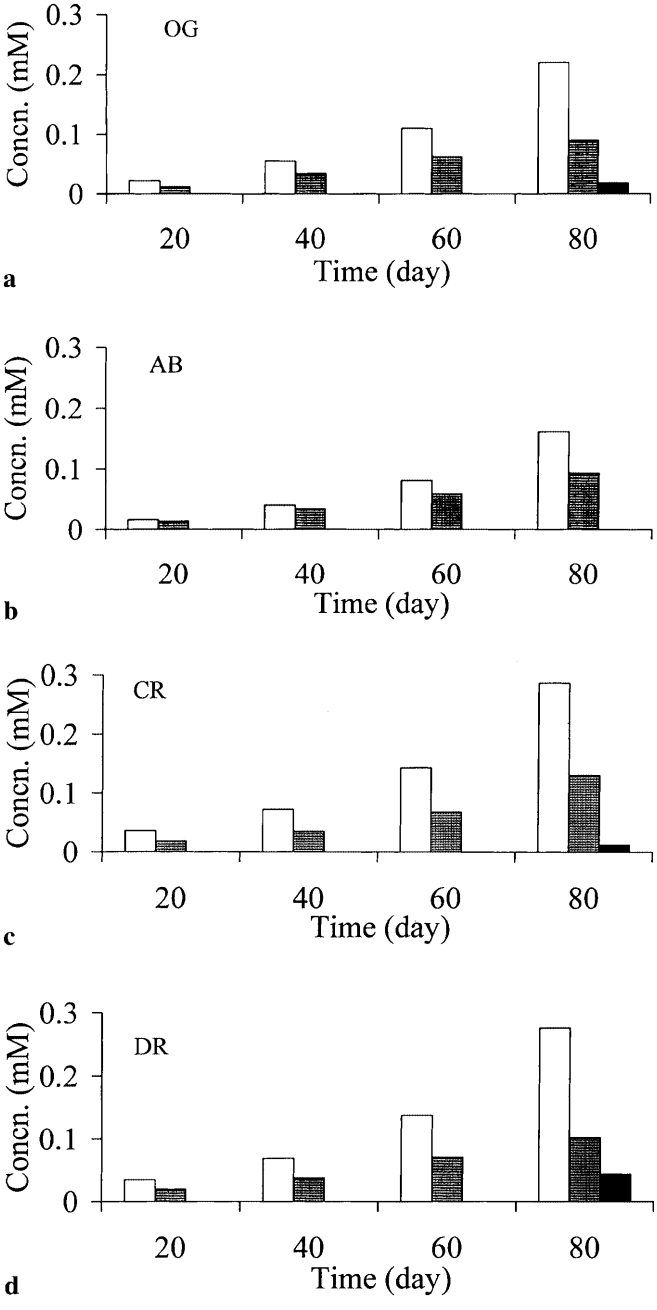

Fig. 2 Unwarranted reduction of azo dyes (OG, Orange G; AB, Amido black 10B; DR, Direct red 4BS; CR.– Congo red) by the bacteria isolated from dye effluent-contaminated soil in a two-stage anaerobic–aerobic bioreactor using glucose as the H-donor: dye (M), amine (EM), unreduced dye (M)

aerobic tank, the amine was completely degraded by the aerobic biomass. At low (up to 50 mg/l) inlet concentrations of OG, amine was not detected in the aerobic tank. However, when the dye concentration was raised to 100 mg/l, a small amount of amine (0.016 mM) was detected in the aerobic tank.

Obviously, the bulk of the immobilized biomass was at the bottom of the column. Even in the effluent of the anaerobic column an increase in the biomass could be observed after 2 months (which stabilized after 4 months; ≤0.1 mg protein/ml effluent). After 3 months. the equilibrium concentration of aerobic biomass in the aerobic stirred tank corresponded to 0.4 mg protein/ml (Fig. 3). The number of cells which could form colonies on mineral agar plates containing aniline as the sole nitrogen source was 6.7×10^7/ml; with amine (aniline) as the sole source of carbon and nitrogen the number was 1.31×10^5/ml.

After breakthrough with OG had been obtained, a similar experiment was conducted with AB. After an adaptation period of 4 weeks, the concentration of the dye was raised from 10 mg/l (0.016 mM) to 100 mg/l (0.162 mM) at an interval of 20 days. AB was completely degraded at lower concentrations. Breakthrough occurred at an inlet concentration of ≥100 ppm (0.162 mM). At breakthrough concentration, a different coloured substance with a λ_{max} of 545 nm and 0.093 mM amine appeared in the anaerobic column efflux. The maximum reduction rate of AB was 51.3 mg/l per day or 14.46 mg dye per gram of glucose utilized. As with OG, in this experiment amine (0.015 mM) remained in the agitated aerobic tank at the breakthrough concentration of AB.

Similar experiments were conducted with DR and CR. With both dyes, breakthrough occurred at ≥200 mg/l. At the influx DR concentration of 200 mg/l (0.276 mM), 37% of the dye was recovered as amine, corresponding to a reduction rate of 112.5 mg/l per day or 32.02 mg dye/g glucose utilized by the anaerobic biomass. With CR at an influx concentration of 200 mg/l (0.287 mM), 45.6% of the dye was recovered as amine in the efflux of anaerobic fixed-bed column, which corresponds to a reduction rate of 134.9 mg/l per day or 38.9 mg dye/g glucose utilized.

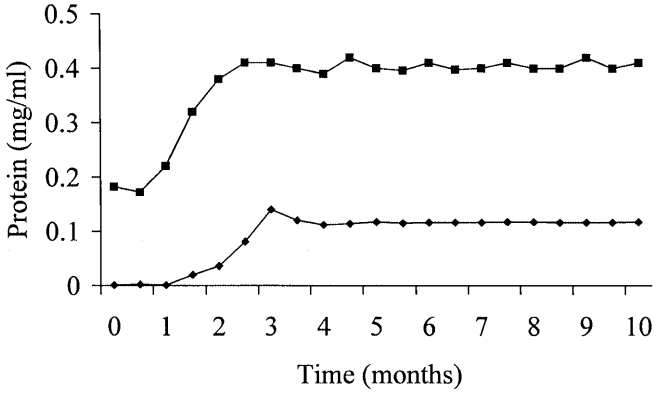

Fig. 3 Cellular protein level in the efflux of the anaerobic–aerobic bioreactor: aerobic (■), anaerobic (◆). Data shown represent the mean value from four estimations

At the breakthrough concentration, the inlet concentrations of non-reduced DR and CR were 43.8 mg/l and 12.7 mg/l, respectively. At a higher influx concentration (200 mg/l) of DR and CR 0.024 mM and 0.044 mM amine, respectively, was detected in the aerobic tank. Furthermore, adsorption of DR and CR to cells present in the efflux of anaerobic column was also observed.

During the entire experiment there was an unwarranted increase in the concentration of amine in the efflux of the anaerobic column when the concentration of the dye in the feed was increased. A small amount (up to 32 mg/l unutilized glucose was observed in the effluent of the anaerobic column when the inlet concentration of dye was low. However, in the aerobic agitated tank (with any of the dyes) no glucose was detected. Ammonia was not found either in the effluent of the anaerobic column or in the effluent of the agitated aerobic tank. During the experiment, when the dye in the inlet solution was changed from one to another, a period of approx. 2–3 weeks was necessary to attain an apparent steady state.

Discussion

Many compounds that are difficult to degrade aerobically seem to be readily degraded anaerobically. In turn, the products of anaerobic biotransformation resist further anaerobic mineralization yet are good substrates for aerobic biodegradation. Thus, for the total mineralization of many recalcitrant pollutants a sequential anaerobic–aerobic treatment strategy is considered best (Zitomer and Speece 1993). As the degradation of recalcitrant compounds with xenobiotic characteristics requires unusual catabolic activities, the microbial degradation of sulfonated aromatics is often accomplished by mixed culture. The present investigation shows that azo dyes can be degraded by a sequential anaerobic–aerobic process. In this experiment with mixed bacterial cultures and with glucose as co-substrate and H-donor, azo dyes were completely decolorized and amine was released under anaerobic conditions. The released amine was mineralized in the aerobic part of the two-stage system. The ability of microorganisms to utilize the amine (aniline) as the sole source of carbon and nitrogen has been confirmed in a subsequent plate-culture study. Growth on agar plates showed that the aerobic biomass of the agitated aerobic tank was composed of organisms capable of utilizing amine (aniline) as the source of nitrogen and organisms able to use aniline as the source of nitrogen and carbon. Furthermore, throughout the experimental period of more than 10 months, as no additional nitrogen source other than the test dye was added into the feed solution of the anaerobic column, the observed growth and

subsequent stabilization of the microbial biomass in the fixed-bed column and the agitated aerobic tank indicate that the organisms used in the study are capable of utilizing azo dyes as the sole nitrogen source.

The appearance of a coloured product having a different absorption maximum (545 nm) at a high influx concentration of AB (620 nm) and the absence of any such compounds at lower influx concentrations indicate that the metabolic reduction of AB starts with the conversion of this dye into another coloured compound which can be metabolically decolorized by the anaerobic biomass used in the study. It also indicates that the HRT of 1.5 days in the anaerobic column is insufficient and therefore should be increased with increasing concentrations of AB for complete decolorization.

This experiment demonstrated that, in the anaerobic pretreatment, the sulfonated azo dyes with a highly electrophilic character could be reduced and that the reduction products were acceptable for aerobic mineralization. Because of a lack of facilities, the metabolic pathway for the dyes with mixed culture could not be deduced and it could not be ascertained as to whether complete mineralization occurred. However, the observed growth and maintenance of the biomass, the unhindered operation of the two-stage treatment system for more than 12 months, and the disappearance of amine in the aerobic tank indirectly indicate either that all the dyes are completely mineralized or that the end-products are not inhibitory or toxic in nature. Unlike previous studies (Kulla et al. 1983; Haug et al. 1991) in which the bacteria used required adaptation and showed narrow specificity to the dye to which they were adapted, the bacterial strains used in this study isolated from the dyeing effluent-contaminated soil neither required adaptation nor showed specificity to any dye. They have naturally developed the capacity to degrade dyes with different structures, as shown in the present study. Therefore, the two-stage anaerobic–aerobic bioreactor system with the mixed culture can be used in the textile industry to treat dye-containing wastewater because, under anaerobic conditions, it reduces a wide range of azo dyes and aerobically oxidizes many different amines and possibly the sulfonated products released during the anaerobic cleavage of sulfonated azo dyes. In view of their continuous exposure to various other constituents present in the dyeing effluents in their natural soil environment, these bacterial isolates are expected to be able to degrade the dyes unhindered when wastewaters from the dyeing industry are used. The wastewater from most of the small-scale dyeing units contain negligible organic matter. However, the combined wastewaters of the textile industries may contain starch and carboxymethylcellulose (CMC) as a result of their utilization in the sizing process. The use of this system in the degradation of azo dyes with organic compounds such as starch, CMC, ethanol, methanol or acetone in place of glucose as the co-substrate is being studied in our laboratory.

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