Biodegradation (2006) 17: 403–413
DOI 10.1007/s10532-005-9011-0

Microbial decolorization of reactive black-5 in a two-stage anaerobic–aerobic reactor using acclimatized activated textile sludge

Sagarika Mohanty, Nishant Dafale & Nageswara Neti Rao*
*National Environmental Engineering Research Institute, Nehru Marg, 440020, Nagpur, India (*author for correspondence: e-mail: dr_nnrao@rediffmail.com)*

Accepted 8 August 2005

Key words: biological decolorization, reactive black 5, textile sludge, two-stage anaerobic–aerobic reactor

Abstract

A two-stage anaerobic–aerobic treatment process based on mixed culture of bacteria isolated from textile dye effluent was used to degrade reactive black 5 dye (RB-5). The anaerobic step was studied in more detail by varying the dye concentration from 100 to 3000 mg l^{-1}. The results showed that major decolorization was achieved during the anaerobic process. The time required for decolorization by >90% increased as the concentration of the dye increased. It was also found that maintaining dissolved oxygen (DO) concentration below 0.5 mg l^{-1} and addition of a co-substrate *viz*., glucose, facilitates anaerobic decolorization reaction remarkably. An attempt was made to identify the metabolites formed in anaerobic process by using high performance liquid chromatography (HPLC) and UV–VIS spectrophotometry. A plate assay was performed for the detection of dominant decolorizing bacteria. Only a few bacterial colonies with high clearing zones (decolorization zones) were found. The results showed that under anaerobic condition RB-5 molecules were reduced and aromatic amines were generated. The aromatic amine metabolite was partly removed in subsequent aerobic bio-treatment. It was possible to achieve more than 90% decolorization and approximately 46% reduction in amine metabolite concentration through two-stage anaerobic–aerobic treatment after a reaction period of 2 days.

Introduction

Textile industries produce multi-component wastes (dyes, starches, enzymes, fats, greases, waxes, surfactants, resins, chlorinated organic compounds etc.) depending upon the wet processes involved (Correia et al. 1994; Delee et al. 1998). Textile dyes are of synthetic origin and consist of complex aromatic molecules (Banat et al., 1996). These dyes impart color to textile processing and dye manufacturing wastewater.

Some of these dyes, particularly reactive azo dyes, are known to resist biodegradation. Therefore, color removal from textile effluents is still a major environmental problem as reactive azo dyes are not readily treated through conventional wastewater treatment systems (Pagga & Brown 1986; Seshadri et al. 1994). Chemical treatments, aerobic and anaerobic biological treatment, anaerobic–aerobic processes in tandem, have been attempted for color removal from textile wastewater (Reife & Freeman 1996). Chemical treatments like adsorption (Ramakrishna & Viraraghavan 1997), flocculation–coagulation and precipitation (Vandevivere et al. 1998), electrochemical oxidation (Lin &Peng 1994), photocatalytic oxidation (Davis et al. 1994; Zielinska et al. 2001), electro-Fenton oxidation (Bose 2002, Huang et al. 2001) though successful in COD reduction and color removal, are not yet affordable.

Aerobic biological systems are less effective to treat dye-bath wastewater (Ganesh et al. 1994). The removal mechanism primarily involves adsorption to activated sludge and aerobic

biodegradation occurs very slowly leaving behind difficult to degrade and/or non-biodegradable materials in the effluent. Interestingly, anaerobic systems are found to deal with this problem to a great extent (Chen et al. 2003; Cooper 1993); but the degradation metabolites are reported to be even more toxic (Brown & Hamburger 1987; Reife & Freeman 1996; Stolz 2001). A combination of anaerobic system followed by aerobic system has proven to be a better solution (Rajaguru et al. 2000; Seshadri et al. 1994) as the toxic amines generated from the former reaction are mineralized by the latter.

However, knowledge of biological decolorization is still inadequate and especially with reference to reactive dyes. Reactive Black 5 dye is widely used in textile dyeing and is reported to be toxic too (Fatemeh et al. 1990; Lars & Mallika 1997). In this study, the optimal conditions for anaerobic decolorization of RB-5 were reported. Based on this result, decolorization of RB-5 was attempted in a two-stage anaerobic–aerobic reactor.

Materials and methods

Chemicals

Reactive Black 5 was procured from a manufacturing unit in Gujarat. The structure of RB-5 is shown in Figure 1. RB-5 has H-acid core molecule with two pendant vinylsulphone groups. The various chemicals for preparing nutrient media were procured from Hi-Media Pvt. Ltd., Mumbai. Ethyl acetate and methanol solvents used for extraction and HPLC analyses were of chromatography grade. Anhydrous sodium sulphate (E.Merck India Pvt. Limited, Mumbai) was used for drying the extracts.

Biomass

Microbial biomass used for the decolorization experiments was activated sludge collected from aerobic treatment unit of a functional textile efferent treatment plant. Initially the sludge was transferred into 100 ml of 10 mg l^{-1} RB-5 dye solution in a flask. The flask was subsequently sealed and incubated for 48 h. The spent of RB-5 solution was drained from the bottom and fresh lot of RB-5 solution fortified with nutrients, was poured into the flask and again incubated. This procedure was repeated at intervals of 48 h. After 10 days, color of sludge bearing dye solution was noticed to decrease. The acclimation procedure was continued using higher concentrations (< 100 mg l^{-1}) of RB-5 which were increased in steps of 25 mg l^{-1} every 5 days till 30 days. By this procedure good quality biomass effective towards decolorization of the chosen dye was developed. The same sludge was used for further batch and continuos mode of decolorization experiments.

Decolorization experiments in batch reaction

Decolorization experiments were carried out in a fermenter vessel of 4.2 l capacity (Electrolab, UK). Initially, the biomass was grown in nutrient rich medium i.e. nutrient broth, inorganic salts (K_2HPO_4, KH_2PO_4, NH_3SO_4) and glucose by inoculating acclimated sludge. The biomass was grown in the fermenter till it attained an adequate mixed liquor suspended solids (MLSS) concentration (approx. 1500 mg l^{-1}). After the development of adequate biomass, which required about 48 h, the dye solution (100 mg l^{-1}) was fed into the fermenter vessel at a rate of 2 ml min^{-1}. Dissolved oxygen, pH and temperature were maintained at 3.0 mg l^{-1}, 6.0 and 30 °C, respectively. The

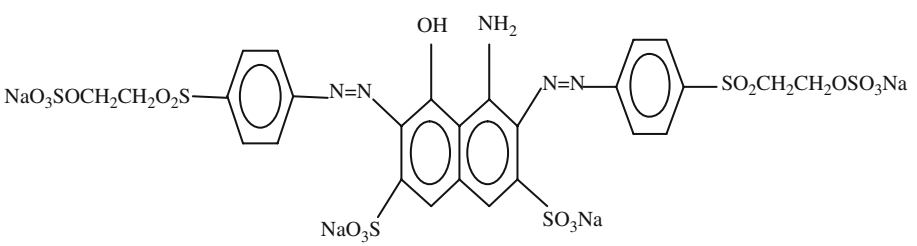


Figure 1. Structure of Reactive Black 5 (RB-5).

contents in the fermenter were stirred at 10 rpm continuously throughout the reaction period. The reaction period was 2 days and all observations were noted during this incubation period. Once the reactor was energized, the dye concentration was increased from 100 to 3000 mg l^{-1} and the decolorization was monitored. A 5 ml sample from each of replicate experiments was removed from fermenter at different time intervals. The samples were centrifuged at 15000×*g* for 10 min. The supernatant was used to measure the absorbance of dye at a wavelength of 597 nm as a function of incubation time.

Optimization of DO concentration

This was optimized by carrying out decolorization as mentioned above but by varying the DO concentration from 0.5 to 3.0 mg l^{-1}. In all the experiments stirring was fixed at 10 rpm such that it merely provided mixing without aeration of the system.

Effect of glucose as a co-substrate

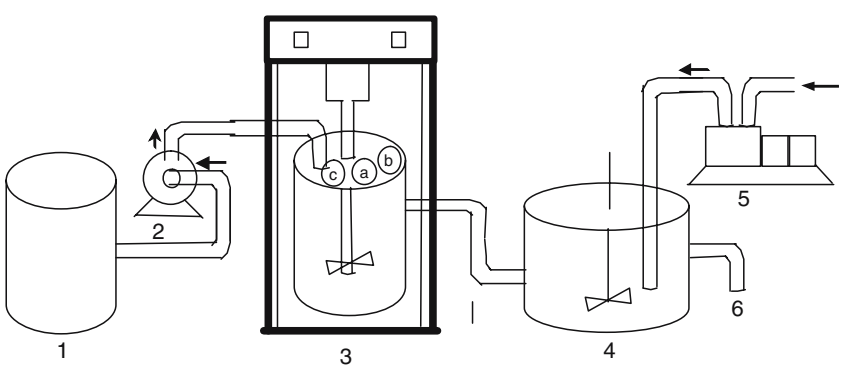
In this case, the batch mode decolorization experiments described above were performed using glucose as an additional carbon source. An arbitrary dose of 2-g glucose per liter of effluent was applied.

Plate assay

Plate assay was performed for the detection of decolorizing activity of bacteria. The nutrient agar and reactive black-5 dye was autoclaved at 121 °C for 15 min. Mix culture (1 ml) was plated on nutrient agar plates containing reactive black 5 (final conc. 500 mg l^{-1}). The plates were wrapped with parafilm and were incubated in anaerobic jar at 37 °C for 7 days. The plates were observed for clearance of the dye surrounding the colonies.

Decolorization in a two-stage anaerobic–aerobic reactor

Figure 2 illustrates a two-stage anaerobic–aerobic reactor used in this study. The anaerobic decolorization unit was as described previously (section 'Decolorization experiments in batch reaction'). The fermenter vessel was completely airtight and the DO concentration was maintained below 0.5 mg l^{-1}. The aerobic reactor was also of the same capacity and the DO concentration was maintained at 3.0 mg l^{-1}. The dye solution (100 mg l^{-1}, RB-5) was initially fed into anaerobic



1. Influent
2. Peristaltic Pump
3. Anaerobic Reactor (Fermenter)
   - (a) pH Probe
   - (b) DO Probe
   - (c) Temp Probe
4. Aerobic Reactor
5. Air Compressor
6. Outlet

Figure 2. Sequential anaerobic–aerobic reactor set up.

reactor at a rate of 2 ml min^{-1} and the effluent was collected into the aerobic rector. The pH was maintained between 6.0 and 7.0. The experiments were carried out for a period of 2 days at room temperature. Samples were collected from both the anaerobic and aerobic reactor tanks at the end of 2 days. The test samples were filtered through 0.45 μm membrane filters and scanned in the range of 200–700 nm on an UV–VIS spectrophotometer.

Analysis

Measurement of dye concentration

Samples were collected at different time intervals (0, 12, 24 and 48 h) and filtered through 0.45 μm membrane filters. The filtrates were scanned in the range of 200–700 nm on an UV–VIS spectrophotometer (Lambda 900 Perkin Elmer). The absorbance of the sample was noted at λ_{max} 597 nm and was used to infer the color content in the sample at different time intervals.

A calibration plot (absorbance versus concentration of RB-5) was drawn and used for estimating the concentration of unknown dye solutions. The plot was linear ($R^2 = 0.92$) between 0–100 mg l^{-1} RB-5 solution ($\varepsilon = 0.0307$ mg l^{-1}). The test samples drawn from experiments with higher concentrations of RB-5 were adequately diluted and absorbance was determined.

HPLC analysis

The analysis of decolorization metabolites from anaerobic reactor was done by extracting the initial (0 h) and final (48 h) samples into ethyl acetate for analysis by chromatographic techniques. A 75 ml of sample was extracted with equal volume of ethyl acetate in three subsequent steps. The extracts were dried over anhydrous Na_2SO_4 and volume was reduced to 5 ml using a rotary evaporator. This was dried under mild flow of nitrogen gas and the residue was dissolved in HPLC grade methanol (5 ml). HPLC analysis was conducted using Model 481, Millipore-Water make Lambda-Max LC spectrophotometer. The stationary phase was RP 18 (Lichrosorb, 2 mm diameter, 250 mm length, 5 μ particles, Germany). The mobile phase was 50% methanol, 0.3% H_3PO_4 and 49.7% water with the flow rate of 1.2 ml min^{-1}. A 10 μl sample was injected and the eluent was detected at 265 and 283 nm, as the case may be.

Results and discussions

Decolorization experiments in batch reaction

The results of decolorization experiments illustrating the effect of DO and co-substrate (glucose) are presented in Table 1. Only 7% decolorization of RB-5 was attained during 48 h under 3.0 mg l^{-1} DO and in the absence of co-substrate. The decolorization was marginally increased by adding co-substrate at the same DO concentration. However, the decolorization improved significantly when DO was brought down progressively while co-substrate was present. Maximum efficiency was obtained when the reactor was supplemented with 2 g l^{-1} of glucose and the DO content was maintained at < 0.5 mg l^{-1}. When the decolorized effluent was exposed to air under brief agitation, the color reappeared partly. However, the reappearance of color was negligible when glucose was used as co-substrate. Therefore, the process of anaerobic decolorization is found to be facilitated by strictly oxygen limited condition and presence of a co-substrate is also critical. The presence of a co-substrate was also reported to be essential for the decolorization (Ozsoy et al. 2005) of several other dyes.

The time overlaid UV–VIS spectra of test samples collected during the decolorization experiment are shown in Figure 3. It is found that the intensity of characteristic absorption peaks of RB-5 at 597, 398 and 330 nm decreased drastically within 24 h and these peaks disappeared completely after 36 h and beyond. Simultaneously, a new peak appeared at 267 nm in all the spectra corresponding to 12–48 h. The intensity of the peak at 267 nm increased upto 24 h, but thereafter it decreased a little and saturated. The decrease in

Table 1. Effect of dissolved oxygen (D.O.) and co-substrate on the decolorization of RB-5 (100 mg l^{-1})

Expt. No.	Parameter			Decolorization (%)
	D.O. (mg l^{-1})	Co-substrate glucose (g l^{-1})	Detention period (h)	
1	3	–	48	7
2	3	2	48	10
3	2	2	48	15
4	1	2	48	70
5	0.5	2	48	96

intensity of 597 nm peak with treatment duration is depicted in Figure 3 (inset). It can be seen that decolorization was very slow during initial 12 h, then maximum decolorization takes place between 12 and 24 h, and finally it attains saturation level beyond 48 h. It can be inferred that more than 90% decolorization of the dye occurs within 24 h. There is further increase in the decolorization efficiency upto 96% in the next 24 h. The UV–VIS spectral changes represent disappearance of RB-5 and formation of a metabolite during anaerobic decolorization reaction.

The UV–VIS spectra corresponding to initial and final samples of decolorization experiments for high concentrations of RB-5 (500–2000 mg l^{-1}) are shown in Figure 4. It is found that decolorization was significant till 2000 mg l^{-1}, although it was somewhat hindered at higher concentrations of the dye (2500 & 3000 mg l^{-1}). Once again, the disappearance of peaks due to RB-5 was accompanied by the appearance of a new peak at 267 nm even at this very high concentration of dye. The mechanism of decolorization may be presumed to be unaffected by the change in concentration of RB-5 till 2000 mg l^{-1}, as can be inferred from similar spectra at all concentrations of the dye. But, the time required for attaining >90% decolorization efficiency increased as the concentration of dye increases. Figure 5 depicts the time period needed to attain more than 90% efficiency as a function of concentration of the dye. The data relevant to decolorization in the presence of glucose as co-substrate was also included. The change in the time period with increase in dye concentration can be approximated as linear. Approximately, 25–33% decrease in treatment time can be realized upon addition of glucose.

Decolorization in a two-stage anaerobic–aerobic reactor

The UV–VIS spectra of samples collected from the anaerobic and aerobic reactors after a reaction period of 2 days are shown in Figure 6. As found earlier in this study, more than 90% decolorization was achieved in the anaerobic tank. The intensity of peak at 267 nm was further reduced after the aerobic treatment. The concentration of the metabolite was reduced atleast by 46%. Aerobic

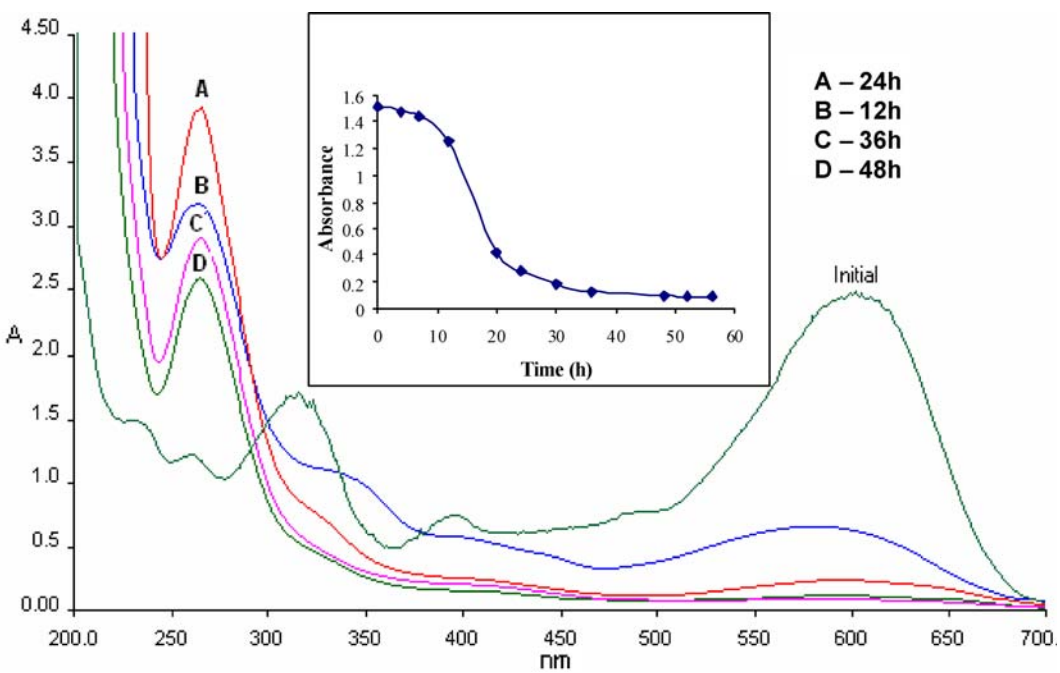


Figure 3. Time overlaid UV–VIS spectra for samples collected during anaerobic decolorization. (Inset) Decrease in absorption at $\lambda_{max} = 597$ nm as a function of treatment period. [RB-5] = 100 mg l^{-1}; $T = 303$ K; [Glucose] = 2 g l^{-1}.

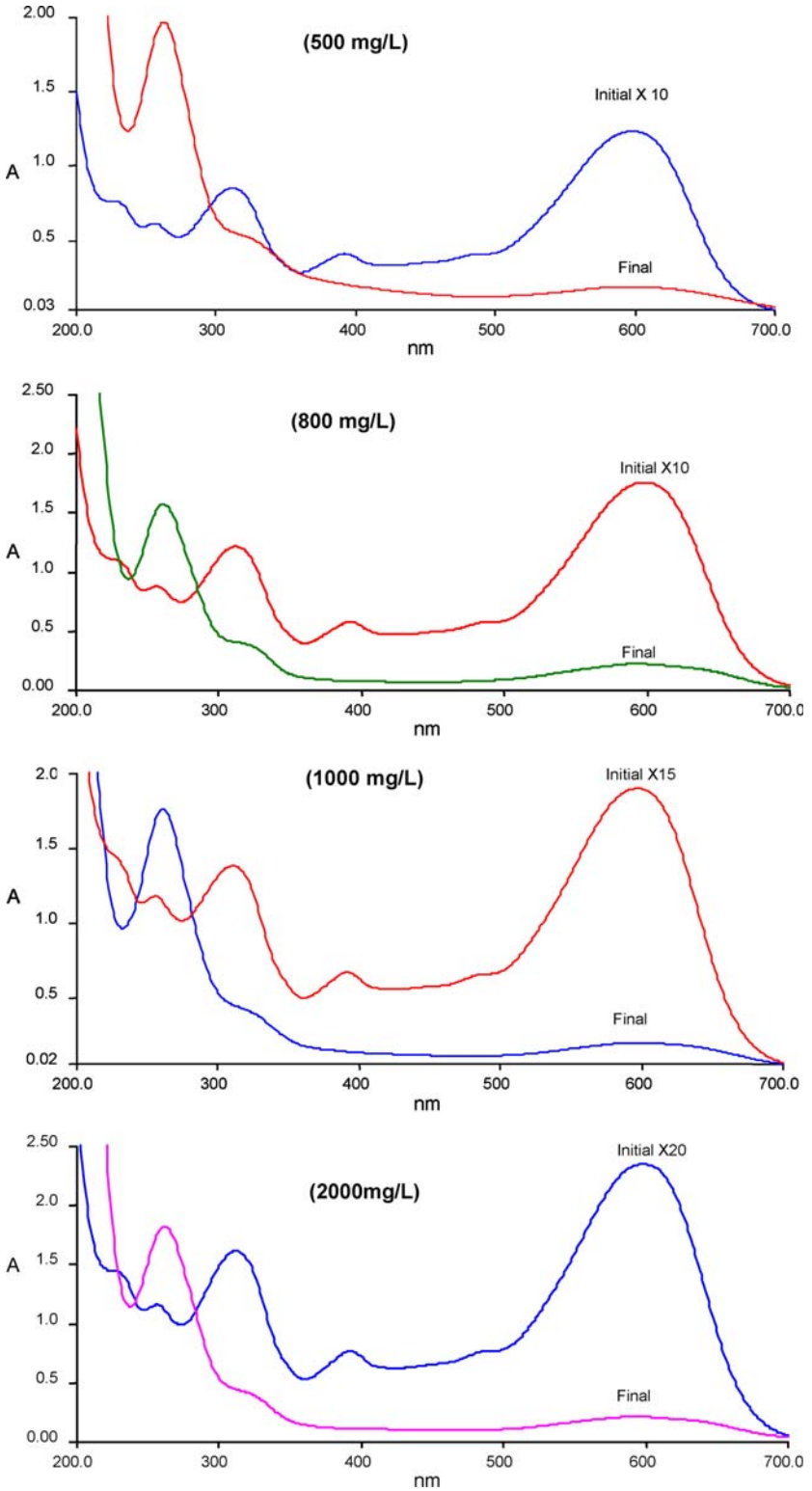


Figure 4. UV–VIS spectra depicting decolorization of defferent concentrations of RB-5 in the range 500–2000 mg l^{-1}. [DO] = 0.5 mg l^{-1}; T = 303 K; [Glucose] = 2 g l^{-1}.

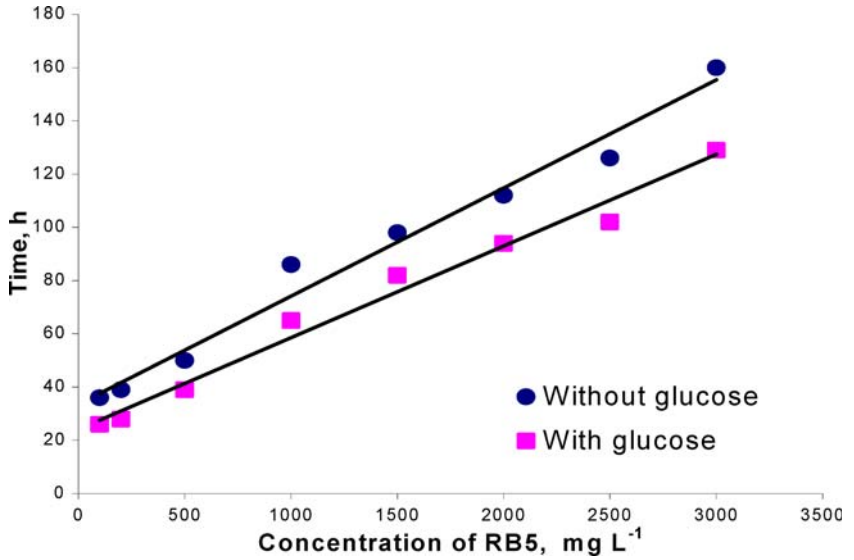


Figure 5. Total time period required for >90% decolorization versus concentration of RB-5. [DO] = 0.5 mg l^{-1}; T = 303 K; [Glucose] = 2 g l^{-1}.

reaction promotes oxidation of the metabolites formed in the anaerobic reaction chamber. This is in accordance with the literature reports (O'Neill et al. 2000).

Decolorizing bacteria

Bacteria responsible for decolorization were isolated from the reactor vessel and grown on agar plate containing RB-5 dye. Clearing zones signifying the decolorization ability of a few bacterial colonies are shown in Figure 7. It is found that only a few bacteria are responsible for decolorization of RB-5. No clear zones were formed in the absence of anaerobic conditions. Identification and characterization of clear zone-showing bacteria are in progress.

Discussion

The results in the foregoing sections suggest that decolorization of RB-5 occurs during anaerobic

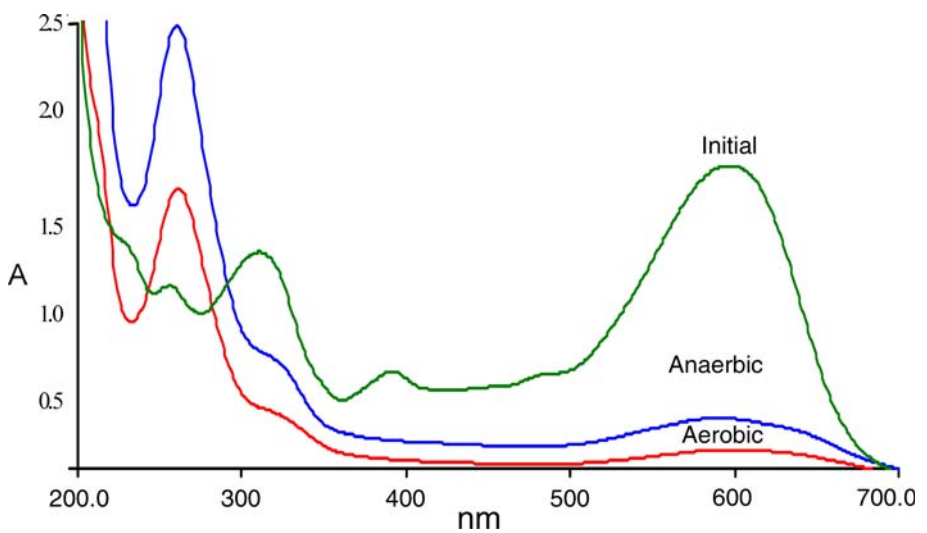


Figure 6. UV–VIS spectra depicting decolorizaion and reduction in metabolite concentration during sequential anaerobic–aerobic treatment of RB-5 (100 mg l^{-1}). $[DO]_{anaerobic}$ = 0.5 mg l^{-1}; $[DO]_{aerobic}$ = 3.0 mg l^{-1}; T = 303 K; [Glucose] = 2 g l^{-1}.

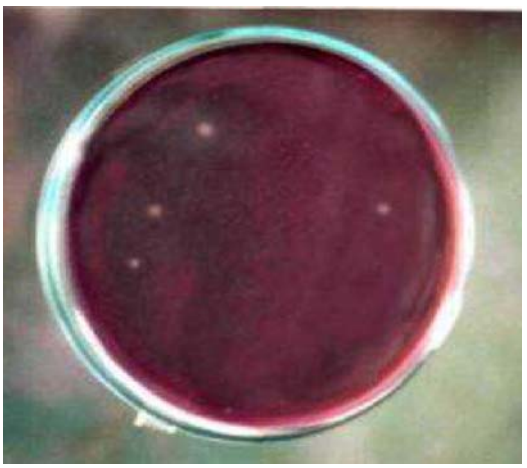
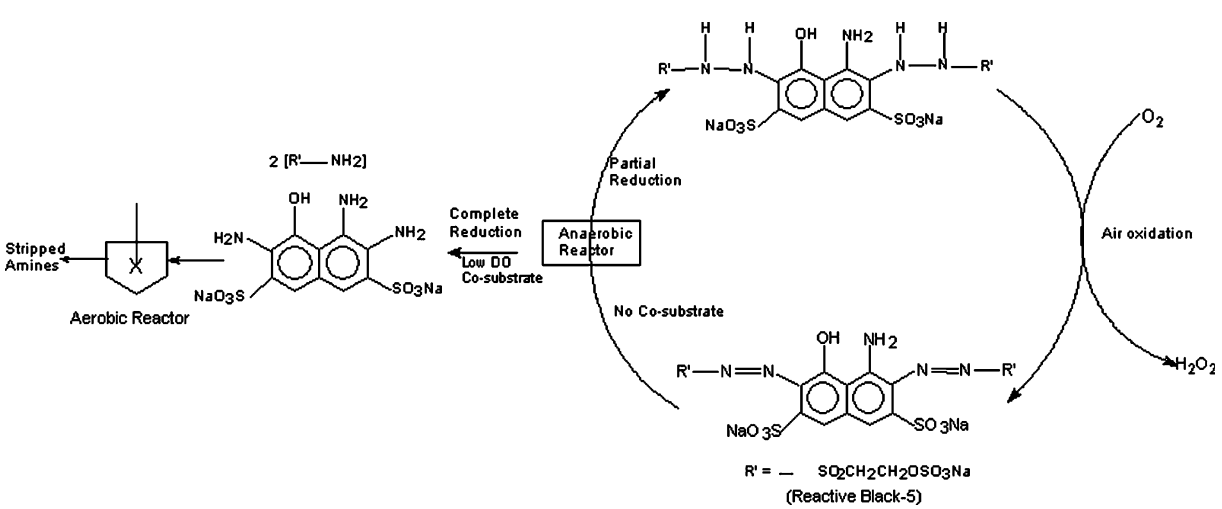


Figure 7. Appearance of clear zones around bacterial colonies due to the reduction of reactive black 5 on agar plates.

reaction set in by the acclimatized activated sludge collected from a functional ETP treating textile effluents. Moreover, only a few bacterial strains were decolorizing type. The decolorization reaction was accompanied by the formation of a metabolite that showed up at 267 nm in UV–VIS spectra. Further, the decolorized effluent regains color at least partly by exposing to air under brief agitation. On the other hand, biodegradation of RB-5 may be expected to comprise two main steps: the reductive cleavage of the azo bond under anaerobic condition and the subsequent aerobic mineralization of metabolites formed in anaerobic process. These observations may be explained with the help of the tentative mechanism shown in the Scheme 1.

In the Scheme 1, cleavage of azo bond (–N =N–) in RB-5 molecule is expected to result in permanent decolorization of the RB-5 dye solutions. Aromatic amines are produced as a result of complete cleavage of –N =N– bond. Prior to this, partial reduction of –N =N– via a two electron–two proton reduction process into –HN–NH– functionality may also occur (Mohanty et al. 2003). This –HN–NH– intermediate appears to be air sensitive and is oxidized back to azo group. This is evidenced through the regeneration of color in the decolorized effluents when exposed to air. Probably, hydrogen peroxide is the byproduct of this oxidation reaction, though it was not attempted in the present study to detect peroxide. Some previous studies suggest the formation of peroxide in similar reaction (Gonzalez-Flecha & Demple 1995; Imlay 2003). The formation of amine product in anaerobic process also is substantiated by HPLC and UV–VIS data. The new peak at 267 nm in UV–VIS spectra (Figures 3, 4 and 6) is suggestive of the formation of amine intermediates. HPLC data further confirms this observation (Figure 8). The HPLC chromatogram indicates the transformation in the structure of the molecule. Some new peaks are found at retention times 7.0, 10.5 and 12 min. These metabolites have been traced to amine compounds by performing HPLC detection at 283 nm in accordance with a previous study (Brown & Hamburger 1987;



Scheme 1. Tentative mechanism for anaerobic decolorizationof RB-5 and metabolite formation.

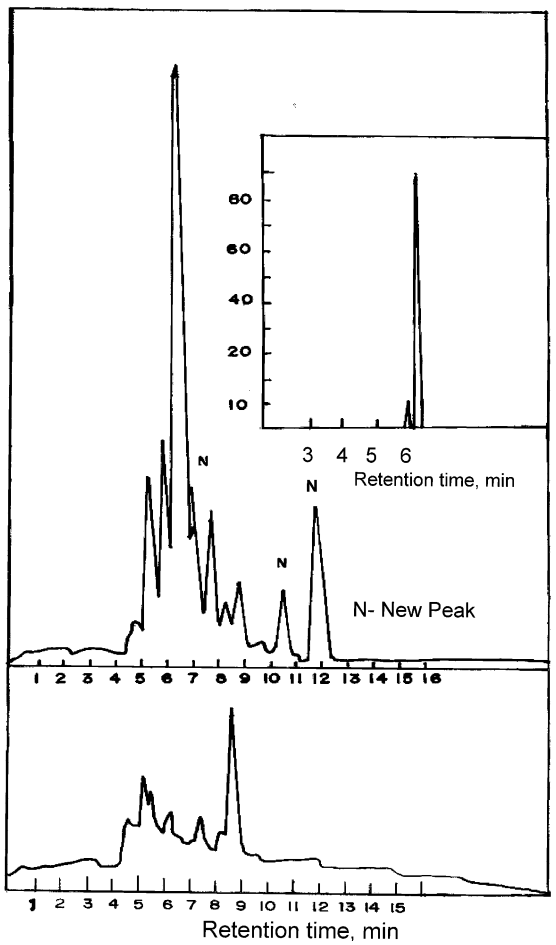


Figure 8. HPL chromatograms ($\lambda_{det.} = 265$ nm) of anaerobically decolorized RB-5 sample. The new peaks are marked as 'N'. (Inset) HPL chromatogram of RB-5 sample ($\lambda_{det.} = 283$ nm) corresponding to aromatic amine metabolite.

McMullan et al. 2001). This indicates that amines are formed as decolorization metabolites. It was observed in the present study that time required for attaining >90% decolorization efficiency increased as the concentration of dye increases. Isik & Sponza (2004) has also reported that decolorization rate was low at high organic loading under anaerobic conditions. However, approximately 25–33% decrease in treatment time can be obtained upon addition of glucose. Under anaerobic condition, azo dyes are readily cleaved via a four electron reduction at the azo linkage generating aromatic amines. The electrons are provided by electron donating carbon sources such as glucose, starch, volatile fatty acids (VFA) etc. Therefore, use of glucose as co-substrate resulted in reduction of treatment time for attaining >90% decolorization. In addition, it is also known that methanogenic and acetogenic bacteria contain unique reduced enzyme co-factors that could also potentially reduce azo bonds (Carliell et al. 1995).

Under anaerobic conditions many bacteria reduce azo dyes reportedly by the activity of unspecific soluble cytoplasmic reductases (azo reductases). Some prominently featured bacteria are: *Bacillus subtilis* (Horitsu et al. 1997; Zissi et al. 1997), *Bacteroides* sp., *Eubacteria* sp., *Clostridium* sp., and *Pseudomonas* spp. For example, *pseudomonas* spp. isolated from anaerobic–aerobic dyeing house wastewater treatment facility degrades most azo dyes (Yu et al. 2001). Anaerobic bacteria (eg. *Bacteroides* sp., *Eubacteria* sp., and *Clostridium* sp.) as well as facultative anaerobic bacteria e.g., *Proteus vulgaris* and *Streptococcus faecalis* are known to decolorize many azo dyes *via* reduction of the azo bond (Bragger et al. 1997; Fatemeh et al. 1990; Gingell & Walker 1971; Wuhrmann et al. 1980). Mixed bacterial cultures from a wide variety of habitats have also been shown to decolorize the diazo-linked chromophore of dye molecules in 15 days (Knapp & Newby 1995). Nigam & Marchant (1995) and Nigam et al. (1996) demonstrated that a mixture of dyes were decolorized by anaerobic bacteria in 24–30 h, using free growing cells or in the form of biofilms. Decolorization takes place due to the production of an exocellular enzyme azoreductase that reduces the azo bond present in the azo dyes and subsequently lead to the formation of amines (Brown & Hamburger 1987; McMullan et al. 2001). It is found in the present study that only a few bacteria present in the textile activated sludge are responsible for anaerobic decolorization of RB-5. Biochemical characterization and identification of these bacteria is currently in progress. Further, a study targeting salt tolerance of system is also underway in order to assess the applicability to real textile wastewater.

Conclusions

The conclusions drawn from this study can be summarized as follows:

The activated sludge from the textile industry can be used successfully for decolorizing RB-5 dye solutions by more than 95% within 2 days

under anaerobic conditions. Presence of a co-substrate (e.g., glucose) and maintenance of oxygen-limited conditions are the essential conditions for attaining maximum decolorization efficiency. Approximately, 25–33% decrease in anaerobic treatment time can be realized with the addition of glucose. The anaerobic decolorization of RB-5 dye occurs as a result of reduction of –N=N– bond accompanied by the formation of aromatic amines.

The amine intermediates formed in anaerobic treatment can be removed by aerobic biological treatment. Thus, more than 90% decolorization and approximately 46% reduction in amine metabolite concentration can be obtained through two-stage anaerobic–aerobic treatment after a reaction period of 2 days.

Only a few strains of bacteria from among many present in the activated sludge dominantly decolorize RB-5.

Acknowledgements

The authors wish to thank Dr Sukumar Devotta, Director, NEERI for encouraging to publish this work. A part of the work was accomplished under the financial support received through CSIR Task Force Project (CORE-008, IWMC).

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