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Effect of oxygen on decolorization of azo dyes by *Escherichia coli* and *Pseudomonas* sp. and fate of aromatic amines

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Abstract

The degradation of two azo dyes; Congo Red (CR) and Direct Black 38 (DB 38), in use in textile industries in Turkey, were investigated using two facultative microorganisms (*Escherichia coli* and *Pseudomonas* sp.) under anaerobic, aerobic, and microaerophilic conditions. Hundred milligram per litre of dyes and 1000 mg glucose-COD/l containing basal medium were used and were incubated for 5 and 9 days. Simultaneous biomass activity and colour removal performance, was monitored during batch assays. The effects of two different microorganisms and aerobic/anaerobic conditions on decolorization were recorded with the monitoring of colour, pH, COD, dissolved oxygen, alkalinity, and volatile fatty acids concentrations. The aromatic amines (as benzidine) arising from the metabolites of anaerobic biodegradation of dyes and the recoveries of these aromatic amines were also monitored. High benzidine recoveries indicated the accumulation of aromatic amines under aerobic conditions. The colour of the CR and DB 38 dyes were removed up to 98 and 72%, respectively, by *E. coli* at the end of anaerobic incubation, while no decolorization occurred throughout the aerobic incubation. Under microaerophilic conditions, the azo dyes CR and DB 38 were decolorized up to 39 and 75% by *E. coli,* respectively. In studies with *Pseudomonas* sp., the colour of the CR and DB 38 dyes were removed up to 100 and 83%, respectively, after 5 days of anaerobic incubation, while 76 and 74% colour removal efficiencies were observed under microaerophilic conditions.
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1. Introduction

Azo dyes can be degraded under anaerobic conditions. Although anaerobic microorganisms can biodegrade dyestuffs, the intermetabolites released from the azo dyes could be toxic. The rate of azo reduction is affected by the structure and the substituents on the aromatic rings [1,2]. The azo dye reduction products are not very likely to be degraded under anaerobic conditions. In other words, cleavage products of azo dyes are generally regarded as recalcitrant compounds in anaerobic environments. Therefore, these compounds are excreted after the anaerobic treatment step. Under aerobic conditions many aromatic amines are readily degraded but some of these could not be mineralized. Razo-Flores et al. [2] reported that the azo dye azosalysilate could be metabolized to aromatic amines, supplying electrons to support reductive azo bond cleavage.

Since some aromatic amines were not degraded under methanogenic/anaerobic conditions, the studies focused on different microbial communities, which could use alternative electron acceptors through removal of colour from the azo dyes. For this reason it is important to highlight the enormous potential of microbial communities in anaerobic environments for decolorizing azo dyes and determining the possibility of degradation of the aromatic amines.

The reduction of azo dyes can be mediated by facultative as well as obligate anaerobes and has been reviewed recently [3]. The role of microbes in reducing azo dyes to toxic or carcinogenic compounds is of considerable concern [4]. Several research groups have

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recently demonstrated the possibility of utilizing microbial communities for the biodegradation of azo dyes from the textile and dyestuff industries [5–10]. Azo dyes can be degraded under aerobic conditions [6,11] or under anaerobic conditions [12,13].

Bacterial degradation is usually initiated by anaerobic reduction of azo linkage to generate aromatic amines under anaerobic conditions and they are generally not metabolized further under these conditions [14,15]. Razo-Flores et al. [2] studied the biodegradation of Mordant Orange under methanogenic conditions. They reported that aromatic amines could be further mineralized under anaerobic conditions. Sarnaik and Kanekar [16] studied the decolorization of methyl violet by *Pseudomonas mendocina*, resulting in 100 mg/l of mineralization of dye to CO_2 and phenol via dimethylation. Zissi and Lyberatos [17] found that *p*-aminobenzene can be degraded by *Bacillus subtilis* under anoxic conditions and aniline was produced as aromatic amine. Chang and Kuo [18] studied the decolorization of 100 mg/l Reactive Red 22 dye using *Escherichia coli* under anoxic and aerobic conditions. They found that the dissolved oxygen level significantly inhibited bacterial colour removal. Sani and Banerjee [19] found 92, 96 and 96% decolorization rates in Magenta, Crystal Violet and Malachite Green dyes, respectively, using *Kurthia* sp. They found higher COD removal efficiencies (56–85%) and decolorization efficiencies (19–33 mg/g). Chen et al. [20] found that 95% red azo dye (Red RBN) was reduced within 20 h at a dye concentration of 100 mg/l using *Proteus mirabilis*. The biosorption of dye was 13–17%. Yu et al. [21] showed that *Pseudomonas* sp. can effectively cleave the azo bond regardless of dye structures.

Anaerobic and facultative microbes play an important role in the metabolism of recalcitrant azo dyes by aerobic microbes. At the same time, the anaerobes also digest and remove a low amount of COD from the wastewater [21]. It is important to investigate the most active microbial strains for azo reduction. Several research groups have recently demonstrated the possibility of utilizing special microbial communities for the decolorization of azo dyes [8,22].

Our investigations, therefore, were directed toward examining the biodegradation and decolorization of two azo dyes, namely Congo Red (CR, Direct Red 28) and Direct Black 38 (DB 38), by two different facultative bacteria (*E. coli* and *Pseudomonas* sp.) under anaerobic, aerobic and microaerophilic conditions. The biomass growths of the aforementioned bacteria, COD, colour, pH and volatile fatty acids (VFA) variations were investigated during 4 and 9 days batch incubation periods. Furthermore, aromatic amines as product of azo cleavage and recovered amines were monitored as benzidine equivalent under anaerobic, aerobic and microaerophilic conditions.

2. Materials and methods

2.1. Dyes

DB 38 and CR, typical azo dyes used in dyeing textiles and cotton yarn, were of commercial quality. These dyes were purchased from a textile factory in Izmir. DB 38 and CR, which are two banned azo dyes in Turkey, were used in dissolved form. Dye structures are depicted in Fig. 1.

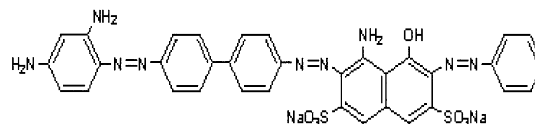
2.2. Synthetic media

Vanderbilt mineral medium was used in all batch experiments [23]. Glucose was used as co-substrate to provide the electrons for the reductive cleavage of the azo dyes, namely DB 38 and CR. Sodium thioglycollate was used to maintain anaerobic conditions. Hundred milligram per litre dyes (37 mg COD/l for DB 38 and 74 mg COD/l for CR) and 1000 mg/l glucose-COD were added separately to the synthetic medium. Furthermore, 2000 mg/l $NaHCO_3$ was added to the medium to maintain a residual alkalinity of 400 mg/l after 5 and 9 days of the incubation period.

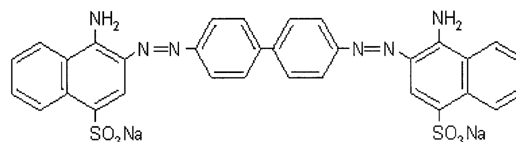
2.3. Cultures and experimental procedure

Two facultative cultures, *E. coli* and *Pseudomonas* sp., were obtained from the Microbiology Laboratory of the Medicine Faculty at Aegean University and inoculated into nutrient broth (1 g/l beef extract, 2 g/l yeast extract, and 5 g/l NaCl) medium. These bacteria were facultative anaerobes capable of growing under both aerobic and anaerobic conditions.

These bacteria were cultivated in nutrient broth medium under aerobic, anaerobic and microaerophilic conditions. The anaerobic incubation was carried out in 3.5 l glass anaerobic bottles, Anaerob*Gen* (OXOID) at



DB 38, Colour Index number: 30235, λmax: 520, COD of 1000 mg/litre of DB 38 solution: 370 mg/litre



CR (Direct Red 28), Colour Index number: 22120, λmax: 497, COD of 1000 mg/litre of CR solution: 746 mg/litre

Fig. 1. The structures of azo dyes (DB 38 and CR) used in this study.

35 °C. The anaerobic cultivation was carried out in dark glass serum bottles with a volume of 165 ml (effective volume 150 ml) and they were sealed with rubber septa and incubated at 35 °C in the incubator mentioned above for the anaerobic conditions. Microaerophilic conditions were maintained in an oxygen range varying between 0.01 and 0.5 mg/l without aeration in flasks with 300 ml effective volume at 30 °C. Aerobic conditions were maintained by an air pump giving a dissolved oxygen concentration of 4–8 mg/l in the flasks with 300 ml of effective volume at 30 °C. Mixed cultures (1 ml aerobic and 1 ml anaerobic culture) were used in the inoculation of 150 ml of synthetic media.

The degree of decolorization of the dyes was determined spectrophotometrically in the clear supernatants of cultures grown aerobically, anaerobically and microaerophilically after being centrifuged at 6000 rpm for 10 min. Results were corrected according to blank (dye-free) samples. Non-inoculated sterile control bottles, which contained only the dye-free synthetic medium was used as blank (control) and were incubated at 35 °C in order to detect the absorbance of dye-free medium. Decolorization efficiencies were calculated from absorbance measurements and curves plotted between dye concentrations and absorbance values.

To determine whether or not extracellular or intracellular enzymes decolorized the dyes, dye containing cultures were centrifuged after incubation, the supernatants were decanted and incubated for decolorization with cell-mass containing samples.

In order to observe the effect of initial COD and dyes concentrations on decolorization, different amounts of glucose-COD (250–2500 mg/l) and CR dye (250–3000 mg/l) concentrations were incubated under anaerobic conditions. The bacteria cells were separated and the COD and dye concentrations of the supernatant monitored for a 12-day period.

The microbial cultures were autoclaved at 121 °C for 15 min to measure the adsorption or abiotic removal of dyes.

2.4. Measurements of azo dye reduction

The chemical reduction of azo dyes was carried out using sodium dithionite. The process for the reduction of DB 38 and CR dyes was as follows: 0.06 g of dye sample was heated to boiling point with 1 M NaOH for 1 h; 0.6 g sodium dithionite was added after 30 min. Hundred milligram per litre of dye was reduced according to the method mentioned by Pielesz [24]. Aromatic amines released from the dyes were measured in diluted reduced dye solutions at an absorbance maxima of 440 nm.

2.5. Analytic techniques

COD was measured by closed reflux colorimetric following Standard methods. The MLSS concentration was measured by membrane filtration technique following Standard methods in membrane filters with a pore size of 0.45 μm [25]. pH and dissolved oxygen were measured in a pH meter (WWT pH 330) and in an oxygen-meter (WWT Oxi 330), respectively. VFA and bicarbonate alkalinity were measured following the methods proposed by Anderson and Yang [26].

Dye concentrations were measured at their maximum absorbance spectra ($\lambda_{max} = 520$ nm for DB 38 and $\lambda_{max} = 497$ nm for CR) in a spectrophotometer, Optima Photomech 301-D UV–VIS. Absorbance values were calculated by subtracting the absorbance values of dye-free control medium from the absorbance values of synthetic media containing dye. The concentrations of the dyes were calculated from the calibration curves of absorbances versus concentrations. Since the metabolites released from the breakdown products of DB 38 and CR dyes are colourless and the absorbance of anaerobic metabolites in control was subtracted, the interference of anaerobic extracellular products to the absorbance was neglected.

In order to determine the concentrations of *E. coli* a calibration curve was prepared between known cell mass (MLSS) concentrations and absorbance of synthetic medium at 600 nm. The absorbance measurements were used to determine the cell mass concentrations of *Pseudomonas* sp. over 4 days of the incubation period at the aforementioned absorbance values.

Amines released from the reduction of azo dyes (CR and DB 38) were quantified as benzidine equivalent. Total aromatic amines (as benzidine equivalent) emitted from the reduction of azo linkage were measured colorimetrically at 440 nm after reacting with 4-dimethylaminobenzaldehyde–HCl, according to the methods described by Oren et al. [27]. In order to measure the total aromatic amine concentrations as benzidine equivalent, firstly a calibration curve was plotted between known benzidine concentrations and absorbance values and secondly the total aromatic amine concentrations were measured from the plot between reduced dye (DB 38 and CR) and absorbances.

3. Results and discussions

3.1. Variations in cell mass concentrations

In order to observe the simultaneous colour removal and increases in cell mass of *E. coli* and *Pseudomonas* sp. concentrations of cultures were measured at the end of 9 and 5 days of incubation period, respectively. The concentration of *E. coli* increased from an initial 100 to

1100 mg/l under aerobic conditions after 9 days of incubation during decolorization of dyes. Maximum MLSS concentrations were 990 mg/l ($A_{600} = 0.383$) and 637 mg/l ($A_{600} = 0.237$) from the incubations under microaerophilic and anaerobic conditions, respectively (data not shown).

The concentration of *Pseudomonas* sp. increased from 150 mg/l ($A_{600} = 0.09$) to 1500 mg/l ($A_{600} = 0.700$) at the end of 4 days of aerobic incubation period. The maximum MLSS concentrations were 1100 mg/l ($A_{600} = 0.400$) and 740 mg/l ($A_{600} = 0.350$) under microaerophilic and anaerobic conditions, respectively (data not shown).

3.2. Effect of anaerobic, aerobic and microaerophilic conditions on decolorization of DB 38 and CR azo dyes by E. coli culture

Fig. 2 depicts the colour removal efficiencies throughout the 9 days of the incubation period under anaerobic, aerobic and microaerophilic conditions for azo dye DB 38. More than 72% of the colour was removed within 3 days under anaerobic conditions while at the same time 24 and 63% decolorization was observed under aerobic and microaerophilic incubations, respectively. In other words, the efficiency of colour removal with *E. coli* in anaerobic incubations was faster than that obtained in aerobic incubations. A maximum of 75% colour removal efficiency was obtained under microaerophilic conditions while a maximum of 80% decolorization efficiency was achieved after incubation with *E. coli* under anaerobic conditions. It has been observed that very high decolorization efficiencies were achieved under anaerobic incubation, although the MLSS concentration of *E. coli* was comparably lower (MLSS = 637 mg/l) than those obtained under aerobic conditions (MLSS = 1000 mg/l). The results of this study showed that the rate and the efficiency of decolorization did not sharply increase with increases of anaerobic cell mass concentration of *E. coli*. Dye removal rate ($E = 80\%$) of 20 mg/(l day) was observed at a MLSS concentration of 600 mg/l while a 19 mg/(l day) dye removal rate ($E = 78\%$) was obtained in a MLSS concentration of 300 mg/l under anaerobic conditions. The maximum decolorization was 76% under both anaerobic and microaerophilic conditions after 9-day incubation periods. The DB 38 azo dye concentration decreased from an initial 100 to 20 mg/l and 30 mg/l while *E. coli* was grown under anaerobic and microaerophilic conditions, after 4 days of incubation. The dye concentration was reduced to 80 mg/l for the same incubation period under aerobic conditions (data not shown). These results confirmed that colour removal could only be effected under anaerobic environments. The decolorization of DB 38 dye by *E. coli* was initiated when the dissolved oxygen concentration became very low and accelerated with the oxygen depletion. The data obtained in this study showed that although the initial cell concentration of *E. coli* was as low as 250 mg/l the decolorization efficiency was not changed under anaerobic conditions (data not shown). The results of this study clearly indicated that decolorization was not dependent on biomass concentration but was significantly correlated with the dissolved oxygen. Therefore, oxygen had an inhibitory effect on decolorization under anaerobic incubations, as reported by Chang et al. [28]. In other words, the microbial reduction of these azo dyes is an enzymic reaction linked to anaerobiosis because it is inhibited by oxygen as mentioned by Chung et al. [12]. Therefore, facultative or obligate anaerobes are necessary for azo dye reduction.

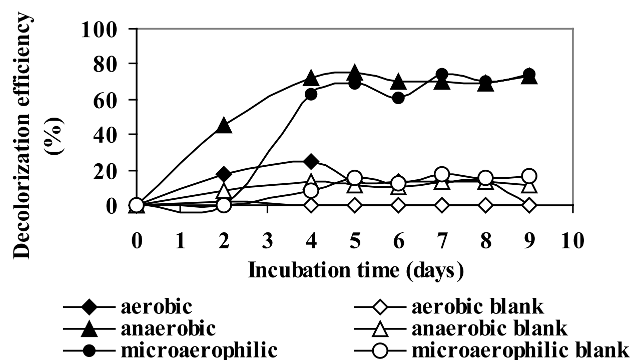


Fig. 2. Decolorization efficiencies with *E. coli* for DB 38 azo dye throughout 9 days of batch incubation period.

Chung and Stevens [8] and Chang et al. [28], also determined that the inhibition by oxygen of enzymic reduction of azo dyes for *Pseudomonas luteola* affected the enzyme azoreductase. This was attributed to competition for NADH utilization by aerobic respiration, which triggers electron transfer from NADH to oxygen to form ATP [28]. Since NADH acts as an electron donor for the reduction of azo bonds that leads to bacterial decolorization of azo dyes, the consumption of NADH by oxidative phosphorylation would result in a negative effect on the azoreductase-driven decolorization. However, Chang et al. [28] determined that the presence of oxygen did not directly inhibit the activity of azoreductase, thus oxygen inhibition is more likely to be a metabolism-dependent event. The data obtained showed that glucose and DB 38 dye could be used and consumed as carbon and energy source by *E. coli* cells resulting in the successful degradation of both overall COD and dyes.

Fig. 3 shows the decolorization efficiencies for CR degradation over 9 days of the incubation period with *E. coli* under anaerobic conditions. The maximum decolorization was 98% under anaerobic conditions while 30 and 39% colour removal efficiencies were obtained under aerobic and microaerophilic conditions during CR degradation. The maximum *E. coli* concentrations

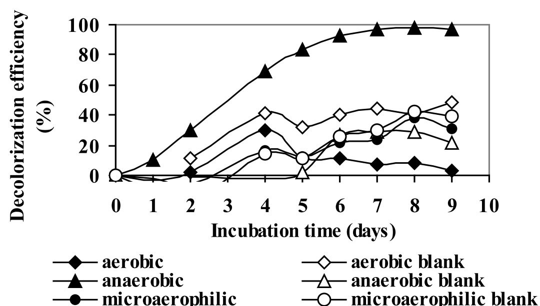


Fig. 3. Decolorization efficiencies with *E. coli* for CR azo dye throughout 9 days of batch incubation period.

reached were 800, 560 and 550 mg/l under aerobic, microaerophilic and anaerobic conditions, respectively (data not shown). Eight percent and 25% colour removal efficiencies were obtained after 7 days of the incubation period under aerobic and anaerobic conditions. On day 7, CR azo dye was completely decolorized resulting in 100% colour removal efficiency in *E. coli* culture. The data obtained in this run showed that the maximum decolorization achieved was 98% under anaerobic conditions and the efficiency of decolorization of CR dye was significantly affected by oxygen.

The CR concentrations decreased to 20 mg/l from an initial 100 mg/l after 5 days of incubation under anaerobic conditions while the CR concentration was reduced to 80 mg/l under aerobic and microaerophilic conditions (data not shown).

3.3. Mechanisms of microbial decolorization of DB 38 and CR dyes by E. coli under anaerobic conditions

Decolorization of a dye solution by bacteria could be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to biodegradation either the major visible light absorbance peak would completely disappear or a new peak would appear. Dye absorption would result in cell materials, which are deeply coloured because of adsorbed dye, whereas those retaining their original colours are accompanied by the occurrence of biodegradation [20]. In this study the abiotic decolorization studies with autoclaved, dead *E. coli* cells showed that no significant colour removal were observed under anaerobic conditions for DB 38 and CR dyes. In addition, as the DB 38 and CR levels were reduced, the broth media returned to its original yellow–white colour. The absorbance peak at 520 nm completely disappeared after cultivation for decolorization of DB 38. In other words, when comparing the UV–VIS scans of dye and incubated dye samples it was observed that the aforementioned spectra were different, suggesting that DB 38 was degraded and exhibited maximal absorbance peak at 350 nm. In addition, as the DB 38 dye was reduced the synthetic medium returned to its original colour. The anaerobic breakdown products of CR dye have a peak at 350 nm while the original CR dyes show maximal absorbance at 497 nm. These results indicate that colour removal by *E. coli* may be largely attributed to biodegradation under anaerobic conditions via intracellular enzymes since the decolorization in the supernatant samples was not significant ($<1\%$) while the colour in cell-mass containing samples completely disappeared. This fact indicates that dye decolorization involves constitutive intracellular enzymes. Therefore, it is postulated that the mechanism of colour reduction by *E. coli* can be explained as follows: the dye was adsorbed to the cell surface, some amounts of the dye were transferred through the cell membrane, the transferred dye was degraded enzymically inside cells and the degraded products diffused out to the broth solution. In this study, at dye concentrations of 100 mg/l, almost all of the dyes were degraded completely by the reductase enzyme inside the cells as reported by Chen et al. [20].

3.4. Effect of initial glucose-COD and CR concentrations on decolorization efficiency in E. coli cultures under anaerobic conditions

In order to minimize the glucose-COD concentrations used in decolorizing the CR dye, the COD concentrations were raised in *E. coli* cultures. The decolorization efficiencies were almost the same as the concentration of COD was increased from 250 to 2500 mg/l. The colour removal efficiencies reached a plateau resulting in a decolorization efficiency of 96 and 100% after 5 days of the incubation period (Fig. 4). Increases in COD did not negatively affect colour removal. It can be assumed that the glucose was used as carbon and energy source and provided sufficient electrons for reductive conditions through the cleavage of the azo bond found in the structure of CR dye. However, increasing the glucose concentration as a donor of reductive equivalent did not

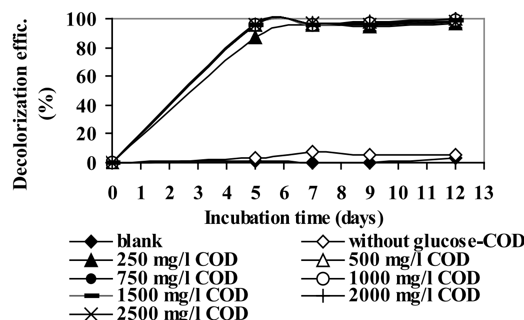


Fig. 4. Effect of initial glucose-COD concentrations on anaerobic decolorization of CR dye.

enhance the decolorization efficiency of either azo dye but seemed to stimulate anaerobic degradation and colour removal.

In order to investigate the effect of increasing CR dye concentrations on decolorization efficiency, *E. coli* was grown on dye concentrations varying between 250 and 3000 mg/l. When CR concentrations were increased from 250 to 3000 mg/l, the colour removal was as low as 5–20% on day 2. Variations of colour removal efficiencies with initial dye concentrations are depicted in Fig. 5. Decolorization efficiencies were almost the same, around 65% on day 5 for 2000 and 2500 mg/l CR dye concentrations. However, with dye concentrations 250, 750, 1000, 1500 and 3000 mg/l, colour removal efficiencies dropped to 10 and 20%. On day 7, 50–80% decolorization efficiencies were obtained for CR concentrations of 3000, 2500, 2000 and 250 mg/l, respectively. Hundred percent and 85% colour removal efficiencies were obtained for CR concentrations varying between 250 and 2500 mg/l, while 55% decolorization removal were obtained for CR concentrations of 3000 mg/l after 12 days incubation. Since high decolorization efficiencies (80–90%) were obtained at CR concentrations as high as 2000 mg/l, it can be concluded that the CR dye can be used as a carbon source like glucose through decolorization as reported by Chang et al. [28].

3.5. Simultaneous COD reduction and aromatic amine production in E. coli cultures containing DB 38 and CR dyes

3.5.1. DB 38 dye

During anaerobic batch culture after 9 days incubation, the COD level was reduced to 427 mg/l from the initial COD concentration of 1000 mg/l, indicating a COD removal efficiency of 57%. Table 1 shows the COD, pH, VFA, dissolved oxygen, alkalinity, the aromatic amine (as benzidine) variations and the aromatic amine recoveries after 9 days incubation under anaerobic, aerobic, and microaerophilic conditions. After the first day, the COD in the batch reactors decreased slowly but was sharply reduced after the second day. The low COD removal efficiencies were either due to the intermediate products of anaerobic degradation or due to the extracellular specific metabolites released from *E. coli* under anaerobic conditions. However, the change in colour in the incubated batch reactors, with no significant decreases in COD, indicated the cleavage of azo compounds, under anaerobic conditions. These results show that anaerobic conditions were not very effective in removing COD. Although no significant decreases in COD concentrations was observed, DB 38 dye was reduced and converted to aromatic amines very efficiently under anaerobic conditions compared to aerobic conditions as reported by Ganesh et al. [13]. The breakdown products of the samples were monitored by detecting the intermediate degradation products such as benzidine and VFA. As shown in Table 1, aromatic amines and VFA accumulation was detected under anaerobic incubations. The catabolism of glucose-COD is ultimately responsible for the production of reduced enzyme cofactors through reductive cleavage of azo bonds, resulting in decolorization. The COD was reduced from 1000 to 129 mg/l ($E=87\%$) and 217 mg/l ($E=78\%$) under aerobic and microaerophilic conditions, respectively, after 9 days of incubation.

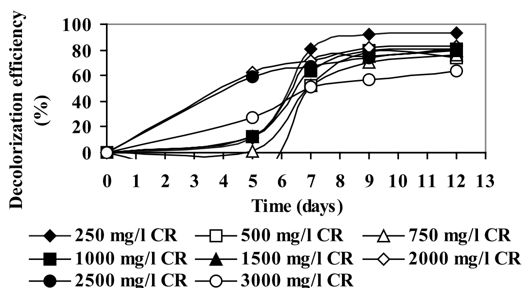


Fig. 5. Effect of initial CR concentrations on anaerobic decolorization.

Aromatic amines as benzidine were recovered in high yields after anaerobic reduction of both dyes. Benzidine was recovered in high stoichiometric percentages (Table 1). Anaerobic degradation of the DB 38 breakdown product (benzidine) was recovered at a relatively high yield of the DB 38 removed (100%) under anaerobic conditions by *E. coli* (Table 1). During this time, benzidine was detected at high concentration (5.51 mg/l), indicating that further metabolism of the aromatic amines did not occur. This shows that the benzidine released from DB 38 was not metabolized after cleavage of the azo bond under anaerobic conditions.

Aromatic amines as benzidine equivalent were not detected under aerobic conditions indicating no breakdown of azo bonds in oxygenated environments. Microaerophilic conditions containing low oxygen (0.01–0.2 mg/l) were found to be suitable for the cleavage of the azo bond, resulting in aromatic amine release because of the breakdown of the DB 38 dye (Table 1).

3.5.2. CR dye

The COD concentration was reduced from 1000 to 463 mg/l ($E=54\%$) under anaerobic conditions, while 85 and 87% COD removal efficiencies were obtained under aerobic and microaerophilic conditions, respectively (Table 1). The CR azo dye degradation products (aromatic amines) were recovered with a yield of 78% under anaerobic conditions. The benzidine concentration was 10.6 mg/l, indicating that aromatic amine accumulation was as a result of the cleavage of the azo

Table 1
Variations in COD, VFA, dissolved oxygen, aromatic amines and benzidine recoveries in DB 38 and CR dyes by *E. coli* and *Pseudomonas* sp.

Microorganisms conditions dyes	Chemical Oxygen Demand (COD)		Aromatic amines (A.A.)				Other parameters				
	COD (mg/l)	COD removal efficiency (%)	Absorbance (A_{440})	A.A. (mg benzidine/l)	A.A. (mg benzidine/l)	A.A. recovery (%)	Initial pH	Final pH	Final alk.[a] (mg $CaCO_3$/l)	TVFA (mg CH_3COOH/l)	D.O variation
E. coli											
Aerobic											
Blanc	101	90					8.05	9.31	1010	–	5.65 ± 2.09
CR	152	85	0.0509	13.7	ND[b]	–	8.09	9.47	740	–	4.49 ± 0.64
DB38	129	87	0.021	5.51	ND	–	8.05	9.24	1350	–	5.40 ± 1.34
Anaerobic											
Blanc	426	57					8.04	6.37	516	603	–
CR	463	54	0.0509	13.7	10.6	78	8.07	6.34	510	635	–
DB38	427	57	0.021	5.51	5.5	100	8.06	6.43	581	586	–
Microaerophilic											
Blanc	150	85					8.06	8.88	ND	–	0.50 ± 0.40
CR	125.5	87	0.0509	13.7	ND	–	8.09	8.94	ND	–	0.50 ± 0.30
DB 38	217	78	0.021	5.51	5.1	93	8.06	8.91	ND	–	0.34 ± 0.30
Pseudomonas sp.											
Anaerobic											
Blanc	390	61						6.31	465	775	–
CR	507	49	0.0509	13.7	6.4	47		6.25	511	624	–
DB 38	485	52	0.021	5.51	4.4	79		6.31	514	684	–
Microaerophilic											
Blanc	136	86					7.77	8.68	ND	–	0.03 ± 0.02
CR	160	84	0.0509	13.7	7.1	52	7.89	8.72	ND	–	0.03 ± 0.02
DB 38	146	85	0.021	5.51	6.8	123	7.68	8.61	ND	–	0.04 ± 0.03

[a] Total alkalinity after incubation, B. alkalinity in anaerobic incubation.
[b] Not determined.

chromophore in the CR dye. Benzidine was not detected under microaerophilic conditions during the incubation of CR dye with *E. coli,* indicating the accumulation of dye without reductive cleavage.

3.6. Decolorization of DB 38 and CR azo dyes by Pseudomonas sp. under anaerobic and microaerophilic conditions

Figs. 6 and 7 show the decolorization assay during 5 days of the incubation period under different dissolved oxygen levels for both dyes. The initial colour removal efficiency was zero and the extent of colour removal 100% at the end of 3 days incubation under anaerobic conditions for CR dye. Seventy six percent and 45% colour removal efficiency was obtained under micro-aerophilic and aerobic conditions at the end of 3 days of incubation under anaerobic conditions for DB 38 dye. Similarly 99 and 49% colour removal efficiency was obtained under anaerobic and aerobic/microaerophilic conditions for CR dye. At this point, it seems reasonable to assume that the *Pseudomonas* sp. caused cleavage of the azo bond of the CR dye, which decomposed to form aromatic amines. Abiotic decolorization studies showed that there was 30% colour removal in autoclaved cells of *Pseudomonas* sp. (Fig. 6). This could be attributed to activation of the azo-reductase due to reductive conditions because of the Na_2S content in the mineral medium used through decolorization of CR under anaerobic conditions. Another reason for abiotic removal could be the reductive effect of sunlight on decolorization of CR dye during the sampling although dark bottles were used as reactor since no dye removal was observed in autoclaved cells of *Pseudomonas* sp.

3.7. Simultaneous COD reduction and aromatic amine production in Pseudomonas sp. cultures containing DB 38 and CR dyes

The aromatic amine concentrations based on benzidine were 6.4 and 7.05 mg/l under anaerobic and microaerophilic conditions, respectively, for CR dye. The yield of benzidine was 47 and 52%, indicating that the breakdown of products CR were partly mineralized further and reduced under anaerobic and microaerophilic conditions, respectively. At this point, it seems reasonable to assume that the *Pseudomonas* sp. caused cleavage of the azo bond of CR and DB 38 azo dyes, which decomposed to form aromatic amines, as observed in the decolorization of reactive red 22 by *Pseudomonas luteola* [28]. However Hu [7] pointed out that decolorization of the azo dye Red G by *Pseudomonas luteola* may be due to incomplete reduction of the azo bond (–N–N–) to form a single bond (–NH–NH–).

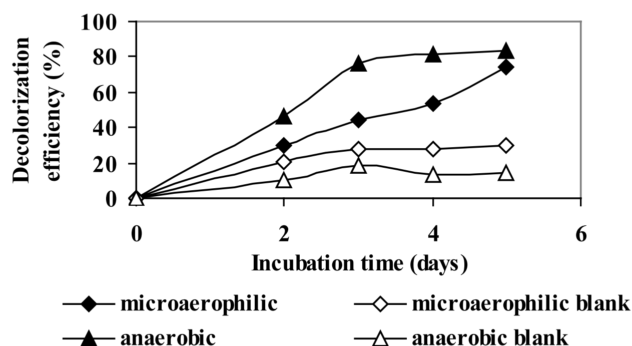


Fig. 6. Colour removal efficiencies for DB 38 azo dye by *Pseudomonas* sp. during incubation period.

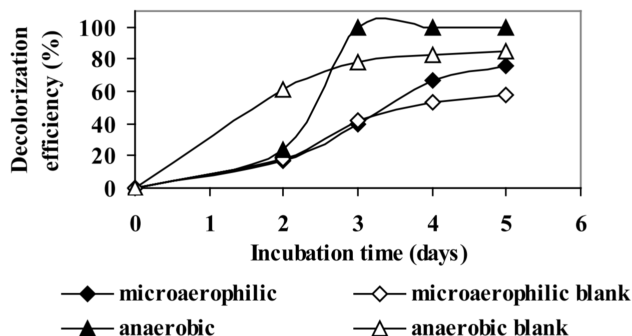


Fig. 7. Colour removal efficiencies for CR azo dye by *Pseudomonas* sp. during incubation period.

The concentrations of aromatic amines from the degradation of DB 38 were 4.36 and 6.79 mg/l, respectively, indicating that benzidine did not decrease and that the aromatic amines produced were not converted further. In other words, the benzidine produced was recovered at very high concentrations in the incubated samples under anaerobic and microaerophilic conditions (yields were 79 and 123%, respectively), indicating that further metabolism of these aromatic amines did not occur because of their accumulation.

It is important to note that decolorization of both azo dyes by *E. coli* and *Pseudomonas* sp. seems to involve the complete breakdown of azo bond to form benzidine (aromatic amine). Since the other decolorization metabolites were not investigated, except VFA as glucose breakdown products, it can be speculated that complete and partial reduction of the azo bond may occur simultaneously as reported by Chang et al. [28].

Very high benzidine recoveries were observed in DB 38 compared with CR dye, indicating the accumulation of aromatic amines in both cultures. The decolorization ratio of CR was higher than that for DB 38 for both cultures. This result may be attributed to the number of azo bonds (diazo dye) and $-NH_2-$ and $-SO_3Na-$ groups in the CR dye. Since the DB 38 dye has tri azo bonds (tri azo dye) and a hydroxyl group, lower

decolorization was detected compared to CR dye, as reported by Ogawa et al. [1].

4. Conclusions

The facultative bacteria *E. coli* and *Pseudomonas* sp., used in this study did not decolorize DB 38 and CR under aerobic conditions but when the same cultures were incubated under anaerobic and microaerophilic conditions significant decolorization was achieved. For maximum colour removals the optimal dissolved oxygen conditions in both dyes were as follows: (0.34 mg/l < DO < 1.54 mg/l for *E. coli* and 0.03 mg/l < DO < 0.04 mg/l for *Pseudomonas* sp.).

CR was completely and rapidly decolorized (100%) by *E. coli* (6 day) and *Pseudomonas* sp. (2.2 day) under anaerobic conditions, whereas only 26 and 40% decolorization was recorded under aerobic and microaerophilic conditions, respectively. DB 38 was decolorized with lower colour removal efficiencies by *E. coli* (73%) and *Pseudomonas* sp. (83%) compared to CR azo dye. Furthermore, the colour of CR was removed faster than that of DB 38 by *E. coli* and *Pseudomonas* sp.

During the mineralization of CR and RB 38 dyes; benzidine, and the VFAs were detected as degradation intermediates of dyes and glucose, demonstrating that aromatic amines were not mineralized further and accumulated at the end of the anaerobic incubation period. The recoveries and the accumulation of aromatic amines were higher in DB 38 dye compared to CR. Only 50–60% of COD was removed under anaerobic conditions while 80–90% COD removal efficiencies were achieved under aerobic conditions, indicating that no significant decrease in COD concentration was observed under anaerobic conditions. However, anaerobic conditions were effective in colour removal.

E. coli and *Pseudomonas* sp. may be promising bacteria for decolorizing the effluents containing the aforementioned dyes in textile industries. Since the aromatic amines are generally not metabolized further under anaerobic conditions an aerobic post-treatment step would be required for the complete mineralization of azo dyes. This could help to remove the possible toxicity originating from the amines.

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