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Research Article

# The Effect of Oxygen on Anaerobic Color Removal of Azo Dye in a Sequencing Batch Reactor

In this study, various amounts of oxygen were added to the anaerobic phase of an anaerobic-aerobic sequencing batch reactor (SBR) receiving azo dye remazol brilliant violet 5R to mimic the input of oxygen into the anaerobic zones of biological textile wastewater treatment plants. The effect of oxygen on the anaerobic biodegradative capability of the mixed microbial culture for remazol brilliant violet 5R was investigated. To investigate the effect of oxygen on anaerobic azo dye biodegradation, the anaerobic phase of the SBR cultures were exposed to a very low limited amount of oxygen for various air flow rates. Initially, an air flow rate of 20 mL/min was applied, further on the air flow rate in the anaerobic phase was increased up to 40 mL/min. System performance was determined by monitoring chemical oxygen demand, color removal rate, activities of anaerobic (azo reductase) and aerobic enzymes (catechol 2,3-dioxygenase, catechol 1,2-dioxygenase). The results of percentage COD reduction at each stage were similar for all runs, giving an overall reduction of 96%. Anaerobic color removal efficiency and azo reductase activity of anaerobic microorganisms were adversely affected by the addition of oxygen. Color removal efficiencies of the anaerobic phases decreased from 80% down to 42 and 38% for the limited oxygen conditions of 20 mL/min and 40 mL/min, respectively. It was observed that the activity of catechol 2,3-dioxygenase and catechol 1,2-dioxygenase, involved in breakage of aromatic rings, increased after they are exposed to oxygen limited conditions compared to fully anaerobic conditions. It was also observed that catechol 1,2-dioxygenase enzyme activity increased by increasing the oxygen level on oxygen limited conditions in the anaerobic zone.

**Keywords:** Azo dye; Azo reductase; Catechol dioxygenases; Oxygen; Sequencing batch reactor

*Received:* May 10, 2009; *revised:* July 10, 2009; *accepted:* July 20, 2009

**DOI:** 10.1002/clen.200900095

## 1 Introduction

The textile industry produces one of the highest amounts of wastewater during its manufacturing process. Textile dying wastewater is generally characterized by its intense coloring, caused by large amounts of unfixed residual dye, and a large amount of refractory COD caused by high molecular synthetic textile auxiliaries and dye. The most important problem in the treatment stage is caused by the physical and chemical properties of dyestuff in wastewater. The largest class of dyes applied in textile processing is the azo dyes which amount to approximately 70% of all dyes produced. Azo dyes are aromatic compounds with one or more -N=N- groups.

Textile processing wastewaters typically contain 10 to 200 mg/L dye, presenting highly colored waters [1]. Because many azo dyes and their breakdown products are toxic to aquatic life, the removal of color from dye containing effluents has been a major concern. Their discharge into surface water leads to aesthetic problems and adversely affects aquatic life.

Characterization of dye containing textile processing wastewaters is very difficult due to the structural dissimilarity of the dyes used and the diversity of dying processes. There are many reports on the use of chemical and physical methods for color removal [2 – 4]. The most commonly used chemical and physical treatment methods for dye containing textile processing wastewaters are chemical oxidation, chemical flocculation and settling, adsorption, membrane filtration and ion exchange. By these existing physical and chemical color removal methods color is generally concentrated in a sludge or colored molecules are partly removed. Moreover, the formation of large amounts of sludge and economical limitations are some disadvantages of these methods. Alternatively, biological treatment methods, generally considered to be environmentally friendly, may present a relatively inexpensive way to remove dyes from wastewaters.

It is known that several microorganisms, such as fungi, bacteria and algae, can decolorize many azo dyes [5]. Generally, bacterial azo dye biodegradation proceeds in two stages. Anaerobic azo dye reduc-

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**Abbreviations:** RBV-5R, Remazol brilliant violet 5R; SBR, Sequencing batch reactor

tion, the reductive cleavage of azo linkages, is the first stage in the complete anaerobic-aerobic degradation of azo dyes, results in aromatic amine accumulation. Aromatic amines, which are formed during anaerobic treatment, are generally colorless and hazardous and many of them can be degraded under aerobic conditions by a hydroxylation and ring opening mechanism. So, the aerobic stage is necessary to achieve degradation of the aromatic amines regarded as recalcitrant compounds in the anaerobic treatment step. Aromatic amine compounds, which are formed as the result of reduction of azo dyes in the first stage, are then mineralized by aerobic oxidation at the second stage. Thus, using both anaerobic and aerobic stages represents both decolorization and mineralization of azo dyes [6].

Since the reduction of azo dyes is an oxidation-reduction reaction in which the dye acts as an electron acceptor, it is assumed that the presence of different electron acceptors (i.e., oxygen) may compete with the azo dye for reducing equivalents and affect the color removal rate. Even though the anaerobic zone has no purposeful input of oxygen, the anaerobic bacteria in it are exposed to oxygen from two sources. One is from the atmosphere, since anoxic zones are typically open tanks, via oxygen transfer at the air/liquid interface. The other is from the aerobic zone via recirculation flow. Since oxygen is a much more preferable electron acceptor than azo dyes, the effect of oxygen concentration on anaerobic treatment performance becomes more important. Our investigations were therefore directed toward examining the effect of oxygen on biodegradation and decolorization of azo dye.

In this study, the effect of oxygen in a range of different concentrations on anaerobic color removal efficiency using simulated textile wastewater containing RBV-5R azo dye in a SBR was investigated. SBR performance was determined by monitoring COD, color, color removal rate and activities of anaerobic enzyme (azo reductase) and aerobic enzymes (catechol 2,3-dioxygenase, catechol 1,2-dioxygenase).

## 2 Material and Methods

### 2.1 Chemicals

In this study, remazol brilliant violet 5R (RBV-5R) was used as the azo dye (see Fig. 1). Since RBV-5R includes the aromatic amine benzidin, it is forbidden both in Europe and in Turkey. However, it is still widely used in the textile industry because of its low price and effective dying efficiency.

### 2.2 Basal Medium and Seed Inoculum

The SBR system was inoculated with sludge collected from the wastewater treatment plant of Kipas Textile Industry in Kahramanmaras, Turkey, and acclimatized to the described basal media and glucose for 90 days. During this acclimatization run dyestuff was added daily into the reactor just after the end of the fill phase, so as to produce initial concentrations of 50 mg dye/L. Since the reactor had a cycle time of 12 h, the night feed, which was prepared concentrated in 100 mL glass bottles, was maintained automatically by timers.

The bacteria are described as facultative microorganisms, which are capable of growing under both anaerobic and aerobic conditions. A sludge age of 15 days was maintained by withdrawing the

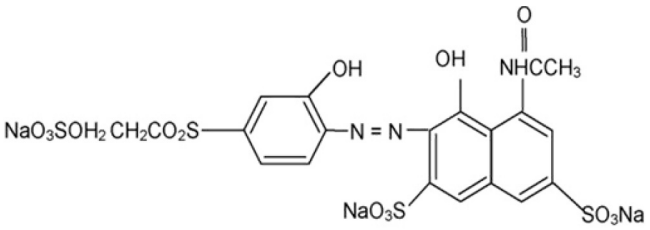


**Figure 1.** The structure of remazol brilliant violet 5R (RBV-5R).

necessary volume of biomass/liquid mixture daily. The mixed liquor suspended solid (MLSS) concentration was kept 5500 mg/L during SBR operation.

Simulated textile wastewater was prepared using a mixture of RBV-5R and the nutrient elements required for microbiological growth. Glucose (contributed to COD of 1085 mg/L) was used as the sole energy source in the SBR reactor. In all experiments, a basal media containing the following compounds was used (mg/L): $NH_4Cl$ (230), $K_2HPO_4$ (37), $KH_2PO_4$ (67), $MgCl_2$ (15), $CaCl_2 \cdot 2\,H_2O$ (22), $FeCl_3 \cdot 6\,H_2O$ (5), $H_3BO_3$ (0.35), $ZnCl_2$ (0.05), $CuCl_2.2\,H_2O$ (0.038), $MnCl_2 \cdot 4\,H_2O$ (0.5), $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ (0.05), $AlCl_3 \cdot 6\,H_2O$ (0.09), $CoCl_2 \cdot 6\,H_2O$ (1), $NiCl_2 \cdot 6\,H_2O$ (0.092), $Na_2SO_3 \cdot 5\,H_2O$ (0.164).

### 2.3 Reactor Operation and Experimental Matrix

The experimental system was composed of a SBR in 12 h total cycles consisting of three discrete runs listed in Tab. 1. The effluent was discharged at a volumetric exchange ratio of 50%. Dye stuff and glucose were added manually at the beginning of the each run of the SBR with a concentration of 50 and 1120 mg/L in the reactor, respectively. The experimental SBR consisted of a 6.5 L vessel (Bioflo 110, New Brunswick Scientific Co., Edison, NJ, USA) with an effective working volume of 5 L. The contents of the SBR were mixed by a single shaft impeller system at a speed of 450 rpm. An internal pH controller (Bioflo 110 system) was used to control the pH between 7.2 and 7.3 by adding 0.2 mol/L HCl and 0.2 mol/L NaOH. The SBR system was operated with a solid retention time (SRT) of 15 days. The reactor was run at a constant temperature of 25 ± 1°C. All inorganic nutrients were provided in excess, making textile dye the limiting substance. Care was taken to totally exclude atmospheric oxygen from the SBR by continually sparging with nitrogen gas during the first run of the study. Oxygen was provided in excess to the aerobic phase of the SBR by air pumps to maintain the aerobic cycles of study runs. A very low, limited amount of oxygen addition to the anaerobic cycles was provided by pumping air at 20 and 40 mL/min flow rates into the system by means of peristaltic pumps. The study was composed of three runs. In the second and third run, the effect of oxygen on anaerobic color removal was investigated; Run 1 was the oxygen free stage (fully anaerobic) which has no oxygen exposure in the anaerobic reaction phase of SBR operation.

The reactor system was operated with simulated textile wastewater for 135 days through Run 1, 2 and 3 under steady state conditions in order to investigate the effect of oxygen on color, COD removal and anaerobic-aerobic enzymes, while the total operation time was 225 days with the acclimation runs. The SBR reactor was operated for 45 days (3 SRT), before starting to operate in every run in order to reach steady state conditions. In other words, the data monitored in all runs indicates the results obtained in steady state conditions. Steady state was defined by color removal, effluent COD

 

**Table 1.** Summary of three operational conditions tested for color removal.

| Cycle times (12 h) | Run 1 | Run 2 | Run 3 |
|---|---|---|---|
| Anaerobic reaction (h) | 8 | 8 | 8 |
| Aerated reaction (h) | 2.75 | 2.75 | 2.75 |
| Settling (min) | 35 | 35 | 35 |
| Draw (min) | 5 | 5 | 5 |
| Fill (min) | 5 | 5 | 5 |
| Air Flow Rate (mL/min) | – | 20 | 40 |

concentrations, anaerobic and aerobic enzymes. After the reactor had achieved stable operating conditions the limited amount of oxygen addition was employed through runs 2 – 3. Measurements of the three cycles were performed in each run. The data illustrated in all figures are the mean values of the measurements for 3 cycles. The operation conditions in runs and in the relevant acclimation runs were similar except for the additional limited amount of oxygen in Runs 2 and 3.

### 2.4 Analytical Procedures

Mixed liquor samples taken during the 8 h cycles of the anaerobic phases of the SBR were clarified by centrifugation (Eppendorf Centrifuge 5415R, Hamburg, Germany). Dye concentrations were measured at the maximum absorbance wavelength of azo dye RBV-5R ($\lambda$ = 560 nm) by spectrometer (Chebios Optimum-One UV-VIS Spectrometer, Roma, Italy) and calculated from the calibration curves of absorbance versus concentration. Biomass concentration was measured as absorbance at 600 nm followed by reference to an experimentally derived standard curve.

The soluble COD was measured calorimetrically. Firstly the samples were centrifuged for 5 min at 10 000 rpm. COD was measured by a reactor digestion method using commercially prepared HACH testing kits (HACH DR/2500). This method was the current US EPA approved method for analyzing chemical oxygen demand (Hach Method 8000) [7].

### 2.5 Enzyme Assays

Azo reductase is known as the enzyme responsible for color removal under anaerobic conditions. Catechol 2,3-dioxygenase and catechol 1,2-dioxygenase are known to be the enzymes responsible for aromatic amine removal under aerobic conditions.

Cell free extracts were prepared for the enzyme assays as described by Çinar et al. [7]. A Chebios Optimum-One UV-VIS Spectrophotometer (Rome, Italy) was used for all enzyme assays. The anaerobic enzyme used as an indicator of anaerobic RBV-5R biodegradation was azo reductase. The activity of azo reductase was monitored by the change of absorbance at 560 nm with time in mixtures of 400 µL of 10 mol/m$^3$ sodium phosphate buffer (pH 7.0), 200 µL NADH (final concentration 2 mol/m$^3$) and 200 µL of the azo dye RBV-5R (20 mg/L). The reaction was initiated by the addition of 200 µL of the cell free extract [9].

The activity of catechol 1,2-dioxygenase was monitored by the change of absorbance at 257 nm with time in mixtures of cell free extract (250 µL), 10 mol/m$^3$ catechol (250 µL) and enough reagent to make a total volume of 3000 µL [10]. The reagent contained a solution of 50 mol/m$^3$ Tris-HCl and 10 mol/m$^3$ EDTA at pH 7.5. The activity of catechol 2,3-dioxygenase was measured using the same method, except for the detection wavelength, which was 375 nm. This is the wavelength at which 2-hydroxy-cis,cis-muconic semialdehyde, which is the product of catechol 2,3-dioxygenase, has an absorption maximum [10].

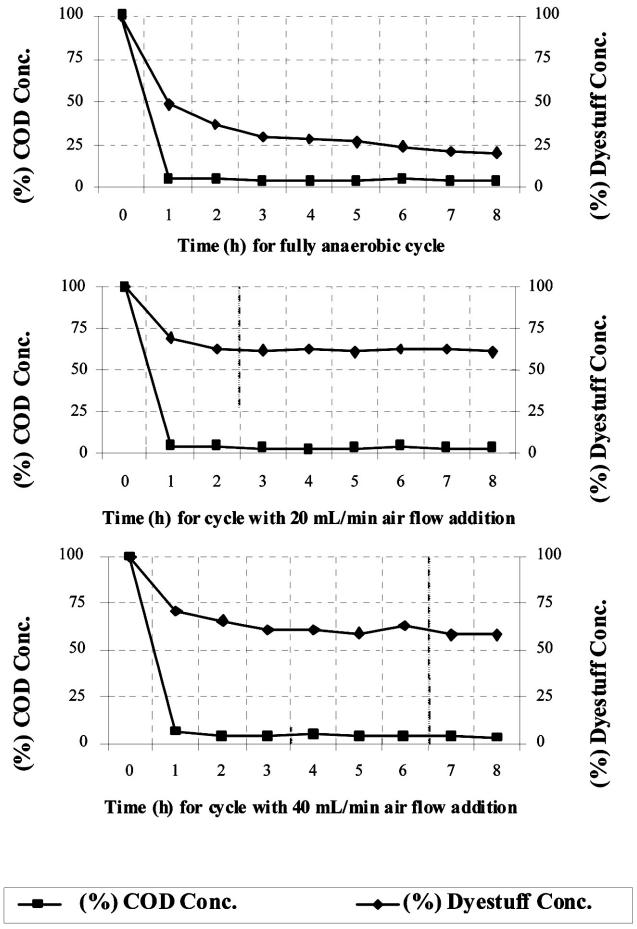


**Figure 2.** The effect of oxygen on COD and color removal efficiency.

The protein concentrations were required to calculate specific enzyme activities. Protein concentration was measured using the bicinchoninic acid-copper reaction in which proteins reduce $Cu^{2+}$ to $Cu^{+}$ in alkaline solution. Bovine serum albumin was used as the standard. A UV-VIS Spectrophotometer (Chebios Optimum-One) with a 1 cm light path was used at a wavelength of 562 nm to determine the protein concentration from a standard curve [11].

## 3 Results and Discussion

### 3.1 COD and Color Removal Efficiency

Anaerobic azo dye decolorization is based on oxidation-reduction reactions in which the azo dye acts as an electron acceptor. A labile carbon source is therefore essential. The main source of COD was glucose added to the system as an electron donor. Electrons released from oxidation of the electron donating primary substrate (glucose), are transferred to the electron accepting azo dye, thereby resulting in color removal. System efficiency for substrate removal

 

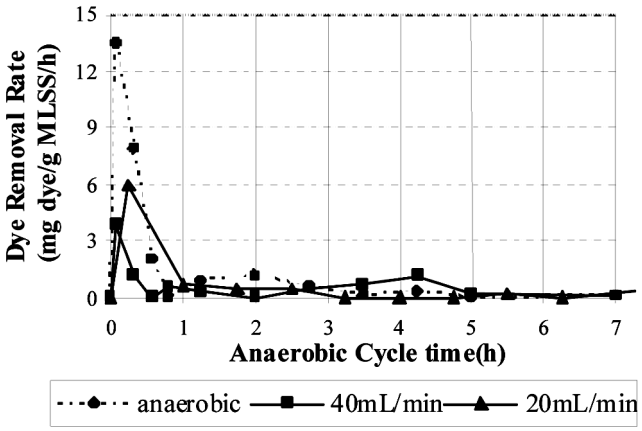


**Figure 3.** The effect of oxygen on dye removal rate.

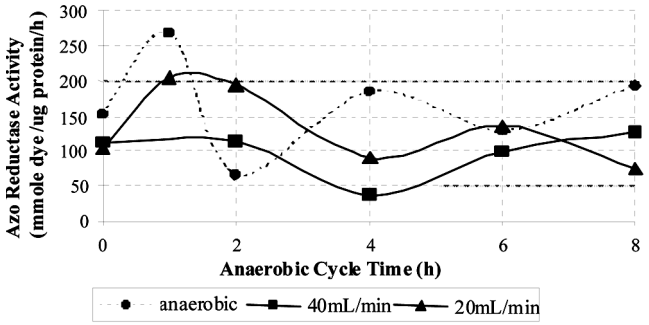


**Figure 4.** The effect of oxygen on azo reductase activity.

was constantly evaluated in terms of soluble COD. The results of Runs 2 and 3 are compared to Run 1 in terms of COD removal efficiency and color removal efficiency, and depicted in Fig. 2.

Results of percentage COD reduction at each stage were similar for all runs, giving an overall reduction of 96%. Most of the COD was removed in the first hours of the SBR. It could be concluded that COD is mainly used by anaerobic organisms for growth and to obtain energy. These results are in agreement with previously published data [12]. The anaerobic stages of SBR studies have shown efficient color removal rates, mostly higher than 70% [13–16]. Meanwhile, COD removal efficiency of the anaerobic phase of the SBR was found to depend on dyestuff type, amount of initial COD concentration, anaerobic cycle time, etc. Nevertheless, there are also reports that there is no efficient COD removal in the anaerobic cycle of an SBR [17, 18].

The higher percentage of color removal in Run 1 compared with Runs 2 and 3 indicates that oxygen has an adverse effect on the dye reduction mechanism. This is because the electrons released from oxidation of the glucose are preferentially used to reduce oxygen rather than the azo dye. Increasing the air flow rate from 20 to 40 mL/min caused a slight increase in effluent color. On the other hand, the SBR system reduced around 1000 mg/L initial COD concentrations to about 40 mg/L for all runs of the study, with no effect of oxygen on COD removal observed. The adverse effect of oxygen on color removal in the anaerobic zone of the SBR was clearly seen, reducing the 50 mg/L initial dye stuff concentrations by up to 10, 29 and 31 mg/L for the anaerobic, limited oxygen conditions of 20 mL/min and limited oxygen conditions of 40 mL/min, respectively. Oxygen is a more effective electron acceptor than azo dyes, which justify the lower decolorization rates as oxygen addition increases into the anaerobic zone [19]. Data obtained from this study showed the no inhibitory effect of limited oxygen levels on color removal as pointed by Ramolho et al. [20].

## 3.2 Color Removal Rate

The other important parameter for evaluating color removal performance within the system is color removal rate. Color removal rate is calculated as dye concentration removed by unit microorganism in unit time (mg dye/g MLSS h). Color removal rates for different operating conditions are presented in Fig. 3. Since the azo dye was mostly removed within the initial phases of the anaerobic zones, no apparent color removal rate was observed after the 5th hour of the anaerobic zone in the SBR.

Color removal reached a maximum rate under fully anaerobic conditions; whereas it dropped under anaerobic conditions with the addition of a limited amount of oxygen. Although there are few published reports regarding color removal rates; Çinar et al. [16] reported that the highest color removal rate was obtained under anaerobic conditions and no color removal was observed under aerobic conditions, since the dye was only removed under anaerobic conditions.

The dye removal rates decreased after the 1st hour of the anaerobic stages of the SBRs. This may have resulted from decreasing glucose concentrations throughout the anaerobic cycle; thereby it lacked the necessary electron donor for reductive dye removal process. The lowest color removal rate in the first 15 min was obtained for the situation in which 40 mL/min of air was supplied to the anaerobic reaction phase.

## 3.3 Anaerobic Enzyme Activity

Electrons released from the oxidation of the substrate are transferred to an azo dye by an enzyme mediated mechanism called azo reductase. Azo reductase in anaerobic bacteria is responsible for the reduction of the azo bond that gives its color to azo dye. Thus, monitoring azo reductase enzyme activity is important for evaluating color removal performance within a system. Figure 4 shows the anaerobic enzyme activities under different operating conditions.

As expected, azo reductase activities gradually increased in the first hours of the anaerobic cycles where maximum color removal rates were achieved and then started to decrease, caused by the consumption of the electron source. Competition of molecular oxygen for available reducing equivalents with the azo dye decelerated the reductive cleavage of the azo bond. Delays observed for the time intervals in which enzyme activity reached a maximum explain the adverse effect of oxygen on azo reductase activity under anaerobic conditions with the addition of a limited amount of oxygen. Azo reductase inhibition by oxygen was reported in previous studies [19, 21, 22]. Maier et al. [23] reported that azo reductase activity could increase in the absence of oxygen. In a similar study on the azo reductase enzyme, which was carried by Hernandez et al. [24], it was reported that the enzyme activity was limited with a ratio of approximately 100% when exposed to oxygen. The highest activity obtained in the study was 268 (mmol dye/μg protein per h) under fully anaerobic conditions.

 

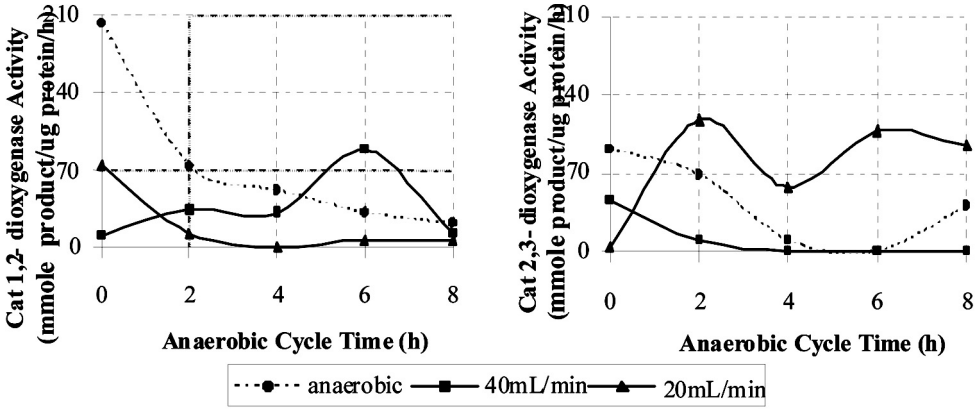


**Figure 5.** The effect of oxygen on aerobic enzyme activities.

### 3.4 Aerobic Enzyme Activity

In the aerobic degradation of aromatic compounds by microorganisms, catechol plays a key role, since it is a ring cleavage substrate in which an exceptionally large number of peripheral pathways converge [25]. Since catechol 1,2-dioxygenase and catechol 2,3-dioxygenase are key enzymes in the catabolism of aromatic compounds, the determination of these enzyme activities is important for monitoring whether aromatic amine degradation occurs during the study cycles. Figure 5 represents the catechol 1,2-dioxygenase and catechol 2,3-dioxygenase enzyme activities under different operation conditions.

Enzymes which are responsible for aromatic amine removal become more active when color removal rates are high which results in more aromatic amine production within the SBR. Since maximum color removal efficiency was obtained from the first run (anaerobic cycle), catechol 1,2-dioxygenase enzyme activity was high at the beginning of anaerobic conditions compared to limited oxygen conditions. Similarly, catechol 2,3-dioxygenase enzyme activity was initially high but then started to decrease towards the end of the anaerobic cycle time, resulting from the adverse effect of anaerobic conditions on aerobic enzymes [8, 10]. Ma and Love [26] reported that both catechol 2,3-dioxygenase and catechol 1,2-dioxygenase rapidly lose their activities while the process starts in the anaerobic reaction phase of the SBR reactor. As seen in Fig. 5, dioxygenase enzymes represent different behaviors in different operation conditions. No apparent catechol 2,3-dioxygenase activity was observed under oxygen limited conditions of 40 mL/min air, conversely catechol 1,2-dioxygenase enzyme activity gradually increased and reached the maximum value at the 6th hour of the cycle time. The effect of oxygen availability on catechol 1,2-dioxygenase activity was also investigated by Viliesid and Lilly [27], and they found that the activity of catechol 1,2-dioxygenase was dependent on dissolved oxygen concentration. The activity of catechol 1,2-dioxygenase was influenced by the oxygen concentration, and the activity of catechol 1,2-dioxygenase is likely to be low in systems with more limited oxygen concentrations. When 20 mL/min. air was given to the system, catechol 2,3-dioxygenase enzyme yielded more activity compared to catechol 1,2-dioxygenase.

Since much of the electron donor source within the system was consumed in the first hour, aromatic amines start to serve as electron donors, resulting from oxygen accumulation in the system. The increase in enzyme activity, in particular in the 6th hour, can be explained in this way. Hence, it can be concluded that bacteria is able to cleave -N=N- bonds reductively and utilize amines as the source of carbon and energy for their growth under oxygen limited conditions.

## 4 Conclusions

In this study, the effect of oxygen on anaerobic color removal efficiency in an anaerobic/aerobic sequencing batch reactor treating simulated textile wastewater was investigated and the following conclusions can be drawn:

The azo reductase enzyme has a role in biological removal of textile dyestuff and it is adversely affected by the presence of oxygen, but a very low limited amount of dissolved oxygen addition has no inhibitory effect.

Molecular oxygen decreased color removal significantly in the biological removal of the dyestuff.

The conversion steps of aromatic amines are carried out dissimilar at different oxygen levels. Catechol 1,2-dioxygenase enzyme increased its activity by increasing the oxygen level, however; catechol 2,3-dioxygenase enzyme decreased its activity by increasing the oxygen level within the system.

Under anaerobic conditions with a very low limited amount of dissolved oxygen addition, aromatic amines may serve as electron donors if there is a lack of any substrate in which the azo dye acts an electron acceptor.

### Acknowledgements

This research was supported by Kahramanmaras Sütçü Imam University Division of Scientific Research Projects, through grant 2008/1-40M, The authors are thankful to the anonymous reviewers for their insightful comments and suggestions.

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