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*Full Length Research Paper*

# Biodegradation of reactive red 195 azo dye by the bacterium *Rhodopseudomonas palustris* 51ATA

**Leyla Çelik[1], Ayten Öztürk[1]* and Meysun I. Abdullah[2]**

[1]Department of Biology, Faculty of Art and Science, Niğde University, Campus, 51240 Niğde, Turkey.
[2]Department of Chemistry, Faculty of Art and Science, Niğde University, Campus, 51240 Niğde, Turkey.

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**The sulphonated reactive red 195 dye (RR195) was used as carbon source and energy by the widely spread, eco-friendly, photoheterothrophic strain 51ATA that belongs to *Rhodopseudomonas palustris*. This bacterium, which was isolated from Lake Akkaya, (Niğde, Turkey), was able to completely degrade and mineralize the dye under anaerobic conditions with 100% efficiency. The degradation efficiency of this strain, in the presence and absence of a co-substrate was investigated. The biodegradation of the dye was monitored by UV-visible, (FTIR) spectroscopy and HPLC. From these analyses, a complete mineralization of the toxic aromatic ring system of the reactive red 195 dye was observed.**

**Key words:** Biodegradation, decolorisation, biomineralisation, azo dye, reactive red 195, *Rhodopseudomonas palustris*.

## INTRODUCTION

Azo dyes and dyes are surface contaminants that threaten water resources (Chung and Cerniglia, 1992; Riu et al., 1997; Zolinger, 2003; Anjaneyulu et al., 2005). Remazol dyes are among important water pollutants, because reactive dyes tend to pass through conventional treatment systems unaffectedly (Stolz, 2001). Since color damages the aesthetic nature of the environment and it is the first contaminant to be recognized in wastewater, several methods have been used to decolorize azo dyes (Wu and Wang, 2001; Cisneros et al., 2002; Ramsay and Nguyen, 2002; Akyol and Bayramoğlu, 2005; Sarayu et al., 2007; Wang et al., 2009). The eco-friendly microbial methods are attractive because of their metabolic pathways and versatility of microorganisms (Singh et al., 2004; Anjaneyulu et al., 2005; Pandey et al., 2007). Moreover**,** microbial methods are cost competitive alternatives to the physical and chemical treatment methods, which are the least desirable, since they are rather expensive with the possibility of generating secondary pollutants or new harmful intermediates (Stolz, 2001). Biological processes including aerobic (Tony et al., 2009), anaerobic (Li et al., 2001; Şen and Demirer, 2003) or sequential anaerobic/aerobic are widely applied in the decolorization of azo dyes (Rajaguru et al., 2000; Supaka et al., 2004). Many researchers (Yeşilada et al., 2003; Verma and Madamwar, 2005; Khalid et al., 2008) have investigated the decolorization of azo dyes by different bacteria and fungi. Decolorization step is responsible for color removal but it does not remove the dye-related hazardous aromatic amines from wastewater. The existence of the colorless aromatic amines in the aqueous ecosystems is of a serious environmental and health concern; therefore, a complete removal of these compounds from the aquatic system is required. However, despite the fact that biodegradation and decomposition or mineralization of azo dyes in waste-water is extremely difficult and incomplete, (Coughling et al., 2002; Kalyani et al., 2009) the sequential anaerobic and aerobic bacterial degradation system is reported to be very efficient in degradation but not in complete mineralization of sulfonated azo dyes (Telke et al., 2008). Since azo bond and the sulphonate moiety, common to many azo dyes often render azo dyes recalcitrant to aerobic biodegradation, the degradation of these dyes were studied mostly under anaerobic conditions

*Corresponding author. E-mail: aytenozturk@hotmail.com. Tel: +90 388 2254208. Fax: +90 388 2250180.

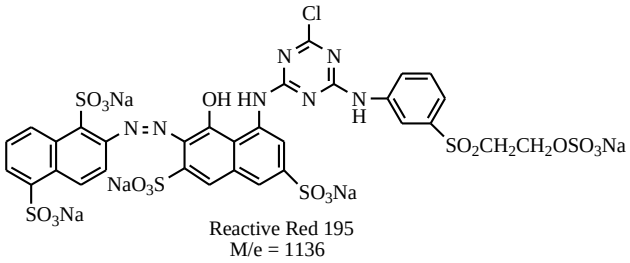


**Figure 1.** Chemical Structure of Reactive Red 195.

(McMullan et al., 2001). However, to the best of our knowledge, up to date, there are no evidences of productive decolorization, biodegradation, and mineralization of sulphonated azo compounds by any anaerobic photosynthetic bacteria except for the decolorization and partial mineralization of azo dyes reported by Wang et al. (2008).

In this article, the utilization of sulphonated azo dyes (Reactive red 195) by the newly isolated anaerobic photosynthetic bacterium *Rhodopsuedomonas* sp.51ATA strain was investigated. Since *Rhodopseudomonas* species can grow anaerobically in the light and aerobically in the dark (Wright and Madigan, 1991; Gibson and Gibson, 1992; Krooneman et al., 1999) and because some *Rhodopsuedomonas* sp. are capable of using benzoates as a sole carbon source, we decided to explore the potential of this strain in the mineralization of the aromatic ring systems. Therefore, in this work we report the complete mineralization of Reactive red 195 dye by *Rhodopsuedomonas palustris* 51ATA strain.

## MATERIALS AND METHODS

### Azo dye and chemical reagents

A commercial dye RR195 obtained from RATEKS-Denizli (Turkey), was used in this study. The dye stock was filtered through 0.2 µm pore filter and then was sterilized. A known concentration of this dye (100 µg /L) was added to each medium. Other chemicals and medium components were of analytical grade reagents.

### Isolation and screening of the bacterium

Sample of waste liquor was collected from the Lake Akkaya, Nigde, Turkey. The sample was enriched by introducing it into a Winogradsky column containing 1 g/L sodium benzoate as the only source of carbon for a period of three months under sun light and at room temperature. A sample (1.0 ml) from the pink- purple anaerobic region was pipetted out and inoculated into the benzoate containing mineral liquid medium under 60 watt lamp for one month. When anaerobic growth was observed, the bacterial colony was purified by traditional roll tube method. A single pink- purple colony was removed and then was inoculated in the MAT soft agar (0.5 %w/v) medium (Selimoğlu et al., 2011). In order to identify this bacterium, physiological and microscopically examination were carried out according to Bergey's Manual of Bacteriology; it was identified as *Rhodopseudomonas palustris* (Boone and Castenholz, 2005).

### Biodegradation of dye

0.1 mg $L^{-1}$ of Reactive red 195 dye (Figure 1) was added to both media, the mineral medium and MAT agar (%0.5 w/v) by the traditional roll tube method and the tubes were inoculated with *R.p.* 51 ATA strain. The tubes were incubated for one month at 25-30°C under light and then were inspected for colonies. A pure colony was then inoculated to both (the mineral and MAT) liquid media, each containing 0.1 mg $L^{-1}$ reactive red 195 dye. When the dye was decolorized, it was further evaluated for its ability to act as carbon source for the strain 51 ATA by determining the growth in a liquid mineral medium containing reagent grade chemicals of the following composition:

The mineral medium contained; 3 g/L $NaHCO_3$, 0.1 g/L $CaCl_2.H_2O$, 1 g/L NaCl, 1 g/L $KH_2PO_4$, 0.5 g/L $MgCl_2.6H_2O$ and did not contain carbon or nitrogen in it, while the MAT medium contained; 3 g/L $NaHCO_3$, 1 g/L $(NH_4)_2SO_4$, 0.1 g/L $CaCl_2.H_2O$, 1 g/L NaCl, 1 g/L $KH_2PO_4$, 0.5 g/L $MgCl_2.6H_2O$, 1 g/L glucose (as a co-substrat), 0.1 g/L yeast extract and 1.0 ml/L of vitamin containing trace elements. In this medium, the dye is considered to act as an additional carbon source with respect to glucose.

### Decolorization process

The aqueous solutions of the azo dye (0.1%) were sterilized by filtration through 0.45 µm pore size membranes and were added individually to both media to attain a final dye concentration of 100 µg $L^{-1}$. The inoculum (added at 1:1000 v/v) was at logarithmic phase culture of strain 51ATA that had been previously grown to a cell density of approximately $1.25\times10^{8}$ cfu $ml^{-1}$ in both media.

Decolorization was measured as a function of decreasing absorbance measured at the maximum absorbance of the dye. A Simadzu A160 and (Selecta) spectrophotometers were used for the UV-visible absorption measurements of the dye. Absorption maximum was recorded at $\lambda_{max}$ 530 nm. Suspensions of strain 51ATA were centrifuged at 10000 rpm for 5 min to pellet the cells, allowing color determination of the supernatant. The maximum absorbance for reactive red 195 dye was at 530 nm. The color loss was based on a standard curve comparing dye concentration to absorbance as percent of uninoculated control. The roll tube method was used for the enumeration of the bacterium culture of the MAT medium and the bacterial density was measured as function of absorbance at 650 nm.

### Extraction from growth medium

The bacterial culture was centrifuged at 10000 rpm for five minutes and the supernatant containing the degradation metabolites were extracted from the aqueous phase with either chloroform or ethyl acetate (3×50 ml) and the organic layer was dried over anhydrous $CaCl_2$, filtered and was evaporated under reduced pressure.

### FT-IR analysis

Biodegradation was monitored by FTIR spectroscopy. FTIR spectra were recorded on PerkinElmer- FTIR spectrum BX 83861. The IR of the dye and the extracted metabolites obtained on the 21st, 45th and the 93rd days were compared.

### HPLC analysis

HPLC analysis was performed on HPLC/ Thermo P1500-SN-4000-UV2000 using 3.9×300 mm Bondapak C-18 reverse phase column. Thirty microliter (30 µl) of each metabolite was manually injected at

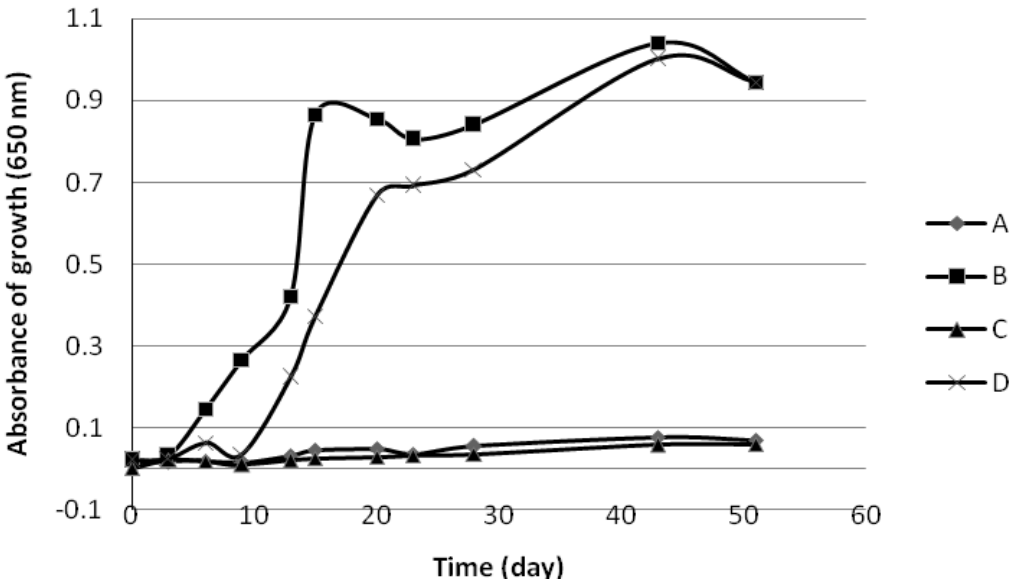


**Figure 2.** Absorbance measurement of bacterial growth in both media. A, growth of mineral medium that contain the dye. **B**, growth of MAT medium that contain the dye. **C**, growth of mineral medium (growth control). **D**, growth of MAT medium (growth control).

a flow rate of 1 ml/min and isocratic mobile phase 60/40 v/v (acetonitrile/water) was used. Detection was performed at 285 nm.

## RESULTS

In this work, we have attempted to study the complete decolorization, biodegradation, and mineralization of Reactive red 195 by the newly isolated anaerobic photosynthetic bacterium *R. palustris* 51ATA strain from Lake Akkaya (Turkey) which is exposed to waste water discharge from many textile and other industries. The anaerobic, photosynthetic microorganisms *Rhodopseudomonas* sp. belongs to the purple non-sulphur bacteria that can degrade benzoates (Gibson and Gibson, 1992; Krooneman et al., 1999). Since *Rhodopsuedomonas* sp. can also grow anaerobically in the light and aerobically in the dark, we decided to make use of this property and use this bacterium in the decolorization, mineralization, and degradation of the sulphonated reactive red 195 dye. Therefore, the decolorization of the dye from aqueous solutions by this bacterium under anaerobic conditions was investigated. Decolorization studies were carried out under light and were followed spectrophotometrically (Figure 2). The decolorization and bacterial growth at certain time intervals were measured as a function of absorbance under two different media (mineral and MAT media). The observed changes in the concentration of the dye in both media (MAT and mineral) are given in Figure 3.

The biodegradation of the reactive red dye 195 was followed by Fourier Transform Infra Red FTIR spectroscopy (Figure 4). The IR of the Reactive red 195 dye showed characteristic absorption bands at $\upsilon_{max}$ ($ccl_4$)/$cm^{-1}$): 3328- 3650 $cm^{-1}$ (N-H, O-H and aromatic =C-H ) stretching, 2924-2955 $cm^{-1}$ (asymmetric aliphatic C-H), 1550 (N=N) stretching, N-C=N stretching were present at 1410 and 1470 $cm^{-1}$, respectively. 1318 $cm^{-1}$ (S=O peaks of the $SO_2$), 1138 $cm^{-1}$(S=O peaks of $OSO_3$), 1039 $cm^{-1}$ (C-O and / or C-N), 1183 $cm^{-1}$ (phenolic C-O), while the other corresponding aromatic peaks and the C-Cl stretching were observed in the finger print region.

HPLC analysis of the reactive red dye 195 was also carried out and the retention times of the individual metabolites were compared with the dye (Figure 5).

## DISSCUSION

The absorbance of the bacterial growth in the mineral and MAT medium at 650 nm was determined (Figure 2). In this curve, A and C showed a very slow bacterial growth in the mineral medium while curve B and D indicated a very fast bacterial growth due to presence of glucose as co-substrate in the MAT medium. However, in each medium, the growth was better when the dye was present (curve A and B). We could conclude that the presence of the co-substrate affects the efficiency of degradation, and the decolorisation was higher in MAT medium compared to mineral medium as shown in Figures 2 and 3.

According to the presence or absence of the co-substrate in both media, the dye decolorisation was slow in the mineral medium due to slow growth of the bacterium and was much faster and more effective in the MAT medium.

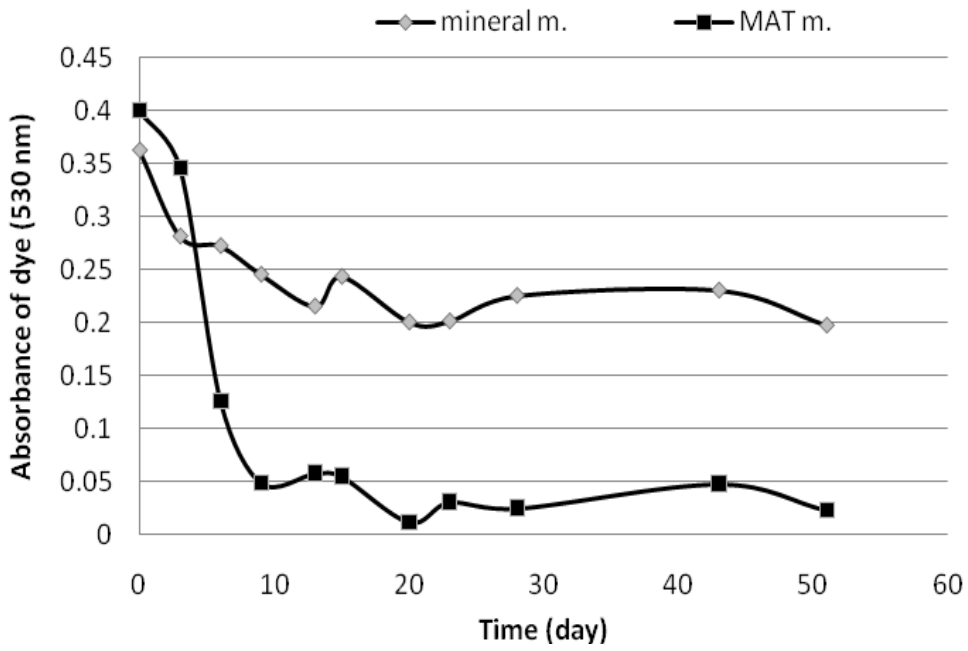


**Figure 3.** Time-course of decolorization in both media.

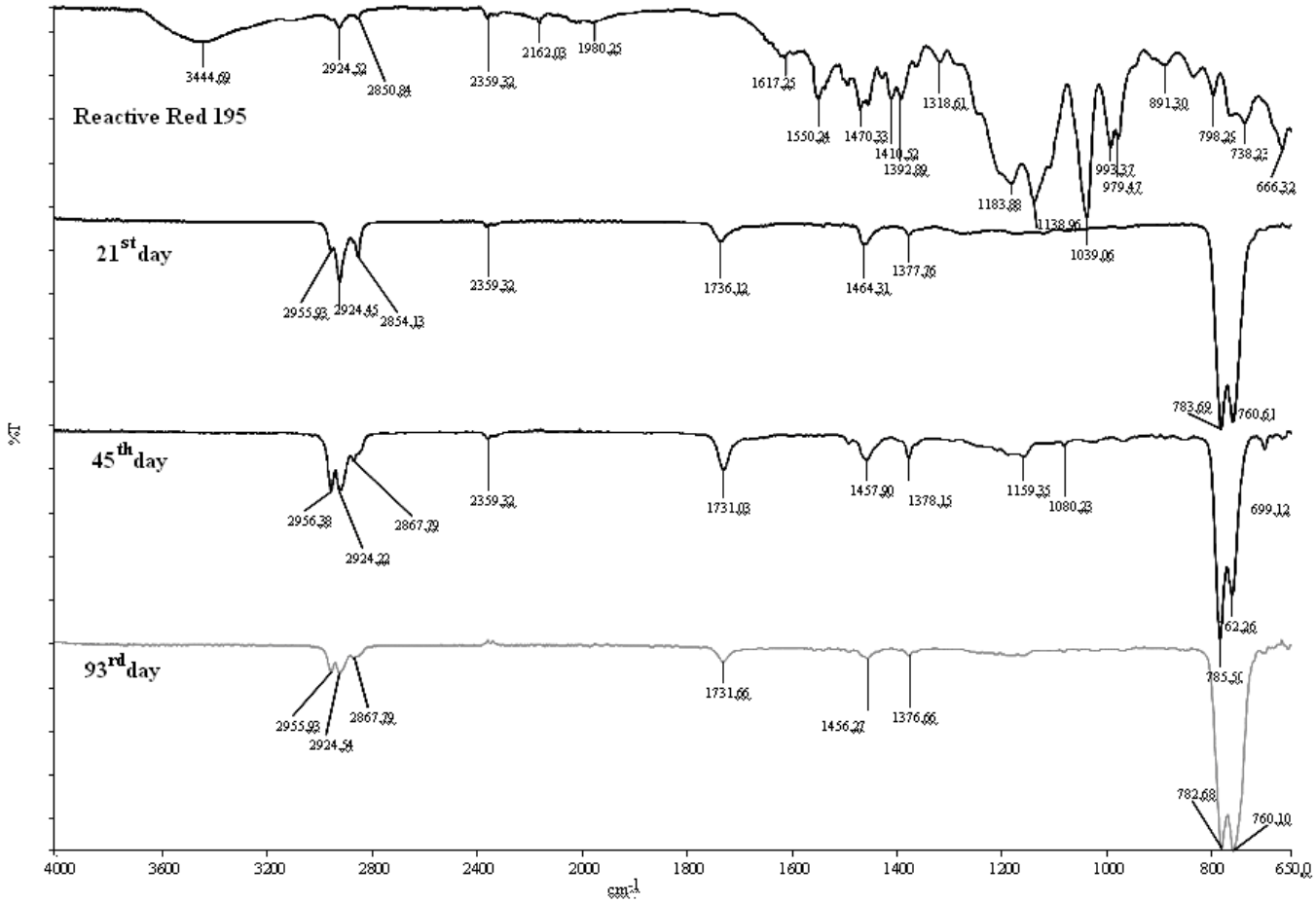


**Figure 4.** FTIR spectra of the dye and metabolites extracted on the 21st , 45th, and 93rd day of anearobic treatment.

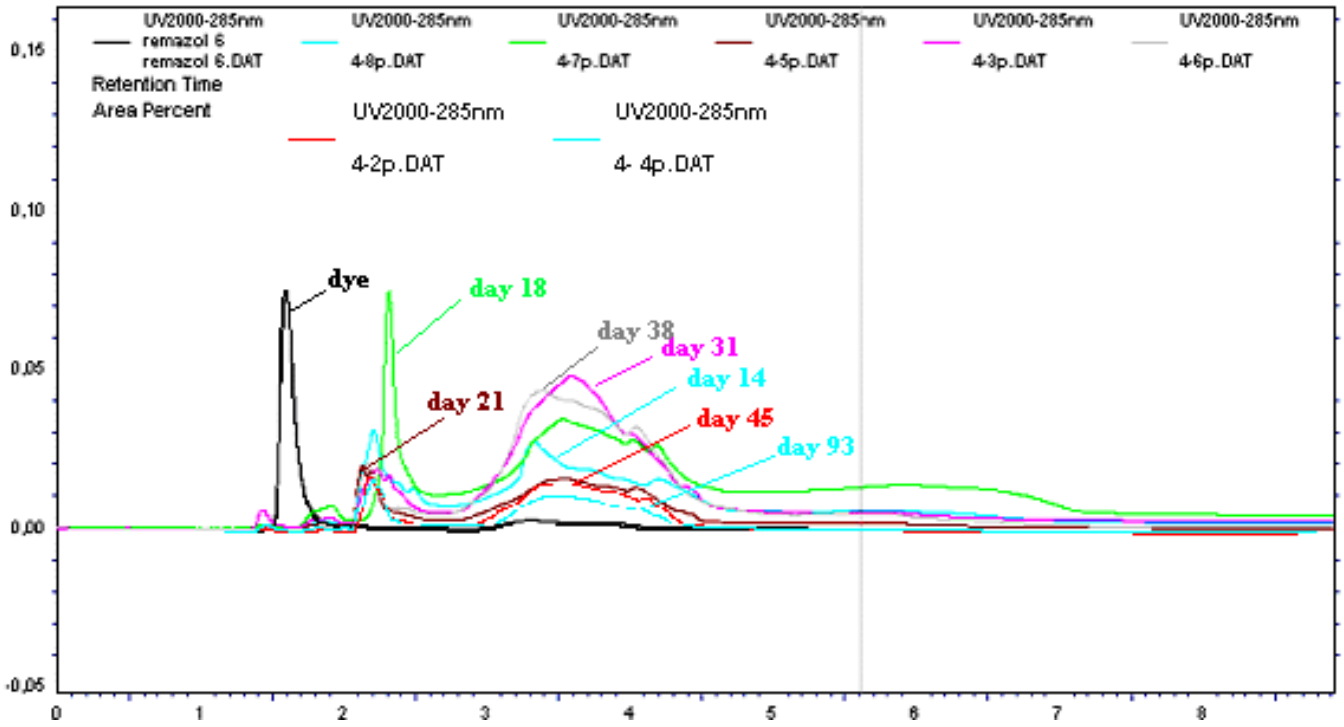


**Figure 5.** HPLC trace of the dye and the extracted metabolites from the MAT medium that contained dye and bacterium. Extracts of the MAT medium after; 14 days (blue), 18 days (green), 21 days (brown), 31 days (Purple), 38 days (violet), 45 days (red line), 93 day (blue), reactive red 195 dye (black).

The decolorisation of the dye in the MAT medium, which contained both glucose that was used as a first carbon source, and the dye, which is considered as a second carbon source, was found to be much faster and more effective (Figure 3). The use of simple carbon source and glucose as a co-substrate is considered as important as the pH has effect in the decolorisation and degradation of the reactive dyes. The addition of glucose has helped in the bacterial growth rate and consequently has increased the rate of decolorisation. These results (Table 1) agree very well with the results obtained by other researchers (Donlon et al., 1997; Bras et al., 2001; Ramalho et al., 2002; Mendez-Paz et al., 2005; Steffan et al., 2005; Jirasripongpun et al., 2007; Zhao and Hardin, 2007; Jadhav et al., 2008; Dawkar et al., 2009; Franciscon et al., 2009).

It is generally assumed that sulfonated azo dyes are not degraded under aerobic conditions (Anjaneyulu et al., 2005); nevertheless, there have been some reports which suggest the conversion of certain sulphonated azo dyes under aerobic conditions. Furthermore, certain carboxylated analogs of sulphonated azo compounds are utilized aerobically as a sole source of carbon and energy by specifically adapted bacteria (Kalyani et al., 2008, 2009).

Biodegradation processes involve anaerobic, aerobic, or sequential combination of the two. Anaerobic degradation of azo dyes is known to yield only azo reduction and decolorisation (Mc Mullan et al., 2001). In general, complete mineralization of azo dyes requires both anaerobic and aerobic bacterial processes. The sequential anaerobic/aerobic treatment processes based on mixed culture of bacteria are widely used because the degradation products that result from anaerobic reduction of azo dyes have to be degraded by aerobic processes (Melgoza et al., 2004; Husseiny 2008). However, as *R.p.* 51ATA can grow under both anaerobic and aerobic conditions, it is considered as ideal choice for this study.

In order to study, the biodegradation of the dye, FTIR analysis was carried out. In the FTIR spectra (Figure 4), the IR of the dye and the extracted degradation products (metabolites) obtained on the 21st, 45th and the 93rd days were compared. The disappearance of many peaks of the dye including the N=N peak at 1550 and the band in the 3328-3650 $cm^{-1}$ region that corresponded to the aromatic =C-H, O-H and N-H peaks was observed and a significant change in the position of the peaks was noticed. Gradually, all the aromatic stretching have disappeared from the spectrum and only the alicyclic C-H, the sulphide stretching of the sulphonated side chain and the strong C-Cl stretchings of the reactive red 195 were observed in the spectrum, thus indicating a complete mineralization of the aromatic ring system by the *R.p.* 51ATA strain.

HPLC analysis of the reactive red dye 195 was also carried out and the retention times of the individual

**Table 1.** Comparison of azo dye biodegradation results with results in literature.

| Azo Dye | Organism used | Dye Conc. | Incubation period | Co-substrate | Final product | References |
|---|---|---|---|---|---|---|
| Mordant orange 1 | Anaerobic granular sludge | 0.001-0.26 mM | 217 days | Glucose, volatile fatty acids | methane | Donlon et al., 1997 |
| DAPABS*,Methyl Orange (Orange 52), HNABSA*, Orange II (Acid orange 7) | *Candida zeylanoides* | 0.2 mM | 20 h | glucose | Aromatic amines | Ramalho et al., 2002 |
| Acid orange 7 | Non-adapted methanogenic granular sludge | 25-300 mg $l^{-1}$ | 35 days | Different cosubstrate (glucose, ex.) | Aromatic amines | Mendez-Paz et al., 2005 |
| Ethyl orange | Microbial consortium | 56.28 µM | 30 days | Glucose, starch | CM* | Steffan et al., 2005 |
| Reactive Red 195 | *Enterobacter* sp., *Serratia* sp., *Yersinia* sp., and *Erwinia* sp | 30 mg mg $l^{-1}$ | 2 days | Glucose, peptone | Aromatic amines | Jirasripongpun et al., 2007 |
| Disperse yellow 3, Disperse Orange 3 | *Pleurotus ostreatus* (fungus) | 200 mg $l^{-1}$ | 5 days | - | Aromatic amines | Zhao and Hardin, 2007 |
| Methyl red | *Galactomyces geotrichum* MTCC 1360 | 100 mg $l^{-1}$ | 1 min.-36 h | molasses | Aromatic amines | Jadhav et al., 2008 |
| Reactive red 141 | *Rhizobium radiobacter* MTCC8161 | 50 mg $l^{-1}$ | 48 h | Urea,Yeast extract | Aromatic amines | Telke et al., 2008 |
| Reactive black 5 | *Rhodopseudomonas palustris* W1 | 50-1000 mg $l^{-1}$ | 15 h | lactate | Aromatic amines | Wang et al., 2008 |
| Navy blue 2GL | *Bacillus* sp.VUS | 50-350 mg $l^{-1}$ | 18-48 h | $CaCl_2$ | Aromatic amines | Dawkar et al., 2009 |
| Reactive yellow 107, Reactive black 5, Reactive red 198 | *Klebsiella* sp. VN-31 | 100 mg $l^{-1}$ | 72 h,120 h, 96 h | Glucose, pyruvate | Aromatic amines | Franciscon et al., 2009 |
| Direct blue 71 | *Klebsiella* sp. VN-31 | 100 mg $l^{-1}$ | 168 h | Glucose, Pyruvate | ND* | Franciscon et al 2009 |
| Reactive red 2 | *Pseudomonas* sp. SUK1 | 100-300 mg $l^{-1}$ | 24 h | - | Aromatic amines | Kalyani et al 2009 |
| Reactive Red 195 | R.p. 51ATA | 100 µg $l^{-1}$ | 93 days | Glucose | CM* | This study |

*CM, Completely mineralized; ND, Not detected; DAPABS,: m-[(4-Dimethylamino) phenylazo] benzenesulfonic acid, sodium Salt; HNABSA, c m-[(2-Hydroxy-1-napthyl) azo] benzenesulfonic acid, sodium salt.

metabolites obtained on the 14th, 18th, 21st, 31st, 38th, 45th and 93rd days were compared with the dye retention time. The co-injection of these metabolites with the dye showed the dis-appearance of the dye implying its mineralization from the solution as it is shown in Figure 5.

## Conclusion

Generally, azo dye mineralization requires both anaerobic and aerobic bacterial processes. Since decolorization does not imply mineralization of the aromatic amines, a complete removal of these amines is required. As *Rhodopsuedomonas* sp. growth is better anaerobically in the light, we have made use of this property in the decolorization, degradation and mineralization of the sulphonated reactive red 195 dye. However, up to date and to the best of our knowledge, no single bacterium has been reported to being used in the complete decolorization, degradation or mineralization of the sulphonated azo dyes. The results obtained from this study showed that:

*1. Rhodopseudomonas palustris* 51ATA strain is an excellent bacterium for the removal of reactive red 195 dye.

2. The presence of dye has increased the rate of the bacterial growth, while the presence of the co-substrate has increased the rate of decolorisation and degradation by increasing the bacterial growth

3. The addition of co-substrate to the medium, affects biodegradation in a remarkable way.

4. Degradation is more effective when the duration of treatment with bacterium is longer than

a month.

Degradation is more effective when either the concentration of dye is low or the concentration of bacterium is high.

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