Decolorization and Degradation of C. I. Reactive Red 195 by *Enterobacter* sp.

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Abstract

Four selected facultative bacteria of *Enterobacter* sp., *Serratia* sp., *Yersinia* sp., and *Erwinia* sp. were investigated for their ability to degrade C. I. Reactive Red 195 in solid and liquid dye medium. Their decolorizations in liquid medium under anaerobic conditions were superior to the aerobic conditions. The best decolorizer, removing dye up to 90%, within 2 days of incubation, was *Enterobacter* sp. The dye degradability of *Enterobacter* sp. was evaluated by the decrease of dye concentration under various conditions. Its decolorizing activity was highly dependent upon the dye concentration, the presence or absence of glucose and/or peptone and pH. Shaking/static and dark/light conditions had no discernable effects on the decolorization. Its degrading activity expressed by the presence of dye metabolites or aromatic amines was analyzed by UV-visible spectrophotometry at 200-400 nm. It was demonstrated that the dye removal by *Enterobacter* sp. was only partial and that it was associated with dye adsorption to cell mass and decolorization rather than further complete biodegradation of the aromatic amines. As some aromatic amines are carcinogenic, selecting an appropriate treatment for complete degradation of azo dyes is an essential component in making environmentally safe water.

Keywords: Azo dyes; *Enterobacter* sp; Decolorization; Anaerobic condition; Aerobic condition; Aromatic amines; Reactive Red 195

1. Introduction

The reactive azo dyes are characterized by having one or more azo bonds (-N=N-), which are bound to substituted aromatic rings. The substituted rings of these dyes are responsible for intense color, water solubility and resistance to degradation under conventional wastewater treatment [1]. Azo dyes are the largest group of synthetic dyes, and 10-15% of these dyes are usually lost in the effluent during synthesis and dyeing processes [2]. The release of azo dyes into the environment has become a major concern in wastewater treatment, since some azo dyes and their metabolites may be mutagens or carcinogens [3, 4]. Many physical and chemical methods, including adsorption, photo-degradation, precipitation, filtration and oxidation, have been used for the treatment of dye-containing effluent. Currently, microbial biodegradation has become a promising approach for dye treatment because it is cheaper, effective and more environmentally friendly.

Many microorganisms are able to decolorize azo dyes aerobically and anaerobically [5]. Degradation of azo dye generally needs two steps involving 1) decolorizing of azo dye by the reduction of azo bonds and 2) degradation of azo dye metabolites or aromatic amines [6]. Bacteria usually degrade azo dyes under anaerobic conditions to colorless toxic aromatic amines, of which, some are readily metabolized under aerobic conditions [7]. In this study, four strains of bacteria isolated from waste disposal sites of textile processing units, were identified according to Bergey's Manual of Determinative Bacteriology [8]. Each strain and mixed cultures of the four

strains were tested for decolorization activity under aerobic and anaerobic conditions using C. I. Reactive Red 195 as a dye model. Then the best decolorizer was studied under various conditions for decolorization such as glucose and peptone concentration, shaking/static, dark/light and initial pH in order to gain information on the efficiency of dye removal.

2. Materials and methods

2.1 Microorganisms and the dye

Four bacterial strains (azo1, azo2, azo3 and azo4) used in this study were isolated from wastewater at the waste disposal sites of textile processing units of Raja-uchino Co., Ltd. Chonburi province. They were identified by biochemical tests according to Bergey's Manual [8]. Aliquots of four pure cultures were kept as seed stocks at -70 °C in nutrient broth containing 30 mgl^{-1} of Reactive Red 195 and 10 % glycerol.

The azo dye Reactive Red 195 was obtained from Gopal Chemicals., Ahmedabad, India. It is commonly used in textile processing. The dye has maximum absorbance (λ_{max}) at 540 nm and its chemical structure is shown in Fig. 1.

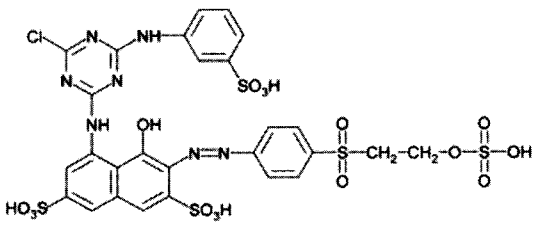

Fig 1. Chemical structure of C.I. Reactive Red 195 used in biodegradation experiments.

2.2 Decolorizer selection under aerobic/anaerobic conditions

Pure cultures of the four strains were recovered from -70 °C freezer and were grown under aerobic conditions in 100 ml of nutrient broth containing beef extract 30g l^{-1}, peptone 50 g l^{-1}, and Reactive Red 195 at 30 mg l^{-1} under shaking at 150 rpm at 37 °C. For the decolorization tests, 1 ml of 24 h cultures ($3.6x10^9$ CFU ml^{-1}) was inoculated in 100 ml of liquid medium (mineral salt medium containing $(NH_4)_2SO_4$ 0.5 g l^{-1} KH_2PO_4 2.66 g l^{-1}, Na_2HPO_4 4.32 g l^{-1}, glucose 1 g l^{-1} and Reactive Red at 30 mg l^{-1}) in order to simulate the waste water in the textile industry. The cultures were incubated for 7 days under either aerobic or anaerobic conditions. For anaerobic conditions, the liquid flasks were gassed with oxygen-free nitrogen gas through the rubber stopper using hypodermic needles after each sampling. Decolorization assay was also observed in agar dye plates, and anaerobic conditions were established using anaerobic jars.

2.3 Decolorization in different culture conditions of the selected decolorizer

The effects of various culture conditions such as glucose concentration (1, 5, 10 g l^{-1}), peptone concentration (1, 2 g l^{-1}), pH (at 7, 8, 9 and 10), agitation (at 150 rpm and static), and dark/light state (covered with foil and uncovered) were also examined similarly in anaerobic liquid medium after a 2 day incubation. All the experiments were performed in triplicate. The un-inoculated control was also incubated under the same conditions to check for abiotic decolorization.

2.4 Color reduction measurements

Decolorization in dye agar plates was observed by fading of dye medium surrounding the bacterial colonies, compared with the original dye medium as a control. For liquid medium, the triplicate samples of the culture broth were analyzed by a spectrometer at λ_{max} 540 nm using the supernatant from the culture medium after centrifugation at 8000 g for 10 minutes. The culture broths were collected for decolorization measurement after two days of incubation except for the selection study, that the samples were taken almost every day. The color removal was reported as percent decolorization and was expressed as:

$$\text{Decolorization (\%)} = (A_0-A_t)\,x100/A_0$$

where A_0 and A_t are absorbency of the dye solution initially and at time (t), respectively.

Decolorization of the dye was also monitored over the range 200 – 400 nm using a scanning UV-visible spectrophotometer (Spectronic Instruments, Inc, USA) to determine azo dye metabolites or aromatic amines degradation.

3. Results and discussion

3.1 Identification of the isolated microorganisms

All pure cultures of azo1, azo2, azo3 and azo4, isolated after 2 times successive enrichment in dye broth containing Reactive Red 195 at 30 mg l^{-1}, grew to white colonies when plated on nutrient agar without dye. However, the colonies were pink on the dye agar plate, and the agar medium became colorless, especially where colonies were dense. The pink colonies were observed after one day of incubation and this suggested the adsorbing of dye into cell mass. The colorless dye media after 7 days incubation also demonstrated the decolorization activity of the tested microorganisms, which could possibly be associated with either bioadsorption or biodegradation, or both. The biochemical and physical profiles revealed their identities to be *Enterobacter* sp., *Serratia* sp., *Yersinia* sp., and *Erwinia* sp. as shown in Table 1. They are facultative bacteria which made it feasible for degradation of Reactive Red 195 under both aerobic and anaerobic condition.

Table 1 Biochemical and physical profiles of the four isolated strains

Characteristics	*Enterobacter* sp. (azo1)	*Serratia* sp. (azo2)	*Yersinia* sp. (azo3)	*Erwinia* sp. (azo4)
Morphology/Motility	Rod/+	Rod/+	Rod/-	Rod/+
Gram staining	-	-	-	-
Catalase	+	+	+	+
Oxidase	-	-	-	-
Oxidative -fermentative	+	+	+	+
Nitrate reduction	+	+	+	-
Urease	-	-	+	-
Indol	-	-	+	+
Methyl red	+	+	+	+
Acetyl methyl carbinol	+	+	+	+
Citrate	+	+	-	+
Amylase	+	+	+	+
H_2S production	-	-	-	+
Gelatinase	-	+	-	+

3.2 Screening for the best decolorizing bacteria

Experiments on growth and dye removal ability of the pure strains on dye agar plates under aerobic and anaerobic conditions revealed that *Erwinia* sp. (azo4) and *Enterobacter* sp. (azo1) grew rapidly with more visible colonies than *Yersinia* sp. (azo3) and *Serratia* sp. (azo2) (data not shown). Decolorization of the dye under anaerobic conditions could be observed clearly within a week, while under aerobic conditions took 2 weeks. Complete fading of dye was clearly seen where colonies were numerous. However, rate of decolorization could not be observed clearly in this solid medium.

Liquid culture in flasks under both aerobic and anaerobic condition provided the results shown in Fig 2 and Fig 3, respectively. We observed that all bacterial strains grew rapidly under aerobic conditions and entered the stationary phase within 5 days, at which *Erwinia* sp. azo4 was the best growing bacteria (data not shown). All these strains under aerobic conditions exhibited a similar pattern of decolorization with initially rising and then

falling before reaching the stationary phase at about 50-60 % decolorization within 6-7 days of incubation (Fig. 2).

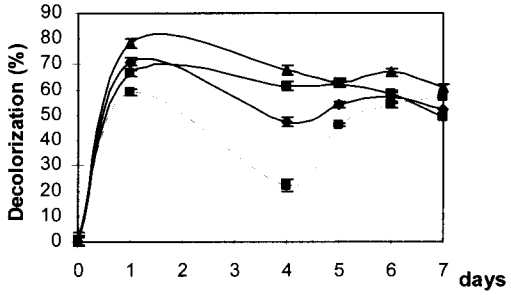

Fig 2. Decolorization of C.I. Reactive Red 195 (30mgl^{-1}) by azo1 (—◆—) azo2 (—▲—) azo3 (—■—) and azo4 (- - -■- - -) in liquid medium under aerobic conditions at 37°C.

For anaerobic conditions, the pure and mixed-strain cultures grew more slowly than that of the aerobic conditions and they reached high cell density within 7 days. The dye concentration in each bacterial culture except azo 4 rapidly decreased to a constant level within 2-3 days of incubation (Fig. 3). The percent decolorization by culture of *Enterobacter* sp. azo1 (91%), *Yersinia* sp. azo3 (87%) and mixed cultures of the four strains (71%) was higher than by pure cultures of *Serratia* sp. azo2 (46%) and *Erwinia* sp. azo4 (5%). Thus, the dye was decolorized more rapidly under anaerobic conditions than aerobic conditions. This finding was consistent with Chen et al. [9]. These bacteria may decolorize the dye under reduction either by electron transport from the cellular metabolic pathways of flavins or quinines, or by external redox mediators as well as by azoreductase enzymes [6].

Therefore, *Enterobacter* sp. azo1 was the best decolorizer among all the tested cultures. It may remove dye by initial adsorption together with decolorizing by electron transport under aerobic conditions and by azoreductase enzymes under anaerobic conditions. However, this bacterial strain was unable to grow in higher dye concentration as it was dead when we used dye concentrations at 50 and 100 mg l^{-1} to test its decolorizing activity. Probably Reactive Red 195 was toxic to the cells at high dye concentration.

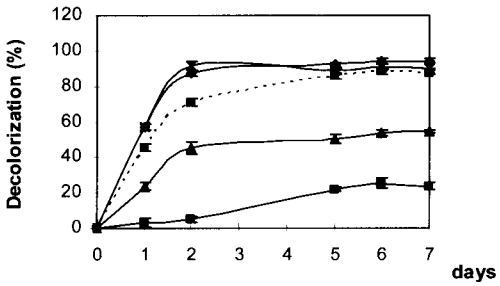

Fig 3. Decolorization of C.I. Reactive Red 195 (30mgl^{-1}) by azo1 (—◆—) azo 2(—▲—) azo3 (—✱—) azo4 (—■—) and mixed culture (- - -■- - -) in liquid medium under anaerobic conditions at 37°C.

3.3 Conditions for decolorization

The effects of nutrient supplementation of either glucose or peptone on decolorizing activity varied from enhancement [2, 9, 10] to inhibition [9, 11]. In this study the optimal glucose concentration for decolorization was at 5 g l^{-1} while higher concentrations of glucose and peptone inhibited decolorizing activity (Table 2). Probably, glucose at 5 g l^{-1} generated more redox mediators that acted as electron donors for the reduction of azo bonds [10, 12].

Table 2. Effect of glucose and peptone concentration on decolorization of Reactive Red 195 by *Enterobacter* sp. azo1 at 37°C under anaerobic conditions.

Glucose and peptone concentration (g l^{-1})	Average decolorization (%)
Glucose 1 g l^{-1}	68
Glucose 5 g l^{-1}	93
Glucose 10 g l^{-1}	84
Glucose 5 g l^{-1} + peptone 1 g l^{-1}	91
Glucose 5 g l^{-1} + peptone 2 g l^{-1}	80
Glucose 10 g l^{-1} + peptone 1 g l^{-1}	61
Glucose 10 g l^{-1} + peptone 2 g l^{-1}	48

Chen et al. [9] reported that metabolism of organic and inorganic nitrogen sources also regenerated NADH or redox mediators for decolorization by *Aeromonas hydrophilia*. In our study, we found no decolorization in the absence of glucose and peptone. This implied

that *Enterobacter* sp. azo1 could not metabolize the dye as sole carbon and energy sources. It needed either glucose or peptone as co-substrate for decolorization. We obtained a imilar level of decolorization in culture when peptone alone at 1g l^{-1} was used, or when glucose was alone. However, the decolorization was decreased when more peptone was added in the presence of glucose. The decolorization inhibition from peptone in the presence of glucose may possibly result from nitrate or nitrite, a reducing equivalent that cells generated from peptone consumption. These metabolites of nitrate/nitrite may compete with the azo dye and result in less decolorization. Therefore, *Enterobacter* sp. azo1 cannot use dye as the sole carbon and energy source. It was able to decolorize dye only in the presence of either glucose or peptone, and peptone would inhibit decolorization if it was used together with glucose.

An optimal pH for decolorization by *Enterobacter* sp. azo1 was selected from the range of 7-10 and compared with unadjusted (pH 7.14) conditions. We found that decolorization was highest at pH 7 after which (pH 8-9) the activity decreased (Table 3). Probably pH 7 was optimal for growth and function of *Enterobacter* sp. azo1. However, culture at pH 10 resulted in slight and significantly higher decolorization than at pH 8 and 9, respectively. It is possible that pH 10 was detrimental to the bacteria and caused release of enzymes or redox mediators to cause dye reduction, or the dye may be reduced by alkaline hydrolysis.

Table 3. Effect of pH on decolorization of Reactive Red 195 by *Enterobacter* sp. azo1 at 37 °C under anaerobic conditions (mean ± S.D.).

Initial pH	7	7.14	8	9	10
% decolo-orization	93±3.51	75±2.36	19±4.02	2±2.53	23±1.33

Shaking/static and light/dark conditions were also studied. We found that shaking at 150 rpm and light exposure did not result in any different decolorization from static and dark conditions (data not shown). This may possibly imply that the flow rate of the effluent and sunlight upon the wastewater treatment pond could likely not effect dye removal. However, several factors in wastewater from the textile industry could make conditions of wastewater treatment ponds different from that of the laboratory study, and so study on the site of the wastewater treatment pond may lead to more conclusive results.

Dye degradation by *Enterobacter* sp. azo1 was analyzed successively under anaerobic and then after aerobic conditions. It was analyzed after 2 days anaerobic incubation and then once again after 5 days aerobic incubation. The spectrum of Reactive Red 195 (at 540 nm) before and after 2 days' anaerobic cultivation, is shown in Fig. 4.

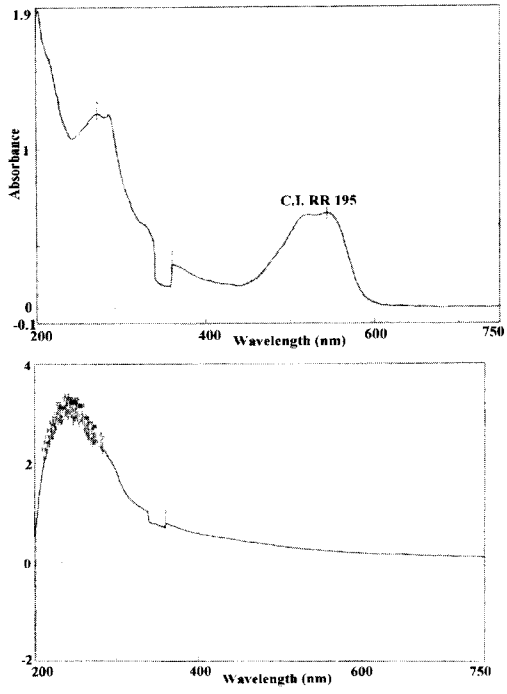

Fig 4. UV-visible spectra of Reactive Red 195 before (top) and after (bottom) 2 day treatment with *Enterobacter* sp. azo1

It revealed that *Enterobacter* sp. azo1 decolorized Reactive Red 195 to colorless products; an absorbance peak of Reactive Red 195 (at 540 nm) disappeared and there were numerous new peaks in the range of 200-300 nm. These peaks were likely to be azo dye metabolites or carcinogenic aromatic amines [3] as there were no such peaks in the culture medium without dye under similar incubating

conditions. Further incubation under aerobic conditions of the decolorized culture revealed that only a few peaks disappeared (data not shown). This implied that *Enterobacter* sp. azo1 could not degrade azo dye metabolites in the UV range completely. Therefore, color removal by *Enterobacter* sp. azo1 was largely attributed to bioadsorption and decolorization with slight degradation of the azo dye metabolites. The remaining aromatic amines azo dye metabolites may bring risk to the environment and degradation of these colorless toxic aromatic amines should be emphasised for the next study in order to ensure the safe release of wastewater contaminated dyes.

4. Conclusion

Enterobacter sp. azo1 was the best decolorizer among all the tested bacteria, with the ability to decolorize dye up to 90% of Reactive Red 195 within 2 days of anaerobic incubation. The decolorization mechanism of *Enterobacter* sp. azo1 involved dye adsorption and dye reduction. Its decolorization depends on dye concentration, glucose, and pH of the dye medium. Decolorization with slight biodegradation of aromatic amines could bring risk and thus degradation of dye metabolites should be monitored for the safe release of waste water.

5. Acknowledgements

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