Decolorization of Reactive Black 39 and Acid Red 360 by *Pseudomonas aeruginosa*

Balci Behzat

ABSTRACT

The aim of this work is to evaluate decolorization of Reactive Black 39 (RB39) and Acid Red 360 (AR360) by *Pseudomonas aeruginosa*, which was isolated from a non-dye-contaminated activated sludge biomass. In the present study, the effect of various physicochemical parameters, initial dye concentration, temperature, pH, inoculum size and yeast extract concentration as an organic source on decolorization were investigated. *P. aeruginosa* was able to decolorize 20 mg/L RB39 completely within 144 hours in the presence of 0.5 g/L yeast extract at 25 °C. Decolorization efficiencies for AR360 were found to be higher than RB39 under the same conditions. Optimal temperature to decolorize RB39 and AR360 was found to be 30 and 25 °C, respectively. The activation energy (E_a) values for decolorization of RB39 and AR360 were found to be 61.89 kJ/mol and 81.18 kJ/mol, respectively. Experience showed that the pH and inoculum size had a considerable effect on decolorization of RB39 and AR360 by *P. aeruginosa*.

Key words | Acid Red 360, decolorization, *Pseudomonas aeruginosa*, Reactive Black 39

Balci Behzat
Department of Environmental Engineering,
Cukurova University,
01136, Balcali,
Saricam,
Adana,
Turkey
E-mail: *behzatbalci@gmail.com*

INTRODUCTION

Dyes usually have a synthetic origin and complex aromatic structures. These synthetic dyes are widely used in tanneries, and in textile, food, pharmaceutical, cosmetics and electroplating factories (Saban 2011). These industrial processes can discharge wastewaters containing dye into water systems (Mohan *et al.* 2002). The presence of the dyes in water systems reduces light penetration into deeper layers, lowering the gas solubility, diminishing photosynthetic activity and reducing the water quality (Myslak & Bolt 1988). Reactive dyes represent the most widely used dyes in the textile industry due to their bright colors, colorfastness and simple and low-cost application processes (Al-Degs *et al.* 2008; Ahmad & Alrozi 2011). A large number of reactive dyes are azo compounds (Raymound & Dunald 1984). Azo dyes are aromatic compounds with one or more –N = N– groups (Saban 2011). Conventional aerobic biological wastewater treatment systems usually cannot efficiently decolorize wastewaters containing dye due to the strong electron-withdrawing group (Nigam *et al.* 1996). Although dyes can be expected to be resistant to biological degradation because of their azoic structures, it has been reported that many bacteria and fungi can degrade numerous dyes under certain conditions (Yoo *et al.* 2000; Chang *et al.* 2001a; Stolz 2001; Hu 2001; Asad *et al.* 2007; Kapdan & Erten 2007; Almeida & Corso 2014; Kumari *et al.* 2015).

Many studies have focused on the decolorization potential of dyes by acclimatized microorganisms, which isolate dye from contaminated areas such as soil and wastewater treatment plants (Alhassani *et al.* 2007; Wang *et al.* 2009).

The main objective of this study was to observe potential decolorization of RB39 and AR360 by non-acclimatized *Pseudomonas aeruginosa*, which was isolated from non-dye contaminated activated sludge. There is no literature on decolorization of RB39 and AR360 by *P. aeruginosa*. Thus the data from this study may contribute to the literature on decolorization of dyes by pure bacterial cultures.

MATERIALS AND METHODS

Dye

The dyes used in this study are RB39 and AR360, obtained from the local textile industry in Turkey. The maximum absorbance wavelengths of RB39 and AR360 are 611 nm and 531 nm, respectively. AR360 (CAS No: 61968-06-7)

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and RB39 (CAS No: 68259-02-9) are azoic structure dyes, which are mainly used for polyamide and wool dying (Lacasse & Baumann 2004).

Isolation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was isolated from an aeration basin of activated sludge at a soft drinks factory located in Adana, Turkey. The isolation of *P. aeruginosa* was conducted in three main stages. Cetrimide agar, which is a type of agar for selective isolation of *P. aeruginosa*, was used in the first stage of isolation. Cetrimide agar is selective for *P. aeruginosa*, however other bacterial species may develop (Cholley *et al.* 2008). In the second stage, a single colony growth on cetrimide agar at 42 °C was transferred to nutrient agar under sterile conditions. Thus a single species of bacterial culture was obtained. In the third stage, 16S rDNA gene was amplified using PA-SS-F GGGGGATCTTCG-GACCTCA and PA-SS-R TCCTTAGAGTGCCCACCCG primers for the single bacterial colony (Spilker *et al.* 2003). The pure culture of *P. aeruginosa* was transferred to a 500 mL Erlenmeyer flask containing 250 mL nutrient broth and incubated at 35 ± 2 °C.

Decolorization experiments

All decolorization experiments were conducted three times in static conditions and the average values were used for calculations. Effect of initial dye concentration (20, 40, 60, 80 and 100 mg/L), yeast extract concentration (0.1, 0.5, 1, 2 and 4 g/L), temperature (10, 15, 20, 25, 30, 35, 40 and 45 °C), pH (3, 4, 5, 6, 7, 8, 9 and 10) and bacteria inoculum size (1, 2, 3 and 4 mL) on decolorization was studied. Tests on the effect of yeast extract concentration, temperature, pH and bacteria inoculum size were performed with 100 mg/L RB39 and AR360. The composition of the basal mineral medium was (g/L): K_2HPO_4 4.35, KH_2PO_4 1.7, NH_4Cl 2.1, $MgSO_4$ 0.2, $MnSO_4$ 0.05, $FeSO_4{\cdot}7H_2O$ 0.01, $CaCl_2{\cdot}2H_2O$ 0.03 (Lobos *et al.* 1992). Microbial culture at nutrient broth was centrifuged at 5,000 rpm for 30 minutes, the supernatant was withdrawn and microbial culture was washed three times with sterile distilled water to remove the nutrient residues. Microbial cultures were inoculated in 250 mL Erlenmeyer flasks including 120 mL basal mineral medium containing dye and yeast extract. The amount of initial bacteria of solutions with 1 mL inoculum size was approximately 5.5×10^6 CFU/mL. Tests on the effect of dye and yeast extract concentration, temperature and pH were performed with 1 mL inoculum size. Glass materials and solutions were autoclaved at 121 °C, dyes were made sterile by using a sterile filter with 0.45 μm pore size. Bacterial culture was autoclaved at 121 °C for abiotic decolorization experiments. Thus biotic and abiotic decolorizations were compared. Abiotic decolorization experiments were conducted under the same conditions as biotic experiments. Controls without culture and autoclaved culture were also conducted. Samples were collected at intervals of 24 hours for all decolorization experiments.

Analytical methods

Dye concentration of supernatant was estimated by measuring absorbance at maximum wavelengths (RB39 = 611 nm, AR360 = 531 nm) and computing concentration from the calibration curve. The calibration curves were prepared with a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. Concentrations of calibration curves ranged between 2.5 and 25 mg/L. The calibration checks were carried out in duplicate. Growth of microorganisms was determined by the plate count agar (PCA).

Statistical analysis

Statistical evaluations were performed using SPSS Statistics 20.0 with confidence interval of 95% ($P \leq 0.05$).

RESULTS AND DISCUSSION

Effect of dye concentration on decolorization

Concentrations of azo dyes have an important effect on the decolorization process. High dye concentrations may negatively affect the decolorization efficiency due to the potential toxicity on microorganisms (Singh 2012). Decolorization efficiencies for AR360 were found to be higher than RB39 under the same conditions. Beside decolorization efficiencies, decolorization rate (mg/L/h) was calculated to understand the effect of initial dye concentration on decolorization, because decolorization rate may be increased with increasing initial dye concentration (Cruz & Buitrón 2001). Although decolorization efficiencies decreased with increasing dye concentrations, the decolorization rate increased with increasing dye concentrations. Decolorization rates and decolorization efficiencies were calculated using Equations (1) and (2), respectively.

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Decolorization efficiencies and rates for initial dye concentrations are given in Table 1. There was no inhibitory effect caused by the studied dye concentrations. Pre-trials showed that it was hard to determine the effect of initial dye concentration with high yeast extract concentrations (1, 2, 4 g/L) due to the high decolorization efficiencies. So effect of initial dye concentration was performed with 0.5 g/L yeast extract. Effect of initial dye concentrations on decolorization with 0.5 g/L yeast extract at 25 °C is shown in Figure 1. Abiotic decolorization of dyes by dead culture was found negligible for all dye concentrations at 25 °C. Biotic and abiotic experiments indicated that biotic processes are the main decolorization mechanism of RB39 and AR360 by *P. aeruginosa*. There was no dye decrease in control groups. Some pure culture decolorization studies used far higher dye concentrations than this study such as 500 and 1,000 mg/L (Wang *et al.* 2009; Tripathi & Srivastava 2011; Kumari *et al.* 2015). However, dye levels in wastewater and aquatic environments are lower than these high concentrations. Dye concentrations in textile wastewater generally range between 10 and 200 mg/L (Singh 2014).

Table 1 | Decolorization efficiencies and rates for initial dye concentrations with 0.5 g/L yeast extract at 25 °C within 144 hours

	Decolorization efficiency (%)		Decolorization rate (mg/L/h)	
Dye conc. (mg/L)	RB39	AR360	RB39	AR360
20	100	100	0.139	0.22
40	61.5	100	0.177	0.319
60	58.3	80	0.235	0.342
80	55	65	0.317	0.352
100	53	56	0.376	0.378

$$\text{Decolorization rate} = \Delta C_t/\Delta t \quad (1)$$

$$\text{Decolorization efficiency} = \frac{C_t - C_0}{C_0} \times 100 \quad (2)$$

where ΔC_t (mg/L) is the change in dye concentration over the time interval Δt (h), C_0 is the initial dye concentration (mg/L).

Effect of yeast extract on decolorization

Decolorizing of RB39 and AR360 increased with increasing yeast concentration from 0.1 to 4 g/L. AR360 was completely removed with 4 g/L yeast extract within 96 hours. Figure 2 shows the decolorization of RB39 and AR360 at different yeast extract concentrations. In many reports, several azo dyes were decolorized under static conditions by many bacterial strains with complex carbon and nitrogen sources such as peptone, yeast extract and glucose (Nigam *et al.* 1996; Yu *et al.* 2001; Chen *et al.* 2003). Some studies investigated the effect of different nitrogen sources on decolorization of azo dyes and reported that the best decolorization was achieved with the yeast extract. They also reported that the decolorization of azo dyes increased with the increase in yeast extract concentration (Chang *et al.* 2001b; Dong *et al.* 2003; Moosvi *et al.* 2005; Mahmood *et al.* 2011). The metabolism of yeast extract is thought to be important for the regeneration of NADH, which behaves as an electron donor during the reduction of azo bonds (Craliell *et al.* 1995). The concentration of

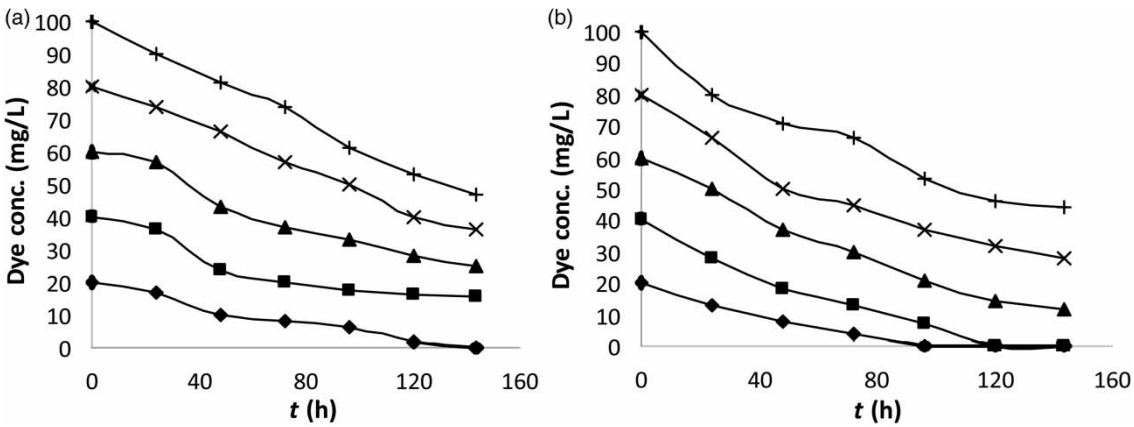


Figure 1 | Effect of initial dye concentrations for RB39 (a) and AR360 (b) on decolorization with 0.5 g/L yeast extract at 25 °C (20 mg/L (◆), 40 mg/L (■), 60 mg/L (▲), 80 mg/L (×), 100 mg/L (+), (RB39 $P < 0.05$, AR360 $P < 0.05$)).

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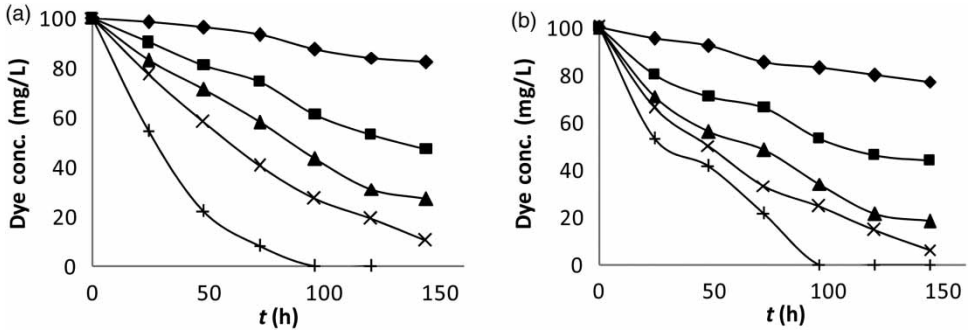


Figure 2 | Effect of yeast extract concentration on decolorization of RB39 (a) and AR360 (b) at 25 °C (0.1 g/L (♦), 0.5 g/L (■), 1 g/L (▲), 2 g/L (×), 4 g/L yeast ext. (+), (RB39 $P < 0.05$, AR360 $P < 0.05$)).

yeast extract has a direct effect on the culture concentration and decolorization increased with increasing culture concentration. While bacterial culture reached 18.6×10^6 CFU with 1 g/L yeast extract and 100 mg/L dye, it reached 27.3×10^6 CFU with 4 g/L yeast extract and 100 mg/L dye within 144 hours at 25 °C. In many studies, carbon and nitrogen sources were used at high concentrations, such as nutrient broth containing 5 g/L peptone and 3 g/L meat extract for decolorization of azo dyes by pure cultures (Alhassani *et al.* 2007; Kalme *et al.* 2007; Ghodake *et al.* 2009). Thus they obtained high decolorization efficiencies in a shorter time than this study. However, these high nutrient concentrations may cause potential negative effects such as high COD and high cost in the possible application of microorganisms for the decolorization of wastewaters containing dye.

Cell growth with different yeast extract concentrations

After inoculation of the cells to the RB39, AR360 and yeast extract mediums it was determined that 12–15 hours were required for the organisms to acclimate to their new environment in the lag phase. During the logarithmic growth phase with 1, 2 and 4 g/L yeast extract, a significant decolorization occurred for 100 mg/L RB39 and AR360. Also, dissolved oxygen dropped to nearly 0.25 mg/L at the logarithmic growth phase. This result indicates that effective decolorization of RB39 and AR360 by *P. aeruginosa* occurs under low dissolved oxygen levels. Decolorization of azo dyes by bacterial cells is initiated by cleavage of azo bonds by azo reductase (Zimmermann *et al.* 1982). The presence of oxygen normally inhibits the activity of azo reductase. NADH acts as the electron donor for the reduction of azo bonds and aerobic respiration prevents the electron transfer from NADH to azo bonds (Chung & Stevens 1993; Chang *et al.* 2001b). Decolorization of 100 mg/L RB39 and cell growth at different yeast concentrations is given in Figure 3.

Effect of temperature on decolorization

The temperature of the environment directly affects bacterial temperature. Microorganisms adapt to the temperature changes by biochemical or enzymatic mechanisms. Eventually, temperature has a considerable effect on microbial processes such as water remediation (Saratale *et al.* 2011). Testing of the influence of temperature on decolorization was performed in the temperature range of 10, 15, 20, 25, 30, 35, 40 and 45 °C. Decolorizing efficiencies of RB39 and AR360 increased with increasing temperature from 10 to 25 °C. Decolorization efficiencies of 100 mg/L RB39 and AR360 at 10 °C were found to be 36 and 44%, respectively, with 2 g/L yeast extract within 144 hours. AR360 was completely removed at 25, 30 and 35 °C. RB39 was completely removed at 30 and 35 °C. Decolorization efficiencies significantly dropped when the temperature increased to 45 °C. Optimal temperature to decolorize RB39 and AR360 was found to be 30 °C and 25 °C, respectively. Decolorization efficiencies of 100 mg/L RB39 and AR360 at different temperatures are given in Figure 4.

Reaction kinetics at different temperatures

To show the reaction rates for different temperatures, data were used in the zero-, first- and second-order reaction equations. The linear equations of zero, first and second order are given as Equations (3), (4) and (5), respectively.

$$C_T = C_0 - k_0 t \quad (3)$$

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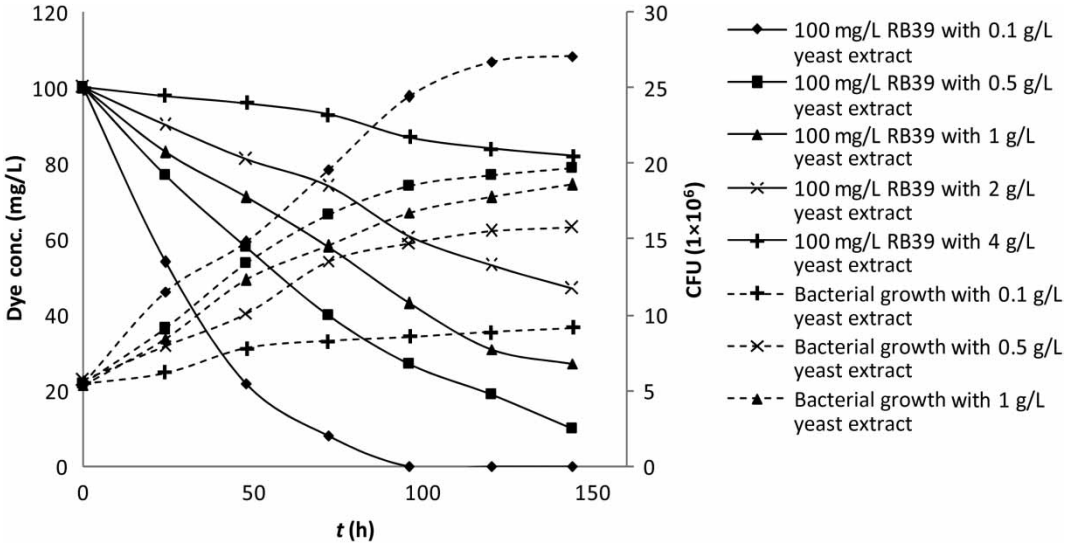


Figure 3 | Decolorization of 100 mg/L RB39 and cell growth at different yeast concentrations at 25 °C.

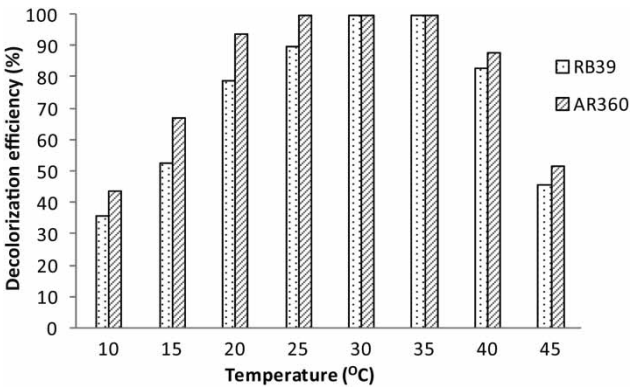


Figure 4 | Effect of temperature on decolorization of 100 mg/L RB39 and AR360 with 2 g/L yeast extract within 144 hours (RB39 $P < 0.05$, AR360 $P < 0.05$).

where C_T is the residual dye concentration (mg/L), C_0 is the initial dye concentration (mg/L), k_0 (mg/L/h) is the rate constant of zero-order reaction kinetic. k_0 can be obtained from the plot of C_T vs t.

$$\ln C_T = \ln C_0 - k_1 t \tag{4}$$

where k_1 is the (1/h) rate constant of the first-order reaction kinetic. k_1 can be obtained from the plot of $\ln C_T$ vs t.

$$\frac{1}{C_T} = \frac{1}{C_0} + k_2 t \tag{5}$$

where k_2 is the (L/mg/h) rate constant of the first-order reaction kinetic. k_2 can be obtained from the plot of $1/C_T$ vs t.

Kinetic constants at different temperatures that depend on the zero, first and secondorder for decolorization of RB39 and AR360 by *P. aeruginosa* are provided in Table 2. As shown in this table, the comparison of regression coefficients (R^2) relevant to zero-, first- and second-order rate constants (k_0, k_1 and k_2 values) showed that decolorization of studied dyes at different temperatures can be explained better by first-order reaction kinetics. First-order rate constants increased with increasing temperature from 10 to 35 °C. First order rate constants decreased over 35 °C temperatures. This can be explained by decreasing enzyme activity above 35 °C.

Activation parameters

From the rate constant k_1 (Table 2), the E_a for decolorization was determined using the Arrhenius equation (Dogan & Alkan 2003) The Arrhenius equation is given as Equation (6).

$$\ln k_1 = \ln A - \frac{E_a}{RT} \tag{6}$$

where T is the absolute temperature (K), R is the universal gas constant (8.314 J mol/K), A is a pre-exponential factor expressing the empirical dependence of the rate coefficient on temperature and E_a is the activation energy (kJ/mol). E_a can be calculated from the slope of the plot of $\ln k_1$ versus $1/T$ (Angelova *et al.* 2008). E_a values for decolorization of RB39 and AR360 were found to be 61.89 kJ/mol and 81.18 kJ/mol, respectively. The Arrhenius plot of $\ln k_1$

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Table 2 | Zero-, first- and second-order constants for decolorization of RB39 and AR360 at different temperatures

	Zero order				First order				Second order			
	RB39		AR360		RB39		AR360		RB39		AR360	
°C	k_0	R^2	k_0	R^2	k_1	R^2	k_1	R^2	k_2	R^2	k_2	R^2
10	0.254	0.944	0.297	0.936	0.003	0.986	0.004	0.965	0.001	0.985	0.001	0.97
15	0.37	0.966	0.462	0.954	0.005	0.996	0.007	0.992	0.002	0.99	0.003	0.984
20	0.555	0.983	0.628	0.943	0.011	0.992	0.009	0.91	0.006	0.92	0.029	0.839
25	0.62	0.965	0.801	0.853	0.014	0.994	0.033	0.988	0.013	0.804	0.073	0.83
30	0.811	0.952	0.815	0.978	0.022	0.983	0.038	0.999	0.018	0.826	0.05	0.879
35	0.92	0.91	1.362	0.841	0.023	0.997	0.062	0.981	0.03	0.788	0.095	0.832
40	0.577	0.97	0.638	0.959	0.015	0.986	0.015	0.986	0.005	0.858	0.011	0.878
45	0.328	0.985	0.37	0.99	0.005	0.994	0.005	0.994	0.001	0.953	0.001	0.977

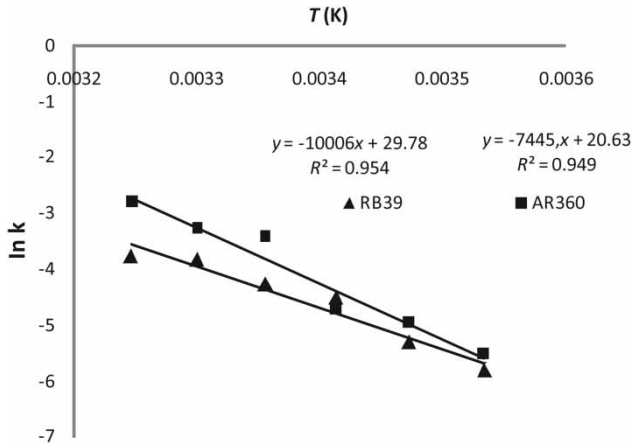


Figure 5 | The Arrhenius plot of decolorization of RB39 and AR360 by *P. aeruginosa*.

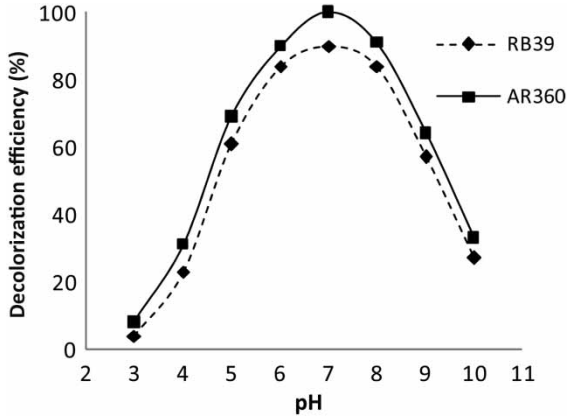


Figure 6 | Effect of pH on decolorization of 100 mg/L RB39 and AR360 with 2 g/L yeast extract within 144 hours (RB39 $P < 0.05$, AR360 $P < 0.05$).

against $1/T$ for the decolorization of RB39 and AR360 by *P. aeruginosa* is shown in Figure 5.

Effect of pH on decolorization

The effect of pH on decolorization was investigated in the pH ranges of 3, 4, 5, 6, 7, 8, 9, and 10. It was found that pH has an important effect on decolorization of RB39 and AR360 by *P. aeruginosa*. *P. aeruginosa* showed good growth at pH 6, 7 and 8, while pH 3, 4 and 10 were found to be inhibitory for the growth. Decolorization efficiencies of 100 mg/L RB39 and AR360 at pH 7 were found to be 90 and 100%, respectively with 2 g/L yeast extract at 25 °C within 144 hours. It was observed that there was no significant decolorization when the initial pH of the medium was below 5 and above 9. Optimal pH to decolorize RB39 and AR360 was found to be 7. Decolorization efficiencies of 100 mg/L RB39 and AR360 at different pH values are given in Figure 6.

Effect of inoculum size on decolorization

Tests on the effect of inoculum size on decolorization were performed by adding 1, 2, 3 and 4 mL of culture to dye containing solutions. It was found that initial amount of bacteria has a significant effect on decolorization. While 100 mg/L RB39 was 90% decolorized with 1 mL inoculum within 144 hours, it was completely decolorized within 144 hours with 2 mL inoculum size. When inoculum size increased to 3 and 4 mL, dyes were decolorized faster. While 100 mg/L AR360 was 96% decolorized with 1 mL inoculum size it was completely decolorized within 72 hours with 4 mL inoculum size. Effect of inoculum size on

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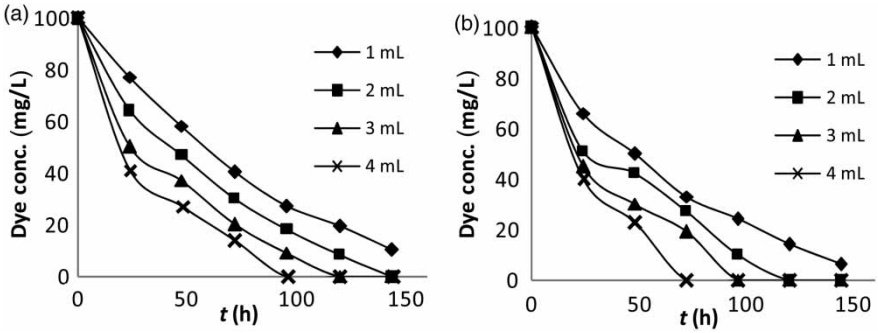


Figure 7 | Effect of inoculum size on decolorization of RB39 and AR360 with 2 g/L yeast extract (RB39 $P < 0.05$, AR360 $P < 0.05$).

decolorization of RB39 and AR360 by *P. aeruginosa* is given in Figure 7.

CONCLUSION

In this study, non-dye acclimatized *P. aeruginosa* was used for decolorization of textile dyes RB39 and AR360. *Pseudomonas aeruginosa* is able to decolorize RB39 and AR360 with 100% decolorization efficiencies under certain conditions. The results indicate the potential application of *P. aeruginosa* for decolorization of effluents containing textile dye. The culture requires yeast extract as the organic source for decolorization. Just 0.5 g/L yeast extract is sufficient for complete decolorization of 20 mg/L RB39 and AR360 within 144 hours. Initial dye, yeast extract concentration, temperature, pH and inoculum size have considerable effects on decolorization of AR360 and RB39 by *P. aeruginosa* ($P < 0.05$). Higher decolorization efficiencies in shorter times may be obtained by using carbon and nitrogen organic sources with high concentrations. Nevertheless, high concentrations of organic sources may cause high chemical oxygen demand and high costs when used as potential applications for the decolorization of textile dye using pure cultures.

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