



Biodecolorization of Acid Red GR by a newly isolated *Dyella ginsengisoli* LA-4 using response surface methodology

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ABSTRACT

Decolorization of Acid Red GR by a newly isolated biphenyl-degrading bacterium, *Dyella ginsengisoli* LA-4 was presented in this paper. The optimal decolorization conditions were determined by response surface methodology (RSM) based on the rotatable central composite design. The results indicated that strain LA-4 possessed the highest decolorizing activity under anaerobic conditions with inoculation amount 6.49%, pH 7.06 and temperature 29 °C. Some ions such as Cu²⁺, Zn²⁺ could inhibit the decolorization, whereas 1 mmol/L of Mg²⁺, Ca²⁺, Co²⁺, Fe³⁺, Ni²⁺ and Mn²⁺ had no effects on the process. It was demonstrated that anthraquinone as a redox mediator could significantly accelerate the reduction process of azo dyes.

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1. Introduction

Azo dyes characterized by the presence of one or more azo groups are widely used in textile, printing, cosmetics, pharmaceutical, food and many other industries because of their synthesis and chemical stability [1]. Disposal of these dyes can cause environment problems, since they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration [2] and also be toxic to some aquatic organisms due to their breakdown products [3]. Many techniques such as physical process, chemical process or their combinations and also biological process are used for decolorization of these dyes, of which biological anaerobic treatment is a relatively efficient, inexpensive and frequently applied method [4–7]. It has been demonstrated that increasing number of microorganisms are being described for their abilities to decolorize and degrade azo dyes under certain environmental conditions [8–10]. Pure fungal cultures have been used to develop bioprocesses for the mineralization of azo dyes. However, there are still some disadvantages which limited their application such as low pH requirement for an optimum activity of the enzymes and the long hydraulic retention time [11,12]. In contrast, bacterial decolorization is normally faster. Previous studies indicated that, bacterial strains like *P. mirabilis*, *P. luteola*, *Pseudomonas* sp. and *K. rosea* had shown

very promising results for dye degradation under anoxic conditions [13–16]. Here we reported bacteria *Dyella ginsengisoli* LA-4, which could decolorize various azo dyes efficiently.

Most azo dyes are fortuitously reduced under anaerobic conditions with relatively low reaction rate [17]. It was demonstrated that the decolorization of azo dyes could be improved by adding redox mediators, which could accelerate the reaction by shuttling electrons from microorganisms or chemical electron donors to the electron-accepting azo dye [18,19]. It was observed that the addition of quinoid redox mediators such as lawsone (2-hydroxy-1,4-naphthoquinone), anthraquinone-2-sulfonate (AQS) and anthraquinone-2,6-disulfonate (AQDS) could really enhance bacterial reduction of azo dyes [17,18].

Until now, the effects of environment factors on microbial decolorization of azo dyes are usually examined with single-factor optimization. Response surface methodology (RSM) is a powerful tool in evaluating the effective factors, building models to study the interaction between the variables and selecting the optimal conditions of variables or desirable responses [20]. Compared with conventional approaches for the same number of estimated parameters, RSM can not only evaluate the interactions effects among tested operating variables but also reduce the number of experiments to be undertaken [21].

A novel biphenyl-degrading bacterium, *D. ginsengisoli* LA-4 was isolated from activated sludge and its characteristics have been investigated in details [22]. In addition, we found that strain LA-4 could also grow anaerobically and decolorize several azo dyes.

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Table 1
Azo dyes used in this study.

| Azo dyes | Chemical structure | λ_{\max} (nm) |
|--------------------------|--------------------|-----------------------|
| Acid Red GR | | 510 |
| Acid Orange G | | 442 |
| Reactive Brilliant X-3B | | 538 |
| Reactive Brilliant KE-3B | | 513 |
| Reactive Brilliant K-2G | | 535 |
| Reactive Brilliant K-2BP | | 513 |
| Direct Fast Blue B2RL | | 582 |

There were almost no reports on the genus *Dyella* with respect to biodegradation of environmental pollutants, especially for azo dyes. Therefore, it was necessary to develop this new microbial resource in environmental bioremediation for azo dye decolorization.

In this study, RSM based on rotatable central composite design (CCD) was applied to identify and optimize the decolorization conditions for the decolorization of Acid Red GR by strain LA-4. The effects of NaCl and metal ions were also investigated. Meanwhile, biocatalyst effects of anthraquinone on the anaerobic reduction of azo dyes with the resting cells of strain LA-4 were determined.

2. Materials and methods

2.1. Dyes and chemicals

Azo dyes used in this study (Table 1) were kindly presented by Dye Synthesis Laboratory in Dalian University of Technology, and all of them were the highest purity available. All other reagents were of analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China).

2.2. Bacterial strain and culture conditions

Strain LA-4 [22] used in this study was presented by Dalian University of Technology. Pure culture was maintained in the Defined Basal Salts Medium (DBSM), which contained (g/L): 0.5 NaCl, 0.5 $K_2HPO_4 \cdot 3H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, pH 7.0 and 0.1 biphenyl as carbon source. The decolorization medium used in this study was Luria–Bertani medium (LB), which contained (g/L): 10.0 peptone, 5.0 yeast extract, 10 sodium chloride, pH 7.0.

2.3. Decolorization of Acid Red GR by resting cells of strain LA-4

Strain LA-4 was grown aerobically in 100 mL LB medium at 30 °C, 150 rpm, and the cells were harvested by centrifugation (8000 rpm, 10 min, at 4 °C) and washed at least twice with phosphate buffer solution (pH 7.0), then resuspended in the same buffer to a final concentration of 10 mg/mL (wet cell weight) and stored at 4 °C for subsequent use.

The bacterial suspension was inoculated into 100 mL flasks with 60 mL Luria–Bertani medium containing 85 mg/L dye. Then the effects of aerobic conditions (150 rpm, 30 °C) and anaerobic con-

Table 2
Design with experimental values and predicted values of Acid Red GR decolorization.

| No. | Factor A | Factor B | Factor | Decolorization (%) | |
|-----|----------|----------|--------|--------------------|-----------|
| | | | | Experiment | Predicted |
| 1 | 2 | 9 | 20 | 6.68 | 0.95 |
| 2 | 10 | 5 | 20 | 38.89 | 37.77 |
| 3 | 6 | 3 | 30 | 6.32 | 21.20 |
| 4 | 6 | 7 | 30 | 95.59 | 97.74 |
| 5 | 2 | 5 | 20 | 18.2 | 8.76 |
| 6 | 6 | 7 | 30 | 97.76 | 97.74 |
| 7 | 0 | 7 | 30 | 0 | 29.12 |
| 8 | 2 | 5 | 40 | 50.17 | 25.24 |
| 9 | 6 | 11 | 30 | 7.57 | 6.78 |
| 10 | 6 | 7 | 30 | 96.73 | 97.74 |
| 11 | 6 | 7 | 30 | 97.42 | 297.74 |
| 12 | 2 | 9 | 40 | 30.15 | 17.18 |
| 13 | 12 | 7 | 30 | 97.43 | 93.36 |
| 14 | 6 | 7 | 13 | 2.68 | -2.82 |
| 15 | 10 | 5 | 40 | 88.82 | 80.46 |
| 16 | 6 | 7 | 30 | 97.4 | 97.74 |
| 17 | 10 | 9 | 20 | 20.56 | 31.40 |
| 18 | 10 | 9 | 40 | 78.48 | 73.87 |
| 19 | 6 | 7 | 50 | 4.61 | 55.57 |
| 20 | 6 | 7 | 30 | 96.18 | 97.74 |

ditions (nitrogen gas was filled up in anaerobic bottles to assure the anaerobic circumstance, 30 °C) on the decolorization by strain LA-4 were investigated.

Effects of various parameters, including inoculation amount (2–10%, v/v), pH (3–11), temperature (15–50 °C), NaCl concentration (0–7%, w/v) and metal ions (1 mmol/L of Ca²⁺, Mg²⁺, Fe³⁺, Zn²⁺, Cu²⁺, Mn²⁺, Co²⁺ and Ni²⁺) were estimated. Sampling interval was 20 h in each experiment. Experiments were performed in 20 mL anaerobic bottles containing 15 mL LB medium. Nitrogen gas was filled up in anaerobic bottles to assure the anaerobic circumstance before decolorization.

2.4. Optimization of decolorization conditions by response surface methodology

RSM was applied to identify the relationship between the response functions and process variables as well as to optimize the decolorization conditions. Design Expert 7.1.3 software was used to analyze the obtained results. In this study, three independent variables or factors were chosen to maximize the dye decolorization rates, including inoculation amount (factor A), pH (factor B) and temperature (factor C).

For statistical calculations, the relation between the coded values and real values were as described in the following Eq. (1):

$$X_i = \frac{U_i - U_0}{\Delta U} \quad (1)$$

where X_i is the independent variable coded value; U_i is the real value of the independent variable; U_0 is the real value of the independent variable on the center point; ΔU is the step change and the

Table 3
Estimated regression coefficients of inoculation amount (A), pH (B) and temperature (C).

| Term | Estimated coefficient | Standard error coefficient | F-value | P-value |
|----------------|-----------------------|----------------------------|------------|---------|
| Intercept | 97.74 | 8.54 | | |
| A | 21.42 | 4.54 | 22.26 | 0.0008 |
| B | -3.61 | 4.01 | 0.81 | 0.3898 |
| C | 14.73 | 4.22 | 12.21 | 0.0058 |
| AB | 0.36 | 5.67 | 3.998E-003 | 0.9508 |
| AC | 6.55 | 5.67 | 1.33 | 0.2751 |
| BC | -0.064 | 5.67 | 1.262E-004 | 0.9913 |
| A ² | -16.22 | 5.06 | 10.26 | 0.0094 |
| B ² | -20.94 | 3.15 | 44.23 | <0.0001 |
| C ² | -26.13 | 3.60 | 52.83 | <0.0001 |

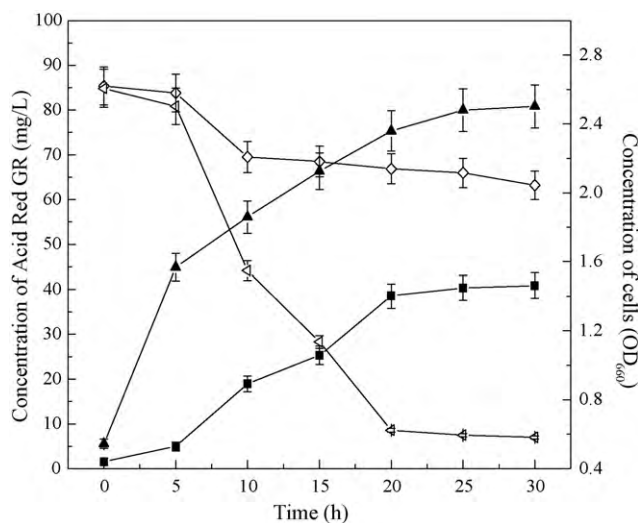


Fig. 1. Growth of strain LA-4 and Acid Red GR decolorization under aerobic and anaerobic conditions. (◇) Aerobic decolorization; (△) anaerobic decolorization; (▲) aerobic growth; (■) anaerobic growth.

central point was set with α of 1.414. Data from the central composite experimental design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models.

The significance of the model equation and model terms was evaluated by F test. The quality of the polynomial model equation

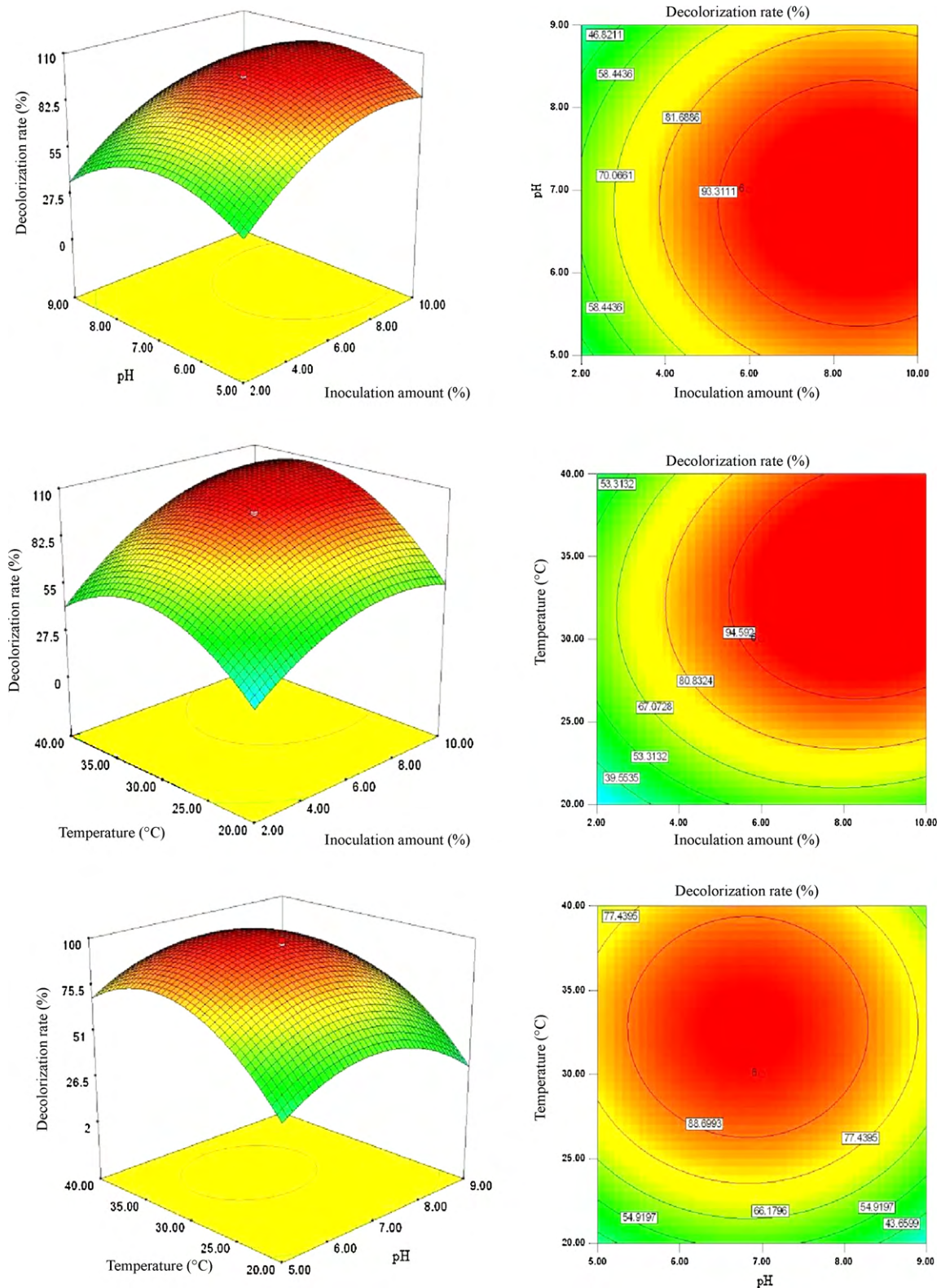


Fig. 2. Three-dimensional surface graph and its corresponding contour plot of response surface methodology: (a) pH and inoculation amount; (b) temperature and inoculation amount and (c) pH and temperature.

was expressed by determination coefficient R^2 , adjusted R^2 and “adequate precision”. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. The optimal values were obtained by solving the regression equation and analyzing the response surface plot.

2.5. Effects of anthraquinone on different azo dyes decolorization by resting cells

The effects of anthraquinone on the reduction of Acid Red GR and anthraquinone concentration (0.0–1.2 g/L) were investigated.

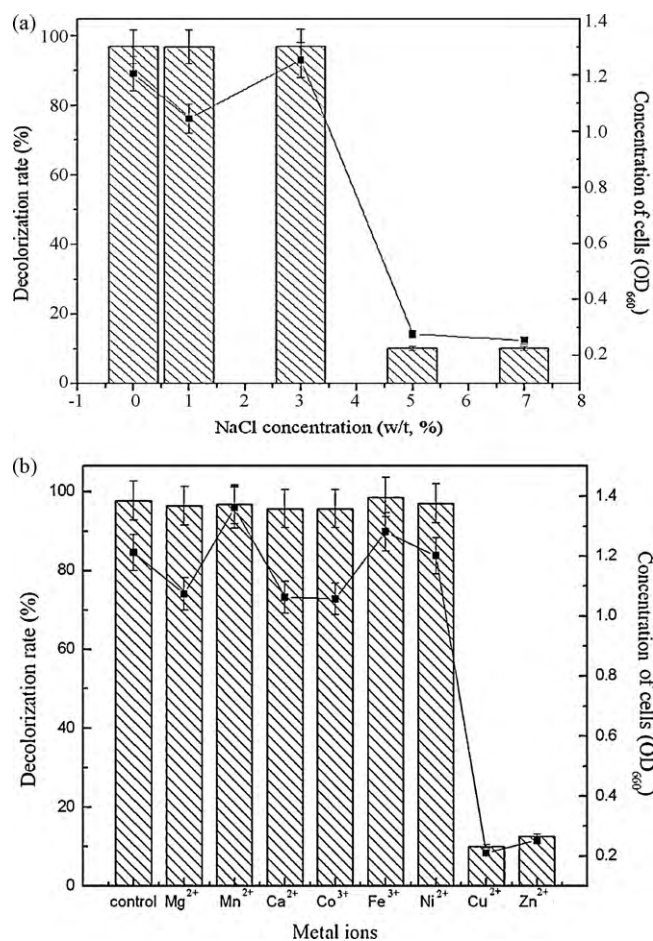


Fig. 3. Effects of NaCl (a) and metal ions (b) on decolorization of Acid Red GR by strain LA-4. (▨) decolorization rate; (■) growth.

In addition, other azo dyes such as Reactive Brilliant Red X-3B, K-2BP, K-2G, KE-3B, K-2BP, Acid Red B, Acid Orange G and Direct Fast Blue B2RL were tested for the effects of anthraquinone on the decolorization by strain LA-4. The experiment only with strain LA-4 was conducted as mentioned above.

2.6. Assays

Dye concentration was measured by monitoring the changes of maximum absorbance (Table 1) with a UV–vis spectrophotometer (JASCO V-560, Japan). Then the decolorization rate was calculated. The growth of strain LA-4 under aerobic conditions was measured by UV–vis spectrophotometer (JASCO V-560, Japan) at 660 nm. All of the experiments were performed in duplicates and the average values were used in calculations.

3. Results and discussion

3.1. Decolorization conditions of Acid Red GR by strain LA-4

Decolorization of Acid Red GR was studied under anaerobic and aerobic conditions (150 rpm) with an initial dye concentration of 85 mg/L. It was observed that decolorization rate of Acid Red GR was more than 89% under anaerobic conditions but only 22% under aerobic conditions, while the growth of strain LA-4 was better under aerobic conditions (Fig. 1), indicating that strain LA-4 was a facultative anaerobe. Oxygen was favorable to the growth of the bacterium but deleterious to the yield process of the related enzyme. It was speculated that under aerobic conditions, the aerobic respiration

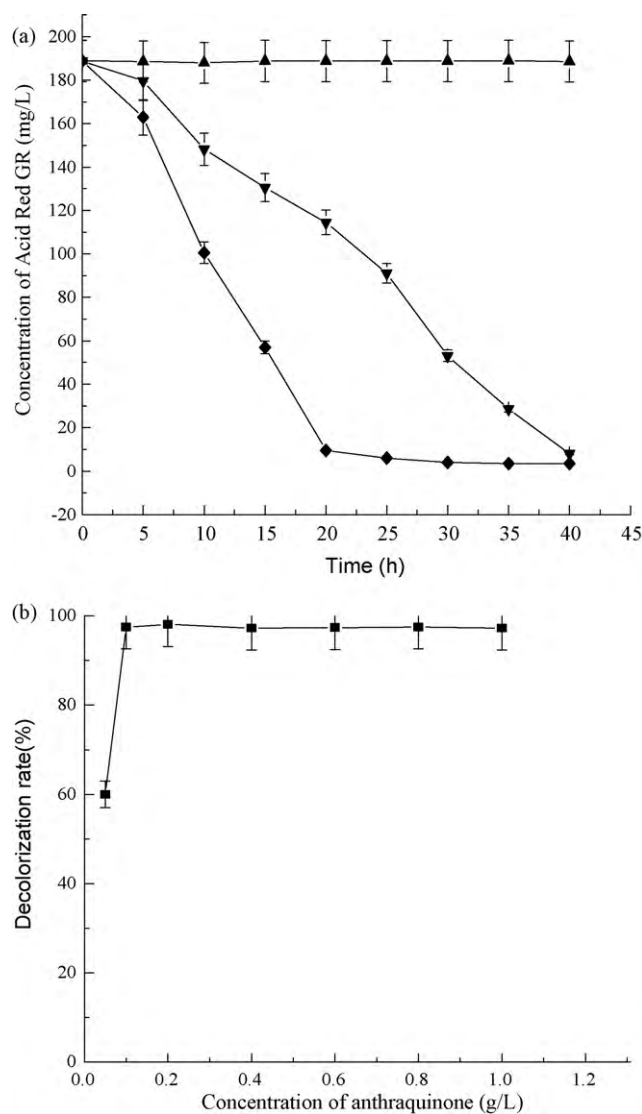


Fig. 4. Effects of anthraquinone on Acid Red GR decolorization, (a) decolorization of Acid Red GR with and without anthraquinone, (▲) anthraquinone/Acid Red GR; (▼) LA-4/Acid Red GR; (◆) anthraquinone/Acid Red GR/LA-4 and (b) effects of anthraquinone concentration on decolorization.

of the strain might dominate the utilization of NADH and deprive the azoreductase from obtaining electrons from NADH to decolorize azo dyes [23]. Therefore, anaerobic conditions were adopted to investigate bacterial dye decolorization in further experiments. It was also exhibited that the exponential growth phase of strain LA-4 was observed between 5 and 20 h of incubation under anaerobic conditions. After 20 h of incubation, the percentage of dye removal was more than 89%. The metabolic pathway of Acid Red GR by *Shewanella decolorationis* strain was proposed by Xu et al. [24]. According to this report, it was predicted that the corresponding decolorization products of Acid Red GR by strain LA-4 were aniline, 1,4-diaminobenzene and 1-amino-2-naphthol.

3.2. Optimization of decolorization conditions by response surface methodology

Using the rotatable central composite design method, a total of 20 experiments with different combinations of inoculation amount (factor A, 2–10%), pH (factor B, 5–9) and temperature (20–40 °C) were performed. Dye decolorization was used as the response (Y, %) and calculated at different sampling intervals (Table 2). Initial Acid

Red GR concentration was 200 mg/L. By applying multiple regression analysis, the following second-order polynomial equation was obtained as Eq. (2):

$$Y = 97.74 + 21.42A - 3.61B + 14.73C + 0.36AB + 6.55AC - 0.064BC - 16.22A^2 - 20.94B^2 - 26.13C^2 \quad (2)$$

where Y was the predicted response, and A , B , and C were the coded values of the test factors.

An ANOVA for response surface quadratic model shown in Eq. (2) was checked by F test and summarized in Table 3. The coefficients, which indicated the significance of each factor and the mutual interaction between factors, were checked with P -values. Generally, the corresponding coefficient was considered significant with smaller P -values [25]. For decolorization conditions, A , C , A^2 , B^2 and C^2 were significant factors due to the P -value, which was less than 0.05. Whereas B , AB , AC , BC were not significant due to the P -value (more than 0.05).

According to the ANOVA, the model was significant ($P < 0.05$). The determination coefficient R^2 (0.9208) indicated a good fitness of the model. The closer the R^2 to 1, the better was the correlation between the experimental and predicted values [26]. The value of adjusted R^2 (0.8496) suggested that 84.96% of the variability in the response could be explained by the model. Therefore, the model can be used for further analysis.

The response surface graph of dye decolorization was shown in Fig. 2. The response surfaces and contour plots shown in Fig. 2 were based on the regression model of Eq. (2) holding one variable at constant of its zero level, while varying the other two variables within their experimental range. The inoculation amount (A) and temperature (C) showed the significant effects on dye decolorization with the P -value was 0.0008 and 0.0058, respectively (Table 3).

Based on Fig. 2a, with the increase of inoculation amount, dye decolorization rate was increased. As the inoculation amount was higher than 6%, the decolorization rate reached high level without any changes. As shown in Fig. 2b, with the increase of temperature, the decolorization rate increased at low temperature and then decreased at high temperature. It was investigated that pH was not a significant factor. Fig. 2c revealed that decolorization rate was relatively high at a wide range of pH 5–9. The predicted optimum values of inoculation amount, pH and temperature were obtained by applying the regression analysis (Eq. (2)) using “response optimizer” in Design Expert 7.1.3 software. The optimal decolorization conditions were as follows: 6.49% of inoculation amount, pH 7.06, temperature 29 °C and its corresponding maximum decolorization rate were 98.47%.

Three verification experiments were carried out under the optimum conditions obtained through RSM. The average decolorization rate achieved 98.36%. The experimental value closely agreed with the theoretical predicted values of 98.47% obtained from RSM, which also confirmed that the RSM was effective and reliable for optimizing the decolorization rate of dye.

3.3. Effects of NaCl and metal ions on Acid Red GR decolorization

Effects of NaCl on the dye decolorization were determined, and the results were shown in Fig. 3a. Strain LA-4 could grow and decolorize dye with 3% NaCl (w/v). The decolorization rate decreased rapidly when the concentration of NaCl was more than 3%.

The effects of different metal ions on the Acid Red GR decolorization were tested in the presence of 1 mmol/L different metal ions (Fig. 3b). It was shown that metal ions such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{3+} , Co^{2+} and Ni^{2+} had no effects on the dye decolorization, while Zn^{2+} and Cu^{2+} could inhibit the decolorization process.

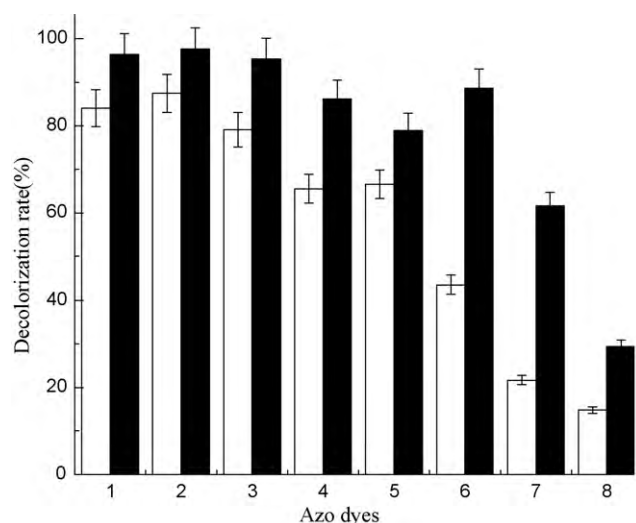


Fig. 5. Effects of anthraquinone on various azo dyes decolorization, (□) without anthraquinone; (■) with anthraquinone, (1) Acid Red; (2) Acid Orange; (3) Acid Red GR; (4) Reactive Brilliant X-3B; (5) Reactive Brilliant KE-3B; (6) Reactive Brilliant K-2G; (7) Reactive Brilliant K-2BP and (8) Direct Fast Blue B2RL.

3.4. Effects of anthraquinone on decolorization of Acid Red GR by resting cells

As shown in Fig. 4a, in the system of anthraquinone/Acid Red GR, the concentration of dye remained unchanged. However, in the systems of Acid Red GR/LA-4 and anthraquinone/Acid Red GR/LA-4, the redox mediator anthraquinone at a concentration of 1.0 g/L enhanced the decolorization rate of Acid Red GR significantly. The corresponding time of completely decolorizing 180 mg/L Acid Red GR by the systems with and without anthraquinone were 20 and 40 h, respectively. Furthermore, it was obvious that the adaptation stage of enhanced system was shorter than that without anthraquinone. Optimal concentrations of anthraquinone were shown in Fig. 4b. The results showed that the highest decolorization rate was obtained when the concentration of anthraquinone was up to 0.2 g/L. However, the effects were not obvious when the concentration of anthraquinone was above 0.2 g/L.

3.5. Effects of anthraquinone on decolorization of various azo dyes

Generally, the industrial effluents consist of a mixture of various dyes. In this study, the reduction of various azo dyes was investigated in the absence or presence of anthraquinone (Fig. 5). Initial dye concentration and the amount of resting cells were 100 mg/L and 6%, respectively. It was indicated that strain LA-4 could decolorize different azo dyes besides Acid Red GR. Due to the effects of the quality, type and position of charged functional groups of azo dyes, different decolorization rate was observed [27]. Compared with the absence of anthraquinone, the addition of anthraquinone resulted in significantly increasing decolorization rate of dyes by strain LA-4, especially for sulfate dye, K-2G, K-2BP and triple azo dye.

4. Conclusions

A novel biphenyl-degrading strain *D. ginsengisoli* LA-4 can effectively decolorize azo dyes under anaerobic conditions. The optimal conditions were determined as inoculation amount 6.49%, pH 7.06 and temperature 29 °C. And the heavy metal ions such as Zn^{2+} , Cu^{2+} could inhibit dye decolorization, while Ca^{2+} , Mg^{2+} , Mn^{2+} , Fe^{3+} , Co^{2+} and Ni^{2+} had no effects on the process of decolorization.

As reported previously, anthraquinone can enhance the azo dyes decolorization. In our study, we also found that anthraquinone really accelerated the decolorization processes, which suggests that *D. ginsengisoli* LA-4 holds the potential for field application.

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