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Identification and Characteristics of A Novel Salt-Tolerant *Exiguobacterium* sp. for Azo Dyes Decolorization

Liang Tan · Yuan-yuan Qu · Ji-ti Zhou · Ang Li · Min Gou

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Abstract A salt-tolerant bacterium was isolated from the surface soil of a pharmaceutical factory, which could efficiently decolorize azo dyes. The strain was identified as *Exiguobacterium* sp. according to its morphological characteristics and 16S rRNA gene sequence analysis. Decolorization of X-3B with resting cells of this strain, which were catalyzed by redox mediator (anthraquinone), was studied, and the conditions were optimized. For color removal and cells growth, the optimal inoculation amount, pH, temperature, salinity, and metal ions were 6% (*v*/*v*), 5.4–7.0, 30–40 °C, 15% (*w*/*v*) NaCl, and 1 mmol L^{-1} Mg^{2+} or Ca^{2+}, respectively. It was exhibited that decolorization process proceeded primarily by enzymatic reduction associated with a minor portion of bio-adsorption to inactivated microbial cells. Anthraquinone could really accelerate the decolorization of X-3B under the optimal conditions.

Keywords Azo dye · Decolorization · Salt-tolerant · Redox mediator

Introduction

Azo dyes, the largest chemical class of dyes with the greatest variety of colors, have been used extensively for textile dyeing and paper printing. Approximately 10–15% of the dyes are released into the environment during manufacturing and usage, which poses a potential health hazard to all forms of life [1–2]. Therefore, treatment of azo dye wastewater has become a matter of great concern, and several advanced treatment methods, such as physical and chemical methods, have been suggested and studied. However, they are not widely applied because of the high cost and production of more toxic intermediates. Compared with these methods, biological methods were environmental-friendly and cost

L. Tan · Y.-y. Qu (✉) · J.-t. Zhou · A. Li · M. Gou
School of Environmental and Biological Science and Technology,
Key Laboratory of Industrial Ecology and Environmental Engineering (Ministry of Education, China),
Dalian University of Technology, Dalian 116024, China
e-mail: yuanyuanqu@yahoo.cn

less. Most of azo dyes are resistant to aerobic microbial attack in conventional-activated sludge treatment [3]. However, sequencing anaerobic–aerobic treatment has been found effective in color removal from water [4]. Cleavage of azo bonds occurs under anaerobic conditions to form corresponding amines, which can be further mineralized under aerobic conditions [5–6]. In such combined system, the anaerobic and facultative microbes play an important role to make the recalcitrant azo dyes easily cleaved by aerobic microbes. At the same time, the anaerobes also digest and remove a significant amount of chemical and biochemical oxygen demands [7]. Hence, decolorization with anaerobic microbes is an important step for the biotreatment of dyeing and printing wastewater. Until now, many microorganisms have been proven to possess the ability of decolorizing azo dyes. However, dyeing and printing wastewater always contains high amounts of inorganic salts, which usually inhibit the activities of most microorganisms [8]. Therefore, exploitation of the salt-tolerant bacteria would be an alternative to the conventional biological treatment systems for azo dye wastewater.

As reported previously, most azo dyes, especially for reactive azo dyes, are fortuitously reduced under anaerobic conditions with relatively low reaction rate [9]. This presents a problem for the application of high-rate anaerobic bioreactors for the treatment of dye-containing wastewater because long hydraulic retention times would be necessary to reach a satisfactory extent of dye reduction. However, it can be resolved by using property of redox-mediating compounds to increase azo dye reduction rate by shuttling electrons from microorganisms or chemical electron donors to the electron-accepting azo dye [10–11]. Enzyme cofactors such as flavin adenine dinucleotide and certain quinones are known as effective redox mediators for azo dye reduction. For instance, in a biotic system, quinones accelerated chemical azo dye reduction by sulfide, as well as electrochemical azo dye reduction [12–14].

This paper describes on isolation and identification of a novel salt-tolerant bacterium used for azo dye decolorization under high-salt conditions. Characteristics such as optimal growth and decolorization conditions, decolorization route, and inhibitory dye concentration were investigated. Besides, biocatalyst effect of anthraquinone on the anaerobic reduction of azo dye X-3B with resting cells of this strain was estimated in detail.

Materials and Methods

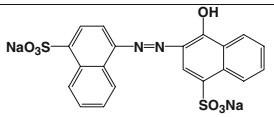
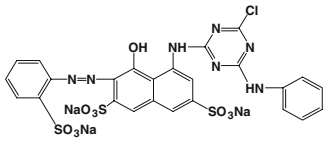
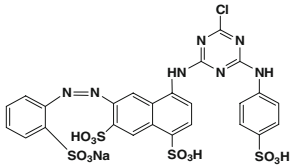
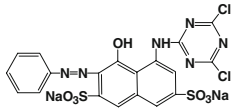
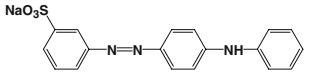
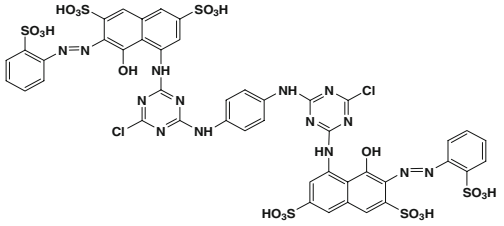
Dyes and Chemicals

The azo dyes used in this study, Reactive Brilliant Red X-3B, K-2G, K-2BP, and KE-3B, Acid Red B, and Acid Orange G, were from Dye Synthesize Laboratory, Dalian University of Technology. The chemical structures of these dyes were shown in Table 1. All other reagents were analytical grade and purchased from Shenlian Ltd. (Dalian, China).

Medium

The salt-tolerant medium was used both for enrichment and pure cultures of decolorization and salt-tolerant organisms. The medium contains (per liter) 4.0 g peptone, 1.0 g NH_4Cl, 1.0 g $NaHCO_3$, 0.2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 50.0 g NaCl.

Table 1 The azo dyes used in this study.

Azo dyes	Wavelength (nm)	Chemical structure
Acid Red B	516	
Reactive Brilliant Red K-2BP	513	
Reactive Brilliant Red K-2G	515	
Reactive Brilliant Red X-3B	538	
Acid Orange G	450	
Reactive Brilliant Red KE-3B	513	

Isolation and Identification of Salt-Tolerant Bacterium for Azo Dye Decolorization

A salt-tolerant azo dye-decolorizing strain was isolated from the surface soil of a pharmaceutical factory in Shulan, Jilin, China. Screening was carried out in serum bottles. After purification by successive single colony isolation on agar plates, strain TL was chosen as the target one with the highest capability for azo dyes decolorization.

Genomic DNA extraction, polymerase chain reaction amplification of 16S rRNA, and sequencing of the amplified 16S rRNA gene were done by TaKaRa Biotechnology

Company, Dalian, Liaoning, China. Related sequences were obtained from the GenBank database by using the BLAST search program. Alignment of sequences was carried out with ClustalX 1.8 software. A phylogenetic tree was constructed by using the neighbor-joining method as implemented within the Mega3.1.

Decolorization of Dyes by Growing Cells of Strain TL

Cells were cultured overnight with enrichment medium containing 200 mg L^{-1} X-3B at 30 °C statically in rubber-stopped serum bottles until color was removed completely and absorbance at 660 nm reached 0.2. The growth-and-degradation curves were plotted at the initial concentration of X-3B, and the amount of pre-cultured cells were about 180 mg L^{-1} and 4% (*v*/*v*), respectively. For optimal growth and decolorization experiments, inoculation amount (2–10%, *v*/*v*), pH (4.3–10.1), temperature (20–45 °C), salinity (20–150 g L^{-1} NaCl), and metal ions (1 mmol L^{-1} of Ca^{2+}, Mg^{2+}, K^{+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, and Al^{3+}) were estimated.

Some other azo dyes, such as Reactive Brilliant Red K-2BP, K-2G, Acid Red B, Acid Orange G (monoazo dyes), and Reactive Brilliant Red KE-3B (disazo dye), were tested for the degradability by growing cells of strain TL under the conditions of 6% (*v*/*v*) inoculation amount, 30 °C, pH 7.0, 50 g L^{-1} NaCl, and 100 mg L^{-1} dyes. After incubation to an appropriate sampling time, OD_{660} was examined to represent the concentrations of bacterium cells, and supernatant after centrifugation (8,000 rpm) was used for decolorization assay.

Effects of Anthraquinone on X-3B Decolorization by Resting Cells

Bacterial cells were collected by centrifugation, washed with phosphate buffer solution (pH 7.0) for at least two times, and re-suspended in the same buffer for decolorization experiment. In optimal decolorization condition experiments, inoculation amount [2–10 g (wet weight) L^{-1}], pH (3.23–10.91), temperature (20–45 °C), salinity (20–200 g L^{-1} NaCl), anthraquinone concentration (0.2–1.0 mg L^{-1}), and metal ions (1 mmol L^{-1} of Ca^{2+}, Mg^{2+}, K^{+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, and Al^{3+}) were estimated.

Decolorization Assay

Decolorization of dyes was determined by monitoring the decrease in absorbance at the maximum wavelength of each dye. The maximum wavelengths of the azo dyes used in this study were listed in Table 1. An UV-visible scanning spectrophotometer (JASCO V-560) was used for absorbance measurement and recording of UV-visible absorption spectra.

Results and Discussion

Isolation and Identification of Azo-Dye-Decolorizing Bacterium

A moderate salt-tolerant azo-dye-degrading bacterial strain, named as strain TL, was isolated after screening and single colony purification. It is a Gram-positive, non-motile, anaerobic short rod (Fig. 1). It formed round orange colonies on the solid medium containing azo dye X-3B, which was smooth on the surface and irregular on the edge.

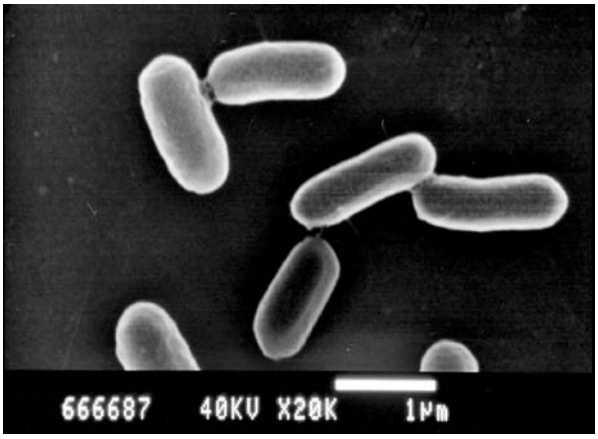


Fig. 1 The scanning electron microscopy (SEM) photograph of *Exiguobacterium* sp. TL

To further identify this strain, the nucleotide sequences of 16S rRNA gene were analyzed and compared with those of related strains in GenBank. The full-length 16S rRNA gene of strain TL (accession number EU159578) showed 99% homology to that of *Exiguobacterium* sp. BTAH1 (Fig. 2).

Until now, studies of *Exiguobacterium* sp. mainly focused on the characteristics of resistance to extreme conditions such as high/low temperature, alkaline environment, and high concentrations of salts [15–20]. However, for the environmental pollutant treatment, members of *Exiguobacterium* sp. were studied on aerobic degradation of some large molecular such as phenanthrene, pesticide, and the organics in tannery wastewater [21–23], and biological reduction of heavy metals such as Cr (VI) and As (III) [24–26]. There has been no report on decolorization of azo dyes by genus *Exiguobacterium*.

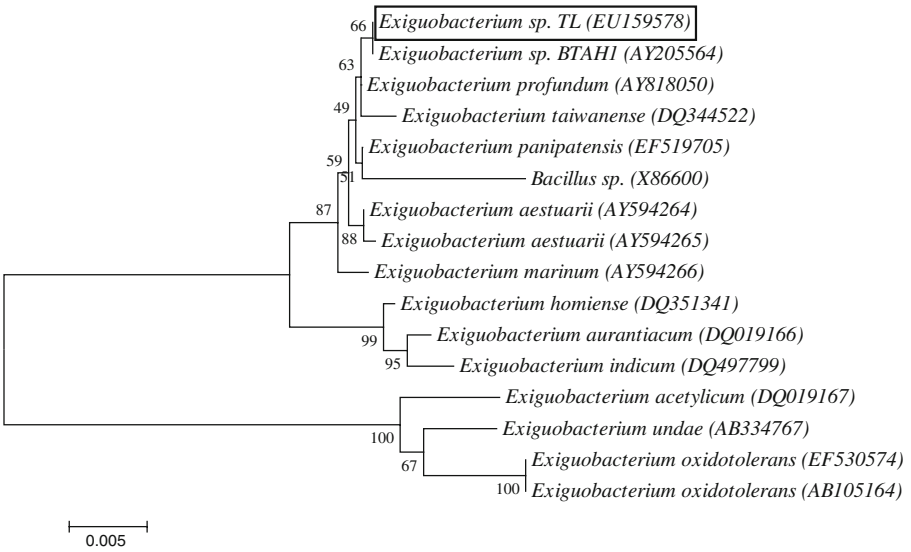


Fig. 2 Phylogenetic relationships of strain *Exiguobacterium* sp. TL and selected relatives. Strain *Exiguobacterium* sp. TL is *underlined* with a bane. Bootstrap values greater than 50% are shown. Accession numbers of previously published sequences are indicated in *parentheses*

Characteristics of Decolorization by Growing Cells of Strain TL

The time course of growth and degradation was shown in Fig. 3. It was indicated that the exponential growth phase was observed between 5 and 15 h of incubation, following a lag phase of 2.5 h. The fastest decrease in dye concentration was observed during the late exponential growth phase. After 22.5 h of incubation, dye removal rate were over 95%.

Effects of inoculation amount, temperature and pH, salinity, and metal ions were also investigated. An inoculation amount of at least 6% (*v*/*v*) could reach highest decolorization rate of 90% within 12 h, at 30–40 °C (data not shown). Besides, strain TL could tolerate acidulous and neutral pH range (5.4–7.0), and the optimal decolorization rate was reached at the initial pH value of 5.4. It was suggested that strain TL was a moderately salt-tolerant bacterium, which reached up to 87% decolorization ratio in 12 h with 15% (*w*/*v*) NaCl. Furthermore, Mg^{2+} and Ca^{2+} could accelerate decolorization by strain TL, but Al^{3+}, Zn^{2+}, Cu^{2+}, and Fe^{3+} inhibited it. It was obvious that strain TL did not reached high decolorization percentage of Reactive Brilliant Red X-3B out of the optimal range of temperature.

Besides, other five azo dyes were tested and confirmed for being completely decolorized within 22.5 h by growing cells of strain TL. Due to the effects of quality, type, and position of charged functional groups, difference in decolorization rates was observed for different azo dyes [27–28]. It was indicated that strain TL could decolorize different types of azo dyes besides X-3B.

Decolorization of X-3B with Different Initial Concentration

Strain TL could tolerate and decolorize 1,000 mg L^{-1} X-3B to less than 200 mg L^{-1} in 25 h (Fig. 4). It was shown that when the concentration was higher than 600 mg L^{-1}, X-3B could not be completely decolorized. Obviously, decolorization was inhibited when the concentration of X-3B were over 600 mg L^{-1}. It was possibly because the intermediates, which were toxic to microorganisms, would be accumulated after decolorizing high concentrations of azo dyes [29]. The UV-visible spectra of X-3B degradation by strain TL is shown in Fig. 5. The absorbance peak at 538 nm completely disappeared, and two peaks at about 362 and 248 nm appeared after decolorization. In addition, as the azo dye was reduced, the culture returned to its original color. It was indicated that dye adsorption would result in cells being deeply colored, whereas cells retaining their original colors were

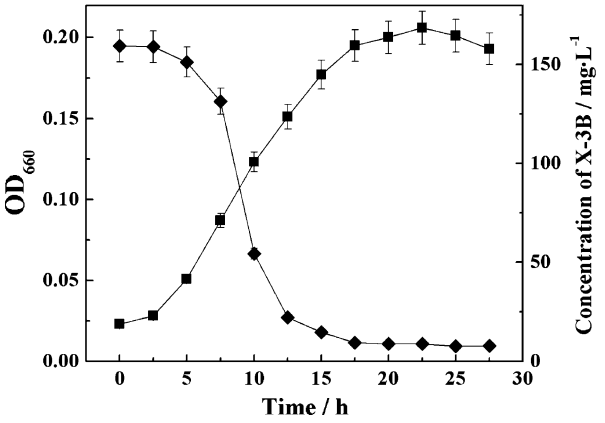


Fig. 3 Time courses of growth and decolorization of X-3B by strain TL. The initial cell density (OD_{660}) was about 0.20. *Square* Growth; *diamond* decolorization

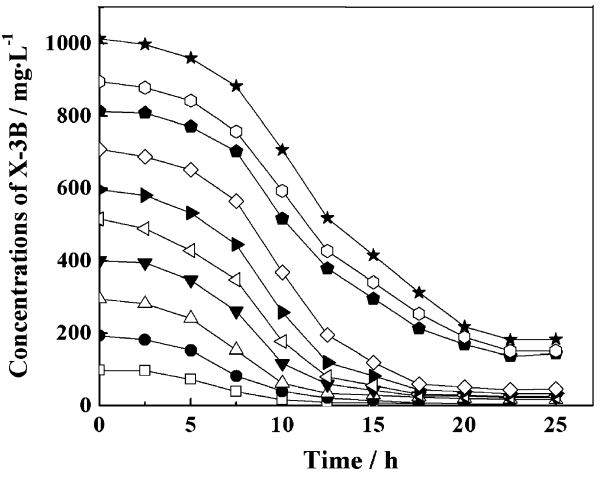


Fig. 4 Decolorization of X-3B by strain TL with different initial concentration. *Star* 100 mg L^{-1}, *empty pentagon* 200 mg L^{-1}, *filled pentagon* 300 mg L^{-1}, *empty diamond* 400 mg L^{-1}, *filled right triangle* 500 mg L^{-1}, *empty left triangle* 600 mg L^{-1}, *filled inverted triangle* 700 mg L^{-1}, *empty triangle* 800 mg L^{-1}, *filled circle* 900 mg L^{-1}, *empty square* 1,000 mg L^{-1}

accompanied by the degradation of dyes [30]. Therefore, the color removal by stain TL might be largely attributed to biodegradation.

Effect of Anthraquinone on Decolorization of X-3B by the Resting Cells of Strain TL

As shown in Fig. 6, the redox mediator anthraquinone at a catalytic concentration of 1.0 mg L^{-1} could enhance the decolorization of X-3B by resting cells of strain TL. The corresponding time of completely decolorizing 75 mg L^{-1} X-3B with and without anthraquinone were 16 and 24 h, respectively. Furthermore, it was obvious that the adaptation phase of enhanced system was shorter than that without anthraquinone. Optimal concentrations of resting cells and anthraquinone were investigated. The results showed that when concentrations of resting cells and anthraquinone were up to 6 g (wet weight) L^{-1} and 0.6 g L^{-1}, respectively, highest decolorization rate, which was higher than 90%, in 12 h was reached. Besides, the effect of other conditions such as temperature, salinity, and pH were also investigated. The best performance of decolorization was observed under 30 °C, less than 5% (*w*/*v*) NaCl and pH 7.0. The results showed that the resting cells of strain TL,

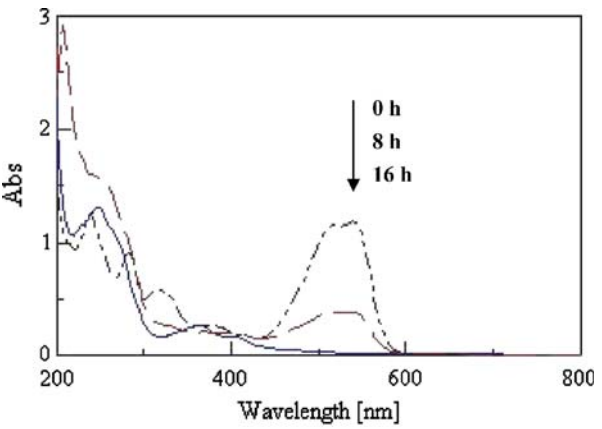


Fig. 5 Variation of UV-visible spectra of X-3B (50 mg L^{-1}) before and after decolorizing by strain TL. *Broken line* 0 h, *dashed line* 8 h, *solid line* 16 h

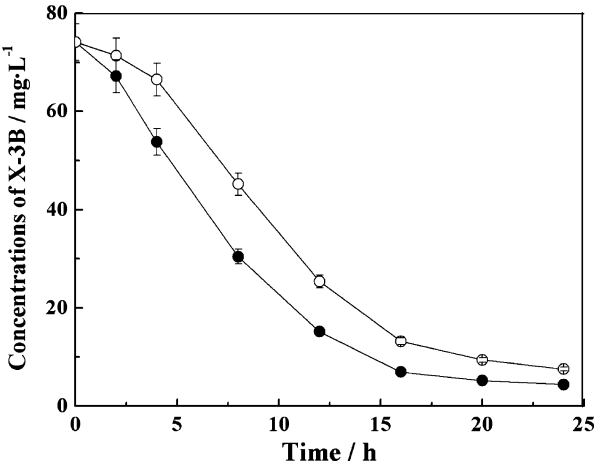


Fig. 6 Decolorization of X-3B by the resting cells of strain TL with and without anthraquinone. *Filled circle* With anthraquinone, *empty circle* without anthraquinone

which was catalyzed by anthraquinone, could keep high decolorization efficiency when the salinity was less than 50 g L^{-1}. As reported, microorganisms possessed different abilities of accumulating certain solutes, which could affect the ability of salt tolerance [31]. It was assumed that when exposed to a high concentration of salt, the osmotic pressure inside strain TL cells was increased, and certain solutes were accumulated at high cytosolic levels. Therefore, strain TL could exhibit high efficiency for color removal under such salt concentration. Besides, it was interesting that strong absorption was observed under acidic conditions because bacterial cells were deeply colored after 16 h, and it was not removed later. About 90% X-3B was removed through absorption at the pH value of 3.23. Moreover, as shown in Fig. 7, the scanning electron microscopy photograph of coexistence system exhibited that bacterial cells were adsorbed on the surface of anthraquinone, and hardly dissociative cells could be seen, indicating that decolorization reactions likely occurred on the surface of anthraquinone (Fig. 7).

The enhancement of redox mediators such as anthraquinone, anthraquinone-2, 6-disulfonate, and active carbon to bio-decolorization of azo dyes was studied by some researchers [10–11, 32], and they all focused on co-metabolism processes. However, little research has been done about the redox-mediator-enhanced process with members belonging to *Exiguobacterium* sp. for azo dye decolorization. This study proved that

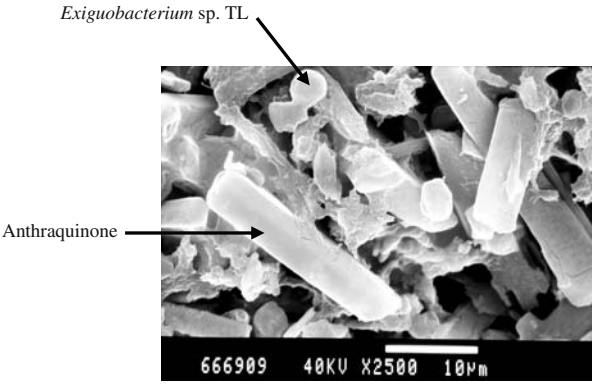


Fig. 7 The SEM photograph of the coexistence system of *Exiguobacterium* sp. TL and anthraquinone

anthraquinone could enhance the resting cells of *Exiguobacterium* sp. TL on azo dye decolorization. About one third of time was saved compared with the anthraquinone free system because adaptation phase was shortened due to the bio-catalyst effect of anthraquinone.

Conclusions

A novel strain with high capability for azo dye decolorization was isolated from one contaminated soil system and identified as *Exiguobacterium* sp. The optimal conditions were determined as 6% (*v*/*v*) of inoculation amount, pH range from 5.4 to 7.0, temperature range from 30 to 40 °C, and the strain could decolorize X-3B in the presence of 15% (*w*/*v*) NaCl. In this study, it was found that the decolorization process by strain TL was mainly due to biodegradation, and only a minor portion of X-3B was adsorbed onto the cells surface. It was also investigated that strain TL could tolerate higher concentration of X-3B, and the decolorization seemed to be inhibited when the initial concentration was more than 600 mg L^{-1}. As reported previously, anthraquinone can enhance the azo dye decolorization. In our study, we also found that anthraquinone really accelerated the decolorization processes under the optimal conditions: pH 7.0, 30 °C, 1 mmol L^{-1} Mg^{2+} or Ca^{2+}, 6 g (wet cells) L^{-1} resting cells, and 0.6 g L^{-1} anthraquinone. It suggested that strain TL was efficient for the field application of azo dye biotreatment.

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