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Biological decolorization of the reactive dyes Reactive Black 5 by a novel isolated bacterial strain *Enterobacter* sp. EC3

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ABSTRACT

Studies were carried out on the decolorization of the reactive dye Reactive Black 5 by a newly isolated bacterium, EC3. Phenotypic characterization and phylogenetic analysis based on 16S rDNA sequence comparisons indicate that this strain belonged to the genus *Enterobacter*. The optimal conditions for the decolorizing activity of *Enterobacter* sp. EC3 were anaerobic conditions with glucose supplementation, at pH 7.0, and 37 °C. The maximum decolorization efficiency against Reactive Black 5 achieved in this study was 92.56%. Ultra-violet and visible (UV–vis) analyses before and after decolorization and the colorless bacterial biomass after decolorization suggested that decolorization was due to biodegradation, rather than inactive surface adsorption. The bacterial strain also showed a strong ability to decolorize various reactive textile dyes, including both azo and anthraquinone dyes. To our knowledge, it is the first time that a bacterial strain of *Enterobacter* sp. has been reported with decolorizing ability against both azo and anthraquinone dyes.

1. Introduction

Large amounts of dyes are annually produced and applied in many different industries, including textiles, cosmetics, paper, leather, pharmaceutical and food [1]. Reactive dyes, which are the only textile colorants designed to bond covalently with cellulosic fibers (i.e., cotton), are extensively used in the textile industry because of their wide variety of color shades, high wet fastness profiles, ease of application, brilliant colors, and minimal energy consumption [2]. The three most common groups are azo, anthraquinone and phthalocyanine dyes [3]. Most of these dyes are toxic and carcinogenic [4]. Disposal of these dyes into the environment causes serious damage, since they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration [5] and also they may be toxic to some aquatic organisms due to their breakdown products [6].

Dyes can be removed from wastewater by chemical, physical and biological methods [7–10]. Although a number of chemical, physical processes like flocculation, chemical coagulation, precipitation, ozonation and adsorption have been employed for the treatment of dye bearing wastewaters, they possess inherent limitations such as high cost, formation of hazardous by-products and intensive energy requirements [11,12]. Conversely, bio-processing can overcome these defects because it is cost saving and environmentally benign. Fungi [13] and algae [14,15] have been used in dye decolorization. Adsorption rather than degradation plays a major role during the decolorization process by fungi and algae, as a result, the dyes remain in the environment. It is well-known that bacteria can degrade and even completely mineralize many reactive dyes under certain conditions [16–19]. Even better, the products of intermediate metabolism produces during the decolorization process, such as aromatic amines, can be degraded by the hydroxylase and oxygenase produced by bacteria [20]. To our knowledge, no bacterial strain has been reported with decolorizing ability against both anthraquinone and azo dyes. Additional work is still required to isolate new microorganisms capable of degrading a wide range of structurally different dyes and to study their physiological characteristics, in order to not only understand the underlying mechanisms in dye biodegradation, but also for biotechnological application. In this study, a newly isolated bacterium, *Enterobacter* sp. EC3, capable of decolorizing anthraquinone as well as azo dyes was isolated and the effects of various parameters (such as oxygen, initial bacterial concentration, dye concentration, glucose concentration, pH and temperature) on dye decolorization by the newly isolated bacteria were investigated.

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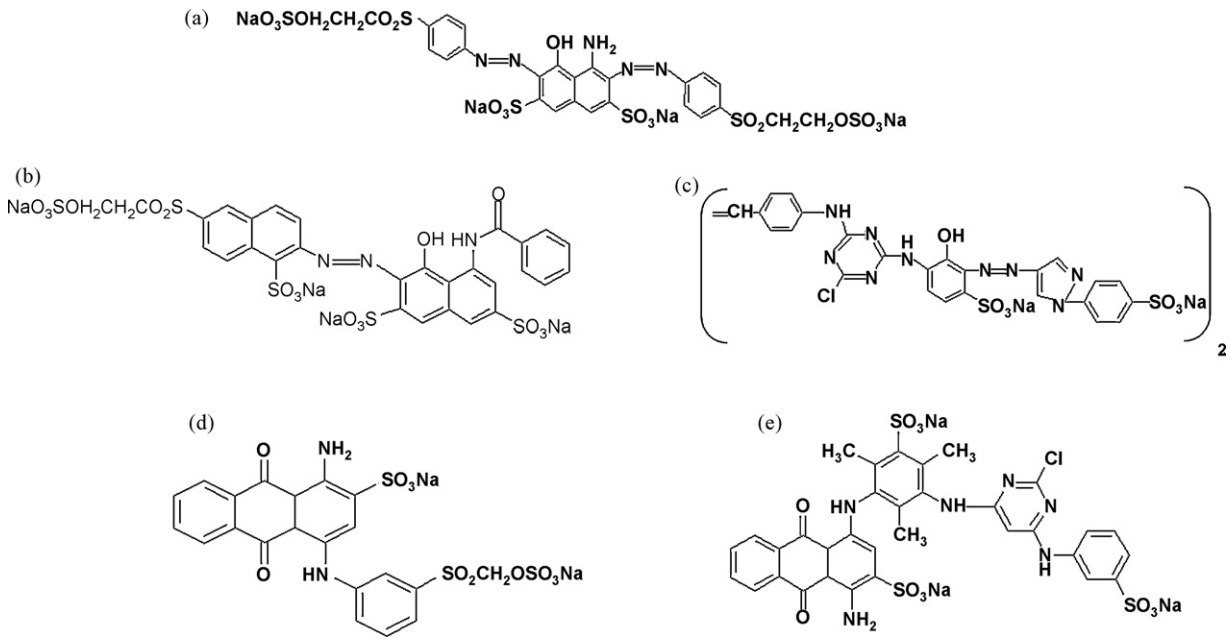

Fig. 1. Chemical structures of dyes used in this study (a, C. I. Reactive Black 5; b, C. I. Reactive Red 180; c, C. I. Reactive Yellow 81; d, C. I. Reactive Blue 19; e, C. I. Reactive Blue 49).

2. Materials and methods

2.1. Synthetic dyeing wastewater

The following five reactive dyes with the purity of 95%, C. I. Reactive Black 5 (Fig. 1a), C. I. Reactive Red 180 (Fig. 1b), C. I. Reactive Yellow 81 (Fig. 1c), C. I. Reactive Blue 19 (Fig. 1d), C. I. Reactive Blue 49 (Fig. 1e), were dissolved in M9 medium [21] (Na_2HPO_4 31.7 g L^{-1}, KH_2PO_4 3 g L^{-1}), NH_4Cl 0.5 g L^{-1}, NaCl 0.5 g L^{-1}, $MgSO_4$ 0.12 g L^{-1}, $CaCl_2$ 4 mg L^{-1}, glucose 8 g L^{-1}, vitamin B 0.15 mg L^{-1}, pH 7.0) at a concentration of 50 mg L^{-1} to confect the synthetic dyeing wastewater. C. I. Reactive Black 5, a commonly used commercial azo dye, was chosen for the screening of degradative bacteria to obtain a high-performance bacterial decolorizer. The other four reactive dyes with different chemical structures, including azo dyes (C. I. Reactive Red 180, C. I. Reactive Yellow 81) and anthraquinone dyes (C. I. Reactive Blue 19, C. I. Reactive Blue 49), were used to detect the decolorizing ability of strain EC3 against structurally different dyes.

2.2. Isolation and identification of decolorizing bacteria

Aliquots (10 g) of activated sludge collected from a textile mill in Xiamen, China, were inoculated into 100 mL of synthetic wastewater with Reactive Black 5, followed by incubation at 32 °C for 5 days under static conditions before being transferred to fresh synthetic wastewater. The process was repeated several times until the synthetic wastewater was decolorized. Then the samples were 10-fold serially diluted and 100 μL aliquots of each dilution were spread onto M9 agar plates with 50 mg L^{-1} Reactive Black 5. After 24 h of incubation at 32 °C, morphologically different colonies were streaked on M9 agar for purification before being inoculated into the synthetic wastewater to check their decolorizing ability. The bacterial strain with the strongest decolorizing ability, designated as EC3, was isolated and preserved at −70 °C in Luria-Bertani medium (LB) with 1.086 M glycerol.

Bacterial cells from cultures of strain EC3 were collected by centrifugation (5000 × *g* for 10 min) and subjected to sequential digestion by lysozyme (2.5 mg mL^{-1}, 37 °C for 1 h) and proteinase K (200 mg mL^{-1} in 0.035 M SDS, 55 °C for 1 h), followed by incubation in 0.027 M Cetyltrimethyl Ammonium Bromide (CTAB) and 0.7 M NaCl at 65 °C for 15 min. After extraction with phenol/chloroform, DNA was recovered by ethanol precipitation and then dissolved in ddH_2O [22]. The 16S rRNA gene was amplified by PCR in a 50 μL reaction system using primers 27F and 1492R under the following conditions: 1× Ex Taq Buffer, 0.2 mM of each dNTP, 0.2 mM of each primer and 1 U Ex Taq polymerase (TAKARA). An initial denaturing period of 5 min was followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and the final extension (72 °C) time was 10 min [22]. Then the PCR products were sent for sequencing (Invitrogen Biotechnology Co., Ltd.). The sequence was input into National Center for Biotechnology Information (NCBI) to identify it by Basic Local Alignment Search Tool (BLAST) search. Physiological characteristics were determined according to the procedures outlined in Bergey's Manual of Determinative Bacteriology [23].

2.3. Effects of different parameters on dye decolorization

The bacterial strain EC3 was cultured in LB broth at 150 rpm, at 37 °C for 12 h before the bacterial cells were collected by centrifugation (5000 × *g* for 5 min) and re-suspended in modified M9 broth.

The bacterial suspension was inoculated in 100 mL Erlenmeyer flasks containing 60 mL modified M9 broth to study the effect of aerobic conditions (150 rpm, 32 °C) and anaerobic conditions (in anaerobic workstations, 32 °C) on the decolorizing performance of the cultures. Anaerobic conditions were performed by incubation in a "bug box" anaerobic workstation (Ruskin Technologies) with mixed gas (N_2:H_2:CO_2 = 80:10:10, v/v).

Effects of various parameters, including initial glucose concentration (0, 1, 2, 4, 8 or 12 mg L^{-1}), dye concentration (25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, 500 mg L^{-1} and 1000 mg L^{-1}), temperature (22–42 °C with 5 °C interval) and initial pH (4.0, 6.0, 7.0, 8.0, 10.0, and 12.0), on dye decolorization were investigated. Experiments were performed in 15 mL glass tubes containing 10 mL medium, and all the pHs in the experiments were adjusted by NaOH and HCl (1 mol L^{-1}). The bacterial cell suspension was inoculated

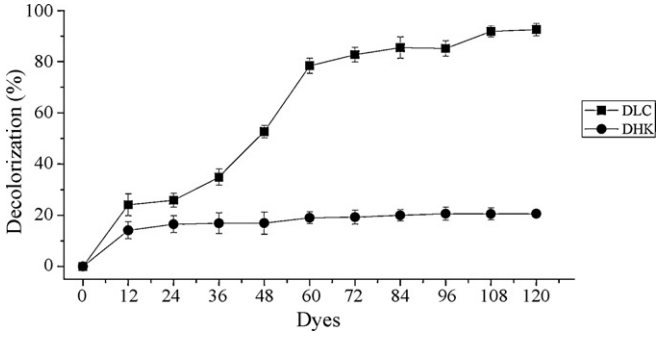

Fig. 2. Decolorization of Reactive Black 5 by living (DLC) and heat-killed (DHK) cells of *Enterobacter* sp. EC3 (DLC, decolorization by living cells; DHK, decolorization by heat-killed cells).

into the tubes with an initial optical density (600 nm) of 0.3. Each experiment was carried out in triplicate.

2.4. Measurement of decolorization efficiency

Samples (0.4 mL) were collected every 12 h and centrifuged at 8000 × *g* for 5 min. Decolorization efficiency was determined by measuring the absorbance of the culture supernatant at 610 nm using a Unicam UV9100-visible spectrophotometer. Decolorization efficiency was calculated using the following equation:

$$\text{Decolorization efficiency}\,(\%) = \frac{\text{OD}_1 - \text{OD}_t}{\text{OD}_1} \times 100$$

where OD_1 refers to the initial absorbance, OD_t to the absorbance after incubation; and t to the incubation time.

2.5. Decolorization mode of Enterobacter EC3 on Reactive Black 5

Decolorization of dyes may take place by adsorption [11] or degradation [24]. In the case of adsorption, dyes are only adsorbed onto the surface of bacterial cells, whereas new compounds come into being when dyes are degraded by bacterial enzymes during the degradation process. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other. If the dye removal is attributed to biodegradation, either the major visible light absorbance peak will completely disappear or a new peak will appear [25]. Dye adsorption can be also easily judged by an evidently colored cell pellet, whereas those retaining their original colors are accompanied by the occurrence of biodegradation [16].

A bacterial cell suspension was autoclaved at 121 °C for half an hour and then added into the medium and the decolorization efficiency tested. Bacterial cells without treatment were added into the medium as a control. After incubation for 48 h, samples from the control culture were centrifuged at 8000 × *g* for 5 min and the supernatant was scanned from 300 nm to 700 nm using a Unicam UV9100-visible spectrophotometer to detect any transformation of compounds in the medium.

3. Results and discussion

3.1. Isolation and identification of decolorizing bacteria

A bacterial strain, which had high decolorization ability against Reactive Black 5, was isolated from an area near the effluent of a textile and dyeing industry. Significant decolorization efficiency (78.4%) was observed after 60 h of incubation and a maximum value (92.6%) was achieved after incubation for 108 h (Fig. 2). The colony of bacterial isolate EC3 was circular, flat, smooth and yellow-white. EC3 was observed to be a Gram-negative rod and its cells were elongated and curved. Sequence analysis of 16S rDNA showed that strain EC3 had highest similarity with the species *Enterobacter cloacae* (99%). Based on the phenotypic characteristics and phylogenetic analysis, strain EC3 was identified as *Enterobacter*. The 16S rDNA sequence of *Enterobacter* sp. EC3 has been deposited in GenBank with the accession number EU780076.

3.2. Decolorization of Reactive Black 5 under aerobic and anaerobic conditions

Decolorization of Reactive Black 5 was more than 92% in anaerobic conditions and only 22% in aerobic conditions (150 rpm), while the growth of *Enterobacter* sp. EC3 was greater under aerobic as compared to anaerobic conditions (Fig. 3). This result suggested that *Enterobacter* sp. EC3 was a facultative anaerobe. Oxygen was favorable to the growth of the bacterium but deleterious to the yield process of the degradation related enzyme. The result accorded with the conclusion that none of these decolorizing bacteria are able to efficiently decolorize dyes under aerobic conditions [17,26]. Dissolved oxygen often inhibited the anaerobic decolorization of azo dyes, because oxygen was a preferable terminal electron receptor over the azo groups. During the dye reduction stage, if the extra-cellular environment is aerobic, the presence of high-redox-potential electron acceptor, oxygen, may inhibit the dye reduction mechanism [27].

3.3. Effects of initial dye concentration, initial glucose concentration, pH and temperature on decolorization

Addition of glucose enhanced the decolorization of Reactive Black 5 by *Enterobacter* sp. EC3 (Fig. 4a). However, lack of glucose inhibited the decolorizing activity of *Enterobacter* sp. EC3 since only 20.11% color removal was observed after 120 h incubation. In experiments with glucose supplementation, results suggested that 1 g L^{-1} glucose was enough for the decolorization although the latent period was 36 h. When the glucose concentrations were 2 g L^{-1} and 4 g L^{-1}, *Enterobacter* sp. EC3 exhibited strong decolorizing activity with about 90% decolorization efficiency in 48 h. With increased glucose concentration (12 g L^{-1}), the "latent period" and low decolorization efficiency reoccurred just like the result achieved without glucose (0 g L^{-1}). The reason why the decolorization efficiency was low when no glucose concentration was added may be that without glucose could not meet the growth requirements of the bacteria. When the glucose concentration was much higher, such as 8 g L^{-1} and 12 g L^{-1}, the bacteria could utilize glucose preferentially, thus resulting in the "latent period" or lower decolorization efficiency. Two opinions have been argued for many years: one deems that dyes are not a carbon source since the anaerobic bacteria obtain energy from the glucose instead of the dyes and glucose can enhance the decolorizing performance of biological systems [28]; while another deems that glucose can inhibit the decolorizing activity [16]. The variability may be due to the different microbial characteristics involved. Our results showed that a certain concentration of carbon source (such as glucose) was necessary for the *Enterobacter* sp. EC3 decolorizing process. Effects of some other carbon sources on bacterial decolorization performance have been studied in former researches. Lactate, peptone, succinate, yeast extract, and formate were proved to enhance decolorization, while sucrose, and dextrin resulted in lower decolorization activities [29].

The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye at higher concentrations, and the ability of the enzyme to recognize the substrate efficiently at the very low concentrations [27]. In our experiments, with increase of the initial dye concentration, the decolorization efficiency over the same time interval

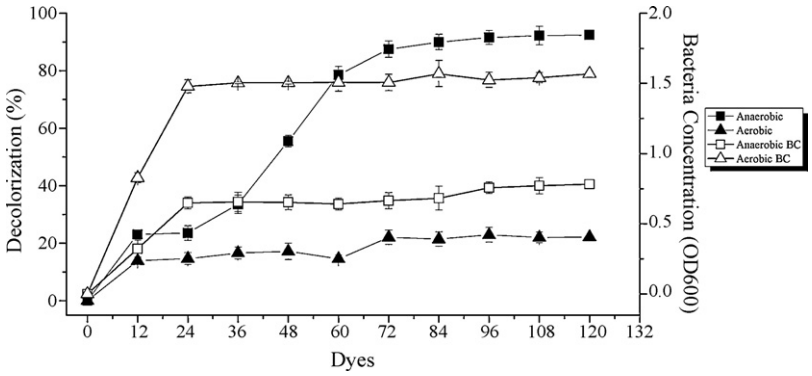

Fig. 3. Growth of *Enterobacter* sp. EC3 cultures at OD600, and dye decolorization in aerobic and anaerobic conditions (aerobic, dye decolorization in aerobic conditions; anaerobic, dye decolorization in anaerobic conditions; aerobic BC, bacterial concentration in aerobic conditions; anaerobic BC, bacterial concentration in anaerobic conditions).

decreased. When the effect of different initial dye concentrations of Reactive Black 5 on decolorization was observed using 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1} and 500 mg L^{-1}, the required times to reach a maximum decolorization efficiency were 72 h, 108 h, 108 h, 108 h and 120 h, respectively (Fig. 4b). It is reported that dye decolorization can be strongly inhibited when a high concentration dyestuff is used to examine the poisonous effect of the dye on the degrading microorganisms [26,30]. To accurately appraise the decolorizing ability of *Enterobacter* sp. EC3, decolorization was investigated by assessing the amount of dyes decolorized. Although the decolorization efficiency was only 35.63% at 1000 mg L^{-1} dye after 120 h incubation, 356.3 mg dye had been degraded, only 44 mg less than the decolorization at 500 mg L^{-1}. This result indicated that *Enterobacter* sp. EC3 showed high decolorizing performance even in high initial dyestuff concentrations.

Our experiments indicated that *Enterobacter* sp. EC3 grown on synthetic dyeing wastewater containing Reactive Black 5 showed a high decolorization rate (92.6%) at pH 7.0 after 108 h of incubation. This could be due to the fact that the optimum pH for the growth of *Enterobacter* sp. EC3 was neutral. Similar decolorization efficiency was observed from pH 8.0 to pH 12.0 in 120 h, whereas the rate of color removal was much lower at acidic (pH 4.0 and 6.0) conditions (Fig. 4c). pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0 [16,31,32]. The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperatures [33]. It should be mentioned that the pH of the wastewater samples used for isolation of *Enterobacter* sp. EC3 was about 8–9. The fact that *Enterobacter* sp. EC3 could decolorize reactive dyes in a relatively wide range of pH, make it suitable for practical bio-treatment of dyeing mill effluents.

It was noticed that with an increase in temperature from 22 °C to 37 °C the decolorization rate increased and a further increase in temperature to 42 °C drastically affected decolorization activity of *Enterobacter* sp. EC3 (Fig. 4d). The optimum temperature for decolorization was found to be 37 °C. Although a lag phase was observed and the decolorization rate was comparatively low at 22 °C, the decolorization efficiency increased to a similar level from 27 °C to 37 °C. Decolorizing activity was significantly suppressed at 42 °C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 42 °C [34,35].

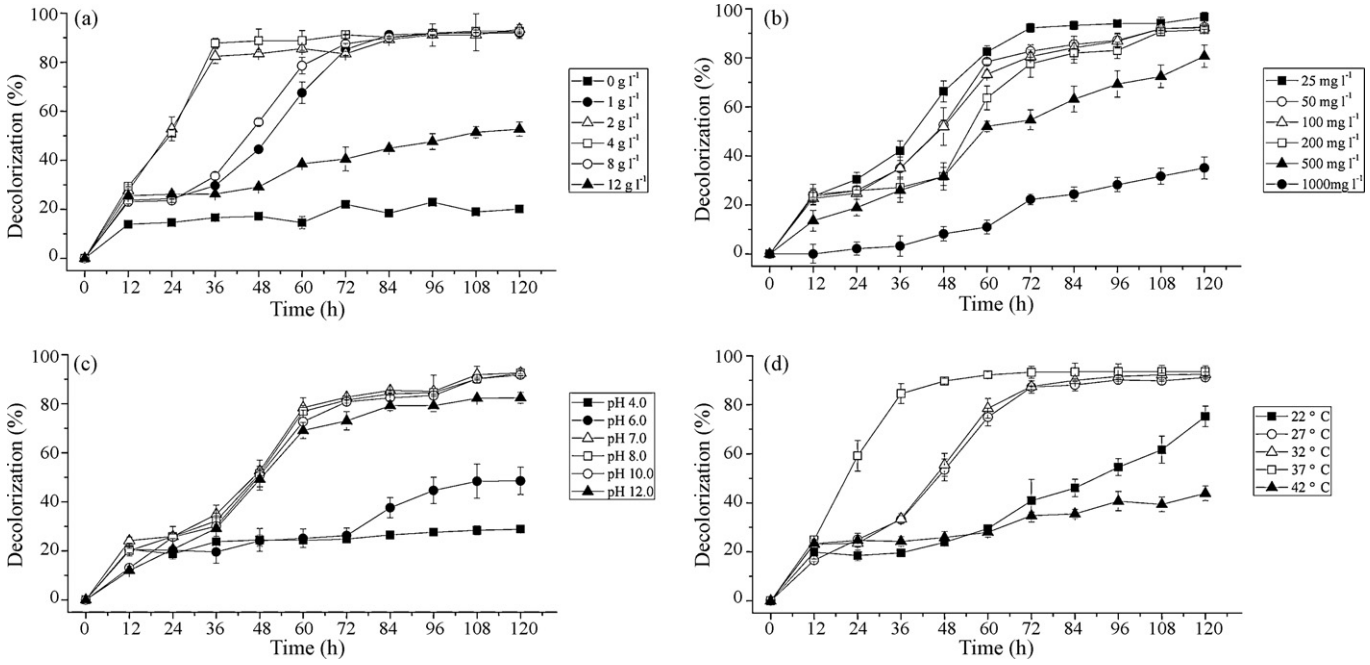

Fig. 4. Effect of initial glucose concentration (a), dyes concentration (b), pH (c) and temperature (d) on decolorization of Reactive Black 5.

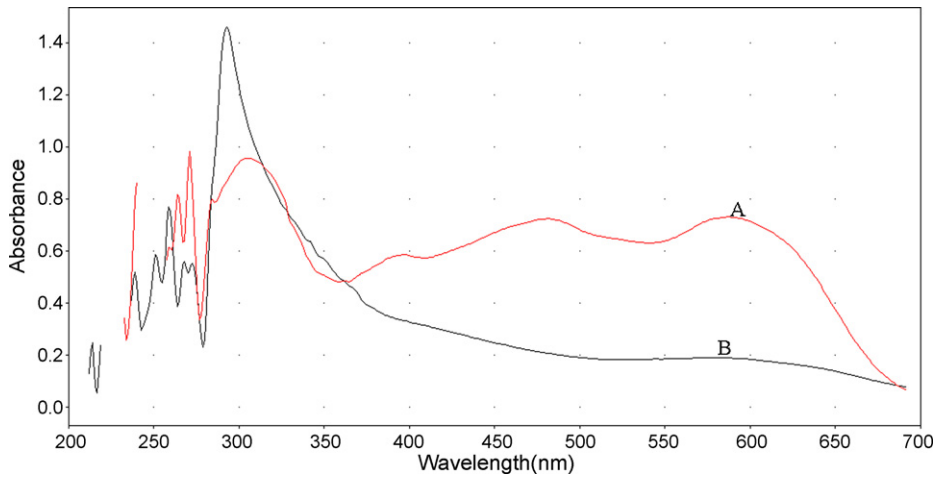

Fig. 5. Variation in the UV–vis spectra of Reactive Black 5 before and after decolorization by *Enterobacter* sp. EC3 (A, 0 h; B, 48 h).

3.4. Decolorization of various textile dyes

Industrial effluent consists of a mixture of various dyes. Ability of *Enterobacter* sp. EC3 to decolorize different dyes was studied. The five structurally different reactive dyes used in this research were all efficiently decolorized by *Enterobacter* sp. EC3 in 120 h. A maximum decolorization efficiency of 92.56% was recorded in Reactive Black 5, and for the other four dyes, the value varied from 67.68% to 89.73%. The decolorization efficiency against all three kinds of azo dyes was over 80%, and the decolorization efficiency against the two anthraquinone dyes was about 70%. To our knowledge, this is the first time that a bacterial strain of *Enterobacter* sp. was reported to have decolorizing ability against both azo and anthraquinone dyes. The variation in the decolorization of different dyes might be attributable to the structural diversity of the dyes [36]. It is believed that anthraquinone dyes are more recalcitrant than azo dyes [37]. The decolorization efficiency of *Enterobacter* sp. EC3 against all the reactive dyes tested in this study was up to 68%, which suggested that *Enterobacter* sp. EC3 could be used to decolorize complex dyestuff effluent containing various reactive dyes.

3.5. Decolorization mode of Enterobacter sp. EC3 against Reactive Black 5

In the cultures with addition of heat-killed bacterial cells, only 20.42% decolorization efficiency was recorded after 120 h incubation, which might be due to the adsorption by dead bacterial cells, and this was also confirmed by the presence of colored cell pellets. In the control culture, 92.56% decolorization efficiency was achieved in 120 h (Fig. 2) and the cell pellets were not pigmented. Meanwhile, a UV–vis spectral scan (200–700 nm) of supernatants after decolorization showed that the maximum absorbance wavelength shifted from 610 nm to 290 nm in control cultures (Fig. 5). The great changes occurring both in UV and visible spectra indicated that the molecular structure of Reactive Black 5 changed evidently after decolorization. The black color of Reactive Black 5 was caused by the conjugated structure of azo bonds (chromophore) and amino group. It could be presumed that the azo bonds cleaved during the reaction which indicated that the primary chromophore was destroyed. The absorbance peak at UV spectra did not disappear in the end of decolorization, which indicated that Reactive Black 5 was not completely mineralized while some new metabolites formed in the culture

These results provided obvious evidence of biodegradation of reactive dyes by *Enterobacter* sp. EC3 in the decolorization process, and also supported the earlier conclusion that decolorization by bacteria is mainly due to biodegradation, rather than inactive surface adsorption [18,38,39].

4. Conclusions

In this study, a decolorizing bacterial strain, *Enterobacter* sp. EC3, was isolated from activated sludge. The optimal conditions for the decolorizing activity of *Enterobacter* sp. EC3 were anaerobic conditions with glucose supplementation, at pH 7.0, and 37 °C. The maximum decolorization efficiency against Reactive Black 5 achieved in this study was 92.56%. *Enterobacter* sp. EC3 showed decolorizing activity through a degradation mechanism rather than adsorption, and it could tolerate high concentrations (up to 1000 mg L^{-1}) of Reactive Black 5. Because of high degradative and decolorizing activity against various reactive dyes (including both azo and anthraquinone dyes) commonly used in the textile industries, it is proposed that *Enterobacter* sp. EC3 has a practical application in the biotransformation of various dye effluents.

Acknowledgements

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