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Decolorization of azo dyes by free and immobilized bacterial-fungal consortium QM

Liang Yin*, Jiaxian Feng, Jiayin Ye, Chaofeng Zhu

School of Life Science, South China Normal University, Guangzhou 510630, China, Tel./Fax: +86-20-85211372; emails: yliang@scnu.edu.cn (L. Yin), 755628752@qq.com (J. Feng), 929583304@qq.com (J. Ye), dfd3000@tom.com (C. Zhu)

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ABSTRACT

A developed bacterial-fungal consortium QM consisting of two strains were isolated from textile effluent and identified as *Geotrichum candidum* and *Bacillus cereus*. The study of decolorization of azo dyes showed that mixed consortium QM had higher decolorization efficiency than individual strain in a wide range of pH, temperature, dye concentrations and salinity. Decolorization rate of different azo dyes by consortium QM reached above 80% in 24 h. Consortium QM enhanced the decolorization efficiency of azo dye due to synergistic effect between the two strains, which was related to oxidation/reduction potential (ORP) decrease caused by *Geotrichum candidum*. The UV-Vis and HPLC analysis revealed that azo dye was degraded by the consortium QM. Furthermore, the consortium QM immobilized in alginate beads and nylon sponges was used for repeated-batch and continuous decolorization. It was observed that over 60% azo dyes were removed by immobilized consortium after five cycles of repeated batch decolorization. Immobilized consortium QM could decolorize average 80% dyes in the packed-bed bioreactor with hydraulic retention time (HRT) of 25 h during 20 d of continuous operation. These results showed that consortium QM has a potential for the application of textile effluent decolorization.

Keywords: Bacterial-fungal consortium; Azo dye; Decolorization; Immobilization

1. Introduction

Synthetic dyes are widely used in several industries including textile dyeing, paper printing, color photography, pharmaceutics and cosmetics. Most of them are azo dyes which are aromatic compounds with one or more azo groups (–N=N–) [1]. It is estimated that l0%–15% of the dyes are released into the environment during their synthesis and dyeing processes [2]. The strong color of azo dyes even at very small concentrations has a huge influence on the aquatic ecosystem [3]. In addition, many azo dyes and their degradation products are carcinogenic and mutagenic to human and animals. Thus, the dye removal of the effluent has become a major environmental concern [4,5].

Compared with conventional physical and chemical dye removal methods, microbial decolorization of azo dyes has recently received much attention as it is cost-effective, environmentally friendly, and it produces less sludge [6,7]. The adaptability and the activity of selected microorganisms play an important role in the effectiveness of microbial decolorization. Many microorganisms, including bacteria, fungi, even yeasts, have been found to be able to decolorize azo dyes by bioadsorption or degradation [8]. Fungal enzymes are non-specific towards different structures of dyes and thus oxidize a wide range of them. The bacterial biodegradation to azo dyes is associated with its intracellular and extracellular oxidoreductive enzyme system such as azoreductase. Mixed consortium can provide advantages over pure strain as they involve the combined and inductive effects of various enzymes which can work synergistically [9–13]. Some reports proved synergistic action of bacterial-fungal consortium enhances degradation and detoxification of contaminants [14,15]. However, literatures on the degradation of azo dyes by fungal-bacterial consortium are few. It is required to develop different fungal-bacterial

*Corresponding author.

consortium for decolorization and degradation of azo dyes. In industrial operations, immobilized microbial cell systems could provide additional advantages over freely suspended cells such as good operation stability, high cell density, reuse of the biomass, much more tolerance to toxic chemicals of high concentrations and higher efficiency of biodegradation per unit volume of the reactor [16,17]. Immobilized microbial systems have valuable application in effluent treatment. However, most of the microbial immobilization for dye decolorization focused on a single strain of bacteria or fungus and the immobilization of bacterial-fungal consortium is missing.

In the present study, a fungal–bacterial consortium QM consisting of *Geotrichum candidum* and *Bacillus cereus* was developed for decolorization of azo dyes. The relationship between decolorization efficiency and ORP (oxidation/redox potential) was explored during decolorization. The environmental factors of decolorization by this consortium, including pH, temperature, agitation speed, salinity and dye concentrations were investigated. The intermediate metabolites of decolorization of azo dye were analyzed using UV-Vis and HPLC. In addition, immobilization of bacterial-fungal consortium QM was investigated. Bacteria Q1 and fungus M3 were immobilized in alginate beads and nylon sponges, respectively. Repeated-batch and continuous decolorization assays were carried out to evaluate the potential of continuous operation of immobilized consortium.

2. Materials and methods

2.1. Materials

Azo dyes including Direct Navy Blue R (DNBR), Direct Red 156 (DR156), Direct Blue 25 (DB 25), Acid Yellow 36 (AY 36), Reactive Red 180 (RR180) in commercial purity were obtained from a textile dying plant in Guangzhou, China. DNBR is a model azo dye for decolorization experiments. All the other chemical regents are of analytical grade.

2.2. Isolation and identification of decolorizing microorganisms

The sample was inoculated in flasks containing nutrient medium for 24 h. The culture was transferred to decolorization medium (2 g Glucose, 1.0 g NH_4Cl, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4$, 1.0 g NaCl and 0.1 g azo dye per liter) for 48 h. The decolorized culture was plated on the nutrient agar plate and incubated at 30°C for 48 h. Mixed consortium QM with remarkable ability to decolorize azo dye was found. Two microorganisms, bacterial strain Q1 and fungal strain M3, were isolated and purified from mixed consortium. Bacterium Q1 was identified by its morphology, biochemical characteristics and 16S rDNA gene. Fungus M3 was identified by its morphology and 18S rDNA gene.

2.3. Microorganisms and culture conditions

Two strains were isolated in our laboratory. The pure cultures of isolates were maintained at 4°C on nutrient agar slants. The bacterial strain was precultured in nutrient medium (beef extract 3 g, peptone 10 g, NaCl 5 g, pH 7.0 per liter) at 30°C and 180 rpm for 24 h. Cells were harvested by centrifugation, washed with 0.1 M phosphate buffer (pH 7.0), and resuspended in 0.1 M phosphate buffer (pH 7.0) as inoculum (OD_{600} = 0.7~0.8). The fungal strain was precultured in yeast peptone dextrose (YPD) medium (yeast extract 3 g, peptone 5 g, dextrose 3 g per liter) at 30°C and 180 rpm for 48 h. The mycelium of fungus was collected by filtration after 2-d culture, and then washed three times with 0.1 M phosphate buffer (pH 7.0) as inoculum.

2.4. Decolorization assays by single strain and mixed consortium

Bacterial suspension (5% v/v), fungal mycelium (wet weight 5 g 100 mL^{-1}) and mixed consortium including bacterial suspension (2.5% v/v) and fungal mycelium (wet weight 2.5 g 100 mL^{-1}) were inoculated into decolorization medium, respectively. After inoculation, decolorization experiments were performed at 30°C under static condition for 48 h. The ORP of decolorization medium were measured during 48 h of decolorization. The samples were withdrawn at different time intervals, centrifuged at 10,000 rpm for 10 min and analyzed for decolorization rate. Decolorization rate was determined by measuring absorbance of culture supernatants at absorbance maxima of respective dyes.

$$\text{Decolorization rate (\%)} = (A_0 - A_1) \times 100 / A_0$$

where A_0 indicates the absorbance of the dye before decolorization and A_1 indicates the absorption of the dye after decolorization. The decolorization experiments were repeated three times. The mean decolorization rates were calculated.

2.5. Effects of environmental conditions on decolorization

Effects of different environmental conditions, including pH (4–10), temperature (20°C–45°C), dye concentration (50–600 mg L^{-1}), salt concentration (1%–10%) and agitation speed (0–300 rpm) on decolorization by mixed consortium were investigated. The decolorization rate was measured after 24 h of decolorization. All the experiments were performed in triplicate and the mean values of the data were presented.

2.6. UV-Vis and HPLC analysis

The decolorization medium were withdrawn every 24 h during the decolorization of azo dye DNBR by mixed consortium, then centrifuged (10,000 rpm, 10 min) and the supernatant obtained was scanned by UV-Vis spectrometer. The analysis results were compared with that of the control dye. The metabolites were extracted from the supernatant using equal volume of ethyl acetate, dried over anhydrous Na_2SO_4, and evaporated to dryness through rotatary vaccum evaporator. The residue was dissolved in methanol for HPLC. HPLC experiments were carried out on a C18 column. The mobile phase consisted of water and methanol in the ratio of 60:40. The flow rate of mobile phase was 1.0 mL min^{-1}. The samples were filtered with a 0.2-μm membrane filter and about 10 μL of the filtered samples was injected into the HPLC.

2.7. Immobilization

The bacterial strain Q1 was incubated in nutrient medium for 24 h. Cells were harvested at 4°C, 10,000 rpm. Wet cells were mixed with sterile sodium alginate with 2% weight to volume (w/v) ratio, dropped into 0.2 M $CaCl_2$ solution with a syringe to form particles (beads) of 2–3 mm in diameter. These particles (beads) were kept in $CaCl_2$ solution for 12 h. For immobilization of fungal strain M3, 10 nylon sponge cubes (1 cm^3) were placed in a 250 mL Erlenmeyer flask containing spores freely suspended in 100 mL YPD medium and incubated in a rotary shaker at 30°C for 3 d. After fungi colonized the carries, immobilized fungi were transferred aseptically into Erlenmeyer flask containing physiological water and stored at 4°C for decolorization.

2.8. Repeated-batch decolorization by immobilized consortium

Repeated-batch decolorization assays were carried out to evaluate continuous decolorization ability of the immobilized consortium. 1.5 g immobilized bacteria Q1 and 2 g immobilized fungi M3 were added into decolorization medium containing 100 mg L^{-1} azo dyes (Fig. 1). Decolorization experiments were carried out at 30°C under static condition. After 2 d of decolorization by immobilized consortium, decolorization medium was decanted and decolorization rate was measured. Immobilized cells were rinsed with sterile water and then transferred into a fresh decolorization medium. The same process was repeated several times. SEM images of immobilized consortium were observed before decolorization and after four decolorization cycles.

2.9. Continuous decolorization in the packed-bed bioreactor by immobilized consortium

The capacity of immobilized consortium to decolorize azo dyes was studied in the packed-bed bioreactor at laboratory scale. A glass-made packed-bed reactor of a total volume of 250 mL and an internal diameter of 5 cm was used, while 60% of the reactor's total volume was filled with calcium alginate-immobilized bacteria and nylon sponge-immobilized fungi (3:4 mass ratio) (Fig. 1). Decolorization medium containing 100 mg L^{-1} azo dye DNBR was fed constantly through the reactor. The flow rate was maintained at 10 ml h^{-1}, with hydraulic retention time (HRT) of 25 h. The bioreactor was operated in thermostatically regulated room at 30°C. At 24-h interval, samples were taken periodically from the outlet of the reactor and centrifuged at 10,000 rpm for 15 min. The supernatants were analyzed spectrophotometrically at 571 nm to determine the amount of dyes. SEM images of immobilized consortium were observed after 20 d of operation.

3. Results and discussion

3.1. Isolation and identification of decolorizing microorganism

A decolorizing bacterium and a decolorizing fungus were isolated from textile effluent by enrichment culture and isolation technique. Bacterial strain Q1 was Gram-positive and rod-shaped. The cells were 0.5–1.0 μm wide and 1–2 μm long. When grown at 37°C for 24 h on nutrient agar plate, colonies were round, smooth, and approximately 2–3 mm in diameter. Sequence analysis of its 16S rDNA gene showed that strain Q1 was most closely related

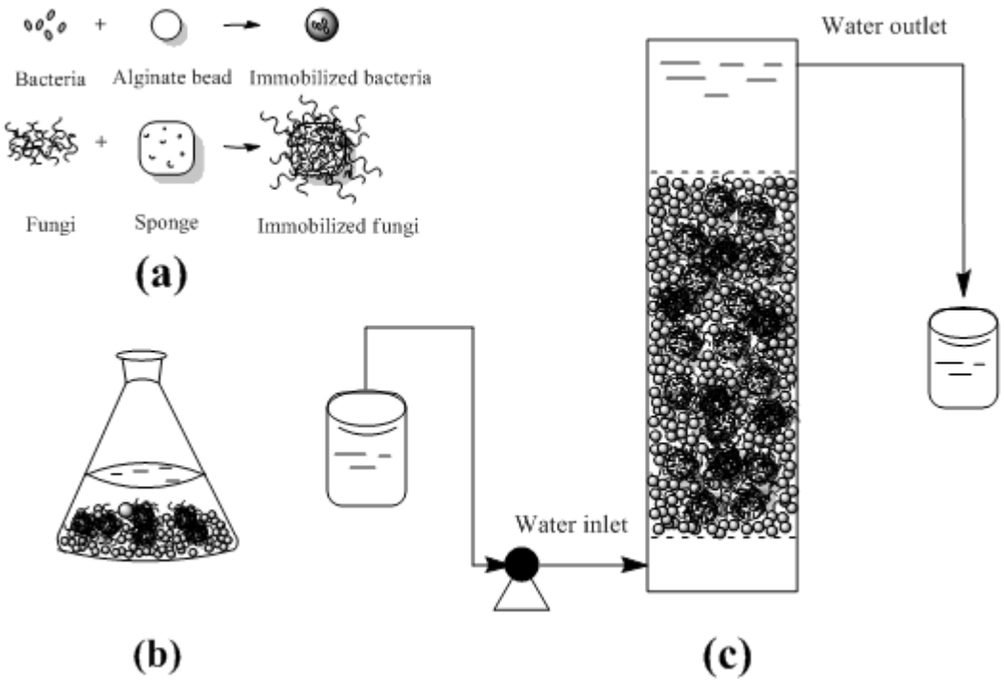

Fig. 1. Schematic illustration of the bioreactor system: (a) immobilization of bacteria and fungi; (b) repeated-batch decolorization; (c) continuous decolorization in the packed-bed bioreactor contained immobilized bacterial-fungal consortium.

to *Bacillus cereus* (Fig. 2). The shape of fungal colonies and its 18S rDNA sequence indicated that fungal strain M3 was closely related to *Geotrichum candidum* (Fig. 3). Thus, strain Q1 and strain M3 were identified as *Bacillus cereus* and *Geotrichum candidum*, respectively (GenBank No. GQ280380 and EU616671, respectively).

3.2. Decolorization by individual strain and mixed consortium

Decolorization by individual strain and mixed consortium was performed at 30°C, pH 7.0 under static condition in decolorization medium containing 100 mg L^{-1} azo dye for 2 d. As shown in Fig. 4, consortium QM consisting of fungus M3 and bacterium Q1 decolorized 94.4% in 24 h and 98.6% dyes in 48 h, whereas individual strain M3 decolorized 11.3% in 24 h and 18.5% dyes in 48 h, and strain Q1 decolorized 38.5% dyes in 24 h and 45.7% in 48 h. The results showed that the consortium QM had better decolorization ability than individual strains. Fig. 5 presented the results of decolorization of azo dyes with different structures by mixed consortium. Mixed consortium was able to decolorize 85% of these azo dyes in 24 h, suggesting mixed consortium had strong decolorization ability for different azo dyes. Some researchers have reported such synergistic effect between different microorganisms on decolorization. It was pointed out that coculture of several microorganisms had more advantages than single strain on chemical pollutions biodegradation [15,18–20].

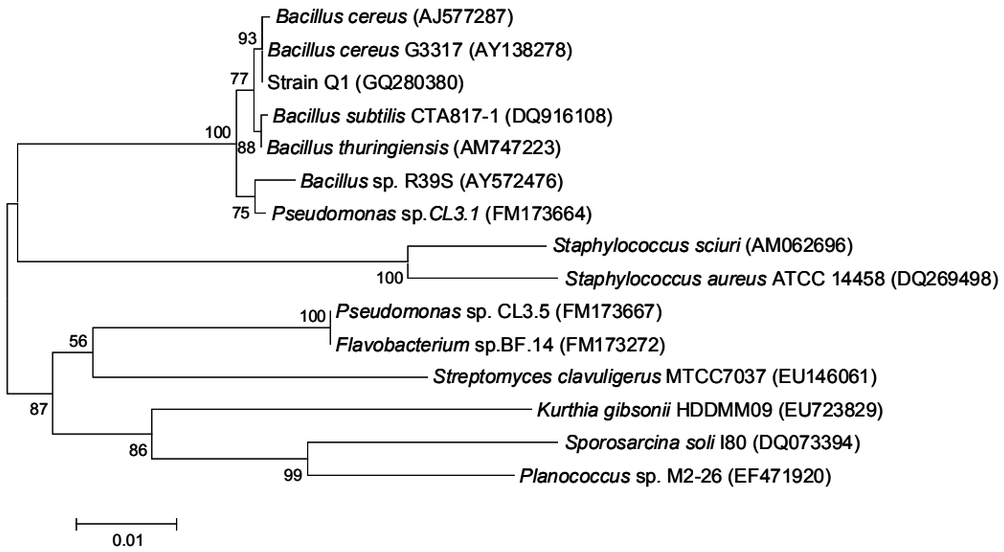

Fig. 2. Phylogenetic tree of strain Q1 and related species constructed on basis of 16S rDNA sequences.

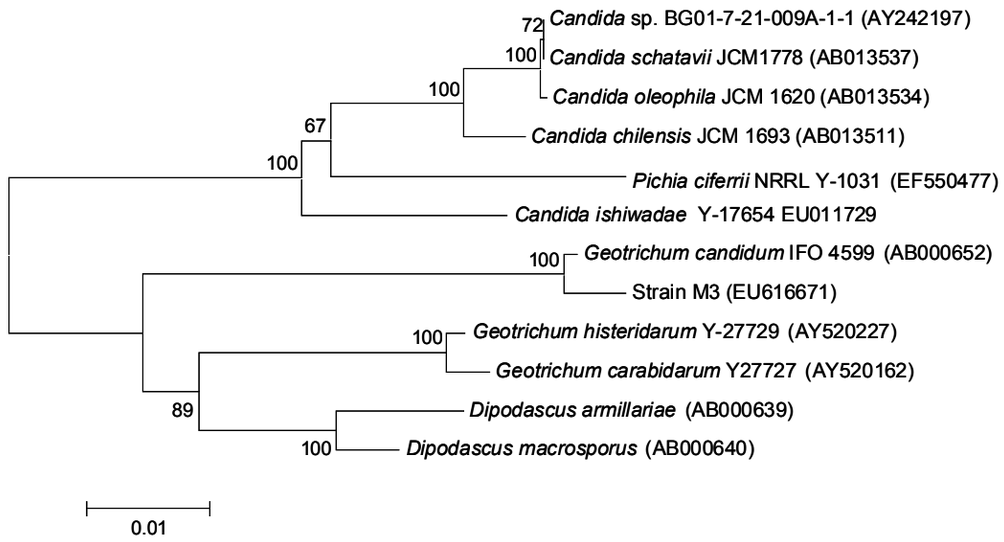

Fig. 3. Phylogenetic tree of strain M3 and related species constructed on basis of 18S rDNA sequences.

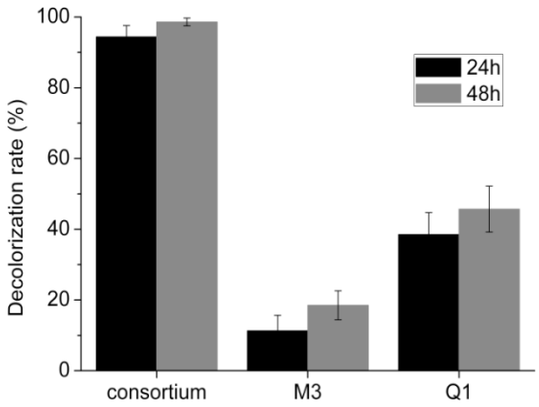

Fig. 4. Decolorization of azo dyes by individual strain and mixed consortium.

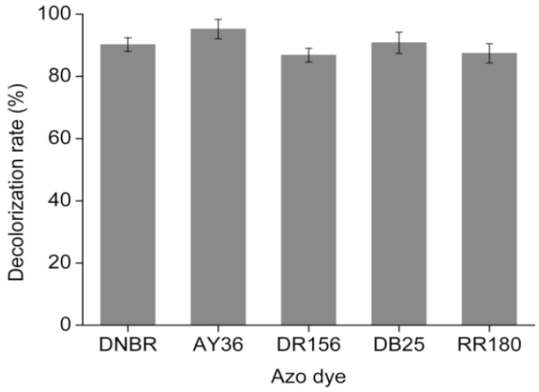

Fig. 5. Decolorization of azo dyes of different structure by mixed consortium.

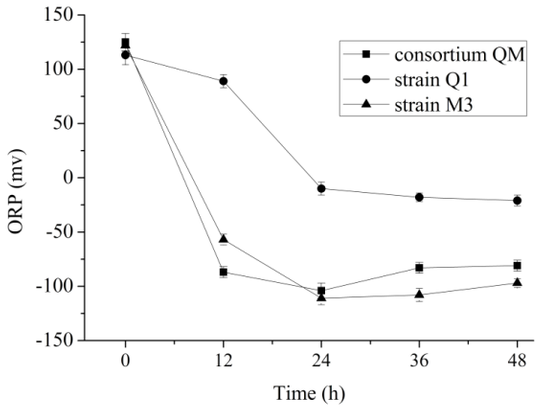

Fig. 6. ORP during decolorization of azo dyes by individual strain and mixed consortiums.

Fig. 6 showed changes of ORP during 48 h of decolorization by individual strain and mixed consortium. ORP of bacterium Q1, fungus M3 and mixed consortium was 89 mv, 111 mv and 104 mv after 12 h decolorization, and after 24 h of decolorization, it was 10, –111 and –103 mv, respectively, suggesting strain M3 can significantly decrease ORP in coculture of strain Q1 and strain M3. Some literatures reported that low ORP condition was beneficial to decolorization of azo dye by bacteria [21,22]. Synergistic effect of decolorization by coculture of two strains was probably because low ORP environment caused by strain M3 enhanced decolorization ability of strain Q1. But Zhou et.al [23] considered that the synergistic effect of fungi and bacteria for decolorization was probably that the dye was easier to transfer into their cells for further degradation due to fungal adsorption of azo dye. The further research for synergistic effect on decolorization by mixed consortium QM was required.

3.3. Effects of environmental conditions on decolorization

Effect of temperature on dye decolorization by mixed consortium was shown in Fig. 7(a). The results indicated that temperature had a great influence on microbial decolorization of azo dye. The decolorization rate of azo dye by consortium QM reached 23.8% at 20°C and 94.3% at 30°C. Consortium QM could still remain high decolorization efficiency (over 80%) at temperatures ranging from 25 to 45°C. However, decolorization efficiency decreased sharply when temperature was less than 25°C or more than 45°C.

Effect of pH on decolorization of azo dye by mixed consortium was shown in Fig. 7(b). The effect of pH on decolorization activity of consortium QM was also significant. Consortium QM was able to efficiently decolorize azo dye at a wide range of pH (6–10). The maximum decolorization rate (93.8%) was achieved by mixed consortium at pH 7. However, there was a sharp decrease in decolorization ability at pH below 6 or above 10. Mixed consortium had less sensitivity towards changes in pH than individual strain. The result was similar to previous reports [12,24].

Effect of oxygen on cell growth and microbial decolorization is one of the most critical factors to be considered. As shown in Fig. 7(c), decolorization rate of consortium QM reached highest in the static condition. With increase in agitation speed, decolorization efficiency of mixed consortium QM decreased, suggesting that oxygen inhibited decolorization of consortium QM. The presence of oxygen could increase ORP in the decolorization medium and lead to decrease of decolorization efficiency. The result was in accordance with ORP investigation (Fig. 6). Bacterial decolorization was due to the cleavage of the azo bond (–N=N–) under the reducing conditions [25].There is the probability that oxygen inhibits the enzymatic reduction of azo bond, since aerobic condition may prevent the electron transfer from NADH to azo bonds [26].

Considering presence of salt in dying effluent, effect of salinity on decolorization by mixed consortium was studied at NaCl concentrations from 1% to 10%. As shown in Fig. 7(d), the highest decolorization efficiency (90.5%) was achieved by mixed consortium at 1% of NaCl concentration. Decolorization rate by mixed consortium decreased gradually with increase of NaCl concentration. This was probably attributed to the decrease of enzyme activity and high osmotic pressure under high saline condition [27,28]. More than 80% dyes were decolorized by consortium QM at less than 6% of NaCl concentration. The results showed that mixed consortium had a certain salt tolerance on decol-

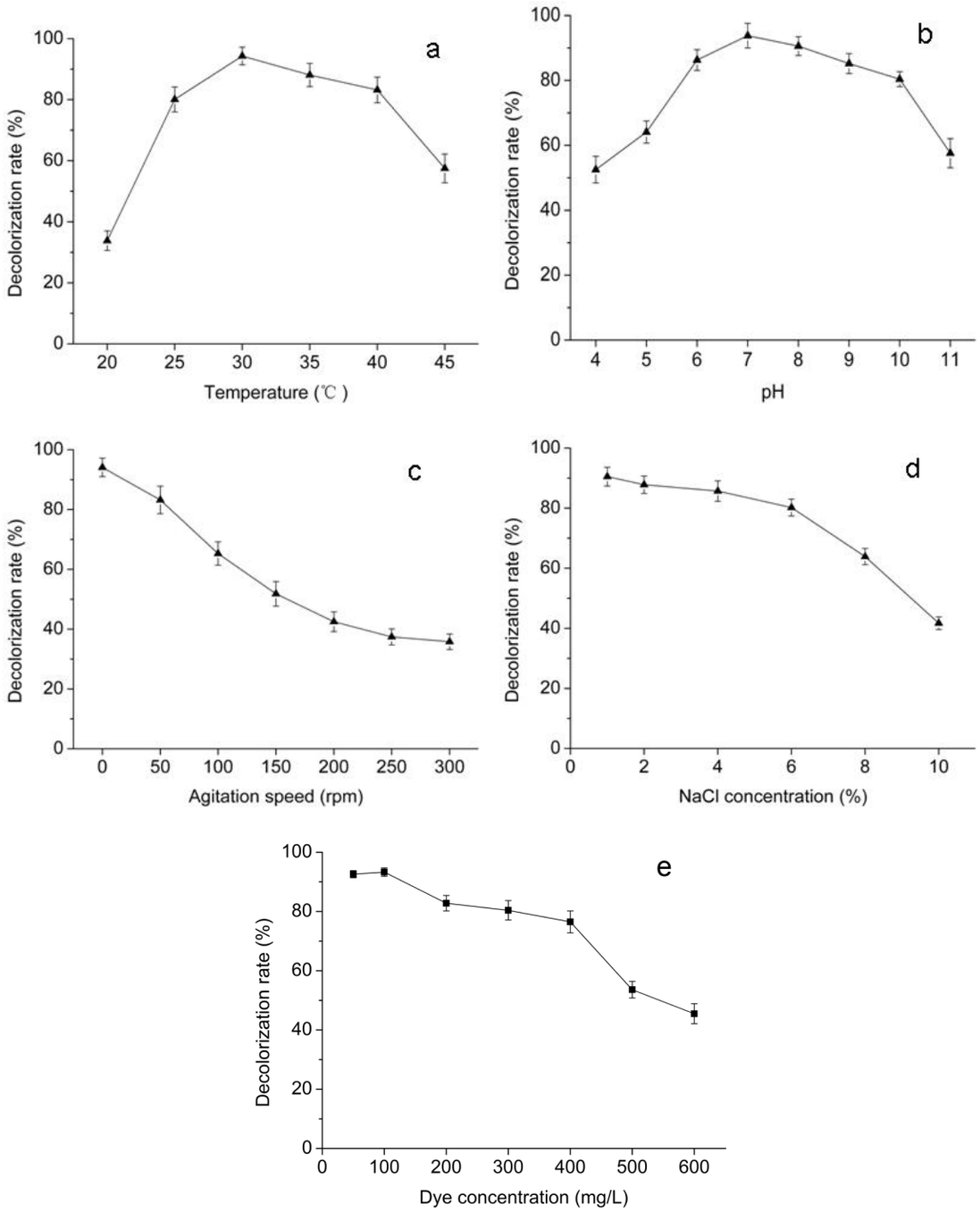

Fig. 7. Effects of environmental conditions on decolorization by mixed consortium: (a) temperature; (b) pH; (c) agitation speed; (d) salinity; (e) dye concentration.

orization, suggesting potential for decolorization of effluent containing salt.

Decolorization for azo dyes ranging from 50 to 500 mg L^{-1} by mixed consortium after 24 h was shown in Fig. 7(e). Strain M3 and strain Q1 could not decolorize any azo dye at presence of 400 mg L^{-1} azo dye (data not shown), whereas 76.5% decolorization rate was achieved by mixed consortium at the same concentration. The decolorization rate of mixed consortium reached 45.5% at 500 mg L^{-1} dye concentration, suggesting that high concentration dyes inhibit decolorization of mixed consortium due to toxicity of azo dye or metabolic products to cells [29]. Mixed consortium QM had a certain decolorization capability of effluent containing high concentration azo dye.

3.4. UV-Vis and HPLC analysis

The decolorization of dye by mixed consortium was monitored spectrophotometrically. Fig. 8 showed the change of UV–Vis absorption spectrum of dye with increasing decolorization time. UV–Vis spectral analysis of Direct Navy Blue R showed the major visible light absorbance peak at 571 nm. The absorbance of samples withdrawn after decolorization by mixed consortium decrease gradually. After 2 d of decolorization, the visible light absorbance peak for the dye at 571 nm almost disappeared and new absorbance peak appeared at 261 nm. According to Asad et al. [30], decolorization of dyes by microorganisms could be due to adsorption, or degradation by microbial cells. In case of adsorption, the UV–Vis absorbance peaks decrease relatively in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or new peaks appear. UV–Vis spectral results confirmed that decolorization dye was due to degradation by mixed consortium, whereas not adsorption. Decolorization experiment using inactive bacterial-fungal consortium in the same condition proved few adsorption of dyes by microbial cells (data not shown). The result of HPLC analysis of the control dye was shown in Fig. 9(a). A single major peak for Direct Navy Blue R appeared at a retention time of 1.81 min. After 2 d of the decolorization, the HPLC chromatogram showed almost disappearance of the single peak for the control dye at 1.81 min and two new peaks at 1.23 and 1.54 min (Fig. 9(b)). After 5 d of decolorization, the peaks at 1.23 and 1.54 min decreased obviously and a serial of new peaks appeared at 0.98, 0.81 and 0.75 min (Fig. 9(c)). The new peaks indicated the production of certain metabolites, confirming the degradation of Direct Navy Blue R by mixed consortium. Previous studies indicated that decolorization of azo dye by bacteria was attributed to reductive cleavage of azo compounds to aromatic amines in anaerobic conditions, which were difficult to be mineralized by bacteria but could be further degraded by fungi due to ligninolytic enzymes [31–33]. Consortium QM could achieve partial or complete mineralization of azo dye, suggesting great importance in the treatment of dying effluent since aromatic amines produced with the cleavage of azo bond were supposed to be recalcitrant and cause secondary pollution [34].

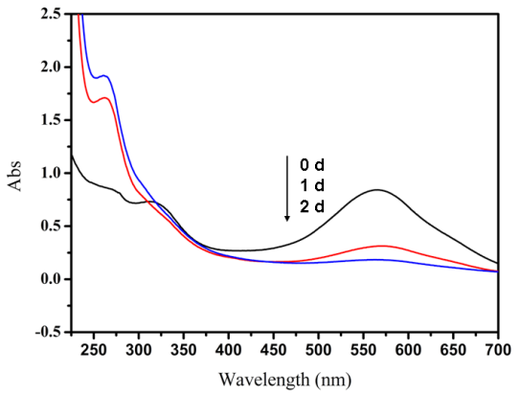

Fig. 8. UV–Vis spectrum of decolorization for Direct Navy Blue R by bacterial-fungal consortium at different decolorization times: 0, 1 and 2 d.

3.5. Repeated batch decolorization by immobilized consortium

Immobilized microorganism has many advantages compared with conventional free cells. Immobilized microorganism was widely applied in the field of effluent treat-

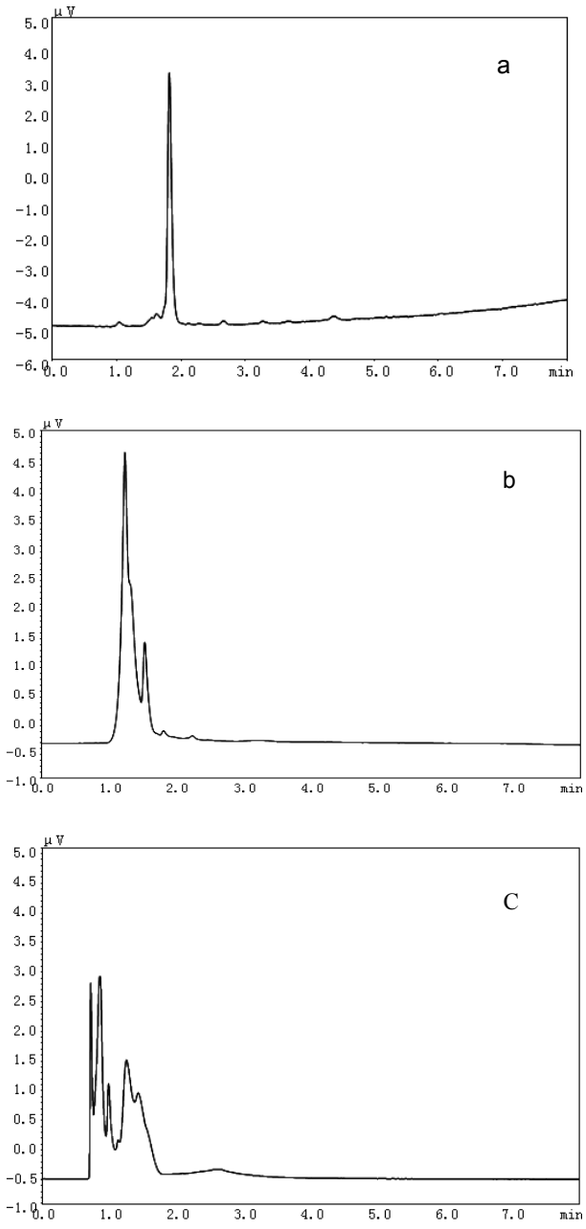

Fig. 9. HPLC analysis of metabolites of dye decolorization by bacterial-fungal consortium: (a) the control dye Direct Navy Blue R; (b) degradation products of the dye after 3rd day of decolorization; (c) degradation products of the dye after 5th day of decolorization.

ment [16,17,35]. In order to evaluate the potential for the continuous decolorization of immobilized consortium, repeated-batch decolorization experiments were performed. As shown in Fig. 10, decolorization rate of immobilized consortium could reach over 90% in the first cycle and second cycle. In the fourth batch, 78.2% dyes were decolorized by immobilized consortium. After 5 runs, decolorization efficiency of immobilized consortium still remained over 60%, suggesting immobilized consortium had good continuous decolorization ability. In SEM images (Figs. 11(a) and 11(b)), it was observed that the amount of bacterium Q1 in alginate beads increased after five cycles of decolorization, indicating bacterium Q1 kept growing in immobilized beads during repeated batch decolorization. In SEM images (Figs. 11(d) and 11(e)), it was found that mycelia morphology of fungus M3 in carriers remained integrity after five cycles.

3.6. Continuous decoloirzation in the reactor by immobilized consortium

Fig. 12 showed color removal in the reactor by immobilized consortium during 20 d of operation. Decolorization rate increased with operation time, which reached a maximum (91%) after the third day. In initial operation time of bioreactor dye decolorization was partially contributed to the bioadsorption of immobilized consortium [36]. The reactor maintained high decolorization efficiency (above 80%)

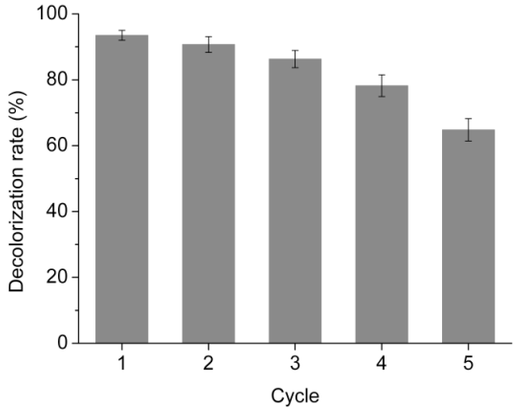

Fig. 10. Repeated-batch decolorization of azo dye by immobilized bacterial-fungal consortium.

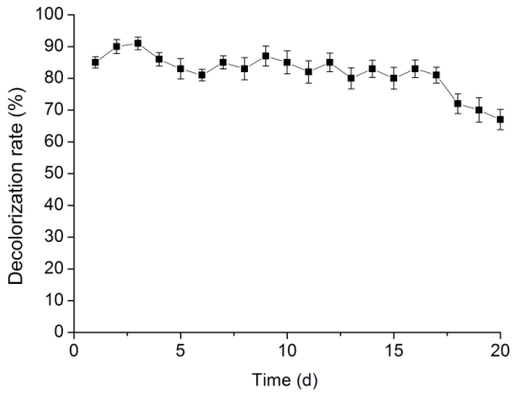

Fig. 12. Continuous decolorization of azo dye by immobilized bacterial-fungal consortium in packed-bed bioreactor.

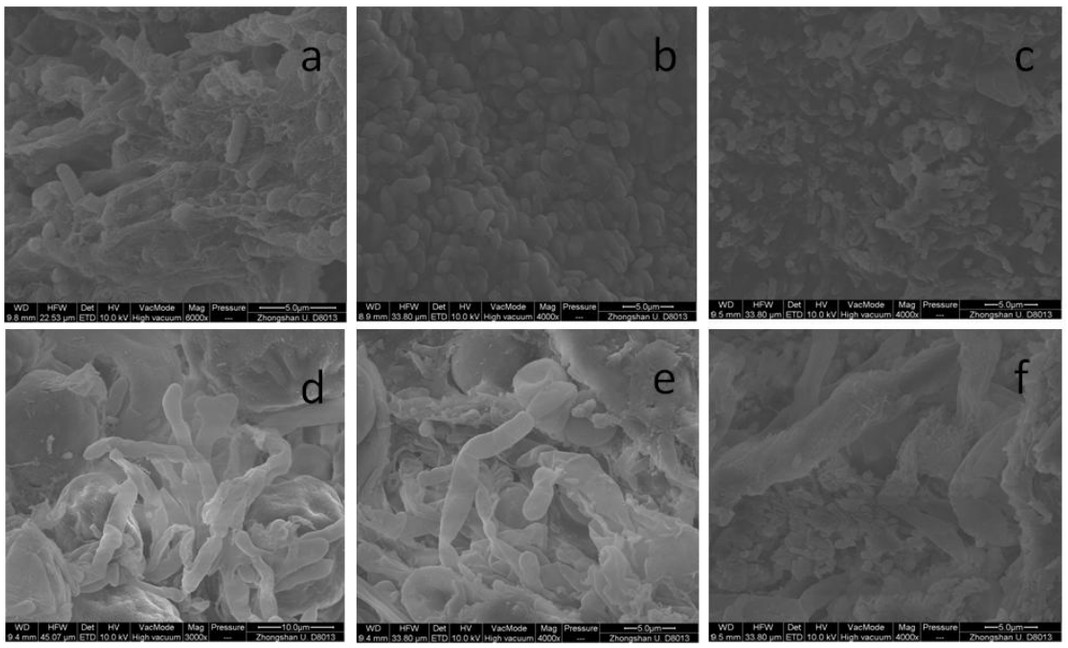

Fig. 11. SEM images of immobilized microorganisms: (a) bacteria before decolorization; (b) bacteria after repeated batch decolorization; (c) bacteria after continuous decolorization; (d) fungi before decolorization; (e) fungi after repeated batch decolorization; (f) fungi after continuous decolorization.

until 17 d. After 18 d decolorization rate decreased to 72%. It was observed that the amount of the bacteria immobilized in calcium-alginate beads did not decrease (Fig. 11(c)) and mycelia morphology of the fungi immobilized in nylon sponges remained intact after continuous decolorization in the bioreactor (Fig. 11(f)). The bioreactor packed with immobilized bacterial-fungal consortium achieved an average decolorization efficiency of 80% for 20 d of operation. To date, this is the first report that continuous decolorization of azo dye was carried out with immobilized bacteria in calcium-alginate bead and immobilized fungi in nylon sponges, which were filled in a packed-bed bioreactor. The results indicated that immobilized bacterial-fungal consortium would be suitable for removing azo dyes from dyeing effluent.

4. Conclusions

This study investigated the decolorization ability by consortium QM consisting of fungus M3 and bacterium Q1, which were identified as *Geotrichum candidum* and *Bacillus cereus* respectively. Compared with individual strain M3 and strain Q1, mixed consortium containing both strains showed better decolorization capability in broad ranges of temperature, pH, salinity and dye concentration, suggesting synergistic effect between *Geotrichum candidum* M3 and *Bacillus cereus* Q1 on decolorization of azo dye in complex environments. In addition, the present study found that synergistic effect of decolorization was related to ORP decrease caused by *Geotrichum candidum* M3, which should be further explored in the future research. The UV-Vis and HPLC analysis of decolorization showed that azo dyes were further degraded to metabolic products by consortium QM. We also found the fungal-bacterial consortium immobilized in alginate beads and nylon sponges decolorized over 60% azo dye after five cycles of repeated batch decolorization. Immobilized bacterial-fungal consortium could decolorize azo dye efficiently in the packed-bed bioreactor with continuous mode. The results indicated that mixed consortium QM might be a practical alternative in the treatment of textile effluent containing azo dyes.

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