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Azo dye decolorization by a new fungal isolate, *Penicillium* sp. QQ and fungal-bacterial cocultures

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ABSTRACT

A new azo dyes-decolorizing fungi strain QQ was isolated from activated sludge. It was identified as *Penicillium* sp. based on 26S rRNA gene sequence analysis. The study indicated that strain QQ could aerobically decolorize Reactive Brilliant Red X-3B by the way of bioadsorption, and nutrient-poor medium was more beneficial for adsorption. Decolorization rate was inversely proportional to the size of mycelial pellets. The optimum pH was observed at 4 or 5 for X-3B decolorization. There was still 70% color removal when salinity increased to 6%. By contrast with aerobic decolorization, the degradation of azo dyes occurred under anaerobic conditions, and some azo dyes could be absolutely decolorized. Furthermore, the decolorization of azo dyes by fungal-bacterial cocultures was investigated. The results demonstrated that strain QQ and *Sphingomonas xenophaga* QYY cocultures performed better than any single strain did. Weak acidity conditions and the presence of small amount of surfactant could enhance the ability of consortium to decolorize azo dyes.

1. Introduction

Almost 7×10^5 tones of dyes are produced around the world every year, and most of them are azo dyes consisted of one or more azo groups (R_1-N=N-R_2), which are extensively used as industrial raw materials [1]. It is quite undesirable to discharge azo dye with different colors into the environment due to their color pollution and toxic intermediates [2]. Compared with chemical and physical methods, biodegradation has been the main focus on degradation of azo dyes, which can produce lower costs and fewer toxic resultants [3,4]. Many microorganisms, belonging to bacteria, fungi, even yeasts, have been found to be able to decolorize azo dyes by bioadsorption or degradation.

Among these microorganisms, bacteria and fungi play key roles in treating dye wastewater, and it has been demonstrated that they decolorize dyes with different types of enzymes. Up to now, white rot fungi, particularly *Phanerochaete chrysosporium*, have been reported to be the most common microorganism. It has been accepted that some extracellular lignin enzymes produced by white rot fungi, such as lignin peroxidase (LiP), laccase and manganese peroxidase (MnP) are responsible for the degradation of azo dyes [5]. In addition, there are also several other reports about decolorization of azo dyes by *Penicillium* genus, though the mechanism of decolorization is still unclear [6,7]. By contrast, bacteria have been proved to cleave azo bonds by azoreductase. Recently, azoreductase activity has been detected in many bacteria, such as *Sphingomonas xenophaga* BN6, *Pigmentiphaga kullae* K24 and *Caulobacter subvibrioides* C7-D, and so on [8–10].

There is little application for practical treatment although many pure cultures are available to decolorize azo dye, which can be supported by three reasons [11]. Firstly, fungi cannot use azo dye as sole carbon and energy source, and their growth is time-consuming. Secondly, low efficiency of bacteria degrading azo dyes is achieved under aerobic conditions, because oxygen is a more efficient electron acceptor compared with azo dye. Thirdly, single strain cannot adapt the complex and variable environment conditions. Therefore, mixed microbial populations are expected to perform better than single microorganism does [12]. However, there is little information about using fungal-bacterial consortium to decolorize azo dye.

In this work, we isolated an azo dye-decolorizing *Penicillium* sp. strain, of which the ability to decolorize azo dyes was investigated under both aerobic and anaerobic conditions. Also, this isolate and a bacterial strain (*S. xenophaga* QYY) were combined to develop a cocultures system to improve the decolorization efficiency. It was suggested that strain QQ and strain QYY cocultures would be an alternative for azo dyes wastewater treatment.

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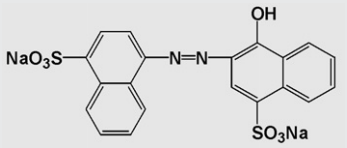
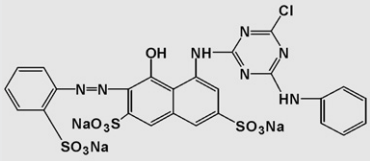
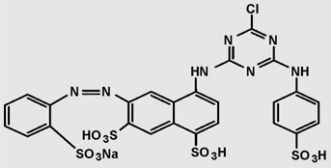
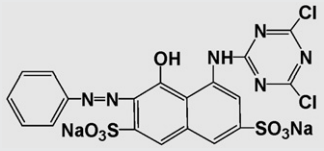
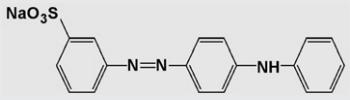
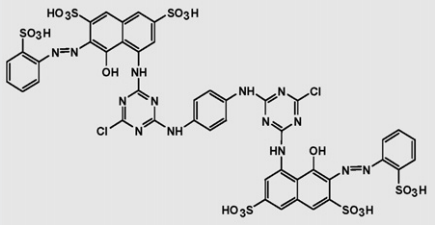
Table 1
Characteristics of main azo dyes used in this study.

Azo dye	Color index name (C.I.No.)	Molecular structure	Wavelength (nm)
Acid Red B	Acid Red B 14 (14720)		516
Reactive Brilliant Red K-2BP	Reactive Red 24 (18208)		535
Reactive Brilliant Red K-2G	Reactive Red 15		515
Reactive Brilliant Red X-3B	Reactive Red 2 (18200)		538
Acid Orange G	Acid Orange 10 (16230)		450
Reactive Brilliant Red KE-3B	Reactive Red 120 (25810)		530

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. The other reagents were of analytical grade.

2.2. Isolation and identification of an azo dye-decolorizing fungus

Activated sludge was collected from the wastewater treatment plant of Dalian Petrochemical Company/Petrochina Co. Ltd. in Liaoning province of China. After enriching and screening, a fungal strain that could decolorize Reactive Brilliant Red X-3B was isolated and it was designated as QQ. Genomic DNA of strain QQ was extracted by grinding mycelial pellets with liquid nitrogen. Purified DNA was used as template to amplify the D1/D2 domain of the 26S rRNA gene by PCR using the D1/D2 forward and reverse primer. Then the PCR product was sequenced by TaKaRa Biotechnology (Dalian) Co. Ltd. 26S rDNA sequence analysis was performed with the aid of Clustal W1.8 software package by using the neighbor-joining method and Jukes-cantor distance correction matrix method.

2.3. Microorganisms and culture media

Two microorganisms, *S. xenophaga* QYY and strain QQ were used in this study. Strain QYY was previously isolated in our laboratory with the ability of degrading bromoamine acid (BAA) [13]. Fungal strain QQ was obtained from activated sludge as described above. Four kinds of culture media used were as follows:

(a) Mineral salts medium (MS), which contained ($g\,l^{-1}$): $(NH_4)_2SO_4$ 2.0, KH_2PO_4 2.0, Na_2HPO_4 1.3, $FeCl_3$ 0.25.
(b) The composition of Luria–Bertani medium (LB) was ($g\,l^{-1}$) 10 tryptone, 5 yeast extract, 10 NaCl, pH 7.2.
(c) Modified Martin Broth (MMB), which contained ($g\,l^{-1}$): $(NH_4)_2SO_4$ 1.0, KH_2PO_4 1.0, $MgSO_4$ 0.5, sucrose, 10.0.
(d) Potato Dextrose Medium (PDM), Potato Tryptone Medium (PTM) and Potato Sucrose Medium (PSM) were Potato Medium (PM) added with $10\,g\,l^{-1}$ dextrose, tryptone and sucrose, respectively.

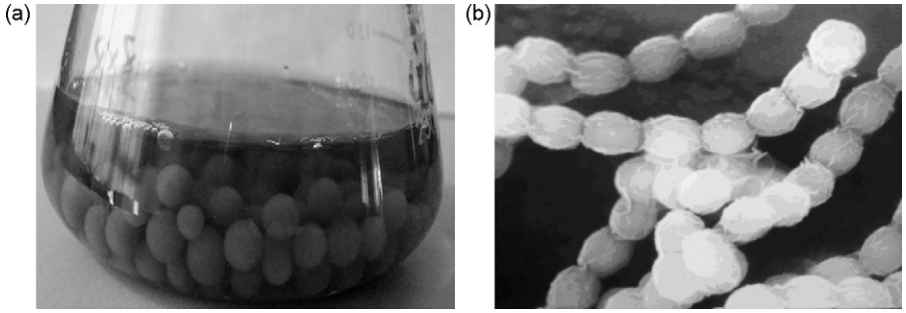


Fig. 1. Morphological characteristics of strain QQ. (a) The photo of mycelial pellets growing in liquid culture medium; (b) the electron microscope picture of spores (1000× folds).

2.4. Aerobic decolorization of X-3B by strain QQ

Aerobic decolorization of X-3B was performed in 250-ml flasks with 100 ml medium containing 100 mg l^{-1} dye. 500 μl spore suspension (with OD_{600} value of 0.2) of strain QQ was inoculated into the medium and the culture solution was incubated at 30 °C under 150 rpm. The influence of environmental factors on decolorization rate, such as the types of culture media (referred to Section 2.3), pH (2–9, MMB medium) and NaCl concentration (0–6% (w/v), MMB medium), were investigated.

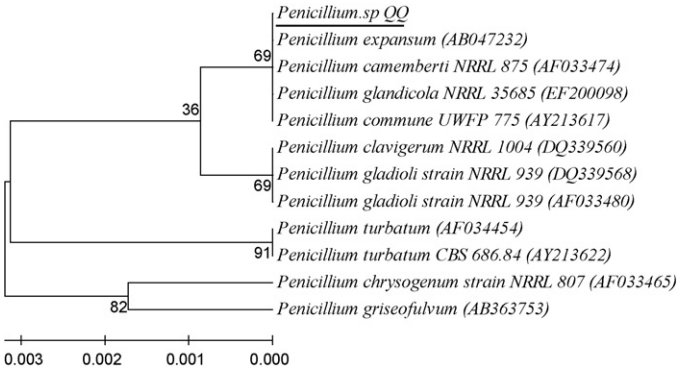


Fig. 2. Phylogenetic tree of *Penicillium* species. The strains selected were as follows: *P. expansum* (AB047232), *P. camemberti* (AF033474), *P. glandicola* (EF200098), *P. commune* (AY213617), *P. clavigerum* (DQ339560), *P. gladioli* (DQ339568), *P. gladioli* (AF033480), *P. turbatum* (AF034454), *P. turbatum* (AY213622), *P. chrysogenum* (AF033465), *P. griseofulvum* (AB363753).

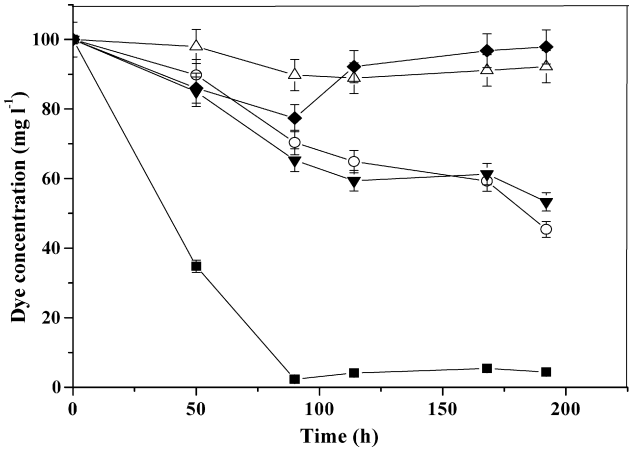


Fig. 3. Decolorization of 100 mg l^{-1} X-3B by strain QQ in different media under aerobic conditions. (■) Modified Martin Broth (MMB); (○) Potato Sucrose Medium (PSM); (△) Potato Tryptone Medium (PTM); (▼) Potato Dextrose Medium (PDM); (♦) Potato Medium (PM).

2.5. Anaerobic decolorization of azo dyes by strain QQ

The ability of strain QQ to decolorize other azo dyes was carried out under anaerobic conditions. Spore suspension of strain QQ was firstly inoculated to MMB medium without dye to form mycelial pellets with size of 3–4 mm. Then equal weight of mycelial pellets (wet cells) was added into several 10-ml anaerobic bottles containing 5-ml MS medium with 50 mg l^{-1} azo dyes. Nitrogen gas was filled up in anaerobic bottles to assure the anaerobic circumstance before decolorization. The total process of decolorization was observed.

2.6. Decolorization of azo dyes by fungal-bacterial cocultures

Utilizing fungal-bacterial cocultures to decolorize X-3B and Acid Red B was investigated. The wet cells of strains (0.4 g of stain QQ, 0.4 g of strain QYY) were simultaneously inoculated into MS medium with 50 mg l^{-1} azo dyes. And the culture was cultivated for 3 days under anaerobic conditions. The effects of consortium on decolorization were also observed under different pH, NaCl concentration, and surfactants (SDS and LAS).

2.7. Analytical methods

The culture was collected at intervals during decolorization process, and then centrifuged 3 min at 12,000 rpm (Beckman centrifuge Avanti J-30I, USA). Dye concentration was measured by monitoring the changes of maximum absorbance with a UV–vis spectrophotometer (JASCO V-560, Japan). The decolorization rate (*P*%) was calculated using the following equation:

$$P\% = \frac{A_0 - A_1}{A_0} \times 100\% \quad (1)$$

where A_0 and A_1 represent the initial and residual concentration of azo dye, respectively. All of the experiments were performed in duplicates and the average values were used in calculations.

3. Results and discussion

3.1. Isolation and identification of strain QQ

A new fungal isolate QQ capable of decolorizing azo dye X-3B was isolated from activated sludge. White and smooth mycelial pellets were harvested when strain QQ was cultured in liquid medium (Fig. 1a). However, a large number of bluish green spores formed around the white colony after 2–3 days incubation on potato agar plates. It was observed (Fig. 1b) that the spores were chain spherical by scanning electron microscope (JEM-1200EX, Japan). In addition, the sequence of 26S rDNA D1/D2 domain revealed a 100% homology with that of *Penicillium expansum* (Fig. 2). Thus, strain QQ was

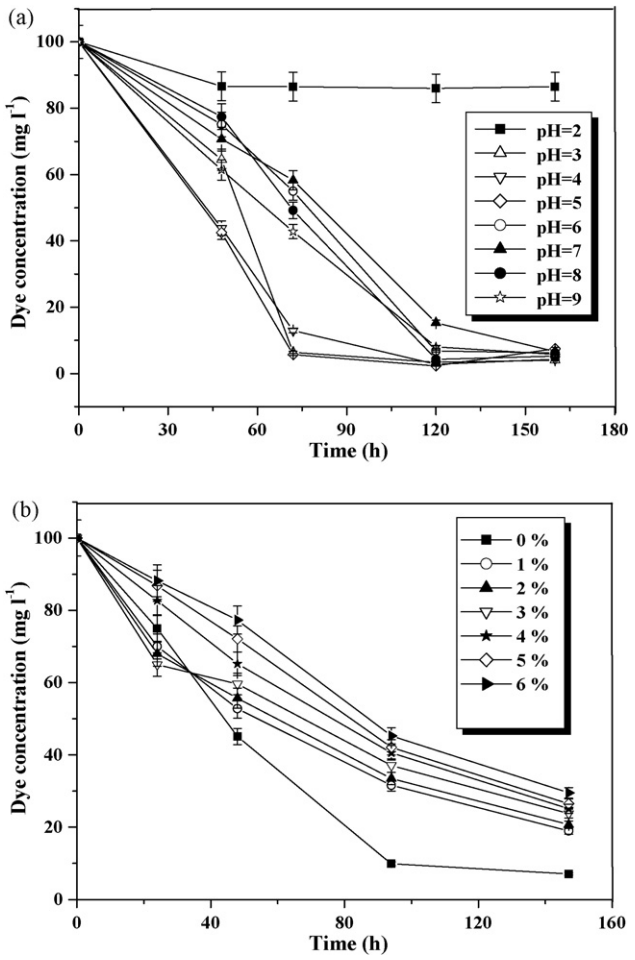


Fig. 4. Effects of (a) pH and (b) NaCl concentration on aerobically decolorization of X-3B by strain QQ.

identified as *Penicillium* sp. (GenBank No. EU572723) on the basis of morphology and 26S rRNA gene sequence analysis.

3.2. Effects of media on aerobic decolorization of X-3B by strain QQ

It was found that the decolorization of X-3B occurred along with the spores growing into mycelial pellets under aerobic conditions. The types of culture media had great influence on decolorization process. As shown in Fig. 3, nearly 97.7% color was removed after 90 h in MMB medium, and it was much higher than that in PM medium added with various carbon sources. However, no growth of spore was observed when X-3B was used as sole carbon and energy source, which consisted with the previous report [11].

In our study, bioadsorption rather than biodegradation could be inferred as the major reaction during aerobic decolorization of X-3B. Therefore, the adsorption and desorption of azo dye were suggested to make changes of residual concentration of X-3B in cultures, which was decreased as time passed and then again increased except for PSM medium (Fig. 3). The different phenomenon in PSM medium was probably because that the adsorption capacity of strain QQ could not reach its maximal value within the tested time. Recently, using microorganisms as adsorbing agents has received more attentions because they own wide ranges of binding sites for azo dyes [14]. Many functional groups located on the surface of microbial cells, such as amino, carboxyl, phosphate and hydroxyl group are responsible for the adsorption of azo dyes [15]. As previously reported, mycelial biomass and the

Table 2
Size of mycelial pellets and decolorization rate in different media.

	Types of media				
	MMB	PDM	PSM	PM	PTM
Diameter of mycelial pellets (mm)	1	2–3	3–4	4–5	5–6
Decolorization rate (%)	97.7	34.7	29.6	22.6	10.2

exposure of functional groups induced by different media could cause different decolorization efficiency. In this study, we found that the size of mycelial pellets should be another significant factor because its size was inversely proportional to decolorization rate (Table 2). This phenomenon could be explained that large pellets coiled with tighter mycelium could supply more smooth surfaces than the smaller ones, which was unbeneficial for the sites exposure to azo molecules. According to the discussion above, nutrient-rich potato media were unfit for decolorization by strain QQ.

3.3. Effects of pH and salinity on aerobic decolorization of X-3B by strain QQ

pH was an important factor for bioadsorption process, because it could affect dye structure and electric charges related to bioadsorption on the surface of cells [16]. As shown in Fig. 4a, the optimal pH for aerobic decolorization of 100 mg l^{-1} X-3B was around 4 or 5 in 45 h with 57% removal. And 25–38% of color was removed at other pH values (except for pH 2). Therefore, it was obvious that weak acid condition was profitable for decolorization, which was due to the sulfonic groups in the structure of X-3B. Sulfonic groups were prone to combine H^+ under acid conditions, thus X-3B was

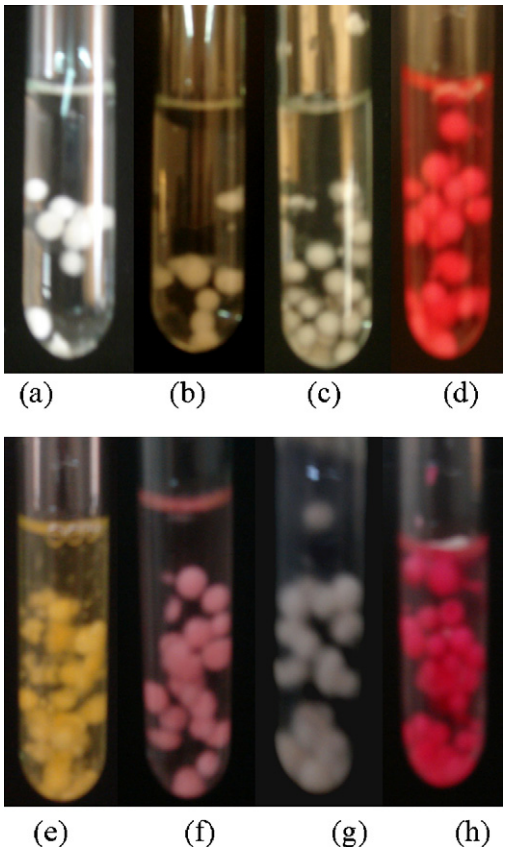


Fig. 5. Decolorization of various azo dyes by strain QQ under anaerobic conditions. The azo dyes tested were as follows: (a) Reactive Brilliant Red X-3B; (b) Acid Red B; (c) Direct Fast Black G; (d) Reactive Brilliant Red K-2BP; (e) Methanil Yellow G; (f) Reactive Brilliant Red K-2G; (g) Acid Orange G; (h) Reactive Brilliant Red KE-3B.

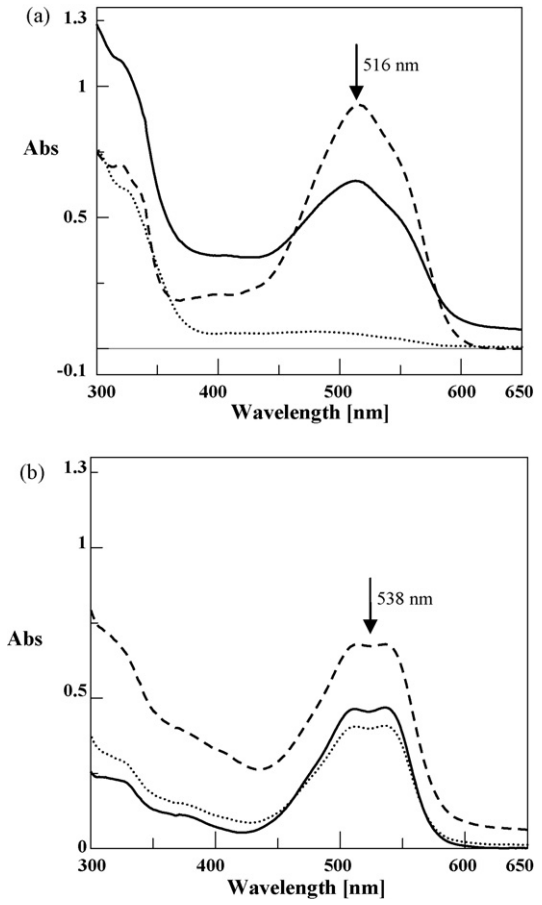


Fig. 6. UV–vis spectra of (a) Acid Red B decolorization and (b) X-3B decolorization by single strain and fungal-bacterial cocultures. (- - -) Single QYY strain; (—) single QQ strain; (···) fungal-bacterial cocultures. The initial concentration of Acid Red B and X-3B were both 50 mg l^{-1}, and residual concentrations of dyes were detected after 3 days.

transformed from ionic state to non-ionic state. This could cause the electrostatic repulsion between dyes and cells to decline and the adsorption capacity of X-3B to increase. According to Xiao's description on removal of X-3B by a fungal strain HX, the optimal pH was also 5 [17]. However, for strain QQ there was only 15% color removal around pH 2. The explanation might be that the spore inoculated could not form mycelial pellets to adsorb X-3B effectively in strongly acid solutions. Therefore, the subsequent experiments were carried out at pH 5.

Considering the existence of salinity in dye wastewater, the influence of NaCl concentration on decolorization of X-3B by strain QQ was studied. In order to avoid inhibiting the growth of strain QQ, small NaCl concentration (0–6%, w/v) was primarily investigate. Fig. 4b shows the effects of NaCl on decolorization of azo dye. Under low NaCl concentration (0–3%), decolorization rate of X-3B was enhanced with increasing salinity at the initial 24 h. Xin et al. also reported that the decolorization of Reactive Brilliant Blue KN-R by *Penicillium terrestre* GX2 was improved as salinity increased from 0% to 2% [18]. The possible reason was that the fungal pellets became smaller under higher NaCl concentrations, which produced more surface areas for adsorption. On the other hand, the common ion effect caused from NaCl could reduce the solubility of azo dyes and increase the chance of strain QQ to contact with dyes. Furthermore, according to the coexistence principle of common ion effect and salt effect, it was speculated that salt effect instead of common ion effect played the major role after 24 h, so that the change tendency of decolorization rate was

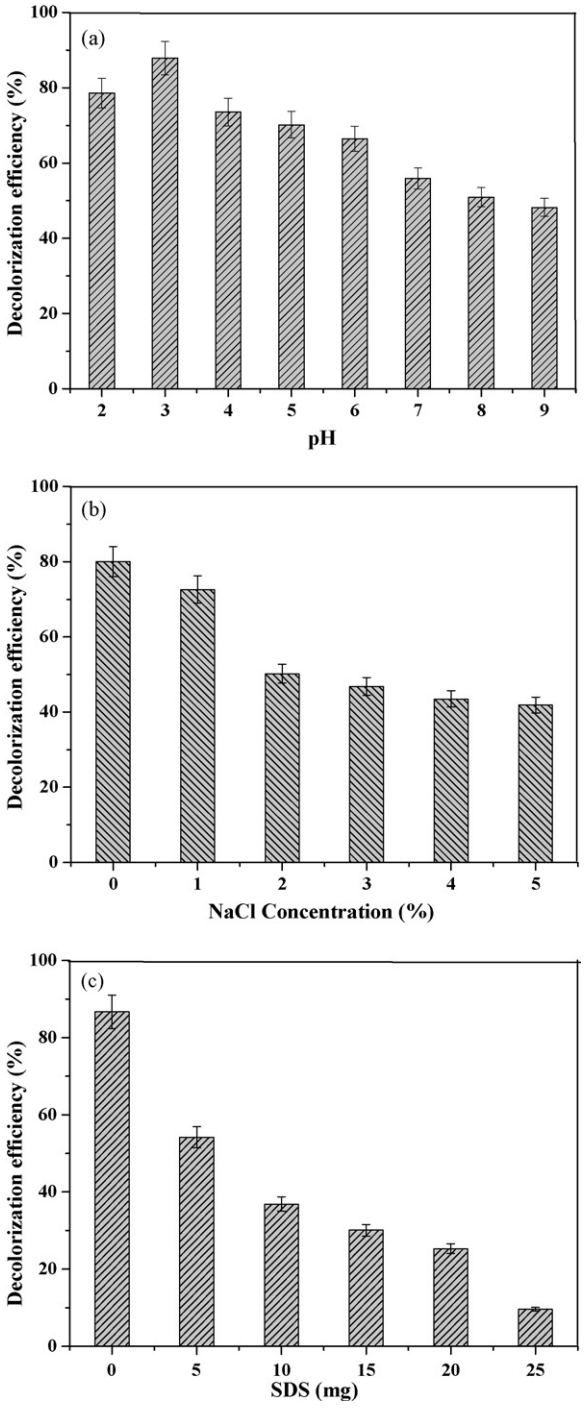


Fig. 7. Effects of environmental factors on decolorization by fungal-bacterial cocultures. (a) pH; (b) NaCl concentrations; (c) SDS.

reversed (Fig. 4, 0–3% NaCl). However, higher salinities (4–6%) could inhibit the decolorization, although there was still about 70% dye removal (Fig. 4b). It was probably because the growth of strain QQ was restrained by increased osmotic pressure on cells. Therefore, controlling appropriate salinity and adsorption time could obtain better dye-decolorizing efficiency.

3.4. Anaerobic decolorization of azo dyes by strain QQ

Because aerobic bioadsorption could not metabolize azo dye, decolorization of azo dyes by *Penicillium*. sp QQ was performed under anaerobic conditions. Azo dyes tested included X-3B, Acid

Red B, Direct Fast Black G, Reactive Brilliant Red K-2BP, K-2G and KE-3B, Methanil Yellow G, and Acid Orange G. It was notable that strain QQ turned into the color of azo dye in the initial stage of decolorization due to adsorption. However, only the mycelial pellets in four cultures (including X-3B, Acid Red B, Direct Fast Black G and Acid Orange G) could decolorize dye absolutely and then become colorless (Fig. 5). Owing to the fact that these mono-azo dyes possess simpler structures and smaller molecular weights, they were easier to be attacked or degraded by microorganisms. From the shift of maximum absorption wavelengths of dyes (UV–vis spectra not shown), it could be inferred that all azo dyes were biodegraded by *Penicillium* sp. QQ under anaerobic conditions. However, accumulation of aromatic amine was detected by HPLC technology in each culture, which confined incomplete biodegradation of azo dyes by this fungal isolate.

3.5. Decolorization of azo dyes by fungal-bacterial cocultures

Simple azo dye including X-3B and Acid Red B was used to test the decolorization by fungal-bacterial cocultures under static conditions. As expected, cooperative strains exhibited better performance than any single strain (Fig. 6). Especially for Acid Red B, more remarkable decolorization effect was observed. It was speculated that the degradation of intermediates, i.e. aromatic amines by bacteria could decrease the inhibition on fungi.

The effects of various environmental factors on decolorization by cocultures, such as pH, NaCl concentration and surfactants were shown in Fig. 7. All of the decolorization rates reached 50% at tested pH values and the best one 87.8% was obtained at pH 3. It was also suggested that weak acidity was beneficial for decolorization of consortium. In addition, decolorization rate dropped to 42% when salty concentration changed from 0% to 5%, which meant that salty environment had more serious influence on consortium. The presence and types of surfactants (SDS and LAS) were considered to study the impact on X-3B decolorization (0.25 g) by consortium. The results suggested that both SDS and LAS could depress the decolorization of X-3B, but there was no significant difference between the inhibition effects from SDS and LAS. Furthermore, very few SDS (0.025 g) could result in the sharp decrease (about 80%) of decolorization efficiency. So it was advised that the pre-treatment of surfactants was necessary for disposing soluble azo-dye wastewater.

4. Conclusions

In the present study, a new azo dyes-decolorizing fungi strain *Penicillium* sp. QQ was isolated from activated sludge. Under aerobic conditions strain QQ could quickly decolorized X-3B by the way of adsorption, while degradation of azo dyes was occurred under anaerobic conditions. The decolorization rate of azo dyes by *Penicillium* sp. QQ and *S. xenophaga* QYY cocultures was higher than that of by any single strain, which had more practical significance for the treatment of dye wastewater. However, the further research on the mechanism of color removal by cocultures should be carried out.

Acknowledgements

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