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Bioaccumulation versus adsorption of reactive dye by immobilized growing *Aspergillus fumigatus* beads

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Abstract

The removal of reactive brilliant blue KN-R using growing *Aspergillus fumigatus* (abbr. *A. fumigatus*) immobilized on carboxymethylcellulose (CMC) beads with respect to initial dye concentration was investigated. Bioaccumulation was the dominant mechanism of the dye removal. According to the UV–vis spectra and the results of three sets of experiments, it could be concluded that the bioaccumulation using immobilized growing *A. fumigatus* beads was achieved by metabolism-dependent accumulation and metabolism-independent adsorption (15–23% proportion of overall dye removal), which included biosorption by mycelia entrapped in them and adsorption on immobilization matrix. The transmission electron microscope (TEM) images showed the intracellular structures of mycelia and the toxicity of dye. It was found that the fungus had a considerable tolerance to reactive brilliant blue KN-R at initial dye concentrations of <114.7 mg/l. Though at high initial dye concentrations the growth of mycelia was inhibited significantly by the dye molecules in the growth medium, the bioaccumulation capacity was not markedly affected and the maximum bioaccumulation capacity was 190.5 ± 2.0 mg/g at an initial dye concentration of 374.4 mg/l. The bioaccumulation rates were not constant over the contact time.
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Keywords: *Aspergillus fumigatus*; Bioaccumulation; Biosorption; Immobilization

1. Introduction

The use of non-conventional low-cost adsorbents including biomass for the removal of dyes was highly recommended in a recent review by Crini [1]. Among different adsorbents, growing cells can remove different types of dyes via active bioaccumulation and is regarded as an alternative biological treatment [2–4]. Bioaccumulation is defined as the accumulation of pollutants by actively growing cells by metabolism- and temperature-independent and metabolism-dependent mechanism steps [5]. Moreover, direct use of growing cells as biosorbents can avoid the need for separate biomass production processes (e.g., cultivation, harvesting, drying, processing and storage prior to use) and can be very efficient under appropriate conditions that favor active growth of cells (i.e., moderate dye concentrations, solution pH, and salt concentrations).

The use of freely suspended biomass may be plagued with operational difficulties, including the required separation of the suspended biomass from the aqueous medium and the possibility of pipelines and filters clogging [6]. In general, the natural mycelium pellets are not mechanically strong and vary in size, leading to problems like channeling in column operation.

Immobilization of microorganism can overcome the operational difficulties described above. It also provides additional advantages such as high cell density, strong endurance of toxicity, lower operating costs, simple maintenance management and lower residual sludge. Applications of immobilized actively growing biosorbents have been shifted to wastewater treatment [7–9]. In recent years, several studies have shown that the mechanisms for the decolorization of dyes using immobilized microorganisms may include both biodegradation and adsorption [10,11]. However, which mechanism dominates the overall removal of the dye remains unknown.

A recent study demonstrated that *Aspergillus fumigatus*, a naturally abundant and readily alive microorganism, could be used as biomass for biosorption of heavy metals [12]. This organism was recently isolated in our laboratory [13–15], and was

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found to be very effective for the removal of diverse dyes. For example, it was shown that more than 91.2% of reactive brilliant red K-2BP and 100% of reactive yellow X-R at an initial dye concentration of 200 mg/l could be removed within 96 and 12 h, respectively, by *A. fumigatus* mycelium pellets [13]. It indicated that *A. fumigatus* may possess great potential for the treatment of dye wastewater.

In our prior studies [16,17], we found a natural polymer CMC was best suited for immobilizing the fungus and converted it to biomass beads that could be used for removal of dye from aqueous phase. In this study, we selected reactive brilliant blue KN-R, a common dye used in dyeing industries in China, as the target dye chemical for representative of non-biodegradable anthraquinone dyes due to their solubility resulting from ionized groups in their chemical structure in solution. A series of experiments were initiated to evaluate the removal efficiency of the dye by immobilized growing *A. fumigatus* beads under different initial dye concentrations. Our goals were to evaluate toxicity of dye for the fungus, to elucidate the mechanism of dye removal, and to quantify the concentration dependence of the removal efficiency. The experimental results would provide insight information on design of batch and flow systems under different influent dye concentrations for maximizing decolorization rate and decolorization efficiency in future engineering applications.

2. Materials and methods

2.1. Dye and immobilization matrix

The reactive brilliant blue KN-R (C.I. Reactive Blue 19), in commercial purity, was obtained from Guangzhou Yongzhi Dyeing Chemical Industry Co., Ltd. in Guangzhou, China. It was used without further purification. Its chemical structure is shown in Fig. 1. CMC sodium salt (CMC–Na) with 300–600 mPa s at 2.0 wt.% and with sodium content of 6.5–6.8 wt.%, which was used as the immobilization matrix, was provided by Fuchen Chemical Enterprise Co. (Tianjing, China).

2.2. Fungus and medium

The fungus, *A. fumigatus*, was isolated from biosolid collected from an activated-sludge system that was operated for treating dye wastewater treatment generated in a dyeing factory located in Guangzhou, China.

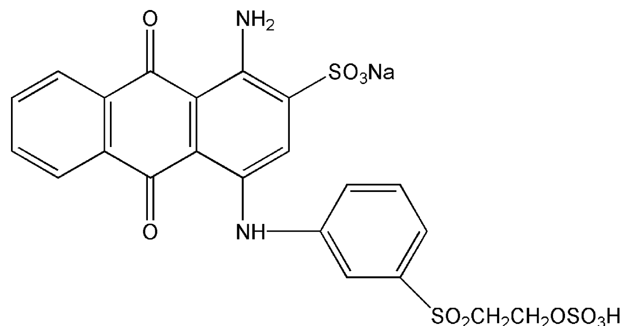

Fig. 1. Chemical structure of reactive brilliant blue KN-R.

Enriched culture medium used in this study had following compositions (g/l): glucose (15), KH_2PO_4 (1), $(NH_4)_2SO_4$ (1), $MgSO_4{\cdot}7H_2O$ (0.5), NaCl (0.5) and yeast extract (1). Basic culture medium comprised of 5 g/l of glucose, 1 g/l of KH_2PO_4, 1 g/l of $(NH_4)_2SO_4$, 0.5 g/l of $MgSO_4{\cdot}7H_2O$, 0.5 g/l of NaCl. Basic culture medium contained 0.05–0.8 g/l of dye constitutes dye-containing culture media for bioaccumulation. The pH of both enriched and basic culture media were 5.18 and 6.45, respectively, which were within the range of aqueous suitable pH for the growth of fungus. The solution pH was thus not adjusted.

2.3. Immobilization method

CMC solution (2.5% 100 ml) was thoroughly mixed with 1 ml of spore suspension at a concentration of about 1×10^8 ml^{-1}. The CMC–spore mixture was injected drop-wise to a $FeCl_3$ solution (0.05 mol/l) using an injector with a 16-sized pinhead to form beads. The spore-immobilized beads were cured in the $FeCl_3$ solutions for 1 h to enhance their mechanical stabilities. The cell-free immobilized beads were prepared following the above procedures except that the spores were excluded.

Aseptic technique was demanded in the process of spore-immobilizations. All the solutions except the $FeCl_3$ solutions (filter sterilization) were autoclaved at 121 °C for 20 min.

The above-prepared spore-immobilized beads were collected, rinsed thrice with sterilized water and transferred into enriched culture medium. The inocula were incubated on a rotary shaker at 30 °C and 150 rpm for 3 days. Thereafter, the freshly prepared beads, namely immobilized mycelia beads were harvested and rinsed in the same way, stored in physiological saline at 4 °C.

2.4. Bioaccumulation and three set of experiments

Water on the surface of stored immobilized mycelia beads was blotted up by using filter paper. The immobilized mycelia beads (1.0 g) were transferred into 50 ml of dye-containing culture media and incubated on a rotary shaker at 30 °C and 150 rpm. The immobilized mycelia beads incubated in dye-containing culture media were called immobilized growing *A. fumigatus* beads in this study.

To confirm joint function in the process of decolorization of dye-containing culture media, the three sets of experiments were performed as follows: in the first set of experiments, the immobilized mycelia beads were transferred into 50 ml of basic culture medium at 2% of dosage and incubated for 48 h on a rotary shaker at 30 °C and 150 rpm. The incubated beads were then transferred into conical flasks containing 50 ml of dye aqueous solutions after rinsed thrice with distilled water. In the second set of experiments, 1 ml of spore suspensions at a concentration of about 1×10^8 ml^{-1} were transferred into 100 ml of enriched culture medium. The spore–media mixture was then incubated at 30 °C for 3 days on a rotary shaker set at 150 rpm. After that, the incubated free cells were harvested and autoclaved at 121 °C for 20 min. After rinsed thrice with distilled water, the killed free cells were transferred into the conical flasks containing 50 ml

of dye-containing culture media at 1% of dosage. In the third set of experiments, the cell-free immobilized beads were transferred into conical flasks containing 50 ml of dye-containing culture media at 2% of dosage. Thereafter, the above-prepared conical flasks in the three sets of experiments were placed on a rotary shaker at 30 °C and 150 rpm for 48 h. The absorbance of supernate of solutions containing residual dye was measured.

In the toxicity of dye experiments, the concentrations of dye remaining in the solutions were determined at the end of 48 h. The immobilized mycelia beads containing dyes were rinsed thrice using distilled water and oven-dried at 60 °C for 12 h. The net dry mass of mycelia was calculated by the dry mass of cultured immobilized mycelia beads subtracted the dry mass of dyes accumulated in immobilized beads and that of corresponding cell-free immobilized beads.

To find out the bioaccumulation efficiencies with contact time at various initial dye concentrations, the concentrations of the remaining dyes in the solutions were measured at several hours' intervals.

All the experiments were performed at least in triplicates and the average values were used in calculations.

2.5. Preparation of TEM samples

The immobilized mycelia beads before and after bioaccumulation were cut into cuboids for pretreatment. The pretreatment process was modified by the method reported in the literature [18]. The cuboids were fixed in 4.0% glutaraldehyde at 4 °C. After 4 h of fixation, the cuboids were rinsed 6 times at 20 min interval with 1 mol/l phosphate buffer. After rinsed, the samples were postfixed for 2 h in 1% osmium tetroxide. The cuboids were rinsed for 6 times at 10 min interval with 1 mol/l phosphate buffer again. After dehydration in ethanol concentration series (50, 70, 80, 90, 100%, v/v), the cuboids stayed for 10 min in propylenoxide and were saturated in durcupan/propylenoxide mixture (1:3) for 1 h, in a durcupan/propylenoxide (1:1) mixture for the next 1 h and in a durcupan/propylenoxide mixture (1:3) again for another hour. Samples were stored in durcupan without catalysator overnight. Polymerization was carried out at 70 °C in gelatinous capsules overnight. Ultrathin sections were cut on TESLA BS 490 Microtome. The ultrathin sections were stained with uranyl acetate and lead citrate and photographed in the transmission electron microscope Tecnai-12.

2.6. Analytical methods

The absorbance of dye solutions was measured calorimetrically at maximum absorbing wavelength of dye (591 nm) using an ultraviolet–visible spectrometer (TU-1800SPC, Shanghai, China).

The bioaccumulation efficiency (P) was expressed as the percentage of change in the concentration of bioaccumulated dye at time intervals to the initial dye concentration by immobilized growing *A. fumigatus* beads.

Adsorption efficiency (P') expresses the adsorption ability [12]. P or P' was calculated utilizing the following equation:

$$P(\text{or } P') = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

where A_t was the absorbance of supernate of culture media after bioaccumulation or adsorption at time t; A_0 was the absorbance of supernate of culture media before bioaccumulation or adsorption. The data calculation methods for the three sets of experiments were referred to the above-mentioned Eq. (1).

The bioaccumulation capacity, Q (mg/g) could be calculated as follows:

$$Q = \frac{V(C_0 - C)}{m} \quad (2)$$

where m was the net dry mass of mycelia entrapped in immobilized growing *A. fumigatus* beads after bioaccumulation (g); C_0 was the initial dye concentration (mg/l); C was the concentration of residual dye after bioaccumulation (mg/l); and V was the volume of dye-containing culture media in bioaccumulation system (1).

3. Results and discussion

3.1. Endurance ability of dye concentration

The toxicity of dyes may play a crucial role in dye removal by using live mycelia. Enough attention on biotoxicity of reactive brilliant blue KN-R to *A. fumigatus* should be paid in order to determine whether the dye removal process is biologically feasible.

Fig. 2 shows indirectly the toxicity of reactive brilliant blue KN-R to the immobilized mycelia by the concentration of

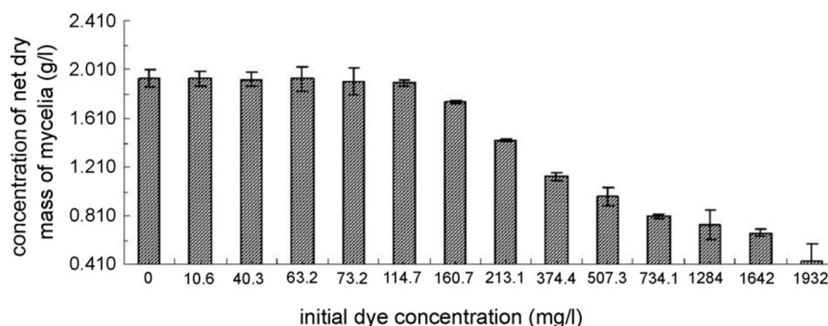

Fig. 2. The concentration of net dry mass of mycelia after bioaccumulation for 48 h.

net dry mass of mycelia cultured in dye-containing culture media at different initial dye concentrations for 48 h. As represented in Fig. 2, the concentration of net dry mass of mycelia hardly reduced at initial dye concentrations in the range of 10.6–114.7 mg/l. It indicates that the fungus has a considerable tolerance to reactive brilliant blue KN-R at this initial dye concentrations range. When the initial dye concentration increased to 160.7 mg/l, an apparent reduction in the concentration of net dry mass of mycelia was observed. The concentration of net dry mass of mycelia decreased with increasing initial dye concentrations. It may result from the increasing toxicity of dye to fungus through inhibition of metabolic activities. Nevertheless, the concentration of net dry mass of mycelia was ≥0.410 g/l, which is the initial concentration of dry mass of mycelia immobilized on beads. Thus, though the dye was toxic to the fungus at initial dye concentrations >114.7 mg/l, the immobilized mycelia could still grow until the initial dye concentration reached 1932.4 mg/l, at which the growth of mycelia was completely inhibited.

3.2. Mechanism of dye removal

3.2.1. Bioaccumulation

Prior studies showed that the mechanism for the removal of dyes using growing cells may be due to either biodegradation or bioaccumulation. A shift of the peak absorbance from maximum absorbance of the original dye solution always occurs, and an extra peak absorbance appears in the process of biodegradation of dye [10,19]. In this study, to elucidate the dye removal mechanisms, the immobilized growing *A. fumigatus* beads were contacted with culture media containing about 300 mg/l dye for 72 h. The UV–vis spectra of the fermented broth along with the initial dye-containing culture media diluted appropriately were recorded. As represented in Fig. 3, no difference in the characteristic spectra of the solutions was observed before and after incubation. This suggested that no new byproduct was produced in the process of decolorization of dye-containing culture media by the immobilized growing *A. fumigatus* beads. In addition, it was observed that the immobilized growing *A. fumigatus* beads after contacted with dye appeared blue (data not shown), which was the color of reactive brilliant blue KN-R. Moreover, the color became gradually darker with contact time. It indicated that the structure of dye, especially that of chromophore of dye, was not transformed during bioaccumulation. Furthermore, it was testified that the decolorization efficiency of dye-containing culture media without carbon or nitrogen resources was low and hardly increased with contact time in our previous study [16]. It indicated that reactive brilliant blue KN-R could not be carbon or nitrogen resources for the growth of mycelia. These meant that the chemical structure of dye was not biodegraded by mycelia in the process of decolorization of dye-containing culture media using growing *A. fumigatus* immobilized on CMC beads. Therefore, it could be concluded that bioaccumulation was likely the dominant mechanism.

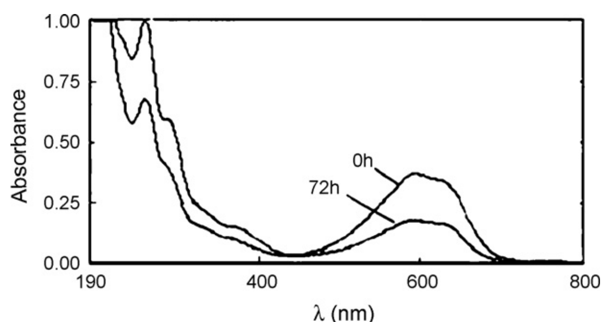

Fig. 3. UV–vis spectra of the fermented broth and the initial dye-containing culture media.

3.2.2. Adsorption and bioaccumulation

Bioaccumulation of metal by actively growing cells involves a combination of metabolism- and temperature-independent and metabolism-dependent steps. The initial rapid accumulation step, i.e., metabolism- and temperature-independent, is thought to involve ion binding at the surface. Bioaccumulation is followed by a second process, which is much slower and can accumulate larger quantities of components than the first metabolism-independent process [3]. The steps of bioaccumulation of dye may be similar to it. To understand the steps in the process of bioaccumulation of dyes from the dye-containing culture media, the abilities of bioaccumulation using immobilized growing *A. fumigatus* beads and sets of dye removal efficiencies at various initial dye concentrations within 48 h were carried out. The data are presented in Table 1.

In the first set of experiments, the biomass of the immobilized *A. fumigatus* beads incubated in basic culture medium for 48 h were equal to or more than corresponding biomass entrapped in immobilized growing *A. fumigatus* beads contacted with dye-containing culture media for 48 h due to dye toxicity (refer to the data in Fig. 2), but the adsorption efficiencies were all lower than those at corresponding initial dye concentrations using immobilized growing *A. fumigatus* beads. So the part of dye removal efficiencies by immobilized growing *A. fumigatus* beads which exceeded that by corresponding immobilized *A. fumigatus* beads could be caused by metabolism-dependent accumulation. Moreover, the adsorption efficiencies data suggest that metabolism-independent adsorption should be inferiorly responsible for the overall removal of the dye from the aqueous solution via bioaccumulation (15–23% proportion). In the second set of experiments, the killed free cells showed effective dye removal by biosorption. It suggests that the bioaccumulation by immobilized growing *A. fumigatus* beads includes a metabolism-independent biosorption process by mycelia entrapped in them. In the third set of experiments, the cell-free immobilized beads could also remove dye from dye-containing culture media. Enlightened from the adsorption between Cd(II) and CMC [20], it may be explained by the electrostatic attractions between the cell-free immobilized beads and the dye. The immobilization matrix CMC is a natural polymer containing hydroxyl, which may be ionized into negatively charged anions in an aqueous solution. The functional groups like amidogen in the dye may be ionized into positively charged amidogen in dye-containing culture medium. Hereby, the dye was adsorbed in the cell-free immobilized

Table 1
Bioaccumulation and three sets of experiments

Initial dye concentration (mg/l)	Bioaccumulation efficiency (%)	Biosorption and adsorption efficiency (%)			Bioaccumulation capacity (mg/g)[d]
		1st[a]	2nd[b]	3rd[c]	
70.3	100.0 ± 0.0	23.1 ± 1.5	93.2 ± 4.5	4.0 ± 0.2	34.1 ± 0.05
213.1	98.3 ± 0.3	15.3 ± 0.8	66.9 ± 3.2	2.9 ± 0.12	148.5 ± 1.6
374.4	57.6 ± 0.5	10.4 ± 1.0	50.4 ± 4.6	2.1 ± 0.025	190.5 ± 2.0
570.3	31.7 ± 1.2	5.0 ± 0.2	23.3 ± 2.1	1.4 ± 0.05	188.0 ± 3.8
734.1	20.6 ± 1.0	0 (Approx.)	19.7 ± 0.8	0 (Approx.)	188.1 ± 7.8

[a] The first set of experiments. Dye aqueous solutions were used as dye removal systems because there was a lack of nutrition for metabolism of mycelia in these solutions.
[b] The second set of experiments. Killed free cells were used as a biosorbent to identify the biosorption of dye. The cells dosage was 1%.
[c] The third set of experiments. Cell-free immobilized beads were used to identify adsorption by immobilization matrix.
[d] The unit mg/g was mg/g (of dry biomass). As calculated, 1.0 g (wet mass) of immobilized mycelia beads contains 0.031 g of immobilized matrix in the experiments.

beads by attraction. The adsorption efficiencies data indicate that adsorption on immobilization matrix also contributes to the total dye removal from dye-containing culture media by bioaccumulation. Therefore, it can be concluded that the bioaccumulation using immobilized growing *A. fumigatus* beads is achieved by metabolism-dependent accumulation in majority and metabolism-independent adsorption, which includes biosorption by mycelia entrapped in them and adsorption on immobilization matrix. Nevertheless, the biosorption mechanism by mycelia is complicated. As reported, biosorption involves a combination of active and passive transport mechanisms starting with the diffusion of the adsorbed component to the surface of the microbial cell. Once the chemicals have diffused to the cell surface, it will bind to sites on the cell surface which exhibit some chemical affinity for the component. Generally, such adsorption is fast, reversible, and not a limiting factor in bioremoval kinetics when dealing with dispersed cells. Biosorption is often followed by a slower binding process in which additional component is bound, often irreversibly [18]. The mechanism of biosorption by mycelia entrapped in the immobilized growing *A. fumigatus* beads needs be explored further.

As presented in Table 1, it should be noted that, although the growth of mycelia may be inhibited significantly at high initial dye concentrations as shown in the above dye toxicity study, the bioaccumulation capacity was not markedly affected and in fact the maximum bioaccumulation capacity was 190.5 ± 2.0 mg/g at an initial dye concentration of 374.4 mg/l. Table 1 also shows that the trending of bioaccumulation capacities as a function of the initial dye concentrations was increased. The initial dye concentration provides an important driving force to overcome all mass transfer resistance of all molecules between the aqueous and solid phases [21]. So the higher dye removal capacities by biosorption using dried dead biomass can be obtained at higher initial dye concentrations due to the greater driving force [22]. Thus, the metabolism-independent biosorption capacities increased with increasing initial dye concentrations. Consequently, though the total accumulation quantities decreased due to greater metabolism inhibition resulting from the greater toxicity of dye at higher dye concentrations, the bioaccumulation capacities increased in trend with increasing initial dye concentrations.

3.3. TEM images of mycelia entrapped in immobilized mycelia beads before and after bioaccumulation

Fig. 4 represents the TEM images of the interior structures of mycelia entrapped in matrix before and after bioaccumulation in culture media containing 200 mg/l dye. It shows that *A. fumigatus* mycelia own septum and vary in diameter and length. In addition, the acute angle embranchments of mycelia were visualized. However, there were differences between mycelia before and after bioaccumulation. The parts of cell walls were damaged on the surface of some mycelia after bioaccumulation (Fig. 4(d)), whereas on the surface of mycelia before bioaccumulation they were intact. The damaged part cell walls may be due to the toxicity of dye in culture media containing 200 mg/l dye.

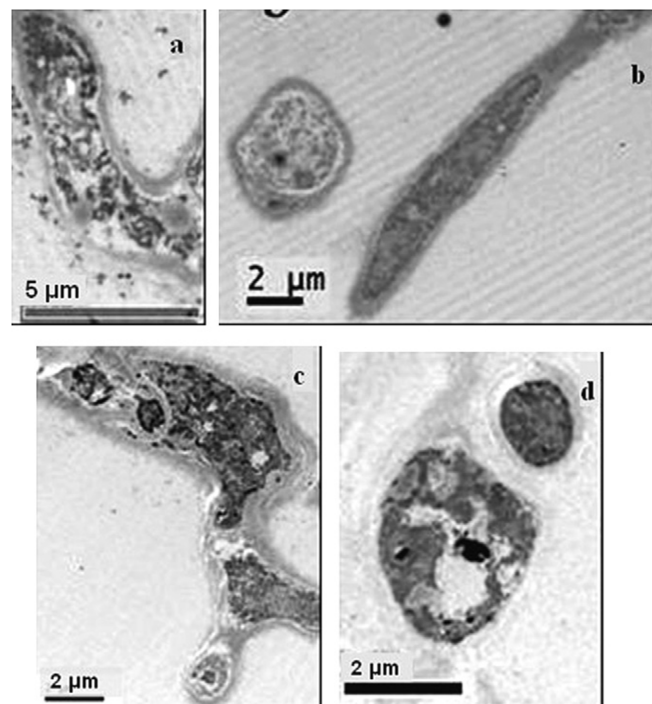

Fig. 4. TEM images of mycelia entrapped in immobilized mycelia beads. (a and b) Before bioaccumulation and (c and d) after bioaccumulation.

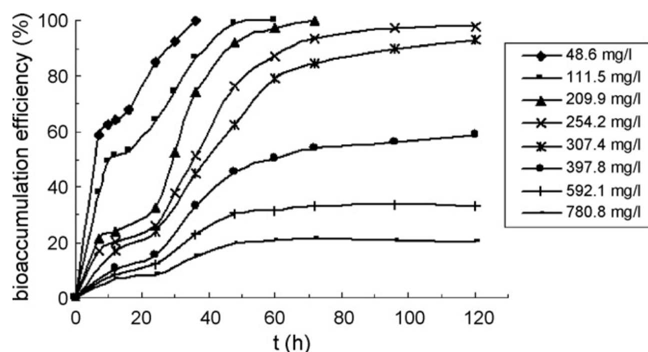

Fig. 5. Bioaccumulation with contact time at various initial dye concentrations.

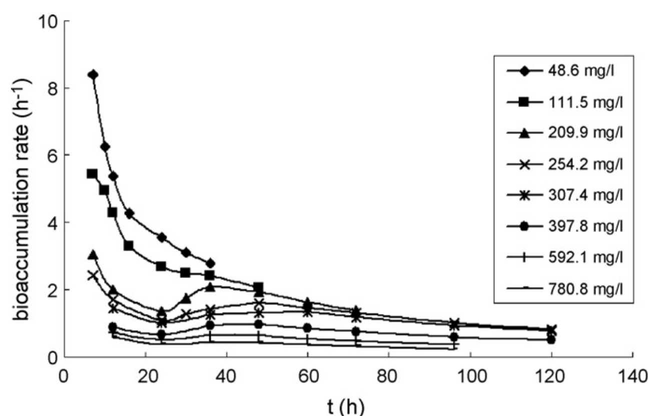

Fig. 6. Bioaccumulation rate with contact time at various initial dye concentrations.

In particular, it was observed that there were residual dyes in the TEM samples after the samples had been dehydrated using ethanol concentration series (50–100%, v/v) in the process of TEM samples preparation. The residual dyes can be preliminarily assumed owing to chemical biosorption and intracellular accumulation of dye. But further research should be done to prove this assumption.

3.4. Bioaccumulation with contact time at various initial dye concentrations

In view of the toxicity of dye, the bioaccumulation at various initial dye concentrations was investigated in order to determine their extents of dye removal. Fig. 5 shows the bioaccumulation efficiencies with contact time at initial dye concentrations in the range of 48.6–780.8 mg/l. Standard deviations were all found to be within $\pm$ 3.0% for the results of bioaccumulation efficiencies. Hence, the error bars were not shown in Fig. 5.

Fig. 5 represents that complete decolorization of the dye-containing culture media by bioaccumulation was achieved within 72 h at the initial dye concentrations of <209.9 mg/l. Decolorization of dye-containing culture media was incomplete at the initial dye concentrations of >209.9 mg/l. However, the bioaccumulation efficiencies both exceeded 93% at an initial dye concentration of 254.2 mg/l in 72 h and at an initial dye concentration of 307.4 mg/l in 120 h. They decreased to a significant extent (20.6%) with increasing initial dye concentrations.

Fig. 6 shows that the bioaccumulation rates were not constant over the contact time. Bioaccumulation rate (h^{-1}) is defined as the ratio of bioaccumulation efficiency to bioaccumulation time. In all the systems examined, the bioaccumulation rates were initially rapid in several hours and then gradually slowed down. They became rapid again at 24 h until 72 h at initial dye concentrations $\geq$209.9 mg/l. According to joint function of dye bioaccumulation, it is not difficult to explain the irregular bioaccumulation rate over the contact time. The relatively rapid bioaccumulation rate in initial several hours may result from rapid adsorption by entrapped mycelia and immobilization matrix. The following period of slower bioaccumulation rate may be in the mycelia adaptation phase after the immobilized mycelia beads were transferred into a new dye-containing culture media. The bioaccumulation rate became slower again after 72 h as the nutriments in the dye-containing culture media were almost used up.

4. Conclusions

The performed studies demonstrate that bioaccumulation is likely the dominant mechanism of decolorization of dye-containing culture media using immobilized growing *A. fumigatus* beads. It is achieved by metabolism-dependent accumulation and metabolism-independent adsorption (15–23% proportion of overall dye removal), which includes biosorption by mycelia entrapped in them and adsorption on immobilization matrix. The toxicity test showed that the fungus could tolerate the dye at initial concentrations of $\leq$114.7 mg/l. At higher initial dye concentrations, the microbial growth was inhibited significantly, but the bioaccumulation capacity was not markedly affected and in fact, the measured maximal bioaccumulation capacity of 190.5 $\pm$ 2.0 mg/g was at an initial dye concentration of 374.4 mg/l. Our study showed that complete decolorization of the dye-containing culture media by bioaccumulation was achieved within 72 h at the initial dye concentrations of <209.9 mg/l, and that the bioaccumulation rates were not constant over the contact time in all the systems examined due to the complicated mechanism of bioaccumulation. The bioaccumulation rates were initially rapid in several hours and then gradually slowed down. They became rapid again at 24 h until 72 h at initial dye concentrations $\geq$209.9 mg/l.

From the above results, we believe that this dye bioaccumulation biotechnology has a good perspective in the future application due to the efficient dye removal and no toxic byproducts produced in the whole process of dye removal. It overcomes the problem of decolorization by biodegradation, which may produce new toxic byproducts owing to incomplete mineralization.

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