RSC Advances

Synergistic removal of dyes by Myrothecium verrucaria immobilization on chitosan-Fe membrane

Journal:	*RSC Advances*
Manuscript ID:	RA-ART-06-2015-011320.R1
Article Type:	Paper
Date Submitted by the Author:	18-Jul-2015
Complete List of Authors:	Wen, Yuezhong; Institute of Environmental Science, Zhejiang University, Liang, Ying; Zhejiang University of Technology, Shen, Chensi; Donghua University, Wang, Hongkai; Zhejiang University, Fu, Dongmei; Dalian Institute of Chemical Physics, Wang, Hongyu; Zhejiang University of Technology,

Synergistic removal of dyes by *Myrothecium verrucaria* immobilization on chitosan-Fe membrane

Yuezhong Wen [a*], Ying Liang [b], Chensi Shen [c], Hongkai Wang [d*], Dongmei Fu [e], Hongyu Wang [b]

[a]Institute of Environmental Science, Zhejiang University, Hangzhou 310058, China

[b]College of Civil Engineering and Architecture, Zhejiang University of Technology, Hangzhou 310032, China

[c]College of Environmental Science and Engineering, Donghua University, Shanghai 201620, China

[d]Institute of Biology, Zhejiang University, Hangzhou 310058, China

[e]Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

---

[*] Corresponding author:

Tel.: +86-571-8898-2421. Fax: +86-571-8898-2421.

E-mail address: wenyuezhong@zju.edu.cn (Y.W.) and hkwang@zju.edu.cn (H. W.)

ABSTRACT

A green efficient strategy for the combination of biodegradation and adsorption methods is urgently desired nowadays to degrade dyes in an economic and effective way. In this study, a kind of novel, environmentally friendly and efficient hybrid for the fungus and chitosan-Fe membrane has been fabricated by the alginate approach. *Myrothecium verrucaria* I-5 and C-1, deuteromycete fungus capable of producing laccase to degrade dyes, have been used. It is found that the chitosan-Fe membrane can excellently immobilize the fungi mycelia and enzyme, and after the immobilization, the fungi and enzyme still maintain a high activity after the dyes decolorization. Also, the immobilized fungi membranes have been proved to have a highly efficiency for dye decolorization. Simultaneously, the decolorization ability of the crude enzymes, which are extracted from I-5 and C-1 medium, and the decolorization ability of chitosan-Fe membrane are also investigated, respectively. The results demonstrate that the dyes can be removed by the fungi and the membrane synergistically. In addition, different kind of dyes are successfully removed with chitosan-Fe immobilized fungi membrane. These results demonstrate that dyes are first adsorbed on the chitosan-Fe membrane, decreasing the toxicity to the fungus, and then bio-degraded by the laccase produced from the fungi on the membrane.

Keywords: Fungi; Chitosan-Fe; Membrane; Immobilization; Decolorization

1. Introduction

Dyes are extensively used in varies applications in food, pharmaceutical, paper, cosmetic, textile and leather industries, and they generate a considerable amount of colored wastewater [1, 2]. Dyes with azo-based are the largest group of synthetic dyes known and most of them are toxic to the aquatic biota even carcinogenic to humans, so they must be removed before the effluents are discharged into the water bodies [3]. Although a wide range of methods has been developed for the removal of synthetic dyes from wastewater to decrease their negative impact on the environment, such as adsorption [4, 5] chemical oxidation [6], microbiological or enzymatic decomposition [7], electrochemical treatment [8], some limitations still exist. Compared with other wastewater treatment methods, adsorption has been found to be initial cost flexibility, simplicity of design, ease of operation [9]. At the same time, it is well known that biodegradation is an environmentally friendly and cost competitive alternative way to remove the environment pollutants [10]. Many researchers reported the combination of fungi and chemical method for synergistic treatment of wastewater. Ma et al. utilized Pseudomonas putida immobilized on activated carbon fiber to treat phenolic water and found that through adsorption and biodegradation two stages, the high concentrated phenol wastewater could achieve good removal efficiency [11]. Ong et. al. used a granular activated carbon biofilm for textile effluent treatment and achieved high removal efficiency because of the simultaneous adsorption and biodegradation processes [12]. Compared with the traditional methods, combination methods are much more efficient, and most of them are reproducible and

environmentally friendly especially with the biodegradation process. Therefore, a green efficient strategy for the combination of biodegradation and adsorption process is urgently desired nowadays to synergystic degrade dyes in an economic and effective way and avoid the secondary pollutions production.

For biodegradation, there are several studies about the decolorization of dyes by immobilized fungi. Compared with the free fungal cells, immobilized fungi offer several advantages. For example, they could resilient to environmental perturbations, such as pH or exposure to toxic enviroment, easy separation of cells from liquid medium, and protection from shear damage [13, 14]. There are two types of cell immobilization, entrapment and attachment [15]. Entrapment is used extensively, and during this process, alginate is the most commonly used polymer as the medium because it has the ability to form the gel mildly when it encounters some divalent cations such as Ca^{2+} (preferably used due to the biocompatibility) and Ba^{2+} (except Mg^{2+}) [16, 17]. Most of the previous studies have concentrated on the immobilization of the white-rot in Ca-alginate beads to decolorize dyes [13]. In recent years, some deuteromycetes have been reported as potential laccase producers offering advantages such as short growth period, simple life cycle and ease of genetic modification. *Myrothecium verrucaria* is a deuteromycete fungus that produces high level of a novel laccase [18].

For adsorption, chitosan is used extensively as absorbent after modification. It is one of the most representative biopolymers, receiving considerable interest for pollutants removal due to its excellent metal-binding capacities and low cost, and it

is known as an ideal support material for enzyme immobilization because of its many characteristics such as hydrophobicity, biocompatibility [19, 20]. When they chelate with metal, their chemical stability and high capacity in adsorption processes have substantial been improved. Iron-chitosan composites and Fe-crosslinked chitosan complex applied to remove Cr (VI), As (III) and As (V) in wastewater has been reported, and some metal ion complex was proposed to efficiently adsorb ionic dyes through strong chelating interactions [21, 22]. In our previous study, we prepared chitosan-Fe (III) hydrogel by chelation procedure to adsorb dyes under alkaline condition with a high maximum adsorption capacity of 294.5 mg/g [23]. In this study, in order to better load the fungi, we prepared the metal chelating chitosan in the form of a membrane to expand the load area and facilitate the inoculation of the fungi.

The *Myrothecium verrucaria* were immobilized on the chitosan-Fe membrane by using alginate to attain eco-friendly and efficient materials. The aim of the present work is to investigate the possibility for the synergistic removal of dyes by biological and physical effect, to identify the chitosan-Fe membrane adsorb the pollutants from the waste water first, and then the enzyme produced from the fungi that growing on the membrane degrade the dyes subsequently.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Zhejiang Golden-shell Biochemical Co., Ltd, Zhejiang, China (deacetylation degree = 91.0%). $FeCl_3 \cdot 6H_2O$, H_2SO_4 (18.4 mol/L),

NH_4OH, sodium alginate, $CaCl_2$ were obtained from National Medicines Corporation Ltd. of China. ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) was purchased from Aladdin. The nutrient oat was ordinary sugar-free oat purchased from supermarket. C. I. Acid Red 73 (AR 73), Acid Blue 113 (AB113) and other dyes were commercial products, their structural formulae were presented in **Table S1** in supporting materials. Distilled water was used throughout this study.

Pure fungus strain I-5, C-1 were obtained from Dr Hongkai Wang (College of agriculture & biotechnology, Zhejiang University, Hangzhou, China) who collected and separated them from decayed agricultural mulch on Xinjiang province, maintained on potato dextrose agar (PDA) plates at 4°C and subcultured every 3 months.

2.2. Methods

2.2.1. Preparation of chitosan-Fe membrane

Chitosan powder was dissolved in 0.1mol/L $FeCl_3$ aqueous solution, and the mixture was magnetic stirred for 2 h at room temperature. Subsequently, glycerol was added into the mixture and stirred for 30 min, and then ultrasonicated to drive bubbles in the mixture away. The mixture was then poured into the culture dishes (D = 6 cm) equally, laid aside in the oven horizontally and dried at 50 °C, then transferred to the fume hood. 2 mL $NH_4{\cdot}OH$ was added to each of the culture dishes, and the dish was shook mildly to guarantee the $NH_4{\cdot}OH$ sufficient contact with the membrane. The redundant NH_4• OH was then decanted, and 2 mL H_2SO_4 (10%, v/v) was added to each culture dish, crosslink 10 min, and the color of the membrane changed to canary.

Then the membrane was thoroughly washed with tap water to remove the unreacted H_2SO_4, then washed with the distilled water until the pH of the washed water was neutral. Thus, the Chitosan-Fe (CS-Fe) membrane was prepared and kept in the distilled water piece by piece. To get the dry weight of CS-Fe membrane, laid them aside in the oven horizontally and dried at 50 °C and then weighed, each of them were about 0.2 g.

2.2.2 Activation and fermentation

Fungus I-5 and C-1 were activated on PDA plates at 28 °C for seven days. 1 cm^2 mycelia was inoculated to 50 mL potato dextrose broth, and then transferred to a thermostatic shaker maintained at 28 °C, 150 rpm. Sampling every day to analyze the variation of laccase activity during the fungus growth [24].

2.2.3. Fungi species identification

To identify the species of the fungus, fungus cultivated on the PDA plates were scraped into the centrifuge tube for extracting DNA by grinding, separating and depositing, and then PCR amplification. The DNA extractive was analyzed with agarose gel electrophoresis (AGE). The DNA mass was measured by using AGE analyzer in the mixture of 1×Tris acetate-EDTA (TAE) buffer and agarose gel (1.5%) stained with ethidium bromide (EB). DNA was collected and purified after AGE and sent to Shanghai Sangon Biotech Corporation to measure internal transcribed spacer (ITS) sequence. The resulting sequences were verified by blast searches in National Center for Biotechnology Information (NCBI). The accession number of the strain C-1 is KT305923, the accession number of the strain I-5 is KT305924.

2.2.4. Immobilization

Medium

Oat was wrapped with absorbent cotton gauze and immersed into the water to extrude the nutrition and impurities out, then sodium alginate (4%, w/v) was added to the oat solution, the mixture was stirred at room temperature for 2 h. Then sterilized in high-pressure steam sterilization pot at 121 °C for 20 min.

Inoculation and immobilization

Chitosan-Fe membranes were spread in sterile culture dishes (6 cm), ultraviolet germicidal irradiated for 30 min, then 7 mL medium was added on the membrane by using the disposable sterilized syringe. After the ultraviolet germicidal irradiation for 30 min again, the I-5 or C-1 was inoculated, and stirred gently with the sterile spear. Meanwhile, the membrane with fungus but without chitosan-Fe and the membrane with chitosan-Fe but without fungus were prepared as the contrast test. The mixture medium of sodium alginate (defined as SA) and sodium alginate with oat (defined as SA-oat) were used for the immobilization matrix and nutrition donor. We defined the membranes prepared above respectively: the membrane inoculated with strain I-5 and with mixture medium and CS-Fe membrane was abbreviate to CS-Fe-SA-oat-I-5,the same definition of CS-Fe-SA-oat-C-1, the membrane inoculated with strain I-5 or C-1 and with mixture but without CS-Fe membrane were abbreviated to SA-oat-I-5 or SA-oat-C-1, the membrane with mixture medium and CS-Fe membrane but without strain was abbreviate to CS-Fe-SA-oat. Subsequently, all of these membranes were transferred to the biochemical incubator and cultivated 2-3 d at 28 °C, and taken out

before spore-bearing. At last, 0.2 mol/L calcium chloride solution was used to immobilize the membrane to prevent the medium dissolve out.

2.2.5. Crude enzyme extract

The mixture of the mycelia and the broth were placed in the centrifuge tube. After centrifugation at 10,000 g, 4 ℃ for 10 min, the supernatant was used as the crude extract, and stored at 4 ℃.

2.2.6. Assessment of laccase activity

Laccase activity was determined spectrophotometrically at 436 nm (ε_{436} = 29,300 $M^{-1}cm^{-1}$), using ABTS as the substrate, at 30 ℃ in 0.2 mol/L sodium acetate buffer (pH 4.5). The reaction solution consisted of 2670 μL sodium acetate buffer (0.2 mol/L, pH 4.5) and 600 μL ABTS (10 mmol/L). After immersed in a constant 30 ℃ bath for 5 min, the reaction solution was added 30 μL enzyme solution. The change in the absorbance due to the oxidation of ABTS was monitored at 436 nm and recorded after 3 min with Shimadzu UV-2401PC UV-vis spectrometer (Tokyo, Japan).

For the fungus membrane immobilization, the membranes with 0.6 cm^2 in size were suspended in 3 mL ABTS solution (10 mmol/L ABTS in 0.2 mol/L pH 4.5 sodium acetate buffer). The absorbance was measured once per minute [25] and recorded after 5 min. One unit (U) of the enzyme activity was defined as the amount of the enzyme that oxidized 1 μmol of ABTS min^{-1} [26].

2.2.7. Decolorization studies

In order to examine the de-colorization performance, five kinds of membranes (CS-Fe-SA-oat-I-5, CS-Fe-SA-oat-C-1, SA-oat-I-5, SA-oat-C-1, and CS-Fe-SA-oat)

were put into the dyes，the membranes used here were prepared the same as before in section **2.2.4**. All the de-colorization experiments were performed in 100 mL flasks, which were sealed and agitated at 150 rpm in a thermostatic shaker maintained at 28 °C. The typical reaction mixture was initiated with 50 mL of dye at 50 mg/L and one piece of the membrane. Dye concentrations were analyzed using a Shimadzu UV-2401PC UV-vis spectrometer (Tokyo, Japan) at its maximum absorbance. The removal of dyes was calculated by equation (1):

$$\text{Removal rate (\%)} = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

Where C_0 is the initial concentration of dye in the liquid-phase (mg/L), C_t is the residual concentration of dye in the liquid-phase (mg/L)

At the end of each batch, the membranes were rinsed twice with distilled water and transferred into fresh dye solution for the next decolorization batch experiment to evaluate the reusability of the prepared membrane [13]. Determined the removal rate of the dye after each batch and repeat the process for three times.

For the membrane desorption experiments, the membranes were fixed in 25 mL 1mol/L NaOH for 1 h, then 1 mol/L HCl was added to neutralize the solution. Then the absorbance of the solution was measured with the spectrophotometer.

Enzyme decolorization experiments were carried out to evaluate the effect of the enzyme for the removal of the dyes. 0.5 mL of crude enzyme were added in 10 mL of 50 mg/L dye solution, and the residual concentration of the dye was measured at different time intervals.

2.2.8. Characterization of the membranes

Surface and cross-section morphology was studied with an electron microscope. The scanning electron micrographs (SEMs) of CS-fungus composite membranes and fungus membranes were obtained with Hitachi TM-1000 at a voltage of 25.0 KV. Before analysis, the samples need to be pretreated. They were first fixed with 2.5% glutaraldehyde in phosphate buffer (0.1mol/L, pH 7.0) for more than 4 h, then washed with phosphate buffer, then postfixed with 1% OsO_4 in the phosphate buffer for 1-2 h and washed with the buffer. Then the samples were hydrated with different ratio of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%), and then transferred to the mixture of the alcohol and iso-amyl acetate, then transferred to the pure iso-amyl acetate overnight. In the end, the samples was dehydrated in Hitachi model HCP-2 critical point dryer with liquid CO_2. The dehydrated samples were coated with gold palladium in Hitachi Model E-1010 ion sputter and observed in Hitachi Model TM-1000 SEM.

3. Results and discussion

3.1. Characterization of the membranes

Scanning electron micrographs (SEMs) of surface and cross-section of the membranes (CS-Fe-SA-oat-I-5, CS-Fe-SA-oat-C-1, SA-oat-I-5, SA-oat-C-1) are shown in **Fig. 1**. It illustrated that the surface of the membranes was covered with thickly mycelia. For the membranes with CS-Fe, the mycelia growth was denser than that without CS-Fe. Although I-5 and C-1 were both *Myrothecium verrucaria*, and they were both rod shaped, there were enormous difference in their morphology, for example, there were many anxiolytic branch on the rod of the fungi C-1. It can be

seen from the cross-section SEMs that the membrane is divided into three layers, the surface is mycelia layer, the middle is the mixture of the mycelia and SA-oat (mixture of sodium alginate and oat), and the bottom is the CS-Fe membrane. When sodium alginate was blended with oat, the structure of the cross-section was glossy and no porous was observed, which was different with the fungus immobilized on the alginate beads [14].

3.2. Fungi species identification

The image of the agarose gel electrophoresis analysis is shown in **Fig.2**. It shows that the DNA bands of I-5 and C-1 are parallel and both of them are about 550 bp. The ITS are shown in **Table S2** in supporting materials, the comparison consequence with above are the same. A BLAST (Basic Local Alignment Search Tool) search result showed that ITS sequence of this fungus was highly homologous (99% percent similarity) to that of *Myrothecium verrucaria.*

3.3. Assessment of laccase activity

Laccase contains four neighboring copper atoms, which are distributed among different binding sites in the molecule and are differentiated by specific characteristic properties allowing them to play an important role in its catalytic mechanism [27, 28]. Laccases are used in many applications, and the most important use of this enzyme is to decolorize recalcitrant dyes [29, 30]. **Figure 3** shows the change of enzyme activity with time in AB113 solution after using SA-oat-Fungus membrane to decolorize the dyes. It can be seen that the enzyme activity presents an increase trend because SA-oat-fungus membrane could not hold the nutrition of oat, leading to the oat came

out of the membrane and immersed in the solution. While when the dyes were treated with CS-Fe -SA-oat-Fungus membrane, no enzyme activity was determined after decolorization, which idicated the favorable immobilization of enzyme on CS-Fe membrane. It could be verified in **Fig.4**, the enzyme activity on CS-SA-oat-Fungus membrane was higher than SA-oat-Fungus membrane. There are many reports about the enzyme immobilization on chitosan because of its many characteristics like hydrophobicity and biocompatibility.

The laccase activity on the membranes before and after the reaction with different dyes were also determined, and the same trend was observed as shown in **Fig. 4**. It can be seen that the membrane with CS-Fe maintains higher activity than that without CS-Fe, and after the reaction, the laccase activity on CS-SA-oat-fungus membranes is still higher than that only on the SA-oat-fungus membrane.

3.4. Single batch decolorization experiment

To determine the decolorization time of different dyes (AR 73 and AB 113), single batch decolorization experiment was done by using five kinds of membranes, and the results were shown in **Fig. 5**. It can be seen obviously that it takes a much longer time to treat AR73 than AB113, and the removal of the AR73 with the CS-SA-oat-fungus membrane reaches equilibrium for about 12 h while only about 4 h for AB113. The CS-SA-oat-fungus membrane could effectively decolorize the basic dyes better than the CS-SA-oat and SA-oat-fungus membranes no matter in AR73 or AB113, and the dyes removal with the CS-Fe-SA-oat-fungus membranes were above 98% for AR73 and above 82% for AB113. It could be seen in **Fig. 5a** and **5b** that during the removal

of AR73, the absorption of the CS-Fe membrane plays the main role in the decolorization, although the medium weakens the adsorptive property of the CS-Fe membrane. The dyes removal on SA-oat-fungus membranes could reach 50% after 12 h. Because of the participation of the fungus, their consumption of the medium during growth and their metabolite enzyme gathering on the CS-Fe membrane greatly improved the decolorizing performance.

Fig. 5c and 5d show that during the removal of AB113, degradation of the fungus play a main role in decolorization. The membrane without CS-Fe could also reach equilibrium in 4 h, a little later than the CS-Fe-SA-oat-fungus membrane. The membranes with fungus and CS-Fe show a bit superiority than only with fungus for dyes removal efficiency. The dyes removal with CS-Fe-SA-oat-I-5 and CS-Fe-SA-oat-C-1 could reach 88% and 84%, respectively, while with the SA-oat-I-5 and SA-oat-C-1, the dyes removal could reach 86% and 82%, respectively. Considering the restriction of time and the influence of the medium, the CS-Fe membrane has not entirely evaluated for its adsorption during the removal of AB113, the total removal of dyes with CS-Fe was 55% after 4 h reaction.

We could conclude from the above results in the single batch experiment that during the removal of dyes, the CS-SA-oat-fungus membranes synergistic removal the dyes by the fungus and the chitosan-Fe membrane. The chitosan-Fe membrane adsorbed the dyes first, then the fungus product enzyme degraded the dyes adsorbed on the membrane. When the dyes are resistant to be degraded, the dyes adsorption on the membrane plays a main role and the fungus react with the dyes gently to remove

the dyes, while when the dyes are easy to be degraded, the degradation of the fungus play a main role to remove the dyes.

3.5. Crude enzyme decolorization

In the previous study, *Myrothecium verrucaria* NF-05 laccase has been purified and used for the degradation and decolorization of the recalcitrant dyes [31], and there also several studies about the decolorization of dyes by laccase proteins [32, 33]. Inspired with the above previous study results, we proposed that the *Myrothecium verrucaria* C-1 and I-5 obtained from the extracellular enzyme production also could decolorize dyes. The crude enzyme were used to decolorize dyes of AR73 and AB113. The laccase activity before adding to the dyes were 892.833 and 311.945 U/mL for I-5 and C-1, respectively, and the results of dyes removal with the crude enzyme were shown in **Fig. 6**. It can be seen from Fig. 6 that the AB113 decolorization could reach equilibrium at 1.5 h, and the removal could get above 70%, while for AR73, the removal is only 50% until to the third day. These phenomena were similar to that of the fungus decolorization of dyes. We could conclude that for the different dyes, the crude enzyme shows the different activity for the dyes removal, and the similar results were also reported by Mohorci and his co-workers [34]. It also has been reported that laccase could not degrade some recalcitrant dyes because of the low efficiency of electron transfer between the substrate and active site of the laccase protein, which is usually deeply located inside the enzyme molecule [18].

3.6. Desorption

In the decolorization experiments, we found that on the membranes which

inoculated with fungus, the zone around the inoculated point at the backside of the membranes especially on the CS-Fe membrane were pale in color than other zone. We propose that dyes absorbed on the membrane and made the color zone. So we designed the desorption experiment to desorb the dyes from the membrane. In order to compare the effect of dyes absorption on different membrane, CS-SA-oat-fungus membranes and CS-SA-oat membranes were used. Furthermore, from the absorbance change of the dyes on the different membranes, the decolorization of the dyes on the membrane could be verified. 1 mol/L NaOH was used as the desorption solution according to the result of our previous study [23]. The dyes used in the experiment become darker in color under alkaline condition resulted in the increase of the absorbance. HCl was used to adjust the pH after the desorption process. **Figure 7** show us that for different kinds of dyes. The decolorization for CS-SA-oat-fungus membrane is apparent lower than that for CS-SA-oat membrane.

3.7. Repeated batch experiments

The reusability of the I-5 and C-1 membranes for the dyes decolorization was investigated in repeated batch, and the membranes without fungus or without CS-Fe membrane were used as controls. As shown in **Fig. 8**, for both AR 73 and AB 113 removal, the membrane with fungus and CS-Fe shows significant more positive reusability for the dyes decolorization than the controls. For AR 73, after twice batch, the removal with SA-oat-I-5 membrane is negative, and with the SA-oat-C-1 membrane, the removal rate is negative even after the first batch, which means the SA-oat-I-5 and SA-oat-C-1 membrane lost their activity in short time. It is because

the fungus took a long time (about 3-5 d) to decolorize AR 73, and before the next batch, many pollutants were aggregated on or in the membrane. When the next batch began, due to the vibration of the shaker, the SA-oat medium could not firmly grasp the dyes already on the membrane, so the dyes dispersed again in the solution, leading to the increase of the concentration. At the same time, due to the toxic effect of the accumulated dyes, the activity of the fungus thereby weakened, and therefore decreased the degradation efficiency. For the CS-SA-oat-fungus, because of the existence of CS-Fe membrane, most of the dyes are adsorbed on the membrane tightly, and hardly dispersed in solution, so the adversely effect of the dyes to the fungus was decreased, thereby the removal ability was improved. For AB 113, as shown in **Fig. 8 c, d**, all the curves are positive, and they show excellent removal at every batch. As disscussed in the above single batch experiment, *Myrothecium verrucaria* has higher activity to degrade the AB 113 in short time, so before the membrane were transferred to the next batch after 48 h, the dyes have been almost completely degraded on the memebrane, thus both of the CS-SA-oat-fungus and SA-oat-fungus membrane showed positive effect for the dyes removal at every batch.

3.8. Scalability experiment

The complicated structures of dye molecules, which vary with respect to the organic chains and the numbers and positions of functional groups, are directly related to their adsorption behaviors [23], and the fungus also have different degradation behaviors with different dyes. To evaluate the removal capacity of different dyes by the membranes, the same removal condition used for AR 73 and AB 113 were applied

to the removal of a variety of anionic dyes in 24 h. The acid dyes, such as C.I. Acid Black 1, C.I. Acid blue 193, C.I. Acid blue 40, C.I. Acid blue 25 and Silk Scarlet, and the reactive dyes, such as C.I. Reactive Yellow 2 and C.I. Reactive Red 24 were chosen as the prototypical dye pollutants, and the results were shown in **Fig. 9.** It can be seen that the membrane with the combination of fungus and CS-Fe could decolorize most of the dyes effectively, and the membrane with fungi I-5 is superior to the membrane with fungi C-1, which is corresponding to the results in the above enzyme decolorization experiment. The removal of most of the dyes could get more than 80%, and higher removal was observed for dyes decolorization with CS-SA-oat membrane.

We combined **Fig.7** and **Fig.9** to analyze the ability of degradation. In **Fig. 9**, the CS-SA-oat-fungus membrane could remove almost all of the dyes at a high removal rate. In **Fig.7**, after desorption, the desorption concentration are a much smaller part of the removal concentration, and the ratio is much lower than the CS-SA-oat membrane, we thought the losing part was degraded by the fungus. Therefore, the dye de-colorization mechanism is the synergistic combination of adsorption process (for chitosan-Fe) and biodegradation (for fungi).

4. Conclusion

In this study, *Myrothecium verrucaria* was found to have the ability to degrade different dyes and produced high level of laccase which can be used to degrade dyes only in the case of the existence of adequate nutrition. The mixture of sodium

alginate and oats were used to meet the immobilization and nutrition needs. It was found that the membrane can excellently immobilize the fungi mycelia and enzyme, and after the immobilization, the fungi and enzyme maintain a high activity after dye decolorization. With the chitosan-Fe on the membrane, the laccase maintain high activity before and after dye decolorization than without chitosan-Fe on the membrane which confirms that the chitosan-Fe has the immobilization ability for the laccase. Different kind of dyes are successfully removed with chitosan-Fe immobilized fungi membrane, with the synergistic effect of adsorption and biodegradation. The dyes are first adsorbed on the chitosan-Fe membrane, decreasing the toxicity to the fungus, and then bio-degraded by the laccase produced from the fungi on the membrane.

Acknowledgments

The authors acknowledge financial support from the Cultivation Fund of the Key Scientific and Technical Innovation Project，Ministry of Education of China (No. 708052), the National Natural Science Foundation of China (No. 21407021 and 21107108), the Shanghai Yang-Fan Program of Science and Technology Commission of Shanghai (No. 14YF1405000), the Fundamental Research Funds for the Central Universities, and DHU Distinguished Young Professor Program.

References

1. R. G. Saratale, S. S. Gandhi, M. V. Purankar, M. B. Kurade, S. P. Govindwar, S. E. Oh and G. D. Saratale, *J. Biosci. Bioeng.*, 2013, **115**, 658-667.

2. G. Crini, *Bioresour. Technol.*, 2006, **97**, 1061-1085.

3. S. Ertugrul, M. Bakir, G. Donmez, *Ecol. Eng.*, 2008, **32**, 244-248.

4. S. Chatterjee, S. Chatterjee, B. P. Chatterjee, A. R. Das, A. K. Guha, *J .Colloid Interface Sci.*, 2005, **288**, 30-35.

5. C. S. Shen, Y. Z. Wen, X. Kang, W. P. Liu, *Chem. Eng. J.*, 2011, **166**, 474-482.

6. C. S. Shen, S. F. Song, L. Zang, X. Kang, Y. Z.Wen, W. P. Liu, L. S. Fu, *J. Hazard. Mater.*, 2010, **177**, 560-566.

7. H. Wu, S. Wang, H. Kong, T. Liu, M. Xia, *Bio. Techno*l., 2007, **98**, 1501-1504.

8. V. K. Gupta, R. Jain, S. Varshney, *J. Colloid Interface Sci.*, 2007, **312**, 292-296.

9. G. Crini, P.-M. Badot, *Prog. Polym. Sci.*, 2008, **33**, 399-447.

10. F. I. Hai, K. Yamamoto, F. Nakajima, K. Fukushi, *Chemosphere*, 2009, **74**, 810-817.

11. X. Ma, N. Li, J. Jiang, *J. Environ. Chem. Eng.*, 2013, **1**, 466-472.

12. S. A. Ong, E. Toorisaka, M. Hirata, T. Hano, *J. Environ. Sci.*, 2008, **20**, 952-956.

13. D. Daassi, T. Mechichi, M. Nasri, S. Rodriguez-Couto, *J. Environ. Manage.*, 2013, **129**, 324-332.

14. K. Enayatzamir, H. A. Alikhani, B. Yakhchali, F. Tabandeh, S. Rodriguez-Couto, *Environ. Sci. Pollut. Res.*, 2010, **17**, 145-153.

13. S. R. Couto, *Biotechnol. Adv.*, 2009, **27**, 227-235.

14. X. Liu, W. Yu, Y. Zhang, W. Xue, W. Yu, Y. Xiong, X. Ma, Y. Chen, Q. Yuan, *J. Microencapsul.*, 2002, **19**, 775-782.

15. Y. A. Morch, I. Donati, B. L. Strand, G. Skja k-Braek, *Biomacromolecules*, 2006, **7**, 1471-1480.

16. D. Zhao, D. Z. Cui, J. S. Mu, X. Zhang, Y. Wang, M. Zhao, *Biocatal. Biotransform*, 2014, **32**, 214-221.

17. F. Zhao, B. Yu, Z. Yue, T. Wang, X. Wen, Z. Liu, C. Zhao, *J. Hazard. Mater.*, 2007, **147**, 67-73.

18. R. S. Juang, F. C. Wu, R. L. Tseng, *Adv. Environ. Res.*, 2002, **6**, 171-177.

21. J. M. Nieto, C. Peniche-Covas, J. Del Bosque, *Carbohydr. Polym.*, 1992, **18**, 221-224.

22. M. T. Klepka, N. Nedelko, J. M. Greneche, *Biomacromolecules*, 2008, **9**, 1586-1594..

23. C. S. Shen, Y. Shen, Y. Z. Wen, H. Y. Wang, W. P. Liu, *Water Res.*, 2011, **45**, 5200-5210.

24. L. Shi, H. Yu, T. Dong, W. Kong, M. Ke, X. Zhang, *Process Biochem.*, 2014, **49**, 1097-1106.

25. J. Hou, G. Dong, Y. Ye ,V. Chen, *J. Membrane Sci.*, 2014, **452**, 229-240.

26. G. Rancano, M. Lorenzo, N. Molares, S. R. Couto, M. A. Sanroman, *Process Biochem.*, 2003, **39**, 467-473.

27. K. S. Shin, C. J. Kim, *Biotechnol. Lett.*, 1998, **20**, 569-572.

28. B. Volesky, S. Schiewer, M. Flickinger, S. Drew, Edited by MC Flickinger and SW Drew, John Wiley & Sons, New York, NY, USA, 1999.

29. A. Mishra, S. Kumar, A. Kumar Pandey, *Int. Biodeterior. Biodegrad.*, 2011, **65**, 487-493.

30. M. J. Zhu, F. Du, G. Q. Zhang, H. X. Wang, T. B. Ng, *Int. Biodeterior. Biodegrad.*, 2013, **82**, 33-39.

31. D. Zhao, X. Zhang, D. Cui, M. Zhao, *PLoS One*, 2012, **7**, e38817.

32. K. S. Wong, Q. Huang, C. H. Au, J. Wang, H. S. Kwan, *Bioresour. Technol.*, 2012, **104**, 157-164.

33. X. Zeng, Y. Cai, X. Liao, X. Zeng, S. Luo, D. Zhang, *Process Biochem.*, 2012, **47**, 160-163.

34. M. Mohorcic, S. Teodorovic, V. Golob, J. M. Friedrich, *Chemosphere*, 2006, **63**, 1709-1717.

FIGURE CAPTIONS

Figure 1. SEM images of the fungus membranes with or without CS-Fe membrane. a. Surface of CS-Fe-SA-oat-I-5 membrane b. Surface of SA-oat-I-5 membrane c. Surface of CS-Fe-SA-oat-C-1 membrane d. Surface of SA-oat-C-1 membrane e. Cross-section of CS-Fe-SA-oat-I-5 membrane f. Cross-section of CS-Fe-SA-oat-C-1 membrane.

Figure 2. DNA mass (5 μL) in 1.5% TAE agarose gel and ethidium bromide (EB) staining electrophoresis of fungus I-5 and C-1: M is Trans2K Plus Ⅱ DNA marker, 1 is DNA mass of fungi I-5, 2 is DNA mass of fungi C-1

Figure 3. Laccase activity curve in solution after SA-oat-fungus membrane decolorization AB113 (T = 4 ºC, pH 4.5) (a) I-5 (b) C-1

Figure 4. Laccase activity on membrane (T = 4 ºC, pH 4.5, membrane area 0.6 cm^2)

Figure 5. Single batch decolorization experiment (Initial concentration 50 mg/L, 50 mL, T = 28 ºC, pH 7.0±0.2)

(a) I-5 AR73 (b) C-1 AR73 (c) I-5 AB113 (d) C-1 AB113

Figure 6. Dye removal by fungus enzyme production (Dyes AB 113, initial concentration 50 mg/L, 50 mL, T = 28 ºC, pH 7.0±0.2)

(a) I-5 (b) C-1

Figure 7. Desorption experiment for different dyes (Initial concentration 50 mg/L, 50 mL, T = 28 ºC, pH 7.0±0.2)

Figure 8. Dye removal in different cycles (Initial concentration 50 mg/L, 50 mL, T = 28 ºC, pH 7.0±0.2)

(a) I-5 AR73 (b) C-1 AR73 (c) I-5 AB113 (d) C-1 AB113

Figure 9. Different dyes removal rate by CS-Fe-SA-oat fungus membranes (Initial concentration 50 mg/L, 50 mL, T = 28 ºC, pH 7.0±0.2)

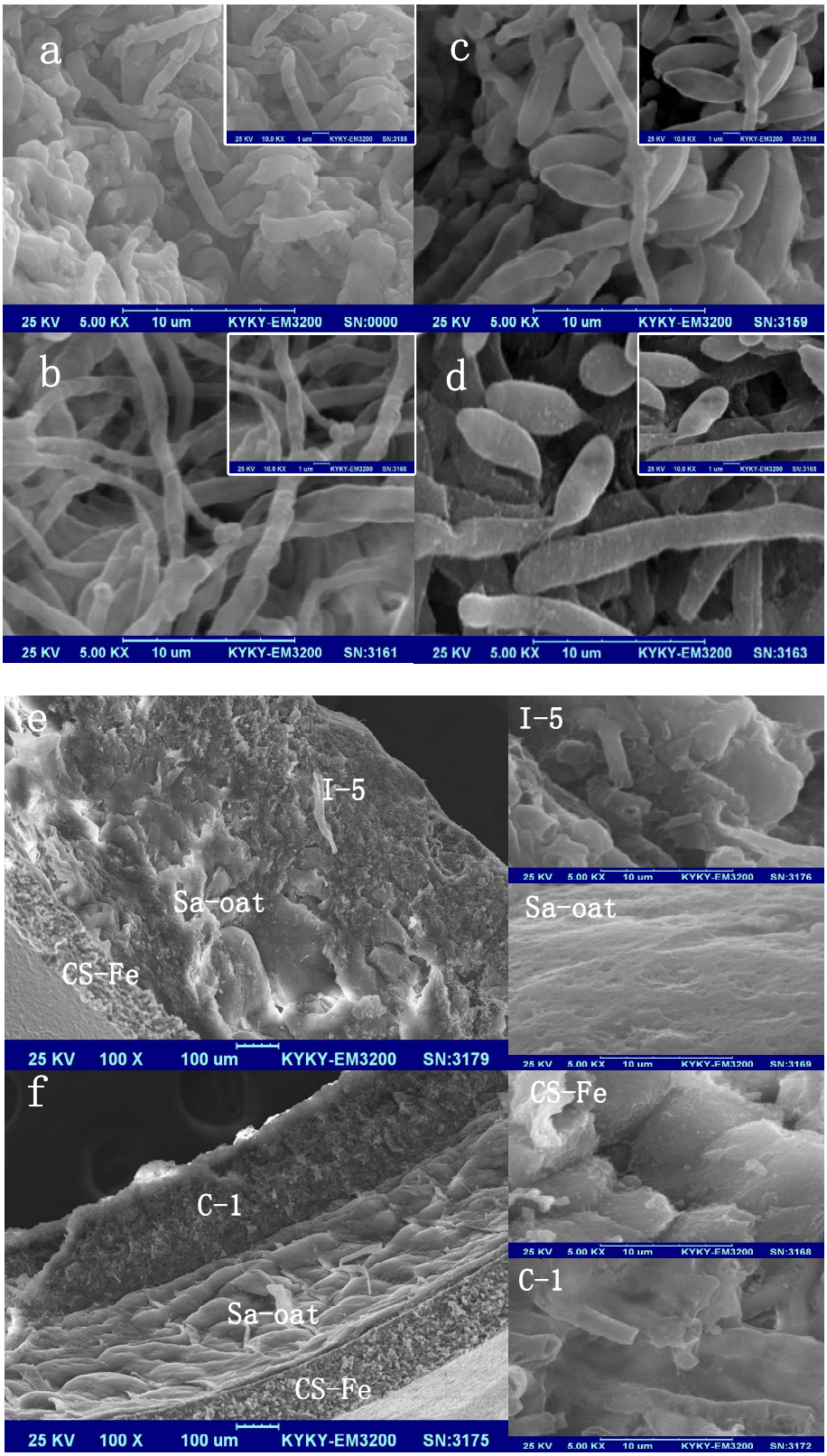

a
25 KV 5.00 KX 10 um KYKY-EM3200 SN:0000
c
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3159
b
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3161
d
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3163
e
I-5
Sa-oat
CS-Fe
25 KV 100 X 100 um KYKY-EM3200 SN:3179
I-5
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3176
Sa-oat
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3169
f
C-1
Sa-oat
CS-Fe
25 KV 100 X 100 um KYKY-EM3200 SN:3175
CS-Fe
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3168
C-1
25 KV 5.00 KX 10 um KYKY-EM3200 SN:3172

Figure 1

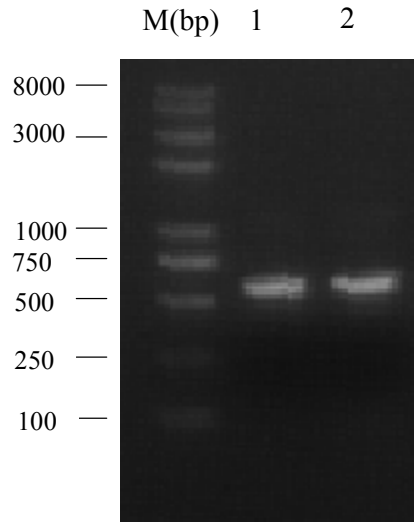
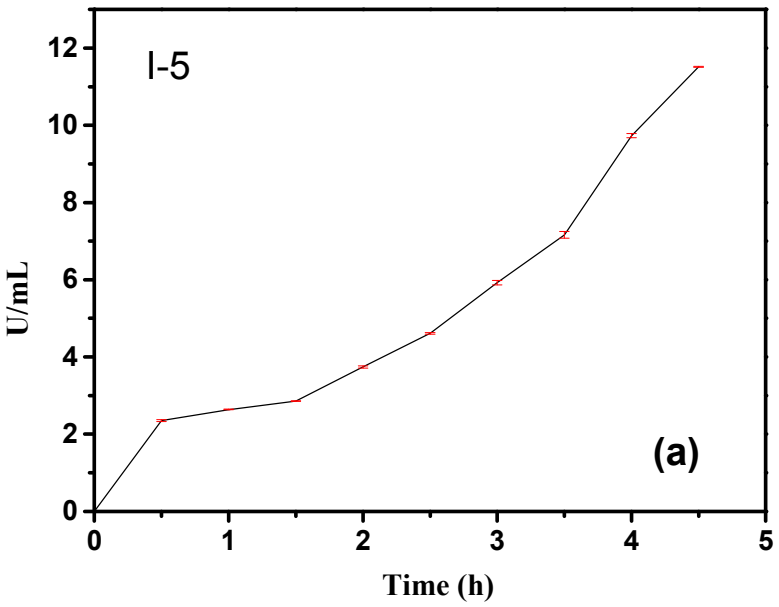
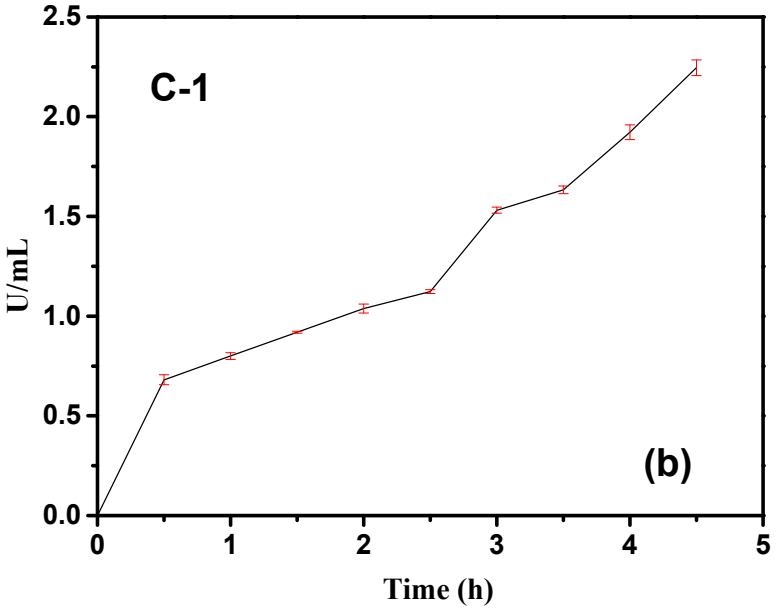


Figure 2



1



2

3 **Figure 3**

4

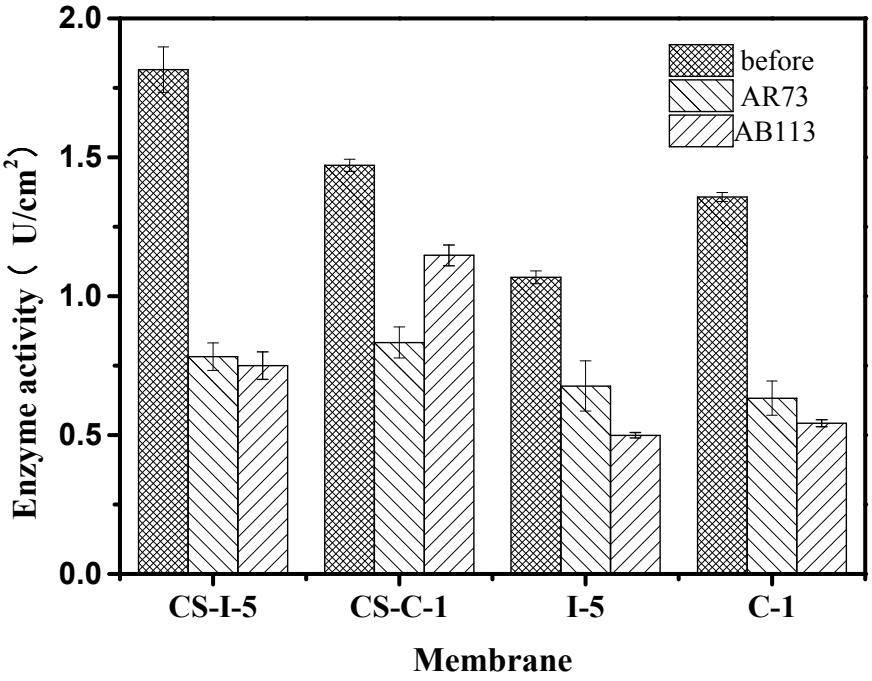

2.0
1.5
1.0
0.5
0.0
Enzyme activity（ U/cm^2）
before
AR73
AB113
CS-I-5
CS-C-1
I-5
C-1
Membrane

Figure 4

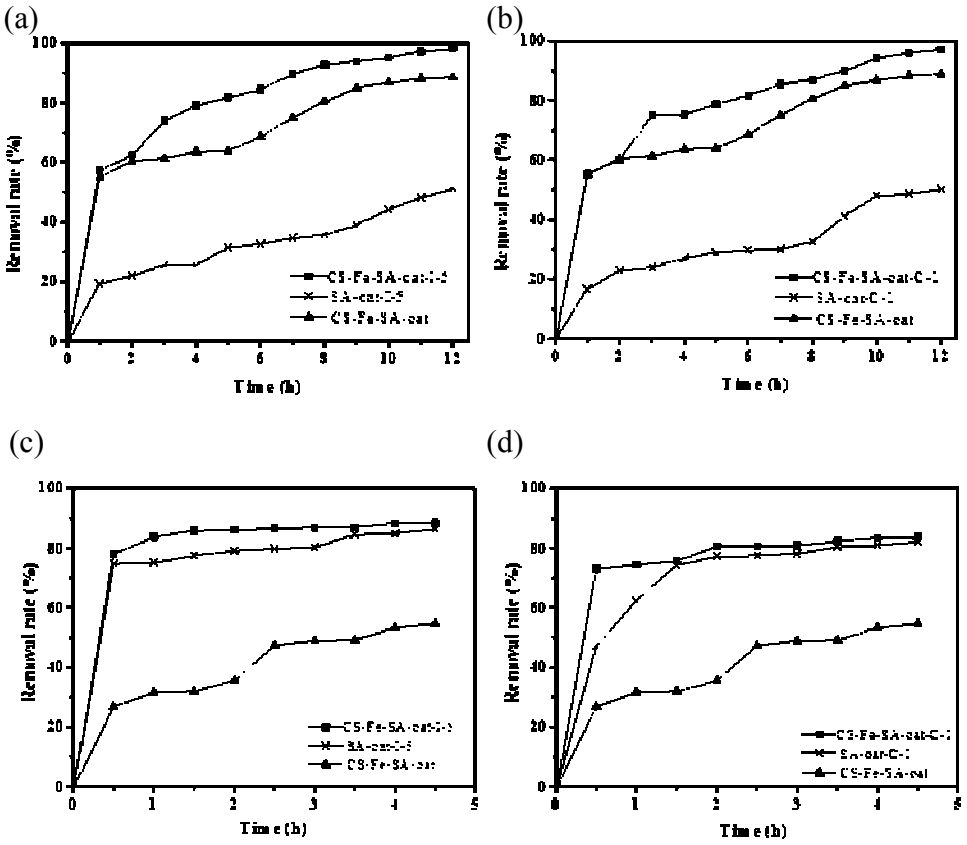
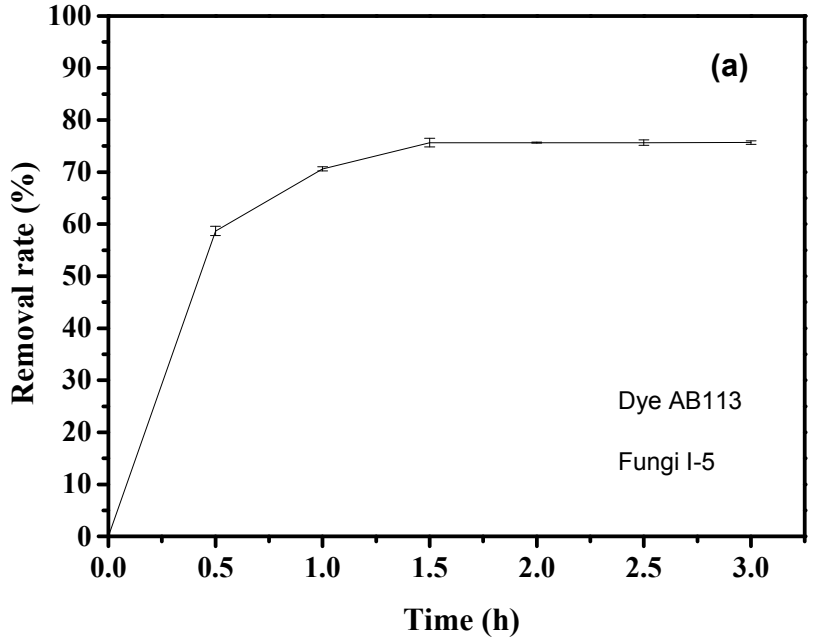


Figure 5


(a)
Removal rate (%)
Time (h)
Dye AB113
Fungi I-5
0
10
20
30
40
50
60
70
80
90
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0

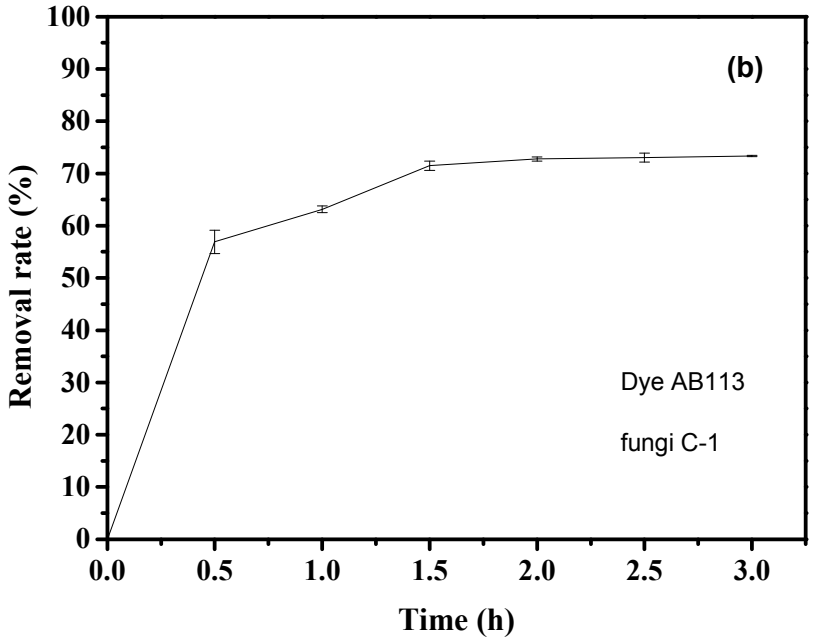

(b)
Removal rate (%)
Time (h)
Dye AB113
fungi C-1
0
10
20
30
40
50
60
70
80
90
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 6

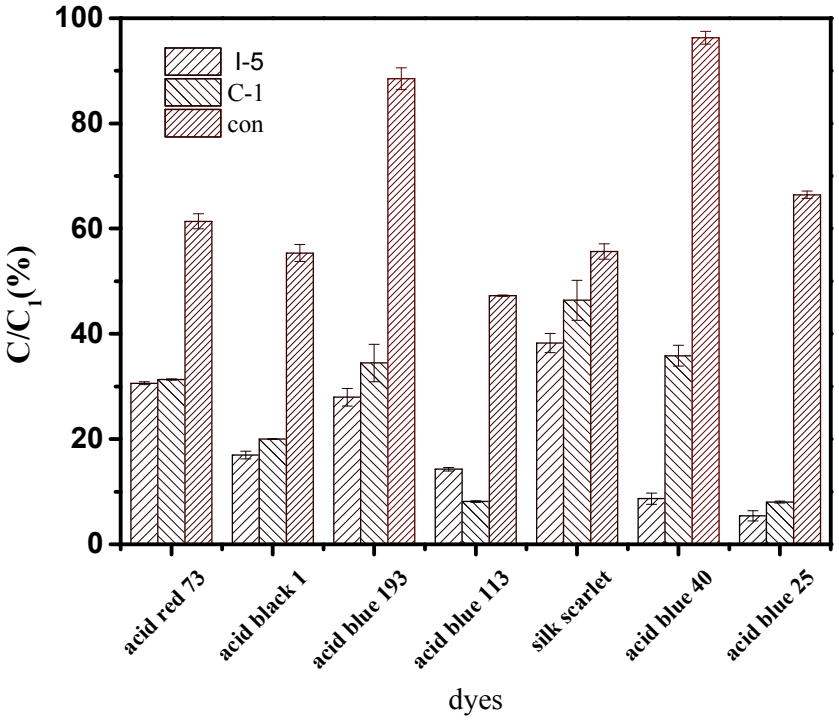
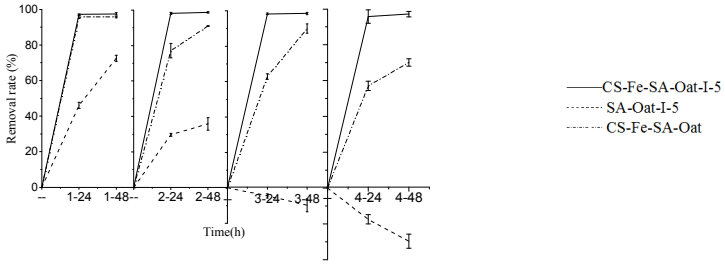
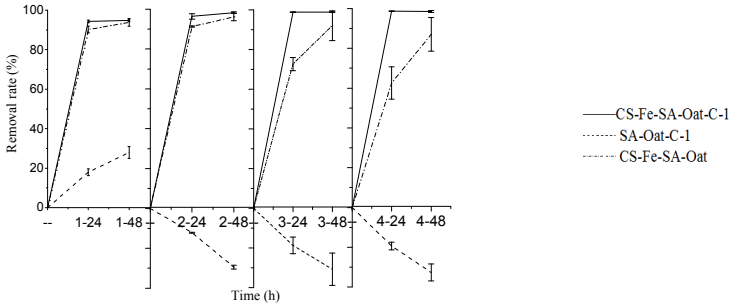

100
80
60
40
20
0
C/C1(%)
I-5
C-1
con
acid red 73
acid black 1
acid blue 193
acid blue 113
silk scarlet
acid blue 40
acid blue 25
dyes

Figure 7

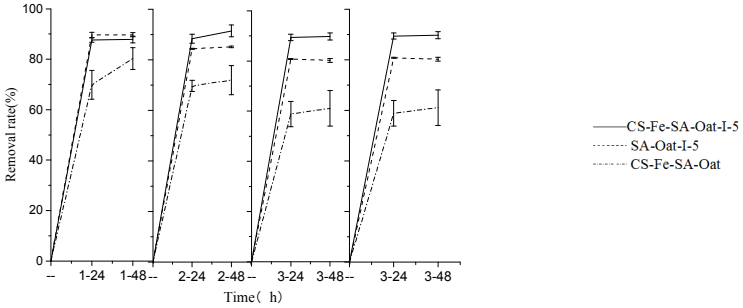
(a)



(b)



(c)



(d)

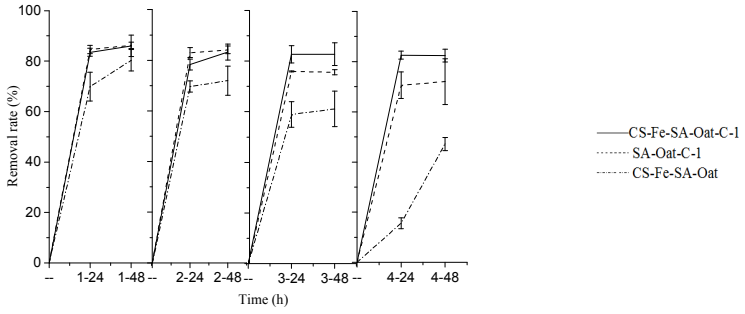


Figure 8

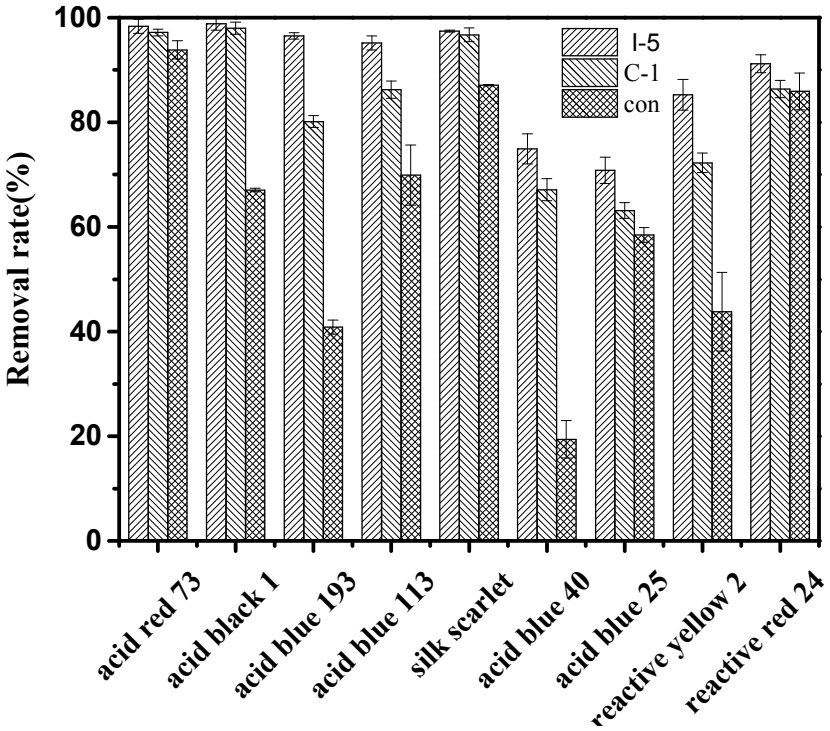

Removal rate(%)
100
80
60
40
20
0
I-5
C-1
con
acid red 73
acid black 1
acid blue 193
acid blue 113
silk scarlet
acid blue 40
acid blue 25
reactive yellow 2
reactive red 24

Figure 9