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Application of immobilized laccase in the removal of oil from wastewater

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ABSTRACT

The removal of oil from simulated oily wastewater by catalyzed oxidation with immobilized laccase was investigated. The effects of several main factors, such as pH, the amount of laccase, temperature, and reaction time, were discussed in detail. The results indicated that the optimum condition for immobilized laccase-catalyzed removal of oil was as follows: 25 mL simulated oily wastewater with the oil concentration of 120 mg/L in phosphate buffer (0.1 M, pH 4.5), reacted with 0.7696 g immobilized laccase and 100 mg/L chitosan at 25℃ for 6 h. The oil removal efficiency was as high as 86% under the optimum conditions. Mg^{2+} and Cu^{2+} ions had little effect on the oil removal efficiency of immobilized laccase, but Fe^{2+} reduced the oil removal efficiency. The effect of the support on oil removal efficiency could be neglected. The immobilized laccase demonstrated a reliable and stable reusability.

Keywords: Immobilized laccase; Catalyzed oxidation; Oily wastewater; Chitosan; Metal ions

1. Introduction

Oil in water can exist in the form of free or suspended oil, dispersed oil, emulsified oil, and dissolved oil. Leakage of oil products can cause problems in industrial circulating water system, even if the oil concentration is very low. The leakage of oil results in bacterium and biological clay, which get attached to the surface of the equipment and pipe. Consequently, it decreases heat transfer efficiency, blocks pipe easily, and increases flow drag. Therefore, the corrosion of the equipment and pipe is exacerbated [1]. Furthermore, the oil in the industrial circulating water can greatly affect the performance of water stabilizing agents, and decreases the efficacies of corrosion inhibitors, scale inhibitors, and dispersers [2–3].

Up to now, numerous techniques have been developed for oil removal. The traditional methods for the oily wastewater treatment include, gravity separation, salting out method, electrolytic method, dissolved air flotation, and chemical treatment [4–6]. However, these methods are frequently less efficient, especially when the oil droplets are finely dispersed and their concentration is very low. Other commonly used methods include advanced oxidation, membrane technique, or the combinations of the above [7–9].

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However, the applications of these techniques are limited to both technical and economic reasons.

The enzymatic degradation of substrates has several advantages, such as the convenient operation, mild reaction conditions, high efficiency, wide operating range, and elimination of recontamination. Therefore, it may be applied as a proper method for the treatment of oily wastewater [10–13]. The catalyzed oxidation, including oil, phenol, and aromatic amines by free laccase, has been widely studied [14–16]. Laccase is a multi-copper oxidase which is able to catalyze the one-electron oxidation of several aromatic substrates with the simultaneous reduction of dioxygen to two molecules of water [17]. The fairly broad substrate range includes polyphenols, polymethoxybenzenes, methoxy-substituted monophenols, benzenethiols, aromatic amines, and other easily oxidizable compounds [18]. However, there are some drawbacks of using free enzymes to detoxify wastewater, and the main ones are their instability towards thermal and pH denaturation, proteolysis, and inactivation by inhibitors [19]. Immobilization of enzymes to solid supports often improves stability and allows their reuse. Although several attempts using immobilized laccase have been made [20], no report on its application in the removal of oil from wastewater has been published as far as we know.

Our previous study demonstrated that free laccase can catalyze the oxidation of the emulsified and dissolved oil from oily wastewater [21]. In the present paper, the effectiveness of immobilized laccase on ceramic-chitosan composite supports (CCCS) under various reaction conditions like, medium pH, amount of immobilized laccase, reaction temperature and time, and additive concentration were investigated. The influence of metal ions in the wastewater and the reuseabililty of the immobilized laccase in the laccase-catalyzed oxidation were also researched.

2. Materials and methods

2.1. Materials

Laccase (EC 1.10.3.2) was purchased from Sigma-Aldrich Co. Ltd. (USA), and the nominal activity quoted by the manufacturer was 4 unit/mg dry solid. One activity unit (U) of laccase was defined as the amount of enzyme required to catalyze 1 μmol of substrate per minute [22]. The second distilled diesel oil was provided by Yanshan Petrochemical Co. Ltd. (China). In order to prepare the simulated oil-containing solutions, diesel oil was emulsified in 0.2 M phosphate buffer (pH 4.5). Petroleum ether (boiling range: 60–90℃) was obtained from Beijing Chemical Factory (China), and its transmissivity was greater than 80% after purification. Chitosan was purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Polyaluminum chloride (PAC) of technical grade was obtained from Tianjin Fuchen Chemical Factory (China). All other chemicals were of analytical grade, and no further purification was required.

The support was cordierite porous ceramics which was from Kaiming Catalyst Co. Ltd. (Hangzhou, China). The pores were full-length with 1.5×1.5 (mm) cross-section. The interval between adjacent pores was 0.4 mm. The bulk density of the support was about $700\,kg/m^3$. The specific surface area of the support measured with a surface area apparatus (BET method) was $24.6\,m^2/g$.

2.2. Analytical techniques

The activity of free or immobilized laccase was determined at 25℃ by a UV spectrophotometer (HACH DR5000, USA) using 0.5 mM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the substrate (prepared with 0.2 M acetate buffer, pH 3.0). The reaction mixture consisted of 8.0 mL acetate buffer and 0.1 mL free laccase solution (or 0.1 g of immobilized laccase). Briefly, the reaction was initiated by adding 2 mL substrate into the mixture, and it was maintained at 25℃ for 4 min. During the reaction, the increase in the absorbance of 420 nm in one minute interval was measured. The molar extinction coefficient of ABTS was $36{,}000\,M^{-1}\,cm^{-1}$ [21].

The oil concentrations in the wastewater were determined with the UV spectrophotometer according to the Chinese National Standard [23]. The oil concentration was measured with the UV spectrophotometer at the wavelength of 225 nm, at which the diesel oil has the maximum absorption. The measurement was performed following the extraction of oil from reaction mixtures or wastewater by petroleum ether. The oil removal efficiency was expressed as the percentage of removed oil concentration.

All experiments for the activity of free and immobilized laccase, and the oil concentrations in the wastewater were replicated for three times.

2.3. Experimental procedures

2.3.1. Preparation of immobilized laccase

The modifying solution was prepared by adding 0.26 mL γ-methacryloxypropyl trimethoxy silane (KH-570) into 30 mL anhydrous alcohol/water (1:1, v/v) solution at pH range from 3.5 to 4.0. The ceramic support was added into the modifying solution at 30℃, stirred for 5 h and then washed with anhydrous alcohol and distilled water, respectively. The modified ceramic was then obtained by drying 12 h at 50℃.

In order to obtain the chitosan solution, chitosan powder was added into 1% (v/v) acetic acid solution at room temperature and stirred for 10 min. Subsequently, the modified ceramic was incubated in the chitosan solution at 30℃ for 1.5 h, and then removed and dried for 12 h in a desicator at room temperature. The same process of incubation and drying was repeated for three times to obtain the CCCS.

Firstly, 1 g of modified ceramic or CCCS was added into 25 mL of 6% glutaraldehyde solution for cross-linking at 30℃ and stirred for 15 min. Then, it was washed by distilled water and dried for 12 h in a desicator, at room temperature. Finally, it was transferred into 5 mL laccase solution (1.25 mg/mL) for immobilization at 4℃ for 24 h and washed with phosphate buffer for three times.

2.3.2. Catalyzed oxidation of oil by immobilized laccase

Wastewater of oil concentration of 120 mg/L was treated by immobilized laccase at a pH of 4.5. The reaction mixture was incubated at 25℃ for certain time periods with shaking at 100 rpm.

Prior to the analysis of the remaining oil concentration, the floc in the reaction mixture was flocculated with flocculating agents of $Al_2(SO_4)_3$ (80 mg/L) and PAC (80 mg/L) at a pH of 8.0. Subsequently, the mixture was centrifuged for 30 min at 3,000 rpm. The supernatant was collected and it was immediately analyzed to determine the residual oil concentration with the colorimetric method described above.

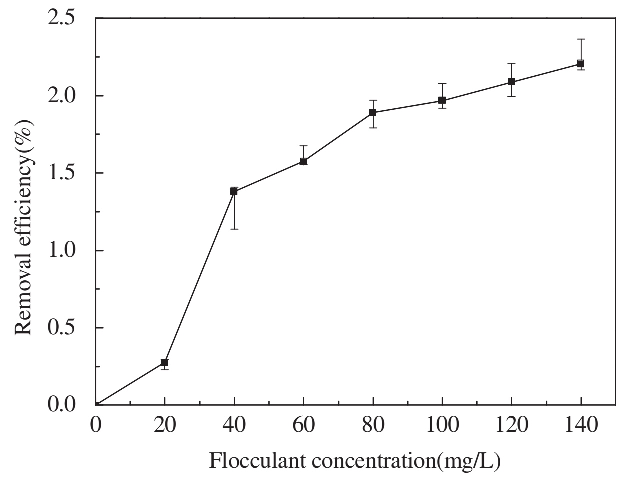

Fig. 1. Effect of flocculant concentration. Reaction conditions: 25 mL of 120 mg/L oily wastewater; without immobilized laccase; pH of 4.5; temperature of 25℃; reaction time of 6 h.

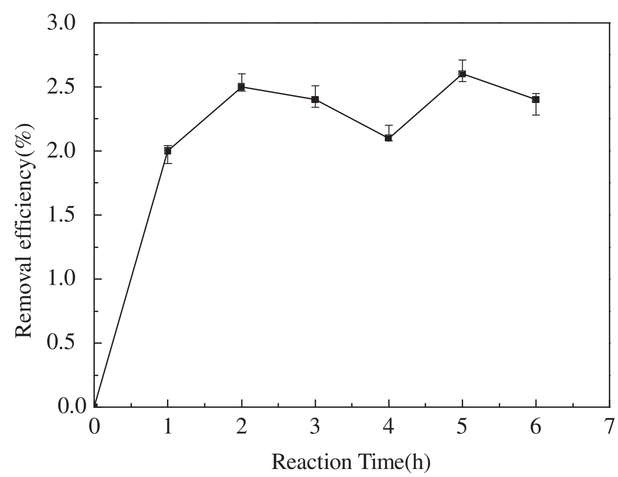

Fig. 2. Effects of support. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.8001 g support without laccase; temperature of 25℃; pH of 4.5.

3. Results and discussion

3.1. Effects of flocculant and support

The effect of flocculant concentration on oil removal efficiency was investigated. Fig. 1 shows that the oil removal efficiency was only 2.2% at the largest flocculant concentration of 140 mg/L, indicating that flocculant exerted a negligible effect during the process of oil removal. The oil removal efficiency was only 1.9% at the actual applied flocculant concentration of 80 mg/L, so the influence of flocculant on the analytical precision of the remaining oil concentration could be neglected.

The support of immobilized laccase has the capability of adsorption. Therefore, it is necessary to investigate the effect of support on oil removal efficiency. Fig. 2 shows that the removal efficiency was almost kept constant at the average value of 2.3% with the change of reaction time. Therefore, the adsorption of oil to the support could also be neglected, so the obtained results of oil removal efficiency below should be mainly caused by the catalysis effect of immobilized laccase.

3.2. Effect of pH

The effect of pH on oil removal efficiency was also investigated (Fig. 3). In the study on the properties of immobilized laccases [24], the optimum working pH value ranged from 2.0 to 8.0. Therefore, the solution of pH was selected from 2.0 to 8.0, here.

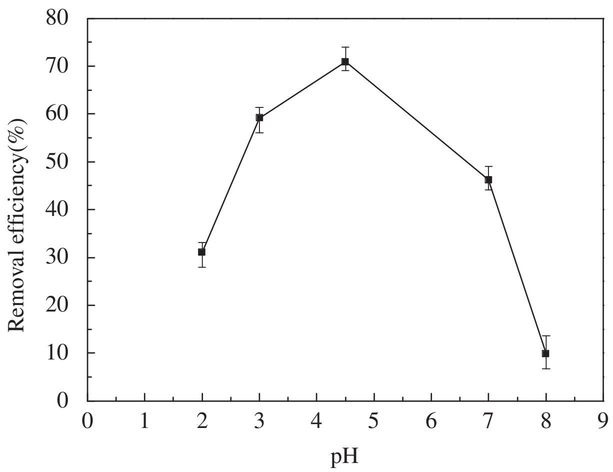

Fig. 3. Effect of pH. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.4688 g immobilized laccase; temperature of 25℃; reaction time of 6 h.

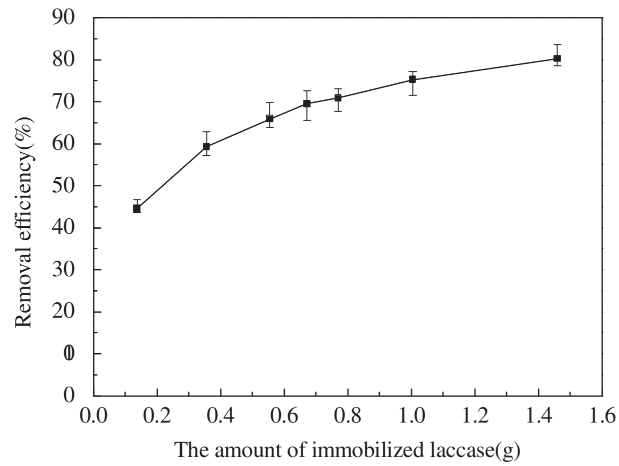

Fig. 4. Effect of the amount of immobilized laccase. Reaction conditions: 25 mL of 120 mg/L oily wastewater; pH of 4.5; temperature of 25℃; reaction time of 6 h.

The oil removal efficiency increased from 31 to 71% when the pH was increased from 2.0 to 4.5. When the pH was increased from 4.5 to 8.0, the oil removal efficiency decreased from 71 to 10%. Therefore, it could be concluded that the optimum pH for oil conversion was approximately 4.5, which was lower than that of free laccase [21]. So immobilization of the laccase had obviously influence on the optimum pH.

3.3. Effect of amount of immobilized laccase

Normally, oil removal efficiency should increase when the amount of immobilized laccase increases, but the suitable amount should be investigated considering the balance between the removal property and economical efficiency. Fig. 4 shows the effect of amount of immobilized laccase on oil removal efficiency. The results show that the oil removal efficiency increased with the increase in the amount of immobilized laccase. When the amount of immobilized laccase increased from 0.1374 to 0.7696 g, the oil removal efficiency had an increment of 26%. However, when the amount of immobilized laccase increased from 0.7696 to 1.4590 g, the oil removal efficiency had an increment of only 9.4%. Therefore, it can be deduced that the reasonable amount of immobilized laccase should be 0.7696 g here.

3.4. Effect of reaction time

The effect of reaction time on oil removal efficiency was investigated in the range of 1–15 h (Fig. 5). It is shown that the oil removal efficiency increased

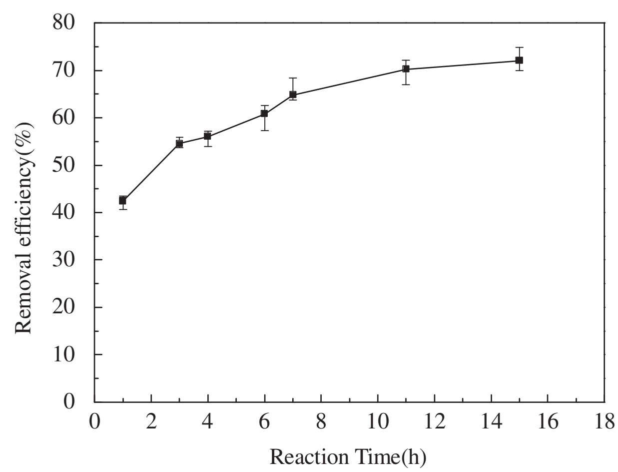

Fig. 5. Effect of reaction time. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.7696 g immobilized laccase; pH of 4.5; temperature of 25℃.

when reaction time elapsed. The oil removal efficiency increased quickly in the initial period and reached 64% within the first 6 h. After that, the oil removal efficiency increased slowly. The mechanism of laccase catalyzing other substrates, such as biphenols, aromatic amines, and some dyes, was that free radical products were converted from the substrate on contact with laccase, and the polymers were led by coupling reactions of the free radical products [25,26]. The oil removal in this case is believed to have a similar mechanism. At first, large amount of oil molecules could contact with the active laccase on the surface of the support, so large amount of free radical products and polymers formed. As a result, the polymers were removed from water subsequently. Therefore, the oil removal efficiency increased as investigation time was increased. However, the polymerization products inhibit the generation of free radical products after a certain time period [25], leading to an inhibited subsequent reaction. Therefore, the increment rate of oil removal efficiency gradually decreased. It can be seen that the oil removal efficiency increased little after 6 h, therefore the reaction time of 6 h was adopted in all further experiments.

3.5. Effect of reaction temperature

Temperature plays a key role during enzyme catalysis. Therefore, it is necessary to evaluate the optimum temperature to obtain higher catalytic efficiency in the reaction mixture. Fig. 6 shows the oil removal efficiencies in the reaction mixture at various temperatures. The results indicated that the oil removal efficiencies were highly dependent on the temperature. It can be seen that the optimal temperature for immobilized laccase was 25℃ because of the maximum efficiency of 68% and the relative low operation temperature, although an oil removal efficiency peak of 63% appeared at 50℃.

The appearance of oil removal efficiency peak can be attributed to the two competing phenomena [26] which were the increase in the rate of thermal deactivation of the enzyme and the increase in the rate of catalytic reaction. It is noticeable that two peaks of oil removal efficiency appear in Fig. 6. In the research of the relationship between temperature and laccase activity, similar phenomenon of two peaks was also observed [27]. Naturally, the two peaks of oil removal efficiency can be attributed to the two peaks of the laccase activity. Furthermore, other factors, such as substrate, immobilization support, and conditions of operation, can also cause the fluctuation in the oil removal efficiency because of their various temperature characters.

It can also be seen that the oil removal efficiency could be kept over 50% in the temperature ranges between 15–30℃ and 43–57℃, which are satisfactory for operation control convenience.

3.6. Effect of metal ions

Metal ions are also an important group of modulators to the enzymatic activity, and they have been observed, in some instances, affecting the enzyme activity and treatment efficiency [25]. Because some metal ions, such as Fe^{2+}, Cu^{2+}, and Mg^{2+}, frequently exist in industrial wastewater, it is necessary to investigate their potential impacts on laccase-based treatment. The effect of Fe^{2+}, Cu^{2+} and Mg^{2+} ions on oil removal efficiency was investigated here (Fig. 7).

It can be seen that Mg^{2+} and Cu^{2+} had little influence on the oil removal efficiency, so they should not cause significant problems during the treatment of real wastewater with laccase. However, Fe^{2+} reduced the oil removal efficiency much. Most of metal ions do not inhibit the enzymatic activity of laccase *in vitro*, but can reduce the substrate conversion [28]. Although the mechanism of the metallic ions interfering with the oil conversion remains unclear, it was reported that Fe^{2+} and Cu^{2+} ions can interrupt the electron transport systems of laccase, leading to the inhibition of substrate conversion [29]. Synthetically

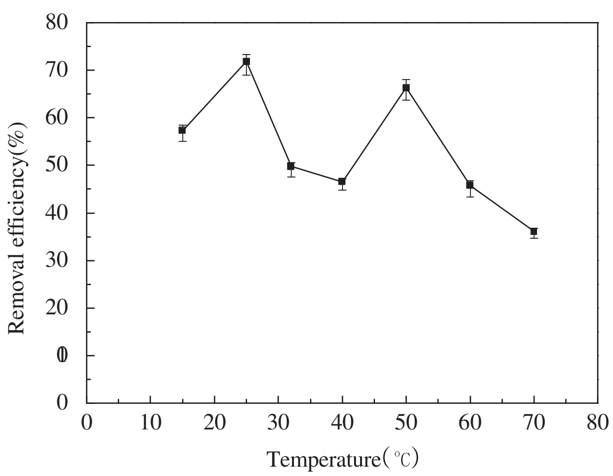

Fig. 6. Effect of temperature. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.7604 g immobilized laccase; pH of 4.5; reaction time of 6 h.

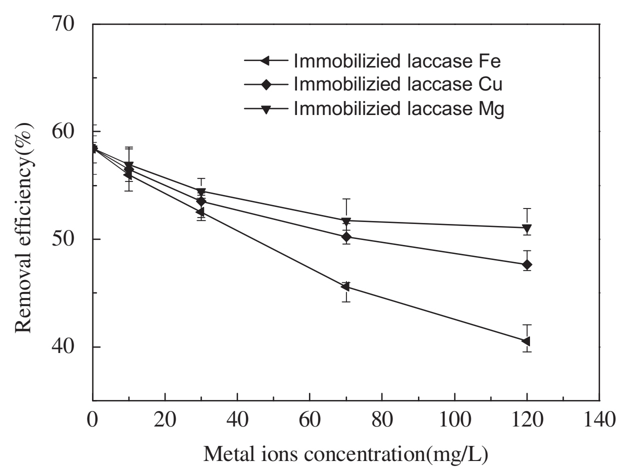

Fig. 7. Effect of metal ions. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.7640 g immobilized laccase; temperature of 25℃; pH of 4.5; reaction time of 6 h.

consider the theories in Refs. [28,29] and our results; it can be deduced that metal ions have different influences on the catalysis efficiency at different circumstances, so their influences have to be evaluated before the application of laccase. In our case of immobilized laccase treating wastewater, the reduction caused by Fe^{2+} ions should be noticed.

3.7. Effect of reaction additive concentration

Many investigations reported that some additives can improve the efficiency of enzyme-catalysis by forming a protection layer in the vicinity of active centers of enzyme [30–34], restricting the attack of free radicals formed in the catalytic reaction. In recent years, chitosan has been widely studied and applied to wastewater treatment, chromatographic support, and enzyme immobilization [27,35–37] because of its nontoxicity, biocompatibility, and biodegradability [38]. In our pervious work, it was discovered that chitosan could intensify the effectiveness of free laccase catalyzing oil [21]. Therefore, chitosan was selected as the additive here.

The effect of different chitosan concentration on oil removal efficiency was investigated (Fig. 8). It can be seen that oil was not significantly removed when only the additive was used. The oil removal efficiency significantly increased as the chitosan concentration was increased up to 100 mg/L. When the chitosan concentration was 100 mg/L, the oil removal efficiency reached its maximum value of 83%. Further addition of chitosan resulted in a slight reduction of the oil removal efficiency, which was consistent with the previous report [39].

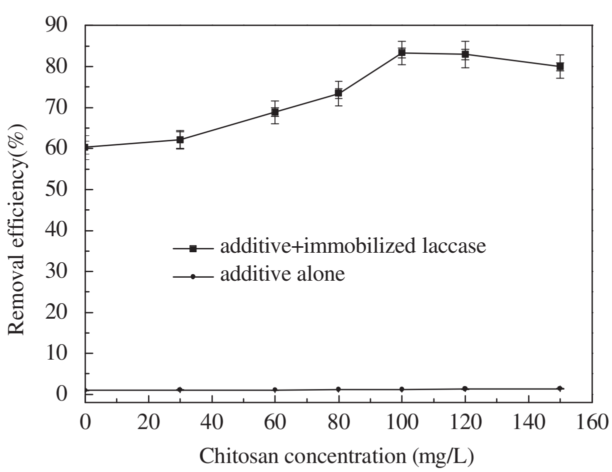

Fig. 8. Effect of additive concentration. Reaction conditions: 25 mL of 120 mg/L oily wastewater; 0.7621 g immobilized laccase; temperature of 25℃; pH of 4.5; reaction time of 6 h.

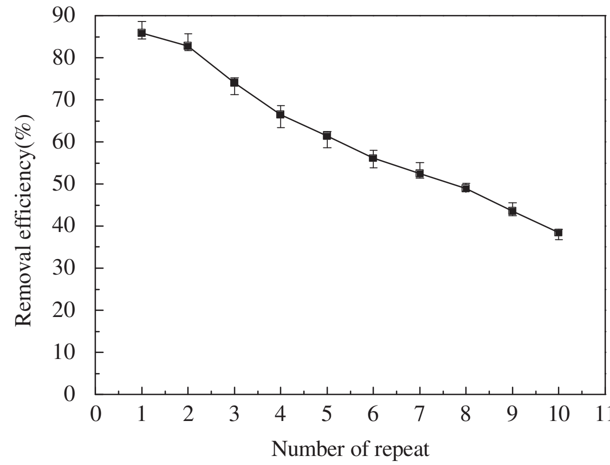

Fig. 9. Effect of repeated use. Reaction conditions: 25 mL of wastewater with initial oil concentration of 120 mg/L; 0.7609 g immobilized laccase; 100 mg/L chitosan; temperature of 25℃; pH of 4.5; reaction time of each batch test of 6 h.

3.8. Effect of repeated use

The operational stability of immobilized laccase in a repeated batch process was evaluated here (Fig. 9). It can be seen that the oil removal efficiency was still as high as 50% after the immobilized laccase was repeatedly used for seven times. Noticing the low initial oil concentration of 120 mg/L, the oil removal efficiency of 50% can be considered being satisfactory. The protein shedding from the CCCS should be the main cause for the loss of activity. The immobilized laccase demonstrated a reliable and stable reusability.

4. Conclusions

The impact of the reaction conditions upon the oil removal efficiency of the immobilized laccase catalysis was investigated. The optimum condition was as follows: 25 mL simulated oily wastewater, with the oil concentration of 120 mg/L in phosphate buffer (0.1 M, pH 4.5), reacted with 0.7696 g immobilized laccase and 100 mg/L chitosan at 25℃ for 6 h. Under this condition, the oil removal efficiency was as high as 86%. Mg^{2+} and Cu^{2+} ions had little effect on the oil removal efficiency of immobilized laccase, but Fe^{2+} reduced the oil removal efficiency obviously. The effect of the support on oil removal efficiency could be neglected. The immobilized laccase demonstrated a reliable and stable reusability.

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