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# Immobilization of Laccase from *Trametes hirsuta* onto CMC Coated Magnetic Nanoparticles

S. Sadeghzadeh[a,b], S. Ghazvini[a], S. Hejazi[a], S. Yaghmaei[a], Z. Ghobadi Nejad*[b]

[a] *Chemical & Petroleum Engineering Department, Sharif University of Technology, Tehran, Iran*
[b] *Biochemical & Bioenvironmental Research Center, Sharif University of Technology, Azadi Avenue, Tehran, Iran*

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*ABSTRACT*

In this study, $Fe_3O_4$/CMC magnetic nanoparticles were synthesized through co-precipitation method. Afterward, laccase from *Trametes hirsuta* was immobilized onto Carboxymethyl cellulose (CMC)-coated magnetic $Fe_3O_4$ nanoparticles by covalent bonding between carboxyl groups of carboxymethyl cellulose and amine group of laccases. Also, the resulted magnetic nanoparticles and immobilized laccase were characterized by Fourier-transform infrared spectroscopy (FTIR), scanning electron microscope (SEM) and dynamic light scattering (DLS) analysis. Moreover, the vital factors in enzyme immobilization, such as contact time, amount of N-hydroxysuccinimide (NHS), and the amount of nanoparticles were optimized, which successively 48 h, 0.01 g, and 0.0125 g were achieved for 0.01g of N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC). Activity recovery of 51 ± 0.8% was achieved by optimizing the immobilization process. The results also indicated that the loading of laccase onto carboxymethyl cellulose-coated $Fe_3O_4$ nanoparticles was approximately 120 (mg/g). Finally, the immobilized laccases on magnetic support could save nearly 50% of their initial activity after five consecutive cycles.

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## 1. INTRODUCTION

Enzymes are natural catalysts, and they have a good accordance with green chemistry [1]. They are non-toxic, biocompatible, biodegradable ,and also sustainable. They are produced from renewable resources like fungi and bacteria [2]. Furthermore, the mild reaction conditions could make them more feasible and cost-effective [3].

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is a lignin-modifying enzyme which has good potential for industrial and bioremediation processes [4]. It is also a multicopper oxidase catalyzing the oxidation of aromatic compounds with concomitant reduction of dissolved oxygen molecules to water [5]. In comparison with other lignin-modifying enzymes (such as manganese peroxidase, lignin peroxidase, and versatile peroxidase), laccase could be more preferable. Laccase needs only dissolved oxygen as a co-substrate, while others need hydrogen peroxidase. Therefore, laccase might be more green option [6, 7]. Furthermore, the oxidation of the phenolic compounds with laccase forms large insoluble polymers that are easily removed by filtration or sedimentation in the aqueous phase [8].

Despite all mentioned advantages, using free enzymes is undesirable due to its high sensitivity to industrial operating conditions, as well as its poor stability and reusability, which increase costs. Also, using free enzyme can reduce the enzyme's catalytic activity and stability due to its likely interaction with the reaction products [9-11]. Therefore, enzyme immobilization could circumvent these weak spots and leads to ease of its application in both batch and continuous systems [12]. Different types of immobilization like adsorption [13], covalent bonding [14], entrapment [15, 16], encapsulation [17], and co-polymerization have been implemented in some studies

*Corresponding Author Email: z_*ghobadi@che.sharif.edu* (Z. Ghobadi Nejad)

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[18]. Immobilization on a solid support is regarded as the most broadly applicable method [19]. In this method, the size of supports plays a crucial role in biocatalysts performance. The small particles lead to more reactivity due to higher surface to volume ratio. On the other hand, handling of small particles is relatively hard, but large particles might imply mass transfer limitations. Immobilization on magnetic nanoparticles could be a promising solution to overcome these obstacles [20, 21]. Magnetic nanoparticles provide small support size and ease of separation and dispersion simultaneously. Amongst magnetic nanoparticles, $Fe_3O_4$ nanoparticles are the most prevalent support due to their lower toxicity and good biocompatibility [22, 23]. The direct usage of bare $Fe_3O_4$ nanoparticles faces some problems such as surface modification and also relatively high reactivity upon direct contacting to certain media. Covering the surface of $Fe_3O_4$ nanoparticles could be a novel approach to circumvent the mentioned drawbacks [24]. Carboxymethyl cellulose (CMC) has a great potential to play a linkage role, and it is also a natural polymer with biocompatibility and good environmental stability [25, 26]. Therefore, CMC was selected for coating the $Fe_3O_4$ nanoparticles. To the best of our knowledge, it is the first study about laccase immobilization on CMC/$Fe_3O_4$ nanoparticles.

## 2. MATERIALS & METHODS

**2. 1. Materials** Laccase from *Trametes Hirsuta* was purchased from Novozyme company. Carboxymethyl cellulose (CMC), guaiacol, N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich company. Also, $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, and ammonia 25% were purchased from Merck company.

### 2. 2. Methods

**2. 2. 1. Preparation of $Fe_3O_4$/CMC Nanoparticles** To prepare $Fe_3O_4$/CMC nanoparticles, 5 mmoles of $FeCl_2.4H_2O$ and 10 mmoles of $FeCl_3.6H_2O$ were dissolved in 100 mL deionized (DI) water with continuous magnetic stirring under nitrogen atmosphere. Afterward, 0.1 g CMC was added to the solution, which was heated up to 80ºC under magnetic stirring. Finally, 7 mL of ammonia solution was added drop-wisely to the solution and stirred for 3 h. Similarly, bare $Fe_3O_4$ NPs were also prepared in the same conditions as above without adding CMC. The synthesized magnetic nanoparticles were washed multiple times with DI water and dried in an oven at 60ºC for 24 h [27].

**2. 2. 2. Surface Activation of $Fe_3O_4$/CMC Nanoparticles with EDC/NHS** In this step, impact of NHS amount (0.0025, 0.005, 0.01, 0.025, 0.04, and 0.055 g) and $Fe_3O_4$/CMC amount (0.003, 0.005, 0.0075, 0.01, 0.0125, 0.015, and 0.0175 g) were studied. 0.0125 g of $Fe_3O_4$/CMC NPs was dispersed in 1 mL DI water and ultrasonicated for 10 minutes, Afterward, 1 mL of EDC solution (0.01 g/mL) and 1 mL of NHS solution (0.01 g/mL) were added to the prepared mixture. The final mixture was put in an incubator and stirred for 1.5 h at room temperature. At the end, the nanoparticles were separated by an external magnetic field and washed twice with DI water and kept for further use.

**2. 2. 3. Laccase Preparation** Laccase from *Trametes hirsuta* is of industrial grade, which contains considerable amounts of preservatives in the enzyme granules, and they should be removed from granules structure. For this purpose, a proper amount of granules was dispersed in DI water and shaked for 20 minutes at room temperature then centrifuged for 20 min at 4000 rpm. The supernatant involves the main parts of extracted enzyme, so it was used as the enzyme solution. The resultant enzyme solution had an activity of 50 U/mL.

**2. 2. 4. Immobilization of Laccase on $Fe_3O_4$/CMC Nanoparticles** The immobilization of laccase on $Fe_3O_4$/CMC nanoparticles was operated in batch mode. To achieve proper immobilization, the effect of various contacting times (24, 48, and 72 h) was studied. Therefore, the surface activated $Fe_3O_4$/CMC nanoparticles were dispersed in 2 mL phosphate buffer solution (0.1 M, pH value of 6), then was mixed with 1 mL of enzyme solution and kept at 4°C for 48 h.

**2. 2. 5. Laccase Activity Measurement** The activity of free and immobilized laccase was measured through the colorimetric method using UV_2450 spectrophotometer (Optizen pop, Japan). Guaiacol used as a substrate, and the enzyme activity was determined by monitoring the oxidation rate of Guaiacol in 15 minutes. The reaction mixture consisted of 2 mL Guaiacol (10 mM), 6 mL phosphate buffer solution (0.1 M, pH, 6) , and 2 mL of enzyme solution. The molar extinction coefficient of Guaiacol is 26.6 (1/(m.M)) at 470 nm [28, 29]. Activity recovery was selected as the response function for laccase immobilization procedure. Activity recovery of the immobilized enzyme was calculated by Equation (1) [30]:

$$\text{Activity Recovery (\%)} = [(\text{FEA (U)})/(\text{IEA (U)})]\times 100 \quad (1)$$

In Equation (1), FEA, and IEA are abbreviation of free enzyme and immobilized enzyme activities, respectively. Protein concentration was determined by Lowry method [31].

**2. 2. 6. Characterization** Scanning electron microscopy (SEM) analysis of magnetic nanoparticles and immobilized laccase was carried out using TeScan-FeSEM-MiraIII under a high vacuum 15 kV. Fourier transmission infrared spectroscopy (FTIR) analysis of $Fe_3O_4$ and $Fe_3O_4$/CMC magnetic nanoparticles was also carried out using Bruker-Tensor 27. Moreover, Dynamic Light Scattering (DLS) analysis of $Fe_3O_4$/CMC nanoparticles was studied utilizing Malvern Instruments Zeta Sizer Nano Series.

## 3. RESULTS AND DISCUSSION

**3. 1. Characterization of $Fe_3O_4$/CMC Nanoparticles and Immobilized Laccase** In FTIR spectra of bare $Fe_3O_4$ nanoparticles, the absorption at 560 $cm^{-1}$ is relevant to bending vibration of Fe-O bonds [32], and also the observed peak at 3421 $cm^{-1}$ is assigned to O-H groups [33]. Figure 1 also shows the spectra of $Fe_3O_4$/CMC nanoparticles. The peak at 1057.45 $cm^{-1}$ is related to the stretching vibration of O-C-C esteric groups. Also, the observed absorption at 1422 $cm^{-1}$ is associated with $COO^-$ groups [27], which are evidence to the presence of carboxylic acid groups and successful surface coating of $Fe_3O_4$ with CMC.

The surface morphology of $Fe_3O_4$/CMC nanoparticles and immobilized laccase was studied by SEM analysis. SEM image of $Fe_3O_4$/CMC nanoparticles showed that they had spherical shape with sizes of about 20 nm (Figure 2a). Moreover, Figure 2b demonstrates SEM image of immobilized laccase. It shows the porous structure of immobilized laccase over $Fe_3O_4$/CMC nanoparticles.

The size distribution of $Fe_3O_4$/CMC nanoparticles was evaluated by DLS analysis. Figure 3 demonstrates a uniform size distribution. The results revealed that nearly all of nanoparticles had sizes in the range of 170 to 270 nm. The observed size distribution with DLS analysis had no accordance with SEM image (about 20 nm). The high surface energy of magnetic nanoparticles is the main reason of this phenomenon. They were agglomerated to decrease their surface energy, and reached to equilibrium.

**3. 2. Effect of Contact Time on Activity Recovery** Time plays a crucial role in every chemical and biochemical reactions; so, contact time is one of important parameters in enzyme immobilization. The effect of contact time on laccase immobilization on $Fe_3O_4$/CMC nanoparticles was investigated. Contact time of 24, 48, and 72 h was selected as the candidate. By increasing time from 24 h to 48 h, activity recovery was increased and reached 39.1 ± 0.7%, but further increasing had no positive effect on activity recovery, significantly (Figure 4). Since there was not significant difference between 48 and 72 h; then, 48 h was selected

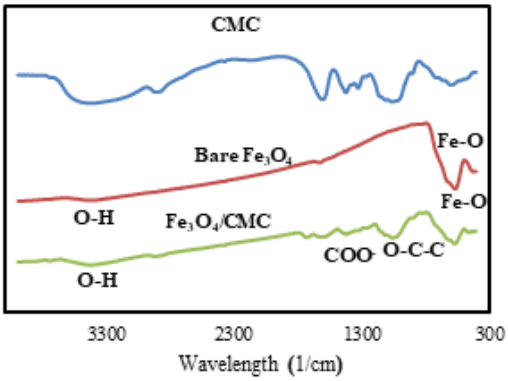


**Figure 1.** FTIR Spectra of CMC, $Fe_3O_4$, $Fe_3O_4$/CMC

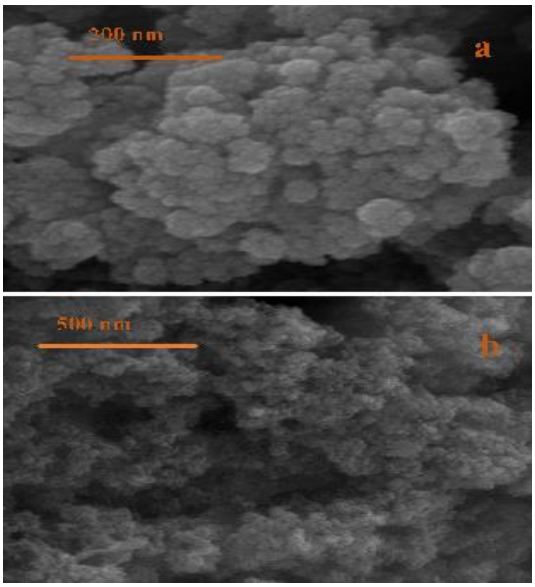


**Figure 2.** (a) SEM Images of $Fe_3O_4$/CMC in 15 kV-200 nm, (b) SEM Images of Immobilized Laccase 15 kV-500 nm

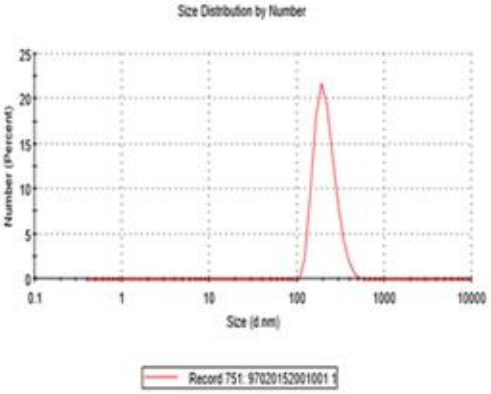


**Figure 3.** DLS Analysis of $Fe_3O_4$/CMC Nanoparticles

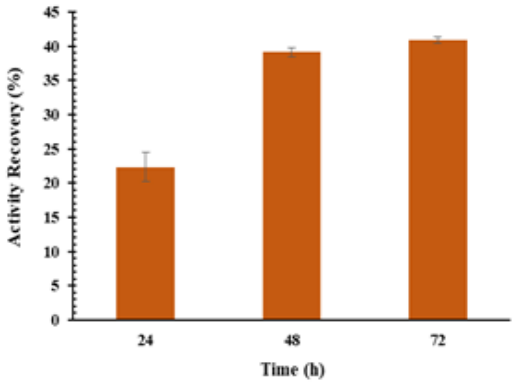


**Figure 4.** Effect of Contact Time on Activity Recovery of Immobilized Laccase (means of triplicate ± standard deviation), pH = 6, Temperature = 4°C, 0.01 g EDC, 0.03 g NHS, 0.01 g NPs

as the optimal condition in order to accelerate immobilization process. Therefore, 48 h was selected as the optimal condition. The similar pattern of results was reported by Kumar et al. [34] and Patel et al. [35].

### 3. 3. Effect of NHS Amount on Activity Recovery

The amount of NHS is a crucial parameter in enzyme immobilization. For this reason, the effect of different amounts of NHS on activity recovery was studied in the range of 0.0025 to 0.055 g. As shown in Figure 5, 0.01 g was the optimum amount of NHS. By increasing the amount of NHS from 0.0025 g to 0.01 g, the activity recovery was ascended, but further increasing resulted in decrease in activity recovery, slightly. The similar pattern of results was reported by Wissink et al. [36]. Inline results have been reported using equal amounts of

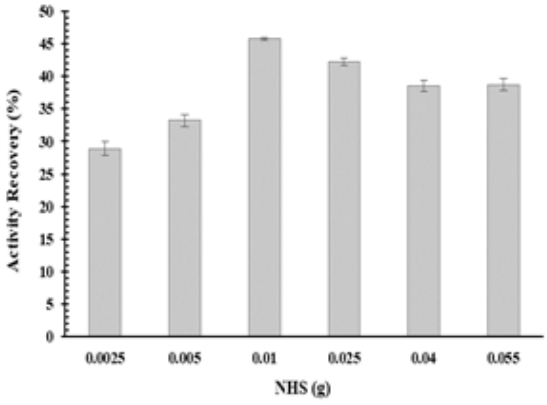


**Figure 5.** Effect of NHS Amount on Activity Recovery of Immobilized Laccase (means of triplicate ± standard deviation), pH = 6, Temperature = 4°C, 0.01 g EDC, 0.01 g NPs

EDC and NHS by Sam et al. [37]. On the other hand, Zhang et al. reported 0.44/0.37 for EDC to NHS ratio [38].

### 3. 4. Effect of $Fe_3O_4$/CMC Nanoparticles Amount on Activity Recovery

The other important factor in enzyme immobilization is the amount of support; so, the effect of different values of $Fe_3O_4$/CMC nanoparticles was investigated in the range of 0.003 g to 0.0175 g. Figure 6 shows a bell shape pattern. It means that by increasing the amount of nanoparticles up to 0.0125 g, the activity recovery was increased. This phenomenon might be due to the lack of available surface and carboxylic acid groups; but, further increase led to decreasing in activity recovery. It could be agglomeration of nanoparticles, which made some enzymes unavailable.

By optimizing the immobilization procedure, the activity recovery was reached 51 ± 0.8%. Moreover, the amount of laccases successfully attached to the NPs was determined by measuring the difference between the concentration of free enzymes before and after the immobilization process. The results showed that by immobilizing the laccase onto $Fe_3O_4$/CMC nanoparticles, 120 mg of laccase was successfully attached to 1 g of supports, while as Park et al. [39, 40] reported 30.4 (mg/g) and 8.6 (mg/g) for their enzyme loading on supports, respectively.

### 3. 5. Reusability of Immobilized Laccas

Reusability capacity of immobilized enzyme is one of the crucial parameters in immobilization procedure. The reusability of immobilized laccase was examined by consecutive activity measurement. After each cycle, immobilized laccases were separated by the external magnetic field, and the reaction media was decanted for activity assay. The separated immobilized laccases were washed with buffer, and the fresh reaction media was charged. The initial activity of immobilized laccase was considered to be 100% as the reference value. The reusability of immobilized laccase was studied up to 5 successive cycles. Immobilized laccases could save nearly 55% of their initial activity after 5 cycles (Figure 6). They lost about 40% of their activity in the second batch and the further batches had no significant effect on immobilized laccase activity. The reusability capacity of immobilized enzyme in this study was lower in comparison with some studies. Sondhi et al. [33] and Nadar et al. [34] reported that their immobilized enzymes could save more than 90% of their activity after 5 cycles. The decreasing of immobilized laccase activity in second batch might be due to enzyme leaching because of non-covalent bonding.

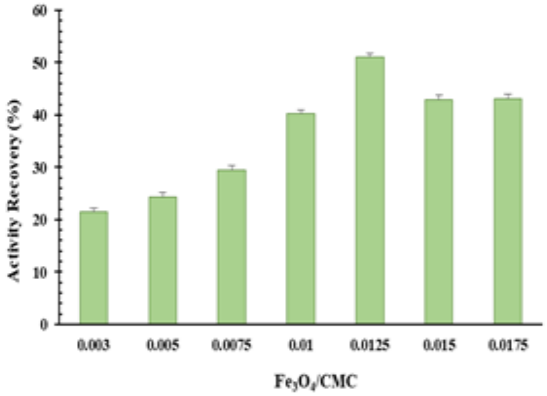


**Figure 6.** Effect of $Fe_3O_4$/CMC Nanoparticles on Activity Recovery of Immobilized Laccase (means of triplicate ± standard deviation), pH = 6, Temperature = 4°C, 0.01 g EDC, 0.01 g NHS

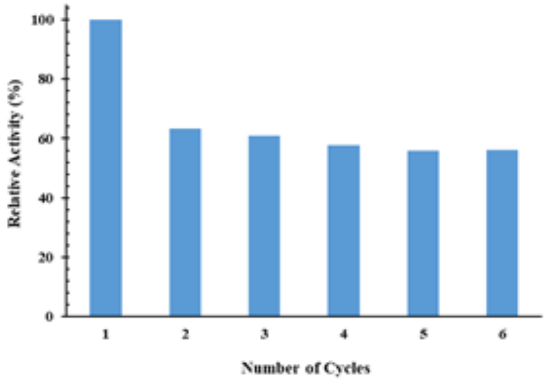


**Figure 7.** Reusability of Immobilized Laccase

## 4. CONCLUSION

In conclusion, laccase was successfully immobilized onto $Fe_3O_4$/CMC nanoparticles. The critical parameter in laccase immobilization such as the amount of NHS, the amount of EDC, contact time, and weight of nanoparticles were optimized. The laccase loading was about 120 (mg/g) of nanoparticles. Immobilized laccases could save more than 50% of their initial activity after 5 cycles. Therefore, applying this immobilization method can provide magnetic nanoparticles for environmental applications, especially in wastewater treatment. According to the enzyme reusability, this method can provide economic benefits.

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## Persian Abstract

**چکیده**

در این پژوهش، ابتدا نانوذرات مغناطیسی اکسید آهن ($Fe_3O_4$) و نانوذرات اکسید آهن پوشش داده شده با کربوکسی متیل سلولز ($Fe_3O_4/CMC$) به روش هم رسوب دهی شیمیایی و به صورت درجا سنتز شدند. سپس، آنزیم لاکاز از کپک *Trametes hirsuta* بر روی نانوذرات آهن پوشیده شده با کربوکسی متیل سلولز *(CMC)* از طریق پیوند کوولانسی بین گروه کربوکسیلیک اسید کربوکسی متیل سلولز و گروه آمین آنزیم لاکاز به صورت کووالانسی تثبیت شد. نانوذرات سنتز شده و آنزیم تثبیت شده بر روی نانوذرات مغناطیسی به وسیله‌ی آنالیز طیف سنجی تبدیل فوریه مادون قرمز، میکروسکوپ روبشی الکترونی و پراش نور دینامیک مشخصه‌یابی شدند. هچنین، پارامترهای تاثیرگذار در تثبیت آنزیم از قبیل زمان تماس، مقدار *NHS* و مقدار نانوذرات مورد بررسی قرار گرفتند و به ترتیب مقادیر ٤٨ ساعتت، ۰/۰۱ گرم و ۰/۰۱۲۵ گرم به ازای ۰/۰۱ گرم از *N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC)* به دست آمدند. بازیابی فعالیت بعد از بهینه سازی پارامترهای تاثیرگذار در تثبیت آنزیم به مقدار ۰/۸ ± ۵۱٪ رسید. همچنین مقدار تثبیت آنزیم لاکاز بر روی نانوذرات $Fe_3O_4/CMC$ حدود (*mg/g*) ۱۲۰ بود. به علاوه اینکه آنزیم لاکاز تثبیت شده توانست تقریبا ۵۰ درصد از فعالیت اولیه خود را بعد از ۵ چرخه متوالی استفاده حفظ کند.