RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Applications of HRP-immobilized catalytic beads on the removal of 2,4-dichlorophenol from wastewater

Shuai Wang He Fang Yukai Wen Minhua Cai

*Wei Liu Shengbin He Xiaoping Xu**

College of Chemistry, Fuzhou University, Fuzhou, 350108, P.R. China

* Corresponding author, professor Xiaoping Xu, Tel.: +8613067237982
E-mail: xu@fzu.edu.cn (Xiaoping Xu); 1185668602@qq.com (Shuai Wang)

Abstract: 2,4-Dichlorophenol, as a highly toxic pollutant, is widely existed in wastewater discharged from several industries. In this work, the use of immobilized horseradish peroxidase for 2,4-Dichlorophenol removal from wastewater was investigated. Sodium hydroxide and hydrochloric acid treated PAN-based beads were modified with ethanediamine and chitosan, and then were activated with glutaraldehyde. The emzyme was immobilized onto the activated beads by covalent crosslinking. The surface of immobilized bead was determined by EDX(SEM) and FTIR. The removal of 2,4-Dichlorophenol has been studied using immobilized beads in the beakers equipped with magnetic stirrer. Optimum pH of the free and immobilized enzyme were determined as 6.0 and 7.0 respectively, and the optimum ratio of hydrogen peroxide to 2,4-dichlorophenol is 1. The experimental results showed that about 90% of 2,4-dichlorophenol was removed by the enzyme-immobilized capsules, and the immobilized enzyme had lower efficiency in the removal of 2,4-Dichlorophenol in comparison with the free enzyme. However, the immobilized beads had shown good operational stability.

Key words: immobilization horseradish peroxidase 2,4-dichlorophenol biodegradation

1. Introduce

Phenolic compounds, recognized as endocrine disruptors, are the most frequent persistent pollutants found in the effluents. They are widely distributed as environmental pollutants in different concentrations in wastewater and usually disposed from various industries, such as oil refinery, petrochemical plants, bleaching processes, coal conversion processes and phenolic resin industries[1-3]. Most of phenolic compounds, especially phenolic organohalogens, are high toxicity, poorly degradable, are likely to impose toxic and carcinogenic effects upon humans even at low concentrations[4-5]. Therefore, Wastewaters containing phenols and other toxic compounds need to be treated carefully before discharge into the environment. Several treatment methods have been widely used for removing phenolic compounds from wastewaters, including activated carbon adsorption, photocatalytic degradation, solvent extraction, biological treatment and electrochemical methods[6-7]. In recent years, bioremediation strategies seem to be a green alternative to the physical and chemical treatment of wastewater [8]. Upsurge of interest has focused on using enzymes from different plants and fungi[9], such as tyrosinase[10], laccase[3] and peroxidase[11], which catalyze the oxidation of many phenolic compounds resulting in the production of quinones or free radicals. These quinones or free radicals usually give rise to the formation of water insoluble oligomers and polymers, which are simply isolated by sedimentation or filtration[12].

Horseradish peroxidase (HRP, EC 1.11.1.7), a widely studied representative of class III heme peroxidase[13-14], has been successfully utilized in many researches,

such as in chemiluminescence assays, immunoassays, bioremediation and wastewater treatment[15]. In the presence of HRP, the oxidation of phenolic compounds are catalyzed by adding the hydrogen peroxide to form the corresponding radicals, and the radicals spontaneously interact to form insoluble polymers and oligomers rapidly, which can be easily removed from the wastewater. However, low stability and poor reusability of free enzyme have limited its further application[3]. Many researches have proved that immobilized technology is a most straightforward and effective method to achieve efficient and continuous application of enzymatic oxidation[16-18]. Adsorption is a simplest and less expansive method of immobilization, which could provide higher retention activity of enzyme but for a shorter time than covalent binding[19]. It means that adsorption of the enzymes are more likely to be gradually released from the carriers. Therefore, the immobilization of support surface by using a crosslinking agent as glutaraldehyde is mostly required[20-21]. For example, Magnetic Fe3O4/SiO2 Particles were successfully used to immobilize horseradish peroxidase by covalent crosslinking for the effectively removal of 2,4-Dichlorophenol[7,22].

In the present work, we are interested in the application of enzyme-like catalytic substrate in the field of the removal of 2,4-dichlorophenol. PAN-based beads, as carriers, were used for the horseradish peroxidase by covalent crosslinking with glutarldehyde.

Here, the aim of this work was to study the performances of enzyme-immobilized beads on the phenolic compounds removal from wastewater and to establish some optimal operational conditions. What's more, some variables(pH, H_2O_2, time)

involved in the oxidative process to remove 2,4-dichlorophenol from wastewater were evaluated particularly. Finally, the reusability experiment of the enzyme-immobilized beads was also applied and evaluated. As far as we know, until now, there has not been any reports concerned with such strategy.

2. Materials and methods

2.1. Materials

Polyacrylonitrile (PAN) was a product of Heowns Biochem Technologies LLC, China. 2,4-dichlorophenol, hydrogen peroxide, sodium hydroxide(NaOH), hydrochloric acid, ethylenediamine, N,N-Dimethylformamide (DMF), glutaraldehyde, 4-aminoantipyrine and phosphoric acid were all obtained from Xilong Chemical CO.,Ltd. Horseradish peroxidase(HRP) ((EC 1.11.1. 7), >250U/mg) was purchased from Shanghai SANGON Biological Engineering Co., Ltd., China. All chemicals were of analytical grade and were used without further purification. Solutions were prepared with distilled water(was prepared by Fuzhou University). In this experiment, 2,4-dichlorophenol buffer solution was assumed as industrial wastewater.

2.2. Preparation of PAN-based beads

The preparation of PAN-based beads method was carried out according to the following steps. A solution of 8% (w/w) polyacrylonitrile was prepared in DMF under constant mechanical stirring. After dissolving polyacrylonitrile, the gel was stored in the room temperature followed by continuous stirring to obtain a homogenous gel. PAN-Based beads were prepared by extrusion using a simple one step process similar to that described by Nigma[23]. The homogenous gel was dropped through a silicon

tube, using a peristaltic pump, into a beaker containing 200ml of distilled water. 10cm dropping height was chose to avoid the droplets sticking together. The obtained PAN-based beads were spherical beads with the same size, and the diameter of beads was about 3mm. Prior to the removal of beads, the mixtures was diluted more than five-fold by adding required amount of distilled water. After being incubated to stabilize the gel membrane, the PAN beads were filtrated and stored in the distilled water for further utilization.

2.3. Modifications of PAN-based beads

The modification of PAN-based beads were carried out by sequential processing of acrylonitrile copolymer membranes with NaOH, ethylenediamine, glutaraldehyde and chitosan, respectively, which had been described by Gabrovska[24] and Nicolucci [25].

2.4. Glutaraldehyde activation and horseradish peroxidase immobilization

The modified beads were immersed in 10% water solution of glutaraldehyde for 60min at 4°C. Then, the beads were thoroughly washed with distilled water. After that the beads were kept in the solution of horseradish peroxidase for 20h with concentration of 1mg/ml at 4°C. Finally, the resulting beads were washed thoroughly once again. After immobilization, the color of the initial white beads were turned into brownish red, and almost no changes in the sizes of the beads. The immobilized beads were stored in phosphate buffer (0.1M, pH 7.0) at 4°C.

2.5. Characterization of the beads

Scanning electron microscopy and energy dispersive X-ray: The initial/immobilized beads were

dried and coated with gold under reduced pressure. A scanning electron microscope(SEM, Nova Nano 230) was employed to observe the morphology of the samples and perform EDX measurements. SEM analyses were performed using an acceleration voltage of 20 kV. EDX measurements were obtained between 0 and 20 keV.

Fourier Transform Infrared Spectroscopy(FTIR): FTIR spectra of the cleaned-beads and modified/ immobilized beads were obtained by using a FTIR spectrophotometer. The FTIR spectrum was recorded using an attenuated total reflectance technique with a spectrometer in the range of 4000~400cm^{-1}.

2.6. Assays of protein loading and horseradish peroxidase activity

The amount of protein bound on the modified beads was determined by the method of Bradford assay[26]. The amounts of immobilized protein were calculated by subtracting the amount of enzyme determined in the residual solutions and washings from the total amount of protein. As described by Nicell[27] and Rao[28], a colorimetric assay was used to measure the activity of free and immobilized HRP at 510nm. This method led to a change of the solution coloring, and the intensity of the coloring is proportional to the concentration. And the retention of activity was defined as the ratio of the reactivity of immobilized enzyme to the same quantity of free enzyme. Here, the same quantity of free enzyme refers to the corresponding amount of immobilized enzyme.

2.7. Studies on the removal of 2,4- dichlorophenol

In order to determine the time required for completion of enzymatic reaction and

efficiency of removal, experiments were conducted to assess the free/immobilized horseradish peroxidase catalyzed removal of 2,4-dichlorophenol(3mM) from aqueous solutions. The experiments were carried out at 25°C in the beakers equipped with magnetic stirrer. 2,4-dichlorophenol and buffer solution were introduced to reaction media followed by addition of enzyme and hydrogen peroxide. The effect of pH on the activity of the free and the immobilized enzymes was determined using the pH range 3.0–9.0 at 25 °C. The results for pH were presented in a normalized form with the highest value of each set being assigned the value of 100% activity[29]. What's more, effects of parameters such as contact time, hydrogen peroxide/phenol from 0.5 to 1.5, and enzyme amount from 0.5 to 1 g/L immobilized beads were studied on phenol removal (% conversion).

The adsorbed phenolic compounds on the non-immobilized beads(without incubation with glutaraldehyde) were carried out in the same manner. In this experiment, the residual 2,4-dichlorophenol concentrations in the assay solutions were measured by a colorimetric method using 4-aminoantipyrine and potassium ferricyanide at 510nm.

2.8. The reusability of immobilized beads

The reusability of immobilized beads was determined by using same immobilized beads for 8 times. After each cycle of reaction, the enzyme immobilized beads were removed and washed with phosphate buffer (0.1 M, pH 7.0) to remove any residual substrate within immobilized beads. After that, the immobilized beads were transferred into fresh reaction medium and the retained activity was determined.

The initial activity was considered as 100% for the immobilized enzyme.

3. Results and discussion

3.1. Characterization studies

3.1.1. SEM and EDX measurements

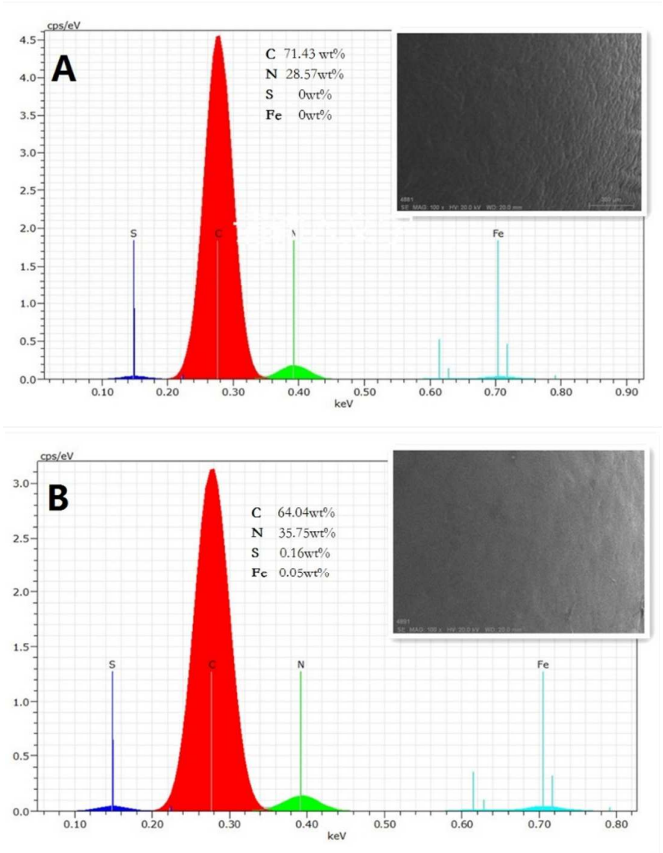


Fig. 1. EDX spectra and SEM photographs of: (A): initial bead, (B): enzyme immobilized bead.

EDX was used to analyze the changes in elemental composition between initial and immobilized beads. Fig. 1 presents different SEM images as well as the corresponding EDX spectra of beads. The testing area of EDX analysis was the attached SEM images[30]. The results showed that the element N increased by approximately 10 Wt.%, followed by the decrease of C(more than 7 Wt.%) in the area of the initial beads in Fig. 1A, compared with the enzyme immobilized beads in Fig. 1B. In addition, the EDX shows that no S and Fe elements was existed in the initial

beads, and it can also be noticed the appearance of S and Fe peaks in the enzyme immobilized beads. The S and Fe elements accounted for 0.16 Wt.% and 0.05 Wt.% respectively, which probably ascribed to the presence of horseradish peroxidase on the beads. These changes indicated that the horseradish peroxidase was successfully immobilized onto the beads.

3.1.2. FTIR-ATR spectroscopy

The surface of the initial beads has nitrile groups (-CN) that provide a reactive surface for modification. Those nitrile groups were modified to form carboxylic group by using NaOH and HCl. The modified beads were aminated by using ethylenediamine, then activated with glutaraldehyde. This biofunctional molecule can react with the amino groups created on the bead surface after activation with ethylenediamine, and the second aldehyde on each glutaraldehyde molecule is available for reacting with amino groups present on the another molecules[10]. From this mechanism, the horseradish peroxidase was covalently immobilized on the PAN-based beads.

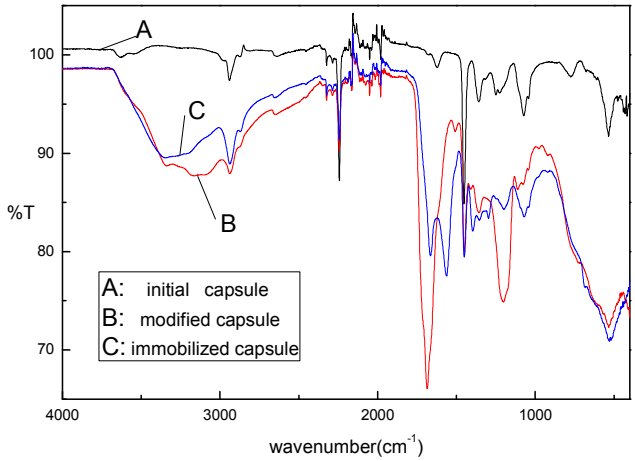


Fig. 2. FTIR-ATR spectrum of initial /modified/immobilized bead.

FTIR spectra of initial/modified/immobilized bead surfaces were presented in

Fig. 2 to verify this mechanism. For the native beads, the adsorption peaks at 2243 cm^{-1} is attributed to the stretching vibrations band of nitrile groups(–CN). The bands at 2939 and 1454 cm^{-1} are attributed by stretching and bending vibration band of methylene(-CH2-) on the bead surface. On the other hand, after modification with NaOH new absorption peaks are observed, the peak at 3400 and 1690cm^{-1} can be ascribed to the presence of O–H bond and C=O bond of carboxylic acids. These two stretching peaks indicated the presence of -COOH after being treated by NaOH. After the horseradish peroxidase was immobilized, the immobilized beads exhibited two characteristic peaks at 1230cm^{-1} and 3343cm^{-1} due to the presence of acylamino, which was the combination of -CHO and $–NH_2$. Furthermore, the present of the bonds of C=N (1664cm^{-1}) and C–N (1071cm^{-1}) indicate that the amidination reaction and the immobilization of horseradish peroxidase were both happened[31]. These characteristic bands confirmed that the enzyme was immobilized on the beads by covalent bonding.

3.2. Immobilization of horseradish peroxidase onto the activated beads

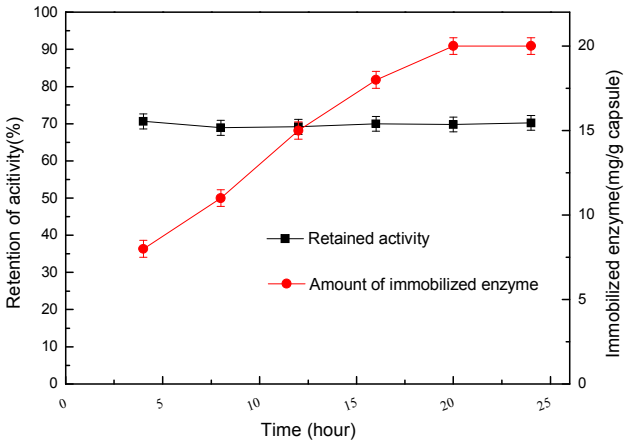


Fig. 3 Effects of time on the immobilized horseradish peroxidase and retained activity.

The immobilization of horseradish peroxidase on the modified beads was carried

out by crosslinking with glutaraldehyde. The enzyme molecules were immobilized with enzyme-containing solution for conjugation on the activated beads. Under the different immobilization time, the efficiency of protein loading and the activity performance on immobilized beads were determined and shown in Fig. 3. As seen from the Fig.3, firstly, the amount of immobilized enzyme was increased along with increasing hours. Since more functional amine groups on the modified beads can be reacted with more aldehyde groups for attachment of intending protein. Then the immobilized horseradish peroxidase reached a constant amount of immobilized enzyme(20mg/g beads) at 20h, and no longer increased over time. Presumably, the amount of bound protein depended on the content of aldehyde groups on the activated beads, and the aldehyde groups for horseradish peroxidase achieved saturation after 20h of immobilization reaction. What's more, the retained activity of the immobilized enzyme on the beads was found to be 70% compared to the free enzyme, and essentially unchanged in the different immobilization times. This phenomenon might be largely attributed to the effects of glutaraldehyde. It is well-known that glutaraldehyde has a very complicated influence on the immobilization of enzyme. As a bridge between beads and the enzyme, glutaraldehyde will result in HRP loading by covalent cross-linking and at the same time causes the changes of the three dimensional conformation of the enzyme[32], resulting reduced enzyme activity recovery. As described about the definition of retention of activity above, it is not affected by the amount of immobilized enzyme. So the values kept constant with the change of the reaction time. To summarize, the amount of maximum bound protein

was 20mg/g of PAN-based beads, and the recovered activity of enzyme was about 70% that corresponds to 3500 U/g beads.

3.3. Effect of pH on the activity of free and immobilized horseradish peroxidase

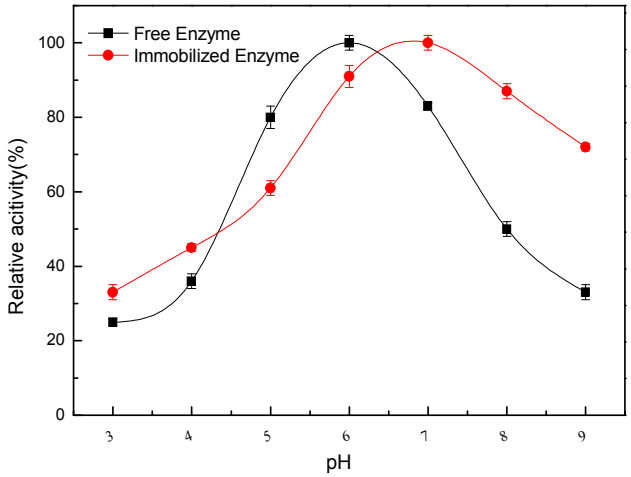


Fig. 4. Effect of pH on the activity of free/immobilized horseradish peroxidase.

For the free/immobilized enzyme, pH is one of the most important influence factors on the retention of activity. So, it is necessary to investigate the pH effects on the activity of free/immobilized enzyme. In this paper, the effects of pH on the activity of free and immobilized HRP was obtained by incubating both the free and immobilized enzyme at 25°C in the pH range 3.0–9.0 followed by measuring the enzyme activity at 510 nm, and Fig. 4 showed the results of these measurements. As seen in this Fig. 4, optimal pH for free enzyme is about 6.0, but for immobilized enzyme is about 7.0. The reason for this difference may be that many amino acid groups or/and the active centers were bared because of the changes in the structures of the immobilized enzyme. Thus, in order to enable the enzyme to function properly, the pH should be raised to a certain amount. Moreover, from Fig.4, the pH profile of immobilized horseradish peroxidase displays

significantly improved stability in the tested pH range compared to that of free enzyme, which implied that the immobilized enzyme is more resistant to alkaline and acid conditions than the free enzyme[22]. This could be due to the stabilization of horseradish peroxidase molecules resulting from multi-point covalent bonding on the PAN-based beads support[33-34]. So, the condition of pH close to 7.0 has been used for the treatment of phenolic effluents.

3.4. hydrogen peroxide concentration

Increasing phenolic compounds removal percentage could be obtained by choosing an appropriate hydrogen peroxide concentration. Some authors[35-36] have tried to introduce an optimal molar ratio of hydrogen peroxide to phenolic compounds resulting in higher removal efficiency, and they also found that the optimum peroxide concentration is totally depends on initial 2,4-dichlorophenol concentration and differ from case to case. In this paper, several experiments were carried out by using three different 2,4-dichlorophenol concentrations (1, 3 and 5mM)and hydrogen peroxide varying from 0.5 to 7.5mM. In all assays, abundant immobilized beads were introduced to reaction medium. Fig. 5 shows the results obtained in these series of experiments, where the maximum conversion plotted against the ratio of hydrogen peroxide to 2,4-dichlorophenol. From the Fig.5, the optimum ratio of hydrogen peroxide to 2,4-dichlorophenol is 1, and the behavior of the phenol removal efficiency was similar in all 2,4-dichlorophenol concentrations. The amount of 2,4-dichlorophenol removed was sharply increased with an increase in hydrogen peroxide up to an optimal point, which shows that hydrogen peroxide is a limiting

factor in this range. Furthermore, after 2,4-dichlorophenol conversion reached its optimum point, adding excess hydrogen peroxide gave rise to significantly reduced the conversion efficiency. The reason for this phenomenon might be that an excess amount of hydrogen peroxide results in needless intermediate products which inhibit the activity of enzyme, and/or that an excess of hydrogen peroxide act as an inhibitor of horseradish peroxidase activity by irreversibly oxidizing the enzyme ferriheme group essential for peroxidase activity[37]. We also found that there was some removal of 2,4-dichlorophenol in the absence of H_2O_2. The most likely reason was that some of hydrogen in the water were acted as the ultimate electron acceptor in the absence of H_2O_2, which resulting in some removal of 2,4-dichlorophenol.

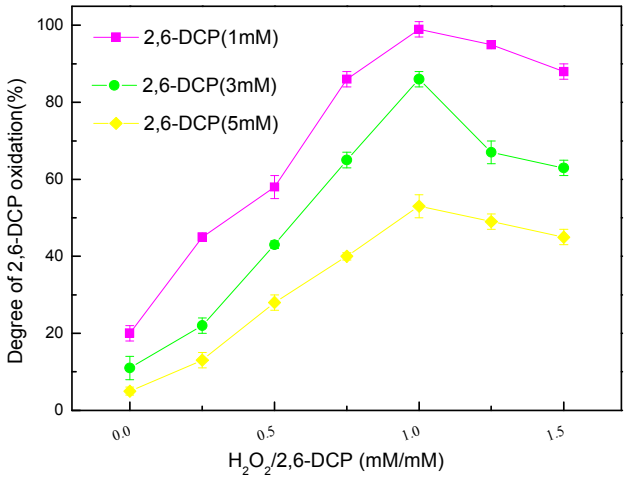


Fig. 5. Effects of hydrogen peroxide concentration on the degree of 2,4-DCP oxidation.

3.5. Removal of 2,4-dichlorophenol from wastewater using immobilized horseradish peroxidase

Phenolic compounds (2,4-dichlorophenol) degradation studies were performed at 25◦C in a series of backers (each one containing 100ml of 3mM 2,4-dichlorophenol, along with hydrogen peroxide). Each backer were combined with enzyme

mixture(free or equivalent immobilized enzyme) and incubated for 12 h with shaking at 100 rpm. Every 2 hour, a 1 ml sample was taken from solution and was analyzed for pH and the residual phenol concentration. Fig.6 shows the percentage removal of 2,4-dichlorophenol by free and immobilized horseradish peroxidase as a function of contact time. It showed the comparison between free and encapsulated enzyme for phenol removal efficiency versus time which are near. And about 90% of 2,4-dichlorophenol were removed after 12 h contact with immobilized horseradish peroxidase compared to free horseradish peroxidase which removed 2,4-dichlorophenol about 99%. This indicate that encapsulated enzyme had lower efficiency in comparison with the same concentration of free enzyme.

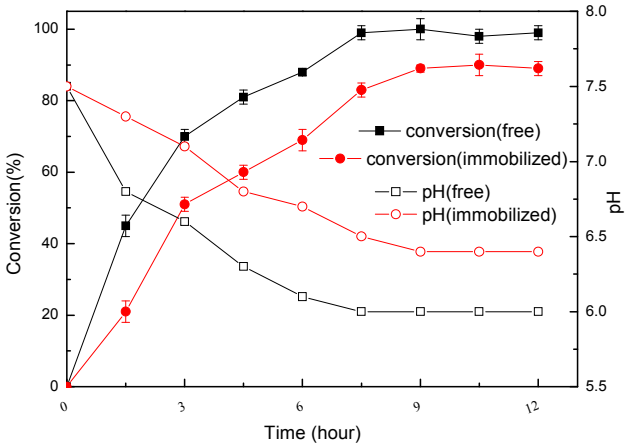


Fig. 6. Evolution of 2,4-DCP oxidation, pH values during 2,4-DCP removal .

The amount of adsorbed 2,4-DCP on the non-immobilized beads has been detected to be 0.05±0.01mmol/L. From the results of adsorption, we know that the adsorption quantity was very little in comparison with the initial concentration(3mM). So, in this experiment, the amount of adsorbed phenolic compound could be ignored. In addition, the pH was also measured at different times of treatment (Fig. 6). As shown in the Fig. 6, throughout the experiment, the pH value decreased gradually as a

result of dehalogenation. This decrease in the pH value did not affect the removal process, because the enzyme can retains its activity in a wide pH range. According to the previous reports[7,9], this change could be associated with the enzymatic transformation of 2,4-DCP and the chloride ions liberation from this halogenated compound. The chloride substituents were released because of a consequence of an oxidative coupling reaction, which is an initial step leading to further decomposition of these organic compounds. As a consequence of dehalogenation, HCl was formed finally, which resulting in the changes of pH.

3.6. Reusability of the Horseradish peroxidase-immobilized on PAN-based beads

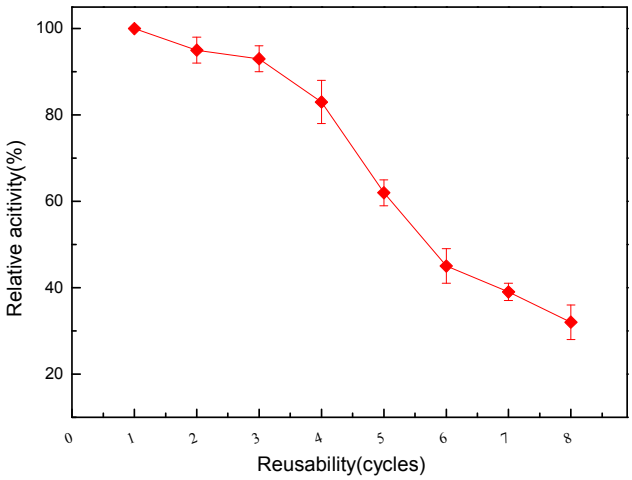


Fig. 7. Reusability of HRP-immobilized beads.

The reusability of immobilized enzyme was one of the most important key features. The reusability was observed by assessing the remained catalytic activity of immobilized enzyme after successive service times[24, 38]. After each reaction period, the same enzyme immobilized-beads were separated and then rinsed thoroughly with phosphate buffer (0.1 M, pH 7.0) to remove any residual reactant within the immobilized beads. The cleansed beads were used for subsequent batches. As shown

in the Fig.7, the relative activity of immobilized enzyme decreased with the increase of the number of reuses. The immobilized beads were reusable up to three cycles almost without any changes in their retention activity. However, they retained around 35% of its original activity after 8 times repeated use. Clearly, the activity loss of covalently immobilized enzyme was very higher. This phenomenon might be caused by the subunit release from immobilized-beads during reuse and washing operations, and/or accumulating of radicals which resulting in enzyme inactivation.

4. Conclusions

In this paper, the preparation and application of immobilized horseradish peroxidase on the PAN-based beads for 2,4-DCP removal from aqueous solution was investigated. Horseradish peroxidase was immobilized on the modified beads by crosslinking with glutaraldehyde. The maximum amount of bound protein onto the beads was about 20mg/g beads, and the retained activity of the immobilized enzyme was about 70%. The performance of 2,4-DCP removal was found to be highly dependent on pH(optimum pH of immobilized enzyme is 7.0) and hydrogen peroxidase(optimum ratio of hydrogen peroxide to 2,4-dichlorophenol is 1). The experimental results obtained also revealed the high effectiveness in 2,4-DCP removal. And the pH value decreased in the reaction medium because of the pollutant dehalogenation. What' more, the reusability experiment showed that the immobilized beads can be used up to three cycles without serious deficiency in their relative activity, which provided an economic advantage for large-scale biotechnological applications. In conclusion, this study helps to design a considerable potential method

for efficient, continuous and safe treatment of phenolic effluents.

References

[1] L. Pramparo, F. Stuber, J. Font, A. Fortuny, A. Fabregat, C. Bengoa, *J. Hazard. Mater.*, 177 (2010) 990–1000.

[2] G. Moussavi, B. Barikbin, M. Mahmoudi, *Chem. Eng. J.*, 158 (2010) 498–504.

[3] Y.Y. Liu, Z.T. Zeng, G.M. Zeng, L. Tang, Y. Pang, Z. Li, C. Liu, X. Lei, M.S. Wu, P.Y. Ren, Z.F. Liu, M. Chen, G.X. Xie, *Bioresour. Technol.*, 115 (2012) 21–26.

[4] L. Meijer, J. Weiss, M.V. Velzen, A. Brouwer, A. Bergman, P.J.J. Sauer, *Environ. Sci. Technol.*, 42 (2008) 3428–3433.

[5] G.M. Zeng, Z. Yu, Y. Chen, J. Zhang, H. Li, M. Yu, M. Zhao, *Bioresour. Technol.*, 102 (2011) 5905–5911.

[6] G.S. Chuang, A.C. Chao, P.Y. Ho, H.Y. Li, *Biochem. Eng. J.*, 21 (2004) 161–170.

[7] J. Huang, Q. Chang, Y.B. Ding, X.Y Han, H.Q. Tang, *Chem. Eng. J.*, 254 (2014) 434-442 .

[8] L.F. Qiu, Z.X. Huang, The treatment of chlorophenols with laccase immobilized on sol–gel-derived silica, *World J. Microbiol. Biotechnol.*, 26 (2010) 775–781.

[9] V.A. Angelini, J. Orejas, M.I. Medina, E. Agostini, *J. Hazard. Mater.*, 185 (2011) 269-274.

[10] G. Bayramoglu, A. Akbulut, M.Y. Arica, *J. Hazard. Mater.*, 244-245 (2013) 528-536

[11] L.C. Lopes, M.T. Barreto, K.M. Gonçalves, H.M. Alvarez, M.F. Heredia, R.O. de Souza, Y. Cordeiro, C. Dariva, A.T. Fricks, *Enzyme Microb. Technol.*, 69 (2015) 10-18.

[12] T. Maruyama, J. Uchida, T. Ohkawa, T. Futami, K. Katayama, K. Nishizawa, K. Sotowa, F. Kubota, N. Kamiya, M. Goto, *Lab. Chip.*, 3 (2003) 308–312.

[13] K. Chattopadhyay, S. Mazumdar, *Biochemistry*, 39 (2000) 263–270.

[14] L. Zhang, W.S. Zhao, Z.L. Ma, G.R. Nie, Y.C. Cui, *Eur. Polym. J.*, 48 (2012) 580–585.

[15] N.C. Veitch, *Phytochemistry*, 65 (2004) 249–259.

[16] R.A. Sheldon, *Adv. Synth. Catal.*, 349 (2007) 1289–1307.

[17] Y. Pang, G.M. Zeng, L. Tang, Y. Zhang, Y.Y. Liu, X.X. Lei, M.S. Wu, Z. Li, C. Liu, *Bioresour. Technol.*, 102 (2011) 10733–10736.

[18] Q. Chang, H.Q. Tang, *Microchim. Acta*, 181 (2014) 527-534.

[19] C. Menale, C. Nicolucci, M. Catapane, S. Rossic, U. Bencivenga, D.G. Mita, N. Diano, *J. Mol. Catal. B: Enzym.*, 78 (2012) 38–44.

[20] G. Bayramoğlu, M. Yakup Arica, *Mat. Sci. Eng. C-Mater.*, 29 (2009) 1990–1997.

[21] Q. Chang, L.H. Zhu, G.D. Jiang, H.Q. Tang, *Anal. Bioanal. Chem.*, 395 (2009) 2377-2385.

[22] Q. Chang, H.Q. Tang, *Molecules*, 19 (2014) 15768-15782.

[23] S.C. Nigma, I.-F. Tsao, A. Sakoda, H.Y. Wang, *Biotechnol. Technil.*, 2 (1988) 271–276.

[24] K. Gabrovska, T. Nedelcheva, T. Godjevargova, O. Stoilova, N. Manolova, I. Rashkov, *J. Mol. Catal. B-Enzym.*, 55 (2008) 169-176.

[25] C. Nicolucci, S. Rossi, C. Menale, T. Godjevargova, Y. Ivanov, M. Bianco, L. Mita, U. Bencivenga, D.G. Mita, N. Diano, *Biodegradation*, 22 (2011) 673-683.

[26] M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248-254.

[27] J. A. Nicell, H. Wright, *Enzyme Microb. Technol.*, 21 (1997) 302–310.

[28] S.V. Rao, K.W. Anderson, L.G. Bachas, *Biotechnol. Bioeng.*, 65 (1999) 389-396.

[29] M.Y. Arica, G. Bayramoğlu, *J. Mol. Catal. B: Enzym.*, 27 (2004) 255–265.

[30] Z. Wang, Z.L. Chen, J. Chang, J.M. Shen, J. Kang, Q. Chen, *Chen. Eng. J.*, 262 (2015) 904–912.

[31] S.F. Li, J.P. Chen, W.T. Wu, *J. Mol. Catal. B-Enzym.*, 47 (2007) 117–24.

[32] P. Ye, Z.K. Xu, J. Wu, C. Innocent, P. Seta, *Macromolecules*, 39 (2006) 1041–1045.

[33] Y. Li, J. Quan, C. Branford-White, G.R. Williams, J.X. Wu, L.M. Zhu, *J. Mol. Catal. B: Enzym.*, 76 (2012) 15–22.

[34] M.A. Duarte-Vazquez, M.A. Ortega-Tovar, B. Garcia-Almendarez, C. Regalado, *J. Chem. Technol. Biotechnol.*, 78 (2003) 42–47.

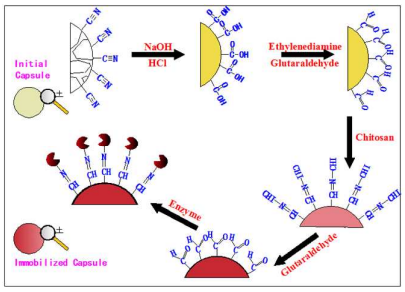
[35] N. Caza, J.K. Bewtra, N. Biswas, K.E. Taylor, *Water Res.*, 33 (1999) 3012–3018.

[36] Y.M. Wu, K.E. Taylor, N. Biswas, J.K. Bewtra, *J. Environ. Eng.*, 125 (1999) 451–458.

[37] M.A. Duarte-Vazquez, B. Garcia-Almendarez, C. Regalado, J.R. Whitaker, *J. Agric. Food Chem.*, 49 (2001) 4450–4456.

[38] S.F. Li, Y.H. Fan, J.F. Hu, Y.S. Huang, W.T. Wu, *J. Mol. Catal. B-Enzym.*, 73 (2011) 98-103.

A table of contents entry.



Graphical abstract

Novel HRP-immobilized beads with the excellent catalytic activity were successfully fabricated to remove 2,4-dichlorophenol from wastewater.