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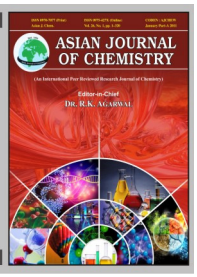

Immobilization of Soybean Hulls Peroxidase on Activated Carbon

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To lay a scientific foundation for the development of an immobilized enzyme-based process for oxidizing dissolved organics in aqueous solution, the immobilization process of soybean hulls peroxidase (SBP) on activated carbon was investigated. Soybean hulls peroxidase (SBP, EC 1.11.1.7) was extracted from soybean hulls in phosphate buffer solution (0.1 M, pH 6.0) and then purified. The molecular weight of the soybean hulls peroxidase is 37 kDa. The soybean hulls peroxidase was immobilized onto activated carbon, the immobilization of soybean hulls peroxidase at pH 5.0 and higher temperature (50 ºC) gave rise to the highest immobilization yield. Equilibrium adsorption models were applied to quantify the effectiveness of immobilization. Adsorption of soybean hulls peroxidase on activated carbon followed the Langmuir model better than the Freundlich model. Fourier transform infrared spectra (FT-IR) provide evidence of the soybean hulls peroxidase enzyme immobilized onto the carbon matrices. The morphology of the native activated carbon and the immobilized soybean hulls peroxidase was confirmed by scanning electron microscopy (SEM).

Keywords: Soybean hulls peroxidase, Immobilization, Activated carbon.

INTRODUCTION

In the environmental fields, enzymes as biocatalysts have been known to apply on the process of pollution treatments and other environmental remediation. Peroxidases, a kind of enzyme, can catalyze the oxidation of the variety of organic and inorganic compounds to form polymeric products of free radicals. All these catalytic reactions require the presence of peroxides such as hydrogen peroxide (H_2O_2) or related compounds to activate the oxidizing reaction. The large family of peroxidases includes Horseradish peroxidase (HRP, EC 1.11.1.7), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), soybean peroxidase (SBP, EC 1.11.1.7) and others are obtained from a large number of animals, microorganisms and plants sources[1]. At present, there is a considerable amount of peroxidase research has focused on Horseradish peroxidase. However, as many enzymes, the cost of purification and the poor thermal, environmental stability limit the large-scale applications of this peroxidase.

Meanwhile, it encourages the researchers to search for less expensive sources of much more other enzymes. The isolation of a highly stable and active peroxidase would develop a catalyst with broad commercial and environmental appeal. Therefore, more researches have focused on soybean (*Glycine max*) hulls peroxidase (SBP) because it is less expensive than Horseradish peroxidase. Soybean hulls peroxidase also belongs to class III of the plant peroxidase superfamily. It was isolated and highlights the advantages over peroxidases from other sources. The purification of soybean hulls peroxidase within using soybean whole seeds as the source has been previously reported by Sessa and Anderson[2]. But at the beginning of 1990s, Gillikin and Graham[3], Gijzen *et al.*[4] extracted soybean hulls peroxidase from the soybean seed hulls as a main by-product of the food industry. They found that soybean seed coat tissues provide low cost and abundant source of peroxidase which can be considered as a lower price alternative to Horseradish peroxidase. Taking into account that soybean hulls is an agricultural by-product found in large quantities and at low cost in many countries, soybean hulls peroxidase is a very attractive candidate for bioremediation.

Soybean hulls peroxidase, as an alternative to Horseradish peroxidase, has received more attention because of the outstanding thermal stability[5]. Unlike Horseradish peroxidase or other plants peroxidases, soybean hulls peroxidase retains catalytic activity over wide ranges of temperature. It has an unusually high thermal stability, being active at 70 ºC where most plant peroxidases are denatured. McEldoon *et al.*[6] also found that soybean hulls peroxidase is a highly thermostable enzyme and its melting temperature of 90.5 ºC is highly typical compared with other plant and microbial peroxidases. Soybean hulls peroxidase's high thermostability makes it an intriguing catalyst for commercial and environmental applications.

Fig. 1 is a possible model of the soybean hulls peroxidase catalytic process adapted by reported Horseradish peroxidase (HRP) reactions[7]. As shown in Fig. 1, SBP_i and SBP_{ii} are the intermediate oxidized by H_2O_2, meanwhile, create free radical of chemical pollutants. Both SBP_i and SBP_{ii} may form a third compound SBP_{iii}, which is the inactivated form of soybean hulls peroxidase[8]. Thus, instead of polymerizing to form large molecular weight products, the free radicals or H_2O_2 could rapidly in active the purified enzyme. The contaminants are oxide to corresponding free-radical formation. And then, peroxidase can catalyze the free-radical polymerized spontaneous[9]. These polymerized products are insoluble and less toxic than initial pollutants; it can be easily removed through a simple filtration of flocculation. All these catalytic reactions require the presence of peroxides such as H_2O_2 or related compounds to activate the oxidizing reaction. Accordingly, H_2O_2, which was environmentally friendly and did not give rise to any waste products[10].

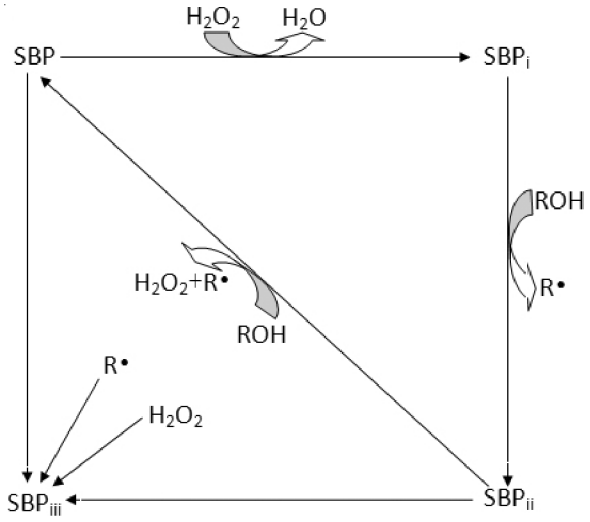

Fig. 1. A model of possible peroxidase catalytic process adapted by Buchanan *et al.*[7]

However, the application of enzymes is restricted because of certain properties like non-reusability, instability, high cost and sensitivity to denaturation. These restrictions, which remain as a challenge for the application of free enzymes, are eased by the use of immobilized enzymes. As catalysts they are not changed during the reactions, it is cost-effective to use them more than once. However, it is difficult to separate them from reaction solutions. Therefore, if they can be attached to the solid support in some way, they can be used again after the products have been removed. For the recovery and reuse of peroxidases in the treatment of wastewaters, the immobilized technology is desirable because of the high cost of enzymes. Moreover, immobilized enzymes allow continuous reaction. Significant enhancements in catalytic efficiency may be obtained if compounds such as chitosan, gelatin and polyethylene glycol (PEG) are added to the reaction mixture[11-15].

Types of carrier on Horseradish peroxidase (HRB) and soybean hulls peroxidase (SBP) immobilization are given in Table-1.

Various techniques had been used to immobilize enzymes, including adsorption and covalent bonding and several approaches have been made for the preparation of immobilized enzymes for the reason that they have shown several advantages over enzymes in bulk solution. Among these methods, adsorption has been considered as a simple and an economical mechanism for immobilization. When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. In other words, attach the enzyme but do as little damage as possible. Considerable knowledge of the active site of the enzyme will prove helpful in achieving this task. It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate or a competitive inhibitor of the enzyme.

TABLE-1
TYPES OF CARRIER ON HORSERADISH PEROXIDASE (HRB) AND SOYBEAN HULLS PEROXIDASE (SBP) IMMOBILIZATION

Peroxidase	Immobilization carrier	Summary
HRP	Polyethylene glycol (PEG)[18,19]	The PEG significantly increased the catalytic efficiency of HRP thereby improving the cost competitiveness of the process.
	PEG and Chitosan[19]	The application of PEG and chitosan effectively protected the enzyme from inactivation resulting in 4-fold and 25-fold reductions in enzyme requirements, respectively.
	Magnetite[20]	When immobilized HRP was used to treat a solution containing various chlorophenols, each chlorophenol was almost 100 % removed and also the removal of total organic carbon (TOC) and adsorbable organic halogen (AOX) reached more than 90 %, respectively.
	Graphite[21]	HRP immobilized in the graphite felt shows a well-defined cyclic voltammetry in the presence of H_2O_2. Immobilized HRP in an electrochemical reactor was found to be an efficient tool for removing 2,4,6-trinitrotoluene.
HRP	Microporous inorganic support[22]	This study indicates the feasibility of an electroenzymatic process to degrade azo dye compounds in wastewater material for HRP immobilization.
HRP	Cinnamic carbohydrate esters[23]	The results show that cinnamic carbohydrate esters represent an appropriate support for peroxidase immobilization and could be used for several peroxidase applications.
HRP & SBP	Aldehyde glass[24]	HRP and SBP were covalently immobilized onto aldehyde glass through their amine groups. The percentages were higher with immobilized HRP than with free HRP. At the same time, it can be affirmed that immobilized SBP is less susceptible to inactivation by H_2O_2 than free SBP.
HRP & SBP	Glutaraldehyde activated aminopropyl Glass beads[12]	The immobilization made HRP and SBP a protective effect on the stability of peroxidases and the phenol conversions. However, more phenol was removed with SBP than with HRP.
SBP	PEG[25]	The absence of PEG increased the amount of SBP required by 33 % for 95 % removal.
SBP	Silica sol-gel/alginate[13]	The SBP were entrapped within silica sol-gel/alginate particles. Upon entrapment, an initial enzymatic rate of polymerization enhances five times compared to the free enzymes.

The surface on which the enzyme is immobilized is responsible for retaining the structure in the enzyme through hydrogen bonding or the formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability. Activated carbon is predominantly an amorphous solid with a large internal surface area and pore volume. Enzyme loading and activity expression is largely dependent on the size of the pores in the carrier bead and the accessible surface. However, in addition to these mentioned carriers, activated carbon (AC) is an important enzymatic carrier[16,17]. There is no more literature shows that this substance can be applicated on the Horseradish peroxidase or soybean hulls peroxidase immobilization. activated carbon would be a suitable choice for further soybean hulls peroxidase immobilized activated carbon.

EXPERIMENTAL

Soybeans (*Glycine max*) were purchased from local supermarket and stored at room temperature. BPA, guaiacol, H_2O_2 (30 %) were purchased from Sigma-Aldrich Fine Chemicals. Other agents and chemical were used of analytical reagent grade and without further purification. A commercial activated carbon (MBC271) particle purchased from Fisher Scientific.

Extraction of soybean hulls peroxidase: In this study, soybean hulls peroxidase enzyme isolated from soybean hulls as the reported procedure[3] described. Firstly, the dry soybean seeds were washed by distilled water and then soaked in distilled water for approximately 24 h or until the seed coats were easily removed. Secondly, this tissue was extracted in buffer which is usually phosphate buffer (Na_2HPO_4/NaH_2PO_4 buffer solution, 0.1 mol/L, pH 6.0) at a ratio of probably 10 mL extraction buffer per 1 g tissue. The most important is that all purification steps must be performed at 4 ºC or on ice. Thirdly, the samples were centrifuged at 6000 rpm for 15 min, filtered through filter paper to remove the soybean hulls fragment and centrifuge again at 15000 rpm for 10 min. The deposition was re-dissolved in the phosphate buffer solution. The final supernatant was stored at 4 ºC and used for the characterization of crude soybean hulls peroxidase extract and followed experiments. The supernatant containing the soybean hulls peroxidase enzyme was used for further studies.

Soybean hulls peroxidase activity assay: Soybean hulls peroxidaze is able to oxidize guaiacol to tetra-guaiacol and form a tea-brown dye, in the presence of H_2O_2. The oxidized product has apparent absorbance in the wavelength of 436 nm. This property has been used to measure the activity of soybean hulls peroxidase, which was measured using phosphate buffer of pH 6, H_2O_2 as substrate and guaiacol as chromogen. Then 1 mL of each enzyme sample was added to 3 mL of substrate (guaiacol and H_2O_2). After 1 min of incubation, the absorbance of the assay mixture was measured at 436 nm on an UV-visible spectrophotometer. In this experiment, soybean hulls peroxidase activity unit (U) is defined by the absorbance value increase 0.001 at OD 436 nm/min, at room temperature and pH 6 conditions.

In these studies, we use the soybean hulls peroxidase concentration to evaluate the soybean hulls peroxidase activity in the solution.

$$\text{Relative SBP activity } (\%) = \frac{C_0 - C_f}{C_0} \times 100\ \%$$

where C_0 and C_f are the initial and final concentrations of soybean hulls peroxidase, respectively.

Effect of time and temperature of immobilization: The immobilized yield of soybean hulls peroxidase on activated carbon in different temperature and pH value conditions was detected. This experiment immobilized the soybean hulls peroxidase on activated carbon at 1, 2 and 4 h. And then detect the remained soybean hulls peroxidase activity in the solution to obtain the immobilized yield of soybean hulls peroxidase on activated carbon. At first, the different pH value for immobilization was screened by determining its high yield. The solution pH value was adjusted using HCl/NaOH solution and the enzyme activity was evaluated from pH 4 to 8. The enzyme activity at which maximum immobilization capacity attained was determined using enzyme relative immobilized activities and the initial concentration of soybean hulls peroxidase is activity $5 \times 10^4\ L^{-1}$.

Adsorption isotherms: In this experiment, the Freundlich and Langmuir adsorption isotherms were used to determine the immobilization constants. The immobilization isotherms of soybean hulls peroxidase onto MBC271 were studied at the optimum temperature 50 ºC and pH 5 conditions. The immobilization experiment was performed in 100 mL conical flasks and the contents were agitated at 100 rpm at preset temperature for 4 h in order to attain equilibrium.

After equilibration, the final concentration (C_f) was measured at 436 nm by spectrophotometric method.

$$\text{Removal } (\%) = \frac{(C_i - C_f)}{C_i} \times 100 \quad (1)$$

$$\text{Amount adsorbed } (q_e) = \frac{(C_i - C_f)}{m} \quad (2)$$

where C_i and C_f are the initial and final concentrations (mg/L) of soybean hulls peroxidase, respectively and m is the mass of activated carbon (in mg/L).

Fourier transforms infrared spectra (FT-IR): Perkin-Elmer FT-IR analyzer (Spectrum Bx) was used to exam the surface of the activated carbon and immobilized soybean hulls peroxidase on activated carbon. The samples were scanned in the spectral range of 4000-400 cm^{-1}. The FT-IR analysis was used for the investigation of the different surface functional groups. The samples were mixed with KBr of spectroscopic grade and made into pellets at high pressure. Fix the mass ratio of KBr and carbon each run, which helps to compare the relative amounts of each response.

Scanning electron microscopy (SEM): The surface morphology of free activated carbon MBC 271 and immobilized soybean hulls peroxidase on it are detected by SEM. Surface morphology was taken using a HITACHI S-5200 scanning electron microscope under condition, 5.0 kV, 5-50 k SE.

RESULTS AND DISCUSSION

Characterization and pre-treatment of activated carbon: The BET surface area and total pore volume were determined using nitrogen gas adsorption analyzer. The characterization

of MBC 271 such as surface area and pore volume is given in Table-2. Before immobilization, the MBC271 was crushed and sieved and then was washed well with deionized water several times to remove soluble inorganic compounds. And then the MBC271 were oven dried at 100 ºC overnight to drive off the moisture and stored in glass bottles for further immobilized experiment.

TABLE-2
CHARACTERIZATION OF MBC 271

No.	Parameters	MBC271
1	S_{BET} (m^2 g^{-1})	1187
2	S_{mic}/S_{BET} (%)	99.0
3	S_{mes}/S_{BET} (%)	1.0
4	Micropore volume, V_{micro} (cm^3 g^{-1})	0.3921
5	Mesopore volume, V_{meso} (cm^3 g^{-1})	0.0252
6	Total pore volume, V_{total} (cm^3 g^{-1})	0.4198

Effect of temperature and pH value on immobilization: Most of the enzymes will inactivate in the relatively high temperature conditions. Moreover, most enzymes have their optimum work temperature ranges. Based on soybean hulls peroxidase's special properties in thermodynamic performance, the temperature for immobilized soybean hulls peroxidase on MBC 271 was chosen to be pH 5 which is tested in previous experiments. And the relative immobilized enzyme activity was designed at 30, 40, 50 and 60 ºC. The results were shown at Fig. 2 which indicated that at 50 ºC and pH 5, soybean hulls peroxidase can be 100 % immobilized on MBC271. From above results, it can be concluded that the optimum condition was temperature 50 ºC and pH 5, MBC271 has highest immobilized yield.

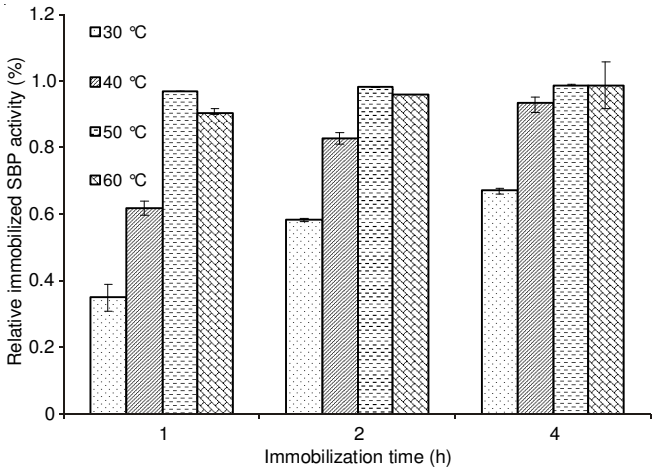

Fig. 2. Effect of immobilization time and temperature on relative immobilized soybean hulls peroxidase activity

Equilibrium isotherms of soybean hulls peroxidase on activated carbon: In this experiment, we study the adsorption on the conditions of pH 5 and 50 ºC which is the optimum condition of immobilization.

$$q_e = \frac{K_L C_e}{1 + bC_e}$$

$$q_e = K_F C_e^{1/n}$$

where q_e is the activated carbon concentration at equilibrium (U g^{-1}), C_e is soybean hulls peroxidase concentration at equilibrium (U mL^{-1}), K_L is the Langmuir isotherm constant (L g^{-1}), b is the Langmuir isotherm constant (L U^{-1}), K_F is the Freundlich constant (U g^{-1}) (L $U^{-1})^{1/n}$.

For this adsorption, we observed that the correlation coefficient R^2 of Langmuir and Freundlich are 0.9741 and 0.8984, respectively. Besides results of R^2, from the Fig. 3, we can conclude that MBC271 followed the Langmuir model of adsorption, it suggested that the occurrence of monolayer adsorption rather than multilayer adsorption.

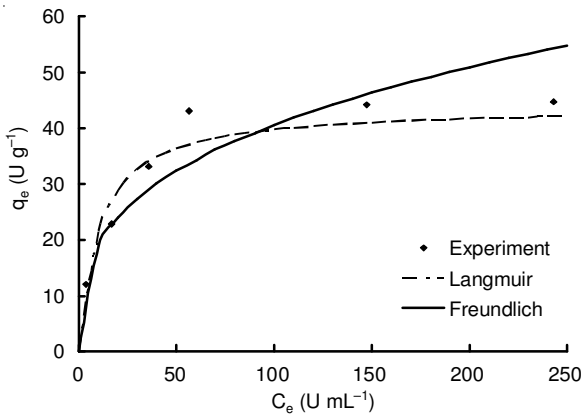

Fig. 3. Langmuir and Freundlich isotherm plots for the immobilization of soybean hulls peroxidase on MBC 271. (Conditiopns: pH 5 and temperature 50 ºC)

Fourier transforms infrared spectra: The FT-IR spectrum of free activated carbon and immobilized soybean hulls peroxidase on activated carbon are shown on Fig. 4. Both free activated carbon and immobilized activated carbon have a wide band at 3700-3200 cm^{-1}, centered at 3400 cm^{-1}, which can be due to O-H stretching or adsorption of water. This bond is attributed to the presence of strong hydrogen bonds. The bands near 1640 and 1570 cm^{-1} corresponds to the amino acid groups. This is concluded that the soybean hulls peroxidase enzyme immobilized onto the carbon matrices.

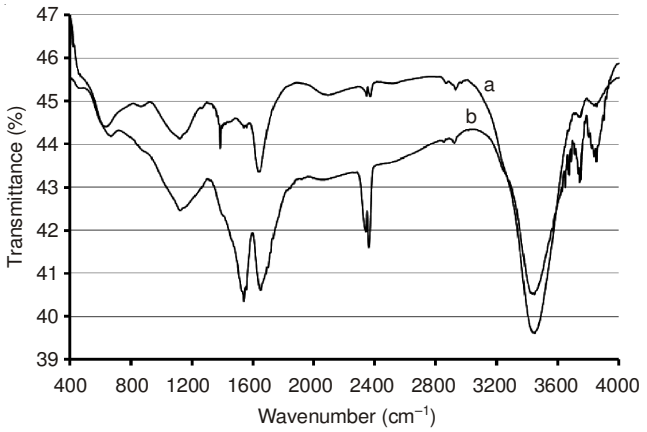

Fig. 4. FT-IR spectrum of (a) MBC271 and (b) Immobilized soybean hulls peroxidase on MBC271 samples

Scanning electron microscopy: The immobilization of soybean hulls peroxidase on MBC271 is evident from SEM microphotographs (Fig. 5). This SEM analysis of immobilized

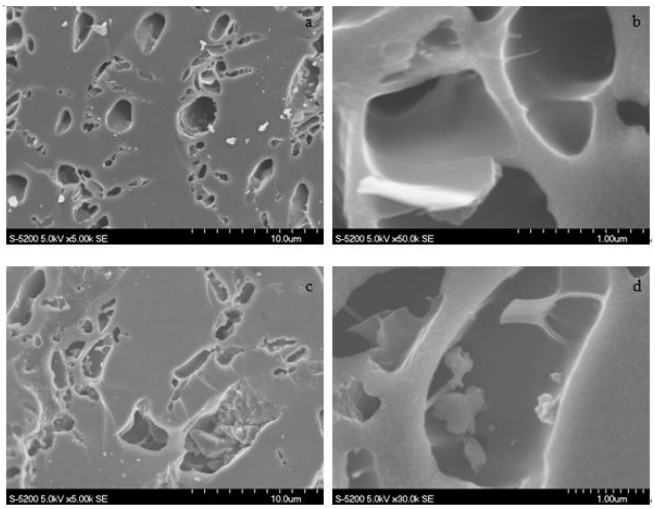

Fig. 5. Surface morphology of free and immobilized activated carbon by SEM analysis. (a) and (b): Free MBC 271 and (c) and (d): Immobilized soybean hulls peroxidase on MBC 271

enzyme on activated carbon confirmed the enzyme is already located at the pore surface area of activated carbon. It is seen in the micrograph that the enzymes are well bound to the wall of the pores in the carbon matrix after immobilization. From this result, we can see the enzymes are existed at the outer pore surface area of the activated carbon. It clearly demonstrates the presence of activated carbon highly porous nature and enzyme located on activated carbon. However, based on the size of soybean hulls peroxidase protein (106.45 × 106.45 × 105 Å) and activated carbon pore size, the soybean hulls peroxidase is difficult to enter into the deep pores of the carbon matrix and may block the entrance of the pore. In the mean time, we also found increased outer surface of activated carbon will increase the immobilized enzyme amount on activated carbon. The increase in stability and the high activity of enzyme immobilized activated carbon would be encouraging for its choice in biotechnology and enzymology applications. The key point of the immobilization is to increase the amount of immobilized enzyme on activated carbon.

Conclusion

The immobilization of soybean hulls peroxidase on MBC 271 was investigated in this study. In conclusion, crude soybean hulls peroxidase can be efficiently immobilized onto activated carbon at relative higher temperature (50 ºC) and lower pH value (pH 5). Immobilized soybean hulls peroxidase can be applied on treatment of high temperature and low pH value effluents directly. Equilibrium adsorption models were applied to quantify the effectiveness of immobilization. Adsorption of soybean hulls peroxidase on activated carbon followed the Langmuir model better than the Freundlich model. Fourier transform infrared spectra provide evidence of the soybean hulls peroxidase enzyme immobilized onto the carbon matrices. The morphology of the native activated carbon and the immobilized soybean hulls peroxidase was confirmed by FT-IR and SEM. The stability and activity of immobilized soybean hulls peroxidase encourages that can be widely applied to organic pollutants removal in biodegradation and reusage in water/wastewater treatment. This study is only focused on laboratory experiment. The key point of the immobilization is to increase the amount of immobilized enzyme on activated carbon and studies of reusage of immobilized soybean hulls peroxidase are needed in future experiment because it can be easily removed from soybean seeds and dissolve in water solution.

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