

Study of Horseradish Peroxidase Fixed on Mesoporous Materials as a Chemical Reaction Catalyst

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Abstract. Nanostructured mesoporous materials is a new type of porous materials, which has been widely used. It has excellent capability in enzymes immobilization, but modification on the chemical bonds of the enzyme reduce the enzymatic activity and rarely used in chemical reactions. The horseradish peroxidase was immobilized on the mesoporous materials with appropriate aperture and its activity and stability was evaluated when catalyzing the nitration reaction of amines and oxidation reaction of thiourea. The optimum mesoporous material to fix the horseradish peroxidase can be obtained by mixing polyoxyethylene - polyoxypropylene-pol, yoxyethylene(P123), 1,3,5-trimethylbenzene(TMB), and tetramethoxysilane (TMOS) at a ratio of 10:1:1, whose surface area and pore volume and pore diameter calculated by BET and BJH model were $402.903\text{m}^2/\text{g}$, $1.084\text{cm}^3/\text{g}$, $1.084\text{cm}^2/\text{g}$ respectively. The horseradish peroxidase, immobilized on the mesoporous materials, was applied for catalyzing the nitration reaction of anilines and oxidation reaction of thiourea, produced a high product yield and can be recycled. Thus, it is a strong candidate as a catalysts for oxidation reactions, to be produced at industrial scale, due to its high efficiency and low cost.

1. Introduction

In recent years, nanostructured mesoporous materials, a new type of porous materials, which have adjustable aperture, large pore volume and pore diameter, has been widely used [1,2]. Mesoporous materials is one of the excellent materials for enzymes immobilization because their pore diameter is applicable [3, 4]. The enzymes immobilized on the mesoporous material have displayed high catalysis activity, because their stability is improved largely. Immobilized enzymes are extremely economical and storable, which allows their applications in other industrial production [5]. It is worth to note that no modification on the chemical bonds of the enzyme has been introduced by our system, therefore, it doesn't affect the enzymatic activity.

Horseradish peroxidase is a type of oxidoreductase [6], which can catalyze a variety of organic hydrogen donor and inorganic hydrogen-donor in nitration and polymerization and the halogenation reaction, ect [7,8]. In general industrial production, nitration of anilines and oxidation of thiourea use inorganic catalysts [9]. However, the conventional method has high requirement, low efficiency, and causes environmental pollution. Therefore, in this paper, we used the horseradish peroxidase immobilized on the mesoporous materials with appropriate aperture to catalyze nitration reaction of amines and oxidation reaction of thiourea [10]. By establishing the suitable reaction conditions and



calculating the reaction yield, our study demonstrated that immobilized horseradish peroxidase is a potential catalyst. The method of immobilizing enzymes also can be applied e in other industrial production.

2. Results and Discussion

2.1. Synthesis and Characterization of Mesoporous Materials

Nanometer mesoporous materials with three different apertures were synthesized as following procedures. The reactant P123 as a template and 1, 3, 5-trimethylbenzene (TMB) as an expanding agent were reacted with Tetramethoxysilane (TMOS) as a precursor to synthesize a series of mesoporous materials. All reactions took place in acidic (HCl) conditions to hydrolyze. Combination of P123 and neutral inorganic silicon materials were achieved by hydrogen bonding. The formation of hexagonal skeleton attributed to the silanol hydrolysis and condensation. The amount of TMB entered in surfactant micelles determines the degree of pore enlargement. When the amount of the surfactant remains the same, the density of the polymer molecules micelles arrangement would decrease and pore size would increase with the increase of the TMB concentration, that make it easier for TMB to enter the pore. However, when the concentration of TMB exceeded to a certain range, the hydrophilic and hydrophobic effect of micelle reached equilibrium, resulting in difficulty for TMB to enter the interior of the micelle, and the pore size will be remained even when the amount of TMB increased. In reverse, an excessive amount of TMB caused lower solubility of surfactant that was difficult to combine with tetramethoxysilane, so it is not conducive to the synthesis of mesoporous materials.

We chose the optimum aperture mesoporous material to immobilize horseradish peroxidase from three products. Detection of fixed enzymes showed the optimal ratio of templating agent P123 and expanding agent TMB is 10: 1. The mesoporous materials and immobilized enzyme of mesoporous materials were characterized by Nitrogen adsorption - desorption isotherms (Figure 1, 2).

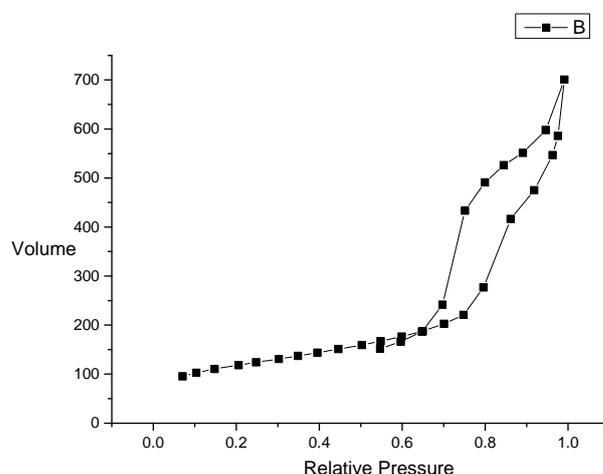


Figure 1. Mesoporous material's nitrogen adsorption - desorption isotherms.

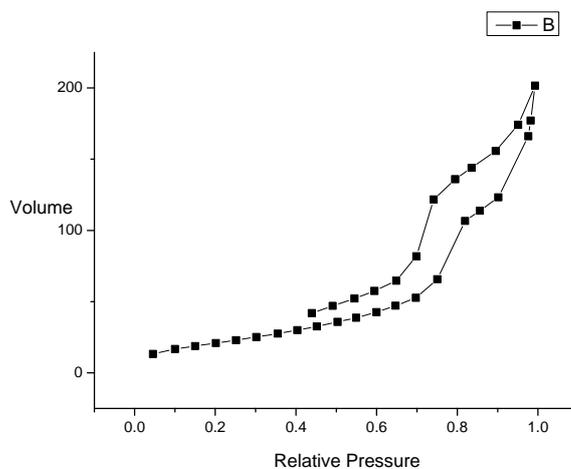


Figure 2. Immobilized enzyme of mesoporous material's nitrogen adsorption - desorption isotherms.

The above figure indicates, when the pressure is relatively low, with the value of partial pressure increasing, the amount of adsorbed nitrogen gradually increase since the hole surface monolayer and multilayer adsorption of nitrogen increase. When the value of the relative pressure is than 0.5, the amount of nitrogen adsorption suddenly increase, so the curve trend became steeper which resulted from the effect of capillary condensation. When the value of the relative pressure is more than 0.95, the curve became much steeper which was due to form nitrogen accumulation in the large particles of mesoporous material. The two curves of the detected sample showed a typical of H1 hysteresis loop belong to Class IV adsorption isotherm.

When P123: TMB = 10: 1, the specific surface area, the pore volume and the pore size of nano-mesoporous material is 402.903m² / g, 1.084cm³ / g, and 10.76nm, respectively, based on BET and BJH model.

2.2. Detection of the activity of the immobilized horseradish peroxidases

After measuring standard density of 6 groups horseradish peroxidase for 30 times at the 402nm, we obtained the standard curve of horseradish peroxidase solution absorbance shown in Figure 3.

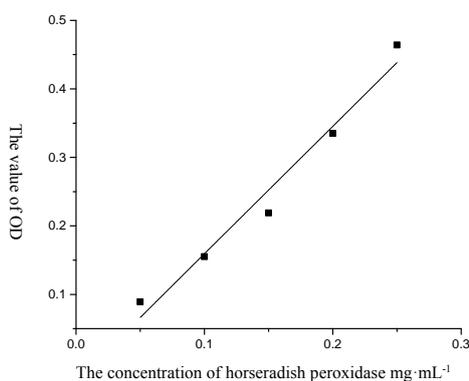


Figure 3. The standard curve of horseradish peroxidase.

Our calculation demonstrated that the highest amount of remaining non-immobilized horseradish peroxidase in the supernatant was 2.23mg and immobilized horseradish peroxidase was 2.73mg (the maximum %CV is 54.6%) using the formula obtained from the standard curve ($y = 1.784x -$

0.0127). The result showed that when the ratio of P123 and TMB is 10: 1, the pore size of the mesoporous material is suitable for fixing horseradish peroxidase.

Worthington method was utilized to assay the enzymatic activity. The absorbance of reactant, hydrogen peroxide, of horseradish peroxidase (in 410nm at 25 °C and pH = 7.0) was detected every other 5 minutes, and the vitality of the immobilized horseradish peroxidase was 136.8U / mg.

2.3. Nitrification of anilines catalyzed by immobilized horseradish peroxidase

(1) Nitration reaction of anilines

The result of nitration reaction of anilines by HPLC is shown in Figure 4, 5, 6. The figure 4 is the HPLC curve of nitration reaction of aniline. Peak 1 showed the same retention time with pure standard of aniline indicating it is unreacted aniline. The retention time of peak 2 and peak 3 were consistent with the standard of o-nitroaniline and p-nitroaniline respectively. The remaining peak is properly caused by polymer products with high molecular weight. The figure 5 showed the nitration reaction of 4-methyl aniline. The peak 1 is unreacted 4-methyl aniline, and the peak 2 is the product 4- methyl -2-nitroaniline. Figure 6 is the nitration reaction of 2-methyl aniline. The peak 1 is the product 2- methyl -4- nitroaniline, and the peak 2 is unreacted 2-methyl aniline.

We determined that the nitration reaction of anilines can occur in the presence of horseradish peroxidase, H_2O_2 , NO_2^- . If there are any lacks of any of the components, the nitration reaction would not react. To ensure the reaction was complete thoroughly, we controlled the time in about 10 minutes even it was stopped in 8 minutes. The reaction was added to a phosphate buffer to ensure horseradish peroxidase catalyzed reaction at the optimum pH environmental conditions. We also investigated the influence of pH in the reaction. The yield of the product of nitration reaction maximized at pH 7, however when pH is above 9 or below 3, the reaction substantially does not occur because of inactivation of enzymes. Moreover, we evaluated whether the reaction product yield is associated with the concentration of H_2O_2 . The result showed the accumulation of product increased when the concentration of H_2O_2 is increased, but when the DOX of H_2O_2 was added at a high concentration, the products of the nitration reaction did not significantly increase. In the experiment, we used the H_2O_2 at a concentration of 2 fold higher than anilines in order to ensure the concentration of H_2O_2 does not affect the reaction. The concentration of nitrite was 12-fold higher than anilines to make the reaction process smoothly. If the concentration of nitrite is too high, it will cause the inactivation of the enzyme that may not conducive to the reaction.

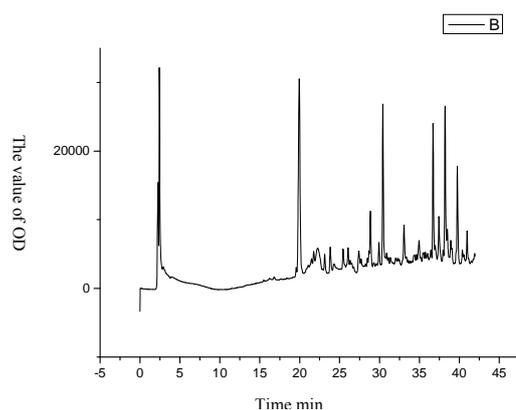


Figure 4. HPLC spectrum of Nitration reaction of aniline.

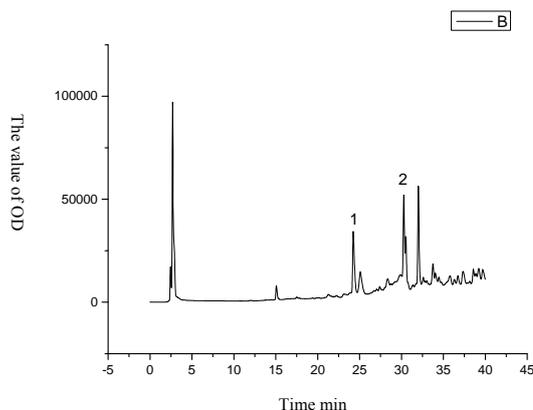


Figure 5. HPLC spectrum of Nitration reaction of P - toluidime.

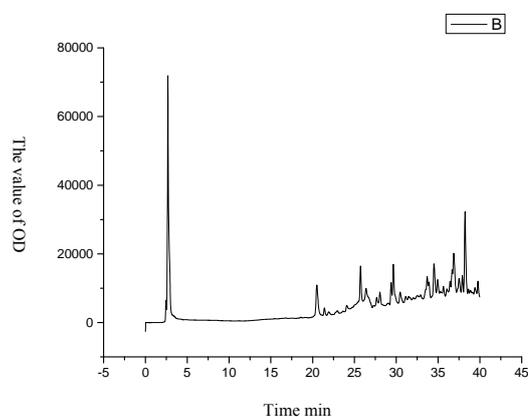


Figure 6. HPLC spectrum of Nitration reaction of O - toluidime.

(2) Oxidation of thiourea

The figure 7 showed that we obtained pure thiourea dioxide from oxidation of thiourea catalyzed by immobilized horseradish peroxidase. The pH of the oxidation is 6.2 to maintain the activity of horseradish peroxidase, but sulfur dioxide urea was easily decomposed to sulfite when pH is higher than 6. Sulfite has strong reduction capability that can react with H_2O_2 to increase adverse reaction. We obtained crystalline compound from the oxidation of thiourea and analysis by infrared spectroscopy showed that crystalline compound is 10.9 g pure thiourea dioxide, so the reaction yield was 50%. The yield was lower in that the pH of the reaction environment was not optimal. Further improvements of the yield of reaction could be achieved by adjusting pH conditions.

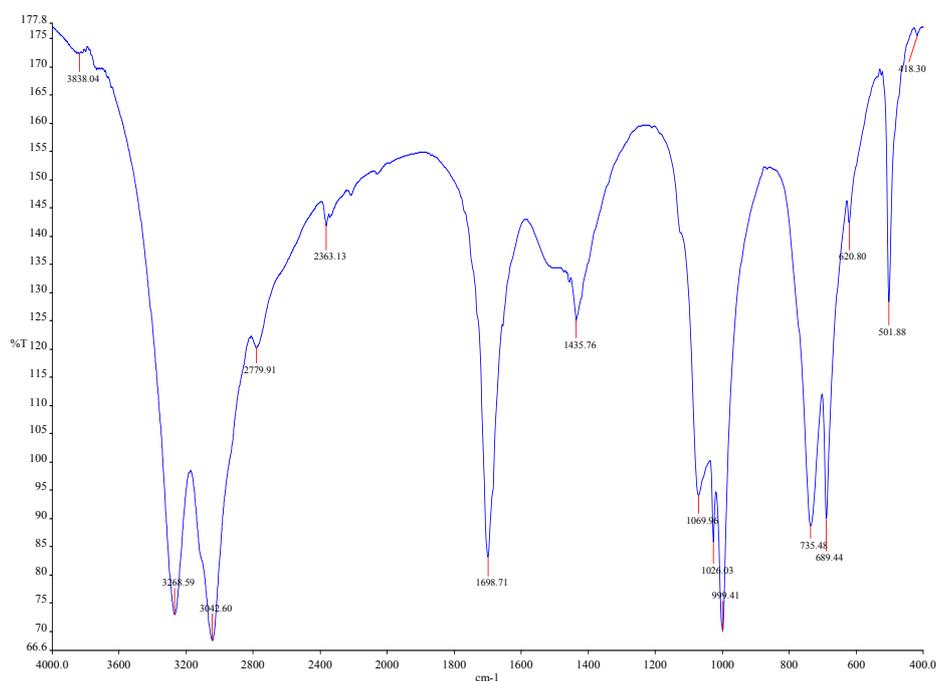


Figure 7. The FTIR of product of Oxidation of thiourea.

3. Experimental Section

(1) Preparation of SBA-15 Mesoporous Silica

2 g of copolymer poly (ethylene glycol)-block-poly (propylene glycol)- block-poly (ethylene glycol) (EO20PO70EO20, Pluronic P123 with the molecular weight of 5,800) was completely dissolved in 75 mL of 1.6 M HCl at 35 °C by stirring, 0.2 g of 1,3,5-Trimethylbenzene (TMB) as a swelling agent was then added by stirring for 1 h, followed by addition of 4.25 g of tetraethyl orthosilicate (TEOS) as the silica source. The mixture was further stirred for 24 h at 35 °C and aged without stirring for 48 h at 100 °C, while the solids were recovered by filtration and air-dried at room temperature. Finally, the product was calcined under 600 °C for 2 h to remove remaining triblock copolymer.

(2) Characterizations of SBA-15 Mesoporous Silica

The X-ray diffraction (XRD) measurements of calcined SBA-15 were performed on a X'Pert MPD pro diffractometer (PANalytical, ALMELO, The Netherlands) using CuK α radiation ($\lambda = 1.5418 \text{ \AA}$) in the range of $0.3 \sim 3^\circ 2\theta$ with step of 0.03° per second. The specific surface area, total pore volume, and pore size distribution of the SBA-15 were estimated by nitrogen adsorption and desorption under 77K with a Micromeritics ASAP 2020 instrument (Norcross, GA, USA), and BJH pore diameter was obtained based on the Barrett-Joyner-Halenda (BJH) calculation. The mesoporous structure was imaged by a JEOL transmission electron microscope (TEM, JEM-2010 at 200 kv, Tokyo, Japan) and by a JEOL field emission scanning electron microscope (SEM, JSM-7000F at 15 kV, Tokyo, Japan).

(3) Activity

First, 5mg horseradish peroxidase immobilized on 20mg mesoporous material and 8mL 0.1 M phosphate buffer (pH 6.2) were mixed on ice bath for 6 hours, then rotated at 3000rpm for 15 minutes. After the supernatant was separated from the precipitate, it was washed and precipitants three times with buffer until no horseradish peroxidase was found in the supernatant. The activity of immobilized enzyme was measured by ultraviolet spectrophotometric using WorthingTon method.

(4) General Procedure for the Preparation of Compounds

The immobilized enzyme was diluted in buffer solution, fixed on mesoporous materials. The nitration reaction was in anaerobic environment. Added 10mL sodium nitrite (60mmol - L⁻¹), and 10mL hydrogen peroxide, 10mL amine compounds (5mmol L⁻¹) and react for 10 minutes. Subsequently, the oxidation of thiourea need 100mL buffer solution, 15.2g Thiourea, and 46mLH₂O₂,

and stirred for 1.5 hours after the end of the reaction, then precipitate for 1 hour to complete the crystalize.

4. Conclusion

We obtained the most suitable nano-mesoporous materials for fixing horseradish peroxidase by mixing P123 and TMB at a ratio of 10:1. The activity of enzyme immobilized on nano-mesoporous materials activity was 136.8U / mg, which was reduced slightly when compared with free enzyme, but it does not affect the catalytic reaction. In nitration reaction of anilines, we found the reaction proceed rapidly when catalyzed by immobilized horseradish peroxidase and took about 10 minutes. The concentration of hydrogen peroxide is 2-fold higher than anilines, while the concentration of nitrite is maintained at less than 10-fold higher than anilines. Oxidation of thiourea catalyzed by immobilized horseradish peroxide smoothly occurred and reduced the amount of hydrogen peroxide affecting the yield of the reaction. Without adding additives such as ammonium acetate largely reduced production cost. Further improvements of the yield of reaction could be achieved by adjusting pH conditions.

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