Catalytic degradation of 2-chlorophenol with laccase in Triton X-100 reverse micelles

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Abstract
The degradation of 2-chlorophenol was catalyzed with laccase in the reverse micelles system by using Triton X-100. The effects of water content, pH, temperature, 2,2-azino-bis-(3-ethylbenzothiazo line-6-sulfonic acid) (ABTS) mediator and type of surfactant on degradation rates were discussed. Results demonstrated that optimum water content (w_0 = [H_2O]/[surfactant]) of the reaction was 20, the optimum pH was 4.0, the optimum reaction temperature was 45℃ and the addition of ABTS greatly favored degradation. Under these optimal conditions, the degradation rate reached a maximum of 33.27%. Additionally, the degradation rates after 10 h with the use of different surfactants cetyl trimethyl ammonium bromide (CTAB), sodium bis(2-ethylhexyl) sulfosuccinate (AOT), and Triton X-100 were 3.4%, 15.2%, and 33.37%, respectively, thereby indicating that the reverse micelles of the nonionic surface active agent Triton X-100 can more effectively catalyze the degradation of 2-chlorophenol.

Keywords: reverse micelles, laccase, 2-chlorophenol, Triton X-100.

Introduction

Chlorophenols are important pollutants in the environment with carcinogenicity, teratogenicity, mutagenicity, widely present in the water and soil environment[1]. Research have shown that chlorophenols mostly have strong bioaccumulation. Some have significant effects of endocrine disruption, can interfere normal secretion and role of human thyroid hormone and adrenaline. Its main source is pesticides, disinfectants, industrial emissions of wastewater, etc.[2] Although chlorophenol is a refractory and toxic organic substance, it is also an important raw material in industry.[3] Consequently, the resulting contamination has a significant impact. Biodegradation is an important approach for chlorophenols degradation. [4] The study found that laccase produced from white-rot fungi can degrade a variety of chlorophenols[5].

Laccase is a single-electron polyphenol oxidoreductase.[6] Its advantage is to direct catalytic oxidation of chlorophenols and other organic pollutants with the absence of H_2O_2 and other secondary metabolites. [7] However, laccase is hydrophilic and has a low redox potential and poor stability, thereby limiting its application to the degradation of hydrophobic organic compounds.[8] Therefore, the maintenance and improvement of enzyme activity has become the focus of the current research.[9] In order to achieve this goal, the molecular engineering will be used to modify the enzyme, and the micro environment of the enzyme reaction can also be changed, the latter is the enzyme catalytic medium engineering. [10] The research of media engineering has gone through the process from water to organic solvent and finally to reverse micelles. [11] While studies show that organic solvent maybe inhibit the catalytic performance of enzyme. [12] Because the reverse micelle system can simulate the natural environment of the enzyme, most of the enzyme can maintain the catalytic activity and stability [13].

Usually, aromatic environmental pollutants have poor water solubility, such as: PAHs, bisphenol A and chlorophenol. The solubility of them in the reaction system can be improved by using reverse micelles, which can improve the efficiency of the degradation and transformation. Meanwhile, the applicability of laccase to chlorophenol degradation can be improved via it [14]. It's a thermodynamically stable, transparent or translucent, macroscopically uniform, and microscopically heterogeneous system that is formed through the dissolution of a surfactant in an organic solvent. [15] Reverse micelles can simulate the natural environment of enzymes. Because of the shielding effect of surfactant micelles, the coated enzyme in the reverse micelles will not be exposed to organic solvents, thereby most of enzymes can retain stability and catalytic activity in reverse micelles.[16]

Consequently, reverse micelles can enhance the dissolution of chlorophenol in a reaction system and thus can increase the contact between chlorophenol and laccase. As a result, the degradation rate can be increased. As shown in figure 1, the reverse-micelle system contains a surfactant and organic solvent, which can favor desorption of laccase and hydrophobic organic pollutants. These desorbed substances will be stored in the water core. Thus, the stability and catalytic activity of laccase will be improved, and space will be formed for the oxidation reaction catalyzed by laccase.[17,18]

In this paper, the catalytic degradation of 2-chlorophenol, a representative pollutant, with laccase extracted from *Trametes versicolor* was performed in a reverse-micelle system fabricated with nonionic surface active agent Triton X-100. The effects of w_0, pH, reaction temperature and addition of ABTS on degradation rates were discussed. Meanwhile, the influence of surfactant type on degradation was studied by using cationic surface active agent CTAB, anionic surface active agent AOT and nonionic surface

active agent Triton X-100. Finally, the degradation pathway of pollutants was discussed. In order to understand the degradation of 2-chlorophenol with laccase in reverse micelles system and further explore the application of enzyme in reverse micelles system, it is necessary to provide the theory foundation for clarifying the mechanism of 2-chlorophenol hydrolysis in reverse micelles system.

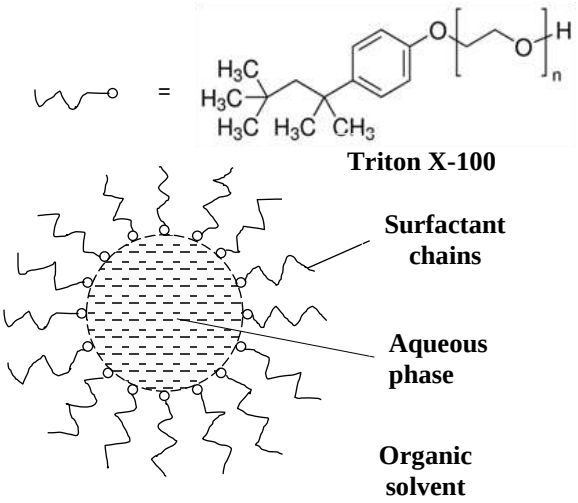

Fig. 1: Diagrammatic sketch of Triton X-100 reverse micelles

Experimental

Materials and Instruments: Experimental drugs: laccase (*Trametes versicolor*, Sigma Co., Ltd.), ABTS(Sigma Co., Ltd.), 2-chlorophenol (Sinopharm Co., Ltd.), 2-chlorophenol (AR, Sinopharm Co., Ltd.), Triton X-100 (Sinopharm Co., Ltd.), cetyl trimethyl ammonium bromide (CTAB, Sinopharm Co., Ltd.), bis-(2-ethyl hexyl), sodium bis(2-ethylhexyl)sulfosuccinate (AOT, Sigma Co., Ltd.), iso-octane (AR, Sinopharm Co., Ltd.), n-hexyl alcohol (AR, Sinopharm Co., Ltd.), acetic acid (AR, Sinopharm Co., Ltd.), and sodium acetate (AR, Sinopharm Co., Ltd.). Additionally, an UltiMate-3000 high-performance liquid chromatograph (HPLC, Dionex Co., Ltd.), a WHY-2 thermostatic water-bath, ultrasonic-cleaner, and a PHS-2F pH-meter (LeiCi Co., Ltd., Shanghai) were also employed.

Fig. 2: The left is laccase-containing reversed micelles, and the right is laccase / ABTS mediator-containing reversed micelles

Degradation of pollutants by laccase in reverse micelles: A certain amount of Triton X-100 was added to a mixture of iso-octane/n-hexyl alcohol (1:2; v:v). The Triton X-100 concentration in the mixture was 0.05 M. Next, an amount of 2-chlorophenol was dissolved in the iso-octane/n-hexanol/Triton X-100 mixture (a reverse-micelle system). Last, a certain volume of solution containing laccase and ABTS mediator was added to the mixture, wherein the laccase content was 0.3 U/mL based on the optimal water content, as shown in figure 2. The oxidation reaction was conducted in the thermostatic water-bath–ultrasonic-cleaner for 10 h. Changes in the 2-chlorophenol content were determined by HPLC.

HPLC analysis: The 2-chlorophenol concentration was gauged by HPLC. The measurement conditions described below. Thermo SCIENTIFIC C18 reverse column (4.6*250 mm) used; a mobile phase of 75 wt % methanol/25 wt %; flow velocity is 1.0 mL/min; a detection wavelength of 285 nm and the sample size is 20μL.

Results and Discussion

Effect of water content on the degradation of 2-chlorophenol: Water content w_0 (w_0= [H_2O]/[surfactant]) is an important factor in the catalysis of laccase in reverse micelles systems. [19] In reverse micellar enzyme catalytic reaction, the presence of a moderate amount of water is the key factor to maintain the catalytic activity and stability of the enzyme. Water content is a good parameter to characterize the reverse micelles, which has a linear relationship with the size of micelle formation. The radius of reverse micelles increases with the increase of w_0. Therefore, it is an important index for the evaluation of reverse micelles. At 0.05 M Triton X-100, 0.3 U/mL laccase, 4.0 pH, 45℃ and a certain amount of ABTS mediator, the degradation rates of 2-chlorophenol at a water content of 10 to 40 were separately determined (Figure 3). The pH was regulated with a 0.1 M NaAc-HAc buffer solution.

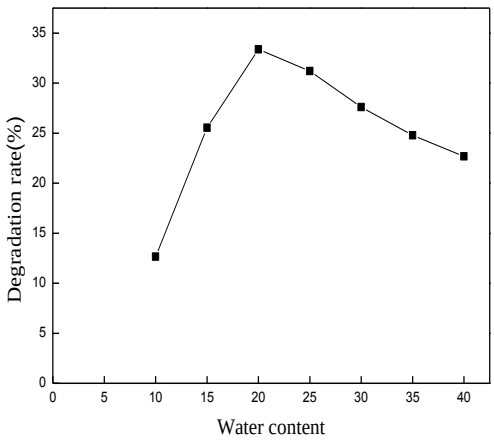

Fig. 3: Effect of water content on the degradation of 2-chlorophenol

As illustrated in fig.3., the optimum water content of the reaction was 20. The water in reverse-micelle systems consists of free and combined water, wherein the latter is formed by the core water combining with the surfactant. Thus, at a low w_0 level, most of the water in the system is combined water, thereby resulting in insufficient core water and a shrunken water core. Accordingly, the space for the conformational change of the enzyme was condensed, rendering it difficult to be converted into a more active conformation. Thus, the enzyme activity was inhibited. On the other hand, the free water content was also low at the low water content; therefore, the molecules of the enzyme could not be effectively dissolved, resulting in the exposure of laccase to the organic environment and a loss of activity. In contrast, when the water content was exceedingly high, the amount of free water in the water core was increased, which improved the immobility of the enzyme. The more active conformation was loosened due to the disturbance of the immobility, thus resulting in decreased laccase activity. [20,21] So water content w_0 was set to 20 in the following experiment.

Effect of pH on the degradation of 2-chlorophenol: The pH of the water cores amid the reverse micelles was almost the same as that of the NaAc-HAc buffer solution, which affected the type of charges over an enzyme, active conformation, and solubility of enzyme molecules. In addition, the intermolecular interactions between the enzymes and the surfactant, as well as the catalytic performance of laccase, are also affected by the pH. [22] At 0.05 M Triton X-100, 0.3 U/mL laccase, w_0=20, 45℃ and a certain amount of ABTS mediator, the degradation rates of 2-chlorophenol catalyzed with laccase at different pH values (3.0 to 6.0) are depicted in figure 4.

At a pH of 3.0 to 4.0, the rates increased with the raise in pH. At pH > 4.0, the degradation rate was negatively correlated with the pH. Thus, the optimum pH for the degradation was 4.0, which is consistent with that in an aqueous system.[23] The pH of the water cores amid the system was almost not affected by the surfactant probably because Triton X-100 (nonionic surfactant) was hardly ionized in the water. Laccase molecules could present and maintain its natural conformation similar structure when the pH of water cores was 4.0, so as to play the best catalytic activity. So, the pH was set to 4.0 in the following experiment.

Effect of temperature on the degradation of 2-chlorophenol: Temperature is an important factor affecting the reaction rate. [24] It can also affect the activity of laccase. So it is necessary to explore the effect of temperature (30~60°C) at 0.05 M Triton X-100, 0.3 U/mL laccase, w_0=20, 4.0 pH and a certain amount of ABTS mediator. The temperature is controlled by thermostatic water-bath.

As shown in Figure 5, the optimum reaction temperature was 45°C. The optimum temperature of laccase from *Trametes versicolor* is 40°C. [25] Reaction temperature on the enzyme activity has two aspects: on the one hand, the increase of temperature accelerated the rate of enzyme catalyzed reaction which is the same as the general chemical reaction; on the other hand, the increase of temperature made the enzyme protein gradually degenerated. We found that the optimum reaction temperature of Triton X-100 reverse micelles system was slightly higher than the optimum temperature of laccase. At reaction temperature < 45°C, the degradation rate was positive correlation with the temperature. As the temperature increases, the spatial structure of laccase will change, resulting in the rapid inactivation.[26] So the temperature was set to 45°C in the following experiment.

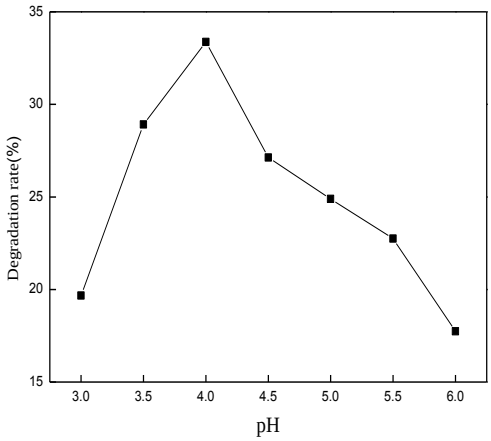

Fig. 4: Effect of pH on the degradation of 2-chlorophenol

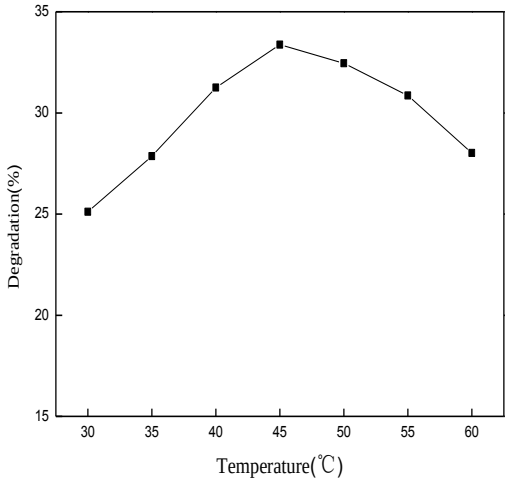

Fig. 5: Effect of temperature on the degradation of 2-chlorophenol

Effect of ABTS mediator on the degradation of 2-chlorophenol: The catalytic oxidation efficiency of laccase is greatly improved by the help of some small molecular mediators. ABTS is this type of common medium. Adding ABTS mediator to the reverse-micelle system under the optimized conditions: w_0=20, pH=4.0 and T=45℃. On the

other hand, a control group was prepared by substituting equiv. molar water for the ABTS.

In Figure 6, the degradation rates of 2-chlorophenol were low when not adding ABTS. After 10 h, the rate was only 6.1%. In contrast, the rate increased significantly with the addition of ABTS. The degradation rate after 10 h reached 33.37%. In the process of degradation, the color of the laccase /ABTS mediator system gradually changed from pale blue to pink in reverse micelle, and the color of the laccase system almost did not change. Catalytic oxidation of laccase mainly includes the following aspects: electron transport in the enzyme molecules, reverse action of product and reduction of enzyme by oxygen molecule. There into, electron transport in the enzyme molecules is a key factor influencing the degradation rate. ABTS is a small molecular mediator, which is mediated by electron transport. [27-29]

It can be used as the carrier of electron transfer, so the electron transfer rate can be improved. Thus, the reaction rate of the system can be increased. The ABTS elevated the low redox potential of laccase, and the small ABTS molecules eliminated the steric hindrance between 2-chlorophenol and the laccase macromolecules. Therefore, the degradation of 2-chlorophenol was favored.

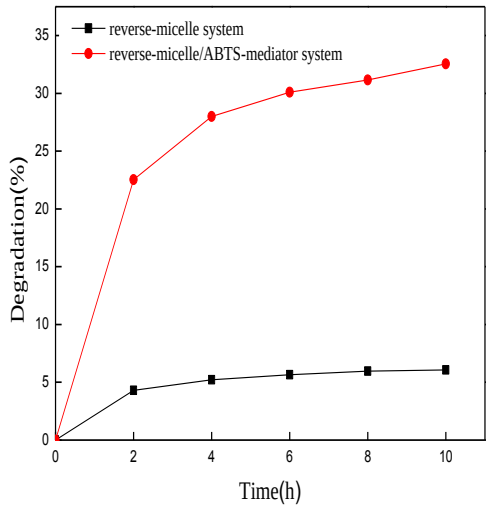

Fig. 6: Effect of ABTS mediator on the degradation of 2-chlorophenol

Effect of various surfactants on the degradation of 2-chlorophenol: Surface active agent refers to a substance which can significantly reduce the surface tension of a solvent at a very low concentration. The cationic surface active agent CTAB, anionic surface active agent AOT, and nonionic surface active agent Triton X-100 were separately applied to build different reverse micelles systems for the degradation of 10 mg/L 2-chlorophenol. As illustrated in Figure 7, after 10 h, the degradation rates in the three systems were 3.4%, 15.2%, and 33.37%, respectively. The positive effects of the three surfactants are ranked as follows: Triton X-100> AOT> CTAB, which demonstrates that the type of surfactant also has an impact on the catalytic performance of laccase. [30]

The value of laccase isoelectric point (pI) is about 3.5 or less and the pH value of reaction system is 4.5, [31] so the charge of laccase surface is negative. Probably, the catalytic activity of laccase was changed by the strong electrostatic attraction between the negative charge of laccase and the positive charge of CTAB. Even resulting some laccases degeneration, degradation rate of 2-chlorophenol is low. In the AOT reverse micelles, between the negative charge of the anionic surfactant and laccase shows strong electrostatic repulsion. However, AOT reverse micelles degradated contaminants better than CTAB, this shows that electrostatic repulsion has little impact on the catalytic performance of laccase compared to electrostatic attraction. While nonionic surface active agent Triton X-100 without charge showed best degradation. Therefore, it is reasonable to infer that electrostatic effect has a certain inhibition on the degradation reaction of laccase. What's more, the differences may be a result from the ionization of an ionic surfactant, which suppresses the catalytic activity of laccase.

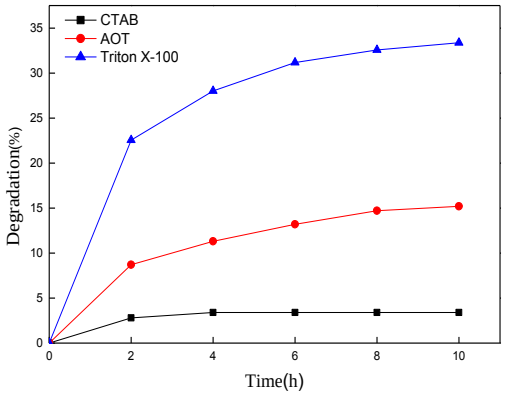

Fig. 7: Effect of different surfactants

The pathways of 2-chlorophenol oxidation degradation: Chlorophenol has strong toxicity and stability because of its aromatic ring structure and chloro atom. [32] The π electrons of benzene ring and P electrons of chlorine atoms form stable conjugated system. Meanwhile, the presence of chlorine atoms inhibits the enzyme activity, thereby increasing their anti-biodegradation ability. [33] Laccase has abroad substate specificity [34] and is widely applied to degrade various kinds of contaminations.

The substrate of laccase has a wide range of properties, but it is specific to oxygen. The enzyme catalytic process [35] generally includes: enzyme molecules acting on substrates, electron transfer in the enzyme molecules, reduction of enzyme molecules by oxygen molecules. In the case of phenolic compounds, the laccase utilizes oxygen molecules as electron acceptors to remove. From the substrate oxidation, the removal of a hydrogen atom from the hydroxyl in the position of o- and p- of polyphenolic

compound by single electron extraction method, the formation of free radicals.

The free radicals are unstable and undergo further polymerization or depolymerization reactions, causing a series of non-enzymatic reactions, such as rearrangement, dimerization, cleavage of side chains and aromatic rings or formation of quinones. In the presence of oxygen, the reduced laccase is oxidized and the oxygen molecules are reduced to water. [36] Accordingly, degradation experiments of 2-chlorophenol were investigated by laccase in reverse micelles. The color of the system changed from pale blue to pink with the process of reaction in reverse micelles containing laccase and ABTS mediator. Pale blue is the color of mixing laccase and ABTS. Usually, the phenol in the air for a long time will become pink, is due to the formation of benzoquinone. [37,38] So pink may mean the formation of benzoquinone. As shown in Figure 8, the dechlorination reaction of 2-chlorophenol gradually happened in catalytic oxidation of laccase and generate benzoquinone and other intermediate products. [39,40]

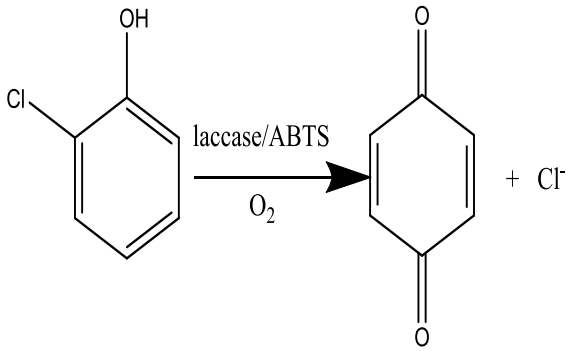

Fig. 8: The pathways of 2-chlorophenol oxidation degradation

Conclusion

The degradation of 2-chlorophenol was catalyzed with laccase in the reverse micelles system by using a nonionic surfactant Triton X-100. Optimum conditions for highest degradation rate (33.37%) were the following: pH=4.0, T=45℃ and water content of 20. Moreover, the addition of ABTS had a notable positive effect on the catalytic performance of laccase. After 10 hours, the rates with the use of CTAB, AOT, and Triton X-100 were 3.4%, 15.2%, and 33.37%, respectively, thereby indicating that the degradation of 2-chlorophenol with laccase was more facilitated in reverse micelles. While the degradation rate was not high, the experiment furnished reference for the utilization of Triton X-100 reverse. Therefore, depth study of the reaction mechanism and improving the degradation efficiency is necessary.

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