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Determination Method of Manganese Peroxidase During Straw Degradation

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Abstract

With the development of research on the utilization of straw as a biomass resource by scientific researchers, there are more and more reports on the analysis of manganese peroxidase in its biodegradation process, which plays an important role in the process of straw biodegradation. There are many methods of analysis and detection. However, the principle of these methods and the accuracy, sensitivity, stability and operational practicality of detection are different, which affects the application range of detection methods. In this paper, different experimental methods for the detection of manganese peroxidase were followed up in the literature. Sodium lactate reaction system, tartaric acid sodium tartrate reaction system, malonic acid buffer reaction system, phenol red method, 2, 6-Dimethoxyphenol (2, 6-DMP) method, guaiacol method and 2, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) method were compared and analyzed. It can provide reference for researchers to accurately and effectively detect the activity of manganese peroxidase in different situations.

Keywords: straw; manganese peroxidase; test method

Introduction

As natural lignocellulose, straw is composed of cellulose, hemicellulose and lignin.In the straw, lignin has a network structure, which is used as a support framework to surround and reinforce cellulose and hemicellulose [1]. As lignin is wrapped with cellulose as a physical barrier, its degradation must precede cellulose decomposition. In the biodegradation process of cellulose, it is the key to first break the lignin shell [2]. The white rot fungi of basidiomycete are the most effective degraders of lignin, and the manganese peroxidase produced by them plays an important role in the degradation of lignin. Manganese peroxidase (MnP) was first obtained from lignin lysis medium of Phanerochaete Chrysosporium by [3]. In many fungi, MnP is the key enzyme for the initial degradation of lignin, because MnP can produce Mn3+ in a strong oxidation state. Mn3+ acts as a diffusible redox medium to cleave the aromatic ring in lignin polymers. Then, under the cooperative action of other enzymes, which eventually leads to the breakdown of the macromolecule. MnP is essentially a pentameric protein containing Fe3+ and a highly helical structure containing one auxiliary helix and ten main helices. In the crystal structure of MnP, there are a large number of acidic amino acids and about 17% neutral sugars. In the presence of Mn2+ and H2O2, MnP can oxidize and decompose aromatic ring polymers 4-10]. Because MnP has the unique ability to oxidize and degrade aromatic compounds, it has been studied and applied in many aspects [4]. The catalytic cycle of MnP is shown in Figure 1. Due to the strong electronegativity of the oxygen atom in H2O2 molecule, the double electrons on the heme in MnP molecule first migrate to the oxygen-oxygen bond, and MnP is oxidized to the active oxygen complex tetravalent iron porphyrin complex (MnP compound 1). Then, Mn2+ loses an electron and MnP compound 1 is reduced to MnP compound 2. Finally, MnP compound 2 is reduced to its initial state through single-electron transfer. When Mn2+ is oxidized to Mn3+, Mn3+ and organic acid chelate to form a high redox potential chelate, which can react with a variety of substrates in single-electron oxidation. For example, phenolic substrate and aromatic amine substrate can be oxidized and dehydrogenated to form free radical products, where benzene ring can be oxidized to aromatic ring positive ion free radicals [10]. These free radical products can react with oxygen molecules to form peroxides, thus cutting off chemical bonds with high bond energy in lignin. Including Cα-Cβ Key fracture, Cα Oxidation and aryl-alkyl C-C bond breaking [10]. MnP can also oxidize non-phenolic structures in lignin molecules under certain conditions β-O-4 key, C-C key and β-Aryl ether bonds, etc., to produce small molecular substances such as vanillin and protocatechuic acid [9,11]. Over the years, researchers have explored and established several methods for the determination of manganese peroxidase activity in practice. This paper will describe and analyze the research work in this field.

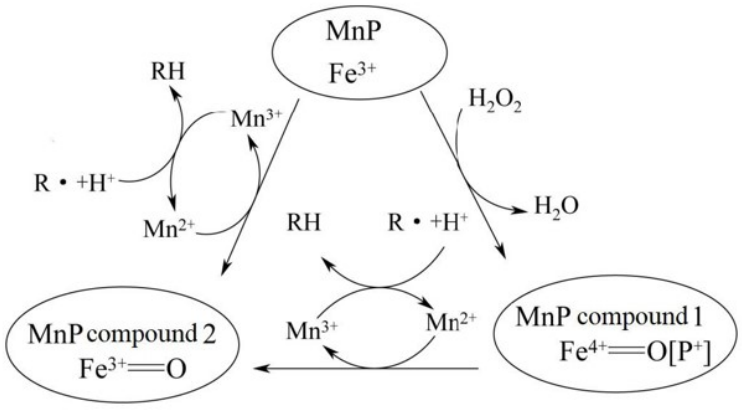

Figure 1: Schematic diagram of catalytic mechanism of MnP [9] RH-Phenol, organic acid, nitroaromatic compound, chlorinated aromatic compound, methoxy aromatic compound; R·- Free radicals formed by RH; [P+]-Porphyrin P-free radical.

Determination of Manganese Peroxidase Activity

Detection Method of Sodium Lactate Reaction System

[12] optimized the reaction conditions for the determination of manganese peroxidase activity, and finally determined the specific experimental steps. A volume of 2 mL of 50 mmol·L^{-1} lactic acid-sodium lactate buffer solution (pH 4.5), 0.1 mL of 1.6 mmol·L^{-1} MnSO4 solution and 0.8 mL of appropriately diluted enzyme solution were added to 10 mL centrifugal tube. The reaction was initiated by adding 0.1 mL of 1.6 mmol ·L^{-1} H2O2 at room temperature. After 3 minutes, the tube solution was quickly put into ice water. 200 μL reaction solutions were added into 96-well plate and the absorbance at 240 nm was measured by microplate reader (PerkinElmer Enspire). One Unit was defined as 1 μmol complex Mn^{3+}-lactate formed per L per min. The enzymatic activity of MnP was calculated according to the following formula. Molar extinction coefficient of Mn^{3+}-lactate is 7800M^{-1}.cm^{-1} at 240 nm [13].

Calculation formula:

$$U/L = \frac{\Delta OD \times V_1}{\varepsilon_{240} \times V_2 \times \Delta t} \times 10^6$$

ΔOD is the difference of absorbance between the start and end. V1 is the total volume of enzyme reaction system. V2 is the amount of enzyme solution. ε is the molar extinction coefficient of Mn^{3+}-lactate. Δt is reaction time (3 min). ε can be determined according to Beer-Lambert law. When the unit of c is mol/L and the unit of b is cm, then absorbance A = εbc, ε is the molar absorption coefficient. [14] reported that the reaction and determination condition was 40^{0}C and absorbance at 270 nm. MnP was assayed in a mixture of 0.9 mL of 50 mM of sodium lactate buffer (pH 4.0) containing 0.3 mM of manganous ions (Mn^{2+}) and 0.1 mL of crude enzyme solution, at 40°C. The reaction was started by addition of 40 μM H2O2 and absorbance at 270 nm (ε =8100 M^{-1} cm^{-1}) was monitored. One Unit was defined as 1 mol complex Mn^{3+}-lactate formed per mL per min. There are also some similar reports, which will not be listed and analyzed here.

Detection Method of Tartaric Acid-Sodium Tartrate Reaction System

The reaction system was tartaric acid-sodium tartrate buffer solution for the determination of manganese peroxidase activity in *Trametes sp*. was reported by [15]. MnP was assayed in a system that is 0.8 mL of 20 mmol·L^{-1} tartrate-sodium tartrate buffer (pH 4.5), 0.05 mL of 2 mmol·L^{-1} MnSO4 solution, 0.1 mL crude enzyme solution and 0.05 mL of 2 mmol·L^{-1} H2O2. After 2 minutes, the change in light absorption values at 238 nm per unit time was determined. One Unit was defined as 1 μmol complex Mn^{3+}-tartrate formed per L per min. Reaction time is not given, ε_{238}=6500 M^{-1} cm^{-1}. This literature reported the transformation of HOPDAs with 10 different substituents by manganese peroxidase was analyzed by UV-V is spectroscopy, and the steady-state kinetic parameters of manganese peroxidase were determined in 1-15 minutes. If researchers want to use this method, they need to study the reaction time, and it is recommended to consider the change of enzyme activity in 2-5 minutes. The calculation formula and molar light absorption coefficient determination method are the same as the above lactic acid method.

Detection Method of Malonic Acid Buffer Reaction System

The reaction system was malonic acid buffer solution for the determination of manganese peroxidase activity was reported by [16]. In the 4 mL reaction system a volume of 3.7 mL 50 mmol·L^{-1} malonic acid buffer solution (pH 4.5), 0.1 mL of 4 mmol·L^{-1} Mn^{2+} solution, 0.1 mL of 4 mmol·L^{-1} H2O2 and 1 mL of crude enzyme were preheated to 30^{0}C. The absorbance at 270 nm was measured and calculated the enzyme activity (ε_{270} =11590M^{-1} cm^{-1}). The vitality of its experimental sample MnP was measured up to 1775 U/L. The definition of enzyme activity, calculation formula and determination method of molar absorption coefficient are the same as the above lactic acid method and phenol red method described below.

Detection Method of Phenol Red Substrate

Phenol red substrate is also one of the commonly used methods for the analysis and determination of manganese peroxidase. The method was used to analyze the characteristics of producing manganese peroxidase and the ability of degrading lignin of *Pichia pastoris* [17]. MnP was assayed at 624 nm. The sample was the centrifugal supernatant of the experimental sample. The reaction mixture contains 0.5 mL of 10 mM MnSO4 solution, 1 mL of 100 mM sodium malonate buffer (pH 4.5), 0.5 ml of 0.25 mM phenol red, and 1 mL of sample enzyme solution. The initial absorbance was measured at 624 nm and then 100 mM H2O2 was added to the sample to start the reaction at 30^0C. React for 5 minutes, immediately terminate the reaction with 1% NaOH solution, measure the absorbance at 624 nm, and calculate the difference between the two times. 1 min oxidized 1 μmol substrate enzyme quantity as an enzyme active unit (U).

Calculation formula:

$$U/L = \frac{\Delta OD \times V_1}{\varepsilon_{624} \times V_2 \times \Delta t} \times 10^6$$

ΔOD is the difference of between the start and the end. V1 is the total volume of enzyme reaction system. V2 is the amount of enzyme solution. ε is molar absorption coefficient of oxidation product from phenol red at 624 nm under the total volume of reaction mixture of blank sample. Δt is reaction time. The reaction system was sodium succinate and sodium lactate solution for the determination of manganese peroxidase activity was reported by [18].

The reaction is divided into two reagents (A and B). The concentration of each substance in reagent A ($mmol{\cdot}L^{-1}$ pH 4.5) was 60 $mmol\ .\ L^{-1}$ sodium succinate, 60 $mmol\ .\ L^{-1}$ sodium lactate, 0.12 $mmol\ .\ L^{-1}$ phenol red, 0.12 $mmol\ .L^{-1}$ MnSO4, and 3.6 $mg\ .mL^{-1}$ gelatin. Reagent B was 6 $mmol{\cdot}L^{-1}$ H2O2 solution. The reaction mixture contained 2.5 mL of reagent A, 0.5 mL of the sample solution, 50 μL of reagent B. After the reaction at 30^0C for 2 min, 100 μL of 5 $mol.L^{-1}$ NaOH was added to terminate the reaction, and the absorbance change at 610 nm was measured. One enzyme activity unit (U) is defined as the amount of enzyme required to oxidize phenol red to produce 1 mmol of product per minute. The literature gives ε_{610}=4460 $M^{-1}\ cm^{-1}$. The reaction degree of the substrate of this method can be determined by NaOH solution stopping the reaction. The reaction process cannot be observed continuously. The calculation formula is the same as above.

Detection method of 2, 6-Dimethoxyphenol (2, 6-DMP) Substrate

The 2, 6-DMP as the substrate for the determination of manganese peroxidase activity was reported by [19]. The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM MnSO4, and up to 650 μL of culture broth in a total volume of 1 mL. The reaction was started by adding 0.4 mM H2O2 and was corrected for background oxidase activity. Chi et al. reported that the total reaction volume of the determination system was 1mL. The reaction mixture contained 840 μL of 50 $mmol{\cdot}L^{-1}$ sodium malonate buffer solution, 50 μL of 10 $mmol\ .L^{-1}$ MnSO4 solution, 50 μL of 10 $mmol\ .\ L^{-1}$ 2,6-DMP, 50 μL of Sample solution, 10 μL of 10 $mmol{\cdot}L^{-1}$ H2O2. The absorbance change at 470 nm was measured with 1 ml of deionized water as control. The enzyme activity unit (U) was defined as the conversion of 1μmol 2,6-DMP per mintue. In calculation, ε_{470}=49600 $M^{-1}\ cm^{-1}$ [20].

In 2, 6-DMP methods, the reaction system was tartaric acid-sodium tartrate buffer for the determination of manganese peroxidase activity was reported by [21]. The total reaction volume of the determination system was 3 mL. The reaction mixture contained 2440 μL of 50 $mmol{\cdot}L^{-1}$ tartrate-sodium tartrate buffer (pH 5.0), 20 μL of 50 $mmol.L^{-1}$ MnSO4, 30 μL of 40 $mmol{\cdot}L^{-1}$ H2O2, 10 μL 50 $mmol{\cdot}L^{-1}$ 2, 6-DMP and 500 μL appropriately diluted enzyme solution. The change in the absorbance at 469 nm was determined at 30^0C for 2 minutes. Enzyme viability was defined as the amount of enzyme required to convert 1 μmoL 2, 6-DMP

within 1 min as one enzyme viability unit (U) at 30^{0}C. In calculation, ε_{469} =27500 M^{-1} cm^{-1}. The calculation formula and determination method of molar absorption coefficient are the same as the above phenol red method described.

The reaction system in 2, 6-DMP methods was acetic acid-sodium acetate buffer for the determination of manganese peroxidase activity was reported by [22]. The reaction mixture contained 2.0 mL of 20 mM acetate-sodium acetate buffer (pH 4.5), 0.8 mL of 40 mM MnSO4 solution, 25 μL of 40 mM 2, 6-dimethoxyphenol, 0.1 mL of enzyme solution and 75 μL of 10 mM H2O2. H2O2 was added to start the reaction. After 3 min of water bath at 30^{0}C, the change of reaction absorbance at 469 nm was measured. The molar extinction coefficient of the oxidation product of 2,6-dimethoxyphenol at 469 nm is 55000 $M^{-1}.cm^{-1}$. The amount of enzyme required to convert 1 μmol of the substrate per minute was defined as an enzyme living unit (U).

Detection Method of Guaiacol Substrate

When guaiacol is used as a substrate for the determination of manganese peroxidase activity, guaiacol can be oxidized by MnP in the presence of the catalyst, and the colored oxidation product can be determined at 465 nm. For the determination of manganese peroxidase by guaiacol method (Table 1), sodium tartrate, sodium succinate, sodium acetate, sodium lactate and sodium lactate can be selected as buffer solutions. The pH ranged from 4.5 to 5.0. The common final concentrations of guaiacol are 0.4, 0.5 and 4.0 $mmol.L^{-1}$. The reaction temperature is between 30 and 40 ^{0}C, and the measurement wavelength is 465nm.

Buffer solution and Concentration ($mol.L^{-1}$)	PH	Substrate concentration	Temperature	Reaction time	Wavelength	Solution
Sodium tartrate 0.10	5	4 $mmol.L^{-1}$	30 °C	2min	465nm	0.5mL[23]
Sodium succinate 0.10	4.5	4 $mmol.L^{-1}$	4.0 °C	5min	465nm	0.2mL[24]
Acetic acid sodium acetate 0.003	4.5	0.5 $mmol.L^{-1}$	30 °C	5min	465nm	1.0mL[25]
Lactic acid sodium lactate 0.05	4.5	0.4 $mmol.L^{-1}$	40 °C	5min	465nm	0.2mL[26]

Tabe1: Reaction conditions for the determination of manganese peroxidase activity by guaiacol substrate method

In detection method of guaiacol substrate, the reaction system was sodium tartrate buffer solution for the determination of manganese peroxidase activity was reported by [23]. Assay mixture contained 500 μL of enzyme solution, 300 μL of 4 $mmol{\cdot}L^{-1}$ guaiacol solution, 1500 μL of 0.1 $mol{\cdot}L^{-1}$ sodium tartrate buffer solution (pH 5.0), 60 μL of 10$mmol{\cdot}L^{-1}$ MnSO4, 60 μL of 5$mmol{\cdot}L^{-1}$ H2O2 and 580 μL of H2O. The change of absorbance at 465 nm [ε= 12100 $M^{-1}.cm^{-1}$] was measured at 30^{0}C for 2 minutes. MnP activity was defined as the amount of enzyme required to catalyze the oxidation of 1$\mu mol.L^{-1}$ guaiacol within 1minute as 1 unit of enzyme activity (U).

The reaction system was sodium succinate buffer solution for the determination of manganese peroxidase activity was reported by [24]. The reaction mixture contained 0.7 mL of 4 $mmol{\cdot}L^{-1}$ guaiacol, 2 mL of 100 $mmol{\cdot}L^{-1}$ sodium succinate buffer solution (pH 4.5), 0.2 mL of 2.5 $mmol{\cdot}L^{-1}$ MnSO4 solution and 0.1 mL of 2.5 $mmol{\cdot}L^{-1}$ H2O2 . After the solution is mixed and shaken, preheat it in a water bath at 40^{0}C for 5 minutes. Pour the above mixed reagent into a cuvette with a volume of 3 mL. During sample determination, 0.2 mL of different crude enzyme solution samples are added to the reaction system, and the absorbance is recorded immediately once every 30 seconds for a total of 3 minutes. Enzyme activity was defined as 1 enzyme activity unit (U) required for a change in absorbance of 0.01 per minute.

The reaction system was acetic acid and sodium acetate buffer solution system for the determination of manganese peroxidase activity was reported by [25]. The reaction mixture system (2.0 mL) containing 0.5 $mmol{\cdot}L^{-1}$ guaiacol, 0.25 $mmol{\cdot}L^{-1}$ MnSO4, 3 $mmol{\cdot}L^{-1}$ acetic acid and 1.5 $mmol{\cdot}L^{-1}$ sodium acetate was adjusted to pH 4.5. Then the reaction was initiated immediately by

adding the final concentration of 0.25 mmol·L^{-1} H2O2, and bathed at 30^0C for 5 minutes. The change of absorbance value of reaction solution at 465 nm is determined. Enzyme activity was defined as 1 enzyme activity unit (U) required for a change in absorbance of 0.01 per minute at 465 nm.

The reaction system was sodium lactate buffer solution system for the determination of manganese peroxidase activity was reported by [26]. The enzyme solution was incubated with 400 μM guaiacol, 50 mM Na-lactate buffer (pH 4.5), 200 μM MnSO4 , and 100 μM H2O2. The extinction coefficient of oxidized guaiacol at 465 nm is 12100 M^{-1} cm^{-1}. Mn^{2+}-dependent oxidation activity was calculated by subtracting the activity in the absence of Mn^{2+} from that in the presence of 200 μM MnSO4. The assay was repeated four times.

Detection Method of ABTS Oxidation Method

ABTS oxidation method is divided into A reaction mixture and B reaction liquid. A reaction mixture contained 100 mmol·L^{-1} sodium succinate (PH 4.5), 100 mmol·L^{-1} sodium lactate buffer (PH 4.5), 6 mg.mL^{-1} egg white, 200 mol.L^{-1} MnSO4, and 80 μg.mL^{-1} ABTS. B reaction liquid is 10 μmol.L^{-1} H2O2. 1 mL of reaction mixture contained 500 μL of liquid A and liquid B, then that was initiated by adding 75 ng enzyme solution (1-10 μL) at room temperature. The initial rate of ABTS oxidation was determined by spectrophotometry based on the increase of absorbance at 415 nm [27]. The calculation formula and the principle of the measurement method of molar absorption coefficient were the same as those of sodium lactate reaction system and phenol red substrate.

Analysis and Discussion

The important criteria for evaluating a method are accuracy, sensitivity, convenience, low cost and stability. At present, MnP activity is often measured by Sodium lactate reaction system, tartaric acid sodium tartrate reaction system, malonic acid buffer reaction system, phenol red method, 2,6-Dimethoxyphenol (2,6-DMP) method, guaiacol method and ABTS method and other methods.

Among the methods for the determination of MnP enzyme activity, the manganese ion method is the most common and widely used. The wavelength of Mn^{2+} reaction system is 240 nm and 270 nm when sodium lactate buffer solution is used, and 270 nm when sodium malonate buffer solution is used. This is mainly because the extinction coefficient of the chelate formed by malonic acid and Mn^{3+} reached the maximum at 270 nm [ε_{270} =11590M^{-1}·cm^{-1}], while the maximum value of sodium lactate chelate is at 240 nm [ε_{240} =6500M^{-1} cm^{-1}] and 270 nm [ε_{270} =8100M^{-1} cm^{-1}]. The chelate formed by Mn^{3+} and malonic acid has a greater extinction coefficient. Therefore, when other conditions are unchanged, malonic acid is used as buffer, it is more sensitive to the change of substrate concentration. Therefore, malonic acid buffer solution is more suitable for Mn^{2+} method [28].

Guaiacol (C7H8O2) can be oxidized under the combined action of MnP and Mn^{2+}. The extinction coefficient of oxidation products of guaiacol at 465 nm is ε_{465} =12100M^{-1} cm^{-1}, so under the condition that the pH (pH 4.5-5.0) for measuring MnP activity is optimal, the enzyme activity value calculated by measuring the absorbance change of oxidation products at 465 nm is more accurate [28].

In the determination of MnP activity with 2,6-DMP as substrate, MnP interacts with H2O2 first to oxidize Mn^{2+} to Mn^{3+} instead of directly interacting with 2,6-DMP. Subsequently, Mn^{3+} oxidizes 2,6-DMP to generate quinone radicals, and Mn^{3+} is reduced to Mn^{2+}, completing a cycle. Quinone intermediates react to form dimers. The maximum absorption wavelength of the dimer is located at 469 nm, and the extinction coefficient is ε_{469} =49600M^{-1} cm^{-1}. It takes 4 moles of Mn^{3+} to make 1 mole of quinone dimer [28]. This method is more sensitive than the above Mn^{3+}sodium lactate buffer solution system method, Mn^{3+}sodium malonate buffer solution system method, and guaiacol method, and is more suitable for the analysis and detection of manganese peroxidase

at the initial stage of the growth and development of fungi.

In some literatures, the unit of enzyme activity is expressed by increasing OD value per milliliter of filtrate per minute. Based on the literature reports, the color reaction law of substances and Lambert`s law, it is suggested that One Unit was defined as 1 μmol complex Mn^{3+}-lactate/tartrate/ malonate formed per L per min or catalyze the oxidation of 1μmol.L^{-1} substrate within 1minutes as 1 unit of enzyme activity (U). In addition, it is recommended that researchers conduct full wavelength scanning during enzyme activity determination to determine the maximum absorption wavelength, and also measure the molar absorption coefficient ε. With regard to the concentration and dosage of manganese sulfate and hydrogen peroxide, a pre-experiment should be carried out while referring to the methods in the literature to determine the applicable dosage or concentration of your sample.

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