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Lignin degradation in corn stover catalyzed by lignin peroxidase from *Aspergillus oryzae* broth: Effects of conditions on the kinetics

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ABSTRACT

In the present study, we aimed to explore the action mechanism of various factors on oxidation degradation reaction of lignin in the corn stover (CS) catalyzed by LiP from *Aspergillus oryzae* CGMCC5992 broth and optimize the oxidative degradation reaction conditions. The Michaelis-Menten (MM) analogous model suitable for oxidation degradation reaction of lignin containing two parameters, including the apparent maximum rate ($\mu_{a,max}$) and apparent Michael constant ($k_{a,m}$), was deduced. The reaction condition to obtain the maximum $\mu_{a,max}$ based on the orthogonal array designs was as follows: pretreatment temperature of 120 °C, pretreatment time of 5 min, reaction temperature of 30 °C, enzyme amount of 3.75 U/100 mL, 50 mmol/L sodium lactate-hydrochloric acid buffer of pH 1.5, H_2O_2 concentration of 20 mM and H_2O_2 amount of 1 mL, respectively. Under the optimized condition, the maximum $\mu_{a,max}$ achieved 1.68 (mg/mL)/min, and the $k_{a,m}$ was 0.37 mg/mL. The oxidation degradation of lignin in lignocellullosic biomass in a dilute solution system followed the MM analogous model. The study laid the foundation for improving pretreatment efficiency using a combination of *Aspergillus oryzae* CGMCC 5992 liquid-state fermentation and H_2O_2 treatment.

1. Introduction

Lignocellulosic biomass becomes a promising alternative as a feedstock for biofuel production due to its characteristic advantageous, such as large abundance and small environmental footprint [1,2]. Lignocellulosic biomass contains lignin, and about 70% of its dry weight is polysaccharides. The polysaccharides can be saccharified into pentoses and hexoses. These sugars are further fermented and converted into many valuable chemicals and food products [3]. Lignin contains an aromatic system composed of phenylpropane units [4].

However, the efficiency of enzymatic saccharification of cellulose in lignocellulose biomass to produce glucose from cellulose remains low due to the presence of lignin encircling hemi-cellulose and cellulose as well as the crystal structure of cellulose and hemicellulose. Lignin limits access of enzymes to the polysaccharide in lignocellulose biomass [5–7]. The crystal structure reduces the access surface area between enzymes and polysaccharides. Therefore, biomass pretreatment prior to enzymatic saccharification is a necessary step for overcoming the structural and steric barriers to improve the enzymatic saccharification efficiency.

Till now, many pretreatment methods have been developed, such as microwave, ionizing radiation, steam explosion, acid or alkali dilution and oxidation or a variety of their combinations [8]. These techniques not only require some special equipments for high temperature, high pressure and corrosion resistance, but also produce high cost of pretreatment and second pollution [9,10], which hinder the commercialization of these methods.

Biodegradation of lignin is considered to be the most promising method for the pretreatment of lignocellulosic biomass. However, the relatively low efficiency, high carbohydrate loss, large cover area and long fermentation cycle make bio-pretreatment difficult to be industrialized. Therefore, the commercialization of bio-pretreatment methods remains obscure before these shortcomings have been overcome. Enzyme involved into lignin biodegradation includes lignin peroxidase (LiP), laccase (Lac) and manganese peroxidase (MnP). In our previous study, *Aspergillus oryzae* CGMCC5992 has been proved to possess the ability to remove chemical oxygen demand from vinasse [11]. When gallic acid is used as the substrate, it can synthesize LiP, MnP and Lac [12].

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List of abbreviations

$k_{a,m}$	Apparent Michaelis constant and
$\mu_{a,max}$	Apparent maximum rate
k_m	Michaelis constant
μ_{max}	Maximum rate
LiP	Lignin peroxidase
Lac	Laccase
MnP	Manganese peroxidase
CS	corn stalk
MM	Michaelis-Menten
ANOVA	Analysis of variance
PDA	Potato dextrose agar medium
PD	Potato dextrose medium

Furthermore, this strain has been proved to secret LiP, endo-1,4-β-D-glucanase and proteinase in the presence of corn stover (CS) [13]. Zyr has suggested that LiP is involved in the degradation of lignin and aromatic compounds [14]. In addition, we have degraded lignin in the CS through combination of the solid-state fermentation (SSF) and enzymatic oxidative degradation, and we have found that *A. oryzae* CGMCC5992 can produce higher amounts of active MnP and LiP using 3% H_2O_2-pretreated CS as matrix and increase disintegration of lignin [15]. Although such a combination method saves the treatment time from 50 days to 10 days and increases the lignin removal ratio from 57.8% to 80% compared with SSF [15], the method can not be applied to the industrialized production of biofuels due to the requirement of large cover area and long fermentation cycle. Recently, the combination of liquid-state fermentation and enzymatic oxidative degradation has been applied in pretreatment of CS, and the sugar yield reaches a maximum of 46.28% [16]. Meanwhile, this method has some advantages, including cost economy, quickness, convenience, safety, no requirement of special equipment and high sugar yield.

For the optimization of a biodegradation process, a kinetic model is important to describe the phenomena of the reaction system. For an enzymatic oxidative degradation process, a kinetic model that can compatibly describe the enzymatic reaction is essential in the reactor design. The Michaelis–Menten (MM) model for enzyme kinetics is suitably applied in a homogeneous system. However, many bio-catalytic reactions occur in a heterogeneous system with effects of mass-transfer and reaction involved, including accessibility and reactivity of soluble enzymes on insoluble substrates, enzyme deactivation and so on [17,18]. In the past, alternative models have been proposed to simulate enzymatic reactions in a heterogeneous system, such as the jammed Michaelis model, the fractal Michaelis model and the model by shrinking particle theory [17–19]. However, the application of those models has some disadvantages because (i) the models consist of complicated equations that should be solved, (ii) the models contain many parameters that could not be uniquely determined, and (iii) some parameters are arbitrarily chosen rather than from a fitting process by experiments.

In the present study, we mainly aimed to use the classical MM model with minor modification to fit the data of the oxidative degradation of lignin in the CS catalyzed by LiP from *A. oryzae* CGMCC5992 broth; to verify whether slightly modified MM equation fitted the data of oxidative degradation in the heterogeneous system; to disclose the reaction mechanism of the oxidative degradation of lignin in the CS by LiP from *A. oryzae* CGMCC5992 broth; and to optimize the condition for the maximum reaction rate.

2. Materials and Methods

2.1. Materials

CS was purchased from a local farm (Zhenjiang, China), dried at 105 °C to constant weight, ground into a fine powder and sieved through a 0.25-mm sieve. All of the chemicals used were of analytical or reagent grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

2.2. Microorganism

A. oryzae CGMCC5992 was isolated by our laboratory and deposited in China General Microbiological Culture Collection Center. The strain was maintained on the potato dextrose agar (PDA) slants at 28 °C for 4 days, stored at 4 °C and passaged once every 2 months.

2.3. LiP preparation

LiP was prepared according to a method described by Zhang et al. with minor modifications [16]. A total of 1×10^6 spores from the *A. oryzae* CGMCC 5992 strain were aseptically inoculated into 100 mL potato dextrose (PD) medium in a 250-mL Erlenmeyer flask and then incubated at 35 °C for 24 h at 125 rpm in a rotary shaking incubator to produce a mass of pellet. This pellet culture was used as seed in the liquid-state fermentation.

Briefly, 10 mL seed culture was aseptically inoculated into a 250-mL Erlenmeyer flask containing 100 mL minimal medium (pH 6.8–7.0) consisting of (g/L): 30 CS powder, 2.5 glycerol, 2.5 maltose, 15 yeast extraction, 45 $(NH_4)_2SO_4$, 0.4 $FeSO_4 \cdot 7H_2O$, 0.4 $CuSO_4 \cdot 5H_2O$, 1 $MnSO_4 \cdot H_2O$, 0.6 $MgSO_4 \cdot 7H_2O$, 0.1 VB_{12} and 4 glycine. After inoculation, the flask was incubated at 35 °C for 72 h at 125 rpm in a rotary shaking incubator. Subsequently, the broth was centrifuged, and the supernatant was collected to determine the LiP activity and used to catalyze the oxidative degradation reaction of lignin from CS.

2.4. Determination of the initial reaction rate of CS oxidative degradation reaction

Fig. 1 shows the flow chart. In addition to the special instructions, the flasks consisting of 70 mL sodium lactate-hydrochloric acid buffer (pH 1.5, 50 mM) and various concentrations (0.5 g, 0.75 g, 1 g, 1.25 g, 1.5 g, 1.75 g and 2 g) of substrate (CS) were pretreated at 115 °C for 5 min. After being cooled to 35 °C, 3.75 U/100 mL LiP (15 mL broth) was added to the flasks, and the volume of reaction mixture was adjusted to 100 mL with sodium lactate-hydrochloric acid buffer. The initial guaiacol concentration before reaction was determined as C_0. The flasks were preheated in water bath at 35 °C for 5 min at 120 rpm. Then, 1 mL of 20 mM H_2O_2 stock solution was added to initiate the oxidative degradation of lignin, and the reaction was maintained for 1 min. Subsequently, the reaction was terminated by rapidly cooling in the ice bath. The guaiacol content in each flask was determined as C_1. The initial reaction rate (μ) at various CS concentrations (0.50%, 0.75%, 1.00%, 1.25%, 1.5%, 1.75% and 2.00%) was calculated according to an equation as follows:

$$\mu = \frac{C_1 - C_0}{\Delta t} = \frac{C_1 - C_0}{1\ \text{min}} \quad (1)$$