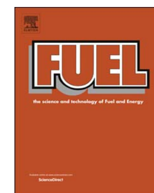
Fuel 214 (2018) 416–422

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel



Full Length Article

An esterase from *Penicillium decumbens* P6 involved in lignite depolymerization

Yi Yang[a], Jinshui Yang[a], Baozhen Li[a], Entao Wang[b], Hongli Yuan[a,*]

[a] State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China
[b] Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico, DF, Mexico

ARTICLE INFO

Keywords:
Penicillium decumbens
Lignite
Depolymerization
Esterase
Humic acid

ABSTRACT

In this study, lignite was degraded using a purified esterase from *Penicillium decumbens* P6 for the first time. The esterase was purified using ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The recovery and purification yield of the enzyme were 15% and 83 folds, respectively. The molecular weight of the purified enzyme was about 45 kDa. Both crude and purified esterases were studied for lignite depolymerization. The tendency of depolymerization by crude enzyme was consistent with the enzyme secreted in the medium. Along with the increased purified esterase concentration from 8 to 50 mg/ml, A_{450} value increased from 0.38 to 2.08. The contribution of esterase to the depolymerization was about 40% in the crude supernatant. Compared with aHA (crude lignite humic acid), bHA (esterase degraded lignite humic acid) has a lower percentage of aromatic carbon and ester groups, but a higher percentage of aliphatic carbon. bHA can promote the growth of asparagus lettuce. The results demonstrated that lignite was depolymerized by the purified esterase and evidenced the potential of esterase application in conversion of lignite into compounds with high bioactivities.

1. Introduction

With a lignin-like structure, lignite is defined as a low rank coal with low calorific value, high moisture and high sulfur content, which limits its usage and conversion [1]. The direct combustion of lignite results in low thermal efficiency and low industrial profit. In addition, the piling up of lignite in the open air for a long time causes energy waste and environmental pollution [2]. The high content of humic acid and fulvic acid and the great reserve (130 million tons) of lignite in China make it a potential natural resource [3]. Hence, research on conversion of lignite into industrial and chemical products with high additional value or into liquid fuel is rationally developed [4].

Enzymatic conversion has been considered as an economic and environment-friendly way for transforming macromolecular coal into simpler and low molecular weight products [5]. Lignin peroxidases, manganese peroxidases and laccases are the most widely studied oxidative enzymes participating in the coal solubilization [6,7], and they require low molecular weight cofactors and mediators to depolymerize the lignin [8]. In addition to the oxidative enzymes, hydrolytic enzymes also showed a great potential to depolymerize lignite. In lignite, carboxylic ester groups amount to approximately 2.5–5% of the total organic matter, corresponding to one to two ester bonds per 100 lignite carbon atoms [9]. A direct correlation between lignite solubilization and enzymatic hydrolytic activity was evidenced in the deuteromycete *Trichoderma atroviride* [10]. Hölker et al. [11] reported that *T. atroviride* could cleave both the carboxylic esters and the phenolic ether bonds during the solubilization of ^{14}C-labelled lignite. However, unlike the peroxidases, no low molecular weight co-factors and mediators were known in the activation of esterases, and the steric hindrance might be an obstacle for esterases to permeate the macromolecular coal network and to depolymerize the coal [12]. Therefore, more research is needed to determine the actual role and the mechanism of esterases in coal degradation.

In our previous work, the fungal strain *Penicillium decumbens* P6 capable of degrading lignite effectively presented inducible esterase activity in the lignite degradation [3], that implied a possible role of esterase in the depolymerization of lignite. In addition, the contents of humic acids and water-soluble humic materials greatly increased and the molecular mass of humic acid decreased in the fungal depolymerized lignite [13]. Moreover, the fungal transformed lignite humic acids have a better bioactivity than the crude lignite humic acids [14,15]. It is well known that humic acids contribute substantially to global soil

Abbreviations: CP/MAS ^{13}C NMR, ^{13}C nuclear magnetic resonance with cross-polarization/magic angle spinning; aHA, lignite humic acid; bHA, esterase degraded lignite humic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DEAE, Diethyl-Aminoethanol; SDS, Sodium Dodecyl Sulfonate; EDTA, Ethylene Diamine Tetraacetic Acid

* Corresponding author at: Center of Life Science, China Agriculture University, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, China.
E-mail addresses: yang11yi@aliyun.com (Y. Yang), yangjsh1999@163.com (J. Yang), bzhli@cau.edu.cn (B. Li), hlyuan@cau.edu.cn (H. Yuan).

https://doi.org/10.1016/j.fuel.2017.11.035
Received 16 August 2017; Received in revised form 26 October 2017; Accepted 11 November 2017
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fertility and have been widely utilized in agriculture, for example, they can promote plant growth and metabolism [16] and reduce some plant diseases [17].

To prove the roles of esterase in the enzymatic attack on lignite, we performed the present study using purified esterase from *P. decumbens* P6 culture. The crude lignite humic acids (aHA) and esterase degraded lignite humic acids (bHA) were quantified and characterized using ^{13}C nuclear magnetic resonance with cross-polarization/magic angle spinning (CP/MAS ^{13}C NMR) and Fourier transformed infrared spectrophotometry (FT-IR). The humic acids produced by esterase depolymerization presented a significantly positive effect on the growth of asparagus lettuce. These results evidenced the key role of esterase for *P. decumbens* P6 in hydrolyzing carboxyl within the lignite structure.

2. Materials and methods

2.1. Lignite samples and fungal strain

Lignite sample was collected from the Huolingele Minerals Administration Coalmine, Inner Mongolia of China. Air-dried lignite sample was pulverized and sieved with a 70 mesh screen. The sample preparation and elemental analysis of the crude lignite humic acid were performed according to the previous reported methods [13]. The fungal strain *P. decumbens* P6 (CGMCC 0866) used throughout the study was maintained at 4 °C on the yeast extract – malt agar (YMPG) slants, consisting of: glucose, 10 g; malt extract, 10 g; peptone, 2 g; yeast extract, 2 g; asparagines, 1 g; $KH_2PO_4{\cdot}3H_2O$, 2 g and $MgSO_4{\cdot}7H_2O$, 1 g in 1 l distilled water. All of the chemicals used in this study were reagent grade purchased from the Beijing Chemicals and Reagent Corp., China, unless otherwise stated.

The fungus was cultured on YMPG agar plates at 28 °C for 1 week, then the spores were washed and suspended in 0.9% of sodium chloride solution. Aliquots of 2 ml of the spore suspension (10^6 spores/ml) were inoculated into 500 ml flasks containing 200 ml liquid medium (10 g wheat bran; 5 g urea; 2 g $KH_2PO_4{\cdot}3H_2O$; 1 g $MgSO_4{\cdot}7H_2O$ and 1 g asparagine in 1 l of distilled water) which had been optimized for esterase production. Powdered lignite was added into the medium at the ratio of 1% (w/v) as an inducer for enzyme production. The inoculated flasks were cultivated by shaking at 150 rpm and 28 °C for 10 days. Culture samples in aliquots of 1 ml were taken every day, and centrifuged at 4 °C, 8000 rpm for 10 min. Supernatants were used for analyzing the esterase activity with the method [18]. When the esterase activity reached the maximum level (after 10 days of incubation), whole cultures were harvested for purification studies as mentioned subsequently.

2.2. Purification of esterase and determination of molecular weight

After 10 days of cultivation, the supernatant was separated from the mycelium by centrifugation of the culture at 8000 rpm for 20 min at 4 °C, and then filtration with Whatman No. 1 filter paper. Proteins in the cell-free extract were precipitated at 4 °C with ammonium sulfate (80%) for about 2 h following by centrifugation at 8000 rpm for 20 min under 4 °C. The sediment was resuspended in 20 ml of 20 mM Tris-HCl buffer (pH 8.0) and dialyzed against 2 l of the same buffer for 24 h to remove $(NH_4)_2SO_4$. Then, the protein solution was loaded on the columns listed bellow to separate and purify acetyl xylan esterase.

Firstly, the protein solution was loaded on a DEAE (Diethyl-Aminoethanol) Sepharose anion exchange column (GE) pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was washed with the same buffer until A_{280} (absorbance at 280 nm) of effluent became zero and then eluted with a linear NaCl gradient (0–1 M) at a flow rate of 1.5 ml/min. The elute was collected into fractions of 1 ml. Esterase activity in the fractions was detected with the previously described method [18] and fractions presenting esterase activity were pooled and lyophilized for the next step of purification.

The dried samples were dissolved in 1 ml of 50 mM phosphate buffer (pH 7.0) and were loaded onto a Sephadex G-75 gel filtration column (GE) that had been pre-equilibrated with the same phosphate buffer. The column was eluted with 50 mM phosphate buffer (pH 7.0) at a flow rate of 1.5 ml/min. The elute was collected into 1 ml fractions for esterase activity detection as mentioned previously [18]. The active fractions were loaded onto the Sephadex G-75 gel filtration column again for further purification. The molecular weight of the purified esterase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Protein sequencing

Protein sequencing was performed according to the method [19]. The acquired amino acid sequence was used in BLAST searching (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for extracting the related sequences in the database. The sequence was aligned together with the extracted reference sequences by MEGA 6.06 software [20].

2.4. Enzyme properties

The esterase activity was determined by the spectrophotometric method [18]. Briefly, 50 μL supernatant of the culture was mixed with 950 μL 4-nitrophenyl acetate in 50 mM phosphate buffer (pH 7.0). The reaction was performed at 37 °C for 15 min. A_{400} (absorbance at 400 nm) was determined with a UV-1800 spectrophotometer (Shimadzu, Japan) to estimate the released *p*-nitrophenol. In control, 50 μL of the supernatant boiled for 30 min were used. One unit of esterase activity was defined as the amount of proteins liberating 1 μmol *p*-nitrophenol per min under the defined conditions.

The optimum pH for esterase was tested at 37 °C in 50 mM citric acid buffer (pH 3.0, 4.0 and 5.0) and 50 mM phosphate buffer (pH 6.0, 7.0, 8.0 and 9.0). The optimum temperature for esterase was determined by standard assay ranging from 30 to 80 °C in the 50 mM phosphate buffer (pH 7.0). The results were expressed as relative activity to the value obtained at either optimal pH assay or optimal temperature assay.

The pH stability of esterase was determined by measuring the residual activity after incubating the enzyme at 37 °C for 1 h in the aforementioned buffer (pH 3.0–9.0). In the thermostability assay, the enzyme solution was incubated in 50 mM phosphate buffer (pH 7.0) at temperatures ranging from 30 to 80 °C for 1 h, and the remaining activity was measured under the optimum conditions. The esterase activity obtained under the optimum conditions was defined as 100%.

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of the purified esterase were determined by measuring the esterase activity at various 4-nitrophenyl acetate concentrations (0.2 mM, 0.4 mM, 0.8 mM, 1.6 mM and 3.2 mM). The K_m and V_{max} of the enzyme were calculated using the Lineweaver-Burk plot constructed by plotting the reciprocal of the substrate concentration on the X-axis and the reciprocal of the esterase reaction velocity on the Y-axis. All determinations were conducted in triplicate.

The effects of metal ions and chemical reagents on the esterase activity were determined. Different metal ions and chemical reagents such as NH_4^+, Al^{3+}, Ba^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Ag^+, Cu^{2+}, Sn^{2+}, Fe^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, Fe^{3+}, urea, SDS (Sodium Dodecyl Sulfonate) and EDTA (Ethylene Diamine Tetraacetic Acid) at 10 mM and 1 mM were added to the purified enzyme solution and then incubated at 4 °C for 2 h. The residual activities were determined under the standard assay conditions. The esterase activity of the enzyme in culture without addition of metal ions or chemical reagents was defined as 100%.

Protein concentration was determined using the Bradford Protein Assay Kit (GenStar, Beijing, China) according to the manufacturer's instructions.

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