International Biodeterioration & Biodegradation 86 (2014) 382–389

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

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

Production optimization and molecular structure characterization of a newly isolated novel laccase from *Fusarium solani* MAS2, an anthracene-degrading fungus

Yi-Rui Wu [a,*], Ding-Lan Nian [b]

[a] Department of Civil and Environmental Engineering, Faculty of Engineering, National University of Singapore, Singapore
[b] Department of Biology, Shantou University, 243 Daxue Road, Shantou, Guangdong, PR China

ARTICLE INFO

Article history:
Received 2 August 2013
Received in revised form
16 October 2013
Accepted 17 October 2013
Available online 13 November 2013

Keywords:
Gene cloning
Fusarium solani
Identification
Laccase
Response surface methodology

ABSTRACT

To investigate the potential of laccase production from strain *Fusarium solani* MAS2, the response surface methodology (RSM) was employed, and the maximum laccase activity of 159.78 U ml^{-1} was obtained at 20 °C and pH 6.5 with 30 mg l^{-1} of initial concentration of anthracene as the sole carbon source. Characterization of this laccase showed the similar properties with other reported laccases; however, the molecular identification, including matrix assisted laser desorption ionization-time of flight-tandem mass spectrum (MALDI-TOF-MS/MS) and gene cloning, demonstrated that this laccase was different from those available laccases, and only shared high homology with a non-identified, hypothetical oxidase from genome-sequenced strain *Nectria haematococca*, which was further annotated to be laccase. Further analysis on its nucleotide and amino acid sequences showed three introns present without detectable N-terminal signal peptide, indicating that this laccase might be synthesized within the cells.

1. Introduction

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are a large group of versatile glycosylated polyphenol oxidases which are mainly produced from the fungal species, but also detected in bacteria, plants, and even some insects (Mougin et al., 2003; Wu et al., 2010b; Zhu et al., 2013). Due to their non-substrate-specific property (Farnet et al., 2008), laccases are extensively involved in a wide range of commercial biotechnologies and environmental applications, such as pulp and paper industries, drug analysis, wine clarification as well as biotransformation of environmental pollutants (Mayer and Staples, 2002).

The anthracene-degrading fungus *Fusarium solani* MAS2, isolated from the polycyclic aromatic hydrocarbon (PAH)-contaminated mangrove sediments, could simultaneously produce laccase during the degradation process (Wu et al., 2010b). As indicated in previous studies (Wu et al., 2010b,c), laccases are crucial for initiating the formation of 9,10-anthracenedione, one of degradative intermediates, from anthracene by strain MAS2. Actually, the production of enzymes by a microorganism is strongly correlated with culture conditions, and even the substrates involved. Thus, one of the most important tasks for biotechnological application (e.g. bioremediation), especially in the large scale production, is to optimize the cultural parameters for microbial growth as well as the secondary metabolites production (e.g. functional enzymes) (Teerapatsakul et al., 2007; Fakhfakh-Zouari et al., 2010). The response surface method (RSM) usually serves as a useful model for studying the effect of several parameters affecting the responses through adjusting them simultaneously and investigating a limited number of experiments (Teerapatsakul et al., 2007; Papadopoulou et al., 2013). This method is more reliable than the classical single variable method, especially to overlook the interactions between different factors, and it does not need tedious and time-consuming experiment and also is less likely to result in the misinterpretation of data (Weuster-Botz, 2000). The application of this statistical method has been employed for the medium optimization on production of different enzymes, including lysozyme (Parra et al., 2005), amylase (Xu et al., 2008), keratinase (Teerapatsakul et al., 2007) as well as laccases from other fungal species (Palvannan and Sathishkumar 2010; Diwaniyan et al., 2012). In order to understand the effect of culture parameters as well as their relationship towards the laccase production, the cultivation conditions for the laccase production from the isolate *F. solani* MAS2 were optimized based on the Box-Behnken design (BBD).

* Corresponding author. Tel.: +65 93759745; fax: +65 65168482.
E-mail address: oxwyr@tom.com (Y.-R. Wu).

0964-8305/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2013.10.015

Different laccases from fungal species have been reported and identified with various molecular structures (Mayer and Staples, 2002; Wu et al., 2010b,c; Grassi et al., 2011). Some laccases from wood fungi have also been purified and well characterized, but laccases from mangrove sediment fungi have not been investigated yet. The previous studies demonstrated that laccase from the *F. solani* MAS2 had a high tolerance against most heavy metal ions, especially the enhancement of enzymatic activity with the addition of mercurial ions (Wu et al., 2010b). However, the data about the molecular structure of this novel enzyme are still unavailable. In this study, therefore, molecular analysis including matrix assisted laser desorption ionization-time of flight-tandem mass spectrum (MALDI-TOF-MS/MS), genomic DNA (gDNA) and complementary DNA (cDNA) cloning were involved in the identification to contribute proteomic and genomic information of this fungal strain to the further industrial or biotechnological applications (e.g. enzymatic treatment of PAH compounds).

2. Materials and methods

2.1. Microorganism and maintenance of culture

F. solani MAS2 used in the present study is an anthracene-degrading fungal strain isolated from PAHs-contaminated mangrove sediments in Hong Kong. It was selected due to the laccase production during the degradation when fed with anthracene as the sole carbon source. The strain was grown and maintained at 25 °C with mineral salt medium (MSM) agar slants (1%) as described by Wu et al. (2010c) without addition of antibiotics (e.g. penicillin G and streptomycin sulfate).

2.2. Inoculum preparation for cultivation

For preparing the inoculums, fungal mycelia with spores were collected from the stored culture slant, and transformed into the fresh MSM agar plate with anthracene as the sole carbon source, the same as the maintenance medium, for growth. After incubation for seven days, three plugs of 6-mm diameter with active fungal cells were used as inocula, and added into 125 ml conical flask with 50 ml of autoclaved MSM liquid medium for the optimization of laccase production. Unless otherwise stated, the flasks were incubated in an orbital shaker with the speed of 150 rpm at 25 °C.

2.3. Experimental evaluation for optimization

Temperature (X_1), pH level (X_2) and initial concentration of anthracene (X_3), the three key factors related to laccase production when degrading anthracene (Wu et al., 2010b), were selected to investigate the effect on the laccase production of strain MAS2. As shown in Table 1, to statistically test the best performance of these factors involved and their relationship, a BBD with 3-factor and 3-level together with the RSM was adopted to establish a total of 15 experimental combinations for analysis. The factors were prescribed into three levels, coded −1, 0, and +1, respectively representing the low, middle and high concentration (or level). The relationship between variables and the responses was calculated by the second order polynomial equation:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j,$$

where Y is the predicted response of the laccase production; X_i and X_j are the input variables affecting the response value Y; A_0 is the offset term; A_i is the linear coefficient; A_{ii} is the quadratic coefficient; and A_{ij} is the interaction coefficient.

All the runs were carried out in triplicate and the average value of laccase activity from each sample was taken as the response value. Design-Expert (Version 7.0, Stat-Ease Inc., Minneapolis, MN, USA) was adopted for the experimental design, data analysis and quadratic model construction. The optimal condition for laccase production involved in solving the regression equation as well as analyzing the response surface contour plots was also obtained from this software.

Table 1
Experimental design combination for the factors and levels used for optimization of laccase production.

Key	Factors	Levels		
		Low (−1)	Middle (0)	High (+1)
X_1	Temperature	20 °C	25 °C	30 °C
X_2	pH	4.5	5.5	6.5
X_3	Initial concentrations of anthracene	30 mg l^{-1}	40 mg l^{-1}	50 mg l^{-1}

2.4. Enzyme activity assay

Factors at three levels were selected and the incubation period (35 d) was formulated according to the previous study (Wu et al., 2010b). After 35 d of incubation, samples received from each flask were centrifuged at 12,000 *g*, 4 °C for 15 min. The supernatant was directly used for the determination of laccase activity. The activity of laccase was determined spectrophotometerically by the oxidation of 0.2 mM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) with a mixture containing 50 μl of supernatant and 950 μl of 100 mM sodium tartrate buffer (pH 4.5), and detected under the wavelength of 420 nm (ε_{420nm} = 36000 M^{-1} cm^{-1}). One unit (U) of laccase activity was defined as the production of 1 μmol product per minute under the condition of 30 °C and pH 4.5 (Wu et al., 2010b). The total protein amount of each sample was also determined through the Lowry procedure by using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

2.5. Effect of pH and temperature on enzymatic activities towards different substrates

Laccase obtained under the optimal conditions was collected from the culture medium, and further purified according to the method of Wu et al. (2010b). The optimum pH of the laccase was investigated over a pH range of 2.0–8.0 at 30 °C using five different substrates, including 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS, 0.2 mM), catechol (10 mM), 2,6-dimethoxyphenol (DMOP, 0.02 mM), guaiacol (5 mM) and 4-hydroxy-3,5-dimethoxybenzaldehydeazine (syringaldazine, 0.01 mM). The buffer systems used for pH study were used by 100 mM sodium tartrate buffer for pH 2.0–6.0 and 100 mM sodium phosphate for pH 7.0–8.0. The effect of temperature was tested from 30 °C to 80 °C with 10 °C increments under their respective optimal pH of different substrates.

2.6. Identification of laccase by MALDI-TOF-MS/MS analysis

The purified laccase was applied to native PAGE analysis as indicated in the previous studies (Wu et al., 2010b), and the band with laccase activity in gels was cut and digested by trypsin according to the methods described by Zhang et al. (2006). The digested peptides from the gel were extracted and dissolved into 1% formic acid for further use. MALDI-TOF-MS/MS analysis was adopted based on the findings of Wu et al. (2009) with some