A comparative study on the degradation of gallic acid by *Aspergillus oryzae* and *Phanerochaete chrysosporium*

Danzhao Guo, Zhicai Zhang, Dan Liu, Huihua Zheng, Hui Chen and Keping Chen

ABSTRACT

Recently, as an emerging persistent dissolved organic pollutant (DOP), gallic acid (GA) and its efficient decomposition methods have received global attention. The present work aimed to compare the effect of *Aspergillus oryzae* 5992 and *Phanerochaete chrysosporium* 40719 on degradation of different concentrations of GA. The *A. oryzae* grew well and achieved a GA removal rate up to 99% in media containing 1–4% GA, much higher than *P. chrysosporium*. The activity of laccase and lignin peroxidase excreted by *A. oryzae* was higher than that by *P. chrysosporium* in the presence of GA. Based on the results of high-performance liquid chromatography–electrospray ionization–mass spectrometry, three relevant intermediate metabolites were determined as progallin A, methyl gallate, and pyrogallic acid, implying that *A. oryzae* could not degrade GA unless the carboxyl in the molecule was protected or removed. In view of the ability of *A. oryzae* to accommodate a high concentration of GA and achieve a high removal rate, as well as the significantly different enzyme activities involved in GA degradation and the underlying mechanisms between the two fungal strains, *A. oryzae* is proven to be a superior strain for the degradation of DOP.

Key words | *Aspergillus oryzae*, degradation, dissolved organic pollution, gallic acid, removal rate

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INTRODUCTION

The main dissolved organic pollutant (DOP) in natural waters is natural organic acids (NOAs), 15–30% of which are hydrophilic small molecules. Gallic acid (GA) is one of the widely distributed hydrophilic small-molecule NOAs (Aiken *et al.* 1992; Wang *et al.* 2009a, 2009b; Chang *et al.* 2011). GA is also an important raw material used in the productions of food, household chemicals, and pharmaceuticals, the waste waters of which all contain a certain amount of GA. GA in the water causes problems as follows: (1) affects the water color, taste, and smell; (2) generates disinfection byproducts during chlorine disinfection, e.g., chloroform and haloacetic acids, which can damage organisms, especially human liver, kidney, and central nervous system (Christense *et al.* 1998), and have carcinogenic, teratogenic, and mutagenic effects too (Wang *et al.* 2010); (3) easily cause membrane fouling during the subsequent process in advanced water treatment; (4) decomposes in water into other organic acids, biogas, etc., consuming water-dissolved oxygen, resulting in hypoxia-induced death of fish, shrimp, and other aquatic organisms; and (5) leads to increased water chemical oxygen demand, etc., resulting in a series of aquatic ecological and environmental problems (Hayatsu *et al.* 1998; Lee *et al.* 2004; Boye *et al.* 2006). Therefore, to efficiently degrade GA has become a hotspot in water pollution control.

Current techniques to remove DOP from water include flocculation, adsorption, nanofiltration, and biological method (Cheng *et al.* 2005; He *et al.* 2006; Williams & Pirbazari, 2007; Lin *et al.* 2008). Flocculation works better for the removal of high molecular weight DOP (MW > 50,000 Da) but has a low removal rate for low molecular weight, highly water-soluble organics, and the safety of using aluminum-based and polyacrylamide flocculants has been questioned too (Wang *et al.* 2009a, 2009b). Although nanofiltration has a high removal rate for DOP in drinking water, it is less effective in depleting certain hydrophilic organic small molecules, and problems such as easy membrane fouling and congestion seriously limit its application (Figaro *et al.* 2006). Biological method can remove 30–50% of the

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DOP, without using chemical substances; it thus becomes a promising approach.

Our previous study has shown that *Aspergillus oryzae* CGMCC5992 can effectively remove organics from vinasse in the distillery industry (Zhang *et al.* 2013). Since vinasse contains a large amount of phenols (Maestro-Durin *et al.* 1993), it is thus of interest to study whether *A. oryzae* CGMCC5992 can degrade phenols. *Phanerochaete chrysosporium* was believed to be the strongest strain in degrading aromatic compounds in wastewater (Renganathan *et al.* 1985; Huynh *et al.* 1985). Therefore, this study investigated the ability of *A. oryzae* to degrade phenols using GA as substrate, as well as the related pathways, with the use of *P. chrysosporium* 40719 as control strain.

MATERIAL AND METHODS

The fungal strains

A. oryzae CGMCC5992 isolated from the sludge of the Yudai River in the grounds of Jiangsu University and *P. chrysosporium* 40719 purchased from the China Center of Industrial Culture Collection were used in this study.

Inoculation and degradation of GA

A. oryzae CGMCC5992 and *P. chrysosporium* 40719 were both handled as below. In total, 1×10^6 spores were inoculated aseptically into a 250 mL Erlenmeyer flask with 100 mL wheat bran dextrose medium containing 20 g/L wheat bran and 20 g/L glucose and were cultured on a rotary shaker at 150 rpm, 32 °C for 1–2 days. The seed culture (10 mL) was then inoculated into other 250 mL Erlenmeyer flasks containing 100 mL basal fermentation medium with the following components (g/L): glucose 13.46, NaH_2PO_4 24.52, $CaCl_2$ 2.18, $MgSO_4$ 9.89, and NH_4Cl 4.68. Then 10, 20, 30, and 40 g/L of GA, respectively, were added to the flasks and cultured at 150 rpm, 32 °C for 8 days.

Sample preparation

During the process of fermentation, the fermentation broth was sampled every 24 h and filtered with carbasius. The filter residue was analyzed for biomass, and the filtrate was centrifuged at 5,000 rpm, 4 °C for 5 min to collect the supernatant, which was stored in a refrigerator at −20 °C and used to analyze for GA content and enzyme activities.

Analytical methods

Analysis of biomass

The fungal growth was determined by measuring the dry weight of the cells. The fermentation broth was filtered with sterile carbasus; the residue was collected and washed until colorless, and as dried at 105 °C to constant weight, and the weight then was designated as biomass.

Analysis of GA content

The GA in the supernatant was analyzed by a high-performance liquid chromatography (HPLC) system (Himadzu LC-20 AT Pump, N2000 station; USA), equipped with a UV detector. The analysis was performed using a RT C18 column (4.6 mm × 250 mm × 5 μm, pore size 100 Å; Australia). The mobile phase was methanol: water containing 0.1% phosphonic acid (15:85, v/v) at a constant flow rate of 0.8 mL/min. GA was detected by absorbance at 270 nm wavelength. The GA peak in samples was confirmed by spiking of pure GA, and the content of GA was calculated by external standard method.

Identification of the metabolite of GA by *A. oryzae* using high-resolution liquid chromatography (LC)–mass spectrometry (MS) and LC-MS^2

LC analysis was performed on an Agilent C-18 column (5 μm, 150 × 2.1 mm, water; Milford, MA, USA), using a high-pressure binary pump, Waters 996 diode array detector, and Agilent 1200 Series autosampler. The elution was at 0.3 mL/min with a linear gradient of acetonitrile (A) and water (B), each containing 0.1% formic acid. The gradient was from 5 to 100% A over 3 min, then to 30% A at 20 min, to 100% A at 25 min (1 min hold), followed by 5% A with a 5 min hold to equilibrate the column. The HPLC system was coupled to a Waters Platform ZMD 4000 ion trap mass spectrometer (USA) operated in the mode of full-scan negative ion electrospray ionization (ESI) (m/z 500–800). The ESI was operated in negative mode; the voltage of sample cone was at 20 V, and the collision energy was at 5 eV.

Enzyme activity determination

Lignin peroxidase (LiP) activity was determined according to Tien & Kirk (1984). The activity of manganese peroxidase

(MnP) was measured at 240 nm using Mn(II) as substrate (Kuwahara *et al*. 1984). Laccase activity was determined spectrophotometrically at 420 nm using guaiacol as substrate (Jiang *et al*. 2011).

Statistical analysis

The data of biomass and enzyme activity tests were statistically analyzed by SPSS 13.0, and all results were presented as mean of the triplicates ± standard deviation. When necessary, the results were analyzed by one-way analysis of variance.

RESULTS AND DISCUSSION

Biomass determination

As shown in Figure 1, the biomass of *A. oryzae* increased with the increase of GA concentration tested in this study during 6 days of culture, but *P. chrysosporium* grew poorly when the concentration of GA was over 2% (Figure 1(b)), indicating that *A. oryzae* 5992 was more tolerant to GA.

Comparison of the GA removal rate between *A. oryzae* 5992 and *P. chrysosporium* 40719

The degradation of different initial concentrations of GA by the two strains is shown in Figure 2. The removal rates of GA by the two strains at the concentrations of 1 and 2% were obviously higher than those at the concentrations of 3 and 4% (Figures 2(a) and (b)), indicating that a lower concentration of GA was more easily degraded by the two strains. The GA removal rate of *A. oryzae* 5992 was obviously higher than that of *P. chrysosporium* 40719 under each concentration of GA (Figures 2(a) and (b)), which was probably related to the higher biomass of *A. oryzae* 5992. The GA removal rate of *P. chrysosporium* reached 99% only with GA concentration lower than 2% (Figure 2(b)), whereas the removal rate of *A. oryzae* quickly increased in a linear manner and reached 99% with all the tested GA concentrations (Figure 2(a)). These results indicated that the ability of *A. oryzae* 5992 to degrade GA was stronger than that of *P. chrysosporium*, especially under a higher concentration of GA.

Enzyme activity determination

To reveal the underlying mechanism of the differential abilities in degrading GA between *A. oryzae* 5992 and, *P. chrysosporium* 40719, the activities of LiP, MnP, and laccase, which are closely related to aromatic compound degradation, were analyzed. The laccase activity of *A. oryzae* 5992 rapidly increased with the increase of GA concentration in the initial 4 days and reached peak value on the sixth day (Figure 3(a)). The laccase activity of *P. chrysosporium* 40719 was detected after 2 days of culture in the medium containing 2% GA (Figure 3(a)). Our results showed that *A. oryzae* 5992 secreted more laccase compared with *P. chrysosporium* 40719.

The LiP activity was detected in the *A. oryzae* 5992 culture on the first day of cultivation (Figure 3(a)), which might be derived from the seed culture. Figure 3(c) shows that although LiP activity of *A. oryzae* 5992 was not stable and fluctuated during the whole fermentation, it was obviously higher in the medium containing 3 and 4% GA than those containing 1 and 2% GA. The LiP activity of *P. chrysosporium* 40719 also appeared unsteady (Figure 3(d)) and was lower than that of *A. oryzae* 5992 at each gradient of GA.

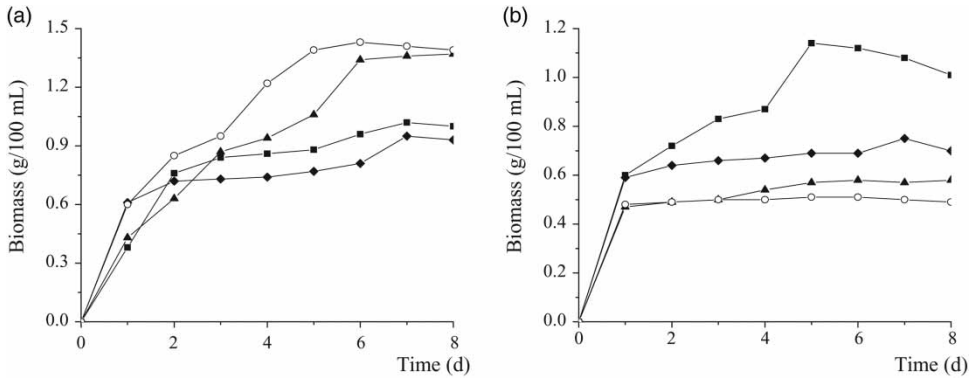

Figure 1 | The biomass of *A. oryzae* (a) and *P. chrysosporium* (b). ◆: 1% gallic acid; ■: 2% gallic acid; ▲: 3% gallic acid; ○: 4% gallic acid.

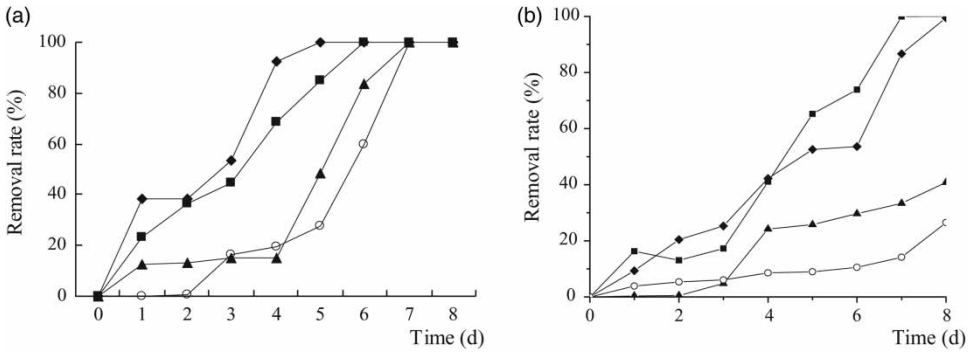

Figure 2 | Comparison of the removal rate of gallic acid by *A. oryzae* (a) and *P. chrysosporium* (b). ◆: 1% gallic acid; ■: 2% gallic acid; ▲: 3% gallic acid; ○: 4% gallic acid.

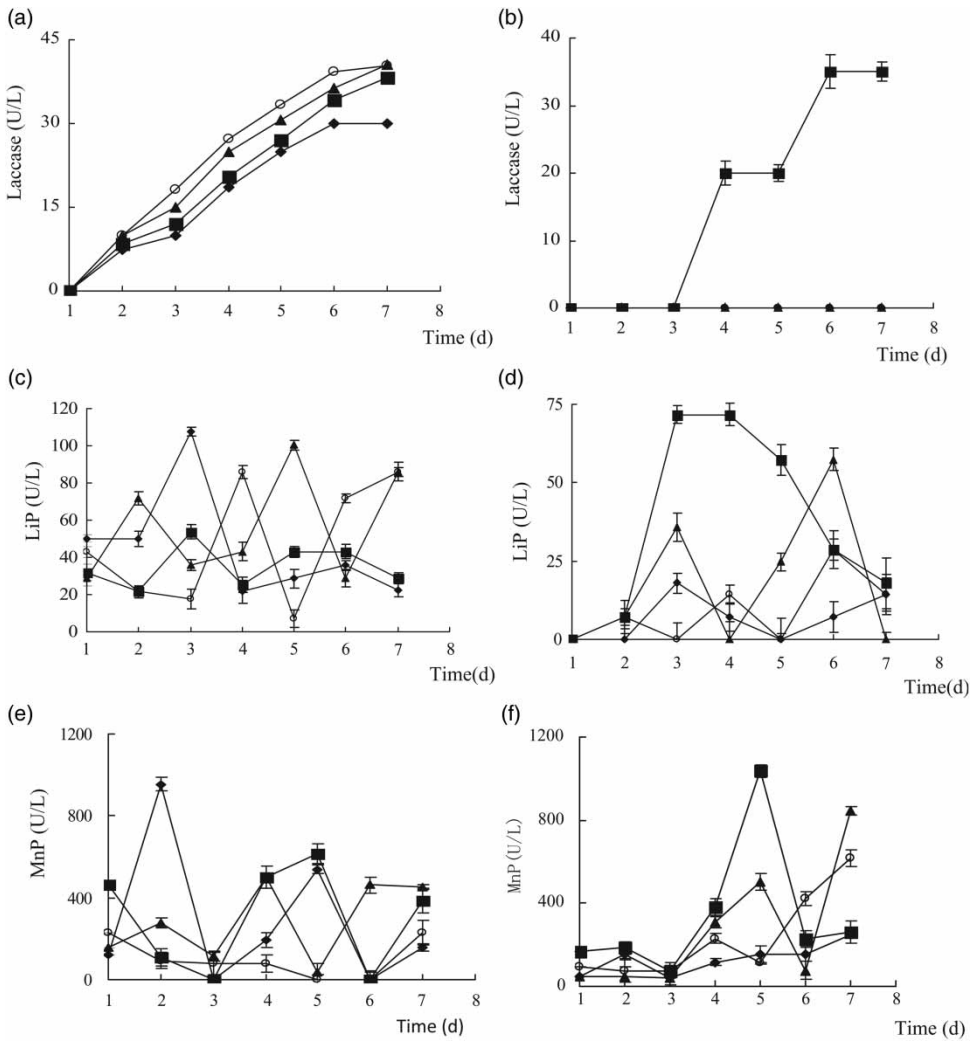

Figure 3 | The results of enzyme activities of laccase produced by *A. oryzae* (a); laccase produced by *P. chrysosporium* (b); LiP produced by *A. oryzae* (c); LiP produced by *P. chrysosporium* (d); MnP produced by *A. oryzae* (e); MnP produced by *P. chrysosporium* (f). ◆: 1% gallic acid; ■: 2% gallic acid; ▲: 3% gallic acid; ○: 4% gallic acid.

The MnP activity of *A. oryzae* peaked in the medium containing 1% GA on the second day (Figure 3(e)), which was far higher than that in the medium containing other concentrations of GA. The MnP activity from *P. chrysosporium* 40719 peaked on the fifth day in the medium containing 1, 2, and 3% GA, respectively, and the maximum activity (615.38 U/L) was obtained in the medium supplied with 2% GA (Figure 3(f)). Many studies have proven that MnP is hardly synthesized by *P. chrysosporium* in a Mn^{2+}-free medium (Rothschild *et al.* 1999; Gold *et al.* 2000; Hofrichter 2002). The wheat bran used to prepare seed culture in this study contained Mn^{2+}, which might have induced the synthesis of MnP during the preparation of seed culture.

MnP, LiP, and laccase are the three key enzymes involved in the degradation of aromatic compounds (Hatakka 1994). Laccase is a polyphenoloxidase that oxidizes phenol, polyphenol, pyrocatechol, and hydroquinol. Moreover, laccase is also capable of removing the methoxy group from the aromatic ring (Gianfreda *et al.* 1999; Kudanga *et al.* 2011). While LiP has the ability to non-specifically catalyze general types of substrates, including phenols, non-phenol aromatic compound, azodye, and anthraquinone dye, MnP is a kind of ectoenzyme relying on Mn(II) and H_2O_2 to catalyze phenol substrates (Evans *et al.* 1994; Susana *et al.* 2006; Tsukihara *et al.* 2008; Ren *et al.* 2009). *A. oryzae* 5992 produced a higher amount of active LiP and laccase but lower MnP compared with *P. chrysosporium* 40719, suggesting that the degradation of GA by *A. oryzae* 5992 relies mainly on LiP and laccase.

Analysis of the intermediate metabolites during GA degradation by *A. oryzae* 5992

The metabolites in the GA-containing fermentation broth of *A. oryzae* at different stages were qualitatively analyzed with LC-ESI-MS. In the TIC spectra of day 0 sample, only one peak at 3.46 min was observed, and based on the quasi-molecular ion m/z 169 ($[M\text{-}H]^-$) and a fragment ion at m/z 124 in the mass spectrum of the peak, it was deduced as the peak of GA. In the TIC (total ion current) spectra of samples on the fourth day and the eighth day, four peaks (retention time: 3.43, 3.551, 5.562, and 13.224 min, respectively) were found to be related to GA metabolism, and the peak at 3.43 min was presumed as GA. The quasi-molecular ion and fragment ion in the one-grade mass spectrum of these peaks are shown in Figure 4(a). In the

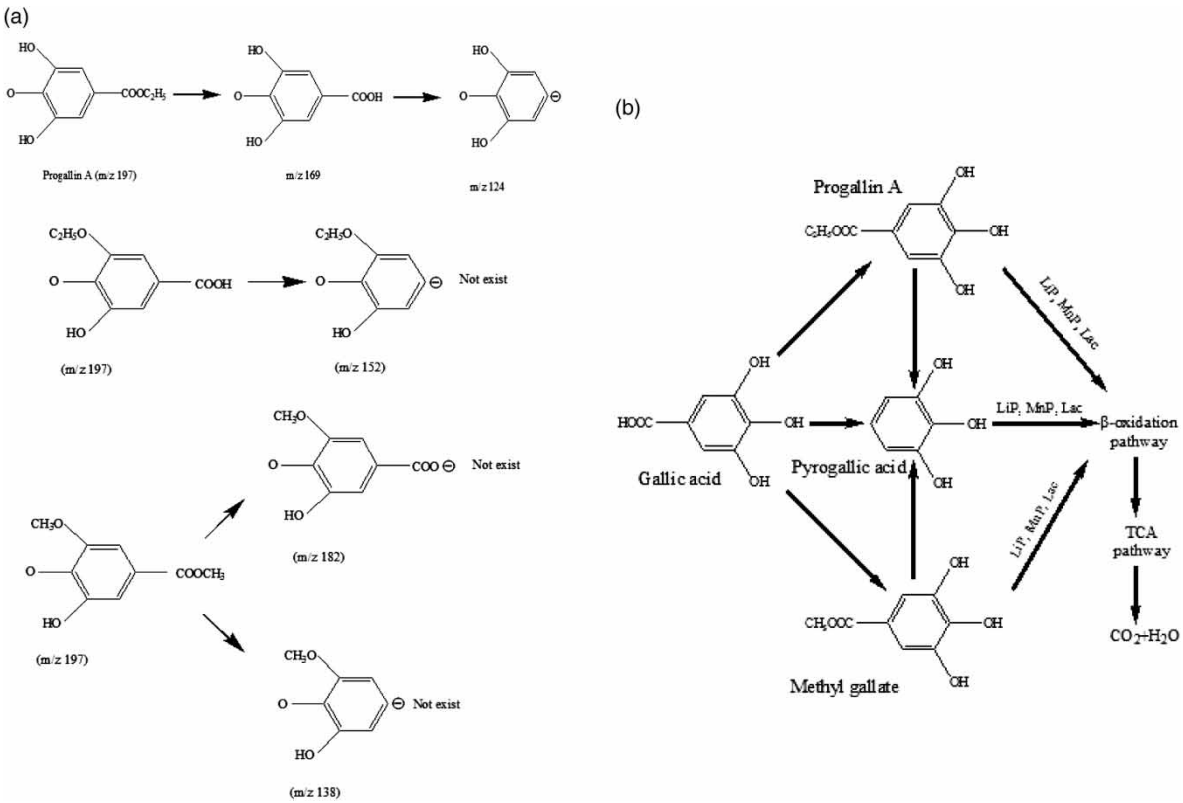

Figure 4 | Diagram of the process of determination of the peak (retention time = 13.224) (a) and the degradation pathway of gallic acid by *A. oryzae* (b).

one-grade mass spectrum of the peak at 13.224 min, the quasi-molecular ion m/z 197 ($[M-H]^-$) and a fragment ion at m/z 169 and m/z 124 were observed, pointing to the possibility of progallin A, since lack of the fragment ion at m/z 153, m/z 182, or m/z 138 in the one-grade mass spectrum (Figure 4(a)) ruled out the possible existence of an ethylated or methylated product of GA. Similarly, the peak at 5.562 min contained the quasi-molecular ion m/z 183, and a fragment ion at m/z 169 was recognized as methyl gallate, and the peak at 3.551 min containing quasi-molecular ion m/z 125 was pyrogallic acid. According to these data, the intermediate degradation metabolites of GA by *A. oryzae* included progallin A, methyl gallate, and pyrogallic acid, while other intermediated metabolites were almost undetectable.

Hypothetical degradation pathway of GA by *A. oryzae* 5992

To further elucidate the producing order of progallin A, methyl gallate, and pyrogallic acid during the degradation process, these intermediates instead of GA were added into the medium, respectively, and the fermentation broth was analyzed by LC-ESI-MS. When progallin A or methyl gallate was added into the medium, pyrogallic acid could be detected but GA could not be detected. When pyrogallic acid was added into the medium, GA, progallin A, and methyl gallate were not detected. These results indicated that the reactions from GA to progallin A, methyl gallate, and pyrogallic acid, and from progallin A and methyl gallate to pyrogallic acid were irreversible (Figure 4(b)). In all these reactions, the fact that no intermediate metabolites other than progallin A, methyl gallate, and pyrogallic acid were detected indicated that the subsequent reaction with these intermediate metabolites was ring-opening, which was possibly a rate-limiting reaction in GA biodegradation (Osono *et al.* 2009). LiP, MnP, and laccase have been proven to be involved in the ring-opening reaction (Pérez *et al.* 2002) that produces unsaturated fatty acid, which can then be oxidized into CO_2 and H_2O through β-oxidation and tricarboxylic acid cycle pathways. Because these subsequent reactions are conducted in the interior of mitochondria, the intermediate metabolites are impossible to detect. Based on this, a metabolic pathway of *A. oryzae* 5992 degrading GA is depicted in Figure 4(b).

Although we did not affirm whether the pyrogallic acid was derived from GA, progallin A, or methyl gallate, and whether the ring-opening reaction occurred with pyrogallic acid, progallin A, or methyl gallate in this study, one thing certain was that *A. oryzae* was unable to open the ring in GA unless the carboxyl of the molecule was protected or removed. It is well known that if a phenolic compound contains methyl group, its degradation by white rot fungi (such as *P. chrysosporium*) only occurs after the removal of the methyl group; however, there is no special requirement regarding the carboxyl in the molecule. This may be the most remarkable difference in the mechanism of GA degradation between the two strains in this study.

CONCLUSION

The results of biomass, removal rate of GA, enzymatic activities, and pathways involved in the degradation of different concentrations of GA in this study revealed that the significant differences in GA degradation between the two strains are the following: (1) *A. oryzae* 5992 grows well in the presence of 1–4% GA and can achieve a 99% removal rate of GA; *P. chrysosporium* 40719 cannot grow when the concentration of GA is higher than 2%; (2) the increase in laccase activity and biomass of *A. oryzae* 5992 is synchronous, but the increase of laccase of *P. chrysosporium* 40719 lagged behind the growth of mycelia; the LiP activity in *A. oryzae* 5992 is far higher than that in *P. chrysosporium* 40719; although the maximum MnP activity shows no significant difference between the two strains, *A. oryzae* 5992 reaches the maximum in a shorter time; and (3) pyrogallic acid, progallin A, and methyl gallate are the three intermediate metabolites of GA degradation, while no other metabolites are detectable, implying that there are some differences in GA degradation pathways between the two strains.

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