Manganese-enhanced degradation of lignocellulosic waste by *Phanerochaete chrysosporium*: evidence of enzyme activity and gene transcription

Chao Huang[a,b], Cui Lai[a,b], Guangming Zeng[*a, b], Danlian Huang[*a, b], Piao Xu[a,b], Chen Zhang[a,b], Min Cheng[a,b], Jia Wan[a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, China.

[b] Key Laboratory of Environmental Biology and Pollution Control, Ministry of Education, Hunan University, Changsha 410082, China.

* Corresponding authors. Address: College of Environmental Science and Engineering, Hunan University, Changsha 410082, China. Tel.: +86 731 88823701; fax: +86 731 88823701.

E-mail addresses: zgming@hnu.edu.cn (G. Zeng); huangdanlian@hnu.edu.cn (D. Huang).

Abstract

Lignolytic fungi initiate lignocellulose decay by producing extracellular oxidative enzymes. For better understanding the enzymatic degradation of lignocellulose by white-rot fungi, we investigated the effect of manganese on the organic matter loss, manganese peroxidase (MnP) activity and manganese peroxidase gene (*mnp*) transcription levels during solid-state fermentation of rice straw with *Phanerochaete chrysosporium*. The results showed that the addition of manganese improved MnP activity and made it reach the peak earlier, promoted fungal growth at the early period (0-9 d) and enhanced the degradation of lignocellulosic waste. The total organic matter loss had a good correlation with fungal biomass during 30 days of cultivation and manganese amendment promoted the ability of *P. chrysosporium* to degrade lignocellulose. Quantitative real-time RT-PCR revealed the differential expression of *mnp1*, *mnp2* and *mnp3*: manganese amendment increased the transcription of *mnp1* and *mnp2* but not *mnp3*. The results indicated that manganese stimulated *mnp* transcription levels and played a post-transcriptional role in MnP production. These findings provide opportunity of development in enzymatic degradation of lignocellulosic waste by *P. chrysosporium* amended with manganese.

Key words Manganese · Lignocellulose · *Phanerochaete chrysosporium* · Manganese peroxidase · Gene transcription

Introduction

Lignocellulose, one of the most abundant renewable sources of carbon, is composed of lignin, cellulose and hemicellulose. Lignocellulosic waste, including agricultural wastes, forestry wastes and agro-industrial residues, can potentially be harnessed to produce value-added products such as biofuels and biochemicals (Da Silva et al. 2014). However, lignin, which accounts for up to 30 percent of the dry biomass weight, is a heterogeneous and highly branched polymer of phenylpropane units that provides strength and rigidity to wood, protecting most of the cellulose and hemicellulose against enzymatic hydrolysis (Pérez et al. 2002). While it is difficult to degrade due to its chemical and structural properties, lignin is identified as a rate-limiting barrier in biodegradation of lignocellulosic waste. Therefore, much attention has been paid to the microorganisms that can effectively degrade lignin by producing ligninolytic enzymes during the last few years (Hobara et al. 2014; Mathews et al. 2015).

White-rot fungi are featured by their distinctive set of extracellular oxidative enzymes that enable them to degrade lignin effectively, along with related compounds found in environmental pollutants, including pesticides (Tang et al. 2008; Zeng et al. 2013a), dyes (Gong et al. 2009) and toxic wastes (Fan et al. 2008; Lai et al. 2016; Xu et al. 2012; Zeng et al. 2013b). *Phanerochaete chrysosporium*, the most in-depth studied white-rot fungus, secretes a series of oxidases and peroxidases that are in charge of producing highly reactive and nonspecific free radicals capable of completely degrading lignocellulose. Extracellular oxidative enzymes in *P. chrysosporium* mainly include lignin peroxidase (LiP, EC1.11.1.14),

manganese peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC1.11.1.16) (Kersten and Cullen 2007; Zhang et al. 2007). Over the last 30 years, extensive development has been achieved in understanding the enzymology and molecular genetics of lignocellulose degradation (Korripally et al. 2015; MacDonald et al. 2012). MnP is the most common lignin-modifying peroxidase produced by almost all white-rot fungi, while LiP is not, suggesting that MnP plays a more crucial part in fungal lignin decomposition than LiP (Floudas et al. 2012).

MnP is a heme-containing peroxidase that can oxidize Mn^{2+} to Mn^{3+}, utilizing hydrogen peroxide (H_2O_2) as an oxidant (Martínez 2002). The generated Mn^{3+} is unstable and can be stabilized by chelating organic acids like citrate and malate (Furukawa et al. 2014). The formed Mn^{3+}-chelator compound is a highly reactive and non-specific oxidant which can oxidize a wide range of monomeric and dimeric phenols such as phenolic lignin model compounds via one-electron oxidative pathways (Wong 2009). For example, a reaction system composed of MnP, oxalate, Mn^{2+}, and H_2O_2 catalyzes C_α-C_β cleavage, C_α oxidation and alkyl-aryl cleavage of phenolic syringyl type β-1 and β-O-4 lignin structures (Higuchi 2004). In *P. chrysosporium*, MnP isozymes are encoded by a family of 3 main structurally related genes (*mnp1*, *mnp2* and *mnp3*) and 2 new genes obtained by BLAST searches of the genome (Martinez et al. 2004). Previous works have revealed differential expression of *mnp* genes in response to culture conditions such as nutrient nitrogen levels (Li et al. 1994), Mn^{2+} (Knop et al. 2014), carbon source and heat shock (Brown et al. 1993). Researches on ligninolytic fungi such as *P. chrysosporium* have demonstrated the correlation between extractable MnP activity and *mnp* transcript level in the presence of manganese, suggesting a

transcriptional role of Mn^{2+}. However, there is so far little information in the literature on the relationships among the regulation of MnP, *mnp* transcript level and lignocellulose decomposition.

The present study seeks to further characterize the manganese-amended enzymatic degradation of lignocellulose in solid-state fermentation (SSF) with *P. chrysosporium*. In addition to investigating the effect of manganese on the loss of total organic matter, as well as the changes of MnP activity and fungal biomass, this work also employs real-time quantitative RT-PCR to quantify the differential expression of *mnp* genes from *P. chrysosporium*. Besides, a correlation analysis between total organic matter loss and fungal biomass, *mnp* gene transcript level and MnP activity was performed.

Materials and Methods

Fungal strain, media, and culture conditions

P. chrysosporium BKM-F-1767 (ATCC 24725) was purchased from the China Center for Type Culture Collection (Wuhan, China). The strain was kept on potato dextrose agar (PDA) slants at 4 °C and transferred to PDA plates at 37 °C before use. Spore suspensions were prepared by diluting fungal spores from plates in sterile water and then adjusted to a concentration of 2.0×10^6 CFU/mL as described previously (Huang et al. 2008b).

Solid-state fermentation and sampling

The rice straw obtained locally was air-dried, chopped and ground to pass a 2-mm-pore-size screen. The main chemical composition of this rice straw was cellulose 39.2%, hemicellulose 25.9% and lignin 12.6%. SSF was conducted in 500-mL flasks containing 30 g straw powder

and 80 mL deionized water in either the presence (treatment group) or absence (control group) of $MnSO_4$ (2 mM final concentration). Each flask was mixed thoroughly and autoclaved for 30 min at 121 °C. After cooling down, it was inoculated with 10 mL spore suspensions at room temperature. The whole fermentation process was operated at 37 °C for 30 days with a constant moisture content of 75% controlled by humidifier. Samples were taken on day 0, 3, 6, 9, 12, 18, 24 and 30. All experiments were performed in triplicate.

Total organic matter and pH determination

The total organic matter (TOM) was determined by a classical loss-on-ignition method. 2 g of sample was oven-dried immediately after collection at 105 °C for 6 h and then heated in a muffle furnace at 550 ± 10 °C for 6 h to a constant weight. The TOM was calculated as the difference between the pre- and post-ignition sample weights. The TOM loss was calculated from the difference of TOM between two adjacent sampling dates. Suspension at a 1: 10 (w:v) ratio of sample-to-water was shaken at 180 rpm for 30 min and then centrifuged at 5000×g for 10 min. The supernatant was used for pH analysis by a Mettler Toledo FE 20 pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China).

Manganese peroxidase assay

Samples were extracted immediately after collection with sterile distilled water at a ratio of 1: 10 (w/v) agitated on a rotary shaker at 180 rpm for 30 min and then centrifuged at 3500×g for 15 min. The supernatant collected was then filtered through a 0.45-μm membrane and used for enzyme activity analysis. MnP activity was monitored by measuring the rate of phenol red oxidation at 431 nm (Roy and Archibald 1993) with a Shimadzu 2550 (Japan) UV-visible

spectrophotometer. The reaction mixture composed of 0.2 mM $MnSO_4$, 0.1 mM H_2O_2, 0.067 mM phenol red, 50 mM sodium malonate (pH 4.5) and a 500 μL aliquot of enzyme extract. One unit (U) of MnP was defined as the amount of enzyme required for oxidation of 1 μmol phenol red per minute at 30 °C.

Fungal biomass estimate

The fungal biomass was estimated by the extraction and quantification of ergosterol (Klamer and Baath 2004). The extraction method consisted of mixing 0.5 g sample, 1 mL cyclohexane and 4 mL 10% (w/v) KOH dissolved in methanol followed by sonication at maximum power for 15 min. The mixture was incubated in a water bath at 70 ºC for 90 min and then 1 mL deionized water and 2 mL cyclohexane were added. Samples were centrifuged at 3000×g for 5 min and the upper phase was transferred to new tubes. The lower phase was washed with 2 mL cyclohexane again and the two upper phases were combined, and evaporated under a stream of nitrogen at 40 °C. The samples were dissolved in 1 mL methanol and filtered through a 0.45-μm membrane and analyzed in an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) with a C18 column and a UV detector set to 282 nm.

RNA extraction and qRT-PCR

Total RNA of samples was extracted from mycelia obtained during SSF using Trizol reagent (Invitrogen Corp., Carlsbad, CA) as described previously (Barrios-González et al. 2008). After treatment with DNase I (Promega, Madison, WI) at 37 ºC for 30 min to remove genome DNA, RNA samples were quantified by spectrophotometry and stored at -70 ºC until use.

The RNA samples were reverse transcribed using the RevertAid^{TM} First Strand cDNA

Synthesis Kit (Fermentas Life Sciences, Ontario, USA) following the manufacturer's instructions. Relative quantification of transcripts *mnp1*, *mnp2* and *mnp3* from *P. chrysosporium* was conducted in real-time PCR using the Maxima SYBR Green qPCR Master Mixes Kit (Fermentas Life Sciences, Ontario, USA) and iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). Primers for the *P. chrysosporium* genes *mnp1* (m77513), *mnp2* (L29039) and *mnp3* (U70998) were designed using Beacon Designer 7.8 (Premier Biosoft, Palo Alto, CA) and synthesized by Sangon Biotech (Shanghai, China). Primer sequences, predicted T_m values and amplicon length are shown in Table 1. β-actin mRNA was used for normalization in the gene expression experiments. The amplification without a template served as a control. The qRT-PCR mixture contained 2.0 μL of cDNA sample, 12.5 μL of 2 × Maxima SYBR Green qPCR Master Mix (Fermentas Life Sciences, Ontario, USA), 0.5 μL of each gene specific primer (10 μM) and 10 μL of nuclease-free water in a final volume of 25 μL. The PCR amplification conditions consisted of 2 min of initial denaturation at 95 ºC followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing at 55 ºC for 30 s, and final extension at 68 ºC. Amplification specificity was confirmed by melting-curve analysis performed from 55 ºC to 95 ºC with stepwise fluorescence acquisition. C_T values were acquired using the automated threshold determination feature of the Bio-Rad iQ5 Software. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), and were expressed as a ratio of target gene expression to equally expressed *β*-actin gene. Data were presented as means of triplicate PCRs.

159 **Statistical analysis**

160 The results were presented with the means values of three replicates and standard deviations
161 of the mean (SD). Difference in parameters were evaluated with a one-way analysis of
162 variance (ANOVA) using a *p*-value of 0.05 to determine significance. Regression analysis
163 was used to examine the relationships between the total organic matter loss and fungal
164 biomass. The analyses were performed using the software package SPSS 18.0 for windows
165 (SPSS Inc., Chicago, IL).

166 **Results**

167 **Time-course of pH and manganese peroxidase activity**

168 As shown in Fig. 1, the pH increased rapidly from initial value of 6.42 ± 0.04 to 7.17 ± 0.12
169 during the first 3 days of SSF in the control, then declined steadily, followed by a slight rise
170 and maintained a stable value of 7.0-7.2 after 9 days. With the supplement of manganese, the
171 pH declined from 6.38 ± 0.06 to 5.98 ± 0.18 during the first 3 days, then rose gradually until
172 day 18 (6.73 ± 0.11) followed by a slight decrease afterward with the final value of 6.51 ± 0.11
173 (day 30). It was clearly observed that the pH in the treatment was lower than in the control
174 during the whole SSF process ($P = 0.044$).

175 The time-course of MnP activity during SSF of straw by *P. chrysosporium* is presented in
176 Fig. 1. MnP activity showed the highest peak value (7.15 ± 0.15 U/g) on day 3 in supplement
177 with manganese, while in the control it reached a peak (6.11 ± 0.76 U/g) on day 6. After 6
178 days of SSF, MnP activity gradually decreased to a low point until day 12 with the value of
179 2.00 ± 0.78 U/g for the treatment and 1.03 ± 0.28 U/g for the control, respectively.

Subsequently, the levels of MnP activity reached a peak on day 18 and followed by gradual decline until the end of SSF (day 30). No significant difference is found between the MnP activity of the two groups after 6 days of SSF ($P > 0.05$).

Biodegradation of organic matter during SSF process

It was suggested that the TOM content decreased during the whole process of SSF (Fig. 2). The TOM levels were 83-84% (w/w) at the beginning of SSF and declined quickly during the first 9 days, followed by a gently descending until day 24 and an obviously decrease from day 24 to 30. There was a significant difference ($P = 0.049$) between the TOM content in the treatment and the control after 30 days, which was $63.80 \pm 3.77\%$ and $68.70 \pm 3.18\%$, respectively. The TOM loss reached the maximum value on day 3 and gradually decreased in the early stage (day 3 to day 12) of SSF, and maintained a low value from day 12 to day 24 followed by a great increase from day 24 to day 30 for both groups. The value of TOM loss was significantly higher in the treatment than in the control on day 3, 6 and 9 ($P = 0.039$, 0.037 and 0.049, respectively). Nevertheless, there was insignificant difference between the TOM loss of two groups after 12 days of SSF.

The dynamic changes of fungal biomass

Fungal biomass is expressed as ergosterol content per gram dry weight of straw in this study. The variation in the ergosterol content of two groups displayed the similar trend (Fig. 3), which increased rapidly during first 3 days followed by a fast decline with the time, then rose from day 12 to 18, day 24 to 30 and decreased from day 18 to 20. Ergosterol content showed the highest peak on day 3 with the value of 0.53 ± 0.02 mg/g and 0.46 ± 0.02 mg/g for the

treatment and the control, respectively, and was comparatively higher in the treatment than in the control at the early period (day 3, 6 and 9) of SSF (P = 0.040, 0.046 and 0.043, respectively), but no significant differences were found after 12 days ($P > 0.05$).

The correlation between total organic matter loss and fungal biomass

To estimate the fungal role in lignocellulose decomposition mostly associated with TOM loss, the relationship between TOM loss and ergosterol content was analyzed (Fig. 4). It was interesting to find a significant positive linear correlation between TOM loss and ergosterol content in the treatment and the control ($R^2 = 0.9594$, $P < 0.001$ and $R^2 = 0.9335$, $P < 0.001$, respectively), and the regression line has a higher slope for the treatment (solid line) than for the control (dashed line).

Quantification of *mnp* gene expression

For analyzing the transcript levels of manganese peroxidase genes affected by Mn^{2+} during SSF, we monitored the time-course (3–30 d) of expression changes of the three *mnp* genes by quantitative RT-PCR using specific primers (Table 1). The transcript level for each isoform varied over time (Fig. 5). Transcript levels of isoforms *mnp1* and *mnp2* displayed a similar overall pattern that decreased at the early stage (3-9 d) and then increased to a higher level during the later phase, which differed from that of *mnp3*. In the case of *mnp3*, the corresponding level did not show an obvious alteration until day 18, whose level was 3.42- and 3.31-fold on day 18 as compared to day 3 in the treatment and the control, respectively. In addition, manganese availability increased the transcript levels of *mnp1* and *mnp2* to values that were 3- to 10-fold and 5- to 12-fold higher than the control, respectively. In turn, *mnp3*

gene expression showed no apparent difference to manganese amendment and seemed to be regulated in a Mn-independent manner.

Discussion

The effect of manganese on pH and manganese peroxidase activity

SSF is defined as the fermentation process in which microorganisms grow on solid substrates in the absence of free liquid (Diaz et al. 2016). It has shown a great promise for its advantage in production of many value added products like enzymes. pH is an important factor in SSF process by affecting the production and secretion of lignocellulose degrading enzymes (Rollins and Dickman 2001). The dynamics of pH during SSF was mainly due to the release of organic acids and ammonia (Hölker and Lenz 2005). In this study, it was observed that the pH in the treatment with manganese was below (P = 0.044) that in the control during the whole period of SSF (Fig. 1), which suggested that the addition of manganese led to the pH decrease. The pH increase from day 0 to day 3 in the control was mainly attributed to the fast mineralization of organic nitrogen and the release of ammonia in this period, which was also observed by Zhang et al. (2015) and Li et al. (2011). In contrast, the addition of Mn^{2+} would improve the secretion and accumulation of organic acids to reduce its activity through complexation thus led to a decrease of pH at the initial stage in the treatment (Horst et al. 1999).

Our previous study confirmed the important role of MnP in biodegrading lignocellulose (Huang et al. 2008a). In order to optimize the production of MnP, we employed the manganese-amended SSF of rice straw to investigate the effect of manganese. The result

suggested that manganese improved the activity of MnP during the first 3 days of SSF and made it reach the peak earlier (Fig. 1). However, no significant effect of manganese was found on the MnP activity on the later phase. One possibility is that lower pH due to the addition of manganese at the initial stage coincided with the optimum pH (3.5 to 6.0) for MnP (Mielgo et al. 2003). Alternatively, the increased fungal biomass resulting from the presence of Mn^{2+} may also contribute to the production of MnP and thus lead to a higher MnP activity. Besides, manganese stimulates the secretion of MnP already synthesized and retained within the cell to the extracellular medium (Mancilla et al. 2010). The decay of lignocellulose by *P. chrysosporium* is induced not directly by the secreted extracellular oxidative enzymes (such as MnP) because of its high-molecular-weight, instead, the reactive oxygen species produced by those enzymes are responsible for it. The initial stage of high MnP production in this study corresponds to the "colonization phase", which is relevant to the initial opening of the cell wall structure and lignin modification by small active substances such as reactive oxygen species, whereas the subsequent stage corresponds to the ''degradation phase" which represents fungal attack on lignin. (Robertson et al. 2008)

Relationship between total organic matter loss and fungal biomass

SSF is considered as an attractive alternative technology for treatment of lignocellulosic waste. TOM loss in SSF of straw is mainly attributed to the fungal decomposition of lignocellulose (Zhang et al. 2014), the decrease in TOM can reflect the degree of lignocellulose degradation. Significant higher TOM loss due to manganese amendment was detected in the early stage (0-9 d) of this experiment (Fig. 2). Manganese acts as a mediator, inducer and substrate for MnP, and promotes MnP production by *P. chrysosporium* (Fig. 1) that leads to enhanced

enzymatic degradation of lignocellulose. The higher TOM loss in the late stage (day 24 to 30) is caused by lignin degradation by *P. chrysosporium* during secondary metabolism. It is interesting to note that TOM loss shows similar trends to MnP activity in the first 12 days, which indicates the role of MnP in TOM loss and the positive effect of manganese on lignocellulose degradation. The different trends of them during the later phase may be attributed to the involvement of other ligninolytic enzymes, such as LiP, the activity of which did not reach the maximal level until day 24 (Supplementary Fig. S1), which was also observed in our previous study (Huang et al. 2010). It is suggested that the two extracellular oxidative enzymes of MnP and LiP may function at different period of lignocellulose modification process, which is supported by a report of Kang et al. (2010) that *mnp* gene is produced prior to other ligninolytic-enzyme-encoding genes.

Ergosterol, the predominant sterol in fungal cell membranes, is used as an indicator of fungal biomass (Robertson et al. 2008). The fungal colonization is mostly associated with the available nutrient and is greatly facilitated at the early stage of SSF as a result of sufficient nutrient. In this work, the results indicated that manganese could promote the *P. chrysosporium* growth and colonization in the initial stage of solid-state fermentation (Fig. 3), which was confirmed by a previous study (Cohen et al. 2002). *P. chrysosporium* is able to degrade and utilize cellulose, hemicellulose and lignin as carbon and energy sources. The degraded materials in substrate during SSF by *P. chrysosporium* vary from stage to stage and are hemicellulose, cellulose, and lignin in turn (Huang et al. 2008b). Their degradation at different stages will affect the growth of *P. chrysosporium* and thus contributes to the dynamic changes of fungal biomass (Fig. 3).

287 Correlation analysis of TOM loss and ergosterol content showed that TOM loss was
288 significantly positive correlated with fungal biomass. As shown in Fig. 4, the linear fit has a
289 higher slope for the treatment (solid line) than for the control (dashed line) indicating that
290 manganese enhances the ability of *P. chrysosporium* to degrade organic matter, which is
291 coincident with the results presented in Fig. 2. It is suggested that manganese amendment
292 results in an increase in production of MnP by *P. chrysosporium* and then leads to promote the
293 enzymatic hydrolysis of lignocellulose.

294 **Quantitative analysis of *mnp* gene expression**

295 Although differential expression of *mnp* genes from *P. chrysosporium* in response to
296 manganese amendment has been previously reported in defined liquid media (Furukawa et al.
297 2014; Gettemy et al. 1998; Janusz et al. 2013), the enzymatic and genetic mechanism
298 underlying the decomposition of lignocellulosic waste by *P. chrysosporium* is quite complex
299 and many uncertainty still remains with respect to the process of lignocellulose mineralization.
300 Quantitative analysis of *mnp* genes transcript in this work revealed the physiological status of
301 *P. chrysosporium* not just its presence. Higher transcript levels of *mnp* genes (*mnp1* and *mnp2*)
302 and extractable MnP activity were obtained due to the addition of manganese, suggesting that
303 the MnP isozymes were differentially regulated by manganese and MnP1 together with MnP2
304 might be the primary MnP isozymes which was in agreement with a previous report (Gettemy
305 et al. 1998).

306 Compared with the cumulative levels of the three *mnp* transcripts, MnP activity reached its
307 peak (on day 6 without manganese, Fig. 1) later than the abundance of *mnp* transcripts which
308 peaked on day 3 at the early fermentation stage. It was suggested that the MnP activity

displayed a slower delay than *mnp* mRNA levels. This might be attributed to a delay between transcription and translation. Furthermore, the enzyme assayed here only referred to the extracellular enzyme, that fraction of the translated enzyme that had not yet been secreted was excluded, thus resulting in an underestimate of the total enzyme produced. Manganese amendment raised *mnp1* and *mnp2* gene transcription level and advanced the peak of MnP activity (Fig. 1 and 5), which indicated not only its function of the transcriptional stimulation but also a post-transcriptional role of manganese in secretion of active MnP from intracellular to extracellular space (Janusz et al. 2013). During the later phase of fermentation, the transcription of *mnp* genes may be attributed to carbon and nitrogen consumption resulting from lignin degradation by *P. chrysosporium* (Furukawa et al. 2014). However, this study only focused on *mnp* genes and cannot fully reveal the fungal metabolism of lignocellulose that should be focused on in future research.

Previous observations have demonstrated that the 5′-upstream region of the ligninolytic enzyme genes contain several putative *cis*-acting elements, such as the CCAAT-boxes, metal responsible elements (MREs), cAMP response elements (CREs), heat shock elements (HSEs), and a binding site for activator protein 2 (AP-2) (Janusz et al. 2013; Tello et al. 2000). The regulation of expression of MnP is dependent on Mn, and putative MREs following the consensus sequence of TGCRCNC have been found in *mnp* genes of *P. chrysosporium* (Fig. 6). These sequences that were identified in mammalian metallothionein genes are responsible for binding and transferring heavy metal iron such as Zn^{2+} and Cd^{2+} (Thiele 1992). In *P. chrysosporium*, paired MREs have been identified in the *mnp1* and *mnp2* promoters, while being absent in the *mnp3* promoter (dotted box in Fig. 6), which originally suggested the role

of MREs in expression of MnP induced by manganese. However, a research focused on the *mnp1* promoter of *P. chrysosporium* described a novel promoter sequence of a 48-bp fragment responsible for manganese dependent regulation of *mnp1* and the putative MREs were not involved (Ma et al. 2004). The same result was found in *T. versicolor* that the MREs sequences were not required for manganese dependent regulation of *mnp* gene expression (Johansson et al. 2002). To date, the role of regulatory elements in the expression of MnP is not fully understood, and need to be further explored to achieve an efficient production of MnP for biotechnological applications.

In conclusion, we studied the manganese-amended enzymatic degradation of lignocellulose in solid-state fermentation by *P. chrysosporium*. The addition of manganese improved the activity of MnP during the first 3 days and made it reach the peak earlier, promoted the fungal growth in the initial stage (0-9 d), enhanced the decomposition of lignocellulose and induced the expression of *mnp1* and *mnp2* but not *mnp3*. The results suggested roles of manganese both in transcriptional stimulation and as a post-transcriptional factor responsible for the production and secretion of MnP. A good correlation between organic matter loss and ergosterol content was found, indicating a fungal role for lignocellulose decomposition. The present work will provide an alternative to enhance the enzymatic degradation of lignocellulosic waste by *P. chrysosporium*. Further efforts are needed in future to improve the conversion of lignocellulosic biomass to reducing sugars that can be fermented by microbes to produce renewable fuel such as ethanol.

Compliance with ethical standards

Funding This work is financially supported by the Program for the National Natural Science Foundation of China (51378190, 51278176, 51408206, 51579098 and 51521006), the National Program for Support of Top-Notch Young Professionals of China (2014), Hunan Provincial Science and Technology Plan Projects (No.2016RS3026), the Program for New Century Excellent Talents in University (NCET-13-0186), the Program for Changjiang Scholars and Innovative Research Team in University (IRT-13R17) and Scientific Research Fund of Hunan Provincial Education Department (No.521293050).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. The article is an original paper, is not under consideration by another journal, and has not been published previously. All authors read and approved the final manuscript.

References

Barrios-González J, Baños J, Covarrubias A, Garay-Arroyo A (2008) Lovastatin biosynthetic genes of *Aspergillus terreus* are expressed differentially in solid-state and in liquid submerged fermentation. Appl Microbiol Biotechnol 79(2):179-186

Brown JA, Li D, Alic M, Gold MH (1993) Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium*. Appl Environ Microbiol 59(12):4295-4299

Cohen R, Persky L, Hazan-Eitan Z, Yarden O, Hadar Y (2002) Mn^{2+} alters peroxidase profiles

and lignin degradation by the white-rot fungus *Pleurotus ostreatus* under different nutritional and growth conditions. Appl Biochem Biotech 102(1):415-429

Da Silva TL, Gouveia L, Reis A (2014) Integrated microbial processes for biofuels and high value-added products: the way to improve the cost effectiveness of biofuel production. Appl Microbiol Biotechnol 98(3):1043-1053

Diaz AB, Blandino A, Webb C, Caro I (2016) Modelling of different enzyme productions by solid-state fermentation on several agro-industrial residues. Appl Microbiol Biotechnol 100(22):9555-9566

Fan T, Liu YG, Feng BY, Zeng GM, Yang CP, Zhou M, Zhou HZ, Tan ZF, Wang X (2008) Biosorption of cadmium(II), zinc(II) and lead(II) by *Penicillium simplicissimum*: Isotherms, kinetics and thermodynamics. J Hazard Mater 160(2-3):655-661

Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martínez AT, Otillar R, Spatafora JW, Yadav JS (2012) The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336(6089):1715-1719

Furukawa T, Bello F, Horsfall L (2014) Microbial enzyme systems for lignin degradation and their transcriptional regulation. Front Biol 9(6):448-471

Gettemy JM, Ma B, Alic M, Gold MH (1998) Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. Appl Environ Microbiol 64(2):569-574

Gong JL, Wang B, Zeng GM, Yang CP, Niu CG, Niu QY, Zhou WJ, Liang Y (2009) Removal of cationic dyes from aqueous solution using magnetic multi-wall carbon nanotube nanocomposite as adsorbent. J Hazard Mater 164(2-3):1517-1522

394 Higuchi T (2004) Microbial degradation of lignin: role of lignin peroxidase, manganese
395 peroxidase, and laccase. Proc Jpn Acad Ser B 80(5):204-214
396 Hölker U, Lenz J (2005) Solid-state fermentation — are there any biotechnological
397 advantages? Curr Opin Microbiol 8(3):301-306
398 Hobara S, Osono T, Hirose D, Noro K, Hirota M, Benner R (2014) The roles of
399 microorganisms in litter decomposition and soil formation. Biogeochemistry
400 118(1-3):471-486
401 Horst WJ, Fecht M, Naumann A, Wissemeier AH, Maier P (1999) Physiology of manganese
402 toxicity and tolerance in *Vigna unguiculata* (L.) Walp. J Plant Nutr Soil Sc
403 162(3):263-274
404 Huang D, Zeng G, Peng Z, Zhang P, Hu S, Jiang X, Feng C, Chen Y (2008a)
405 Biotransformation of rice straw by *Phanerachaete chrysosporium* and the related
406 ligninolytic enzymes. Int J Biotechnol 10(1):86-92
407 Huang DL, Zeng GM, Feng CL, Hu S, Jiang XY, Tang L, Su FF, Zhang Y, Zeng W, Liu HL
408 (2008b) Degradation of lead-contaminated lignocellulosic waste by *Phanerochaete*
409 *chrysosporium* and the reduction of lead toxicity. Environ Sci Technol 42(13):4946-4951
410 Huang DL, Zeng GM, Feng CL, Hu SA, Zhao MH, Lai C, Zhang Y, Jiang XY, Liu HL (2010)
411 Mycelial growth and solid-state fermentation of lignocellulosic waste by white-rot fungus
412 *Phanerochaete chrysosporium* under lead stress. Chemosphere 81(9):1091-1097
413 Janusz G, Kucharzyk KH, Pawlik A, Staszczak M, Paszczynski AJ (2013) Fungal laccase,
414 manganese peroxidase and lignin peroxidase: Gene expression and regulation. Enzyme
415 Microb Tech 52(1):1-12

Johansson T, Nyman PO, Cullen D (2002) Differential regulation of *mnp2*, a new manganese peroxidase-encoding gene from the ligninolytic fungus *Trametes versicolor* PRL 572. Appl Environ Microbiol 68(4):2077-2080

Kang Y, Prewitt L, Diehl S, Nicholas D (2010) Screening of basidiomycetes and gene expression of selected lignin modifying enzymes of *Phlebia radiata* during biodeterioration of three wood types. Int Biodeter Biodegr 64(7):545-553

Kersten P, Cullen D (2007) Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. Fungal Genet Biol 44(2):77-87

Klamer M, Baath E (2004) Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biol Biochem 36(1):57-65

Knop D, Ben-Ari J, Salame TM, Levinson D, Yarden O, Hadar Y (2014) Mn^{2+}-deficiency reveals a key role for the *Pleurotus ostreatus* versatile peroxidase (VP4) in oxidation of aromatic compounds. Appl Microbiol Biotechnol 98(15):6795-6804

Korripally P, Hunt CG, Houtman CJ, Jones DC, Kitin PJ, Cullen D, Hammel KE (2015) Regulation of gene expression during the onset of ligninolytic oxidation by *Phanerochaete chrysosporium* on spruce wood. Appl Environ Microbiol 81(22):7802-7812

Lai C, Wang MM, Zeng GM, Liu YG, Huang DL, Zhang C, Wang RZ, Xu P, Cheng M, Huang C, Wu HP, Qin L (2016) Synthesis of surface molecular imprinted TiO2/graphene photocatalyst and its highly efficient photocatalytic degradation of target pollutant under visible light irradiation. Appl Surf Sci 390:368-376

Li D, Alic M, Gold MH (1994) Nitrogen regulation of lignin peroxidase gene transcription.

438 Appl Environ Microbiol 60(9):3447-3449

439 Li NJ, Zeng GM, Huang DL, Hu S, Feng CL, Zhao MH, Lai C, Huang C, Wei Z, Xie GX
440 (2011) Oxalate production at different initial Pb^{2+} concentrations and the influence of
441 oxalate during solid-state fermentation of straw with *Phanerochaete chrysosporium*.
442 Bioresource Technol 102(17):8137-8142

443 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time
444 quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25(4):402-408

445 MacDonald J, Suzuki H, Master E (2012) Expression and regulation of genes encoding
446 lignocellulose-degrading activity in the genus *Phanerochaete*. Appl Microbiol Biotechnol
447 94(2):339-351

448 Mancilla RA, Canessa P, Manubens A, Vicuña R (2010) Effect of manganese on the secretion
449 of manganese-peroxidase by the basidiomycete *Ceriporiopsis subvermispora*. Fungal
450 Genet Biol 47(7):656-661

451 Martínez AT (2002) Molecular biology and structure-function of lignin-degrading heme
452 peroxidases. Enzyme Microb Tech 30(4):425-444

453 Martinez D, Larrondo LF, Putnam N, Gelpke MDS, Huang K, Chapman J, Helfenbein KG,
454 Ramaiya P, Detter JC, Larimer F (2004) Genome sequence of the lignocellulose degrading
455 fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22(6):695-700

456 Mathews SL, Pawlak J, Grunden AM (2015) Bacterial biodegradation and bioconversion of
457 industrial lignocellulosic streams. Appl Microbiol Biotechnol 99(7):2939-2954

458 Mielgo I, Palma C, Guisan JM, Fernandez-Lafuente R, Moreira MT, Feijoo G, Lema JM
459 (2003) Covalent immobilisation of manganese peroxidases (MnP) from *Phanerochaete*

460 *chrysosporium* and *Bjerkandera* sp. BOS55. Enzyme Microb Tech 32(7):769-775
461 Pérez J, Muñoz-Dorado J, de la Rubia T, Martínez J (2002) Biodegradation and biological
462 treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol 5(2):53-63
463 Robertson SA, Mason SL, Hack E, Abbott GD (2008) A comparison of lignin oxidation,
464 enzymatic activity and fungal growth during white-rot decay of wheat straw. Org
465 Geochem 39(8):945-951
466 Rollins JA, Dickman MB (2001) pH signaling in *Sclerotinia sclerotiorum*: Identification of a
467 *pacC/RIM1* homolog. Appl Environ Microbiol 67(1):75-81
468 Roy BP, Archibald F (1993) Effects of kraft pulp and lignin on *Trametes versicolor* carbon
469 metabolism. Appl Environ Microbiol 59(6):1855-1863
470 Tang L, Zeng GM, Shen GL, Li YP, Zhang Y, Huang DL (2008) Rapid detection of picloram
471 in agricultural field samples using a disposable immunomembrane-based electrochemical
472 sensor. Environ Sci Technol 42(4):1207-1212
473 Tello M, Corsini G, Larrondo LF, Salas L, Lobos S, Vicuña R (2000) Characterization of three
474 new manganese peroxidase genes from the ligninolytic basidiomycete *Ceriporiopsis*
475 *subvermispora*. BBA-Gene Struct Expr 1490(1-2):137-144
476 Thiele DJ (1992) Metal-regulated transcription in eukaryotes. Nucleic Acids Res
477 20(6):1183-1191
478 Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem
479 Biotech 157(2):174-209
480 Xu P, Zeng GM, Huang DL, Feng CL, Hu S, Zhao MH, Lai C, Wei Z, Huang C, Xie GX, Liu
481 ZF (2012) Use of iron oxide nanomaterials in wastewater treatment: A review. Sci Total

Environ 424:1-10

Zeng GM, Chen M, Zeng ZT (2013a) Risks of neonicotinoid pesticides. Science 340(6139):1403-1403

Zeng GM, Chen M, Zeng ZT (2013b) Shale gas: surface water also at risk. Nature 499(7457):154-154

Zhang C, Liu L, Zeng GM, Huang DL, Lai C, Huang C, Wei Z, Li NJ, Xu P, Cheng M, Li FL, He XX, Lai MY, He YB (2014) Utilization of nano-gold tracing technique: Study the adsorption and transmission of laccase in mediator-involved enzymatic degradation of lignin during solid-state fermentation. Biochem Eng J 91:149-156

Zhang L, Zeng G, Zhang J, Chen Y, Yu M, Lu L, Li H, Zhu Y, Yuan Y, Huang A, He L (2015) Response of denitrifying genes coding for nitrite (*nirK* or *nirS*) and nitrous oxide (*nosZ*) reductases to different physico-chemical parameters during agricultural waste composting. Appl Microbiol Biotechnol 99(9):4059-4070

Zhang Y, Zeng GM, Tang L, Huang DL, Jiang XY, Chen YN (2007) A hydroquinone biosensor using modified core-shell magnetic nanoparticles supported on carbon paste electrode. Biosens Bioelectron 22(9-10):2121-2126

Figure captions

Fig. 1 Effect of manganese on pH and manganese peroxidase activity during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD (n=3).

Fig. 2 Effect of manganese on TOM and TOM loss during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD (n=3).

Fig. 3 Effect of manganese on fungal biomass during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD (n=3).

Fig. 4 Relationship between TOM loss and ergosterol content during SSF of rice straw with *P. chrysosporium*.

Fig. 5 Relative transcription levels of *mnp1*, *mnp2* and *mnp3* during SSF of rice straw with *P. chrysosporium*. Gene expression was normalized to β-actin expression. The results are expressed as mean ± SD (n=3).

Fig. 6 Comparison of promoter elements of MnP genes from *P. chrysosporium*.