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ENVIRONMENTAL BIOTECHNOLOGY

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

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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 days), 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.

Keywords 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% 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.

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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 nonspecific 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 three main structurally related genes (*mnp1*, *mnp2*, and *mnp3*) and two 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). Lines of research 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).

SSF 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 the 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 a humidifier. Samples were taken on days 0, 3, 6, 9, 12, 18, 24, and 30. All experiments were performed in triplicate.

TOM and pH determination

The total organic matter (TOM) was determined by a classical loss-on-ignition method. Two grams 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).

MnP 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 is 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 °C 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 °C for 30 min to remove genome DNA, RNA samples were quantified by spectrophotometry and stored at −70 °C until use.

The RNA samples were reverse transcribed using the RevertAid™ 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 Mix 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 messenger RNA (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 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and final extension at 68 °C. Amplification specificity was confirmed by melting curve analysis performed from 55 to 95 °C 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.

Statistical analysis

The results were presented with the mean values of three replicates and standard deviations of the mean (SD). Differences in parameters were evaluated with a one-way analysis of variance (ANOVA) using a p value of 0.05 to determine significance. Regression analysis was used to examine the relationships between the TOM loss and fungal biomass. The analyses were performed using the software package SPSS 18.0 for Windows (SPSS Inc., Chicago, IL).

Results

Time-course of pH and MnP activity

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

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

Table 1 Primers used for qRT-PCR

Primer	Sequence (5′–3′)	T_m (°C)	Amplicon (bp)
mnp1-Fw[a]	TACCTGGTCACGGCTACTCG	67.4	77
mnp1-Rv[b]	TTTCCAACAATCCAGGGCAG	64.8	
mnp2-Fw	GCACCTTTAGTAGATGTCG	59.2	83
mnp2-Rv	GCATTCTGAAAACTTGGTGG	61.7	
mnp3-Fw	GCATGGTACTATCGCTTTCC	62.6	77
mnp3-Rv	ATAGGTGGAGCAGTCAAGAT	62.1	
β-Actin-Fw	ACTCTGGTGATGGTGTCTC	62.6	149
β-Actin-Rv	TGTGGTGTTGAAGGGGTAA	62.7	

[a] Forward primer

[b] Reverse primer

decline until the end of SSF (day 30). No significant difference is found between the MnP activities of the two groups after 6 days of SSF ($P > 0.05$).

Biodegradation of organic matter during the 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 gentle descending until day 24 and an obvious 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 ± 3.77 and 68.70 ± 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 days 3, 6, and 9 ($P = 0.039$, 0.037, and 0.049, respectively). Nevertheless, there was an insignificant difference between the TOM losses 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 the first 3 days followed by a fast decline with the time, then rose from day 12 to 18 and 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 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 (days 3, 6, and 9) of SSF ($P = 0.040$, 0.046 and 0.043, respectively), but no significant differences were found after 12 days ($P > 0.05$).

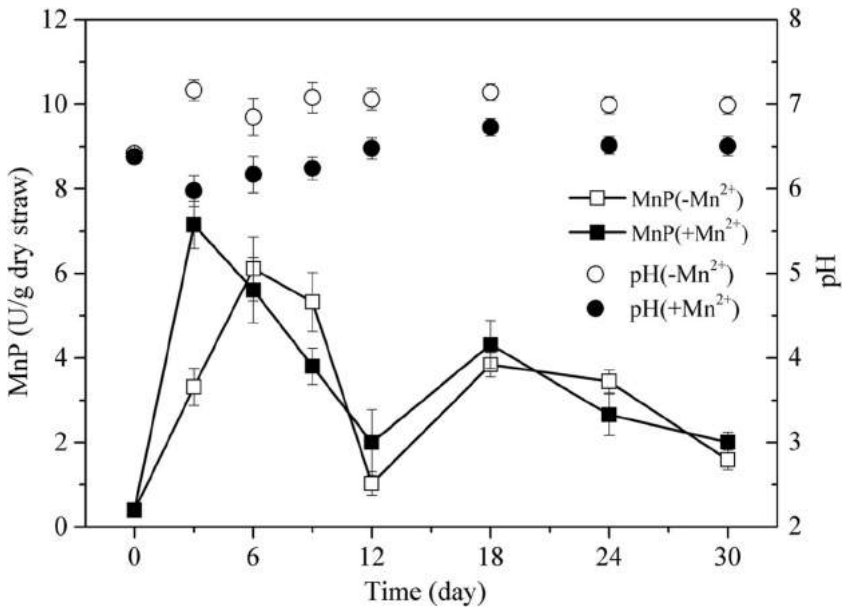

Fig. 1 Effect of manganese on pH and manganese peroxidase activity during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD ($n = 3$)

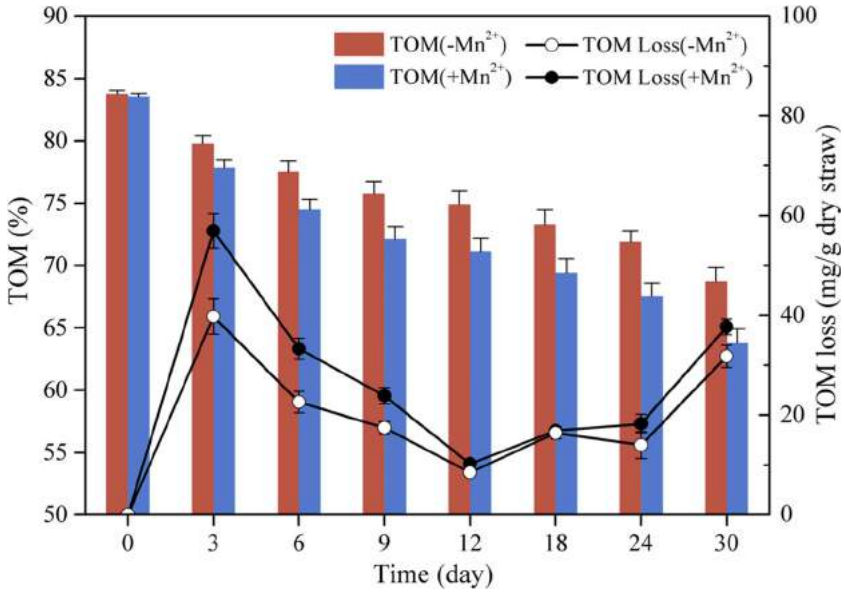

Fig. 2 Effect of manganese on TOM and TOM loss during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD (n = 3)

The correlation between TOM 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 MnP genes affected by Mn^{2+} during SSF, we monitored the time-course (3–30 days) 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 days) 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.

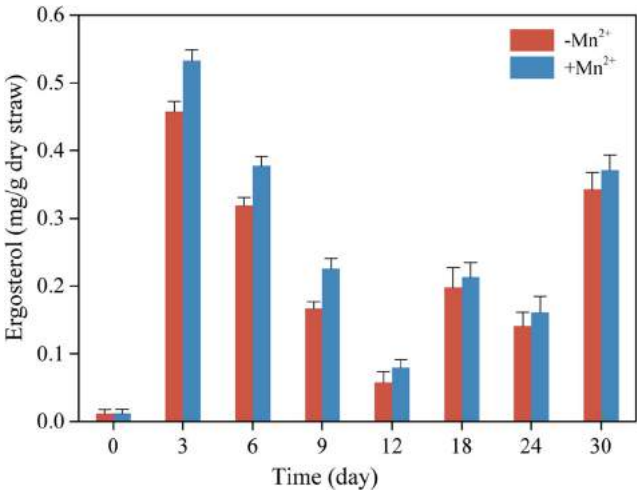

Fig. 3 Effect of manganese on fungal biomass during SSF of rice straw with *P. chrysosporium*. The results are expressed as mean ± SD (n = 3)

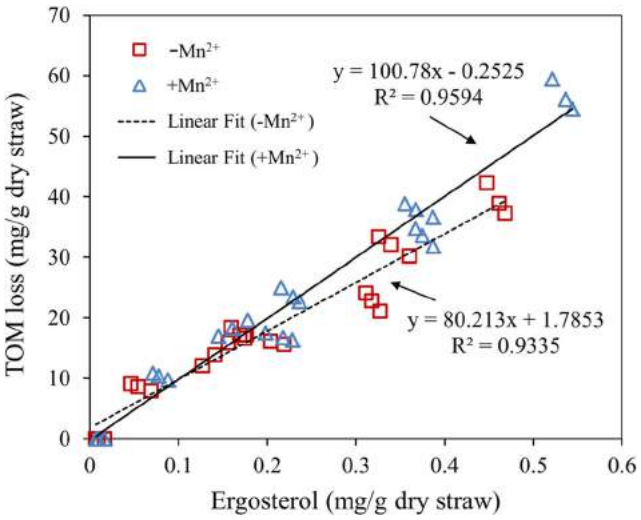

Fig. 4 Relationship between TOM loss and ergosterol content during SSF of rice straw with *P. chrysosporium*

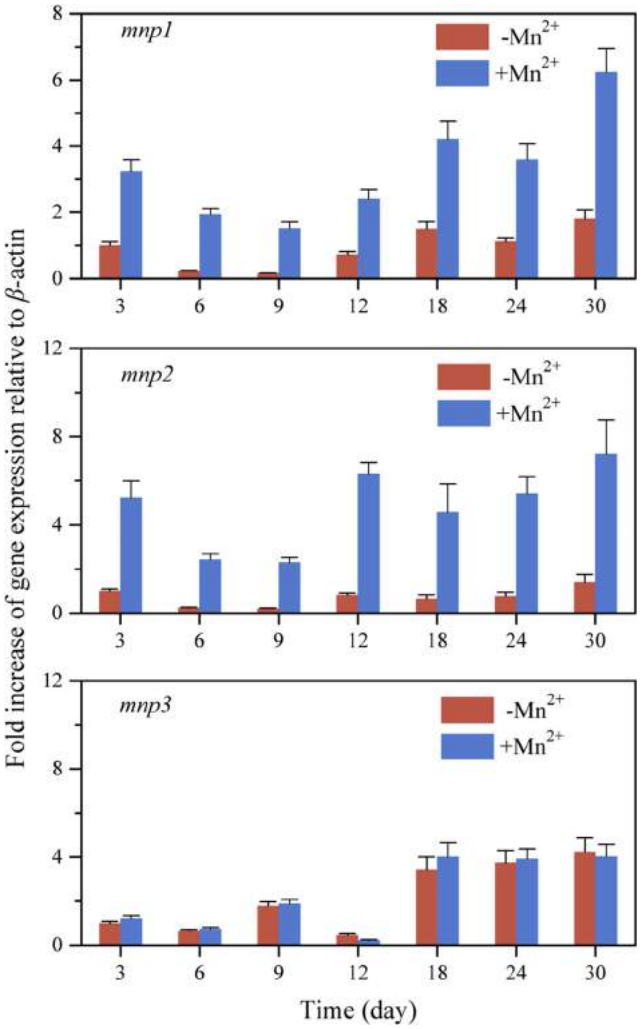

Fig. 5 Relative transcription levels of *mnp1*, *mnp2*, and *mnp3* during SSF of rice straw with *P. chrysosporium*. Gene expression was normalized to β-actin expression. The results are expressed as mean ± SD ($n = 3$)

Discussion

The effect of manganese on pH and MnP 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 the 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 and thus lead 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 TOM 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. Significantly higher TOM loss due to manganese amendment was detected in the early stage (0–9 days) 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 (days 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 periods 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 SSF (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).

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

Quantitative analysis of *mnp* gene expression

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

Compared with the cumulative levels of the three *mnp* transcripts, MnP activity reached its peak (on day 6 without manganese, Fig. 1) later than the abundance of *mnp* transcripts which 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 (Figs. 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 contains 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

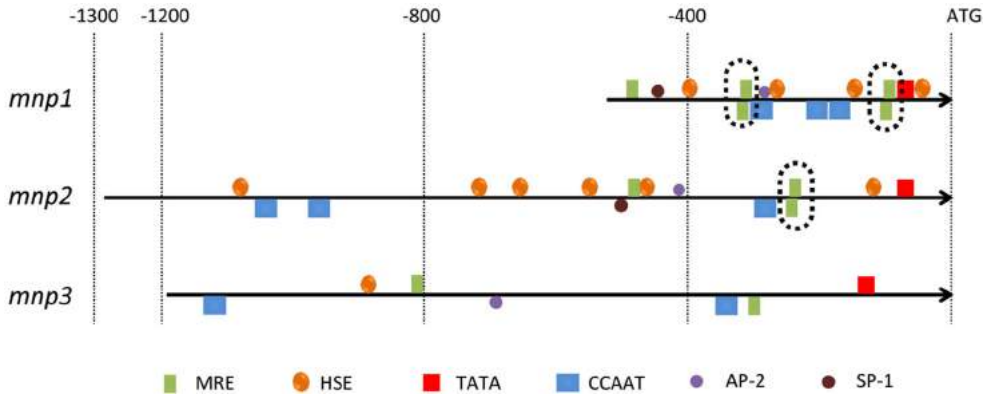

Fig. 6 Comparison of promoter elements of MnP genes from *P. chrysosporium*

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 line of 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 *Trametes versicolor* that the MRE 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 needs 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 SSF 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 days), 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

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