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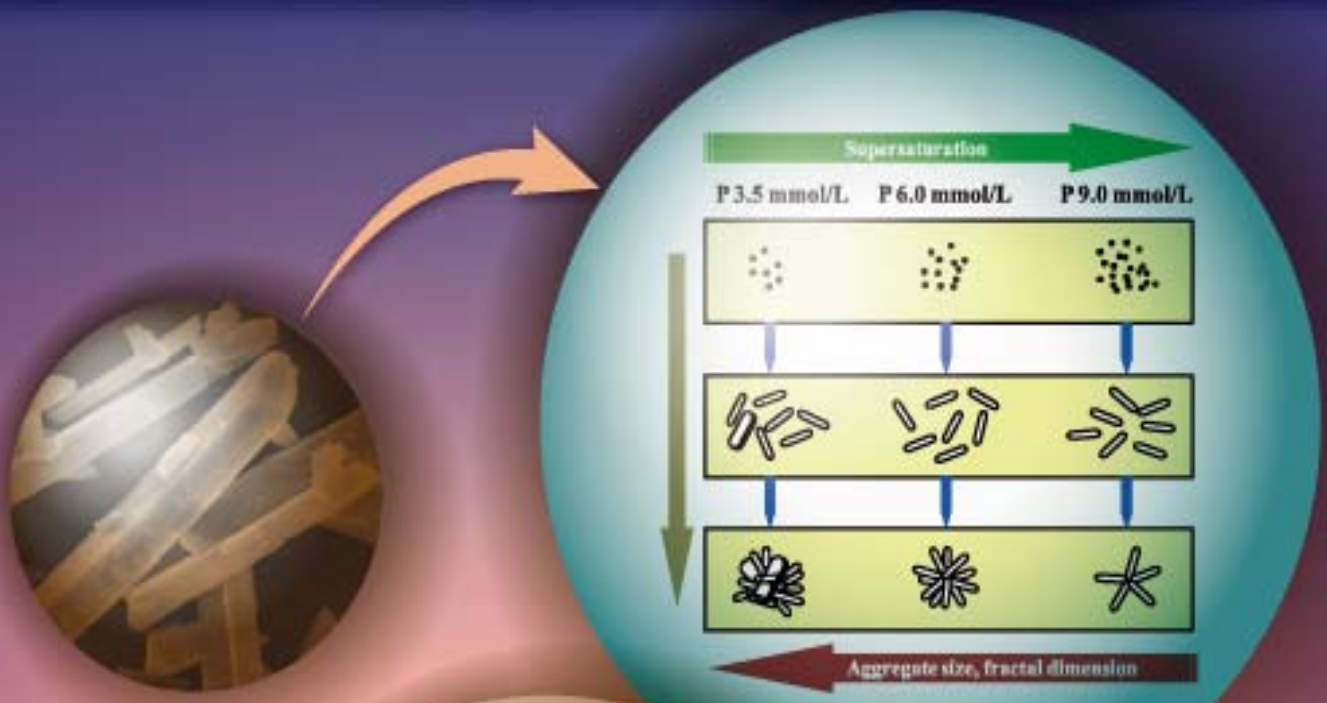

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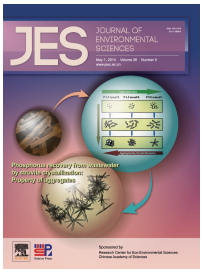

Biodegradation of pyrene by *Phanerochaete chrysosporium* and enzyme activities in soils: Effect of SOM, sterilization and aging

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ABSTRACT

The impacts of soil organic matter (SOM), aging and sterilization on the production of lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium* during the biodegradation of pyrene in soils were investigated. The biodegradation of pyrene by *P. chrysosporium* decreased with increasing SOM content, whereas the maximum activities of LiP and MnP increased, which indicates that SOM outweighed pyrene in controlling enzyme production. Sterilization enhanced the degradation of pyrene due to the elimination of competition from indigenous microbes, whereas aging led to a reduction in the degradation of pyrene primarily through changes in its sorbed forms. Both sterilization and aging could reduce SOM content and alter its structure, which also influenced the bioavailability of pyrene and the enzyme activity. The sterilization and aging processes caused changes in the degradation of pyrene, and the enzyme activities were greater in soils with high SOM contents. MnP was related to the degradation of pyrene to a greater extent, whereas LiP was more related to the decomposition of SOM.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in soils, and they have caused considerable concern because of their known or suspected carcinogenic and mutagenic effects. Therefore, the clean-up of PAH-contaminated sites is desirable in order to protect human health.

Biodegradation is one promising technique for the remediation of PAH-contaminated sites. However, some PAHs, especially those with high molecular weights, resist biodegradation. White rot fungi have attracted considerable attention in the attempt to degrade PAHs because they can degrade PAHs through the release of extracellular enzymes, such as the lignin peroxidase enzyme (LiP) or the manganese peroxidase enzyme (MnP) (Roldán-Carrillo et al., 2003; Valentín et al., 2006; Covino et al., 2010). However, the majority of studies focused on activity of the extracellular enzymes produced by the white rot fungus have primarily been centered on experiments using liquid cultures (Eibes et al., 2005; Rodrigues et al., 2008). However, when fungi enter unsterilized soils, they must compete with indigenous soil microbes for nutrients and the mycelia of the fungi may be attacked by indigenous microbes. Consequently, the generation of enzymes may be influenced by more complex factors in soils. McErlean et al. (2006) reported that the amounts of MnP and laccase produced by *Trametes versicolor* were three to four times greater than those produced by *Pleurotus ostreatus*, *Collybia* sp. and *Rhizoctonia solani* in liquid cultures on day 21, respectively. However, *T. versicolor* and *Collybia* sp. failed to grow in any of the unsterilized soils, whereas *R. solani* and *P. ostreatus* grew well in unsterilized sand, forest and basalt and marl mixed till (field) soils. Furthermore, LiP, MnP and laccase genes in *P. ostreatus* and the laccase

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gene in *R. solani* were detected in soils based on the amplification of PCR products. In a parallel study (Wang et al., 2009), we observed that the activities of LiP and MnP varied considerably during the degradation of PAHs in soils by *Phanerochaete chrysosporium*. The maximum enzyme activity occurred when the degradation entered a plateau. The enzyme activities for the degradation of different PAHs followed the same order of their degradability: phenanthrene > pyrene > benzo[a]pyrene. However, the enzyme activities exhibited a reverse relationship for the degradation of pyrene in different soils, and the activities increased with the soil organic matter (SOM) content. This result indicated that both the PAHs and the soil properties might affect the enzyme activities and consequently influence the degradation of PAHs.

In addition to the biodegradability of contaminants, the forms of the sorbed contaminants in soils are an important factor that influences their degradation (Yang et al., 2009). A large portion of PAHs is biodegradable in aqueous cultures, whereas they resist degradation in the soil phase, which is termed as persistent residue with extremely low bioavailability (Cornelissen et al., 2005). The sorbed forms or the bioavailability of a contaminant are controlled by the properties of the contaminant and the soil, as well as the contact time between the contaminant and the soil. The SOM content is a primary soil property that affects the sorbed forms and bioavailability of organic contaminants (Yang et al., 2011). It has been recognized that a high content of SOM in soil can negatively influence the decontamination rate of organic contaminants by bacteria (Nam et al., 1998; Yang et al., 2011).

More recently, it has been observed that sorbed contaminants become less bioavailable with increased contact time (aging) in the soil (Antizar-Ladislao et al., 2006; Li et al., 2008). The reduction in bioavailability caused by aging has been observed to vary with the contaminants and soils, and no consistent results have been obtained and the underlying mechanisms are poorly understood (Northcott and Jones, 2001; Nam and Kim, 2002; Watanabe et al., 2005). Some researchers have proposed that the contaminant molecules move from easily desorbing and bioavailable sites to difficultly desorbing and less bioavailable sites during aging, and even to irreversible and non-bioavailable sites, which leads to decreased decontamination rates (Northcott and Jones, 2001; Sun et al., 2008). Regardless of the mechanism, the interactions with SOM exert significant effects on the sequestration of contaminants during aging. Fungi could decompose SOM and enhance the bioavailability of contaminants sorbed on it. Consequently, the effects of aging on the degradation of contaminants by fungi might be different than those by bacteria. But, the effects of aging on the SOM and the enzyme activities produced by fungi during the degradation of organic contaminants have been seldom studied.

In addition, sterilization of the soil is usually applied when studying biodegradation in laboratory experiments so that the degradation capacity of the specific augmented species can be elucidated. This situation is not the case when applying bioremediation in field-contaminated sites. Fewer studies have described the effects of soil sterilization on the bioavailability of sorbed contaminants and changes in SOM (Northcott and Jones, 2001; Nam et al., 2003; Mueller et al., 2006; Kelsey et al., 2010). Kelsey et al. (2010) suggested that the uptake of anthracene and *p*, *p*′-DDE by earthworms, *Eisenia fetida*, increased in soils sterilized by both autoclaving and gamma-irradiation. However, sterilization had no effect on the bioaccumulation of *p*, *p*′-DDE by *Lumbricus terrestris*, and the uptake of anthracene increased only in the gamma-irradiated soils. Fourier transform infrared spectroscopy (FT-IR) measurements revealed that the functional groups of SOM changed due to sterilization by both autoclaving and gamma-irradiation. It is interesting to know whether the SOM change caused by sterilization has any influence on the fungi degradation ability and their enzyme activities. Additionally, when fungi are used in unsterilized soils, indigenous microorganisms may interact with the fungus species, which may also lead to a change in the fungi's biodegradation ability and their enzyme activities.

Therefore, in this study, the degradation of pyrene (used as a model PAH) by *P. chrysosporium* and the activities of two key enzymes, LiP and MnP, in three different soils were studied. Soils that were either freshly spiked (unaged) or aged for six months, and sterilized or unsterilized, were examined to elucidate the possible influence of soil type, aging, and sterilization. The knowledge on the level of extracellular oxidation enzymes related to the degradation process under these conditions is important for understanding the degradation mechanisms of PAHs in soils by white rot fungi, and consequently, may be valuable for promoting the efficient use of white rot fungi for the remediation of PAH-contaminated sites.

1 Materials and methods

1.1 Soil preparation

Two soils were collected from gardens in Tianjin and Shijiazhuang, China, which were named soils 1 and 2, respectively. Soil 3 was an artificial soil produced according to OECD guideline 207 (OECD, 1984). The soils were air-dried at room temperature and then passed through a 2-mm mesh sieve before use. For the sterilized soils, forty aliquots of 100 g soil were added to 250 mL beakers and tightly covered with silicone-backed Teflon liners. The soil in the beakers was then autoclaved for 30 min on each of 2 successive days at 121°C and 0.1 MPa. Soil organic matter content and texture were measured and are listed in **Table 1**, and the employed analysis methods can be found

Table 1 Main physicochemical properties of the tested soils and pyrene concentration in different soils before initiating the experiments

Soil	Soil organic matter (%)			Sand (%)	Silt (%)	Clay (%)	Texture	Pyrene concentration (mg/kg)		
	Freshly	Sterilization	Aging					Nonsterilized-unaged soils	Nonsterilized-aged soils	Sterilized-unaged soils
Soil 1	0.30	0.29	0.29	15	64	21	Silt loam	10.0 ± 0.51	9.32 ± 0.50	10.11 ± 0.35
Soil 2	3.56	3.4	3.2	27	39	34	Clay loam	10.11 ± 0.22	9.25 ± 0.43	10.0 ± 0.44
Soil 3	10.0	9.6	9.3	70	10	20	Clay loam	9.93 ± 0.41	8.89 ± 0.31	10.32 ± 0.21

in our previous study (Wang et al., 2009).

1.2 Soil contamination and aging

Pyrene with a purity of 98% was purchased from the Aldrich Chemical Company (Milwaukee, WI, USA). A stock solution of pyrene (1 g/L) was prepared in HPLC-grade methanol. The soils did not initially contain detectable levels of pyrene. To obtain pyrene-contaminated soil samples, twenty 200 g aliquots of sterilized and nonsterilized soils were weighed into 500 mL brown wide-mouth bottles, respectively. The soil samples were spiked with 2 mL of the pyrene-methanol solution (1 g/L) to obtain an initial pyrene concentration of 10 mg/kg. The bottles were placed on a clean bench for 4 hr, with vigorous mixing for 6 sec every 30 min to accelerate the evaporation of methanol and to thoroughly mix the pyrene with the soil. The bottles were then tightly covered with silicone-backed Teflon liners and shaken for 48 hr on a reciprocating shaker that was operated at 120 r/min and (25 ± 1)°C. Ten aliquots of the soil were immediately used in the biodegradation experiment as freshly spiked (unaged) soils, and the remainder of the soils were stored in the dark at (25 ± 1)°C for 180 days to obtain aged soils. The spiked pyrene concentrations were measured using three randomly sampled soils (**Table 1**).

1.3 Biodegradation experiment

Ten grams of the contaminated soils (nonsterilized-unaged soils, sterilized-unaged soils, and nonsterilized-aged soils) were weighed into Petri dishes that had a diameter of 9 cm, and then 2 mL of a *P. chrysosporium* suspension with an OD value of 1 was added followed by 1 g sawdust and 1.5 mL of a liquid nitrogen-limited medium to obtain a soil water-holding capacity of approximately 60%. The Petri dishes were horizontally stirred with a glass rod to thoroughly mix the sample and then capped. The dishes were then incubated in the dark at (28 ± 1)°C. A specific amount of sterilized water was added to the dishes daily to compensate for the water loss due to the evaporation of moisture. Some of the dishes were sacrificed at day 1, 3, 7, 11, 15, and 19, and the pyrene concentration and enzyme activities in the residue were analyzed.

The experiments were conducted in triplicate, and soil samples without inoculation of *P. chrysosporium* were used as controls.

1.4 Humic acid extraction and Fourier transform infrared spectroscopy

Soil 2 was used as an example soil for the analysis of humic acid (HA). In a separate experiment, the HA was fractionated and extracted from the sterilized, nonsterilized-unaged and nonsterilized-aged Soil 2 samples following the methods of Kohl and Rice (1998) and Kelsey et al. (2010). Briefly, 10 g of air-dried and sieved (2 mm) soils were mixed with 200 mL of 0.5 mol/L NaOH and shaken under N_2 for 24 hr at 22°C at 200 r/min. The samples were centrifuged (5000 ×*g*) for 15 min under cryogenic conditions, and the supernatants were isolated from the residual soil. The soil residue was extracted seven additional times by sonication in 50 mL of 0.5 mol/L NaOH. The alkaline extracts were combined and acidified to a pH < 2 and centrifuged to isolate the precipitated HA. The demineralization of the humic samples was performed by repetitively washing with HCl and HF. The samples were washed with distilled water until a neutral pH was achieved, and then they were stored in desiccators for further analysis.

The IR spectra of the HA from Soil 2 before and after sterilization and aging were recorded using a Bruker Tensor 27 FT-IR spectrometer from 400 to 4000 cm^{-1} with a 4 cm^{-1} resolution. A background spectrum of KBr was collected to eliminate contributions from atmospheric CO_2 and H_2O, which would interfere with spectra. The samples were packed into a sample holder, their surfaces smoothed with a glass slide, and were then immediately transferred to the diffuse reflectance cell, which was flushed with N_2 for 10 min to remove water vapor and CO_2.

1.5 Enzyme extraction and analysis

The extraction of LiP and MnP from the soils followed the procedure of Bollag et al. (1987) with minor modifications. The entire soil sample from a Petri dish was transferred to a 20 mL capped glass tube, mixed with 1:1 (*W*/*W*) sterilized distilled water, and then shaken for 30 min at 50 r/min. The solid phase was separated by centrifugation, and the supernatant was frozen, thawed, and centrifuged to remove particles of high molecular weight polysaccharide slime. The supernatant was sequentially filtrated through

Whatman No.1 filter paper (a 0.45 μm membrane filter) and an Amicon YM10 membrane.

The enzyme activities were measured using a UV-visible spectrophotometer (Cary 50 Conc, Varian, USA). The activity of LiP was measured at 310 nm using veratryl alcohol as a substrate (Belinky et al., 2006; Wang et al., 2009). The activity of MnP was measured at 240 nm using Mn(II) as a substrate (Wang et al., 2009). The crude enzyme extract of the control soil, which was not spiked with *P. chrysosporium*, was used as the reference for the enzyme activity assays. The enzyme activity is expressed as U/g dry soil. One unit (U) was defined as 1 μmol of substrate oxidized per minute at 25°C. All assays were conducted in triplicate.

1.6 Analysis of pyrene

The soil samples were mixed with an equal quantity of anhydrous sodium sulfate and then subjected to Soxhlet extraction for 24 hr using a 1:1 (*V*/*V*) mixture of dichloromethane/acetone. The total extract was then concentrated by rotary evaporation (< 45°C, 0.05 MPa) to a volume of approximately 3 mL. The residue extracts were further concentrated to near dryness under a gentle stream of N_2 and then redissolved in 5 mL of acetonitrile. The recovery of pyrene in different soils using this extraction method was measured to be greater than 94%.

Pyrene in acetonitrile was analyzed using a high performance liquid chromatography system (Waters 1525 Binary HPLC Pump, Millennium 32 station, USA) equipped with a fluorescence detector (Waters 2475 Multi λ Fluorescence Detector). Analysis was performed using a Waters Symmetry C_{18} column (μ Bondapak 3.9 mm i.d. × 150 mm × 5 μm, pore size 100 Å, Ireland). An acetonitrile-water mixture (80:20, *V*/*V*) was used as the mobile phase at a flow rate of 1.0 mL/min. Pyrene was detected with excitation wavelength of 333 nm and emission wavelength of 390 nm. All results are presented as the mean of triplicate measurements ± standard deviation. When necessary, the results were analyzed with a one-way analysis of variance (ANOVA).

1.7 Statistical analysis

The data were analysed statistically using SPSS 17.0. Evaluation of significant differences among treatments was performed using one-way ANOVA testing using the Duncan test, with statistical significance of $p < 0.05$.

2 Results and discussion

2.1 Biodegradation in unaged soils

Table 1 presents the initial concentrations of pyrene in the soils before initiating the biodegradation experiment. For the nonsterilized- and sterilized-unaged soils, the initial concentrations of pyrene ranged between (9.93 ± 0.41) and (10.32 ± 0.21) mg/kg dry soil, which are not significantly different.

The biodegradation of pyrene by *P. chrysosporium* in the three nonsterilized-unaged soils proceeded at a rapid rate in the early incubation phase, and then the biodegradation rate slowed, exhibiting a biphasic profile (**Fig. 1**). The percentage of pyrene that was biodegraded markedly varied with the soil types ($p < 0.05$). As the SOM content increased from 0.3% for Soil 1, to 3.56% for Soil 2, and to 10.0% for Soil 3 (**Table 1**), the 19-day percentage degradation of pyrene in the nonsterilized-unaged soils decreased with the increase of SOM, being (66.20 ± 2.72)% in soil 1, (51.16 ± 0.40)% in soil 2, and (44.99 ± 4.01)% in soil 3, whereas the pyrene lost in the corresponding control soils was (20.21 ± 2.43)%, (16.54 ± 2.81)%, and (14.62 ± 1.43)%, respectively (data not shown). When the soils were sterilized, the biodegradation of pyrene slightly increased for Soils 2 and 3 ($p < 0.05$) compared to the nonsterilized soils, whereas no significant change was observed for Soil 1. The 19-day percentage degradation of pyrene was (65.71 ± 4.20)%, (58.57 ± 0.30)% and (51.25 ± 2.42)% for the sterilized-unaged Soils 1, 2, and 3 respectively, whereas (13.23 ± 0.95)%, (11.96 ± 0.80)%, (8.0 ± 1.21)% of the pyrene was lost after 19 days in the corresponding control soils (data not shown). Therefore, pyrene could be

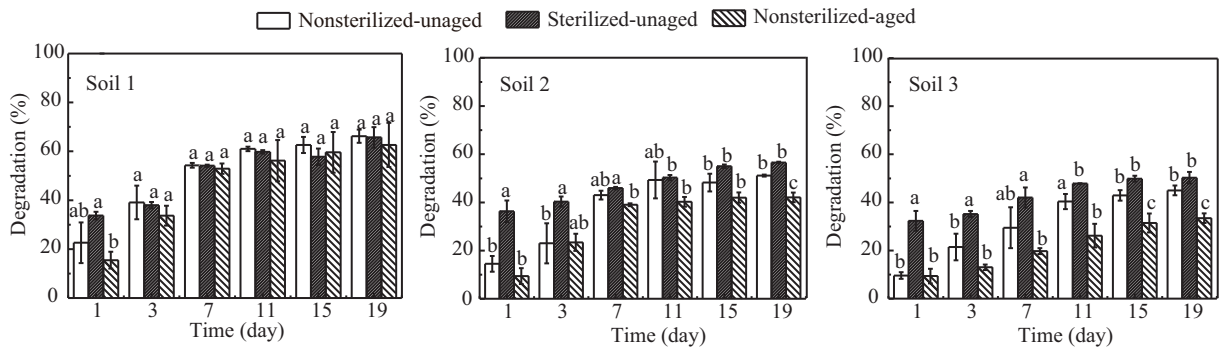

Fig. 1 Degradation of pyrene from nonsterilized-unaged, sterilized-unaged and nonsterilized-aged soils by *P. chrysosporium*. Bars are the standard errors of the means. The differences among treatments were tested by one-way AVONA, treatments with the same letters within the same soils are not significantly different at $p < 0.05$ according to the Duncan test.

efficiently degraded by *P. chrysosporium*, and the pyrene degradation rates in the augmented soils were markedly greater than in the corresponding controls. The degradation of pyrene by *P. chrysosporium*, which was calculated as the differences in pyrene content between the inoculated and the control soils, in different soils decreased with increasing original SOM content. Sterilization increased the degradation of pyrene by *P. chrysosporium* by 0.49%, 7.42%, and 6.26% for Soils 1–3, respectively. Therefore, there were significant differences in the degradation of pyrene by *P. chrysosporium* between the sterilized and nonsterilized soils with higher SOM content ($p < 0.05$).

It has been reported that the bioavailability of sorbed organic contaminants decreases with increasing SOM content, which leads to reduced biodegradation by bacteria (Nam et al., 1998; Yang et al., 2011). This process occurs because SOM is the most important soil component that binds organic contaminants in soil, and soils with higher SOM contents usually contain more active sorption sites that could strongly combine with many organic contaminants and limit their mobilities (Nam et al., 1998; Watanabe et al., 2005). The data in this study confirm that although white rot fungi could decompose SOM, the degradation of pyrene is still controlled by its availability.

After sterilization, the degradation of pyrene by the fungi was enhanced. Pozdnyakova et al. (2008) noted that strains of the genera *Phanerochaete* and *Trametes* formed weak and short-lived mycelia in nonsterilized soils compared to those in sterilized soils. The white rot fungi, *Pleurotus ostreatus*, could survive for a relatively long time in nonsterilized soil only when the soils were supplemented with additional substrates (Morgan et al., 1993; Machado et al., 2005). Hence, the enhanced degradation of pyrene could first be attributed to the elimination of competition with *P. chrysosporium* from indigenous microorganisms in natural soil samples.

In addition to the biological aspect, sterilization decreased the contents of SOM, especially for soils with a higher SOM content (**Table 1**). This finding is consistent with some previous studies, which confirmed that sterilization could induce the reduction of dissolved organic matter in soil (Bunzl and Schimmack, 1988; Kreller et al., 2005; Schaller et al., 2011). The reduction in the SOM content might result in more pyrene being easily released from the soils. To further examine the possible structural change in the SOM after sterilization, FT-IR spectra were obtained for the HA extracted from Soil 2 as an example, as noted in the discussion of biodegradation mechanisms.

2.2 Biodegradation in aged soils

Aging was conducted under non-sterilized conditions to observe whether aging of the soil has an influence on the biodegradation process. After 180 days of aging, the extracted concentrations of pyrene from the aged soils were statistically different than for the corresponding freshly spiked soils. The initial pyrene concentrations ranged from (9.32 ± 0.50) to (8.89 ± 0.31) mg/kg dry soil for the nonsterilized-aged soils (**Table 1**). One possible reason for this result is that the forms of the sorbed pyrene had changed during aging, leading to a reduced amount of extractable pyrene. In the control samples, (17.24 ± 1.23)%, (17.15 ± 2.41)% and (12.46 ± 1.13)% of pyrene was lost by day 19 in nonsterilized-aged Soils 1, 2 and 3, respectively (data not shown). These losses could be attributed to the degradation by indigenous microbes, because the biodegradation reaction was stimulated after the soils were amended with water and nutrients. When the soil was inoculated with *P. chrysosporium*, the 19-day degradation percentage was (62.64 ± 9.01)%, (42.07 ± 2.0)% and (33.44 ± 2.0)% based on the initial measured concentrations (**Fig. 1**), which were 45.40%, 24.92%, and 21.08% greater than the corresponding controls. The 180-day aging caused a significant reduction in the degradation of pyrene ($p < 0.05$) at each harvest time compared to the freshly spiked soils. The aging caused a reduction in the 19-day pyrene percentage degradation of 3.56%, 9.09% to 11.55% for Soils 1, 2 and 3, respectively. Therefore, the reduction in the degradation of pyrene caused by the aging was more obvious in Soils 2 and 3, which contained more SOM (3.56% and 10.0% organic carbon, respectively, **Table 1**) than Soil 1 (containing only 0.30% of organic carbon, **Table 1**). This result appears to indicate that the aging-induced sequestration was more pronounced in the organic matter-rich soils. When a soil contains very little SOM, such as Soil 1, it cannot provide effective binding sites even when given sufficient contact time, and the aging-induced reduction in bioavailability is less and even negligible. A similar result was observed in the biodegradation of phenanthrene by bacterium P5-2 in aged soils with various organic carbon contents, ranging between 0.03% and 14.7% (Nam et al., 1998). The aging effect on the biodegradation rate was only pronounced in the organic matter-rich soils with organic carbon > 2.0%, whereas it was less obvious in soils with < 2.0% organic carbon. The authors consequently proposed that there may be a threshold level of organic matter for the aging effect and that aging could not cause obvious sequestration in soils with < 2.0% organic C content (Chung and Alexander, 1998). Soils 2 and 3 had considerably different SOM contents; however, the aging-induced reduction in the degradation of pyrene in the two soils was quite similar. The SOM in Soil 2 could provide sufficient sorption sites for a certain amount of pyrene, and the diffusion rates of pyrene in Soils 2 and 3 might be similar, leading to a similar aging-induced reduction in the degradation.

The mechanisms for aging are not fully understood. It has been proposed that during aging, hydrophobic chemicals can disperse into the rigid phase of SOM or become entrapped within the nanopores of the soil, where they may be retained and become less accessible (Zimmerman et

al., 2004; Pan and Xing, 2008). However, during aging, whether and how SOM changes have seldom been demonstrated. In this study, the decrease in the SOM content after aging was more obvious in soils with high SOM contents (**Table 1**).

2.3 Enzyme activities in unaged soils

Figure 2 outlines the kinetics of the MnP and LiP activities produced by *P. chrysosporium* during the degradation of pyrene in the nonsterilized and sterilized-unaged soils. The MnP and LiP activities increased slowly during the initial period, and then they rapidly increased during day 7–11. After the peak value, the MnP and LiP activities decreased. This result indicated that the production of enzymes was related to the degradation of certain substrates. When the substrate concentration decreased with degradation, the amount of enzyme decreased. For nonsterilized-unaged Soils 1–3, the maximum value was 0.34, 1.44 and 1.49 U/g dry soil for MnP, and 0.05, 0.13 and 0.41 U/g dry soil for LiP (**Fig. 2**). This result indicated that MnP from

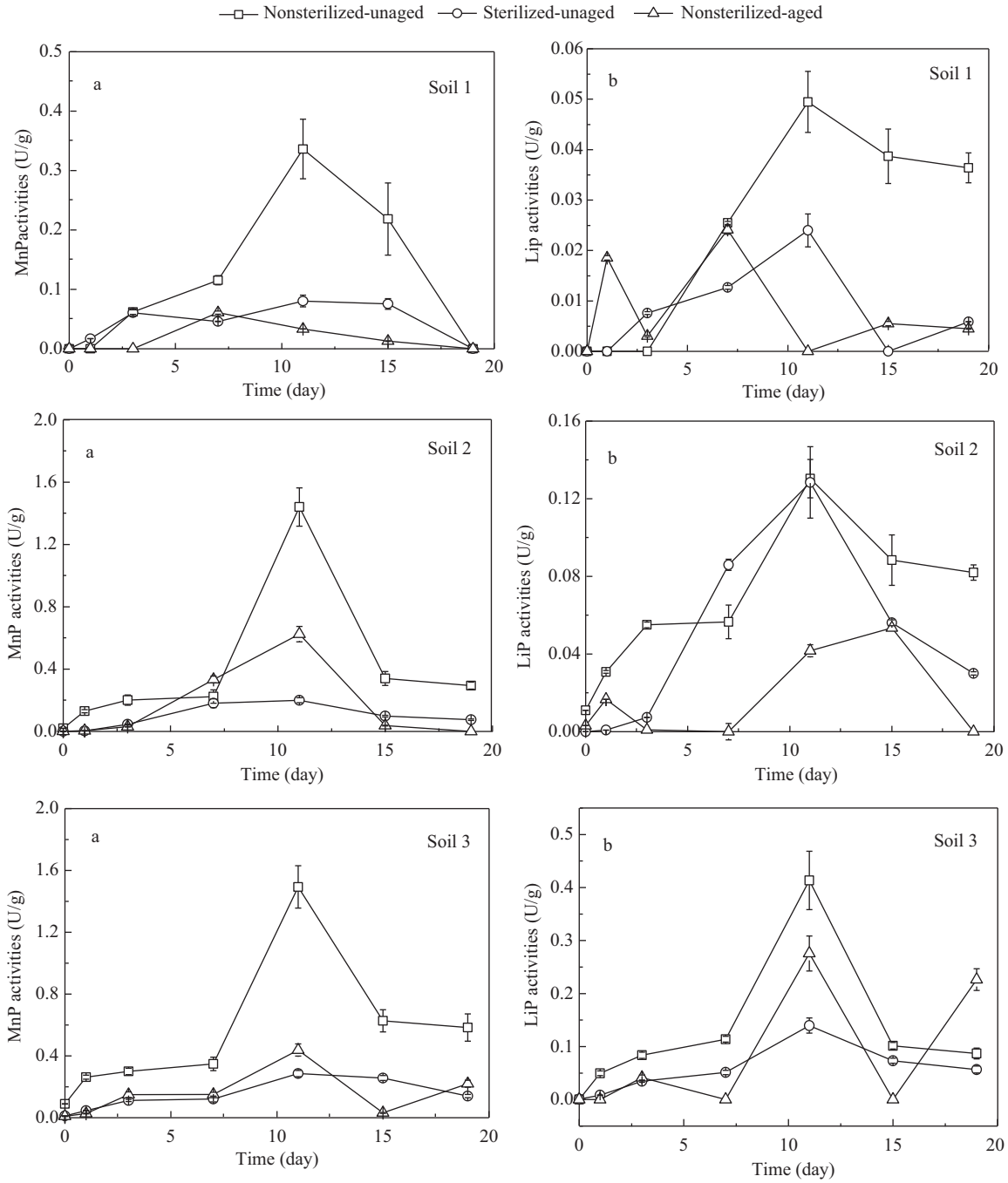

Fig. 2 Maximum enzyme activities of MnP (a) and LiP (b) produced by *P. chrysosporium* in nonsterilized-unaged, sterilized-unaged and nonsterilized-aged soils during degradation of pyrene.

P. chrysosporium played a greater role than LiP in the degradation of pyrene.

For sterilized-unaged Soils 1–3, the highest MnP activity was 0.08, 0.20 and 0.29 U/g dry soil, whereas the LiP maximum value was 0.02, 0.11 and 0.14 U/g dry soil. In both the sterilized and nonsterilized soils, the enzyme activities increased with increasing SOM content ($p < 0.05$), which confirms the good growth of *P. chrysosporium* in "rich" soils (Pozdnyakova et al., 2008). It has been proposed that the extracellular enzymes of white rot fungi are responsible for the degradation of PAHs. In this study, an inverse relationship was observed between the degradation of pyrene and the enzyme activities among different soils. This finding suggests that the bioavailability of pyrene exerts a significant influence on its degradation, which was low in "rich" soils with a high SOM content. The pyrene molecules sorbed in some interior SOM sites are even unavailable to extracellular enzymes, although they are plentiful in "rich" soils.

After sterilization, the enzyme activities, MnP and LiP, decreased compared to the nonsterilized soils, which exhibited the same tendency with SOM content but opposite to pyrene degradation. Although the change in SOM content due to sterilization was small, a significant change might occur for the most labile fraction of SOM, which is the most available for supporting the growth of white rot fungi.

2.4 Enzyme activities in aged soils

The kinetics of the MnP and LiP activities during the degradation of pyrene in the aged soils are shown in **Fig. 2**. For the nonsterilized-aged Soils 1–3, the maximum value was 0.06 U/g dry soil at day 7 for Soils 1, 0.63 and 0.44 U/g dry soil at day 11 for MnP for Soil 2 and 3, and 0.024 U/g dry soil at day 7 for soil 1, 0.04 and 0.27 U/g dry soil for soils 2 and 3 at day 11 for LiP. Hence, the aging induced a reduction in the enzyme activity. This is the first finding of a greater reduction in enzyme activities due to aging in soils with higher SOM contents (Soils 2 and 3), which is in agreement with the aging-induced reduction in the degradation of pyrene in different soils. As discussed above, SOM exerts considerable effect in the binding of hydrophobic organic contaminants (HOCs), and soils with higher SOM contents have a greater capacity to sequester pyrene, which leads to a greater reduction in the bioavailability of pyrene (Weber et al., 1992). Consequently, the fungi could use less pyrene as their carbon source, and the enzyme activities produced by the fungi are reduced.

However, the changes in enzyme activities could also be induced by the soil properties. After aging, both the content and structure of SOM changed (**Table 1** and Section 2.5.1, FT-IR analysis), which became unfavorable for the growth of fungi.

2.5 Biodegradation mechanisms

2.5.1 FT-IR analysis

The HAs from Soil 2 before and after sterilization were analyzed using FT-IR. The bands from the HA functional groups in the FT-IR spectra were analyzed according to the literature (Kelsey et al., 2010; Uslu et al., 2010). Peaks at 1710, 1671 and 1656 cm^{-1} in the spectrum of the HA from the sterilized soil exhibited a stronger absorbance than the HA from the nonsterilized soil (**Fig. 3**), which indicates an enhancement of the C=O functional stretching of peptides. Furthermore, the lignin derivatives, which were indicated by the peak at 1273 cm^{-1}, have an enhanced signal intensity in the spectrum of the HA from the sterilized soil compared with those from the nonsterilized soil. This might be induced by pyrolysis of different biomass to form more lignin derivatives during soil sterilization (Dupont et al., 2008). Finally, the peak at 1090 cm^{-1} was sharper and more intense for the HA from the sterilized soil than that from the nonsterilized soil. This peak corresponds to a carboxyl stretching band of the C–O groups in carbohydrates (Wen et al., 2007). Hence, the intensities of the above bands in the spectra for the HA in the sterilized soil were all greater than those of the HA from the nonsterilized soil, which suggested that the polarity of the HA was enhanced after sterilization. In fact, the polarity of the organic matter functional group exerts great influence on contaminant sorption (Rutherford et al., 1992; Kile et al., 1999). Both the reduction in SOM content (**Table 1**) and the structural change in HA after sterilization could lead to a reduction in soil sorptive affinity for pyrene, which will consequently result in an increased bioavailability of pyrene. Hence, the change in SOM or the sorbed forms of pyrene is another reason for the enhanced degradation of pyrene after sterilization of the soil. A similar result was reported for the biodegradation of anthracene by *P. chrysosporium* DSM 1556, which was higher in sterilized soils than in nonsterilized soil (Andersson and Henrysson,

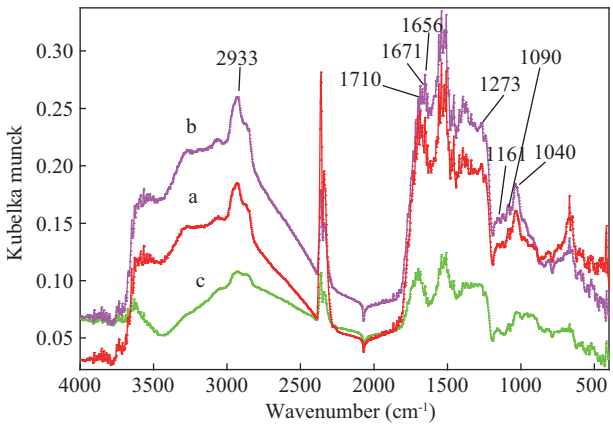

Fig. 3 FT-IR spectra showing the effect of sterilization and aging on the humic acids extracted from Soil 2 subjected to three treatments: (a) nonsterilized-unaged, (b) sterilized-unaged, and (c) nonsterilized-aged.

1996).

The spectra are considerably different before and after aging. Peaks at 2933 cm^{-1}, which are related to aliphatic C–H carbon stretching (Wen et al., 2007; Kelsey et al., 2010), was lower in the HA from the aged soil compared to the HA from the unaged soil. Subsequently, peaks at 1710, 1671 and 1656 cm^{-1} of the HA of the aged soil exhibited weaker absorbance than those of the unaged soil, indicating a reduction in the C=O stretching (Stevenson, 1994; Wen et al., 2007). The peak at 1273 cm^{-1} is due to the C–H stretching and OH deformation of COOH or C–O stretching of aryl esters; however, it was absent in the aged soil. Finally, the peaks in the range of 1161–1040 cm^{-1} in the spectrum of the aged soil exhibited a weaker absorbance of cuticular polysaccharides and OH groups of carbohydrates, including C–C, C–OH and C–O–C functional groups, than those of the unaged soil (Wen et al., 2007). Hence, the aging process made HA more compact and less polar. This result makes the adsorption capacity of SOM for hydrophobic chemicals stronger, which accounts for the reduced degradation of pyrene. This process further decreased the carbon source for *P. chrysosporium*, which resulted in decreased enzyme activities (see discussion of enzyme activities in soils, Section 2.5.2). It should be noted that FT-IR is only a semi-quantitative method to characterize organic functional groups, thus the analysis of FT-IR results in this study is only a supplement to data acquired in batch experiments.

2.5.2 Relationships between enzyme activity and SOM, pyrene degradation

To further understand the relationships between the soil properties, enzyme activities and biodegradation of pyrene by *P. chrysosporium* under different conditions, correlation studies were conducted. The LiP and MnP activities increased with increasing SOM content, with a correlation coefficient (r^2) of 0.9776 between SOM and LiP activity, 0.6820 for MnP activity in the nonsterilized-unaged soils, and 0.9843 and 0.6196 in the sterilized-unaged soils, respectively (data not shown). A similar correlation relationship was obtained between the enzyme activity and SOM content after the soils were aged, with r^2 of 0.9769 and 0.6160 in the nonsterilized-aged soils for LiP and MnP, respectively. When linear correlations using all data (9 soils including freshly, unaged and aged soils) were examined, the correlation between the SOM content and LiP activity was still good (r^2 = 0.9388), whereas a lowered correlation coefficient (r^2) of 0.4515 between the SOM content and MnP activity was obtained. These results confirmed the above hypothesis that the production of LiP and MnP could be activated in soils with higher SOM contents and SOM acts as a nutrient source for fungal growth (Wang et al., 2009). The correlation of LiP with SOM was more significant than that of MnP, which suggests that the production of LiP was more amenable to being controlled by SOM, whereas some other factors, such as the pyrene degradation (see discussion below), may be involved for the production of MnP.

To dynamically compare the enzyme production with the degradation of pyrene, enzyme production rates and maximum pyrene degradation rates were calculated (**Table 2**). For the nonsterilized-unaged soils, the maximum LiP activity was obtained with rates of 0.005, 0.009 and 0.03 U/(g·day) before day 11 for Soils 1–3, respectively, and for MnP, the corresponding rates were 0.026, 0.11 and 0.12 U/(g·day). The maximal degradation rates for the degradation of pyrene by *P. chrysosporium* within the first 7 days were 8.81, 6.52 and 4.75 (%/day) for the corresponding soils (**Table 2**). For sterilized-unaged soils, the maximum generation rates for the two enzymes decreased; however, the pyrene degradation rates increased (**Table 2**). After aging, the maximum generation rates for the two enzymes and the pyrene degradation rates decreased. Negative correlations were obtained between the enzyme generation rates and the pyrene degradation rates in different soils, with the correlation coefficients (r^2) of 0.9783 and 0.8908 for LiP and MnP in the nonsterilized-unaged soils, and 0.9598 and 0.9797 for LiP and MnP in the sterilized-unaged soils. After aging, the corresponding r^2 values were 0.8681 and 0.9413, respectively.

Unexpected negative correlations existed between the pyrene degradation and enzyme generation, which indicates that the influence of the macro-level of SOM may outweigh the effect of micro-levels of organic pollutants in initiating the generation of enzymes by *P. chrysosporium*.

For all soils, MnP had a weaker correlation with SOM,

Table 2 Production rate of LiP and MnP activities and pyrene degradation rate of by *P. chrysosporium* and their correlation coefficient (r^2) in soils by different treatment modes

	Nonsterilized-unaged soil			Sterilized-unaged soil			Nonsterilized-aged soil		
	LiP (U/(g·day))	MnP (U/(g·day))	Pyrene (%/day)	LiP (U/(g·day))	MnP (U/(g·day))	Pyrene (%/day)	LiP (U/(g·day))	MnP (U/(g·day))	Pyrene (%/day)
Soil 1	0.005	0.026	8.81	0.003	0.022	8.92	0.003	0.025	8.25
Soil 2	0.009	0.11	6.52	0.008	0.043	8.41	0.004	0.041	5.98
Soil 3	0.03	0.12	4.75	0.022	0.059	6.49	0.024	0.054	2.64
r^2	0.9783	0.8908		0.9598	0.9797		0.8681	0.9413	

but had better correlation with the pyrene degradation rate than LiP. Hence, this result appears to indicate that MnP is related to pyrene degradation to a greater extent, whereas LiP is related to the decomposition of SOM to a greater extent, which could also consequently benefit the degradation of pyrene by releasing the incorporated pyrene in SOM in all soils, including fresh, sterilized and sequestered soils.

MnP and LiP have a better correlation with the degradation of pyrene in the sterilized-unaged soils than those in the other treated soils. Hence, it is reasonable to speculate that the change of soil properties by sterilization was more favorable for the biodegradation of PAHs than for the production of enzyme activities. Therefore, information on biodegradation efficiency and enzyme activities enables an understanding of the behavior of PAHs in soils and the effects of the properties of the soil and PAHs, sterilization and aging on the enzyme activities. The effect of aging was considerably more pronounced than the effect of sterilization.

3 Conclusions

As the soil organic matter (SOM) content increased, the 19-day degradation percentage of pyrene decreased in all three types of soils treated with different methods, including the nonsterilized- and sterilized-unaged soils and the nonsterilized soils, whereas the maximum LiP and MnP activities increased. These results indicated that SOM outweighed pyrene in controlling enzyme production. Sterilization and aging both decreased the SOM content and altered the SOM structure, as indicated by FT-IR. Furthermore, sterilization resulted in reduced LiP and MnP activities and increased pyrene degradation, and the changes were obvious in Soils 2 and 3, while not significant for Soil 1. Aging decreased the pyrene degradation percentage and LiP and MnP activities. Furthermore, MnP is related to the degradation of PAH to a greater extent in all soils with different treatment modes, whereas LiP is related to the decomposition of SOM to a greater extent during the biodegradation of pyrene by *P. chrysosporium* in soils.

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