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Effects of inoculation with *Phanerochaete chrysosporium* on remediation of pentachlorophenol-contaminated soil waste by composting

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ABSTRACT

The effect of inoculation with *Phanerochaete chrysosporium* (*P. chrysosporium*) during different fermentation phases on remediation of pentachlorophenol (PCP)-contaminated soil waste (PSW) was investigated over 60 days. This was accomplished by evaluating physico-chemical and biochemical properties of composts, as well as bacterial community composition using denaturing gradient gel electrophoresis (DGGE). Results showed that the inoculations could significantly enhance composting efficiency and PCP removal. The best degree of maturity and highest PCP removal occurred in Run C (inoculation during the second fermentation phase) were compared with Runs A (control treatment) and B (inoculation during the first fermentation phase). A positive effect on production of manganese peroxidase (MnP) and lignin peroxidase (LiP) was found in inoculated runs, especially in Run C, while the production of laccase (Lac) was limited by *P. chrysosporium* inoculants. As a result of DGGE analysis, the compost bacterial community composition was altered by different inoculations, as indicated by the differences between the final composts. This study highlights the different effects of the inoculations on remediation performance of PSW. The inoculation during the second fermentation phase is more effective than that during the first fermentation phase.

1. Introduction

Pentachlorophenol (PCP) that is rated as a priority pollutant by the EPA, has been extensively used throughout the world as a wood preservative, pesticide and general biocide in agriculture and industry [1,2]. Large areas of soils and sediments in lakes or rivers have been polluted by PCP. After then, PCP can enter the food chain, which is thought to be teratogenetic and carcinogenic to humans [3,4]. Therefore, it still represents an environmental hazard.

As a promising bioremediation technology, composting has been successfully applied to the remediation of PCP-contaminated soil waste (PSW) [5,6]. In comparison with other technologies, composting has many advantages, which include relatively low capital and operating costs, simplicity of operation and design, and relatively high treatment efficiency [7]. Bioremediation can be enhanced by the manipulation of environmental factors to create an optimum environment for microbial degradation [8,9].

Phanerochaete chrysosporium (*P. chrysosporium*), as a kind of white rot fungus, has demonstrated a high capacity to degrade a variety of structurally diverse organopollutants including PCP [10–12] due to the production of extracellular ligninolytic enzymes, manganese peroxidase (MnP), lignin peroxidase (LiP) and to a lesser extent laccase (Lac) [10,11]. Therefore, it had been the subject of extensive investigation. There were some studies about the biodegradation of PCP in soil, water and some bioreactors by *P. chrysosporium* [12–14]. Recently, the application of inoculation with *P. chrysosporium* to treat hazardous wastes has been shown to be effective in PCP degradation and remediation of PSW at laboratory-scales [6]. However, little information about the effect of inoculation was reported due to lack of further research.

The success of remediation of PSW by composting are determined by microbial activity, as microorganisms play the most critical role in the composting process [15]. Meanwhile, the aim of optimizing composting factors is also to ensure that microorganisms are metabolically active and able to degrade contaminants [8,16]. Although the microbial communities naturally in soil waste usually carry out the composting process, the inoculation of microorganisms could potentially improve this process [17,18]. Therefore, the effect of inoculation on microbial community composition should not be neglected. Usually, microbial communities within contaminated ecosystems tend to be dom-

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Table 1
The common physical and chemical properties of composting materials.

	Moisture (%)	OM ($g\,kg^{-1}$)	TOC ($g\,kg^{-1}$)	TKN ($g\,kg^{-1}$)	TOC/TKN	pH
Soil	26.13 ± 0.58	95.0 ± 5.3	55.1 ± 2.1	2.4 ± 0.1	22.7 ± 0.98	4.73 ± 0.17
Rice straw	11.73 ± 0.29	738.0 ± 39.5	428.0 ± 16.1	8.8 ± 0.5	48.8 ± 2.24	–[a]
Vegetables	79.06 ± 1.66	167.6 ± 7.7	97.2 ± 3.3	5.0 ± 0.2	19.6 ± 0.76	–[a]
Bran	14.06 ± 0.35	817.4 ± 31.4	474.1 ± 20.1	41.2 ± 1.9	11.5 ± 0.53	–[a]

[a] Sample not quantified.

inated by those organisms capable of utilizing and/or surviving toxic contamination [19]. Considering the dominance of bacterial communities in hydrocarbon-contaminated condition [19], the profiling of bacterial communities is enabled by contemporary advances in molecular techniques using the method of polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) [20,21]. Interpretation of the data from DGGE profiles allows assessment of how any treatment impacts on bacterial community composition.

The objective of present study was to assess the effect of different inoculation time on remediation of PSW by composting. Normally, physico-chemical, and biological analyses were used in the evaluation of compost maturity and PCP removal. Meanwhile, the extracellular ligninolytic enzymes' activities correlating with PCP removal were clarified. PCR-DGGE was used to investigate the response of bacterial community composition to the inoculations.

2. Materials and methods

2.1. Fungus and inoculant preparation

P. chrysosporium strain (BKM-F-1767) was purchased from the China Center for Type Culture Collection (Wuhan, China). Stock cultures were maintained on potato-dextrose agar (PDA) slant stored at 4 °C until use. The fungus was transferred to 250 ml Erlenmeyer flasks each containing 100 ml of potato-dextrose medium. A sterile glass bead (8 mm) was added to each flask. Flasks were incubated at 37 °C on a rotary shaker at 135 rpm for a week. The culture was collected by filtration, and then washed thrice with 100 ml of 0.12 M NaH_2PO_4–Na_2HPO_4 buffer. Afterwards, those mycelia preparations (<3 mm) were used as inoculants.

2.2. Composting establishment and sample collection

PCP was purchased from American ADL Co. with a purity >98%. The raw soil was obtained from Yuelu Mountain (Changsha, China). The soil was air-dried and ground to pass through a 2-mm mesh, and then stored at 4 °C. A stock solution of PCP ($10\,g\,L^{-1}$) prepared in acetone was added to sieved soil to achieve the initial PCP concentration of 133 mg $(kg\,dry\,wt)^{-1}$. The PCP-spiked soil was stored in three 2-L specimen containers, and then left uncovered in a fume hood to allow the acetone to evaporate. Rice straw and vegetables, which were dried and then chopped into 10–20 mm pieces, were prepared as other composting materials. Bran was used to adjust the initial C/N ratio. The common physical and chemical properties of composting materials were shown in Table 1.

An experimental composting system with a weight of about 15 kg (dry wt) was set up. The soil waste, which consist of PCP-spiked soil, rice straw, vegetables and bran were mixed at a ratio of 5.3:6:1.7:1 (fresh wt), and then packed loosely in an open box with dimension of 0.65 m × 0.45 m × 0.42 m (length × width × height). The mixture had good heat preservation. The organic matter (OM) content of this mixture was 58%, while the initial C/N ratio was about 30:1. The PCP concentration in the mixture was equivalent to 50 mg $(kg\,dry\,wt)^{-1}$. Moisture was monitored and adjusted to about 60% during the first fermentation phase and about 45% during the second fermentation phase by the addition of sterile deionized water, respectively. To provide some aeration, the mixture was turned twice a week during the first 2 weeks and then once a week afterwards. Three runs, each in triplicate, were set up. Run A was the control without *P. chrysosporium* inoculants. Run B was inoculated with 0.5% *P. chrysosporium* mycelium (fresh wt) in each kg dry mixture during the first fermentation phase (day 0). Run C was inoculated with the same amount of *P. chrysosporium* mycelium during the second fermentation phase (day 15).

The experiment was conducted for 60 days and compost samples were collected every 3 days. At each sampling occasion, three subsamples for parameter analysis were taken from different places of the composting material (about 0.2 m in depth). Samples for total DNA extraction were stored immediately at −20 °C until use.

2.3. Analysis of composting parameters

The temperature in the compost piles was monitored every day. The moisture content was measured after drying the samples overnight at 105 °C. The dried samples were ground and analyzed for total organic carbon (TOC) by dry combustion [22]. The total nitrogen (TN) was measured using the Kjeldahl's method [23]. The germination index (GI) was determined using seed germination and root length tests of *Lepidium sativm* L. according to the method described by Jiang et al. [6]. The aqueous compost extracts were obtained by mechanically shaking the samples with distilled water at the solid: liquid ratio of 1:10 (w/v) for 1 h. The suspensions were centrifuged at 13,000 × *g* for 20 min and filtered through 0.45 μm membrane filters. The filtrates were used for the enzyme activity analyses with an ultraviolet spectrophotometer (UV-2250, SHIMADZU Corporation, Japan).

MnP activity was estimated by monitoring the oxidation of phenol red spectrophotometrically at 610 nm [12]. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of phenol red per minute. LiP activity was estimated by monitoring the oxidation of veratryl alcohol to veratryl aldehyde spectrophotometrically at 310 nm [24]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol veratryl aldehyde per minute. Lac activity was determined by the oxidation of 2,2′-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) spectrophotometrically at 436 nm [25]. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

2.4. PCP extraction and analysis

PCP was extracted from compost samples as described by Scelza et al. [26] with a few modifications. Ten grams samples (fresh wt) were solvent extracted by shaking at 200 rpm with 20 ml acetone and *n*-hexane (1:1, v/v) for 2 h. The solvent was decanted after centrifugation at 10,000 × *g* for 10 min. Isolated samples were allowed to dry at 30 °C for 24 h and subsequently extracted with 10 ml acetone and *n*-hexane (1:1, v/v) for 10 min followed by 10 min of centrifugation at 10,000 × *g* [27]. Solvent extracts were dried under a stream of nitrogen and redissolved in acetonitrile for HPLC analysis. The residual PCP in all the samples was quantified by HPLC (Agilent Technologies, USA) using an UVD detector and an Eclipse XDB-C18 (4.6 mm × 150 mm) column with 5 μm particle size. Analysis was conducted using 68% of acetonitrile and 32% of buffered water (1% acetic acid) as mobile phase with a column temperature at 25 °C and 1.0 ml min^{-1} flow rate. Detection was carried out at 220 nm. PCP concentrations and removal percentage were calculated by reference to appropriate standard PCP solutions.

2.5. Bacterial community fingerprinting by PCR-DGGE analysis

The final compost samples (day 60) from the three runs were used for DGGE analysis. DNA extraction from 0.5 g of each sample (fresh wt) was performed based on the method described by Yang et al. [28] followed by removal of humic substances [1]. DNA was purified with BioTeke multifunctional DNA purification kit (BioTeke Corporation, China) following the manufacturers' instructions.

The 16S rDNA genes were amplified with bacterial universal primers 338f/518r [29]. A GC clamp was attached to forward primer to prevent complete separation of the strands during DGGE. The reaction mixture (50 μl) contained 10 pmol each primer, 200 μmol of each deoxynucleoside triphosphate, 5 μl 10× Ex *Taq* buffer, 2 U Ex *Taq* polymerase, 2 μg of bovine serum albumin (BSA) and 1 μl DNA template. Cycle conditions for the amplification were as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 40 s, extension at 72 °C for 40 s, then a final extension at 72 °C for 10 min included to complete any partial polymerizations with Bio-Rad PCR Thermal Cycler Model (Bio-Rad, USA). The amplified products were analyzed by electrophoresis on a 2% agarose gel, followed by staining with SYBR Green I nucleic acid gel stain, and then were visualized under ultraviolet light.

The DNA fragments of the PCR products were separated on a DGGE gel, which was prepared according to the method of Muyzer et al. [20] with a denaturing gradient from 30 to 65% (where 100% is defined as 7 M urea with 40% deionized formamide). The electrophoresis was performed in an electrophoresis cell D-Code™ System (Bio-Rad, USA) at 60 °C and 120 V for 12 h. After stained with SYBR Green I nucleic acid gel stain for 30 min, the gel was scanned and analyzed for understanding the DGGE profiles.

2.6. Data analysis

All results reported in this study were the means and standard deviations of determinations made on three replicates. Statistical analysis was carried out using SPSS software 16.0. Sample means were compared using Least Significant Differ-