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The effects of bio-available copper on macrolide antibiotic resistance genes and mobile elements during tylosin fermentation dregs co-composting

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ABSTRACT

In this study, aerobic co-composting of tylosin fermentation dregs (TFDs) and sewage sludge with different adding concentrations of copper (Cu) was investigated to inspect the fate of antibiotic resistance genes (ARGs), metal resistance genes (MRGs) and mobile genetic elements (MGEs). Results showed that two concentrations of Cu did affect not only the abiotic factors but the relative abundances of resistance genes. High concentration of Cu inhibited the metabolic capacity of microbial community and the nitrogen-fixing process while had little effect on the degradation of TYL and TOC. The abundance of *ermT*, *mefA*, *mphA* increased partly attributed to the toxic effects and co-selective pressure from heavy metal reflected by MRGs. There was significant correlation among some environmental factors like pH, bio-Cu, organic matters and ARGs.

1. Introduction

Antibiotics fermentation dregs (AFDs), as one kind of organic solid wastes, are by-products of the manufacturing process of antibiotics fermentation (Yang et al., 2016). Although AFDs are rich in some nutrients, they also contain waste culture media within mycelium, trace amounts of antibiotics residual, the degradation products and heavy metals (copper, zinc, ferrum, etc) as well. Meanwhile plentiful of AFDs are generated in the production of antibiotics. It has been reported that in China about 600 tons AFDs had been produced up to 2009 (Zhu et al., 2013). The improper disposing of AFDs would cause the environmental risk of antibiotic resistance genes (ARGs) induced by the antibiotic resistance bacteria (Szekeres et al., 2017). Therefore, AFDs, including the mother liquors and the culture medium have been listed as one of the hazardous solid wastes in 2008 (The People's Republic of China Ministry of Environmental Protection and National Development and Reform Commission, 2008).

Aerobic composting is an environmentally friendly method to reduce the hazardous risk of AFDs and generate organic fertilizer (Qian et al., 2016), meanwhile, almost no secondary pollution produce during this process. The removal rate of penicillin was about 100% during the co-composed process between the penicillin fermentation dregs and sewage sludge (Yang et al., 2016). Also more than 99% of penicillin in fermentation residue were removed after seven days' composting (Zhang et al., 2015). However, antibiotic resistant bacteria (ARB) and ARGs could be induced due to the selective pressure of antibiotics remained in the AFDs and products of composting. In addition, a variety of heavy metals especially Cu and Zn (Wang et al., 2016; Yin et al., 2016) contained in AFDs and composting raw materials did affect ARGs, metal resistance genes (MRGs) and the microbial community composition during composting.

Heavy metals are giving rise to concerns among public health professionals, as they can persist in the environment remaining stable for prolonged periods. Particularly Cu had played a direct role for the spread and selection of antibiotics resistance in bacteria isolated from some environmental medium, especially existed with a relatively high content in AFDs and animal feeding (Li et al., 2017; Cui et al., 2016). Co-selection of antibiotics and heavy metal resistances, which frequently emerged in many environmental system like water and soil, may favor the transmission of multi-resistant bacteria and the spread of resistances into ecological environment (Zhao et al., 2017; Di Cesare et al., 2016). Additionally, Horizontal gene transfer (HGT) transferred by mobile genetic elements (MGEs) such as *integronI1* and *integronI 2* has been reported as the main key force in the dissemination of ARGs (Chen et al., 2013). Numerous researches have demonstrated that the presence of heavy metals and MGEs like Class 1 integron gene should affect the abundance of ARGs, while the effects of heavy metals (e.g. Cu) on ARGs, MGEs during AFDs co-composting process are still unclear

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(Zhang et al., 2016a,b).

In this work, tylosin fermentation dregs (TFDs), a kind of AFDs, were co-composted with sewage sludge to evaluate the effects of ARGs on the process. The objectives of this study were (1) to detect the different types of ARGs, MRGs and MGEs with different Cu treatments in AFDs co-composting; (2) to insepect the effects of Cu on the physico-chemical properties, degradation of antibiotics and the fate of ARGs, MRGs, also MGEs in composting process; (3) to inspect the relationship between ARGs, bio-available Cu and environmental factors. These hope to provide a theoretical basis for assessing the effects of heavy metals and tylosin (TYL) on ARGs in the aerobic co-composting. In addition, the results of this study would also provide insights into the potential effects on ARGs due to the presence of TYL and Cu.

2. Materials and methods

2.1. Composting experimental setup and sampling

Tylosin fermentation dregs collected from an antibiotic production company (Shandong Province, China), and sewage sludge which comprised with primary and secondary bio-solids was got from a municipal wastewater treatment plant (Harbin, China) which comprised with wood chips (1 cm long pieces) were used as the raw material for co-composting. The raw materials were mixed thoroughly with C/N ratio of 25 and moisture content of 65% for optimal composting, then performed in a 42 L rectangular containers as 30 cm height, 40 cm length, 35 cm width, each of them is covered on its all sides and top with 1.5 cm thickness styrene cystosepiment for heat preservation. To provide the favourable aerobic condition, the vent plate with holes of 2 mm diameters was located at the height of 5 cm from the containers' bottom. the ventilation was set as 0.5 L/min kg^{-1} organic matter. Two sampling holes on the side of each container were also set. During the composting process, the materials were fully turned and mixed once a day to ensure a sufficient oxygen supply which could maintain the aerobic conditions in the experiment. Based on the Cu residue level in municipal sewage sludge co-composting, the different treatments were set as: control composting treatment (CK) with only raw materials; adding $CuSO_4$ solution (20 g/L) to attain 200 mg Cu kg^{-1} samples (Cu-L) and 1000 mg Cu kg^{-1} samples (Cu-H) respectively; The composting was conducted for 35 days, the temperature and the moisture were monitored at 9:00 a.m. each day.

A set of samples obtained by mixing two sub-samples were collected on days 2, 4, 7, 10, 22, 35 respectively. Each sample was separated into two parts as one was stored at 4 °C for heavy metal detection and other chemical analysis, while another part of the 2nd, 10th, 35th samples were ready for DNA extraction and Real-time PCR detection after freeze-drying at −80 °C.

2.2. Characterization of abiotic factors in co-composting

2.2.1. Composts analysis

The composting samples were pretreated with air-drying and grinding, then passed through a 200 mesh nylon sieve. The contents of TOC and TN were detected by the elemental analyzer (SSM-5000A, Shimadzu, Japan). The dissolved organic matter (DOM) were extracted with the solid-water ratio of 1:10 (W/V) by Mili-Q water and shaking for 10 h at 25 °C and 140 rpm. The suspension was obtained at 4000 rpm for 10 min and filtrated through a 0.45 μm membrane filter. The concentrations of DOM were determined as DOC (mg/L) using a TOC analyzer (SSM-5000A, Shimadzu, Japan) for these samples.

2.2.2. Detection of bioavailable metals

DTPA-extractable heavy metals were identified as bioavailable heavy metals. The amount of DTPA-Cu in composting process were extracted using a mixed solution (0.05 mol/L diethylenetriaminepentaacetic acid (DTPA), 0.01 mol/L $CaCl_2$, 0.1 mol/Ltriethanolamine (TEA)) with a solid to liquid ratio of 1:5 (w/v) and shaking for 2 h at 25 °C. The centrifugetubes were centrifuged at 4000*g* for 10 min and the supernatant were stored at 4 °C for analyzing by an inductively coupled plasma atomic emission spectrometer (ICP-OES 5300DV).

2.2.3. LC/MS analysis of tylosin

TYL was extracted with 10 mL 90% acetonitrile aqueous solution (v/v, pH 4.0) from the soil samples within 1 g. Vortex for 5 min and ultrasound-assisted extraction for 30 min. Subsequently, the mixture was centrifuged at 14,000*g* for 10 min and then filtered through 0.45 μm filter into a 1.5 mL glass vials before chemical analysis.

Concentrations of TYL were measured by the liquid chromatography combined with ion trap mass spectrometry system (Waters, USA, Open Architecture UPLC, Xevo TQ MS). The Agilent C_{18} column (250 mm × 4.6 mm, 5 m) temperature was set at 30 °C. The flow rate was 0.2 mL/min and the injection volume was 10 μL per sample. The mobile phase was consisted of a mixture solution as 0.1% formic acid in water (solvent A) and methanol (solvent B) with a gradient mode as follows: (i) 0 min (A:B, 45:55,v/v); (ii) 3 min (A:B, 45:55, v/v); (iii) 6 min (A:B, 90:10, v/v); (iv) 8 min (A:B, 45:55, v/v).The mass spectrometer was operated in the positive ionization mode (ESI+). The qualitative and quantitive ion pairs (*m/z*) was 916.8 and 174.1. Other parameters were set as: capillary voltage temperature of 350 °C, positive ion spray voltage of 5000 V, with collision energy and cone energy of 38 eV and 54 V respectively. The gas flow rate was 12 L/min.

Using the above optimized conditions, the concentration of TYL was calculated by the difference of the peak area between the treatments and the standard. The average recovery rates of the blank samples spiked with 10–100 μg/kg of TYL was 91.6%. The detection limit of the TYL was 2 μg/kg.

2.3. DNA extraction and qPCR

DNA was extracted from 0.5 g of each sample using a FastDNA SPIN Kit for Soil (MP Biomedical, France) and operated with the instructions of the manufacturer. The concentration and quality of extracted DNA were checked by NanoDrop ND-1000 (Nanodrop, USA). Seven macrolide resistance genes with three resistance mechanism as *ermB, ermF, ermT* (ribosomal protection); *mphA, mphC* (deactivate); *msrA, mefA* (efflux pump), two heavy metal resistance genes (*copA, pcoA*) and two integrase genes (*intI1, intI2*) were analyzed by Real-time Quantitative PCR detecting system (qPCR).

The relative abundances (RAs) of the genes were expressed as copies 1 g DW. Real-time PCR assays were used to quantify the macrolides resistance genes, MGEs and MRGs. All real-time PCR (RTPCR) assays were performed on a VIIA@7 system (ABI, USA) using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH, Germany). Each reaction (20 μL) contained 5.0 μL DNA sample templates, 10 μL SYBR® Premix Ex Taq II (TliRNaseH Plus), 0.75 μL PCR forward primer (0.1 μM), 0.75 μL PCR reverse primer (0.1 μM) and 3 μL dH_2O.The reaction conditions of q-PCRs were maintained at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, the fluorescence was also measured at the end of each cycle.

2.4. 16 s rRNA gene high-throughput sequencing detection

The abundance and diversity of bacterial communities in TFDs co-composting process were examined by 16 s rRNA high-throughput sequencing. Samples were collected in the 1st, 10th and 35th day. The 16S V4 ~ V5 region was amplified using the primers as: *515F (5′-GTGCCAGCMGCCGCGG-3′)* and *907R (5′-CCGTCAATTCMTTTGAGTTT-3′)* with the average length is 373 bp. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Mix equal volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400 ~ 450 bp were