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Biodegradation of pyraclostrobin by two microbial communities from Hawaiian soils and metabolic mechanism

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ABSTRACT

Pyraclostrobin has been widely and long-termly applicated to agricultural fields. The removal of pyraclostrobin from ecological environment has received wide attention. In this study, using sequential enrichments with pyraclostrobin as a sole carbon source, two microbial communities (HI2 and HI6) capable of catabolizing pyraclostrobin were obtained from Hawaiian soils. The microfloras analysis indicated that only *Proteobacteria* and *Bacteroides* could survive in HI2-soil after acclimatization, whereas the number of *Proteobacteria* in HI6-soil accounted for more than 99%. The percentages of *Pseudomonas* in the HI2 and HI6 microfloras were 69.3% and 59.3%, respectively. More than 99% of pyraclostrobin (C_0 = 100 mg L^{-1}) was degraded by the HI2 and HI6 microorganisms within five days. A unique metabolite was identified by high performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS/MS). A metabolic pathway involving carbamate hydrolysis was proposed. The tertiary amine group of pyraclostrobin was hydrolyzed to primary amine group with the decarboxylation, which facilitated pyraclostrobin detoxification because carboxylester was an important functional group. The metabolic mechanism suggested that *Pseudomonas* expressing carboxylesterase might be able to degrade carbamate chemicals. Therefore, *Pseudomonas* might be an ideal candidate for expression and cloning of carbamate-degrading gene in genomics studies. The current study would have important implications in detoxification and bioremediation of carbamates through the C—N bond cleavage of methyl carbamate.

1. Introduction

Pyraclostrobin, a class of broad-spectrum strobilurins fungicide, was first announced by Syngenta in 2000, and then was ratified in the European Union in 2003 and enrolled at the U.S. Environmental Protection Agency in 2012 [1]. The strobilurin fungicide acts through inhibition of mitochondrial respiration by blocking electron transfer within the respiratory chain, which in turn causes severe disruption of important biochemical processes, and results in cessation of fungal growth. Pyraclostrobin has been widely used for chemical control of various fungi in many countries owing to the low mammalian toxicity, and the broad scope and high potency [1–3]. Although pyraclostrobin has low acute toxicity to mammals, its transformation products (TPs) may produce environmental risks due to the wide usage. Therefore, removal of pyraclostrobin from ecological system has attracted public attention.

It is known that, the conventional chemical or physical technologies have inherent drawbacks due to high operating cost, difficulty in operation and production of secondary pollutants [4]. Instead, bioremediation techniques have been recognized as powerful alternatives to conventional methods for decontaminating soil or water [5,6]. In ecosystem, bacteria play an important role in detoxification of xenobiotics because of their short life cycle, inexpensive cost, eco-friendly process, less secondary pollution and feasibly growth on various substrates [7–12]. Therefore, the microbial influence on pollution control of organic pollutants for the environmental remediation has been received increasing attention by the scientific community. Recent, studies have been focusing on the bioremediation potential of pre-adapted microbes

Abbreviations: DAD, diode array detector; HPLC-QTOF-MS/MS, high performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry; MW, molecular weight; MSM, mineral salt medium; MRM, multiple reaction monitoring; RRLC-QqQ-MS/MS, rapid resolution liquid chromatography tandem triple quadrupled mass spectrometer; TN, total nitrogen; TOC, total organic carbon; TPs, transformation products

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and improvement of biodegradation efficiency by bioaugmentation and biostimulation [13–24].

Although many bacterial strains or microfloras has been isolated for biodegradation of pesticides and their transformation products (TPs), only few studies on biodegradation of strobilurins were reported in the literature. For instance, Lopes et al. isolated and obtained a pyraclostrobin-degrader *Klebsiella sp.* from soybean-grown soil after long-term use of this pesticide [25]. Howell and colleagues found that Cupriavidus sp. and Rhodanobacter sp. exhibited degrading activity against azoxystrobin [26]. Two *Pseudomonas* strains (*Burkholderia* and *Pseudomonas aeruginosa*) were isolated from pyraclostrobin-contaminated natural environment, which were capable of degrading pyraclostrobin and azoxystrobin etc [27,28]. As is known, the microbial metabolism is a major pathway responsible for pyraclostrobin degradation in natural soils. However, the biodegradation mechanism of pyraclostrobin remains unclear by now. It is essential to investigate the degradation products and metabolic mechanism of pyraclostrobin in molecular and biochemical levels, which would provide rationale and guidance for the construction of pyraclostrobin-degrading genes. On the other hand, because bioremediation in nature relies on cooperative metabolic activities of complex microbial populations, biodegradation by a pure strain does not represent the actual behaviors of environmental microorganisms in natural pyraclostrobin-contaminated soils [4]. Therefore, it is necessary to study microorganisms in the pyraclostrobin-contaminated environment.

The main objectives in this study were (1) to isolate and analyze a better-tolerability microbial community capable of catabolizing pyraclostrobin; (2) to investigate the biodegradability of pyraclostrobin by the microbial communities; (3) to elucidate the metabolic pathway and degradation mechanism on basis of the metabolite analyses. The current study would have important implications in detoxification and bioremediation of carbamate chemicals.

2. Materials and methods

2.1. Chemicals and reagents

Pyraclostrobin standard (99.0% purity) was provided by Shanghai Pesticide Research Institute (Shanghai city, China). The HPLC-grade acetonitrile and formic acid were purchased from the Dikma Co., Ltd. (Beijing city, China). All of other reagents and chemicals were analytical-grade. The beef extract, peptone, agarose, acetonitrile and acetone were provided by Aladdin Reagents Co., Ltd., Shanghai, China. Sodium chloride, anhydrous magnesium sulfate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, ferrous sulfate, ammonium nitrate, manganese sulfate, calcium chloride and zinc sulfate were purchased from MYM Biological Technology Co., Ltd. (Beijing city, China). Syringe filter (nylon, 0.22 μm) was provided by Peak Sharp Company, P. R. China.

2.2. Preparation of solutions and medium

The pyraclostrobin stock solutions (5 mg mL^{-1} and 2 mg mL^{-1}) were prepared by directly dissolving the fungicide in acetone and stored at 4 ℃ in dark. Before experiment, the mineral salt medium (MSM) was prepared as the following ingredients: 1 g K_2HPO_4, 1 g $NaH_2PO_4{\cdot}12H_2O$, 1 g NH_4NO_3, 0.25 g $MgSO_4$, 0.02 g NaCl, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}H_2O$ and 0.01 g $ZnSO_4{\cdot}7H_2O$ were dissolved in the distilled water of 1 L. The beef-protein medium was used for bacterial purification, which contained beef extract (3 g), peptone (10 g) and NaCl (5 g) into 1 L distilled water, subsequently adjusted pH to 7. All the suspensions were sealed with sterilized filter membrane and autoclaved for 20 min at 121 ℃, allowed to cool at room temperature, and kept in a refrigerator at 4 ℃ until used.

2.3. Soil collection and enrichment of pyraclostrobin-degrading strains

The soils were randomly collected from subsurface zone (the first 10–20 cm of top soil) in University of Hawaii at Manoa, Honolulu, USA. These soil samples (1 kg) were immediately transported to the Laboratory and sieved through a 40-meshe. These soils were stored at 4 ℃ for no more than one week before used. The parameters of pH, total nitrogen (TN) and total organic carbon (TOC) were detected by Total Organic Carbon Analyzer (TOC Analyzer). The pH of HI2 and HI6 soil samples was 7.26 and 6.97, respectively. The TOC of soil samples were 9.47% (HI2) and 9.72% (HI6), respectively. The content of total nitrogen of HI2 sample (3.35%) was higher than that of HI6 sample (1.99%). The microbial community for both HI2 and HI6 soil samples were analyzed by Lingen biotechnology Co. Ltd.

Approximately 10 g of the soil samples were added to 50 mL sterilized physiological saline solution, and the mixture was shaken for 30 min, and then 1 mL suspension was added to 5 mL sterilized MSM containing pyraclostrobin (100 mg L^{-1}), and the mixture was incubated in dark at 30 ℃ on a rotary shaker at 200 rpm for 7 days for the first period. The successive enrichment subcultures every 7 days were performed in the same MSM supplemented with the same pyraclostrobin concentrations (100 mg L^{-1}). This step was repeated at least five times to eliminate any impurities of organic matter originating from the soil sample and to isolate a mixed culture of pyraclostrobin-degrading microorganisms. After five successive subcultures, the fifth enrichment culture was used for microbial community analysis by Lingen biotechnology Co. Ltd. And the enrichment cultures were conserved at 4 ℃ for further study.

2.4. Microbial community analysis of soil samples and enrichment cultures

Genomic DNA was extracted from the samples by Fast DNA SPIN KIT, a pair of primers 515 F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) were used for the polymerase chain reaction (PCR) of hypervariable V4 region. These samples were individually bar-coded to enable a multiplex sequencing. PCR reactions were conducted in a 20 μL master mix with 4 μL 5 × FastPfu Buffer, 2 μL dNTPs (2.5 mM), 0.4 μL forward primer (5 μM), 0.4 μL reverse primer (5 μM), 0.4 μL FastPfu polymerase (TransGen AP221-02), and 10 ng template DNA. The amplification process was conducted by ABI GeneAmp 9700 series PCR amplifier with 2 min denature at 95 ℃, 25 cycles of 95 ℃ for 30 s; 55 ℃ for 30 s; 72 ℃ for 45 s; with a final extension of 72 ℃ for 10 min. Then these DNA marked with different barcodes were sequenced by Illumina MiSeq platform (Lingen biotechnology Co. Ltd, Nanjing, China). Trimmomatic was used to remove adapters as well as low quality bases (quality score below 20) from the end. A sliding window of 50 bp was used to remove lower quality bases at the end. The paired-end reads would be merged with FLASH if an overlap was detected with a minimal overlap setting of 10 bp. Operational Taxonomic Units were clustered with a similarity cutoff of 97% using Usearch. Taxonomic classifications at phylum, family and genus level of these OTUs sequences were done with RDP classifier. The data were aligned against the SILVA ribosomal RNA sequence database using the BLAST program.

2.5. Biodegradability of pyraclostrobin by microbial consortium

To investigate the biodegradation of pyraclostrobin by the microbial consortiums, 100 μL of the fifth enrichment culture was inoculated into a 10 mL beef extract-peptone medium and incubated at 30 ℃ on a rotary shaker at 200 rpm for two days. The biodegradation studies were conducted in a series of 50 mL sterile glass flasks. An appropriate aliquot of pyraclostrobin stock solution (5000 mg L^{-1} in acetone, filter sterilized beforehand) was added into the bottom of 50 mL flask. After complete volatilization of solvent, the sterile MSM (5 mL) was added and reconstituted by ultrasonic to reach the final concentration of