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Effect of phosphate-solubilizing bacteria on phosphorus dynamics and the bacterial community during composting of sugarcane industry waste

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ABSTRACT

Sugarcane processing generates a large quantity of residues, such as filter cake and ashes, which are sometimes composted prior to their amendment in soil. However, important issues still have to be addressed on this subject, such as the description of bacterial succession that occurs throughout the composting process and the possibilities of using phosphate-solubilizing bacteria (PSB) during the process to improve phosphorus (P) availability in the compost end product. Consequently, this study evaluated the bacterial diversity and P dynamics during the composting process when inoculated with *Pseudomonas aeruginosa* PSBR12 and *Bacillus* sp. BACBR01. To characterize the bacterial community structure during composting, and to compare PSB-inoculated compost with non-inoculated compost, partial sequencing of the bacterial 16S rRNA gene and sequential P fractionation were used. The data indicated that members of the order *Lactobacillales* prevailed in the early stages of composting for up to 30 days, mostly due to initial changes in pH and the C/N ratio. This dominant bacterial group was then slowly replaced by *Bacillales* during a composting process of up to 60 days. In addition, inoculation of PSB reduced the levels of Ca-bound P by 21% and increased the labile organic P fraction. In PSB-inoculated compost, Ca–P compound solubilization occurred concomitantly with an increase of the genus *Bacillus*. The bacterial succession and the final community is described in compost from sugarcane residues and the possible use of these inoculants to improve P availability in the final compost is validated.

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Introduction

The sugarcane industry generates large quantities of solid waste, such as filter cake and ash. Currently, an average of 30 kg of filter cake is produced for every tonne of ground sugarcane. During the 2014/15 harvest in Brazil, a total of 634.77 million tonnes of sugarcane were processed [10], which produced approximately 19.04 million tonnes of filter cake. If this organic waste is not correctly disposed of, it can become a source of environmental pollution and may even cause public health problems [24]. Thus, composting is an important method for management of the waste produced by the sugarcane industry [25,27]. When applied on soil, the resulting compost improves the soil's physical properties, increasing its porosity and water retention capacity. It also alters the chemical and microbiological characteristics of the soil, enriching it with humic substances and macro- and micronutrients [21].

During composting, the microbial community is the most important component involved in recycling the residue but it must overcome a gradual reduction in nutrients and large changes in temperature and water content, as well as oxygen and ammonia levels [34]. Previous studies have described the microbial communities during composting using culture-independent analyses, such as fatty-acid methyl esters (FAMEs), denaturing gradient gel electrophoresis (DGGE), amplified ribosomal rDNA restriction analysis (ARDRA), and high-throughput sequencing technologies [8,15,19,38]. It is therefore of fundamental importance to understand other microbiological aspects, especially the succession of populations during the sequential composting process, in order to improve the physical and chemical characteristics of the final product.

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Highly weathered tropical soils are typically poor in available phosphorus (P) and have a high P fixation capacity [3]. Composting of sugarcane processing waste normally results in high P levels, primarily in organic forms. Thus, compost application provides a steady supply of mineralizable P, and improves its availability in such highly weathered soils [5].

Concurrently, it is also known that phosphate-solubilizing bacteria (PSB) are important for P solubilization of phosphate minerals and mineralization of organic P compounds [30]. However, there are few studies describing the use of PSB to increase the availability of P during composting. However, Billah and Bano [4] reported increases of up to 40% in the quantity of available P when compost piles were inoculated with a strain of *Pseudomonas* sp.

Compost with a higher quantity of available P has great potential for application in tropical soils. Hence, the main objective of the current study was to detail the effect of PSB addition on P availability in compost and to describe the structure of the bacterial communities during this process.

Materials and methods

Compost piles and treatments

The dimensions of the compost piles were 3.2 × 1.6 × 25 m (base × height × length), with a separation of 5 m between them. All compost piles were prepared using two parts of filter cake (1400 kg m^{-1}) to one part of boiler and fly ash (700 kg m^{-1}) together with laying chicken manure (380 kg m^{-1}). For treatments including rock phosphate (RP), a further 2% (42 kg m^{-1}) of powdered (particle size smaller than 0.074 mm) RP was pulverized over the piles. The chemical composition of the RP used was: total P_2O_5 (271.0 g kg^{-1}); citric acid P_2O_5 (46.0 g kg^{-1}); CaO (357.5 g kg^{-1}); Fe_2O_3 (171.3 g kg^{-1}).

During composting, the temperature was monitored daily at five different points in the pile and a minimum humidity of 30% was maintained throughout the composting period. Moisture was conserved weekly by turning the pile over (to improve water evaporation) or by adding water whenever necessary.

The experimental design included full randomization, with four treatments and three repetitions, giving a total of 12 compost piles. The treatments tested were as follows: 1- filter cake + ash (Standard); 2- filter cake + ash + RP (RP); 3- filter cake + ash + inoculant (Standard + Inoculant).; 4- filter cake + ash + RP + inoculant (RP + Inoculant). The composting period was 60 days and the piles were turned over at least every seven days.

Samples were taken every 15 days for chemical and biological analyses from five different levels within the mid 15 m of each pile, and these were mixed to obtain a composite sample for each replicate.

Phosphate-solubilizing strains and preparation of the inoculant

Prior to the experiment described above, bacterial strains were isolated from composting piles of industrial sugarcane residues, located at the same site where the study was later conducted. Sixty-five bacterial strains were isolated either at the start of the composting process (mesophilic phase) or 15 days later, when temperatures were approximately 60 °C (thermophilic phase). Afterwards, strains were selected according to their ability to solubilize P in vitro in an SMRS1 liquid medium [36]. The two best P-solubilizing bacterial strains were selected (Supplementary Fig. S1) and identified as *Pseudomonas aeruginosa* PSBR12 (from the mesophilic phase) and *Bacillus* sp. BacBR1 (from the thermophilic phase) (Supplementary Fig. S2). The accession numbers of their sequences are reported in the GenBank database. Initially, these strains were grown separately in a 13 L Tecnopon fermenter loaded with 10 L of LB medium maintained for 15 h at 300 rpm and 30 °C, with an airflow of 10 L min^{-1}. The number of viable cells was determined using the LB medium microplate method [16]. At the end of fermentation, concentrations of 10^9 cells mL^{-1} of each strain were counted. Before application, equal volumes of each of the bacterial suspensions were mixed and the inoculant was then diluted in 50 L of water, which was applied at a dosage of 8 L mg^{-1} compost and a concentration of 10^8 cells mL^{-1} for each strain. The inoculant was added at the start of the experiment and again 30 days after construction of the piles (DAPC).

Extraction of total DNA and quantification of bacterial communities during composting

Total DNA was extracted from 0.4 g compost samples using Powersoil™ DNA kits (MoBio Laboratories, USA), according to the manufacturer's instructions.

The number of 16S rRNA gene copies was estimated per gram of compost by quantitative PCR using a StepOne™ Real-Time PCR System with SYBR® Green I. The primers Eub338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and Eub518 (5′-ATTACCGCGGCTGCTGG-3′) [29] were used for quantifying, and they generated fragments of approximately 200 bp.

Amplification was performed by applying the following conditions: 95 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The final reaction volume was 25 μL, which contained 12.5 μL of SYBR® Green PCR Master Mix (Applied Biosystems®), 0.20 pmol μL^{-1} of each primer, 0.10 mg mL^{-1} BSA, 50 ng of template DNA and Milli-Q water to complete the reaction volume. Standard curves were obtained by performing amplifications with the number of known copies of the template DNA added to the reaction. In this way, the amplification data for the DNA extracted from the compost were interpolated with the standard curve to determine the number of copies of the gene of interest.

Analysis of the bacterial community structure with terminal restriction fragment length polymorphism

The bacterial community structure was analyzed using terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA gene. The target gene was amplified using the 8fm and 926r primers, with the 8fm primer being labeled with 6-FAM [35]. Enzyme restriction was accomplished using approximately 100 ng of product with 5 U of the *HhaI* enzyme (Fermentas, São Paulo, Brazil), in accordance with the manufacturer's instructions. Afterwards, a precipitation was prepared with ethanol/EDTA/sodium acetate, following the protocol of the BigDye® Terminator v3.1 Cycle Sequencing Kit. Samples were prepared for analysis following the instructions in the BigDye® manual using the GS600LIZ marker (Life Technologies) and they were analyzed in a 3500 Genetic Analyzer (Applied Biosystems, Life Technologies). The results were evaluated using the GeneMapper® 4.1 software with a cut-off line at 50 fluorescence units in order to remove background noise. The table with peak intensities was exported and the bacterial community profiles were grouped based on principal coordinates analysis (PCoA) using PAST 3.03 software [18].

Analysis of the bacterial community structure using high-throughput sequencing of the V4 region of the 16S rRNA gene

High-throughput sequencing of the V4 hypervariable region from the 16S rRNA gene was performed using the primers 515F and 806R [7]. This analysis was carried out according to the sampling time, with four samples for each period. To perform the sequencing,

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