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Changes in labile phosphorus forms during maturation of vermicompost enriched with phosphorus-solubilizing and diazotrophic bacteria

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ABSTRACT

The aim of this study was to assess the effect of N_2-fixing and P-solubilizing bacteria during maturation of vermicompost on phosphorus availability. A bacterial suspension containing *Burkholderia silvatlantica*, *Burkholderia* spp. and *Herbaspirillum seropedicae* was applied at the initial stage of vermicomposting. At the end of the incubation time (120 days), the nitrogen content had increased by18% compared to uninoculated vermicompost. Water-soluble P was 106% higher in inoculated vermicompost while resin-extractable P increased during the initial vermicomposting stage and was 21% higher at 60 days, but was the same in inoculated and uninoculated mature compost. The activity of acid phosphatase was 43% higher in inoculated than uninoculated vermicompost. These data suggest that the introduction of the mixed culture had beneficial effects on vermicompost maturation.

1. Introduction

many tropical soils. P deficiency is mainly caused by strong sorption of PO_4^{3-} to Al and Fe-(hydr)oxides, which turns a large proportion of the total soil P into unavailable forms. An inorganic P fertilizer allowed in organic production is rock phosphate (RP), but due to low solubility, the vast majority of RP is ineffective in releasing sufficient P when applied to soils (Kpomblekou and Tabatabai, 2003).

Organic matter management and nutrient cycling have a central role in achieving sustainability in agricultural systems. Vermicomposting is the non-thermophilic biodegradation of organic material through the interaction between earthworms and microorganisms (Arancon et al., 2004), and the final product, vermicompost (VC), is enriched in humus and available P (Le Bayon and Binet, 2006).

Earthworms have a marked impact on P mineralization and are able to increase the availability of P for plants as a result of their efficient digestive system, while they excrete nutrients through intestinal and cutaneous mucus (Le Bayon and Binet, 2006). As a consequence, earthworms enhance the rate of organic matter transformation and promote high microbiological diversity and activity (Fracchia et al., 2006).

High and diverse populations of native microorganisms favor owed high P bio- of nutrients in cowpea (Kumari and Ushakumari, 2002). Some work has been done with the objective of modifying the microbial community in VC and thus improving the quality of these organic fertilizers (Padmavathiamma et al., 2008). VC is an efficient vehicle and support medium for growth of *Rhizobium*, and its supplementation with native diazotrophic bacteria and mycorrhizas resulted in enhancement of maize growth (Gutiérrez-Miceli et al., 2008a,b). Mohammady Aria et al. (2010) observed that VC inoculated with *Thiobacillus* had a positive effect on the conversion of hard rock phosphate into water-soluble phosphate (WSP).

A number of diazotrophic bacteria, like *Pseudomonas* spp., *Burkholderia* spp., *Agrobacterium* spp., *Azotobacter* spp. and *Erwinia* spp. are able to solubilize phosphate in addition to carrying out biological N_2 fixation. Enhancement of P bioavailability by these microorganisms includes organic acid production, which solubilizes inorganic P (Scervino et al., 2010), and mineralization of organic forms of P by phosphatases that transform P from non-available, organically bound forms into bioavailable phosphate ions (Eivazi and Tabatabai, 1977). Organic amendments enhance soil phosphatase activity, and Saha et al. (2008) showed that application of earthworm casts was helpful in faster transformation of organic P by phosphatases. In the present study, the effectiveness of a selected microbial consortium applied to VC as an alternative technology for increasing P availability from RP for plant nutrition was explored.

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2. Methods

2.1. VC production, inoculant design and rock phosphate

VC was prepared by composting cattle manure and sunflower cake after oil extraction at a 3:1 ratio (w:w) on a dry basis and bedding the materials for 30 days. The mixture was composted thermophilically for 2 months and mechanically turned every 15 days. The composted material (10 kg) was adjusted to 80% moisture content and filled into a 20-L ceramic cylinder. Fifty earthworms (*Eisenia foetida*) per kg^{-1} of compost (dry basis) were introduced into the pot, which was covered with a jute bag to prevent direct exposure to sunlight. The moisture was adjusted weekly to 80% after weighing of the pot. The temperature during vermicomposting was 25–27 °C ± 0.85 °C as determined with a digital thermometer. The pH (H_2O) of the cattle manure was 8.1 and the organic matter composition was: 276 g kg^{-1} total organic carbon (TOC), 158 g kg^{-1} total nitrogen (TN), 17:1 C:N; water soluble phosphorus: 298 mg kg^{-1}. Sunflower filter cake had a pH (H_2O) of 5.9, 341 g kg^{-1} total organic carbon (TOC), 32.4 g kg^{-1} total nitrogen (TN), 10.5 C:N and 448 mg kg^{-1} of water-soluble phosphorus.

A mixture of three N_2-fixing bacteria (*Burkholderia* spp. strain UENF 114111, *Burkholderia silvatlantica* strain UENF 117111 and *Herbaspirillum seropedicae* strain HRC 54), from the Laboratório de Biologia Celular e Tecidual, were used to formulate the microbial inoculant. Both *Burkholderia* strains are able to solubilize phosphate in vitro (Baldotto et al., 2010). These bacteria were grown in DYGS medium (Döbereiner et al., 1995) in a rotary shaker at 120 rpm for 24 h at 30 °C. The bacterial suspension was adjusted to 10^8 viable cells per mL, mixed at an equal volume and 25 mL of the mixed suspension were applied per kg of VC (wet base).

The RP used was igneous apatite rock from Araxá, Minas Gerais State, Brazil, with 24% P_2O_5 and 4% soluble P in 2% citric acid. The RP was added to VC at a ratio of 1 g kg^{-1} (wet base). The treatments were based on a combination of biological enrichment (presence of a mixed bacterial suspension) and RP application (inoculated VC) or the absence of these (non-inoculated VC). Samples were collected immediately after inoculation (zero time) and at 30, 60, 90 and 120 days for analyses.

2.2. Organic matter evaluation

TOC in the VC was estimated using the dichromate oxidation method (Nelson and Sommers, 1982). TNC was determined after digesting the sample with concentrated H_2SO_4 (1:20, w:v) followed by distillation (Bremner and Mulvaney, 1982). The pH was determined with a Thermo Fisher pH-meter equipped with glass electrode (Orion) after shaking in 0.01 mol L^{-1} $CaCl_2$ at a ratio of 1:5 (w:v) for 30 min. Soluble humic substances were extracted by adding 200 mL of 0.1 mol L^{-1} NaOH to10 g of VC and shaking for 16 h at room temperature under N_2 atmosphere. The darkly colored supernatant was separated from the residual VC by centrifugation at 3000*g* for 30 min. The VC residue was re-suspended in 50 mL 0.1 mol L^{-1} NaOH, shaken for 4 h, centrifuged again and added to the previously collected supernatant. This procedure was repeated until a clear solution was obtained. The extracted alkaline solution was acidified to pH 1.0–1.5 with concentrated H_2SO_4 and the humic acid (HA) fraction was separated from the fulvic acid fraction (FA) by centrifugation at 5000*g* for 30 min. The organic carbon (C) content in HA and FA was determined by modified Walkley–Black procedures (Yeomans and Bremner, 1988).

2.3. Available P

WSP was obtained using 1 g of VC and 25 mL of distilled water. The samples were shaken for 2 h (150 rpm), filtered through Whatman filter paper number 42 and the supernatant was analyzed for PO_3^{4-} by spectrophotometry, using the ascorbic acid molybdenum blue method (Murphy and Riley, 1962). To determine resin-extractable P, 0.5 g of VC was shaken for 16 h with 30 mL of deionized water and bags containing 5 g of both an anion and cation exchange resin (Amberlite IRC76). After agitation, the resin was cleaned with fluxed deionized water and the P desorbed with 30 mL 0.5 mol L^{-1} NaCl. P was determined using the ascorbic acid molybdenum blue method.

2.4. Phosphatase assay

Acid and alkaline phosphatases were assayed using 1 g of VC (wet equivalent), 4 mL 0.1 mol L^{-1} modified universal buffer (MUB) (Tabatabai and Bremner, 1969) at pH 6.5 for acid phosphatase and pH 11.0 for alkaline phosphatase and 1 mL 25 mM *p*-nitrophenyl phosphate (Tabatabai and Bremner, 1969). After incubation for 1 h at 37 ± 1 °C, the enzyme reaction was stopped by adding 4 mL 0.5 mol L^{-1} NaOH and 1 mL 0.5 mol L^{-1} $CaCl_2$ to prevent dispersion of humic substances. The absorbance of the supernatant was measured at 400 nm and the enzyme activity expressed as μg *p*-nitrophenol released g^{-1} VC h^{-1}.

Phosphodiesterase activity was measured following the method of Browman and Tabatabai (1978). The substrate was 0.005 mol L^{-1} *bis*-*p*-nitrophenyl phosphate and the concentration of *p*-nitrophenol was measured. Enzyme activity was expressed as μg *p*-nitrophenol released g^{-1} VC h^{-1}.

2.5. Bacteria counts

Estimation of the bacterial population size was obtained by the most probable number method (Döbereiner et al., 1995). Samples of 10 g of the VC were diluted in 90 mL of saline solution (8.5 g L^{-1} NaCl) and after 1 h of agitation (100 rpm), the solution was serially diluted up to 10^{-7} dilution. Aliquots of 0.1 mL were transferred to glass vials containing 5 mL of JMV or JNFB semi-solid medium without nitrogen, respectively, to obtain counts of *Burkholderia* and *Herbaspirillum* (Döbereiner et al., 1995). The vials were incubated at 30 °C for 7 days. Afterward, growth of bacteria was evaluated by the presence of a typical white pellicle on the surface of the medium. The estimated number was obtained by consulting the McCrady table with three replications per dilution.

2.6. Organic acids were analyzed by YongLin Acme 900 Performance Liquid Chromatography (HPLC).

The bacteria strains were grown in the presence 0.5% of RP added to the Digs liquid medium. The culture supernatant was filtered through 0.22 μm nylon filter. The organic acids were separated using RP-18 column. The mobile phase consisted of 0.1% phosphoric acid with a flow rate of 1 mL/min. Organic acids was detected by monitoring absorbance at 210 nm. They were identified and quantitated by comparing the retention times and peak areas with solutions of pure acids.

3. Results and discussion

The total organic carbon content was high in VC at the initial stage of incubation (400 μg g^{-1}) and decreased rapidly during the first 30 days of maturation (Fig. 1A) and stabilized at 25% less C than the initial value. No changes in TOC content were found in inoculated VC. The combination of RP application and bacterial inoculation led to higher recorded TNC (Fig. 1B), with the maximum increase at 60 days of maturation. This increase was 18% above the initial value. The absence of RP and bacterial application

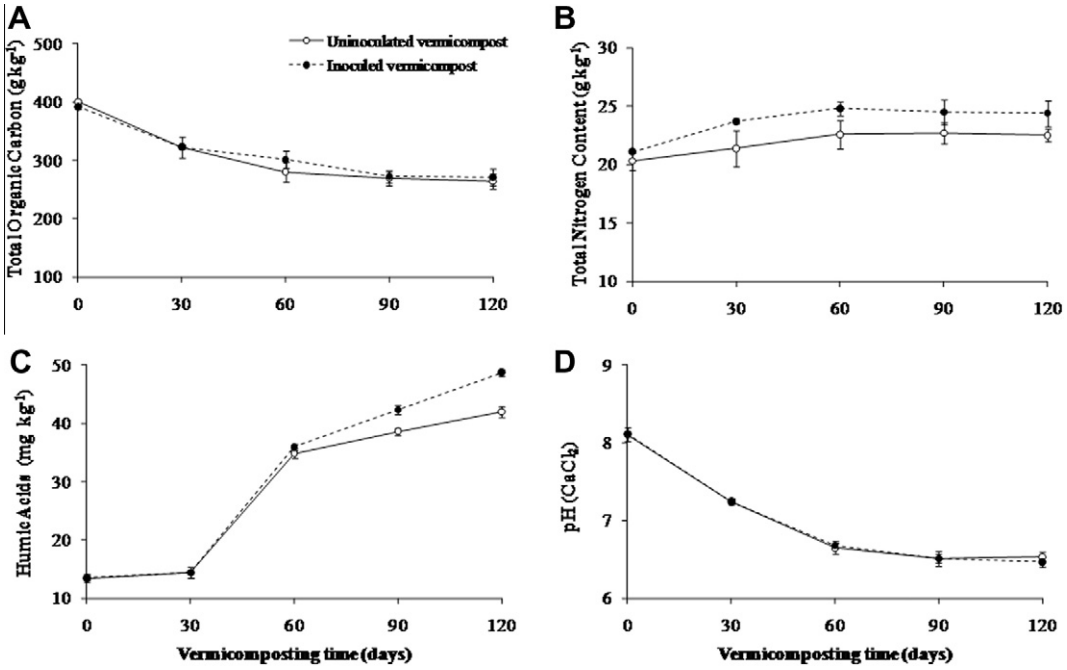

Fig. 1. Effect of microbial inoculation and rock phosphate addition on. (A) TOC (total organic carbon), (B) TNC (total nitrogen content), (C) HA (humic acid) content and (D) pH ($CaCl_2$) of vermicompost. Bars represent standard deviation.

also increased TNC at the end of maturation with respect to the initial value; however, the magnitude of this increase was lower (11%). VC showed increased HA content according to the maturation stage (Fig. 1C). At the end of the incubation time, HA content was 15% higher in inoculated than uninoculated VC resulting in a 10% higher HA/FA ratio (data not shown). Vermicomposting shifted the pH towards acidity (Fig. 1D). For both VCs, the pH decreased rapidly in the first 60 days and reached final values of 6.8–6.6.

According to Frossard et al. (1996), water and resin-extractable P are rapidly available to plants. The content of WSP increased linearly for 60 days in inoculated VC, and reached a maximum of 3.7 mg g^{-1}, then remained constant until the end of incubation (Fig. 2A). For uninoculated VC, the WSP also increased for 60 days, however, after 90 days, WSP decreased abruptly and became 2.4 times less than that of inoculated VC after 120 days. The behavior of resin-extractable P was different, since in the first 30 days, a slow decrease in relation to the value at the initial time was observed in both treatments (Fig. 2B), however, with maturation time, inoculated VC showed an about 15% increase in relation to uninoculated VC between at 60 and 90 days. Maximum resin-extractable P was reached at 60 days in inoculated VC, a value 36% higher than at the initial time. At 120 days, the values were similarly high (around 11.0 mg g^{-1}) in both treatments.

Estimation of alkaline phosphatase (Fig. 3A) and acid phosphatase (Fig. 3B) activity revealed that alkaline phosphatase activity was higher than acid phosphatase activity. Between treatments, the phosphatase activity changed according to the VC maturation stage. Rock phosphate application and use of a bacterial suspension showed the highest alkaline phosphatase activity during the first 90 days, followed by a decrease in activity. At the end of the vermicomposting process, similar values were observed between treatments (17.5 μg *p*-nitrophenol g^{-1} VC h^{-1}) (Fig. 3A). Acid phosphatase activity exhibited different dynamics. At the initial stage of maturation, similar values were observed between the treatments, but after 60 days, a maximum activity (19 μg *p*-nitrophenol g^{-1} VC h^{-1}) was observed in inoculated VC. At the end of the vermicomposting process, this activity decreased but remained

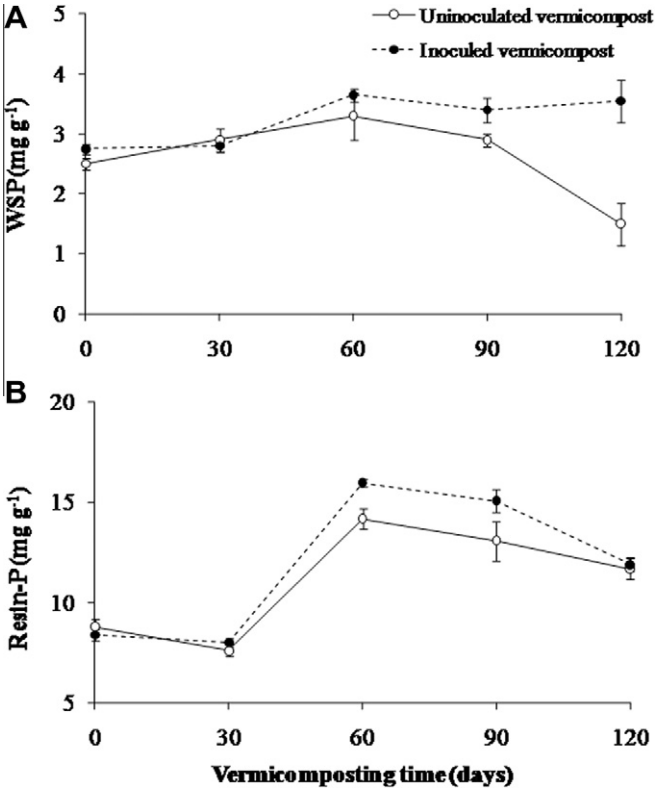

Fig. 2. Available P during vermicomposting with added rock phosphate and microbial inoculation. (A) WSP (water-soluble phosphorus) and (B) resin-extractable P. Bars represent standard deviation.

43% higher than that in uninoculated VC (Fig. 3B). Maximum diesterase activity was observed at the initial stage of incubation, with a maximum value at 30 days, reaching 30 μg *p*-nitrophenol g^{-1} VC h^{-1} in inoculated VC (Fig. 3C). After 30 days, a linear decrease in diesterase activity was observed. At the end of incubation,

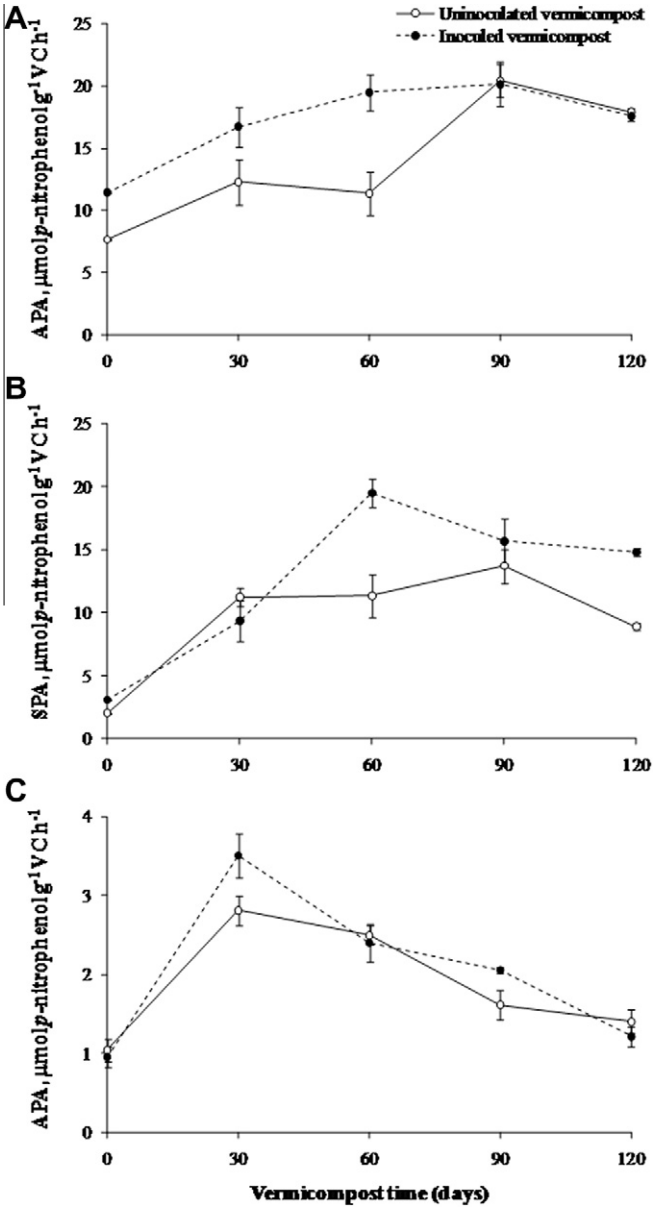

Fig. 3. Phosphatase activities in VC as affected by rock phosphate and microbial inoculation. (A) Alkaline phosphatase; (B) acid phosphatase and (C) phosphodiesterase activity. Bars represent standard deviation.

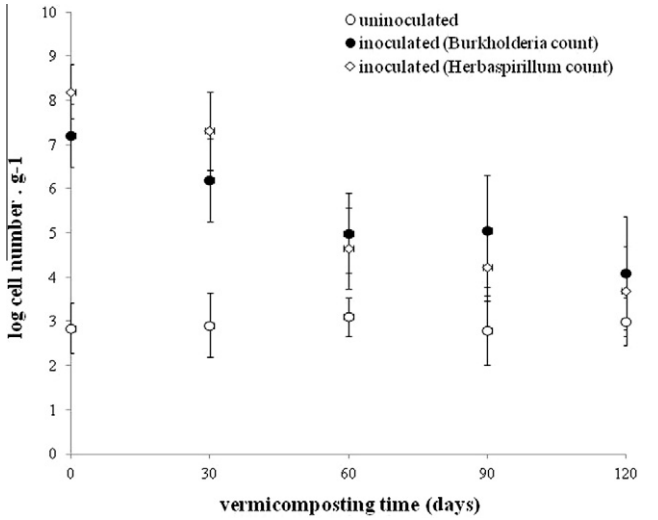

Fig. 4. Estimation of the bacterial population recovered from VC by the most probable number technique over the maturation time. Counts for *H. seropedicae* and *Burkholderia* spp. were respectively performed in JNFb and JMV semi-solid medium without nitrogen. Count for native diazotrophic population was done only in JNFb medium. Means from three replications.

similar values were observed between treatments. In general, inoculated VC displayed higher phosphatase activities for the entire incubation time. The levels of alkaline or acid phosphatase and diesterase were remarkably influenced by the VC maturation stage.

Estimation of the bacterial population revealed a native population of unidentified diazotrophic bacteria, and at the end of vermicomposting, the inoculated VC exhibited higher numbers of diazotrophic bacteria (Fig. 4), which means that the microbial consortium effectively enhanced the population of *Herbaspirillum* and *Burkholderia* as indicated by reisolation and cellular and colony characterization according to Döbereiner et al. (1995).

Management practices with organic materials influence agricultural sustainability by improving the physical, chemical and biological properties of soil; however, use of organic materials as fertilizer is often criticized due to the large volumes required which increase the cost of transport or field work. Deportes et al. (1995) suggested that numerous harmful effects on soil are caused by the application of non-mature compost, i.e., incomplete stabilization of the organic fraction. Vermicomposting is a stabilization process due to the accelerated biooxidation of organic material involving the joint action of high densities of earthworms and microorganisms. Compared to conventional composting, vermicomposting often results in a reduction in mass, shorter processing times, and higher levels of humus with reduced phytotoxicity (Lorimor et al., 2001).

The maturing process involves several changes in chemical content and transformation in the structure of VC, which can be predicted by the C/N ratio and humification index. Despite 30 days of pre-composting of the materials, the introduction of earthworms induced a quick reduction in TOC content in the first 30 days of vermicomposting (Fig. 1A). Over this time, enhanced organic matter stabilization by HA production was observed (Fig. 1C). These differences were attributed to the relationship between ingested microorganisms and the intestinal mucus of earthworms (Trigo and Lavelle, 1993). According to Vinceslas-Akpa and Loquet (1997), earthworms digest long chains of polysaccharides like those present on sunflower cake residues, enhancing microbial colonization. Simultaneously, the structure of lignin changes, probably due to microbial oxidation and demethylation (Stevenson, 1994). Macro-invertebrates produce peroxidases (Neuhauser and Hartenstein, 1978), and these enzymes may enhance the polymerization of aromatic compounds and increase humification, since HA formation can be depicted as an increase in the amount of hydrophobic components and weak interaction forces in humic aggregates.

An enhancement of about 20% in TNC in inoculated VC at the end of the maturation stage in comparison with initial values was observed (Fig. 1B). The increased TNC in VC may be due to the release of nitrogenous products by earthworm metabolism through their cast (excreta), urine and mucoproteins (Padmavathiamma et al., 2008). In the gut of earthworms, it is possible that mucus secreted from the gut epithelium provides an energy source that stimulate biological N fixation in quantities that are significant both for earthworm metabolism and as a source of N for plant growth (Lee, 1985). Previous studies also observed an increase in TNC with microbial enrichment of VC with N-fixing bacteria (Padmavathiamma et al., 2008).

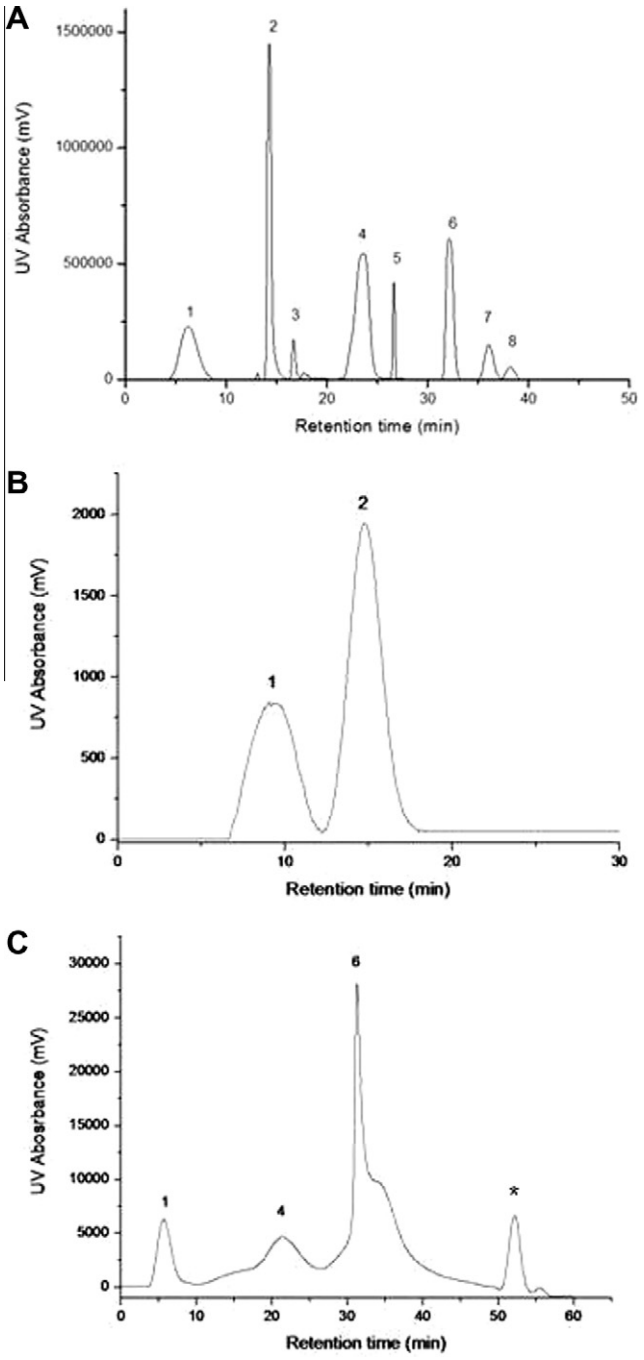

Fig. 5. (A) HPLC chromatogram of standard organic acids (Sigma) with 20 μL of volume injection at 5 mM: 1 – succinic, 2 – oxalic, 3 – citric, 4 – tartaric, 5 – malic, 6 – ascorbic, 7 – formic; 8 – acetic acid. (B) Organic acids secretion by *Burkholderia* strain UENF 114111 and (C) 117111 grown in Digs liquid medium with 0.5% of rock phosphate. ∗ Mean an unidentified organic acid.

The pH of VC decreased significantly in the first 30 days of incubation and slowly decreased until stabilization at 90 days. Decomposition of organic matter leads to formation of HCO_3 and HA (Komilis and Ham, 2006). The first component contributes to a greater drop in pH at the initial stage of incubation and the presence of carboxylic and phenolic groups in HA probably caused a lowering of the pH in more stabilized VC. The combined effect of these two charged ions regulates the pH of VC, leading to a shift in pH from 8.1 to 6.8–6.6 (Fig. 1D).

Water-soluble P values were larger, reaching 3.5 and 1.5 mg g^{-1} in inoculated and uninoculated VC, respectively, after 120 days of vermicomposting (Fig. 2A). Resin-extractable P was higher, with a maximum 16.0 mg g^{-1} at 60 days in inoculated VC, and stabilizing at 11.0 mg g^{-1} in both treatments at the end of the maturation stage (Fig. 2B). Passage of organic residues through earthworms influences P availability (Le Bayon and Binet, 2006) because P is concentrated in their casts through ingestion of P-rich particles. High values of bioavailable P have been found in other VCs and attributed to organic P mineralization due to exudation of organic acids by microorganisms and activation of phosphatases (Gaume et al., 2001). The bacteria strains of *Burkholderia* used in this study secret a significant amount of organic acids in the presence of RP under laboratory conditions being oxalic, citric and tartaric acids the most prominent chemical species (Fig. 5). The ability of microorganism to solubilize P complexes has been attributed to the process of acidification, chelation, exchange reactions and production of organic acids (Gulati et al., 2010). The major mechanism of mineral phosphate solubilizing activity is the secretion of organic acids synthesized by microorganisms (Patel et al., 2011).

The action of enzymes on forms of P is well documented in soil (Quiquampoix and Mousain, 2005), and high phosphatase activities in VC have been reported (Pramanik et al., 2007). In this study, we observed high phosphatase activities throughout vermicomposting, but the kind of enzyme predominating was time-dependent. For example, at the initial stage, phosphodiesterase activity was higher but rapidly decreased with time, while alkaline phosphatase and acid phosphatase activity increased for 90 days, but decreased thereafter. The inoculated vermicompost had shown higher population size of diazotrophic bacteria (Fig. 4) changing the microbial community structure and probably leading to increased demand for phosphate and higher phosphatase activity (Fig. 3).

At the end of the maturation stage, a decrease in resin-extractable P was observed for both treatments and a decrease in WSP was observed in uninoculated VC. P compounds can be associated with organic matter by weak van der Waals interactions or hydrophobic forces that aggregate organic molecules and make these organic molecules insoluble in water (Piccolo, 2002). This aggregation effect could result in an increased organic matter fraction insoluble in water and poor nutrient extractability. It is possible that an increase in the degree of humification shown by enhanced HA content contributes to a reduction in WSP in the uninoculated treatment, while WSP remains elevated in treatments involving microbe inoculation due the high microbial population (Fig. 4). However, despite the decrease in resin-extractable P in both treatments, the markedly high value observed (around 11.0 mg g^{-1}) convert VC into a highly bioavailable P fertilizer, based on the application of the concept of biological enrichment of substrates with the proper combination of beneficial microorganisms.

4. Conclusion

Introduction of select microorganism during vermicompost increased phosphatase activity, P availability and N contents. Such results may represent an important biotechnological tool to increase the value of recycled organic residues.

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