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Microbial Biomass Governs Enzyme Activity Decay during Aging of Worm-Worked Substrates through Vermicomposting

Manuel Aira,* Fernando Monroy, and Jorge Domínguez

ABSTRACT

Vermicomposting is the biooxidation and stabilization of organic matter involving the joint action of earthworms and microorganisms, thereby turning wastes into a valuable soil amendment called vermicompost. Studies have focused on the changes in the type of substrates available before and after vermicomposting, but little is known on how these changes take place, especially those changes related with maturation of vermicompost. This study investigated the effects of aging on the microbiological properties of fresh vermicompost produced from pig slurry by analyzing the substrate after the earthworms had left it. We incubated 16-wk-old vermicompost and sampled it after 15, 30, 45, and 60 d analyzing microbial biomass and activity (assessed as microbial biomass N and basal respiration respectively) and four enzymatic activities (β-glucosidase, cellulase, protease, and alkaline phosphatase). Aging of vermicompost resulted in decreases of microbial biomass and activity. Three of the four enzymes analyzed also showed decrease. An initial increase followed by a rapid decrease in alkaline phosphatase was also recorded. High and significant correlations between microbial biomass and β-glucosidase ($r = 0.62$, $P < 0.001$), cellulase ($r = 0.56$, $P < 0.01$), and protease ($r = 0.82$, $P < 0.001$) were found. Results suggest that there may be two steps involved in the aging dynamics of vermicompost with regards to extracellular enzyme activity; the first step was characterized by a decrease in microbial populations, which resulted in a reduction in the synthesis of new enzymes. The second step was the degradation of the pool of remaining enzymes. This dynamic does not seem to be affected by earthworms because similar decaying patterns of microbial biomass and activity were found in substrate where earthworms were present.

Vermicomposting is a process that involves the oxidation and stabilization of organic wastes through the joint action of earthworms and microorganisms (Domínguez, 2004), thereby turning wastes into a valuable soil amendment called vermicompost. This technique has been widely used to process many different types of residues, including organic and industrial wastes (Edwards and Arancon, 2004). Studies have focused on the changes in the type of substrates available before and after vermicomposting, but little is known on how these changes take place (Edwards and Arancon, 2004). For example, it is not known whether the changes take place continuously during the whole process, thereby improving the vermicompost, or if there is a point at which there is a reversal in the changes, resulting in an impoverished product.

Maturation of the product, which is a critical step during vermicomposting, begins once earthworms leave the substrate and which therefore, can be defined as a microbial-driven process. The microbiological properties of vermicompost, such as microbial biomass content and associated activity, regulate nutrient dynamics during maturation, leading to immobilization or release of nutrients, and therefore affect plant uptake if they are applied to the soil (Schimel and Bennet, 2004). The study of enzyme activities has been shown to be a reliable tool for establishing the maturity of vermicompost (Benítez et al., 2002; Benítez et al., 2005), as well as being of significance in the rate of nutrient cycling including carbon, nitrogen, and phosphorus (Nannipieri, 1994; Tate, 2000). It is also important to study how the aging of microbiological parameters of vermicompost is because they are used for biochemical restoration if enzymes are still active (García et al., 1994; Masciandaro et al., 1997), and it is known that vermicompost with active microorganisms and enzymes significantly improve soil fertility (Dick, 1992; Nannipieri, 1994).

In the present study, the effects of vermicompost aging, especially patterns of changes in microbial biomass and enzyme activities, were investigated because these parameters can control the quality of the resulting vermicompost. Further, it is known that microbial enzyme activity is a useful tool to control functional responses of microbial communities to changes in their environment (Carreiro et al., 2000). It has been reported that the amount and quality of organic amendment applied to the soil will directly affect the quality and functioning of soil, water quality, and thus, the whole environmental quality (García et al., 1994; Masciandaro et al., 1997). In addition, we studied the relationships between microbial biomass and activity with the dynamics of the enzymatic activity. For this, we analyzed β-glucosidase and cellulase, enzymes of the carbon cycle, because they are known to be associated with litter decay and therefore with a turnover of carbon in a wide range of ecosystems (Sinsabaugh, 1994; Sinsabaugh and Moorhead, 1994). We also analyzed alkaline phosphatase activity, involved in the phosphorus cycle hydrolyzing organic phosphate esters to inorganic phosphorus (Alef et al., 1995), which is then available to plants. Protease activity was studied because it catalyzes the depolymerization N-containing compounds into dissolved organic nitrogen (Paul and Clark, 1996), which is a critical step in the nitrogen cycle since polymers are not available to microorganisms because of their large molecular size (Chapin et al., 2002; Schimel and Bennet, 2004).

Departamento de Ecoloxía e Biología Animal, Universidade de Vigo, Vigo E-36310, Spain. Received 4 July 2006. *Corresponding author (aira@uvigo.es).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: N_{mic}, microbial biomass-N; dry wt., dry weight; om, organic matter; PNP, *p*-nitrophenol.

MATERIAL AND METHODS

Experimental Design

The vermicompost was obtained from laboratory-based vermireactors containing the earthworm species *Eisenia fetida* (Savigny, 1826), which were continuously fed with pig slurry. *Eisenia fetida* is an epigeic species, e.g., litter feeding, associated to environments with high organic matter content, and their natural populations live at high densities in patchy distributions (Monroy et al., 2006). The modular design of these vermireactors allows monitoring of the age of substrate in each module (Aira et al., 2006); the substrate from the uppermost modules was chosen to carry out the study because it was the first module with no earthworms, and it was 16 wk old. Samples of vermicompost (1 kg fresh wt.) from five vermireactors were placed separately in five plastic trays (60 × 40 × 10 cm) and maintained at a constant temperature (20°C) in a laboratory chamber. The five vermireactors were set up at the same time in a room with constant temperature (20°C), and each sampling time represented the same aging time for each vermireactor (16 wk old). The modules of vermireactors corresponding to 16-wk-old substrate were sampled because they were the first modules containing no earthworms. Samples from these plastic trays were taken at 0 (16-wk-old vermicompost), 15, 30, 45, and 60 d. The main characteristics of the pig slurry used and the vermicompost obtained from these modules are summarized in Table 1.

Analyses

Microbial biomass N (N_{mic}) was determined by the chloroform fumigation–extraction method with field-moist samples (5 g fresh wt.) (Brookes et al., 1985). The filtered extracts (0.5 *M* K_2SO_4) of both fumigated and nonfumigated samples were analyzed for total extractable N. Total extractable N was determined after oxidation with $K_2S_2O_8$ as described by Cabrera and Beare (1993). N_{mic} was estimated as the difference between the total extractable N extracted from the fumigated and from the nonfumigated samples. This method has been shown to work properly with organic samples from vermicomposting studies (Domínguez et al., 2003). Microbial activity was assessed by measuring the rate of CO_2 evolution from the sample after 6 h incubation (basal respiration). The evolved CO_2 was trapped in 0.02 *M* NaOH then measured by titration with HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$ (Anderson, 1982).

β-Glucosidase activity was assessed by determination of the *p*-nitrophenol (PNP) released, after the incubation of the samples (1 g fresh wt.) with *p*-nitrophenyl glucoside (0.025 *M*) for 1 h at 37°C. Filtered end products were read in a Bio-Rad Microplate Reader at 400 nm (Eivazi and Tabatabai, 1988). Cellulase activity was estimated by determination of the reducing sugars released after incubation of samples (5 g fresh wt.) with carboxymethyl cellulose sodium salt (0.7%) for 24 h at 50°C. Filtered end products were read in a Bio-Rad Microplate Reader at 690 nm (Schinner and von Mersi, 1990). Protease activity was measured by determination of the amino acids released, after the incubation of the samples (1 g fresh wt.) with sodium caseinate (2%) for 2 h at 50°C, using Folin–Ciocalteu reagent. Filtered end products were read in a Bio-Rad 550 Microplate Reader at 700 nm (Ladd and Butler, 1972). Alkaline phosphatase activity was measured by determination of PNP released, after the incubation of the samples (1 g fresh wt.) with *p*-nitrophenyl phosphate (0.025 *M*) for 1 h at 37°C. Filtered end products were read in a Bio-Rad Microplate Reader 550 at 400 nm (Eivazi and Tabatabai, 1977).

Table 1. Physicochemical characteristics (mean ± S.E.) of fresh pig slurry (n = 10) and 16-wk-old vermicompost obtained from vermireactors (n = 5) (dry wt. = dry weight).

Parameter	Pig slurry	Vermicompost
Moisture content, g kg^{-1} dry wt.	860 ± 10	780 ± 25
Organic matter content, g kg^{-1} dry wt.	860 ± 10	650 ± 10
pH	8.3 ± 1.0	7.2 ± 0.1
Electric conductivity, dS m^{-1}	2.5 ± 0.1	13 ± 0.4
Total carbon, g kg^{-1} dry wt.	450 ± 60	360 ± 2
Dissolved organic carbon, mg kg^{-1} dry wt.	11 100 ± 100	1200 ± 100
Total N, g kg^{-1} dry wt.	24 ± 2	27 ± 1
N-NH_4^+, mg kg^{-1} dry wt.	2400 ± 100	30 ± 10
N-NO_3^-, mg kg^{-1} dry wt.	250 ± 50	730 ± 80

Statistical Analysis

Data were analyzed using a repeated measures ANOVA for which incubation time (age) was fixed as a within-subject factor. All variables analyzed fulfilled sphericity assumptions (Mauchly's test). Post-hoc comparisons (Tukey HSD) were made following ANOVA. We also explored the relationship between microbial biomass and activity (N_{mic} and basal respiration) and the four enzymatic activities studied with correlation analysis. All statistical analyses were performed using SPSS 11.5 software.

RESULTS

Microbial biomass of vermicompost (N_{mic}) decreased significantly with aging (ANOVA $F_{4,16} = 336.66$, $P < 0.0001$). Samples incubated for 15 d, although lower than fresh vermicompost, still retain a high content of N_{mic} (10 700 mg kg^{-1} dry wt.), after that it dropped to contents below 2000 mg kg^{-1} dry wt. (30 and 45 d of incubation), with a final content of 315 mg kg^{-1} dry wt. after 60 d of incubation (Fig. 1a).

The microbial activity (measured as basal respiration) of the vermicompost decreased continuously and significantly with aging (ANOVA $F_{4,16} = 8.46$, $P < 0.01$), while a sudden increase was detected in basal respiration at 30 d of incubation (Fig. 1b). However, microorganisms maintained their activity during 45 d of incubation with values of basal respiration over 50 mg CO_2 kg^{-1} organic matter h^{-1}, and then, after 60 d of incubation, strongly dropped to values below 5 mg CO_2 kg^{-1} organic matter h^{-1} (Fig. 1b).

The pattern of activity of the C cycle enzymes, β-glucosidase and cellulose, were different during the aging process until the end where both enzyme activities were significantly decreased (β-glucosidase, ANOVA $F_{4,16} = 27.72$, $P < 0.0001$; cellulase, ANOVA $F_{4,16} = 72.91$, $P < 0.0001$) (Fig. 2a, b). More specifically, the activity of β-glucosidase during aging of the vermicompost was variable with increases and decreases recorded, although the overall general pattern was a decrease in activity (Fig. 2a). In contrast, there was no significant difference in cellulase activity among fresh vermicompost and the vermicompost samples incubated for 15, 30, and 45 d, with the greatest reduction in activity occurring in 60-d-old samples (Fig. 2b). Activity of β-glucosidase was highly correlated with N_{mic} (Pearson $r = 0.62$, $P > 0.001$) but not with basal respiration. On the other hand, cellulase activity showed strong correlations with N_{mic} (Pearson $r = 0.56$, $P < 0.01$) and basal respiration (Pearson $r = 0.53$, $P < 0.01$).

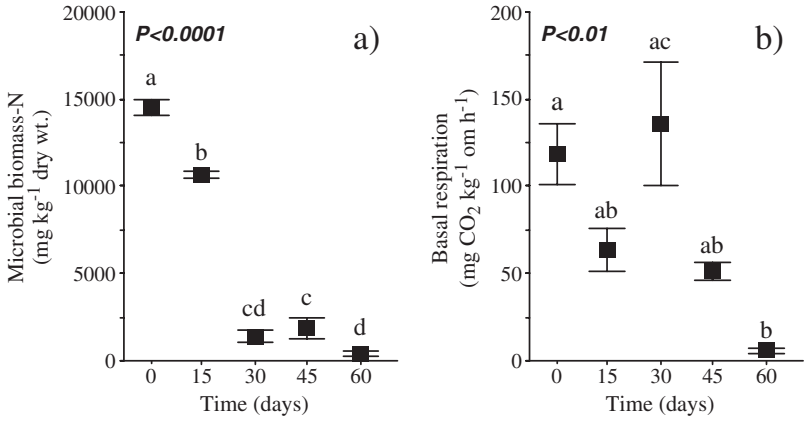

Fig. 1. Changes in (a) microbial biomass N (mean ± S.E.) and (b) basal respiration during incubation of vermicompost (n = 5). Different letters indicate significant differences at $p < 0.05$ (Tukey HSD) (dry wt. = dry weight; om = organic matter).

The activity of protease was significantly affected by aging (ANOVA $F_{4,16} = 36.78$, $P < 0.0001$). Thus, after a slight increase observed after 15 d of incubation, the protease activity strongly decreased to values below 1500 mg tyrosine kg^{-1} dry wt. 2 h^{-1}; this reduction in activity increased with the time of incubation (Fig. 2c). Protease activity showed a high correlation with N_{mic} (Pearson $r = 0.82$, $P < 0.001$) but not with basal respiration.

Alkaline phosphatase activity was also significantly affected by the incubation time (ANOVA $F_{4,16} = 14.26$, $P < 0.0001$). However, in this case the activity of this enzyme peaked in samples of vermicompost incubated for 15, 30, and 45 d, with a mean value of 2800 mg PNP kg^{-1} dry wt. h^{-1}; then the activity decreased to values similar to those corresponding to fresh vermicompost (Fig. 2d). Alkaline phosphatase activity was not correlated with N_{mic} and basal respiration.

DISCUSSION

The results of this study showed that once earthworms have left the organic waste, the process of aging of vermicompost is mainly characterized by microbial processes,

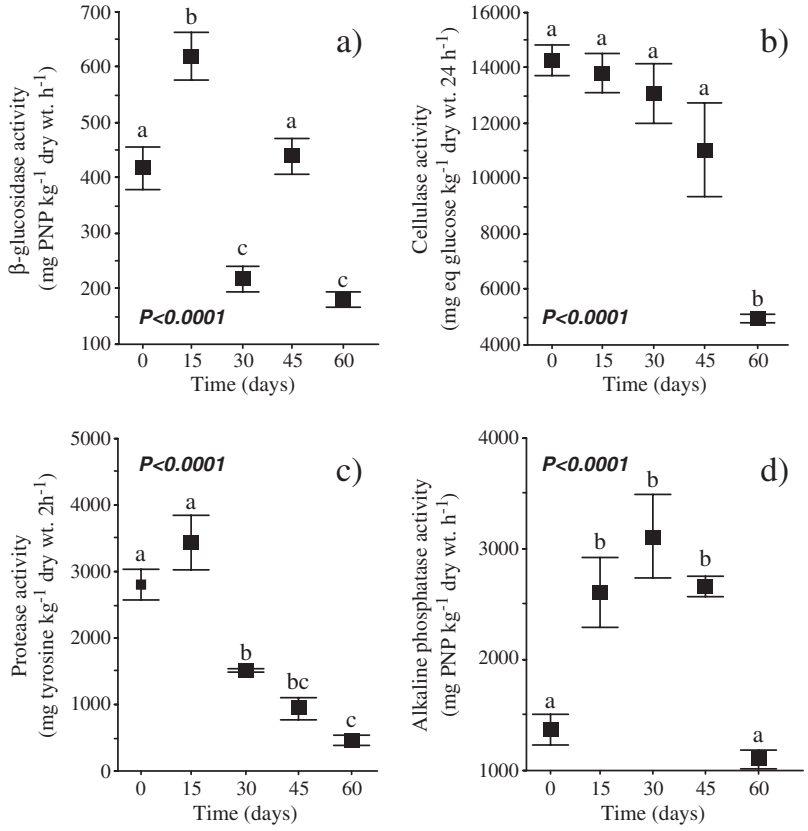

Fig. 2. Changes in (a) β-glucosidase (mean ± S.E.), (b) cellulase, (c) protease, and (d) alkaline phosphatase during incubation of vermicompost (n = 5). Different letters indicate significant differences at $p < 0.05$ (Tukey HSD) (dry wt. = dry weight).

which experience a continual decrease as indicated by microbial biomass N, basal respiration, and some of the enzyme activities measured. Thus, aging clearly decreased protease and cellulase activities, whereas β-glucosidase activity showed an increase-decrease dynamic; the exception was alkaline phosphatase activity which increased during the aging process. Despite different dynamics of aging shown by the four enzyme activities, one of the characteristic findings was that, independently of the previous values reached, the lowest values were recorded in the vermicompost incubated for 60 d, suggesting the existence of a threshold for microbial survival and metabolism which may have affected extracellular enzyme activity. This hypothesis is strengthened by the highly significant correlations found between microbial biomass and three of the enzymes analyzed (β-glucosidase, cellulose, and protease).

Continuous decreases in microbial biomass and activity were expected as the dynamics of these parameters were previously found to follow similar patterns when earthworms are still present in the substrate (Aira et al., 2006). More specifically, the presence of *E. fetida* first promoted an increase and then a decrease in both the microbial biomass and activity after 8 wk during vermicomposting of pig manure (Aira et al., 2006). It is therefore reasonable to expect that this dynamic would continue once earthworms had left the substrate, as indeed was found in the present study. However, in similar studies of vermicomposting of cow manure with *E. andrei* (Atiyeh et al., 2000; Domínguez et al., 2003) the authors found that microbial biomass (N_{mic}) remained stable or even increased during the process, whereas the microbial activity tended to drop.

The interpretation of data arising from enzyme assays is complicated since enzyme activity depends on several factors and different locations of enzymes in the studied system (Nannipieri et al., 2002). We found strong decreases in β-glucosidase, cellulose, and protease activities as those reported by Benítez et al. (1999) during the vermicomposting of sewage sludges, although these occurred within a shorter time scale (10 wk) and with presence of earthworms in the substrate. Further, these kinds of residues have high amounts of heavy metals that are inhibitors of enzyme activity. Thus, the action of earthworms during the first stages of vermicomposting reduced substrate pools for these three enzymes (Aira et al., 2006), since earthworms are able to exploit organic C and N pools. This process went on during maturation without earthworms due to the remaining activity of microorganisms as basal respiration showed. Together with enzyme activity decay, the progressive lack of substrate also reduced microbial biomass, which affected the synthesis of new enzymes as the strong correlation found between the three enzymes and microbial biomass suggests. Moreover, these enzymes can be induced by the substrate (Nannipieri et al., 1990), and hence the decrease may be a consequence of substrate pools being scarcer during aging. The decrease in cellulase activity can be explained by this fact because in a previous study, we found that the earthworm *E. fetida* produced a significant reduction in the cellulose content of pig slurry, a loss of 72 g kg^{-1} of cellulose after 18 wk (Aira et al., 2006); a longer incubation time is expected to result in further reductions. According to this, the pattern of β-glucosidase activity recorded during the vermicomposting of olive waste by Benítez et al. (2005) was the opposite of that of sewage sludge. The olive waste, a lignocellulosic-enriched residue, supplied enough substrate to microorganisms which maintained enzyme production, which would be consistent with the hypothesis of substrate-limiting conditions for enzyme activity. Moreover, since the product of cellulase activity is the substrate of β-glucosidase we should expect an opposite pattern between the two enzyme activities (Nannipieri et al., 2002). Nevertheless, β-glucosidase finally decreased after 60 d despite sufficient substrate due to remaining cellulase activity. This reinforces the hypothesis that intracellular activities, that is, those due to direct action of microbial communities, were key in the total activity of this enzyme.

Phosphomonoesterases catalyze the release of inorganic phosphorus (orthophosphate) from organic phosphomonesters (Alef et al., 1995), and it is known that orthophosphate is an inhibitor of soil phosphatases (Appiah et al., 1985; López-Hernández et al., 1989). Phosphatase activity followed a similar trend to that reported by Benítez et al. (1999, 2005) for the vermicomposting of sewage sludges and olive waste respectively, with an initial increase and a subsequent decrease in the activities; this later drop may be due to an accumulation of inorganic P resulting from enzymatic activity in previous stages of incubation (López-Hernández et al., 1989). Further, the lack of correlation with both microbial biomass and activity may suggest that patterns of this enzyme activity are independent of microbial communities during aging vermicompost, once the pools of extracellular enzymes are synthesized and released.

As it was shown, microorganisms played a significant role in the observed diminishing enzyme activity; microorganisms, which were clearly depressed in biomass and activity in the final stages of incubation, were not able to maintain enzyme production, thereby leading to a reduction in their contribution to the renewal of extracellular enzymes, as it was reflected by the high and significant correlations found between three of the enzymes analyzed and N_{mic}. Extracellular enzymes may remain active and protected in some fractions of organic matter, and in fact extracellular enzymes can remain active for long periods when protected in humic complexes (Benítez et al., 2005), compounds that tend to increase in quantity during vermicomposting (Domínguez, 2004). However, we did not find a pattern of unaltered activity, except for cellulase and phosphatase activities which finally decreased completely, at least partly because the period of incubation was not long enough to achieve the high degree of organic matter stabilization necessary for the formation of humus, the fraction of organic matter in which enzymes would be protected from degradation (Nannipieri et al., 1990, 2002). Therefore, the last decrease observed in enzymatic activity is related to the degradation of the remaining enzymes when fresh vermicompost was removed from vermireactors, particularly after 60 d of incubation when the four enzymes

showed their lowest values. This degradation may be attributed to remaining protease activity during last stages of aging, since this enzyme degrades free proteins, including enzymes.

We propose that there are two steps involved in the dynamics of aging of vermicompost with regards to extracellular enzyme activity; the first step was characterized by a decrease in microbial populations which resulted in a reduction in the synthesis of new enzymes. The second step was the degradation of the pool of remaining enzymes. This dynamic does not seem to be affected by earthworms because similar decaying patterns of microbial biomass and activity were found in substrate where earthworms were present.

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