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Thermostability of Selected Enzymes in Organic Wastes and in their Humic Extract

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Abstract The objective of this study was to evaluate the thermostability up to 70 °C for 1 h of selected enzymes present in fresh and composted sewage sludge (SS and SSC) or municipal solid wastes (MSW and MSWC) and their humic extract. After a thermal treatment at 70 °C, no β-glucosidase activity in any humic extract was detected, whereas in SS, SSC, MSW, and MSWC, it was respectively, 35%, 68%, 17%, and 12% compared to thermally untreated samples. By contrast, *o*-diphenol oxidase activity was even stimulated by thermal treatment in SS samples, but in the humic extracts, this activity decreased by 75–81%. Urease activity in all humic extracts decreased by 70% or more just at 40 °C, whereas for organic wastes, this decrease was observed after treatment at 70 °C. Alkaline phosphatase (AP) activity was affected by thermal treatment only in MSW and MSWC. In humic extracts, AP activity decreased gradually to zero except for the MSW extract, where 45% activity was retained after treatment at 70 °C. In general, thermostability of enzymes in humic extracts was lower than the materials they were extracted from.

Keywords Enzyme stabilization · Compost · Municipal solid wastes · Sewage sludge · Thermostability · Humus–enzyme complexes

Introduction

Many enzymes synthesized by microorganisms are leaked from living cells or released from lysed cells to their environment, but their presence in the soil is ephemeral unless they are absorbed by clay colloids or linked to humic molecules [1]. Extracellular enzymes, which are immobilized in soil organic or inorganic colloids, are thought to play a vital role in microbial ecology. They function as stabilized catalysts for the degradation of high-molecular-weight substrates producing smaller molecules, which can be assimilated by microorganisms [2].

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Nowadays, there is an increasing interest on the possibility of increasing soil-immobilized enzyme activity to transform some organic pollutants into less toxic substances or to release nutrients into the soil solution [3]. Some investigations have been conducted to immobilize enzymes on clay colloids [4–6] or on synthetic humic-like molecules [7], for understanding interactions of enzymes with humic substances [8], and to determine the stability of these enzymatic complexes and evaluate their potential use in the rehabilitation of degraded soils.

However, the stability of humus–enzyme complexes derived from organic wastes such as domestic wastes or sewage sludge (SS) has not been studied. Such organic materials are generated in large amounts by human activities, and they are, therefore, economical materials having stabilized humus–enzymes complexes for extracts with enzymatic activity. The evaluation of the stability of these complexes is necessary to determine their suitability for their use in soil remediation. Enzymes are very sensitive to temperature, and it has been observed that the immobilization of enzymes in humic colloids protect them from denaturation by changes in surrounding temperature [1].

The purpose of this work was: (1) to determine the thermal stability of selected hydrolases (urease [UR], alkaline phosphatase [AP], and β-glucosidase [β-GL]) and an oxidoreductase (*o*-diphenol oxidase [*o*-DPO]) in fresh and composted organic materials (SS and a municipal solid waste [MSW]) and (2) to determine the thermal stability of the same enzymes in the pyrophosphate extract obtained from the abovementioned organic materials. In this way, we will be able to evaluate: (1) which organic material is a better source of thermostable enzymes from an experimental point of view and (2) whether total and extractable enzymatic activities have the same thermostability.

Materials and Methods

Organic Materials

Four different organic wastes were used in this study: SS from a municipal wastewater treatment plant in El Raal—Murcia (SE Spain), the compost produced from the abovementioned SS (SSC), the organic fraction of MSW collected from the treatment plant of Mula (15 km from Murcia city, which receives all the household wastes produced in the metropolitan area of Murcia (300,000 inhabitants), and the compost produced from this organic material (MSWC). MSW was obtained after manual and mechanical separation of most part of the metallic, plastic, and paper materials from the wastes. The composting (industrial scale) of SS and MSW was carried out in static but with mechanical ventilation provided. The maximum temperatures reached (65 °C) was maintained for a minimum of 48 h (to guarantee disinfection of the material), after which the temperature was maintained in the ranges 53–60 °C during most of the process. The moisture level of the material was the optimum (60%) for increasing microbial activity. To improve oxygenation inside of the SS pile during the composting process, a bulking agent (wood chips) was added on a volumetric basis in the proportion of 1:2 (material/bulking agent). The composting process lasted 90 days for both SS and MSW. The main characteristics of these organic materials are shown in Table 1. Six subsamples, taken from each material, were mixed and grounded by a cutting mill to less than 0.5 mm to obtain one homogenized sample of each organic material. Three aliquots of each homogenized sample were used to determine the total enzymatic activity and to extract the humus–enzyme complexes. The three replicates of each material were analyzed, and the mean value was calculated for each parameter.

Table 1 Characteristics of the selected organic wastes.

	SS[a]	SSC	MSW	MSWC
Total organic C, (%)	17 (1.5)	20.6 (0.6)	17 (1.2)	16.6 (1.0)
Electrical conductivity (mS cm^{-1})	6.82 (0.06)	3 (0.03)	6.79 (0.02)	3.93 (0.02)
pH (1:5, om/water)	6.82 (0.09)	7.05 (0.04)	6.73 (0.05)	7.9 (0.08)
Kjeldahl N (%)	4.56 (0.2)	3.39 (0.3)	1.63 (0.2)	2.56 (0.3)
Humic substance C (%)	0.61 (0.2)	1.38 (0.3)	0.34 (0.2)	0.61 (0.1)
Water soluble carbohydrates (mg C kg^{-1})	695 (74.29)	1,524 (149.52)	141 (10.78)	271 (22.84)
P (% P_2O_5)	12.4 (0.2)	10.9 (0.3)	2.04 (0.4)	2.46 (0.5)
K (% K_2O)	0.35 (0.05)	0.38 (0.1)	0.71 (0.4)	0.51 (0.09)
Cu (mg kg^{-1})	177 (0.4)	2.18 (0.09)	283 (0.1)	336 (1.2)
Zn (mg kg^{-1})	581 (52)	523 (42)	628 (60)	1,855 (153)
Cr (mg kg^{-1})	8.4 (0.81)	15 (0.11)	47.9 (3.24)	107 (9.72)
Pb (mg kg^{-1})	28.1 (2.17)	55.5 (4.78)	125 (1.02)	233 (18.5)
Ni (mg kg^{-1})	16.5 (1.29)	21.6 (2.07)	77.2 (6.83)	120 (10.43)

Numbers in parenthesis indicate standard deviation

[a] *SS* Sewage sludge, *SSC* sewage sludge compost, *MSW* municipal solid waste, *MSWC* municipal solid waste compost

Extraction of Humus–Enzyme Complexes

Humus–enzyme complexes were extracted with a 0.1-M, pH 7.1, sodium pyrophosphate solution (*w*/*v* ratio=1:10) by mechanical shaking for 24 h [9]. The extracts were centrifuged and filtrated (through a 0.2-μm Millipore membrane, type DVPP), and then they were dialyzed against distilled water with membranes having 12,000–14,000-Da molecular weigh cutoff and 25-Å pore diameter (Visking® dialysis tube, Serva GMBH, Heidelberg, Germany) to remove inorganic salts that can cause artifacts in the enzymatic activity assays.

Thermal Treatment

Ten grams of organic wastes or 10 ml of the pyrophosphate extract were transferred to glass flasks and placed inside a thermostatic bath at 30, 40, 50, 60, and 70 °C for 1 h [10]. All thermal treatments were carried out in triplicate.

Enzymatic Activity Assays

UR activity was determined by the buffered method of Kandeler and Gerber [11]: 0.5 ml of a solution of urea (0.48%) and 4 ml of borate buffer (pH 10) were added to 1 g of organic material in hermetically sealed flasks and then incubated for 1.5 h at 37 °C. The ammonium content of the centrifuged extracts was determined by a modified indophenol-blue reaction. Controls were prepared without substrate addition prior to incubation to determine the native ammonium content of the organic materials.

AP and β-GL activities were determined following the methods reported by [12] and [13], respectively, using 0.2 g of organic material and 2 ml of modified universal buffer and incubating samples at 37 °C for 1 h. AP activity assay was performed at pH 11 using *p*-nitrophenyl phosphatase as the substrate; β-GL activity was assayed at pH 6 using *p*-nitrophenyl β-D-glucopiranoside as the substrate. Enzymatic reactions were stopped by cooling in ice for 15 min. Then, 0.5 ml of $CaCl_2$ 0.5 M and 2 ml of NaOH 0.5 M (for AP)

or 2 ml of Tris (hydroxymethyl) aminomethane-sodium hydroxide, 0.1 M pH 12 (for β-GL), were added. Controls were performed as samples but adding substrate immediately before the addition of $CaCl_2$ and NaOH. The *p*-nitrophenol (*p*-NP) formed was determined at 398 nm.

o-DPO activity was determined as reported by [14]. To 0.5 g of organic material, 1.5 ml of catechol solution, 0.2 M (substrate), 1.5 ml of proline solution, 0.2 M (reagent), and 2 ml of phosphate buffer (0.1 M, pH 6.5) were added. The mixture was incubated at 30 °C for 10 min, and the enzymatic reaction was stopped by cooling in an ice bath and adding 5 ml of ethanol. After centrifugation, the absorbance of the supernatant was measured at 525 nm. In the control, the substrate was added after incubation and before ethanol addition.

To evaluate the activity of the extractable enzymes, all the above enzymatic activities were measured as described above but using 1 ml of the pyrophosphate extracts.

Statistical Treatment

For each enzymatic activity, data were submitted to a two-way analysis of variance (ANOVA) using the Statgraphics v. 5.0 software package (Statistical Graphics, 1991). A Tukey multiple range test ($p<0.05$) was performed to establish the honestly significant differences (HSD) between means.

Results

β-Glucosidase Activity

Total and extractable β-GL decreased with increasing treatment temperature (Fig. 1). Total β-GL in SS (both fresh and composted) showed higher stability than fresh or composted

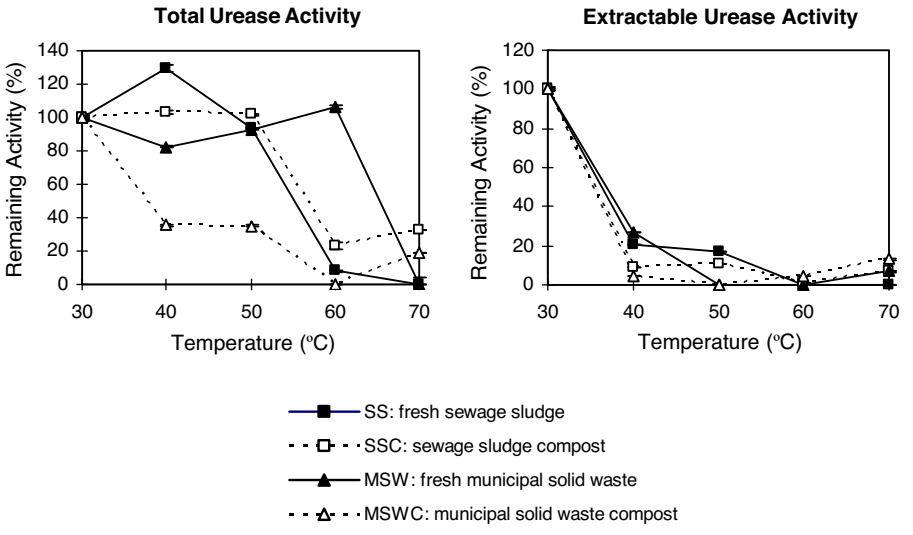

Fig. 1 Effect of temperature increase on the total and extractable β-GL activity of the different organic wastes. *Bars* denoted the standard mean deviation ($n=3$)

MSW (Fig. 1). For instance, at 70 °C, the activity detected in SSC, SS, MSWC, and MSW was 68%, 35%, 17%, and 12%, respectively, compared to the original activity. It should be noted that in SS and SSC, β-GL activity showed only slight variations in the 30–60 °C temperature range, decreasing sharply at 70 °C, whereas in MSW and MSWC, β-GL activity decreased gradually with temperature increase.

In all pyrophosphate extracts, β-GL was thermostable up to 50 °C, but it fell to zero at 60 °C for both MSW and MSWC and at 70 °C for SS and SSC.

o-Diphenol Oxidase Activity

Total *o*-DPO activity decreased with increasing temperature in all the organic materials except SS, where an increase of about 2.5 times was detected both at 60 and 70 °C (Fig. 2).

In the pyrophosphate extracts of SS, SSC, and MSWC, *o*-DPO activity decreased considerably at 50 °C, being only 19% (MSWC) or 25% (SSC and SS) compared to 30 °C (Fig. 2). At 70 °C, *o*-DPO activity was close to the detection limit. In the case of MSW, *o*-DPO activity decreased by 60% from 30 to 40 °C and disappeared at 70 °C.

Urease Activity

In MSWC (Fig. 3) UR activity decreased gradually with increasing temperature, whereas in MSW, UR activity was almost stable up to 60 °C, but at 70 °C, no activity was detected. By contrast, UR activity in SS and SSC was stable up to 50 °C and then decreased sharply.

Concerning thermostability of the extractable UR activity, a strong diminution of this enzymatic activity was observed at 40 °C in all cases. Especially, this decrease has been manifested in the case of MSWC remaining at 40 °C, with only 8.6% of the activity existing at 30 °C.

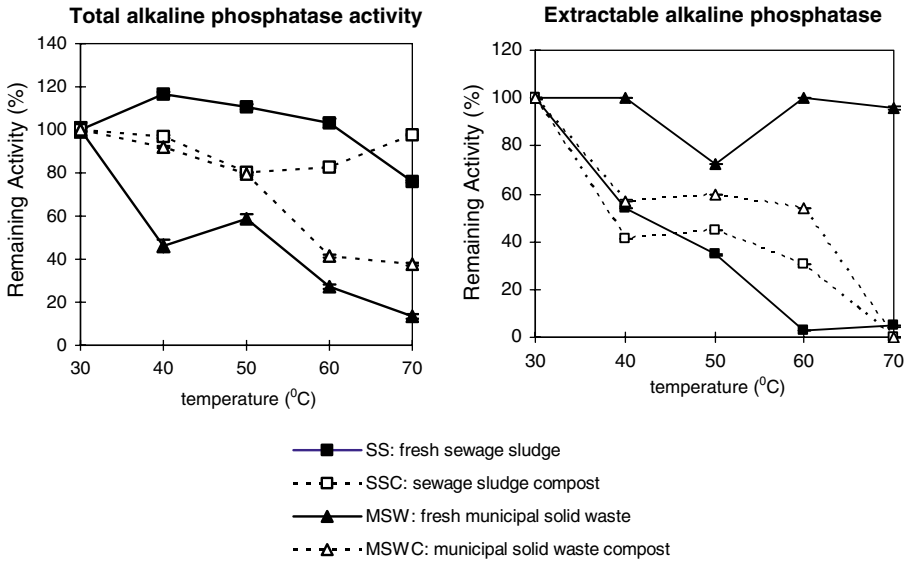

Fig. 2 Effect of temperature increase on the total and extractable *o*-DPO activity of the different organic wastes

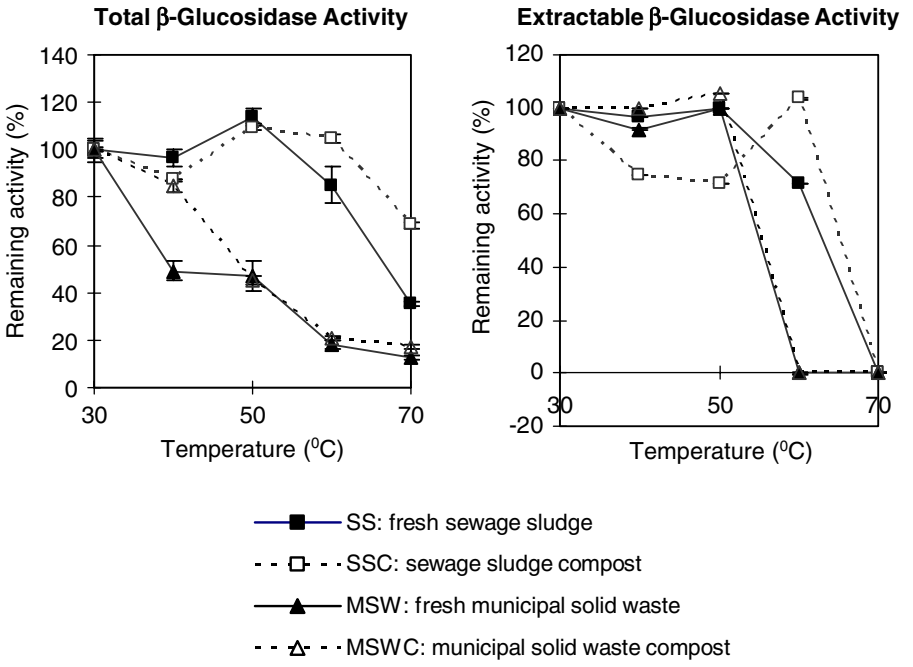

Fig. 3 Effect of temperature increase on the total and extractable UR activity of the different organic wastes

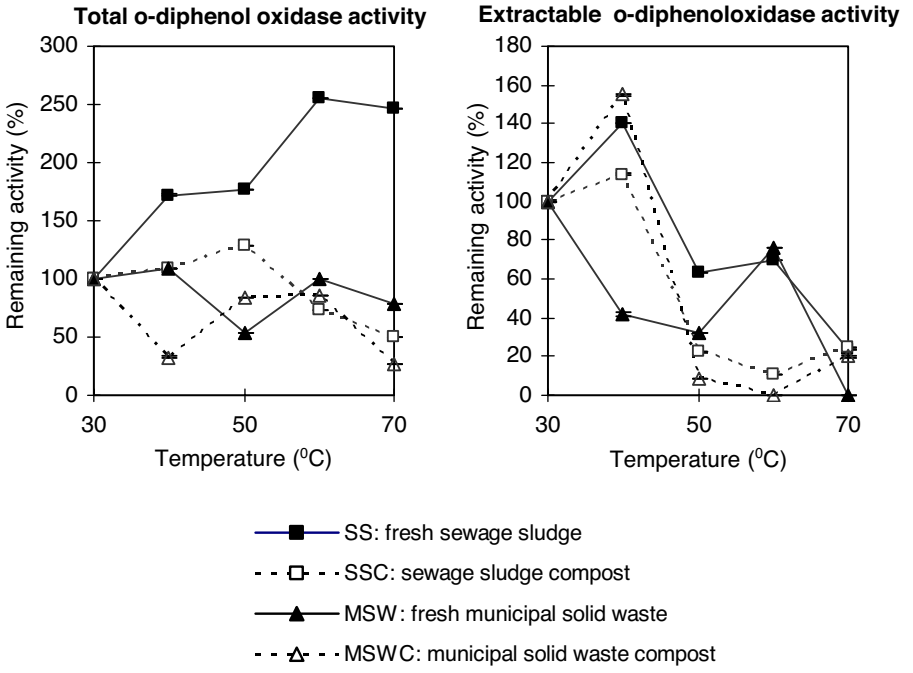

Fig. 4 Effect of temperature increase on the total and extractable AP activity of the different organic wastes

Alkaline Phosphatase Activity

Total AP activity was slightly affected by temperature in SS and SSC samples (Fig. 4). In SS, a decrease of 24% of the initial activity was observed at 70 °C whereas in SSC activity values decreased slightly from 30 to 50 °C, increasing afterward to reach at 70 °C values close to those observed at 30 °C. By contrast, in fresh and composted MSW, AP activity was highly affected by temperature, remaining 13.5% and 38%, respectively, at 70 °C of the activity exhibited at 30 °C.

Extractable AP activity from both SSC and MSWC decreased from 30 to 40 °C and then remained constant up to 60 °C, and at 70 °C, it fell to zero (Fig. 4). In the MSW extract, AP activity showed no significant differences up to 60 °C, then decreasing to about 40% of the activity at 30 °C. In the case of SS, the activity decreased linearly to zero from 30 to 60 °C.

Discussion

It must be emphasized that whereas total β-GL activity was measurable at 70 °C in all studied organic wastes, in the humic extract, it was not detected. In fact, the latter was on average only 10% of total β-GL activity (Table 2). Other authors have reported low levels of UR activity in sodium pyrophosphate extracts and postulated the possible inactivation of ureases by phenolic compounds [13]. On the other hand, the changes in the enzyme activity with temperature can be attributable to changes produced in the tertiary structure of the protein [14].

o-DPO catalyzes the oxidation of *o*-diphenols to quinones, and it is thought to play a major role in soil humification. Synthesis of *o*-DPO by microorganisms is induced by

Table 2 Total and extractable enzymatic activities in the studied organic wastes and percentage of extractable with regards to total activity.

	SS[a]	SSC	MSW	MSWC
Alkaline phophatase activity (μmol *p*-NP g^{-1} h^{-1})				
Total	75.7 (2.8)	12.7 (1.5)	33.8 (1.5)	50.1 (1.0)
Extractable	3 (1.21)	1.65 (1.21)	0.98 (0.03)	1.43 (0.15)
%	3.98	12.99	2.90	2.85
Urease activity (μmol NH_4^+-N g^{-1} h^{-1})				
Total	12.7 (1.3)	5.6 (0.09)	2 (0.3)	2.7 (0.19)
Extractable	0.29 (0.56)	0.11 (1.01)	0.79 (1.27)	0.23 (0.29)
Percent	2.28	1.96	39.5	8.51
β-Glucosidase activity (μmol *p*-NP g^{-1} h^{-1})				
Total	3.2 (0.17)	2.5 (0.158)	2.7 (0.179)	4.3 (0.62)
Extractable	0.06 (0.31)	0.24 (0.41)	0.59 (0.49)	0.37 (0.99)
Percent	1.88	9.6	21.85	9.25
o-DPO activity (μmol ox. catechole g^{-1} 10 min^{-1})				
Total	0.4 (0.02)	0.42 (0.01)	1.3 (0.02)	0.3 (0.02)
Extractable	1.21 (0.47)	0.89 (0.46)	1.42 (0.28)	2.87 (1.35)
Percent	302.5	211.9	109.2	956.67

Numbers in parenthesis indicate standard deviation

[a] *SS* Sewage sludge, *SSC* sewage sludge compost, *MSW* municipal solid waste, *MSWC* municipal solid waste compost

Table 3 Two-way ANOVA results for the selected activities from organic wastes.

Source	β-Glucosidase activity			*o*-Diphenol oxidase activity			Urease activity			Alkaline phosphatase activity		
	temperature (A)	Treatment (B)	A×B interaction	Temperature (A)	Treatment (B)	A×B interaction	Temperature (A)	Treatment (B)	A×B interaction	Temperature (A)	Treatment (B)	A×B interaction
Total enzyme												
F ratio	95.1	106.8	15.1	33.3	836.0	110.3	174.4	39.0	26.0	203.8	428.6	52.3
p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HSD[a]	10.5	8.8		8.6	7.3		11.5	9.6		5.1	4.3	
Extractable enzyme												
F ratio	177.9	5.4	16.5	931.7	113.7	113.7	365.6	6.63	5.33	370.9	179.3	20.1
p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HSD	13.0	10.9		6.7	5.7		8.6	7.2		6.7	5.7	

[a] *HSD* Honestly significant differences, which are obtained using the multiple range test of Tukey

different substrates, including anilines, aromatic compounds, or lignin preparations [15], and investigations have focused on the role of this enzyme in lignin degradation during composting process [16, 17]. These compounds, in a second step, are polymerized and integrated in the humus molecule [12]. The heating of SS produced an increase in *o*-DPO activity, whereas in MSW, it was slightly affected by temperature increase. In the case of the composts, a decrease in this enzymatic activity with increasing temperatures was observed. This fact highlights the influence of the organic matter nature on enzyme thermostability and may be explained by: (1) the separation of inhibitors from the enzyme with the increase of temperature and (2) the unmasking of some active sites produced when the protein conformation change with the temperature increase [1]; (3) similarly to β-GL, this can be attributable to changes in tertiary structure of the enzyme too. Other authors [17] reported activity losses of about 60% after 15 min of preincubation at 70 °C using laccase purified from composted MSW. However, the authors [18] found that the activity of *o*-DPO extracted from a thermophilic strain of bacteria was stimulated by keeping at 80 °C.

In the pyrophosphate extract with a great amount of humic substances, the *o*-DPO activity was higher than that in organic wastes (Tables 2 and 3), but its thermostability was lower. Likely, *o*-DPO did not interact with the humic substance to form humic–enzyme complexes, and its presence in the pyrophosphate extract was especially in the form of free enzyme. Other authors observed that humic substances may alter the polyphenol oxidase stability [19]. As it is known that *o*-DPO is a one of the oxidoreductase enzymes, influenced by the function between oxidation and reduction, the thermal effect on its mechanism could attribute to the deformation of the linkage of complexes, which lead the enzyme to degrade easily.

In terms with UR in the extract, other authors [20] observed that the UR immobilized on montmorillonite or aluminum oxide showed a higher sensitivity to temperature than the free enzyme at 60 °C. Nonetheless, the residual activity at this temperature (60 °C) for the UR–clay complexes was 55% of the initial activity. In our experiment, only 5% of the UR activity detected initially in the humic extract remained at 60 °C. This suggests that UR is more efficiently protected against thermal denaturation in the complex with clay minerals than the complex with humic substance.

From an agronomic point of view, AP in the soil deserves special attention as it catalyzes the transformation of organic P into inorganic P available to plants [21]. It has been indicated that the immobilization of AP in the soil increases its thermal stability with regard to soluble enzyme [22]. However, lower thermostability was observed for AP measured in the humic extract of SS, SSC, and MSWC in comparison with that in the original organic materials (Table 3). The same tendency has been occurred to other authors that have studied AP with clay complexes [4]. They conclude that the drying effect led to a rapid and strong denaturation of absorbed protein.

The immobilization of the enzyme on synthetic or natural humic materials could cause inhibitory effects to the enzyme [23–27]. However, other authors have showed a different perspective about the inhibitory effect of humic substance [28]: The incorporation of the enzyme into humic polymers reduced enzyme activity by phenolic compounds; however, this activity was more stable than the activity of free enzymes once added to the soil. Thus, it should be possible that the interaction with clay colloids changes the mechanism of humus–enzyme complexes in the soil, compared to the absence of the soil. Furthermore, collectively, the data indicate that the enzyme thermal stability is influenced by both the origin of the organic amendment and the degree of stabilization of its organic matter. The research about this topic should be completed with additional experiments consisting in the application of the organic wastes to a degraded soil as organic amendments to test the

stability of the enzymatic activities with time. These studies would help us to understand the linkage between humus and enzymes and the possible use of enzymes as environmental tools for bioremediation.

From this study, it can be concluded that the enzymatic activities of SS and SSC are more thermoresistant than those of MSW or MSWC and that AP, *o*-DPO, and β-GL are more resistant than UR to temperature increase. In general, enzymes of SS were more thermoresistant than those of SSC. Concerning humus–enzyme complexes, in general, it can be said that thermostability of enzymes in humic extracts was lower than the materials they were extracted from.

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