Reconciling apparent variability in effects of biochar amendment on soil enzyme activities by assay optimization

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ABSTRACT

We studied the effects of a biochar made from fast pyrolysis of switchgrass on four soil enzymes (β-glucosidase, β-N-acetylglucosaminidase, lipase, and leucine aminopeptidase) to determine if biochar would consistently modify soil biological activities. Thus, we conducted a series of enzyme assays on biochar-amended soils. Inconsistent results from enzyme assays of char-amended soils suggested that biochar had variable effects on soil enzyme activities, thus we conducted a second experiment to determine if biochar reacts predictably with either enzyme or substrate in vitro reactions. Both colorimetric and fluorescent assays were used for β-glucosidase and β-N-acetylglucosaminidase. Seven days after biochar was added to microcosms of 3 different soils, fluorescence-based assays revealed some increased enzyme activities (up to 7-fold for one measure of β-glucosidase in a shrub-steppe soil) and some decreased activities (one-fifth of the unamended control for lipase measured in the same shrub-steppe soil), compared to non-amended soil. In an effort understand the varied effects, purified enzymes or substrates were briefly exposed to biochar and then assayed. In contrast to the soil assays, except for β-N-acetylglucosaminidase, the exposure of substrate to biochar reduced the apparent activity of the enzymes, suggesting that sorption reactions between substrate and biochar impeded enzyme function. Our findings indicate that fluorometric assays are more robust to, or account for, this sorption better than the colorimetric assays used herein. The activity of purified β-N-acetylglucosaminidase increased 50–75% following biochar exposure, suggesting a chemical enhancement of enzyme function. In some cases, biochar stimulates soil enzyme activities, to a much greater degree than soil assays would indicate, given that substrate reactivity can be impeded by biochar exposure. We conclude that the effects of biochar on enzyme activities in soils are highly variable; these effects are likely associated with reactions between biochar and the target substrate.

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1. Introduction

Biochar, a product of the pyrolysis of organic material, has been credited with many desirable properties as a soil amendment, including soil conditioning (Novotny et al., 2009), enhanced fertility (Van Zwieten et al., 2010), sorption of pollutants (Yu et al., 2009) and hormones (Kim et al., 2007), and an agent of C sequestration (Fowles, 2007; Larson, 2007; Lehmann et al., 2008). Much interest has been driven by high levels of sustained fertility in the Terra Preta soils that feature over 70 times more black carbon ("biochar") than the surrounding soils in the humid tropics of the Amazon Basin (Glaser et al., 2001; O’Neill et al., 2009). Although one study did report enhanced biological N fixation in biochar-amended soils (Rondon et al., 2007), the effects of biochar on the biological activity of soil need greater study to evaluate the potential repercussions of wide application of such material.

Biochar is perceived as being similar to activated carbon which is known to react with or adsorb reactive molecules such as organic compounds (Hilber et al., 2009), potentially modifying their ultimate bioavailability (Yang et al., 2009). However, biochar is in fact a different form of C, and it may not be appropriate to translate the activated carbon literature to biochar applications. Current research into biochar and soil biological activities suggests that there is an initial stimulating effect that diminishes over time (Kuzyakov et al., 2009), as the labile component is metabolized (Smith et al., 2010).

This research examined if biochar would consistently modify soil biological activities. Thus, we conducted a series of enzyme assays on biochar-amended soils. As we will show, inconsistent results...
from enzyme assays of char-amended soils suggested that biochar had variable effects on different soils, enzymes, and assay types, indicating the need for further study to determine if biochar reacts predictably with either enzyme or substrate in in vitro reactions. Thus, we conducted soil-free assays using purified enzymes in which either the enzyme or substrate was exposed to biochar. The enzymes were β-glucosidase, β-N-acetylglucosaminidase, lipase, and leucine aminopeptidase; they were selected to span different substrate types (carbohydrate, lipid, protein). All enzymes were assayed using fluorescence-based approaches, and β-glucosidase and β-N-acetylglucosaminidase were also assayed colorimetrically.

2. Materials and methods

2.1. Soils and biochar

Three soils were studied: a Palouse silt loam (Pachic Ultic Haploxeroll) from a winter wheat field at the Palouse Conservation Field (Pullman, WA; 46.76°N, 117.18°W), a Quincy sand (Xeric Torrissambl) from native shrubsteppe at the Arid Lands Ecology Reserve (Richland, WA; 46.46°N, 119.65°W), and a Warden sandy loam (Xeric Haplocambid) from native shrubsteppe at the USDA-ARS Irrigated Cropping Systems Research Field Station (Paterson, WA; 45.94°N, 119.60°W), and a Warden sandy loam (Xeric Haplocambid) from native shrubsteppe at the Arid Lands Ecology Reserve (Richland, WA; 46.46°N, 119.65°W). The top 5 cm of soil was passed through a 2-mm sieve, and stored at 4 °C. The biochar used was produced by the fast pyrolysis of switchgrass (Panicum virgatum L.; H. Collins, USDA-ARS, Prosser, WA) and sieved through a 66-μm screen. This biochar has been characterized elsewhere (Smith et al., 2010). Briefly, the pH of the biochar was 9.7, it had 52% C, 1.6% N, 3.4 mg water soluble C g⁻¹, and 75 μg water soluble N g⁻¹; the soluble fraction was reported to be dominated by aromatic (amide and methyl) aliphatic and carboxylate (Smith et al., 2010). Subsamples of soil (5 g) were amended with either 0 or 2% biochar (w/w) and all 6 enzyme assays conducted on 1–2 g aliquots after 7 d. Nine replicates were conducted for each treatment. Soils were held at −0.33 bar water content.

2.2. Soil enzyme assays

Four enzymes were selected for this study: β-glucosidase, β-N-acetylglucosaminidase, aminopeptidase, and lipase. Fluorescence-based soil assays for β-glucosidase, β-N-acetylglucosaminidase, leucine aminopeptidase, and lipase were based on protocols using the following respective substrates: 4-methylumbelliferyl β-D-glucopyranoside (Sigma, M3633), 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma, M2133), l-leucine-7-amido-4-methylcoumarin hydrochloride (Sigma, L2145), and 4-methylumbelliferyl heptanoate (Sigma, M2514). Assay protocols were as reported in Saiya-Cork et al. (2002) for β-glucosidase (MUB), β-N-acetylglucosaminidase (MUB), and aminopeptidase. We also conducted colorimetric soil assays for β-glucosidase and β-N-acetylglucosaminidase using the substrates 4-nitrophenyl β-D-glucopyranoside (Sigma, N7006) and 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma, N9376), respectively (Alef and Nannipieri, 1995). For the fluorescence-based assays, standard curves were developed to incorporate both quenching due to soil and quenching due to soil + biochar, and the enzyme activities were calculated against the appropriate curve.

2.3. Purified enzyme assays

The purified enzymes used were: β-glucosidase (Sigma, G4511), β-N-acetylglucosaminidase (Sigma, A2264), aminopeptidase I (Sigma, A9834), and lipase (BioChemika 28602). Biochar-exposed enzyme was produced by vortexing 1 mL of each enzyme (in reaction buffer) with 2 mg biochar, letting it rest for 15 min, then centrifuging at 15,000 × g for 1 min; the supernatant was retained
as “biochar-exposed enzyme.” The 1-mL solution of enzyme contained 25 units (U) for β-glucosidase, aminopeptidase, and lipase. The 1-mL solution of β-N-acetylglucosaminidase contained 10 U.

Substrates were similarly exposed to biochar; 50 μL of substrates (100 μL for the PNP assays) were vortexed briefly with 2 mg biochar, incubated for 15 min, and centrifuged (1 min at 15,000 × g). The supernatants were the “biochar-exposed substrates,” as appropriate. The concentrations of the substrates in these assays were 15 mM for the β-glucosidase (PNP), 200 μM for β-glucosidase (MUB), 10 mM for the β-N-acetylglucosaminidase (PNP), 200 μM for the β-N-acetylglucosaminidase (MUB), 200 μM for the aminopeptidase, and 10 mM for the lipase. The in vitro reactions of the enzymes and substrates were combined for four treatments: 1) pristine enzyme + pristine substrate (control), 2) biochar-exposed enzyme + pristine substrate, 3) pristine enzyme + biochar-exposed substrate, 4) pristine enzyme + pristine substrate with biochar added at the time of the assay. Reaction conditions are provided in Table 1.

2.4. Statistics

All assays were conducted in triplicate; each replicate was itself read in triplicate. For the soil experiment, treatments (0 biochar and 2% biochar) were compared using t-tests. For the in vitro experiment with char-exposed purified enzymes and substrates, means were separated using a general linear models procedure followed by a Bonferroni test in Systat 10 (SPSS Inc, Chicago, IL, USA).

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**Fig. 1.** Biochar effects on soil enzyme activities, following the incubation of 0 or 2% biochar in soil for 7 days. A) Palouse, B) Quincy, C) Warden soils. Units for β-glucosidase (PNP) and β-N-acetylglucosaminidase (PNP) are μg paranitrophenol g⁻¹ soil h⁻¹, units for β-glucosidase (MUB) and β-N-acetylglucosaminidase (MUB) are μmol methylumbelliferone g⁻¹ soil h⁻¹, units for lipase are nmol g⁻¹ soil 10 min⁻¹, and units for leucine aminopeptidase are μmol aminomethylcoumarin g⁻¹ soil h⁻¹. Asterisks note treatments that are significantly different from the control for that enzyme. Significance was set at P < 0.005 for all assays except β-N-acetylglucosaminidase which returned a P value = 0.0148.
3. Results

3.1. Soil enzyme assays

After 7 days, biochar had variable effects on the enzyme activities studied (Fig. 1). Incubating soil with 2% biochar for 7 days increased the activities of enzymes measured by fluorescent substrates in the Palouse soil, compared to non-biochar amended controls (Fig. 1). These fluorescent assay results were not consistent in the other two soils, with only β-N-acetylglucosaminidase increasing in the Quincy soil and β-N-acetylglucosaminidase and β-glucosidase increasing in the Warden soil. Lipase decreased in the Quincy and Warden soils, and leucine aminopeptidase decreased in the Warden soil following the brief incubation with biochar. Except for β-N-acetylglucosaminidase in Warden soil, the colorimetric assays (PNP) were generally decreased in the biochar-amended soils.

3.2. Purified enzyme assays

In soil-free reactions with purified enzymes, both colorimetric and fluorescence assays for β-glucosidase were significantly reduced by the reaction of substrate with biochar prior to the assay (Fig. 2). Neither assay was significantly hindered by the reaction of the enzyme itself with biochar. When biochar was added to the assay at the same time that clean enzyme and clean substrate were combined, the results of the colorimetric assay were not diminished compared to the control; in the same test of the fluorescence-based assay, the results were decreased to the same level as the assay using biochar-exposed substrate (Fig. 2). In contrast to β-glucosidase, the addition of biochar to the β-N-acetylglucosaminidase assay increased the activity (Fig. 2). This increase from the control was significant when the substrate was reacted with biochar for both the colorimetric and fluorometric assay approaches, but for the biochar-exposed enzyme only in the fluorometric assay.

Lipase and leucine aminopeptidase were both assayed using only fluorescence-based approaches (Fig. 2). The activities of both enzymes were significantly reduced when either the enzymes or substrates were exposed to biochar prior to the assay. The activity of leucine aminopeptidase was most reduced when the substrate was exposed to biochar, whereas the activity of the lipase was reduced when either substrate or enzyme was exposed to biochar (Fig. 2).

4. Discussion

4.1. Inconsistency in biochar effects

One of the objectives of this research was to determine if the effect of biochar on soil enzyme activities was predictable in such a way that would help explain how biochar could impact soil functions. It was difficult to understand the inconsistent effects of biochar on soil enzymes; as our data show, these responses vary in direction and magnitude. For example, 7 days after the biochar was

![Fig. 2. Activities of enzymes in reactions where either enzyme or substrate has been exposed to biochar, or in which biochar was added to the assay (CE−CS = clean enzyme and clean substrate; "Control"; EE−CS = exposed enzyme and clean substrate; CE−ES = clean enzyme and exposed substrate; E+S+C = clean enzyme and clean substrate with biochar added to the assay). Within a single graph, bars topped by the same letter are not significantly different (P < 0.01).]
added, the Warden sandy loam was observed to have \(\sim 0.2\)-times the leucine aminopeptidase and \(\sim 7\)-times the \(\beta\)-glucosidase (MUB) activities of the non-amended control soil. Biochar can stimulate overall microbial activity in the short-term (Smith et al., 2010). This stimulation is possibly limited to a specialized subset of the microbial community (Kolb et al., 2009), resulting in some increased enzyme activities. Decreased activities may be due to sorption or blocking of either enzyme or substrate.

Not all biochars behave the same in all soils; depending on the biochar source (Kuzryakov et al., 2009), production method (Amonette et al., 2009), and soil (Kolb et al., 2009), one may see different adsorption behavior and biological activity due to widely varying pH, surface area, pore size distribution, and charge properties (Brewer et al., 2009; Gaskin et al., 2009). However, common features seem to include an initial stimulation of biological activity (Kolb et al., 2009; Smith et al., 2010) and a subsequent persistence of C (Kuzryakov et al., 2009). In this study, when biochar was applied to soil the same of the enzyme activities of the biomass increased after 7 days. This could be due to either stimulation of the microbial activity by the biochar or growth of biomass in response to initially labile biochar-C. Because the fluorescence-based assays incorporate the fluorescence quenching of the assay material (in this study soil and biochar) into the protocol (Marx et al., 2001), these assays are more robust to biochar applications to soil than are traditional colorimetry-based assays. This robustness more accurately reports the activities of enzymes in biochar systems than do the colorimetric assays.

### 4.2. Biochar exposure effects on enzyme activity

The second objective of this research was to modify the soil assays using purified versions of the study enzymes to test the effects of biochar exposure on enzyme activity (Table 1). The reduction in purified enzyme activities observed in both \(\beta\)-glucosidase assays, the lipase assay, and the aminopeptidase assay following the exposures of substrates to biochar is likely due to sorption of the substrates to the biochar; this may either inhibit the enzyme–substrate reaction by blocking reaction sites (Schaffer, 1993), or remove some substrate from solution when the biochar was centrifuged out of the solution. It is unlikely that sorption also occurred when the enzyme was exposed to biochar because the biochar-exposed enzyme showed reduced activity in the fluorescence-based assays of all four enzymes, but not in the colorimetric assay of \(\beta\)-glucosidase. The biochar was removed by centrifugation in the soil-free assays, therefore the difference is not attributable to the quenching reactions observed in the soil with biochar assays, and removal of enzyme from solution by sorption to biochar should have been the same for both types of assay. Therefore, in soil, when biochar stimulated enzyme activities, the degree of stimulation in the 7 days following addition was likely substantial in order to overcome sorption reactions that may impede enzyme function.

The observed elevation of \(\beta\)-N-acetylglucosaminidase activity by its exposure to biochar was puzzling. It was not likely to be an interaction with the liberated para-nitrophenol in the colorimetric assay, as this was the same reporter molecule used in the \(\beta\)-glucosidase (PNP) assay which was not stimulated in this way. Similarly, this was also unlikely to be a stimulation of the fluorescence signal, as the \(\beta\)-glucosidase (MUB) assay was unaffected. We speculate that the biochar may release a small molecule that acts as an allosteric upregulator specific for \(\beta\)-N-acetylglucosaminidase. One such candidate molecule could be ethylene, long recognized for its regulatory activity in microbial processes and hormonal function in plants, which has been recently observed to evolve from some biochars, potentially affecting microbial processes in associated soils (Spokas et al., 2010).

### 4.3. Conclusions

Thus, we conclude that the effects of biochar on soil enzyme activities are variable, depending on the soil, and on the particular enzyme. Additionally, we observed in \textit{in vitro} reactions that biochar reacts with a range of substrates rendering them unavailable to enzyme action. These inconsistencies are illustrative of current knowledge gaps with respect to the interactions of biochar with critical soil biological activities. Further research questions may also include focus on the subset of organisms, or the components of biochar most associated with the biological and chemical stimulation of enzyme activities. As well, biochar is likely to have other effects on the soil microbial community with respect to changes in the soil water dynamics, cation exchange capacity, and dominant C forms. For studying soil enzyme activities, we synthesize the results of the two experiments described here to suggest that future biochar-soil researchers strongly consider using the fluorescence-based assays to most accurately report the activities of soil enzymes in the presence of biochar.

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