1 # Methodological interference of biochar in the determination

2 # of extracellular enzyme activities in composting samples

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4 **Keiji Jindo[13]*, Kazuhiro Matsumoto[2], Carlos García Izquierdo[1], Tomonori**

5 **Sonoki[2], and Miguel A. Sanchez-Monedero[1]**

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7 [1] *Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC).*

8 *Department of Soil Conservation and Waste Management. Campus*

9 *Universitario de Espinardo, 30100 Murcia, SPAIN.*

10 [2] *Faculty of Agriculture and Life-Sciences, Hirosaki University,*

11 *Bunkyo-cho, Hirosaki, Aomori 036-8561, JAPAN.*

12 [3] *Institute of Industrial Science, the University of Tokyo,*

13 *4-6-1 KOMABA MEGURO-KU, TOKYO 153-8505, JAPAN*

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21 *Corresponding author: keijindo@hotmail.com

22 Phone: +34 968396364

23 Fax: +34 968396213

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Abstract

Biochar application has received increasing attention as a means to trap recalcitrant carbon and enhance soil fertility. Hydrolytic enzymatic assays, such as β-glucosidase and phosphatase activities, are used for the assessment of soil quality and composting process, which are based on use of p-nitrophenol (PNP) derivatives as substrate. However, sorption capacity of biochar can interfere with colorimetric determination of the hydrolysed PNP, either by the sorption of the substrate or the reaction-product of hydrolysis into biochar surface. The aim of the present work is to study the biochar sorption capacity for PNP in biochar-blended composting mixtures in order to assess its impact on the estimation of the colorimetric-based enzymatic assays. A retention test was conducted by adding a solution of known amounts of PNP in universal buffer solution (pH=5, 6.5 and 11, corresponding to the β-glucosidase, acid and alkaline phosphatase activity assays, respectively), in samples taken at the initial stage and after maturation stage from 4 different composting piles (two manure composting piles~~-~~: (PM: poultry manure, CM: cow manure~~)~~ and two other similar piles containing 10% of additional biochar (PM+B, CM+B)). The results show that biochar blended composts (PM+B, CM+B) generally exhibited low enzymatic activities, compared to manure compost without biochar (PM, CM). In terms of the difference between the initial and maturation stage of composting process, the PNP retention in biochar was shown ~~more clearly~~higher at maturation stage, caused most probably by an enlarged proportion of biochar inside compost mixture after the selective degradation of easily decomposable organic matter. The retention of PNP was more

pronounced at low pH; (5 and 6.5) than at high pH; (11), reflecting on pH dependency of sorption capacity of biochar and /or PNP solubility.

Keywords: biochar; organic wastes; enzymatic activity, *p*-nitrophenol, adsorption

Introduction

Agricultural use of biochar has been paid attention as an alternative strategy for mitigation of greenhouse gas (GHG) emission as well as improvement of soil properties,. In addition, high sorption character of biochar, similarly to activated carbon, makes it possible to contribute to reduction of several hazards (heavy metals, pesticide, and hydrocarbon) in soil (Yang et al 2009). Furthermore, it has been reported the suitability of biochar as an additional component for enhancing the composting quality by reducing the nitrogen volatilization due to sorption on surface of biochar (Steiner et al 2010), mitigating CH_4 emission due to the higher aeration in composting pile (Sonoki et al., 2012) and improving compost quality such as an intense humification process and more recalcitrant character (Dias et al. 2010; Jindo et al 2012). Lately, the application of compostbiochar blended biocharcompost to soil can promote a synergistic effect on enhancing plant the nutrition content and water holding capacity (Lieu et al.,2012) as well as contributing the reduction of organic pollutants and heavy metal (Beesley et al., 2010).

In terms of the decomposition of organic matter during composting, enzymatic activities such as β-glucosidase and phosphatase are useful tool to reflect dynamics of biodegradation process and provide valuable information about stability and maturity of the compost (Vuorinen 2000; Mondini et al., 2004). These hydrolytic enzymes are measured by colorimetric determination of *p*-

nitrophenol (PNP) which is formed as the reaction-product of hydrolysis of different *p*-nitrophenyl~~nitrophenol~~ derivatives used as a substrate: nitorophenyl-β-d-glucopiranoside (PNG) for β-glucosidase activity, and *p*-nitrophenyl phosphatase (PNPP) for alkaline and acid phosphatase activities.. By contrast, *p*-nitrophenol is a well-known toxic compound in industrial sector, and is treated by absorption ~~in~~ on activated carbon (Tang et al 2007; Ivančev-Tumbas al 2008). Furthermore, the biochar, which has similar absorption character to activated carbon (Hale et al., 2013)~~,~~ interferes with the extraction of soluble organic compounds, leading to underestimation of soil microbial activities (Chan et al., 2007). Even though several works on the relation between microbial measurements and biochar exposure has been reported (Durenkamp et al 2010; Bailey et al 2011; Luo et al., 2013), further research are required for understanding the biochar interaction from the chemical, physical and biochemical point of view. Thies and Rillig (2009) proposed the utilization of spiking assays with specific molecules as internal standard to overcome potential interferences in the estimation of the microbial parameters.

The aim in present work was to study the influence of biochar as a composting component on the retention of the PNP generated from three colorimetric-based enzymatic assays (alkaline and acid phosphatases and β-glucosidase~~glocosidase~~). The retention of PNP was tested in two different composting mixtures (poultry manure (PM) and cow manure (CM)) and other two similar composting mixtures containing biochar as additional component (PM+B, CM+B).

Materials and Methods

Biochar preparation

100   The production of biochar, made from broad-leaved tree (*Quercus*

101   ~~serrate~~ *serrata* Murray), was carried out using a Japanese traditional kiln at

102   atmospheric pressure and a temperature range of 400-600 °C with final

103   temperature of 550 °C . To analyze the physical properties of biochar, we

104   grounded and sieved the biochar less than 0.5 mm in diameter. The elemental

105   content was measured with an elemental analyzer (Thermo Finnigan EA1112,

106   Thermo Fisher Scientific, Inc., MA, USA). The pH of each mixture pH at 1:20

107   (w/v) ratio was measured with a compact pH meter B-212 (HORIBA Ltd., Kyoto,

108   Japan) Microporosity was evaluated by the iodine (I_2) number method and

109   methylene blue (MB) adsorption capacity, respectively, were measured,

110   following the methodology used by Gaspard et al., 2007. Surface area was

111   measured with a BELSORP18PLUS (BEL Japan, Inc., Osaka, Japan). The

112   main characteristics of the obtained biochar are shown in Table 1.~~: pH (H_2O)=~~

113   ~~7.23; C = 791.5 g kg^{-1}; O = 91.5 g kg^{-1}; H = 18.9 g kg^{-1}; ash = 78.7 g kg^{-1}; N =~~

114   ~~37.6 g kg^{-1}; P = 2.3 g kg^{-1}; K = 14.1 g kg^{-1}; Surface area = 255.0 m^2 g^{-1};~~

115   ~~methylene blue (MB) absorption capacity: 8.3 mg g^{-1}; iodine adsorption~~

116   ~~capacity: 100 mg g^{-1}.~~

117

118   *Raw materials and composting process*

119   Composting was carried out at Kanagi experimental farm of Hirosaki

120   University. Two composting mixtures were prepared following initial proportion

121   of organic waste: CM - cattle manure (100.9 kg) mixed with apple pomace (76.8

122   kg), rice straw (9.7 kg) and rice bran (12.7 kg)~~;~~ PM - poultry manure (35.2 kg)

123   mixed with apple pomace (141.8 kg), rice straw (9.9 kg) and rice bran (13.0 kg).

124   Another two composting mixtures (CM+B and PM+B) were prepared by

125   enriching the initial composting mixtures CM and PM with 20kg of biochar. The

126   organic waste mixtures were composted in cone shaped windrows with regular

127   turnings and continuous monitoring of pile temperature and moisture. The

128   principal physicochemical properties of the composting mixtures are described

129   in Table 1, and further information on the composting process and

130   characteristics of the composting mixtures can be found elsewhere (Sonoki et al,

131   2012). The composting process lasted approximately 3 months for all piles. A

132   representative sample of each organic material was taken at the initial stage (I)

133   and after maturation stage (M). These samples were collected from different

134   spots of piles, mixed together, air dried and grounded to 0.5mm.

135

136   *Thermogravimetric analysis (TGA)*

137   Thermal analysis of the organic material was measured using a SDT-

138   2960 simultaneous DSC-TGA thermal analyzer (TA instruments) under static air

139   atmosphere as follows: a temperature equilibrating at 30 $^\circ$C followed by a linear

140   heating rate of 5 $^\circ$C min^{-1} from 30 to 105 $^\circ$C, an isotherm for 10 min and then

141   continued ramping of 5 $^\circ$C min^{-1} from 105 to 680 $^\circ$C. An index of thermal lability

142   of the organic matter (W_2/W_1), shown in Table 1, was calculated from the ratio:

143   Mass loss at 350-550 $^\circ$C (W_2) / Mass loss at 110-350 $^\circ$C (W_1) (Plante et al.,

144   2009).

145

146   *Enzymatic analysis*

147   Alkaline and acid phosphatase and β-glucosidase activities were

148   determined following the methods reported by Tabatabai and Bremmer (1971),

6

149   and Eivazi and Tabatabai (1988) respectively using 0.5 g of organic material,

150   and 2 ml of modified universal buffer (MUB) containing the following substrate:

151   Alkaline phosphatase activity assay was performed at pH 11 using *p*-nitrophenyl

152   phosphatase (PNPP) as substrate, meanwhile acid phosphatase activity assay

153   was performed with the same substrate at pH 5.5; β-glucosidase activity was

154   assayed at pH 6 using *p*-nitrophenyl β-D-glucopiranoside (PNG) as substrate. In

155   the three cases, the suspensions were incubated at 37ºC for 1 hour. Enzymatic

156   reactions were stopped by cooling in ice for 15 min. Then, 0.5 ml of $CaCl_2$ 0.5 M

157   and 2 ml of NaOH 0.5 M (for phosphatases) or 2 ml of Tris (hydroxymethyl)

158   aminomethane-sodium hydroxide (THAM-NaOH) 0.1 M pH 12 (for β-

159   glucosidase) were added. The *p*-nitrophenol (PNP), formed as product reaction

160   from the three enzymatic assays, was determined at 398 nm of

161   spectrophotometer.

162

163   *PNP retention assay during the enzymatic activity analysis*

164   To study the retention of PNP during the analysis of the different

165   enzymatic activities, following spiking assay was performed: Instead of adding

166   the substrates (PNG and PNPP) at the beginning of the procedure, reaction-

167   product (PNP) was added with ~~difference~~ different concentration (0, 50, 100 and

168   150 mg L^{-1}) to buffer solution (pH=5, 6,5 and 11, corresponding to the β-

169   glucosidase, acid and alkaline phosphatase activity assays, respectively). After

170   the incubation, the same procedure as for enzymatic assay, described in

171   previous section was taken place for PNP determination. This procedure

172   allows evaluating the retention of PNP by the biochar during the analysis.

173   Controls were performed similarly by adding the same amounts of PNP after the

174   incubation period and before the measurement of the absorbance in the

175   calibrated spectrophotometer (with an external PNP standard solution). These

176   results were shown in Fig.2. (CM and CM+B) and Fig.3. (PM and PM+B). Lately,

177   the PNP retention was calculated by fitting the amount of PNP measured after

178   the enzymatic determination (PNP_{exp}) and the amount of PNP added in the

179   control (PNP_{add}) to a linear equation ($PNP_{exp} = k \times PNP_{add}$), where k was the

180   slope of the linear fitting. The percentage of PNP recovered in the enzymatic

181   determination was calculated as $100 \cdot k$, whereas the percentage of PNP

182   retention was calculated as $100 \cdot (1-k)$. PNP retention assays were performed in

183   duplicate for all treatments and shown in Table 2.

184

Results and Discussion

Characteristics of the composting mixtures

187   Different composting mixtures were selected at different stages of the

188   composting process to cover the range of organic matter stabilization degree.

189   The different nature of the organic matter at different stages of the

190   biodegradable process and the property of the recalcitrant biochar was

191   assessed by thermogravimetry. (Lyons et al., 2006; Tsui and Juan, 2010;

192   Manya et al., 2013). Basically, the TGS-DSC diagrams are characterized by two

193   main mass losses, showing two exothermic peaks, and these are respectively

194   ~~corresponded~~ which correspond to volatilization of light compounds such as

195   aliphatic molecules or carbohydrates and another to oxidation of high molecular

196   weight components (Fig.1.). Comparing the graph shapes between the samples

197   from initial stage (Fig.1.A and C) and from maturity stage (Fig.1.B and D), the

198   second wave of peak, generated by mass loss at 350-550 ºC, was

199 pronouncedly shown at maturity stage, due to the selective degradation of labile

200 organic materials during the composting process. As a consequence, the index

201 of lability of W_2/W_1 in all samples of maturity stage is higher than those of initial

202 stage (Table 1).

203 The influence of additional biochar into the composting mixture at the initial

204 phase (Fig.1.A and C) is observed by higher peak of second wave in biochar

205 blended composts (PM+B, CM+B), which are described in dotted lines. This has

206 resulted from that biochar originated from hard-wood mostly consists of

207 recalcitrant compounds, which are combusted at W_2 range (350-550 ^0C) in an

208 oxidant atmosphere of air. Consequently, W_2/W_1 ratio at initial time (Table 1)

209 increased in biochar blended piles (PM+B, CM+B) from the piles of non-biochar

210 addition (PM, CM). After maturation stage (Table 1), W_2/W_1 ratio markedly

211 increased in the biochar blended composts (PM+B, CM+B, 2.3, and 1.6,

212 respectively), reflecting the high relative proportion of recalcitrant biochar.

213

214 *Study of the PNP retention on biochar blended compost.*

215   The colorimetric determination of PNP was influenced by the degree of

216 stability of the composting mixtures, which affected the relative proportion of

217 biochar in the mixture. The biochar blended composts showed more retention of

218 PNP, especially in the case of maturity stage (Figure 2 and 3). The amount of

219 PNP retained by the biochar blended composting mixtures (CM+B and PM+B)

220 varied from 41% in the starting composting mixtures up to 74% in mature

221 composts. This result might have attributed to gained dominance of biochar

222 amount inside composting mixtures which was gradually increased during the

223 composting process. The recalcitrance of biochar character was remained

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224 retained until the maturation stage, while labile organic materials in the

225 composting piles were lost due to the selective degradation, as already shown

226 by TGS measurement (Fig.1.). Therefore the effect of the physico-chemical

227 properties of biochar on the compost structure is expected to be more dominant

228 in the mature stage than at the initial stage.

229 The PNP retention by biochar also depends on pH status of the buffer solution,

230 used by each specific enzymatic activity. At high pH condition (pH 11),

231 representing alkaline phosphatase essay, the PNP retention is observed in the

232 range between 15 and 30% of the added PNP (Table 2). However, the same

233 spiking assays performed a low pH (pH 6.5 and 5 from acid phosphatase and β-

234 glucosidase activities, respectively) exhibited high PNP retention from 30%

235 (acid phosphatase determination in PM+B) up to 70% which is the case of the

236 β-glucosidase determination in CM+B. These results are in agreement with the

237 pH dependence of phenol adsorption efficiency by activated carbon reported by

238 several authors (Ayranci and Duman 2005; Tang et al 2007), concluding that

239 the absorption efficiency of activated carbon is lower in alkaline solution than

240 neutral or acid solution. An increase in the amount of OH ions in alkaline

241 solution reduces the diffusion of phenol ions due to an electrostatic repulsion of

242 negatively charged site of the sorbent and phenolic ions. As the pH increases,

243 the surface charge of pyrogenic materials became negative and decreases its

244 sorption capacity (Beker et al 2010). Furthermore, other authors (Zhang et al

245 2010) reported that, regarding the mobility of biochar particle, the lower the pH

246 solution, the lesser transport of the biochar particle.

247   Sorption affinity of pyrogenic material is also influenced by physical properties

248 such as microporosity and surface area, as well as chemical properties such as

249   hydrophobicity in relation with O/C content (Al-Asheh et al., 2004; Ko et al.,

250   2007; Tsui and Juang, 2010). Micropore and mesopore structure, estimated

251   respectively by the iodine number and the methylene blue adsorption, are

252   usually enlarged at high pyrolysis temperature together with surface area..

253   Overall, aAll these biochar properties are dominantly defined by feedstock and

254   the pyrolysis conditions used for the preparation of the biochar (Uchimiya et al.,

255   2010).

256   The PNP retention by the organic matter of the composting mixtures prepared

257   without biochar (PM and CM) was also affected by the pH gradient. Table 2

258   shows that at low pH solution (pH 5) the initial stage of composting, CM has

259   69% of PNP recovery, meaning 31% of PNP was retained. This methodological

260   problem in the determination of the enzymatic activities is well-known in clay

261   mineral soils or soils enriched with organic matter (Tabatabai and Bremer 1971;

262   Trasar-Cepeda et al 1988). The organic material containing large amount of

263   humic substances are known to easily absorb PNP molecules (Chen et al 2009).

264   In order to tackle this obstacle, several authors have recently recommended to

265   test the soil enzymatic assays in samples blended with biochar to ensure the

266   assumption of saturating substrate concentrations, and if necessary to amend

267   the protocols before initiating the assays (Swine (et al., 2013). In practice, and

268   in order to overcome the underestimation by absorption on biochar, Paz-Fierro

269   et al (2012 and 2014) used different calibration curves for each different type of

270   amendment to acquire an accurate measure of soil enzymatic activities. This

271   problem is even more complex in composting samples, where the degradation

272   of labile organic matter causes a progressive enrichment in the proportion of

273   biochar in the mixture. The different proportion of biochar in the starting

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mixtures and the mature compost also requires the adaptation and optimization of the enzymatic assay to the different composting stage.

In conclusion, the presence of biochar limited the validity of enzymatic essays for the colorimetric determination of PNP since PNP was strongly retained in biochar blended compost. It is challengeable to improve the colorimetric methods of PNP determination for biochar interaction, and clear-cut solution has not been found until present day. Further research is necessary in order to correctly quantify enzymatic activity in presence of biochar.~~More other factors are necessary to be considerable for understanding the biochar interaction with enzymatic activity assay.~~

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