Biomass Conversion and Biorefinery (2023) 13:12823–12832
https://doi.org/10.1007/s13399-021-02100-w

ORIGINAL ARTICLE

Valorization of animal bone waste for agricultural use through biomass co-pyrolysis and bio-augmentation

Milkiyas Ahmed[1,2] · Abebe Nigussie[2] · Solomon Addisu[3] · Berhanu Belay[4] · Johannes Lehmann[5] · Shinjiro Sato[1]

Received: 11 August 2021 / Revised: 27 September 2021 / Accepted: 18 November 2021 / Published online: 30 November 2021

Abstract

Thermal treatment of animal bone waste (i.e., pyrolysis) is an alternative technology to sustainably manage slaughterhouse waste for agricultural uses. However, concentration of plant-available phosphorus (P) is limited in thermally treated animal bone (i.e., bone char). This study, therefore, aimed to develop sustainable methods to increase the P fertilizer value of animal bone waste through co-pyrolysis of animal bone with lignocellulose agricultural waste and bio-augmentation. Four types of bone chars were produced using two different pyrolysis temperatures (450°C and 850°C) and pyrolysis techniques (conventional and co-pyrolysis). These bone chars were then bio-augmented with four different phosphate solubilizing microorganisms (PSM). In vitro and incubation experiments were conducted to assess the fertilizing value of the products. The result showed that co-pyrolysis of animal bone with lignocellulose agricultural waste combined with bio-augmentation increased P solubility by 133–167%, at the lower production temperature. P solubility decreased considerably at a higher production temperature. However, it was increased by 16- to 21-fold when co-pyrolysis was coupled with bio-augmentation. Addition of co-pyrolyzed bone char enriched with PSM and organic carbon to soil increased P availability by 34 to 48% and PSM survival rate by 22 to 76%. The findings demonstrated that co-pyrolysis combined with bio-augmentation could be an efficient and low-cost strategy to improve the agricultural use of animal bone and to reduce the dependency on chemical fertilizer. This study has a significant importance particularly for developing countries, where the use of chemical fertilizer is limited due to its high price; and slaughterhouse waste has created an environmental concern.

Keywords Bio-augmentation · Bone char · Co-pyrolysis · *Penicillium* strains · Solubility

1 Introduction

Modern agriculture relies on mineral phosphorus (P) fertilizer produced from rock phosphate (RP), a non-renewable and quickly depleting resource [1]. As demand for P is expected to double by 2050, there is mounting evidence that the world's supply of RP is under threat [2]. In recent years, P scarcity has been identified as a bottleneck in the sustainability of agricultural systems [3, 4]. The lack of mineable P and the prospect of future P shortfalls threaten growth and food security in many developing countries. Currently with increasing urbanization and economic growth, non-competitive agricultural and agro-industrial wastes—such as slaughterhouse wastes—are generated in a large quantity, creating an environmental concern. Slaughterhouse wastes (i.e., animal bones) are polluting the land and water, representing a significant solid waste disposal problem, and incurring management costs in many (per-)urban areas. Thus, sustainable management of animal bone residues—such as thermal treatment (i.e., pyrolysis)—is required to improve the public health and ensure food security through soil fertility management. In Ethiopia, for instance, pyrolysis of animal bones could potentially substitute 25–52% of its annual chemical fertilizer import [5].

✉ Shinjiro Sato
ssato@soka.ac.jp

1 Graduate School of Science and Engineering, Soka University, Tokyo, Japan

2 College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia

3 College of Agriculture and Environmental Science, Bahir Dar University, Bahir Dar, Ethiopia

4 College of Agriculture, Food and Climate Sciences, Injibara University, Injibara, Ethiopia

5 Soil and Crop Sciences, School of Integrative Plant Science, Cornell University, Ithaca, NY, USA

Various researches have shown that pyrolyzed animal bone (i.e., bone char) could be used as a phosphorus fertilizer and thereby could reduce the dependency on chemical fertilizer in a sustainable way [6–8]. However, plant-available P content in pyrolyzed animal bone is often poor because of low solubility of the main P compounds in bone, namely apatite [6, 8]. Therefore, there is a need to develop method to improve the quality of fertilizer developed through pyrolysis of animal bone waste.

Different strategies could be used to improve fertilized value of animal bone waste, such as co-pyrolysis with different lignocellulose agricultural waste; bio-augmentation, i.e., the use of phosphorus solubilizing microorganisms (PSMs). Co-pyrolysis of different lignocellulose agricultural waste with animal bone can significantly reduce Ca-P crystallinity during pyrolysis [9], hence resulting in more amorphous Ca-P forms. On the other hand, PSMs could solubilize mineral bound P via acidification driven by the release of low molecular weight organic acids [10, 11]. Co-pyrolysis of lignocellulose agricultural waste with animal bone could also supply easily available nutrients required by PSM and could improve the performance of PSM to solubilize P and maximize the economics and environmental benefits. To the authors' knowledge, there are no studies to improve the fertilizer value of animal bone waste through combining co-pyrolysis with bio-augmentation. This study therefore aimed to determine whether coupling of co-pyrolysis with bio-augmentation influence the fertilizer value of slaughterhouse waste.

2 Materials and methods

2.1 Bone char-based P fertilizer production: pyrolysis and co-pyrolysis

Sheep bone and sugarcane bagasse waste were oven dried at 70°C for 72 h and milled to particle size <2mm. Different bone char-based P fertilizers were prepared through pyrolysis process at a temperature of 450°C and 850°C with a heating rate of 5°C per minute and 2 h of retention time at the highest heating temperature (BC450 and BC850, respectively). In addition, bone mixed with sugarcane bagasse waste at a mass ratio of 3:1 was co-pyrolyzed under the same pyrolysis condition (Co-BC450 and Co-BC850, respectively).

2.2 Basic characterization of the different bone char-based P fertilizers

Total C and N were measured using a CHN analyzer (2400 series II, Perkin Elmer) as described by Yeomans and Bremner [12]. The pH and electrical conductivity (EC) were measured using auto-electrical pH and EC meter (Seven Easy S20, Mettler Toledo). Total P and Ca [13], 2% formic acid extractable P [14], and water extractable P (AOAC, 2005) were measured to characterize P contents. Water-extractable organic C (WEOC) was analyzed using liquid chromatography organic carbon detection (LC-OCD) method as described by Chinu et al. [15]. Powder X-ray diffraction (XRD) profiles were obtained by using a Bruker D5000, iron-filtered cobalt radiation (40 kV, 40 mA), and a scanning rate of 2° 2θ per min in the range of 10 to 90° 2θ using a fixed divergence slit of 1°. The width at half height of selected diffraction peaks was calculated using Siemens EVA software in order to determine Ca-P crystal formation.

2.3 Bio-augmentation as a strategy to increase P solubility

2.3.1 Phosphorus solubilizing strains and inoculum preparation

Four different *Penicillium* strains: *Penicillium bilaiae* JCM22749, *Penicillium glabrum* JCM22735, *Penicillium expansum* JCM22825, and *Penicillium aculeatum* JCM22556 were purchased from RIKEN Bio Resource Research Center, Tokyo, Japan. All strains were cultivated on Czapek yeast extract agar (CYA) plates for approximately 14 days. Spores were collected by washing the plates with sterile MilliQ water; the suspension was filtered through sterile glass wool to remove the hyphae and then centrifuged at 4000 rpm for 10 min [16]. Spore concentrations in the suspension were adjusted with MilliQ water after determining the spore concentration with hemacytometer.

2.3.2 In vitro bone char-P solubility

An in vitro assay was carried out to determine P solubility from different bone char-based P fertilizers produced at different pyrolysis temperature and pyrolysis techniques augmented with four different *Penicillium* strains. The assay was evaluated using the NBRIP (National Botanical Research Institute's phosphate) growth medium containing (g L^{-1} DI H_2O): glucose (10), $MgCl_2{\cdot}6H_2O$ (5), $MgSO_4{\cdot}7H_2O$ (0.25), KCl (0.2), and $(NH_4)_2SO_4$ (0.1) [17]. A 50-mL centrifuge tube containing 25 mL of NBRIP was prepared and sterilized. The bone chars (BC450, BC850, Co-BC450, and Co-BC850) were then applied at the rate of 1 g P L^{-1}. One milliliter of spore suspension (10^6 spores mL^{-1}) of each strain was mixed with the bone chars. Non-inoculated control receiving an equal amount of inoculant-free NBRIP was also included as a control. The tubes were then shaken on

a rotary shaker for 3, 14, and 21 days at 25°C and 115 rpm with random block design and three replicates.

After each incubation time, samples were centrifuged at 5000 rpm for 5 min, and the supernatant was filtered through a 0.45-µm membrane filter. The supernatant was then analyzed for pH using pH meter, soluble P using flow injection analysis (FIAlab-2500, FIAlab Instruments), and organic acids using high-pressure liquid chromatograph (LC-2030C Shimadzu). Briefly, organic acid concentration was determined using Shim-pack Fast-OA high-speed organic acid analytical column (LC-2030C Shimadzu) in combination with the post-column pH-buffered electrical conductivity detection method. Analytical grade standards of the following organic acids were used: lactic, succinic, malic, citric, formic, gluconic, acetic, butyric, and oxalic acids (Wako Chemical Ltd.). The detected organic acids were identified by comparing the peaks of their retention time and the area under the curve of their chromatogram with the standards.

The growth phenotype of the strains on the surface of bone chars was investigated using scanning electron microscopy (SEM) as described by Baskin et al. [18]. Briefly, the bone char samples from the incubation experiment were washed with distilled water to remove the growth medium component and then attached to carbon double-sided tape, which was placed onto a carbon stub. The samples were then freeze dried by pressing a 36-g copper column block (height of 20 mm, diameter of 15 mm) that had been cooled to –100°C using a cooling unit (FDC10, SUN Technologies, Tokyo, Japan) on the surface of the device from above. The completely dried samples were then coated using an osmium coater (HPC-1SW, VACUUM DEVICE, Ibaraki, Japan) before examination under a low-vacuum scanning electron microscope (JSM-5600, JEOL, Tokyo, Japan) and a field emission electron microscope (JSM-7500F, JEOL).

2.4 Bone char-P solubilization by PSM augmentation under soil condition

2.4.1 Soil incubation experiment

An incubation experiment was conducted using two soils varying in their carbon content to further examine P solubility of the different bone chars bio-augmented with selected *Penicillium* strains in a soil system. Accordingly, two PSM strains were selected for the incubation experiment, namely *P. bilaiae* and *P. expansum*. A control without PSM augmentation was included in the study. A soil used for this experiment was collected from Jimma, Ethiopia (07° 42′ 05″ N, 36° 48′ 40″ E), and was characterized by pH of 4.5 (1:2.5 soil/water ratio), 19 g total C kg^{-1}, and 2.0 g total nitrogen kg^{-1} [19]. Bray II extractable P was 1.4 mg kg^{-1} soil, and the soil had a maximum P-sorption capacity of 456 mg P kg^{-1} soil [9]. The soil was classified as Nitisol [20], and the particle size distribution was 499 g kg^{-1} clay, 482 g kg^{-1} silt, and 19 g kg^{-1} sand. Prior to the incubation experiment, the soil sample was air dried and passed through a 2-mm sieve, and visible root biomass was removed by hand. Fifteen grams of dried soil was added in a 100-mL glass jar. The experiment had 12 treatments resulted from the factorial combination of (i) two bone char types (BC450 and Co-BC450) applied at a rate of 1 g P kg^{-1} of soil, (ii) two soil OC levels (without and with OC amendment), and (iii) bio-augmentation with two PSM (*P. bilaiae* and *P. expansum*) and replicated four times. Sucrose was used to increase soil OC content at the rate of 1% w/w. The following nutrient solutions were also prepared and mixed with the soil (mg kg^{-1}): NH_4NO_3, 100 N; K_2SO_4, 166 K; $MgSO_4{\cdot}7H_2O$, 40 Mg; $MnSO_4{\cdot}H_2O$, 3.4 Mn; $ZnSO_4.7H_2O$, 1.2 Zn; and $CuSO_4{\cdot}5H_2O$, 0.2 Cu [17].

The bone char was bio-augmented with one mL of each fungal spore suspension to provide 10^6 spores (corresponding to 6.66 x 10^4 spore g^{-1} soil) and then added to the soil. The moisture content was kept at 60% water holding capacity (WHC) and incubated in the dark for 6 weeks at temperatures of 25±2°C. The moisture loss was determined gravimetrically once a week, and deionized water was added to compensate the weight loss. The glass jar was covered by parafilm with several holes throughout the experimental period to avoid the development of anaerobic environment. Distractive soil samples were collected on 7, 21, and 36 days of the incubation period and analyzed for Olsen-P. Briefly, 100 mL of 0.5 M $NaHCO_3$ (pH~8.5) was added to 1.0 g soil and agitated on a horizontal shaker at 160 rpm for 30 min. The supernatant was filtered through 0.45 µm filter paper, and the filtrates were then used for colorimetric P determination using a spectrophotometer. The survival rate of the *Penicillium* strains was determined using modified dilution colony forming unit (CFU) plate count technique on yeast extract agar (YEA) [21]. In brief, 3 g of fresh composite soil sample was placed into a flask containing 27 mL distilled water and 2.5 g of glass beads (0.5 mm). The flask was shaken by vertexing for 1 min. Then, 50 µL from the suspension was added to a small tube containing 450 µL distilled water. Series of dilutions (10^{-1}–10^{-4}) were prepared, and 100 µL suspension of two different dilutions was spread on YEA plates. Each dilution was plated in triplicate and then incubated for 3 days at 25°C, and microscopically visible colonies were counted from plates with 20–200 colonies to determine CFU.

2.5 Statistical analyses

Analysis of variance (ANOVA) was used to determine the effect of pyrolysis temperature, co-pyrolysis, and PSM augmentation on P solubility of bone char. If significant differences were found at $p < 0.05$, mean separation was

performed using Tukey's HSD tests. Prior to data analysis, the assumption of homogeneity of variance was checked using Levene's test, while the Shapiro-Wilk test was used to check the normality assumption. All statistical analyses were performed using STATISTICA software version 6.

3 Results

3.1 Characteristics of the different bone char-based fertilizers

The pH values of bone chars produced at 450°C (BC450 and Co-BC450) were lower compared to those produced at 850°C (BC850 and Co-BC850) (Table 1). Co-pyrolysis of animal bone with biomass at 850°C reduced the pH of the product by almost one unit. Total P increased with increasing temperature; however, it was reduced when bone char was co-pyrolyzed with biomass. Most of TP was found to be 2% formic acid extractable, while water soluble P was almost non-existent for all bone chars. The TC, TN, and WEOC of BC850 were less compared to those of BC450. Biomass co-pyrolysis, however, increased TC by two- to five-fold compared to pure bone chars produced at the same temperature. Similarly, biomass co-pyrolysis increased WEOC by 29–730% for both temperatures. The XRD analysis revealed lower peak intensity and broader reflections at lower processing temperatures. On the other hand, sharper diffraction peaks and higher peak intensity were observed in bone char produced at higher pyrolysis temperature (Fig. 1).

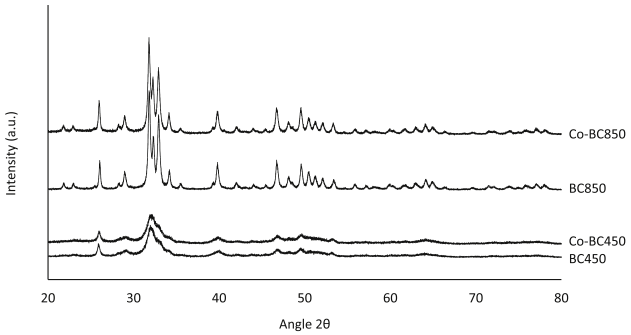

Fig. 1 X-ray powder diffraction spectra from different bone chars produced at 450°C and 850°C without (BC450 and BC850) and with biomass co-pyrolysis (Co-BC450 and Co-BC850)

3.2 In vitro experiment

3.2.1 Phosphorus solubility

P solubility from animal bone waste was altered by processing temperature, biomass co-pyrolysis, and bio-augmentation ($p < 0.001$). Bone chars produced at 450°C (BC450 and Co-BC450) had higher P solubility than bone chars produced at 850°C (BC850 and Co-BC850) (Fig. 2). For instance, *P. bilaiae* increased P solubility by 65–99% from bone char produced at a 450 °C and by more than tenfold from bone char produced at 850 °C (Fig. 2C). Similarly, after 21 days of incubation, *P. expansum* solubilized 188 mg P L^{-1} and 500 mg P L^{-1} at BC450 and Co-BC450, respectively. These values however decreased to 3.53 mg P L^{-1} and 157 mg P L^{-1} at BC850 and Co-BC850, respectively (Fig. 2).

Compared to the conventionally produced bone char, P solubility from animal bone char was also enhanced by co-pyrolysis of animal bone with lignocellulose agricultural waste. Co-pyrolysis coupled with bio-augmentation considerably increased P solubility. At a lower temperature, coupling co-pyrolysis with bio-augmentation increased P solubility by 133–167% (Fig. 2A and B). Similarly, at higher pyrolysis temperature, combining co-pyrolysis with bio-augmentation increased P solubility by 10- to 45-fold (Fig. 2C and D), indicating that co-pyrolysis of bone with biomass significantly improved P solubilization by PSM.

The pH of the medium was also varied among the processing temperatures, biomass co-pyrolysis, and PSM types. For bone chars produced at lower temperature (BC450 and Co-BC450), sharp decreases in pH were observed after day 3 of incubation (Fig. 2A and B). The pH remained the same in BC450, whereas it continued to decrease in Co-BC450 throughout the incubation period (Fig. 2A and B). The pH

Table 1. Basic chemical characteristics of bone chars used in this study. Values are the mean of three replicates ± standard deviation

Bone char†	pH	Total P	Formic P	Water soluble P	Total Ca	TC	TN	WEOC	C/N	Ca/P
		g kg^{-1}								
BC450	7.23	129±1.38	126±1.65	0.44±0.00	145±10.58	131±5.22	19.1±0.83	2.58±1.19	6.88	1.13
Co-BC450	7.30	104±0.94	100±1.35	0.43±0.00	126±4.33	267±6.70	27.6±0.42	3.32±0.54	9.66	1.21
BC850	9.30	165±2.01	123±0.71	0.00±0.00	170±0.93	56.7±1.79	6.50±0.69	0.07±0.03	8.73	1.03
Co-BC850	8.37	146±0.90	112±0.11	0.00±0.00	170±12.99	249±17.36	10.4±0.45	0.58±0.77	24.0	1.17

†*BC450* bone char pyrolyzed at 450°C, *Co-BC450* biomass co-pyrolyzed bone char at 450°C, *BC850* bone char pyrolyzed at 850°C, *Co-BC850* biomass co-pyrolyzed bone char at 850°C

TC total carbon, *TN* total nitrogen, *WEOC* water-extractable organic C, *C/N* carbon to nitrogen ratio, *Ca/P* calcium to phosphorus ratio

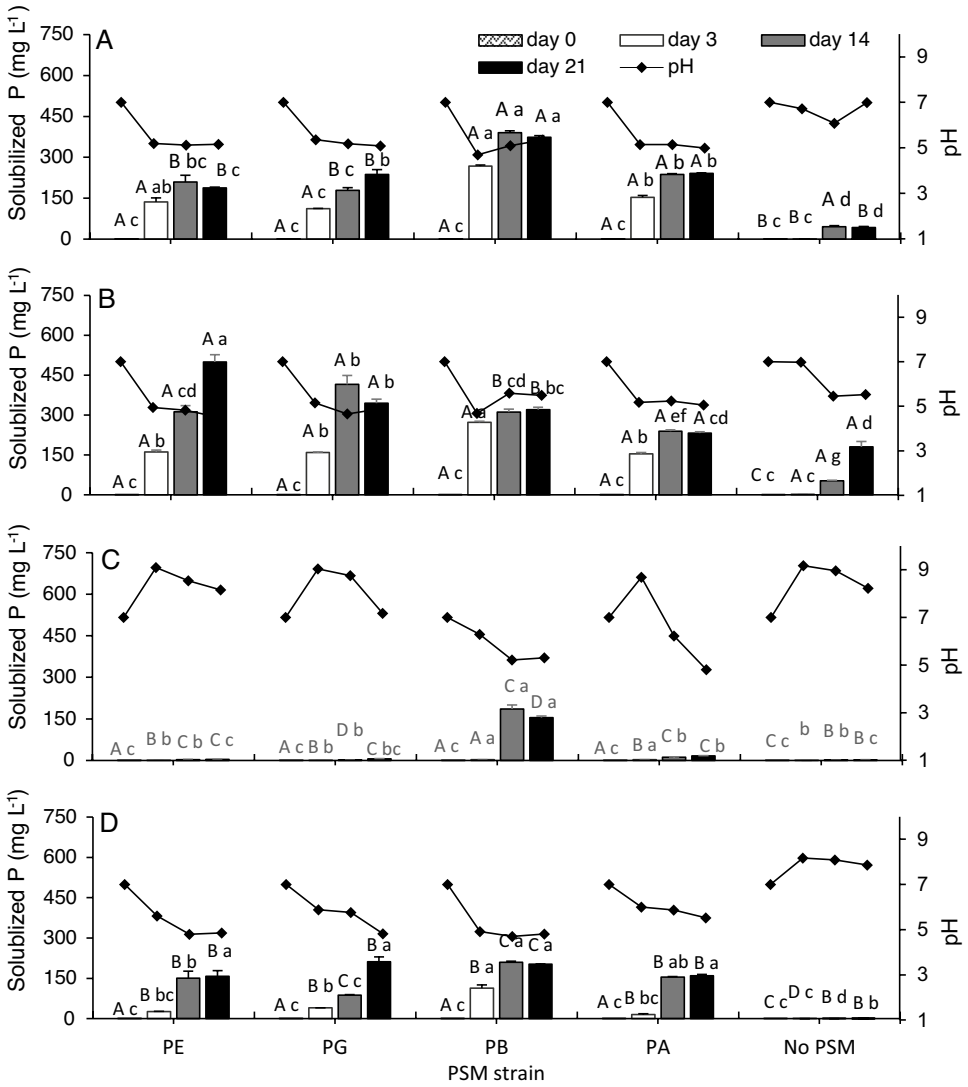

Fig. 2 Change in solubilized phosphorus and pH in different bone chars (**A**) BC450, (**B**) Co-BC450, (**C**) BC850, and (**D**) Co-BC850 augmented with different PSM strains (PE, *Penicillium expansum*; PG, *Penicillium glabrum*; PB, *Penicillium bilaiae*; PA, *Penicillium aculeatum*; and No PSM, control) sampled at different days of incubation. Bars represent the mean of four replicates ± standard error. Capital letters indicate differences across different bone chars within the same PSM levels and sampling dates, whereas lowercase letters indicate differences across different PSM levels within the same bone chars and sampling dates.

was also significantly varied between the bone chars produced at higher temperature (BC850 and Co-BC850). A significant drop in pH was recorded for Co-BC850 with bio-augmentation (Fig. 2D). However, in BC850, only *P. bilaiae*, augmentation resulted in reduced pH (Fig. 2C).

3.2.2 Organic acid production and pH change

One of the mechanisms by which PSM augmentation results in P solubility is through organic acid production and reduction of pH. The type and amount of organic acid produced differed substantially between processing temperature, biomass co-pyrolysis, and by PSM augmentation (Fig. 3). Lactic, pyruvic, acetic, butyric, and formic acids were the predominant organic acids produced but in different amounts (Supplementary Fig. S1). Higher concentration of organic acid production was recorded from bone char produced at 450°C (Fig. 3A and B) than bone chars produced at 850°C (Fig. 3C and D). Very low organic acid production was recorded on BC850; however, it increased very significantly when co-pyrolysis was combined with bio-augmentation (Fig. 3C and D). Pyruvic, acetic, and formic acids were produced in large amounts from BC450 and Co-BC450 treatments by all of the PSMs (Supplementary Fig. S1A and S1B). In BC850 and Co-BC850 treatments however, pyruvic and acetic were most abundant organic acids produced. Among the PSM used in this study, *P. bilaiae* produced large amount of OA, compared to the rest of the PSMs (Supplementary Fig. S1C and S1D).

3.2.3 PSM colonization on different bone chars

Microbial colonization after 21 days of incubation on the surfaces of the different materials was confirmed by SEM images (Fig 4). All the inoculated strains were able to grow and heavily colonize all the bone chars produced at 450°C

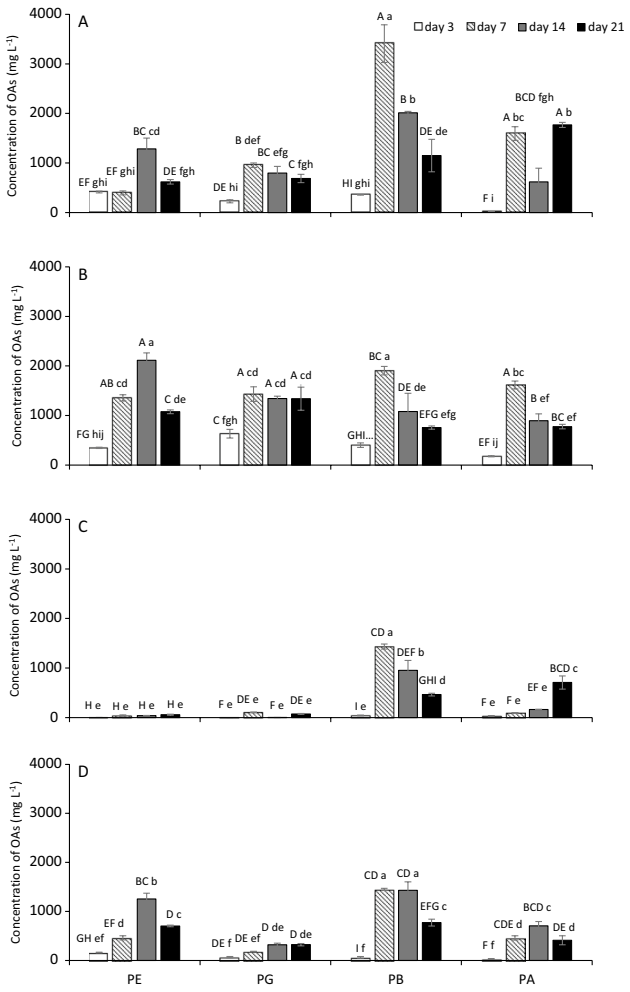

Fig. 3 Concentration of low molecular weight organic acids produced with different bone chars (**A**) BC450, (**B**) Co-BC450 (**C**) BC850, and (**D**) Co-BC850 augmented with different PSM strains (PE, *Penicillium expansum*; PG, *Penicillium glabrum*; PB, *Penicillium bilaiae*; and PA, *Penicillium aculeatum*). Bars represent the mean of four replicates ± standard error. Capital letters indicate differences across different bone chars within the same PSM levels and sampling dates, whereas lowercase letters indicate differences across different PSM levels within the same bone chars and sampling dates

with some regions densely covered with hyphae and spores (Fig. 4A, B, and C). *P. bilaiae* was the only strain that was able to grow on BC850 (Fig. 4D) compared to the rest of the strains where no hyphae growth was detected (Fig 4E). However, increased growth and colonization of all the strains was observed on the biomass co-pyrolyzed bone char (Co-BC850) (Fig. 4F).

3.2.4 Bone char-P solubilization by bio-augmentation under soil condition

The P solubilization potential of the PSMs were significantly affected by soil OC content, bone char type, and incubation period (Fig. 5). During the first 7 days of incubation, addition of *P. bilaiae* on Co-BC450 with OC addition increased P solubilization by up to 23% (Fig. 5A). Similarly, after 35 days of incubation, *P. bilaiae* inoculated with OC addition to soil resulted in significantly higher P solubility than with *P. expansum* or non-inoculated bone chars. The amount of P solubilized by *P. bilaiae* was 42 to 48% higher than those by *P. expansum* and non-inoculated treatments, respectively (Fig. 5C).

3.3 PSM survival rate in soil

Insuring survival and abundance of PSM in a soil system is a key for successful application of PSM to solubilize P from organic waste. The soil incubation experiment demonstrated that bio-augmentation of biomass co-pyrolyzed bone char increased PSM survival by 24–47%, indicating that more suitable environmental condition was created for the PSM through co-pyrolysis of biomass with animal bone (Table 2). Moreover, on soil with higher carbon content, four-fold higher survival rate of the strains was recorded. Among the augmented PSMs, 22–76% higher survival rate was recorded for *P. bilaiae* than *P. expansum*.

4 Discussion

4.1 Increased P solubilization by PSM augmentation in low- than high-temperature bone chars

Much greater P solubilization were observed by PSM augmentation with bone chars produced at lower production temperature (450°C) than those at higher production temperature (850°C) (Fig. 2). This could be due to the higher degree of Ca-P crystallization in BC850 as compared to the poorer crystalline structure observed in BC450 (Fig. 1). Previous studies showed that at lower pyrolysis temperature, some labile C-containing compounds could also remain in bone char, and these compounds may have resulted in Ca-P with poor crystal structure [9]. Additionally, C-containing compounds may also play an important role in supplying C required for the survival and growth of P solubilizing microbes and could have resulted in higher P solubility. On the other hand, most aromatic and organic P forms disappear during higher pyrolysis temperature in favor of inorganic P forms [22]. Such structural difference in P form, presence of C-containing compound, and differences in degree of Ca-P crystallization could have affected P solubility from bone char. This result agrees with Tingting et al. [22] reporting that higher P solubilization by PSM augmentation from sludge biochar produced at 400°C than those produced at 700°C. They also reported that the P species in the char

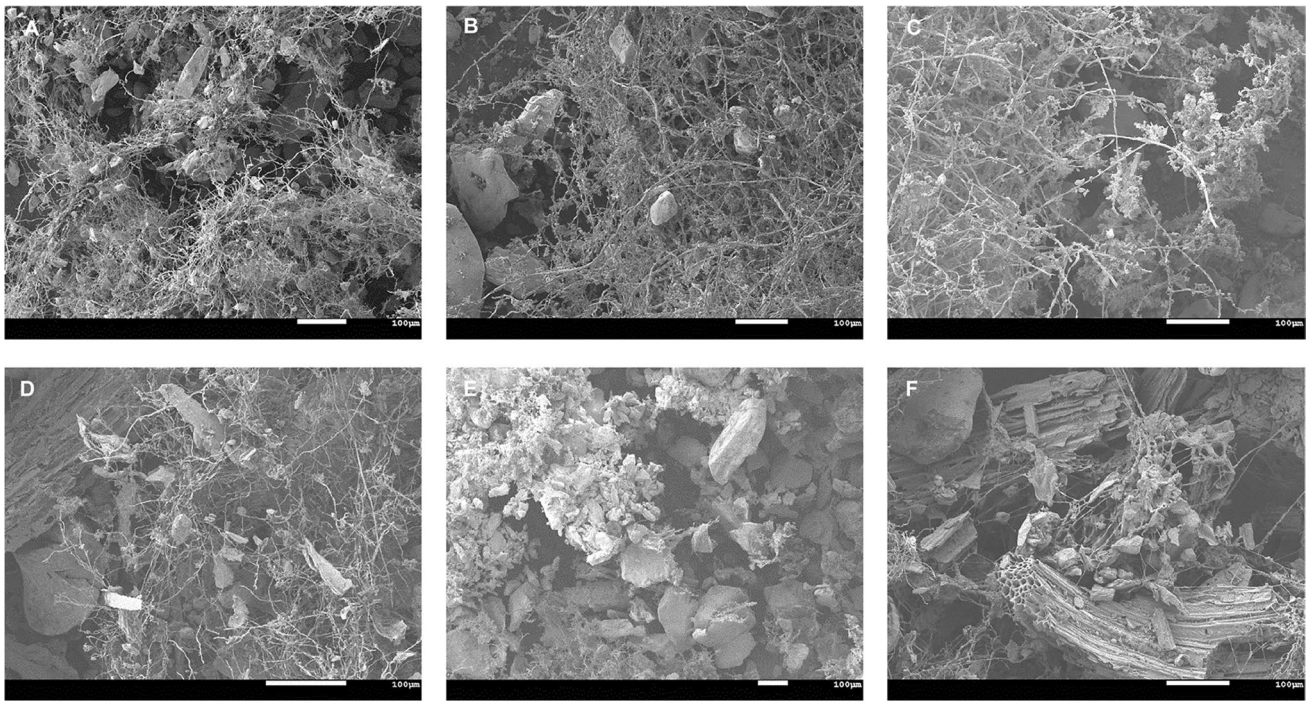

Fig. 4 Scanning electron microscopy (SEM) images of the surface morphology of different bone chars (**A**) *P. bilaiae* growing on BC450, (**B**) *P. aculeatum* growing on Co-BC450, (**C**) *P. expansum* growing on Co-BC450, (**D**) *P. bilaiae* growing on BC850, (**E**) *P. expansum* on BC850, and (**F**) *P. expansum* growing on Co-BC850

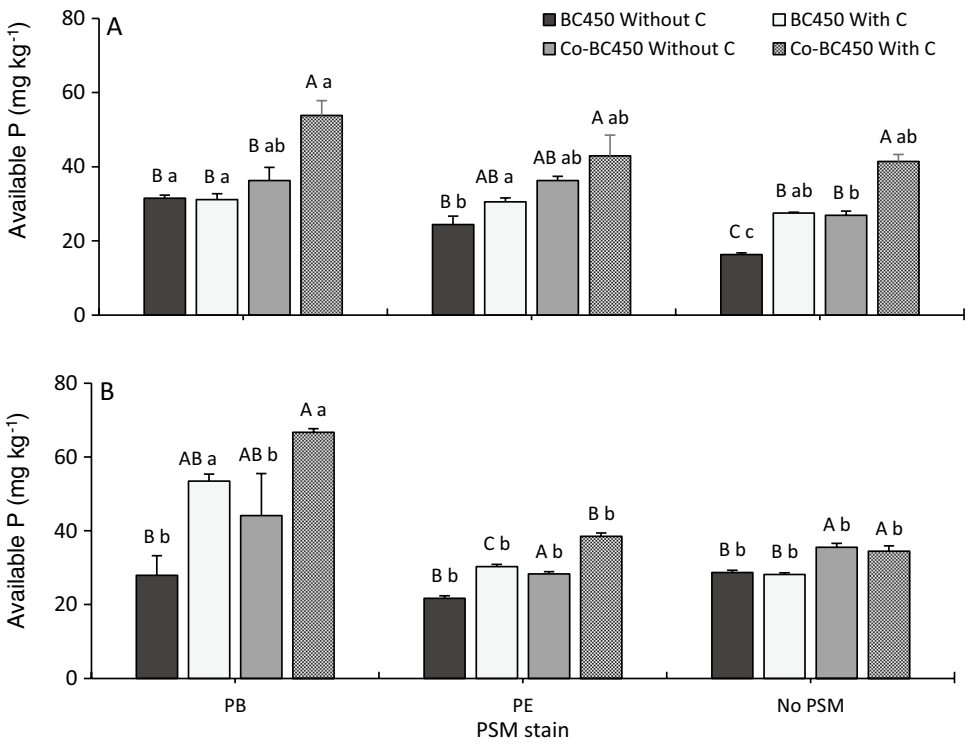

Fig. 5 Phosphorus release from different bone chars by PSM augmentation under soil condition as affected by organic carbon (OC) content of soil over time. Available P after (**A**) 7 and (**B**) 35 days of incubation. Bars represent the mean of four replicates ± standard error. Capital letters indicate differences across different bone chars (BC450 and Co-BC450) without or with OC addition within the same PSM inoculation levels, whereas lowercase letters indicate differences across different PSM inoculation levels within the same bone chars without and with OC addition

produced at 400°C had lower polymerization degree and poorer crystal structure than those produced at 700°C. Similarly, there have been earlier reports on low solubilization of several rock phosphates due to their complex and crystalline structures as compared to the amorphous nature and simple structure of Ca-P [23, 24]. As the phosphate compounds

Table 2. Survival rate of *Penicillium bilaiae* and *Penicillium expansum* added to different bone chars as affected by biomass co-pyrolysis (Co-BC450) after 35 days of soil incubation

PSM strain	Bone char	CFU g^{-1} dry soil	
		Soil with low OC	Soil with high OC
P. bilaiae	BC450	151×10^4 cd	571×10^4 b
	Co-BC450	199×10^4 c	820×10^4 a
P. expansum	BC450	46.6×10^4 e	59.7×10^4 de
	Co-BC450	88.6×10^4 de	213×10^4 c

Table 3. Correlation coefficients of linear regressions between the amount of solubilized P and pH and the total amounts of organic acids (OA) in the medium, respectively, by different bone chars

P source	BC450	Co-BC450	BC850	Co-BC850
Soluble P vs pH	-0.72^{***}	-0.63^{***}	-0.69^{***}	-0.79^{***}
Soluble P vs OA	0.77^{***}	0.56^{***}	0.82^{***}	0.74^{***}

$^{***}p < 0.001$

with poor crystal structure were more vulnerable for PSM, more P could be released from BC450 than BC850 in this study. However, we cannot exclude the possibility that the high initial pH of BC850 bone chars (9.3) produced at higher temperature could have led to increase pH and constrained performance of the PSMs.

4.2 Increased P solubilization by co-pyrolysis and bio-augmentation

The increased solubilized P from Co-BC450 than BC450 and Co-BC850 than BC850 can be explained by the higher TC, TN, and WEOC content in the biomass co-pyrolyzed bone chars (Table 1). High amount of TC and TN could provide the PSM with easily degradable C and N pools and may have contributed for the enhanced organic acid production and P solubilization in biomass co-pyrolyzed bone chars. Also, it is important to note that the liquid NBRIP culture medium contained only one OC source [25, 26]. In contrast, in biomass co-pyrolyzed bone chars, a variety of OC sources required by the strains may have existed and could have contributed to enhanced P solubilization. Enhanced P solubilization with increasing OC concentrations has also been demonstrated previously in other works [16, 27]. The observed increased P solubilization from co-pyrolyzed bone chars suggested that co-pyrolysis of biomass with animal bone could be applied as a low-cost strategy to supply easily metabolizable C and N and improve the performance of PSM to solubilize P.

4.3 Temperature and co-pyrolysis effect on organic acid production and acidification

One of the main mechanisms associated with microbial P solubility is production of organic acids and acidification of local environment. Organic acid production and acidification was also significantly affected by bone char production temperature and biomass co-pyrolysis. Higher amount of organic acid production and acidification were observed in PSM augmentation with bone chars produced at lower production temperature (450°C) (Fig. 3) and biomass co-pyrolyzed bone char. Availability of carbon and nitrogen is the main factor for successful production of organic acid by PSM. The total C and N concentration in bone chars produced at 450°C was 57% and 66% higher than bone char produced at 850°C, respectively. On the other hand, compared to the pure bone char produced at 850°C, PSM augmentation on the biomass co-pyrolyzed bone char resulted in an increased organic acid production and acidification. Biomass co-pyrolysis with bone at 850°C increased the C and N content by 77% and 38%, respectively. This may have provided the PSM with bio-available organic and inorganic carbon and nitrogen pool leading to increased organic acid production. Apart from this, pyrolysis temperature and pyrolysis condition (co-pyrolysis) could cause structural difference in P form [9], and it was well documented that such structural difference in P form affects the nature and amount of organic acids produced by PSM [24, 26, 27].

Correlation analyses revealed negative correlations between concentration of solubilized P and pH and positive correlations with the total amounts of low molecular organic acid concentration for all bone char types ($p < 0.001$; Table 3). The higher concentrations of organic acid (Fig. 3A and B) and solubilized P (Fig. 2A and B) in bone chars produced at lower than higher temperature and from biomass co-pyrolyzed bone char suggested that high organic acid production pattern and P solubilization can be achieved with low pyrolysis temperature and biomass co-pyrolysis.

4.4 Co-pyrolysis and OC addition increased PSM survival rate in soil system

Survival of the inoculum in a soil system is critical for successful microbial augmentation and P solubilization. Bone char often has low OC content; hence, the efficiency of PSM to solubilize bone char-P is expected to be limited, particularly in OC-poor soils. Co-pyrolysis of animal bone with biomass coupled with addition of OC to soil led to higher microbial survival rate (Table 2). The CFU count after 35 days of incubation revealed that the concentration of the spores in the soil was increased by co-pyrolysis and OC addition (Table 2), demonstrating that co-pyrolysis of biomass with bone coupled with OC addition could provide more suitable ecological environment and/or more nutrients

and mineralized organic matter for PSM. Enhanced microbial survival and activity was also reported with addition of biochar to soil [28–30].

The abundance of *P. bilaiae* was 4- to 9-fold higher compared to that of *P. expansum* indicating that the strains differed in their ability to utilize OC (Table 2). The low survival rate observed from *P. expansum* after 35 days of incubation may be the result of lack of OC source as all the added sucrose may have been consumed by the strain. On the other hand, different strains may have different preference for the type of OC source. Such preference was also reported in other studies [16], which investigated the P solubilization activity of different fungal strains inoculated on sewage sludge ashes and biochar in response to addition of different OC sources. The result from this study suggested that biomass co-pyrolysis with bone could enhance the survival and P solubilization potential of PSM.

5 Conclusions

This study provided insights on the effects of pyrolysis temperature, biomass co-pyrolysis, and PSM augmentation on P solubilization from animal bone waste. Co-pyrolysis of animal bone at a temperature of 450 °C coupled with PSM augmentation increased the P solubility of bone char up to 50% of the total P. P solubility by bio-augmentation of pure bone char produced at 850°C was significantly low. However, with co-pyrolysis of biomass, PSM augmentation significantly enhanced P solubility up to 45-fold. Co-pyrolysis of animal bone with biomass also improved PSM survival rate in soil system. The present study demonstrated co-pyrolysis and bio-augmentation as an efficient and low-cost strategy to maximize P fertilizer value of animal bone waste. This plays an important role in enabling farmers in developing countries to enhance crop productivity and reduce production cost through the ability to replace mineral P fertilizer; and it has environmental benefits in the forms of turning non-competitive agro-industrial wastes into valuable products.

Abbreviations CFU: colony forming unit; CYA: Czapek yeast extract agar; EC: electrical conductivity; LC-OCD: liquid chromatography organic carbon detection; NBRIP: National Botanical Research Institute's phosphate; OA: organic acid; PSM: phosphate solubilizing microorganisms; SEM: scanning electron microscopy; WEOC: water-extractable organic carbon; XRD: powder X-ray diffraction; YEA: yeast extract agar

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-021-02100-w.

Acknowledgements This work has been conducted as part of a PhD thesis project supported by Private University Research Branding Project (PLANE3T) funded by MEXT, Japan, and supported by Science and Technology Research Partnership for Sustainable Development (SATREPS; Grant Number JPMJSA2005) funded by Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA). Animal (sheep) bones were supplied from local restaurants in Tokyo, Japan.

Author contribution Milkiyas Ahmed, Shinjiro Sato, and Abebe Nigussie conceived the idea, designed the study, and conducted the statistical analyses. Shinjiro Sato, Solomon Addis, Berhanu Belay, and Johannes Lehmann supervised the development and progress of the work. Milkiyas Ahmed wrote the draft manuscript, and all the authors contributed equally to editing the manuscript. All the authors gave their final approval for publication and have no competing interests or conflict of interest.

References

1. Klinglmair M, Lemming C, Jensen LS, Rechberger H, Astrup TF, Scheutz C (2015) Phosphorus in Denmark: national and regional anthropogenic flows. Resour Conserv Recycl 105:311–324. https://doi.org/10.1016/j.resconrec.2015.09.019
2. Chowdhury RB, Moore GA, Weatherley AJ, Arora M (2017) A novel substance flow analysis model for analysing multi-year phosphorus flow at the regional scale. Sci Total Environ 572:1269–1280. https://doi.org/10.1016/j.scitotenv.2015.10.055
3. Cordell D, White S (2011) Peak phosphorus: clarifying the key issues of a vigorous debate about long-term phosphorus security. Sustainability 3:2027–2049. https://doi.org/10.3390/su3102027
4. Chowdhury RB, Moore GA, Weatherley AJ (2018) A multi-year phosphorus flow analysis of a key agricultural region in Australia to identify options for sustainable management. Agric Syst 161:42–60. https://doi.org/10.1016/j.agsy.2017.12.005
5. Simons A, Solomon D, Chibssa W (2014) Filling the phosphorus fertilizer gap in developing countries. Nat Geosci 7:3. https://doi.org/10.1038/ngeo2049
6. Ahmed, M., Nigussie, A., Addisu, S., Belay, B., Sato, S. (2021) Valorization of animal bone into phosphorus biofertilizer: effects of animal species, thermal processing method, and production temperature on phosphorus availability, Soil Science and Plant Nutrition, https://doi.org/10.1080/00380768.2021.1945403
7. Christel W, Bruun S, Magid J, Jensen LS (2014) Phosphorus availability from the solid fraction of pig slurry is altered by composting or thermal treatment. Bioresour Technol 169:543–551. ISSN 0960-8524. https://doi.org/10.1016/j.biortech.2014.07.030
8. Glæsner N, Hansen HCB, Hu Y, Bekiaris G, Bruun S (2019) Low crystalline apatite in bone char produced at low temperature ameliorates phosphorus-deficient soils. Chemosphere 223:723–730. https://doi.org/10.1016/j.chemosphere.2019.02.048
9. Zwetsloot MJ, Lehmann J, Solomon D (2014) Recycling slaughterhouse waste into fertilizer: how do pyrolysis temperature and biomass additions affect phosphorus availability and chemistry? J Sci Food Agric 95(281-288):390. https://doi.org/10.1002/jsfa.6716
10. Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Aust J Plant Physiol 28:897–906. https://doi.org/10.1071/PP01093
11. Tarafdar JC, Rao AV, Kumar P (1995) Role of phosphate producing fungi on the growth and nutrition of clusterbean (*Cyamopsis tetragonoloba* (L.) Taub). J Arid Environ 29(3):331–337. https://doi.org/10.1016/S0140-1963(05)80112-0

12. Yeomans JC, Bremner JM (1988) A rapid and precise method for routine determination of organic carbon in soil. Commun Soil Sci Plant Anal 19(13):1467–1476. https://doi.org/10.1080/00103628809368027
13. Enders, A., Sori, S., Lehmann, J., Singh, B. (2017) Total elemental analysis of metal and nutrient in biochar. In Biochar: a guide to analytical methods (ed. Singh, B., Camps-Arbestein, M. & Lehmann, J.) 95 – 108 (CRS Press, 2017)
14. Rajan SSS, Brown MW, Boyes MK et al (1992) Extractable phosphorus to predict agronomic effectiveness of ground and unground phosphate rocks. Fertilizer Research 32:291–302. https://doi.org/10.1007/BF01050366
15. Chinu, K., Marjo, Ch. E., Joseph, S. D. Singh, B (2017) Dissolved organic carbon and LC-OCD of biochar. In Biochar: a guide to analytical methods (ed. Singh, B., Camps-Arbestein, M. & Lehmann, J.) 64 – 73 (CRS Press, 2017)
16. Raymond NS, Stöver DM, Peltre C, Nielsen HH, Jensen LS (2018) Use of Penicillium bilaiae to improve phosphorus bioavailability of thermally treated sewage sludge – a potential novel biofertilizer. Process Biochem 69:169–177. https://doi.org/10.1016/j.procbio.2018.03.021
17. Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. https://doi.org/10.1111/j.1574-6968.1999.tb13383.x
18. Baskin T, Orr T, Jercinovic M, Yoshida M (2014) Sample preparation for scanning electron microscopy: the surprising case of freeze drying from tertiary butanol. Microscopy Today 22(3):36–39. https://doi.org/10.1017/S1551929514000522
19. Tigist M., Gebermedihin A., Nigussie A., Amsalu N., Milkiyas A. (2020) Short-term application of biochar increases the amount of fertilizer required to obtain potential yield and reduces marginal agronomic efficiency in high phosphorus-fixing soils. Biochar; https://doi.org/10.1007/s42773-020-00059-x
20. IUSS Working Group WRS. (2014) World Reference Base for Soil Resource 2014. International soil classification system for naming soils and creating legends for soil map. World Soil Resource Report No. 106. FAO, Rome.
21. Trevors JT, Cook S (1992) A comparison of plating media and diluents for enumeration of anaerobic bacteria in a loam soil. J Microbiol Methods 14:271–275. ISSN 0167-7012. https://doi.org/10.1016/0167-7012(92)90060-H
22. Tingting Q., Yang Q., Jun D.C. F, Dong F., Zhou Y. (2019) Transformation of phosphorus in sewage sludge biochar mediated by a phosphate-solubilizing microorganism, Chemical Engineering Journal, Volume 359, 1573-1580, ISSN 1385-8947, https://doi.org/10.1016/j.cej.2018.11.015.
23. Bashan Y, Kamnev AA, de-Bashan, L.E. (2013) Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. Biol Fertil Soils 49:465–479. https://doi.org/10.1007/s00374-012-0737-7
24. Mendes GO, Moreira De Freitas AL, Liparini Pereira O, Ribeiro Da Silva I, Vassilev NB, Dutra Costa M (2014) Mechanisms of phosphate solubilization by fungal isolates when exposed to different P sources. Ann Microbiol 64:239–249. https://doi.org/10.1007/s13213-013-0656-3
25. Jacoby R, Peukert M, Succurro A, Koprivova A, Kopriva S (2017) The role of soil microorganisms in plant mineral nutrition current knowledge and future directions. Front Plant Sci 8:1–19. https://doi.org/10.3389/fpls.2017.01617
26. Scervino J, Papinutti V, Godoy M, Rodriguez M, Della Monica I, Recchi M, Pettinari M, Godeas A (2011) Medium pH, carbon and nitrogen concentrations modulate the phosphate solubilization efficiency of P. purpurogenum through organic acid production. J Appl Microbiol 110(5):1215–1223. https://doi.org/10.1111/j.1365-2672.2011.04972.x
27. Stefanoni Rubio PJ, Godoy MS, Della Mónica IF, Pettinari MJ, Godeas AM, Scervino JM (2016) Carbon and nitrogen sources influence tricalcium phosphate solubilization and extracellular phosphatase activity by Talaromyces flavus. Curr Microbiol 72:41–47. https://doi.org/10.1007/s00284-015-0914-7
28. Lehmann, J., Matthias C. Rillig, Janice Thies, Caroline A. Masiello, William C. Hockaday, David Crowley (2011) Biochar effects on soil biota – a review, Soil Biology and Biochemistry, Volume 43, Issue 9, 1812-1836, ISSN 0038-0717, https://doi.org/10.1016/j.soilbio.2011.04.022.
29. Noyce GL, Basiliko N, Fulthorpe R, Sackett TE, Thomas SC (2015) Soil microbial response over 2 years following biochar addition to a north temperate forest. Biol Fertil Soils 51:649–659. https://doi.org/10.1007/s00374-015-1010-7
30. Zhang L, Jing Y, Xiang Y, Zhang R, Lu H (2018) Responses of soil microbial community structure changes and activities to biochar addition: a meta-analysis. Sci Total Environ 643:926–935. https://doi.org/10.1016/j.scitotenv.2018.06.231