ENHANCING PHOSPHORUS SOLUBILITY AND BIOAVAILABILITY FROM ANIMAL BONES: EFFECTS OF THERMOCHEMICAL AND BIOLOGICAL TREATMENTS

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CONTENTS

ABSTRACT

Modern agriculture relies on mineral phosphorus (P) fertilizer produced from rock phosphate. Large amount of P fertilizers application is required to ensure productivity of agricultural systems. Rock phosphate is a non-renewable and rapidly depleting resource and hence P scarcity is expected to be a bottleneck for the sustainability of crop production in the future. In addition, high production cost of P fertilizers coupled with their heavy metal contamination (e.g., cadmium and uranium) is a challenge to boost agricultural production. Therefore, attention has recently been focused on phosphorus recovery from organic sources such as animal manure, sewage and sludge, crop residues, and animal bone to meet the high demand for P and reduce contaminations associated with mineral P fertiliser production. Recovery and re-use of P from slaughterhouse waste is one of the proposed solutions to secure future P demand. Accordingly, various researches have shown bone char (pyrolyzed animal bone under no or low oxygen condition) as potential P fertilizer with comparatively high P content. However, P contained in bone char is often poorly available to plant. Up to date, there have been considerable variations among the reported studies in the P fertilizing effect of bone char, ranging from positive effect to non-significant effect. The low P solubility from bone char and inconsistency in the past studies calls for more studies to understand factors controlling P solubility and bioavailability in thermally treated bone which may include processing method (pyrolysis vs. combustion) and processing temperature, chemical and structural difference in different animal bone, and soil environments to be applied. In addition to this, different strategies could be used to increase the solubility of P from bone char, such as phosphorous solubilizing microorganisms (PSMs). However, studies on P release pattern from bone char with the presence of PSM are limited and it is largely

unknown whether bone-char production temperature and pyrolysis technique affect the efficiency of PSM to solubilize bone-char P. Therefore, two independent experiments were conducted (i) to determine effects of bone from different animals, thermal processing method, production temperature, and soil pH on P solubility and bioavailability from thermally processed bone and understand the underlying mechanism; and (ii) Investigate how pyrolysis temperature and co-pyrolysis of animal-bone with biomass influence the potential of different PSM strains to solubilize P in bone char and elucidate the survival rate of PSM and their P solubilization potential under different soil organic carbon levels. In the first experiment, P solubility was investigated from thermally treated animal bones produced from: (i) 3 different animals (chicken, sheep, and pig); (ii) 2 thermal processing methods (pyrolysis and combustion); and (iii) 4 production temperatures (300°C, 500°C, 700°C, and 900°C). Chemical extraction, incubation, and pot experiments were performed to evaluate P availability. Infrared splitting factor (IFRS) and width of 604 cm−1 peak (FW85%) calculated from FTIR spectra, coupled with X-ray powder diffractometer (XRD) were used to determine bioapatite crystallinity of thermally treated animal bones. In the second experiment, an in-vitro assay was conducted to investigate the potential of four *Penicillium* strains to solubilize P from bone char produced by different pyrolysis temperatures (i.e. 450^{o}C and 850^{o}C) and pyrolysis techniques (i.e. conventional (BC450, BC850) and co-pyrolysis (Co-BC450 and Co-BC-850)). Incubation and pot experiment were also carried to elucidate if organic carbon (OC) input increases the survival rate of PSM and their efficacy. The result of the first experiment, clearly demonstrated that, P solubility of thermally treated bones largely depended on bone type, thermal processing method, and production temperature as well as soil pH. Chicken bone showed the highest available P content ($p < 0.001$), irrespective

of processing methods and temperatures. In contrast, pig bone exhibited the lowest available P despite its high total P content. Pyrolysis resulted in higher available P content than combustion, particularly at temperatures >500^{0}C. The XRD and FTIR studies confirmed higher degree of crystallization for those produced from pig bone, combustion, and higher temperatures (>700^{o}C). Infrared splitting factor and width at 85% of the height of the 604 cm^{-1} peak were used to assess bioapatite crystallinity, and confirmed a negative correlation between bioapatite crystallization and P availability ($p < 0.001$), indicating the attribution of bioapatite crystallization for low soluble P during combustion and higher temperatures. At low soil pH (pH~4), addition of thermally treated bones increased Olsen-P and plant P uptake by two- to five-folds compared with unamended soils; however, no significant variation was observed at higher soil pH (pH~7). According to the results from the second experiment, solubilized P was significantly influenced by PSM type, processing temperature and biomass addition. All the *Penicillium* strains solubilized 11% - 39% of bone char-P from BC450 within three weeks. In contrast, only *P. bilaiae* was able to solubilize 19% of bone char-P from BC850, while the others solubilized only 2% of bone char-P which was comparable with the control (i.e. non-inoculated). Compared to BC450, addition of *P. expansum* and *P. glabrum* to Co-BC450 resulted in 167% and 133% more solubilized P respectively. Similarly, addition of *P. expansum*, *P. glabrum*, and *P. aculeatum* to Co-BC850 resulted in 10- to 45-fold more solubilized P compared to BC850. Soil C input increased solubility of bone char-P by 34% - 48%; PSM survival rate by 22% - 76%.; and plant P uptake by 29% - 55%. These findings suggested optimum thermal valorization conditions to develop alternative P biofertilizer from slaughterhouse waste; and indicated new approach to increase P fertilizer value of animal-bone using PSM; co-pyrolysis and soil C inputs.

CHAPTER ONE

1. GENERAL INTRODUCTION

1.1. Phosphorus scarcity and the need for an alternative source

Phosphorous (P) is one of the most important plant nutrients, and mainly derived from rock phosphate (RP), a non-renewable and fatally depleting resource (Klinglmair et al., 2015). It is estimated that the world reserves will only last for about 50 to 125 years. High production cost coupled with meager P quality (e.g., heavy metal contamination), are an environmental concern and among the challenges to meet global food demand. Global P demand is projected to double by 2050, (Chowdhury et al., 2018), without an alternative source to complement the demand, it is expected to result in food insecurity around the world, particularly in developing countries (Cordell, 2011).

An alternative and renewable solution to reduce the dependency on RP includes recovery and reuse of P from organic waste disposals (Chowdhury et al., 2018). Recycling of P from organic waste is therefore being considered as a strategy to complement future P demand. Animal bone is one of such renewable P sources with huge potential to secure the demand. It has been reported that developing countries such as Ethiopia for example could secure 28-56% of their annual P demand by recycling animal bone waste (Simons et al. 2014). Recycling P from animal bone waste, that would otherwise generate environmental and health concerns, can therefore can significantly contribute in closing the P loop. Even though high concentration of P is found in animal bone, it is mainly present as apatite, insoluble crystalline calcium phosphate.

1.2. Factors affecting P fertilizing value of animal bone

Recycling P from animal bone by means of pyrolysis, the heating of materials under low oxygen conditions, has been suggested recently as potential P fertilizer with comparatively high P content (Simons et al., 2013; Zwetsloot et al., 2016; Glæsner et al., 2019). However, plant available P in bone char is extremely low. Even though pyrolysis has been forwarded as a

strategy to improve P solubilization from animal bone (Warren et al., 2009; Zwetsloot et al., 2015; Glæsner et al., 2019), there are considerable variations among the reported studies in the P fertilizing effect of bone char, ranging from non-significant effect (Siebers et al. 2013; Zimmer et al. 2019) to positive effect (Warren et al. 2009; Zwetsloot et al. 2015). The low P solubility from bone char and inconsistency in the past studies calls for more studies to understand factors controlling P solubility and bioavailability in thermally treated bone, which may include processing method (pyrolysis vs. combustion) and processing temperatures, chemical and structural difference in different animal bone, and soil environments to be applied. It is, however, remained unanswered how these variations influence P availability from thermally processed bones. Thus, this knowledge gap should be addressed for the potential use of animal bone char as alternative P biofertilizer (Chapter 2).

1.3. Enhancing bone char-P solubility by P solubilizing microorganisms

The use of phosphorous solubilizing microorganisms (PSMs) is promising, sustainable and low cost biotechnological strategy to enhance P solubility from bone char. Previous studies have confirmed the enhancement behavior of PSM on P solubilizing from different P-rich biochars and ash. However, studies on P release pattern from bone char with the presence of PSM and the underline mechanism is limited. It is largely unknown whether bone char production temperature and pyrolysis technique affect the efficiency of PSM to solubilize bone char P. Production temperature and condition can influence the chemical forms in which P exists (Bruun et al. 2017; Glæsner et al., 2019). Lower pyrolysis temperatures, result in calcium (Ca)-P with poorer crystal structure, whereas high pyrolysis temperature can result in structurally persistent and insoluble P forms with higher Ca-P crystallinity. There is a dearth of information on how such differences influence the potential of PSM in solubilizing P from bone chars.

The potential of PSM to increase P solubility and plant P uptake in soil-plant system is found to be limited due to poor survival of the inoculated strain in the soil. A suitable carrier that can provide suitable environment and OC for the inoculant is therefore important to ensure its efficiency. Due to its porous structure, bone char has been suggested to be an ideal carrier for microorganisms (Postma et al., 2010). However, since bone char generally contains less than 10% of OC (IBI, 2015), it can provide no or small amounts of available OC for PSM when it is applied to soil. Nevertheless, co-pyrolysis of biomass with bone and amending OC to soil could be used as a strategy to improve the survival rate of PSM and P solubilization after being introduced to the soil. However, no studies have been conducted so far to elucidate how different OC status in bone char and soil can affect PSM survival rate, P solubilization potential, and plant P uptake and growth (Chapter 3).

1.4. Objectives

Based on the above-mentioned research needs, the objectives of this dissertation were:

(i) To determine effects of bone type, thermal processing method, production temperature, and soil pH on P solubility and bioavailability from thermally processed bone and to understand the underlying mechanism (Chapter 2).

(ii) Investigate how pyrolysis temperature and co-pyrolysis of animal-bone with biomass influence the potential of different PSM strains to solubilize P in bone char and elucidate the survival rate of PSM and their P solubilization potential under different soil OC levels. (Chapter 3).

CHAPTER TWO

2. Valorization of animal bone into phosphorus biofertilizer: Effects of animal species, thermal processing method and production temperature on phosphorus availability

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2.1. INTRODUCTION

Recycling phosphorous (P) from animal bone waste, that would otherwise generate environmental and health concerns, can secure significant fraction of P fertilizer demand. Recovering P from animal bone by means of pyrolysis, the heating of materials under low oxygen conditions, has been suggested recently as one of economically viable and sustainable solution (Simons et al. 2013; Zwetsloot et al. 2016; Glæsner et al. 2019). Even though high concentration of P is found in animal bone, it is mainly present as apatite, insoluble crystalline calcium phosphate. Pyrolysis has been forwarded as a strategy to improve P solubilization from animal bone (Warren et al. 2009; Zwetsloot et al. 2015; Glæsner et al. 2019). However, there are inconsistencies on the findings. For example, Warren et al. (2009) found that pyrolysis of animal bone at 400^{o}C showed more effective P fertilizing effect than rock phosphate. Similarly, Zwetsloot et al. (2016) reported comparable plant P uptake between triple super phosphate (TSP) and bone char pyrolyzed at 750^{o}C. In contrast, no significant differences have been reported between soils with or without bone char amendment (Siebers et al. 2013; Zimmer et al. 2019). This inconsistence in the past studies calls for more studies to understand factors controlling P solubility in thermally treated bone, which may include bone derived from different animals and thermal treatment method, and soil environments to be applied.

The chemical composition and structure of both bone mineral and organic phase vary among animal species (Toppe et al. 2007). Age, genetic, nutrition, and disease also influence bone chemical composition and structure. It is, however, remained unanswered how these variations in structure and chemical composition of different animal species influence P availability from thermally processed bones. In addition, whether thermal processing method (pyrolysis or combustion) and production temperature affect P chemistry of different animal bone and P solubility has so far not been fully investigated. This knowledge gap is important to address for the potential use of animal bone waste as P source.

Previously, the degree of crystallization which explains P solubility during pyrolysis of animal bone has been studied using X-ray powder diffraction (XRD; Glæsner et al. 2019) and X-ray absorption near edge structure spectroscopy (XANES; Glæsner et al. 2019; Zwetsloot et al. 2015). However, XRD and XANES analyses are expensive and time-consuming. On the other hand, Fourier transform infrared spectroscopy (FTIR) has been extensively used to assess crystallinity in archeological bone samples. It requires a low amount of sample (~1mg) and fast and relatively inexpensive analytical procedure. Moreover, the infrared splitting factor (IRSF) and the width at 85% of the height (FW85%) of the 604 cm^{-1} peak, calculated from FTIR spectra have been recently suggested for measurement of apatite crystallinity (Dal Sasso et al. 2018). To the authors knowledge, no efforts have been made to use FTIR spectra for studying apatite crystallinity during thermal processing of animal bone, and correlate P solubility with IRSF and FW85%.

P solubility and availability also depends on soil environments (e.g., soil pH) when P-containing materials are applied to soil. Dissociation of synthetic apatite was observed at low pH in clinical studies (Sader et al. 2013). Previous studies (Siebers et al. 2013; Zwetsloot et al. 2016; Glæsner et al. 2019) demonstrated P availability from bone char on highly weathered acidic soil where high dissociation of apatite was expected. However, there is dearth of information on whether P availability from thermally processed bone varies across different pH soils. Such studies are crucial to identify soil types where thermally processed bone could be used as a potential P biofertilizer.

Therefore, the objectives of this study were (i) to determine the effect of different animal bone, thermal processing method, production temperature on P solubility and crystallinity from thermally processed bone and to elucidate the underlying mechanisms and (ii) to examine the effect of soil pH on P availability over time and plant P uptake when thermally processed bone is incorporated in different pH soils. The hypotheses were (i) P solubility from thermally

processed bone varies among different animal bone due to variation in degree of crystallinity, (ii) thermal processing method and temperature affect apatite crystallization, thereby alter P solubility, and (iii) P fertilization effect of thermally processed bone is more pronounced in low pH soil.

2.2. MATERIALS AND METHODS

2.2.1. Bone char and bone ash production and characterization

Three different animal bones; sheep, chicken, and pig were collected from restaurants in Tokyo, Japan. Sheep and chicken bone had been cooked at restaurants and boiled in water for 1 hr to remove remaining meet residue before experiments. While, pig bone was boiled for over 7 hr at restaurant before collection. The bones were then oven-dried at 70ºC for 72 h, grounded, and passed through a 2 mm sieve. For bone char production, the bones were pyrolyzed in a steel container placed in an electric oven with a heating rate of 5°C per minute under limited oxygen supply, while allowing gases produced during pyrolysis to escape. For bone ash production, the bones were placed in open crucibles to ensure the supply of oxygen during combustion. Bone char and bone ash were produced at temperatures of 300ºC, 500ºC, 700ºC, and 900ºC with the retention time of one hour for bone char production and three hours for bone ash production. The products were then stored at the room temperature until experiments.

Total carbon (TC) and nitrogen (TN) were measured using a CHN analyzer (Perkin Elmer, 2400 series II). Briefly, two milligrams of oven-dried bone char and bone ash were sieved through 150 µm size and placed into tin capsule and analyzed as described by Yeomans and Bremner (2008). The pH and electric conductivity (EC) were measured in a solid-to-solution ratio of 1:10 (w/v) using auto-electrical pH and EC meters (Mettler Toledo, Seven Easy S20) after agitating the mixture in a horizontal shaker for one hour at the speed of 160 strokes min^{-1}. Total P (Wang et al., 2012), 2% formic acid extractable P (Rajan et al. 1992), and water soluble

P (AOAC, 2005; Wang et al., 2012) were measured to characterize P contents of bone char and bone ash. Briefly, 0.2 g of bone char was combusted at 500°C for 8 hr, then digested with 9 mL of HNO_3 and 1 mL H_2O_2 mixture for total P determination. The digestate was filtered through 0.45 μm filter membrane. Formic-P was measured by mixing 0.25 g of bone char and bone ash with 25 mL of 2% formic acid using a horizontal shaker at 160 rpm for 30 min. The mixture was dispersed with ultra-sonication for 10 min. About 0.2 g of bone char and bone ash was mixed with 30 mL deionized water and shaken at 160 rpm for 16 hr for determination of water soluble P. The suspensions were then centrifuged at 5,000 rpm for 10 min and the supernatants were filtered through 0.45 μm filter membrane. The P concentration in the filtrates was then determined using an auto analyzer (FIAlab 2500) to determine total P, formic P and water soluble P.

2.2.2. X-ray Powder Diffraction

Powder X-ray diffraction (XRD) profiles were obtained for all bone char and bone ash samples by using a Bruker D5000, iron-filtered cobalt radiation (40 kV, 40 mA) and a scanning rate of 2^o 2θ per min in the range of 10^o to 90^o 2θ using a fixed divergence slit of 1^o. The width at half height of selected diffraction peaks was calculated using Siemens EVA software.

2.2.3. FTIR Analysis

Based on P characterization of different animal bones (similarity in P solubility between sheep and pig bones), only chicken and pig bones were selected for further spectral analyses. All chicken and pig bone char and bone ash samples were analyzed by FTIR spectroscopy after mixing 1 mg of sample with spectral-grade 100 mg KBr and ground in an agate mortar. The mixture was then compressed at about 7845 kPa for 2 min into a 13-mm sample pellet. A background spectrum was obtained each time before the samples were processed. All spectra

between 4000 and 400 cm^{-1} were collected at a spectral resolution of 2 cm^{-1} and 64 scans. The fingerprint region between 2000 and 400 cm^{-1} was, however, used to assess bioapatite crystallinity. Spectral analysis was performed using Omnic 9 software (Thermo Scientific).

Infrared splitting factor (IRSF) and width at 85% of the height (FW85%) of the 604 cm^{-1} peak were calculated indicate the degree of crystallinity in bone char and bone ash (Dal Sasso et al. 2018). IRSF was calculated as the sum of peak intensities at 604 and 565 cm^{-1} divided by the intensity of the valley between the two peaks (Fig. 2-1a). The width at 85% of the height FW85% of the 604 cm^{-1} peak was also calculated to indicate the degree of crystallinity (Fig. 2-1b). The peak at 1090 cm^{-1} was used to confirm the degree of bioapatite order. All peaks and widths were calculated after baseline correction. The baseline used for calculating IRSF and FW85% were 850-620 and 510-470 cm^{-1}, respectively.

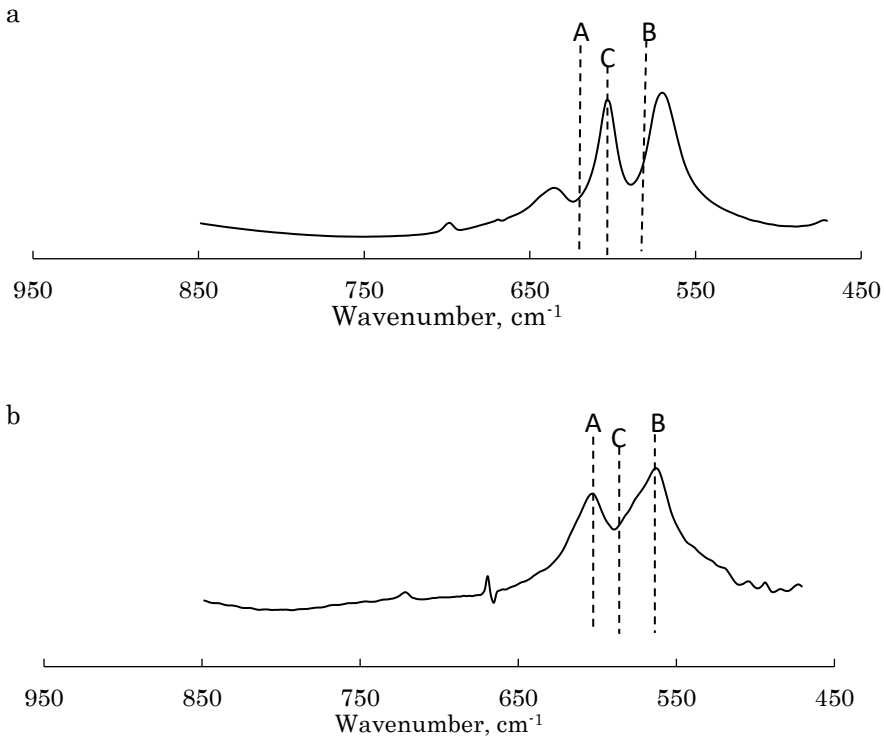

Figure 2-1. FTIR spectra of (a) pig-bone char produced at 900ºC and (b) chicken-bone char produced at 300^0C.

IRSF was calculated using an equation …… $IRSF = \frac{(A+B)}{C}$

2.2.4. Incubation experiment

An incubation study was carried out using two soils with different pH collected from Jimma, Ethiopia (low pH = 4.65) and Tokyo, Japan (high pH = 7.26) (supplementary Table S1). Soil samples were air-dried and passed through a 2 mm sieve. Thirty grams of dried soil was added in 100 mL glass container, moistened with deionized water to 60% of the maximum water holding capacity (WHC), and pre-incubated for 10 d at 20±2°C prior to the commencement of the incubation experiment. Only four different sheep bone char and ash materials were selected as amendment; (i) bone char at 300°C, (ii) bone char at 700°C, (iii) bone ash at 300°C, (iv) bone ash at 700°C. In addition to thermal treated bones, the original feedstock bone meal as well as a soil without amendments (control) was also included as treatment. Sheep bone is one of the most predominant animal wastes in Ethiopia, and two processing temperatures used represent low and high temperatures, respectively. Respective amendment was added to meet 200 mg P kg^{-1} soil based on total P of each amendment and replicated four times. The moisture content was kept at 60% WHC and incubated in the dark for eight weeks at temperatures of 20±2°C. The moisture loss was determined gravimetrically once a week, and deionized water was added to compensate the loss. The container was covered partly throughout the experimental period to avoid the development of anaerobic environment. Destructive soil samples were collected on 1, 7, 14, 28, 42, and 56 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.

2.2.5. Pot experiment

A pot experiment was performed in a greenhouse to elucidate exclusively the soil pH effect by using two soil with different pH of an originally acidic soil (low pH) and limed soil (high

pH). The soil sample was collected from the plowing depth of 20 cm from Jimma, Ethiopia, air-dried, and sieved to 2 mm. Lime was added at a soil to lime ratio of 70:1 (w/w) and incubated for three weeks at the moisture content of 60% WHC. Soil without lime was also incubated for three weeks to avoid the artifacts associated with the incubation. Both lime-treated and untreated soils were then air-dried and mixed with acid-washed sand at a ratio of 2:1 (soil to sand) to ease the root growth (Gómez-Muñoz et al. 2017). Three kilograms of the soil-sand mixture was then filled into plastic pot (0.18 m diameter, 0.2 m height). The same amendments used in the incubation study were used for the pot experiment. The original feedstock bone meal, inorganic P fertilizer (TSP) as well as a soil without amendments (control) were also included as treatment. Respective amendment was added to meet 200 mg P kg^{-1} soil based on total P of each amendment and replicated five times. The moisture content was maintained to 60% WHC throughout the experimental period. Three lettuce (*Lactuca sativa*) seedlings were planted in each pot and thinned to two individuals, then grown for eight weeks. At physiological maturity, the whole plant was harvested, washed, oven-dried at 70°C and weighed to determine dry matter production. The oven-dried plant samples were then ground and passed through a 1 mm sieve for determination of total P concentration.

2.2.6. Statistical analyses

Analysis of variance was used to determine the effect of different animal bone, thermal treatment method, production temperature, and soil pH on P forms, P availability, plant yield, and plant P uptake. If significant differences were found at $p < 0.05$, mean separation was performed using Tukey's HSD tests. All statistical analyses were performed using STATISTICA 6.1.

2.3. RESULTS

2.3.1. Basic properties of bone char and bone ash

The selected properties of bone char and bone ash produced from different animal bone and production temperatures are presented in Table 1. Total C (TC) and total N (TN) tended to decrease with production temperatures. Among different animal bones, pig-bone generally exhibited the lowest values of TC and TN. At the same production temperatures, yield (dry matter), TC, and TN losses were many folds higher during combustion than pyrolysis. For instance, 95% of TC was lost from pig bone at higher pyrolysis temperatures, but it reached to 80% from chicken bone. At higher combustion temperatures, however, TC loss reached to 97%, and was comparable between different animal bones. The C:N ratio of bone-char increased with pyrolysis temperature but decreased slightly with combustion temperature.

The pH increased with production temperature, irrespective of animal species and thermal treatment. The pH value was higher during combustion than pyrolysis, particularly for pig bone. The EC value was the lowest in pig bone, irrespective of thermal treatment and production temperature. It increased with increasing processing temperatures except for pig bone ash. At higher production temperatures (>500^{o}C), sheep bone exhibited the highest EC value, irrespective of thermal treatment.

2.3.2. Phosphorus forms in bone char and bone ash

Total P content varied among different animal bones, thermal processing methods, and production temperatures (Fig. 1). The initial total P content of pig bone meal was 117 g P kg^{-1}, but was about 80 g P kg^{-1} for chicken bone meal and sheep bone meal (supplementary Table S1). This variation among animal species, however tended to decrease with production temperature. Total P increased with production temperature, irrespective of thermal processing

method and animal species. At the same production temperature, combustion process resulted in higher total P content than pyrolysis process ($p < 0.05$).

Large proportion of total P in the thermally-treated bones was extractable with 2% formic acid, except at a combustion temperature of 900°C where only < 1% of P was extractable with formic acid (Fig. 1). Formic-P contributed over 70% of the total P for chicken bone char and sheep bone char, but only 3% of the total P was extractable with formic acid when pig bone char was produced at pyrolysis temperature of 900°C. At production temperature above 700°C, pyrolysis process exhibited higher formic-P than combustion process, irrespective of animal species ($p < 0.05$).

Water soluble P (WSP) was varied among different animal bones ($p < 0.05$), thermal processing methods ($p < 0.01$), and production temperatures ($p < 0.01$; Fig. 2). Chicken bone had the highest WSP, irrespective of thermal processing method and production temperature. At the higher combustion temperatures (~ 900°C), however, very small fraction of P was water soluble. WSP content decreased with production temperature, irrespective of animal species and thermal treatment. Bone char had higher WSP content than bone ash, irrespective of the animal species and production temperature.

Table 2-1 Yield and selected chemical properties of bone char and ash produced from different animal bone and production temperatures

Production temperature (°C)	Yield (%)		pH		Electrical conductivity (μs cm^{-1})		Total carbon (g kg^{-1})		Total nitrogen (g kg^{-1})		Calcium Phosphorus ratio	
	Char	Ash	Char	Ash	Char	Ash	Char	Ash	Char	Ash	Char	Ash
Sheep bone												
300°C	71.3	70.0	6.05	6.32	1026	722	272	271	39.2	43.2	1.03	1.06
500°C	58.7	49.2	7.73	8.75	1357	2345	110	38.5	15.1	6.50	0.94	1.40
700°C	54.0	46.0	9.94	11.6	1575	2705	100	8.3	8.73	1.60	0.93	1.08
900°C	52.0	42.0	10.1	11.1	1654	2750	51.3	7.1	4.30	1.30	1.56	1.40
Chicken bone												
300°C	84.8	70.0	5.96	6.02	521	1104	316	65.7	44.6	14.4	1.10	1.08
500°C	55.0	52.3	7.68	8.02	956	1592	99.7	41.4	14.8	9.63	0.74	1.03
700°C	55.0	44.0	9.22	9.18	1020	1358	78.1	10.7	8.46	1.83	0.97	1.07
900°C	48.0	34.0	9.26	9.09	1118	1020	64.4	9.57	5.06	1.27	1.59	1.34
Pig bone												
300°C	78.6	80.0	6.01	6.05	417	1026	180	94.2	24.7	18.0	0.87	1.11
500°C	75.0	70.0	6.75	7.97	773	914	62.6	17.5	8.16	2.73	1.09	1.23
700°C	72.0	68.0	7.51	10.2	631	889	43.8	8.60	4.56	0.87	1.04	0.89
900°C	58.0	58.0	8.50	10.4	610	708	10.73	4.27	4.03	0.63	1.31	1.33

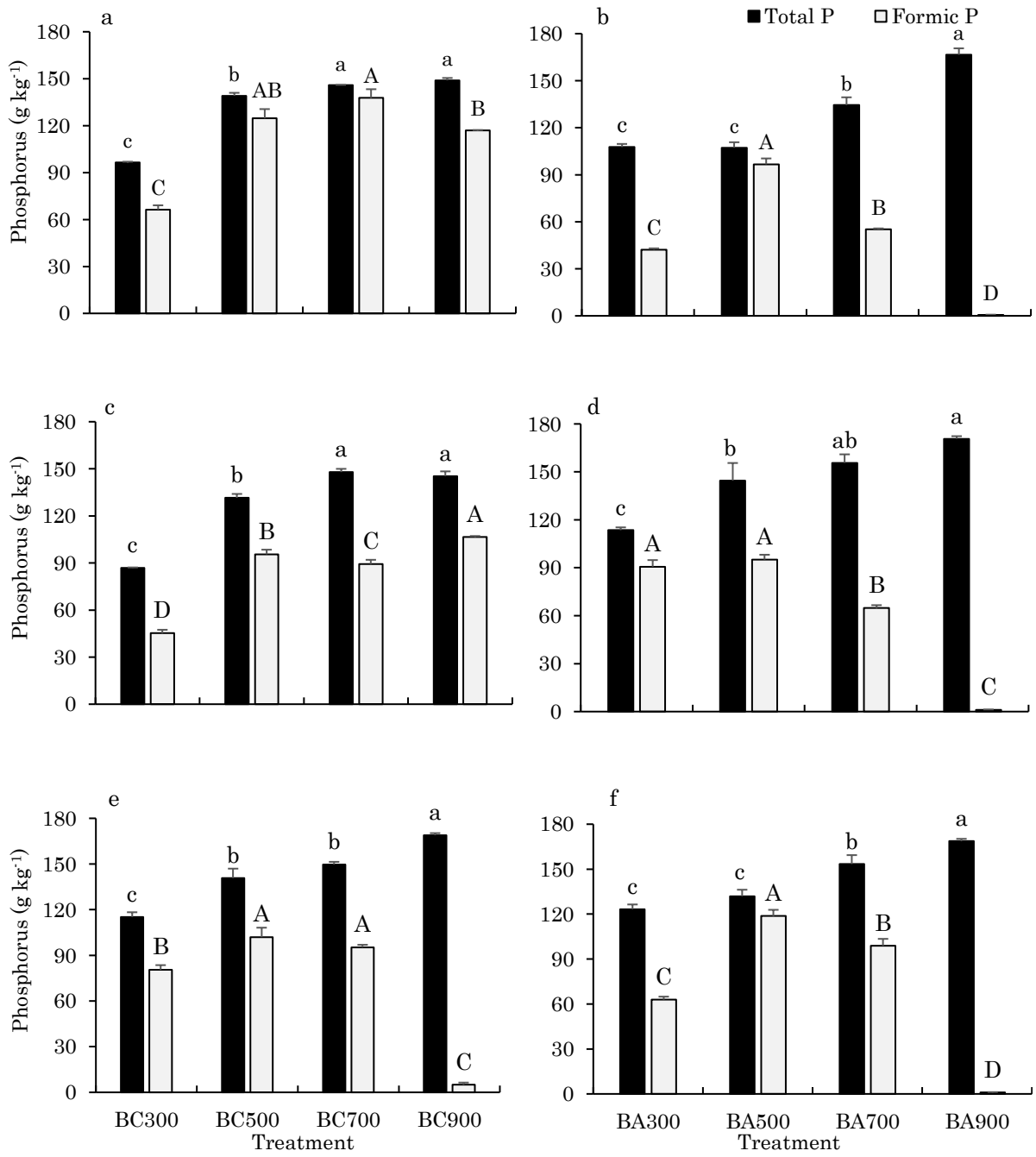

Figure 2-2. Total phosphorus (P) and formic acid-extractable P from (a) sheep bone char, (b) sheep bone ash, (c) chicken bone char, (d) chicken bone ash, (e) pig bone char, and (f) pig bone ash treated at 300°C, 500°C, 700°C, and 900°C. BC and BA represents bone char and bone ash respectively. Vertical bars represent standard errors (n = 3). Same letters indicate no significant differences ($p < 0.05$) in total P (small letter) and formic-P (capital letter) among different temperatures for different animal bones, respectively.

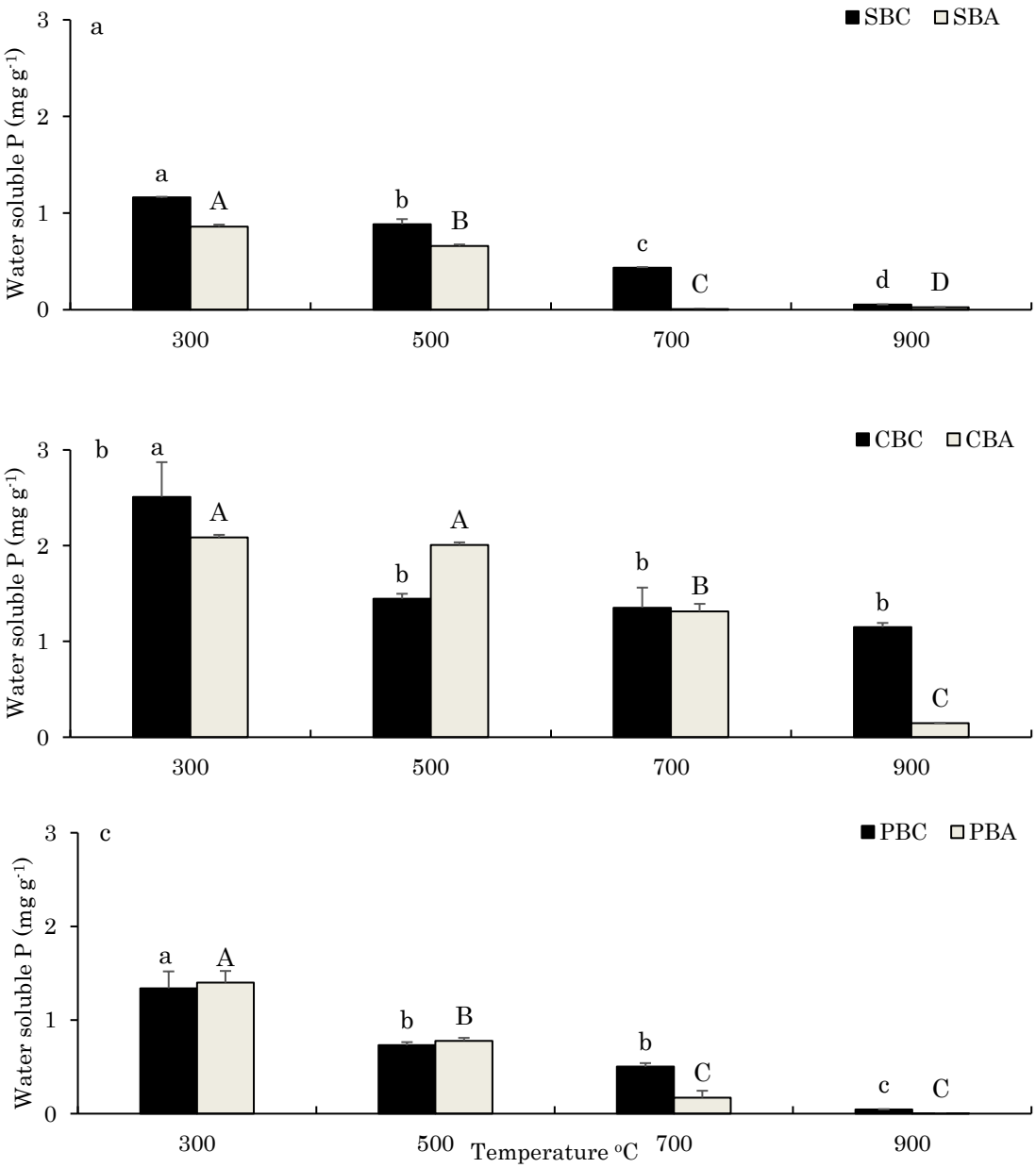

Figure 2-3. Water-soluble phosphorus (P) from (a) sheep bone char (SBC) and ash (SBA), (b) chicken bone char (CBC) and ash (CBA), and (c) pig bone char (PBC) and ash (PBA) treated at 300°C, 500°C, 700°C, and 900°C. Vertical bars represent standard errors (n = 3). Same letters indicate no significant differences ($p < 0.05$) in water P among different temperatures for different animal bone char (small letter) and bone ash (capital letter), respectively.

2.3.3. Degree of apatite crystallization of bone char and bone ash

The XRD pattern clearly showed that processing method and temperature influenced bioapatite crystallinity (Fig. 2-4). The peak intensity around 31° became narrower with increasing processing temperature for all bones. Contrarily, lower peak intensity and broader reflections were detected at lower processing temperatures for all bones. On the other hand, sharper diffraction peaks and higher peak intensity were observed in bone ash than bone char samples (Fig. 2-4). The peak at around 31° was clearly separated into three different peaks particularly for bone char at 900°C and bone ash at 700°C and 900°C. The peak intensity was also differed between animal species. For chicken bone char, relatively little effect was observed on the crystal growth, however pig and sheep bone produced well crystalized diffraction patterns as the peak separation was detected at low temperatures and the peak became increasingly more pronounced at higher temperatures (Fig. 2-4).

FTIR peak at 604 cm^{-1} became sharper and subsequently the band width decreased with increasing processing temperature irrespective of animal species (Fig. 2-5). IRSF and FW85% values calculated from FTIR spectra were used to assess degree of bioapatite crystallization during thermal processing of animal bones. IRSF value increased in all the samples as the temperature increased from 300°C to 900°C (Table 2-2). Similarly, compared to pyrolysis, combustion resulted in higher IRSF value. For example, IRSF value was increased from 4.47 to 6.33 for pig bone char and from 4.09 to 8.41 for pig bone ash. The increase in IRSF is an indication that the hydroxyapatite peaks became narrower and better resolved with increasing production temperature and during combustion. FW85% values, which decreased with increasing temperature irrespective of animal species and processing method (Table 2-2) is also an indication that the hydroxyapatite peaks became narrower and better resolved with increasing production temperature. The value was decreased from 11.2 to 5.61 for chicken bone char and from 9.09 to 5.54 for chicken bone ash. Another indicator for apatite crystallization is

the peak at 1090 cm^{-1} (Fig. 2-6). It appeared as a shoulder at low production temperatures. However, at higher production temperature, the 1035 cm^{-1} and 1090 cm^{-1} bands were clearly separated into two peaks.

Table 2-2 Infrared splitting factor (IRSF) and the full width at 85% of the height (FW85%) of the 604 cm^{-1} peak of chicken and pig bone char and ash

Bone type	Production temperature (°C)	IRSF		FW85%	
		Char	Ash	Char	Ash
Chicken bone	300	3.65	3.85	11.2	9.09
	500	3.50	3.95	10.1	8.42
	700	4.12	5.00	9.77	6.81
	900	5.57	5.27	5.61	5.54
Pig bone	300	4.47	4.09	8.82	8.82
	500	4.58	4.53	9.19	7.89
	700	4.58	6.44	8.00	6.05
	900	6.33	8.41	6.19	4.41

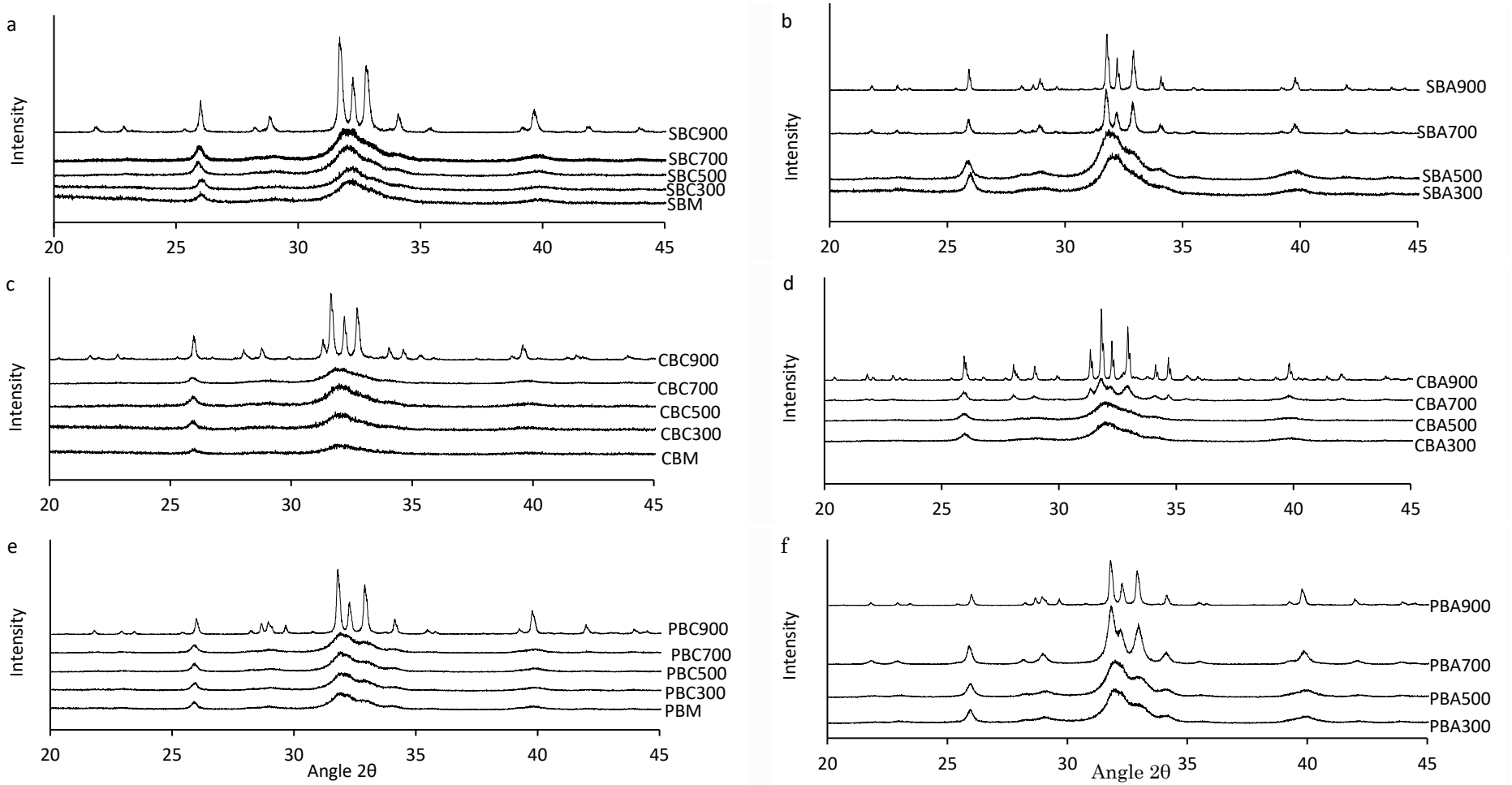

Figure 2-4. X-ray powder diffraction spectra from (a) sheep bone char (SBC) (b) sheep bone ash (SBA) (c) chicken bone char (CBC), (d) chicken bone ash (CBA), (e) pig bone char (PBC), and (f) pig bone ash (PBA) produced at 300°C (300), 500°C (500), 700°C (700), and 900°C (900). BM represents bone meal

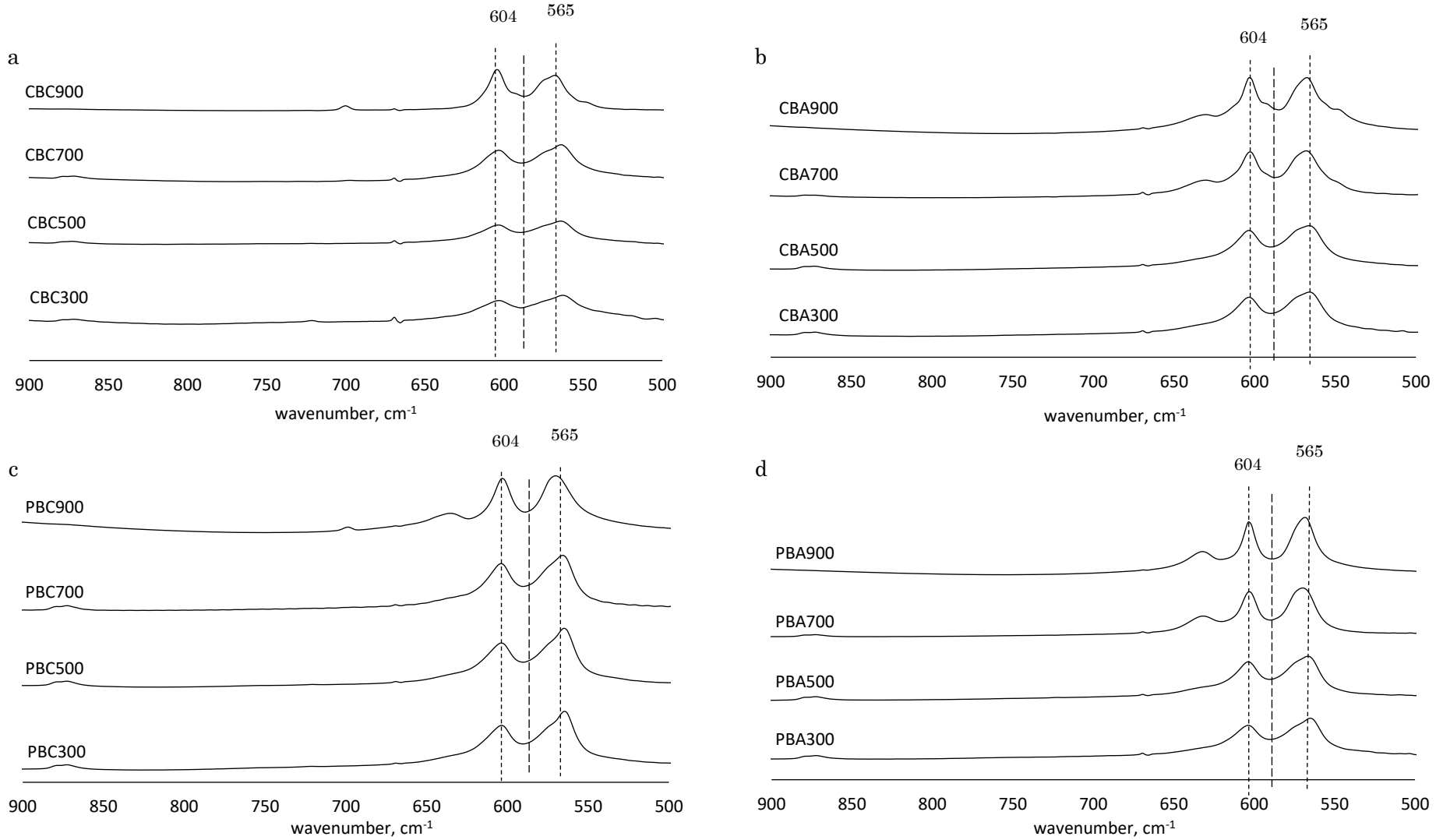

Figure 2-5. FTIR spectra (500 – 900 cm^{-1}) from (a) chicken bone char (CBC), (b) chicken bone ash (CBA), (c) pig bone char (PBC), and (d) pig bone ash (PBA) treated at 300°C (300), 500°C (500), 700°C (700), and 900°C (900). BM represents bone meal

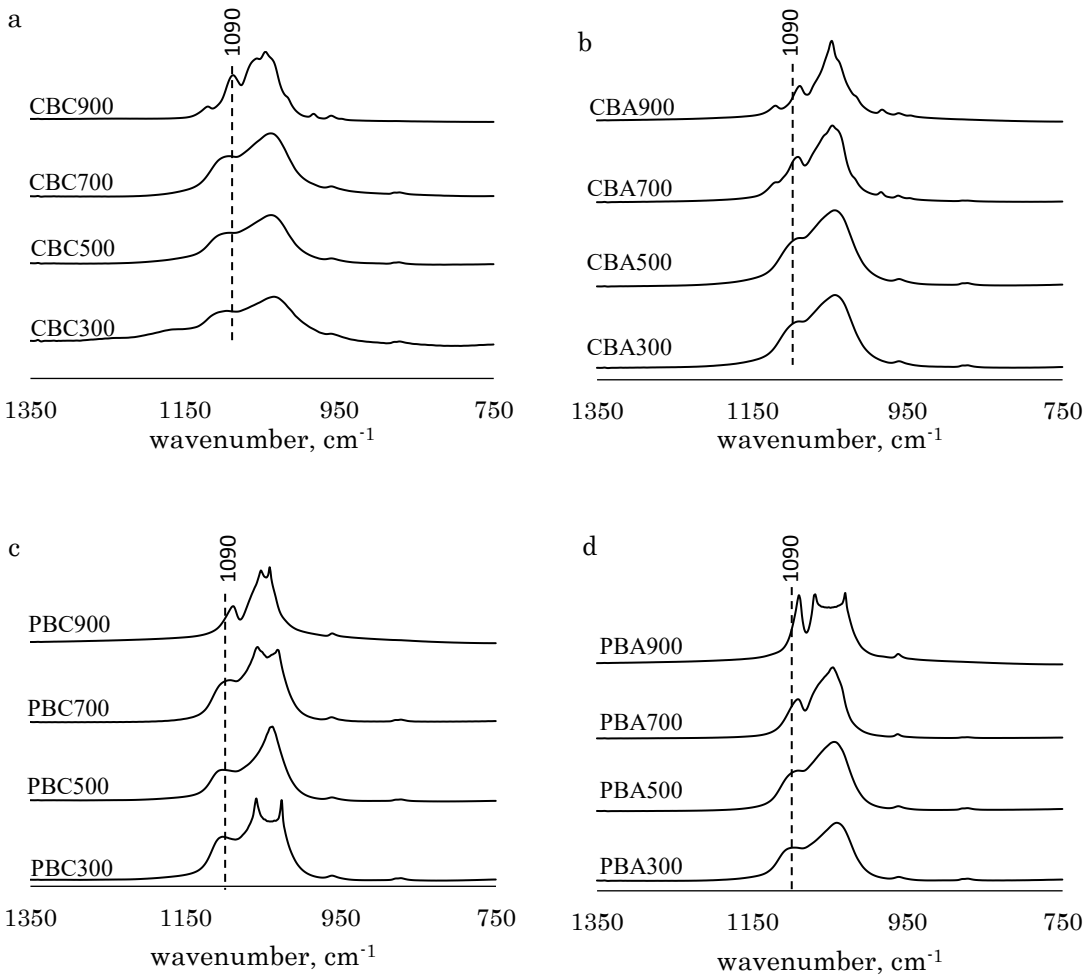

Figure 2-6. FTIR spectra (750 – 1350 cm^{-1}) of (a) chicken-bone char (CBC), (b) chicken-bone ash (CBA), (c) pig-bone char (PBC), and (d) pig-bone ash (PBA) produced at 300°C (300), 500°C (500), 700°C (700), and 900°C (900), respectively.

2.3.4. Effect of soil pH on P availability from bone char and bone ash

Figure 2-7 illustrates the amount of plant available P released from thermally treated bones across different soil pH. The findings unambiguously demonstrated that the P-fertilizing value of thermally treated bones was more pronounced in low pH soil. In low pH soil, thermally treated bones increased Olsen-P content by two- to five-folds compared with the control (i.e. without amendment). After 56 days of incubation, the highest Olsen-P content was observed at pyrolysis temperature of 700ºC (BC700) and combustion

temperature of 300ºC (BA300). No significant variation was observed among the bone meal (BM), bone ash produced at 700ºC (BA700), and bone char produced at 300ºC (BC300). At higher pH soil, in contrast, addition of thermally treated bones slightly increased Olsen-P content (~ 10%) compared with the control. At high pH soil, Olsen-P was not significantly differed among thermally treated bones.

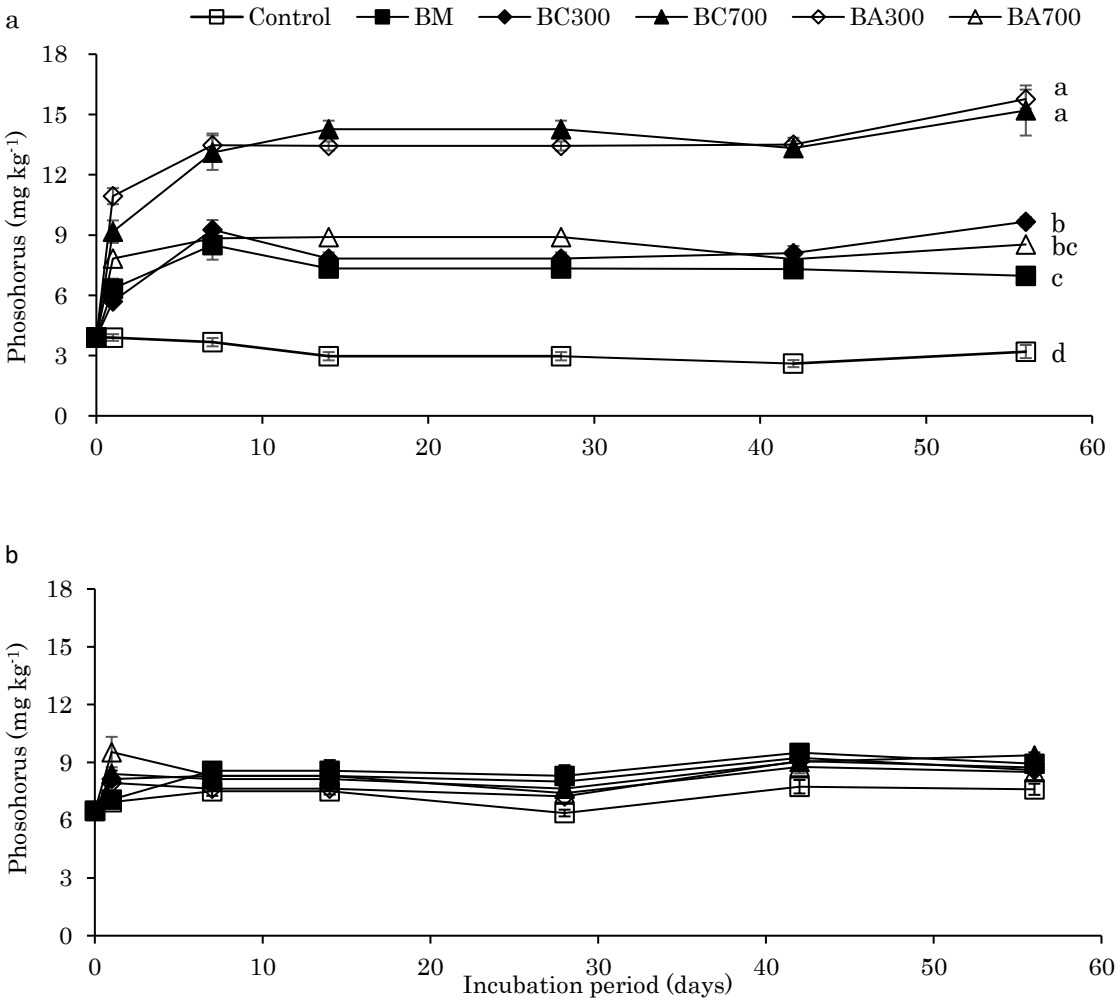

Figure 2-7. Olsen extractable phosphorus (P) of sheep bone char and bone ash amended to (a) low pH and (b) high pH soils during 56 d of incubation period. Control represents soil only without amendments. BM, BC, BA represent bone meal, bone char, and bone ash amendments, respectively. 300 and 700 represent production temperatures. Vertical bars represent standard errors (n = 4). Same letters indicate no significant differences ($p < 0.05$) in Olsen-P among treatments on Day 56 for each soil.

In accordance with the incubation experiment, the pot experiment also showed the significant effect of soil pH on the P-fertilizing value of thermally treated bones (Fig. 2-8). At low pH soil, addition of thermally treated bones increased P uptake by ten-folds compared with the control. Interestingly, thermally treated bones resulted significantly in higher P uptake than TSP ($p < 0.05$) in low pH soil. The highest P uptake was observed with BC700 and BA300 amendments, which agreed with the incubation experiment results. In high pH soil, TSP resulted in the highest P uptake, and no-significant difference was observed among thermally treated bones and the control.

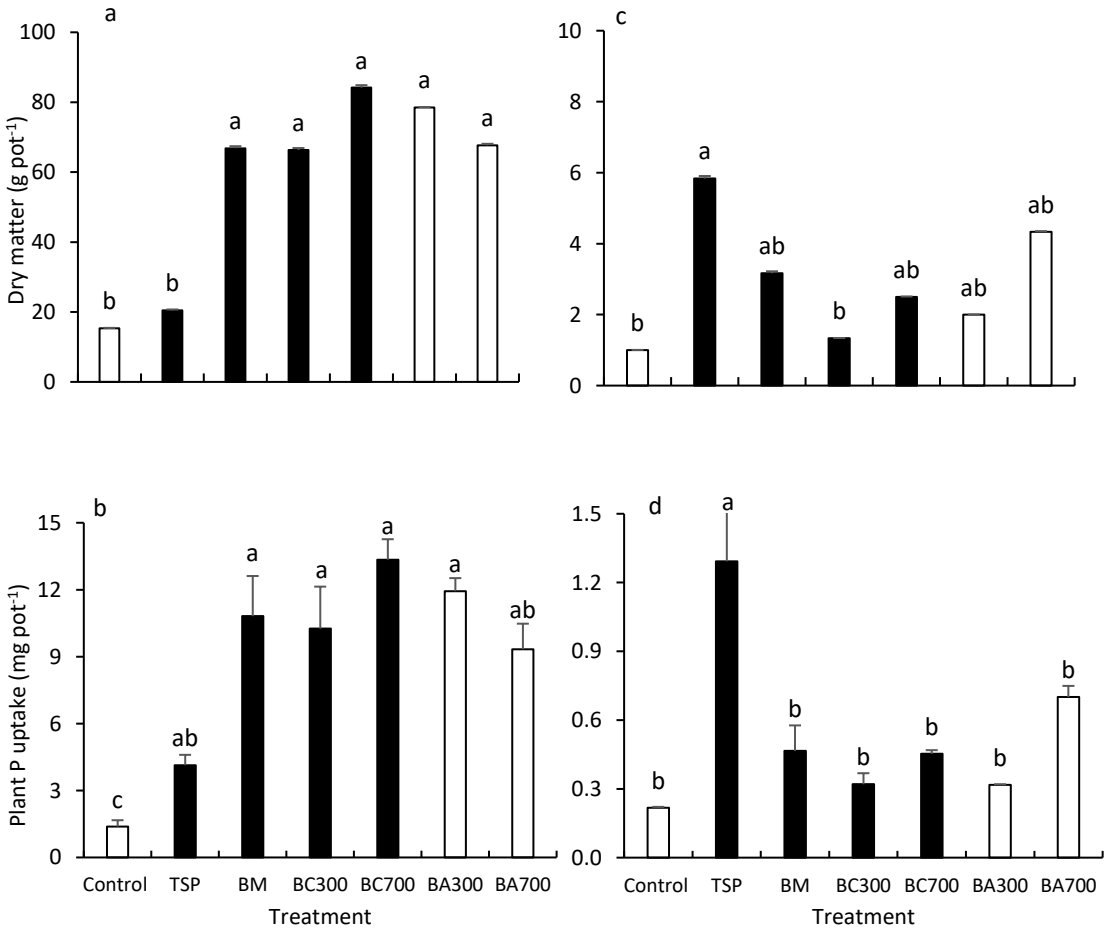

Figure 2-8. Plant yield and plant phosphorus (P) uptake of lettuce grown in low pH soil pH (a and b, respectively) and high pH soil (c and d, respectively). Control represents soil without amendments. TSP = triple superphosphate. Vertical bars represent standard errors (n = 3). Same letters indicate no significant differences ($p < 0.05$) in yield and P uptake among treatment.

2.4. DISCUSSION

2.4.1. Effects of different animal bone, thermal processing method, and production temperature in bioapatite crystallinity and phosphorus availability of thermally processed bone

The findings confirmed our hypothesis that bioapatite crystallinity and P availability from thermally processed bones depended on animal species, thermal processing method, and production temperature. Water soluble P (Fig. 2-3) was overall higher from chicken bone than sheep and pig bone irrespective of processing method and temperatures. Recently, bioapatite crystallinity has been suggested as a mechanism to explain variation in P availability from bone char (Zwetsloot et al., 2015; Glæsner et al., 2019). These previous studies, however, used qualitative methods to assess the degree of apatite crystallization. Our study could quantitatively confirm the degree of apatite crystallinity using IRSF and FW85% calculated from FTIR spectra relative to P availability. Significant negative correlations were observed between IRSF and both formic P ($p < 0.05$) and water soluble P ($p < 0.001$), and significant positive correlation between FW85% and water soluble P ($p < 0.001$; Table 2-3). Furthermore, IRSF values of chicken bone were overall lower by 25% across all processing methods and processing temperatures than those of pig bone, and FW85% values of chicken bone were overall higher by 12% than those of pig bone (Table 2-2), indicating lower degree of bioapatite crystallinity or higher P availability in chicken bone than pig bone. On the other hand, the XRD traces also showed one broad peak in chicken bone whereas sheep and pig bone exhibited more than one and somewhat sharp peaks at lower temperatures ($\leq$ 500°C; Fig. 2-6), indicating low crystalline bioapatite formation in chicken bone than sheep and pig bone. Therefore, the observed higher values of formic P and water soluble P in chicken

bone than pig bone could be partly explained by the distinctly lower degree of bioapatite order. The pig bone used in this study had been boiled for over seven hours during cooking before being used for the experiments, which may have removed fatty meat residue, leaving a purer source of Ca-P mineral. However, the difference observed between chicken and sheep bone could be ascribed to their inherent characteristics. Therefore, pig bone may have resulted in high bioapatite crystals, thus lower P solubility at the highest processing temperature. Further studies are, therefore, suggested to elucidate the observed variation among different bone in terms of degree of bioapatite order and available P, and effects of inherent characteristics and pre-treatment of bones (i.e. boiling, extraction of fat) on crystal formation and P availability.

Whether thermal processing method influences apatite crystal formation has not been fully understood. Distinct diffractogram was observed between bone char and bone ash, irrespective of animal species (Fig. 2-6). Three distinct peaks around 31 degrees in XRD patterns shows crystalline hydroxyapatite formation (Glæsner et al., 2019). For bone char, the three distinct peaks were observed only with temperatures of 900^{o}C, however this pattern was observed for bone ash with temperatures $\geq$ 700^{o}C, indicating that crystallization of bioapatite occurred from lower temperature in bone ash compared to bone char. Higher crystallization of bioapatite with combustion than pyrolysis and higher temperature may be because of higher breakdown of organic P compound transforming to inorganic P with combustion and high temperature (Zwetsloot et al., 2015). Furthermore, previous archeological studies confirmed that the FTIR peak at 604 cm^{-1} became sharp and subsequently the band width decreased during the formation of bioapatite crystals (Dal Sasso et al., 2018). We found similar patterns for bone ash compared to bone char (Fig. 2-4). Subsequently, IRSF values of bone char were overall

lower by 12% across all processing temperatures than those of bone ash, and FW85% values of bone char were overall higher by 21% than those of bone ash (Table 2-2), confirming lower degree of crystallization or higher P availability during pyrolysis than combustion. The higher contents of formic P and water soluble P in bone char than bone ash could, therefore, be ascribed to low bioapatite crystallization during pyrolysis. In agreement with our finding, Christel et al. (2014) also showed higher water soluble P in pig-manure biochar than pig-manure ash. However, further manipulative studies are required to explain how thermal processing methods influence apatite crystal formation and thereby P availability.

Irrespective of thermal processing method, bioapatite peaks by XRD spectra became narrower and better resolved with increasing production temperature (Fig. 2-5), indicating higher degree of bioapatite crystallization. Similarly, at lower processing temperatures ($\leq$ 500ºC), IRSF values were overall lower by 40% across all the different animal none and processing methods than those of higher processing temperatures ($\geq$ 700ºC). On the other hand, FW85% values were overall higher by 40% for lower temperatures compared to higher temperatures. These IRSF and FW85% values unambiguously demonstrated lower bioapatite order or higher P availability at lower production temperatures. In archeological studies, an FTIR peak at 1090 cm^{-1} has been used extensively as an indicator for the degree of apatite order (Saso et al., 2018). At lower production temperatures, the peak at 1090 cm^{-1} appeared as a shoulder (Fig. 2-6) indicating lower bioapatite order. At higher production temperature, in contrast, 1035 and 1090 cm^{-1} bands were clearly separated into two distinct peaks, confirming higher ordered bioapatite. Lower bioapatite crystallinity can, therefore, explain the higher amounts of formic P and water soluble P from thermal processed bones with lower production temperatures. In

agreement with our findings, Zwetsloot et al. (2015) and Glæsner et al. (2019) reported higher crystalline bioapatite with higher pyrolysis temperatures. Similarly, Deydier et al. (2005) showed higher bioapatite crystallinity of bone ash at higher combustion temperatures. As discussed earlier, our study confirmed that FTIR spectra and subsequent IRSF and FW85% could be used for the assessment of bioapatite crystallinity and P availability in thermally treated bones, which is relatively fast and inexpensive analytical procedure.

Previously, Ca:P molar ratio has been used as an indicator for Ca-P crystallinity and P availability from bone char (Zwetsloot et al., 2015). Ca:P molar ratio was varied among thermal processing methods and production temperatures in this study showing that Ca:P molar ratios of bone char and ash at 900°C were the highest (Table 2-1), which corresponded with the lowest water soluble P for the same bone char and ash (Fig. 2-3). Generally, Ca:P molar ratio was higher with higher temperatures and with combustion than pyrolysis process. Similarly, we found a negative correlation ($r = –0.49$; $p < 0.01$) between Ca:P ratio and water soluble P (Table 2-3). In fact, water soluble P increased with decreasing processing temperature, irrespective of animal species and processing methods in this study (Fig. 2-3). The highest formic P was found at around 500°C, irrespective of the thermal processing method (Fig. 2-2). This finding corresponds with Wang et al. (2012) who found the maximum formic P at 450°C during pyrolysis of manure. The lowest formic P contents was, however, observed for bone ash produced at 900°C for all animal species (Fig. 2-2), which could be attributed to high degree of bioapatite order.

Table 2-3. Pearson's correlation coefficients between bioapatite crystallization index and different phosphorus forms

	Water soluble P	Formic extractable P	Total P
IRSF†	–0.79***‡	–0.52*	0.59*
WF85%†	0.71***	0.43ns	–0.74***
Ca:P ratio†	–0.49**	–0.27ns	0.38ns

† IRSF, WF85%, and Ca:P ratio represent infrared splitting factor, the width at 85% of the height of the 604 cm^{-1} peak, and molar ratio of calcium and phosphorous, respectively.
‡ ns, *, **, and *** represent non-significant, significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

2.4.2. Effects of soil pH on phosphorus bioavailability of thermally processed bone

Both incubation and pot trials showed that P fertilization effects of thermally processed bone was more pronounced in lower pH soil (Figs. 2-7 and 2-8), as hypothesized. As compared with the control, the addition of thermally processed bone resulted in three- to five-fold more Olsen-P (Fig. 2-7a) and nine- to thirteen-fold more P uptake (Fig. 2-8b) in low pH soil, irrespective of thermal processing method and temperature. The high P fertilization effect of thermally processed bone in acidic soil can be explained by the dissolution of bioapatite minerals at low pH (Warren et al., 2009). In accordance with our findings, Glæsner et al. (2019) assessed P solubility from bone char across different soil pH and found more P dissociation in low pH soil (4 to 5) compared to high soil pH (7.2). Warren et al. (2009) also tested P availability from bone char in 12 different soil types and reported pH~6.1 as the cut-off point for dissociation of P from bone char. Nevertheless, these previous studies were solely based on incubation

experiments using soils with different mineralogy. However, the effects of soil pH on P bioavailability from thermally treated bone were clearly demonstrated in this study using soils having similar properties except for soil pH through both incubation and bioassay experiments.

Thermally processed bones resulted in higher P uptake even compared to the mineral fertilizer (TSP) in low pH soil (Fig. 2-8b). In acidic soils, P can be quickly adsorbed and immobilized as particularly iron (Fe)- and aluminum (Al)-P minerals, which is typical characteristics of many tropical soils (Glæsner et al., 2019). Those Fe-P and Al-P compounds can release P when soil pH is increased up to ~6.5 through for example liming. Thermally processed bone could also act as a liming material (Vamvuka et al., 2018) and subsequently increase P availability and uptake. Therefore, one of the contributing factors for higher P uptake from thermally treated bones than TSP could be the liming effect by the amendments, which TSP did not show. Rapid adsorption of mineral P was highly probable in our study since the same soil as used in this study had the maximum P sorption capacity of 456 mg P kg^{-1} soil (Zwetsloot et al., 2016).

The plant biomass in the limed soil was many folds lower than those in the un-limed soil (Figs. 2-8a and 2-8c). Similarly, Brod et al. (2015) compared the P fertilizing value of bone meal at different soil pH and found two-fold lower plant biomass in the lime-treated soils. The dissolved free phosphate species might react with calcium, and subsequently forms insoluble calcium phosphate compounds particularly when soil pH becomes more than approximately 7 (Siebielec et al., 2014). In fact, pH in the limed soil was increased by liming to 7.3 in this study. Moreover, application of lime increases microbial activities in acidic soils (Xue et al., 2010) and thereby enhances net P immobilization.

From both incubation and pot experiment in low pH soil the highest plant available P and P fertilization effects of thermally processed bone was observed at pyrolysis temperature of 700ºC and combustion temperature of 300ºC (Figs. 2-8a and 2-8b). At high production temperature, pyrolysis resulted in lower crystalline bioapatite compared with combustion process (Figs. 2-4 and 2-6), and the formic P from BC700 was 150% higher than BA700 (Fig. 2-2), hence higher P availability in bone char than bone ash is expected. On the other hand, at low production temperature, bone ash (BA300) showed higher Olsen extractable P (Fig. 2-7a) and P uptake (Fig. 2-8b) than bone char (BC300) despite no large differences in terms of Ca-P crystallization (Figs. 2-4 and 2-6) and formic P (Fig. 2-2). The highest fertilizer effect from BA300 than BC300 could be ascribed to a considerable breakdown of organic P compounds by combustion compared to pyrolysis at lower temperatures. Similarly, substantial breakdown could also occur with pyrolysis at 700ºC compared to 300ºC transforming organic to inorganic P, thus more available from BC700 than BC300. In agreement with our findings, Zwetsloot et al. (2016) and Glæsner et al. (2019) found low available P from bone char produced at a lower pyrolysis temperature (< 400 ºC).

2.5. CONCLUSION

P solubility of thermally treated bones largely depended on animal species, thermal processing method (combustion vs. pyrolysis), and production temperature. In this study, FTIR spectra, coupled with XRD diffractogram, were used to assess crystallinity of bioapatite crystals during thermal processing of animal bone. Accordingly, XRD traces, IRSF, and FW85% confirmed higher crystalline bioapatite formation with higher production temperatures and during combustion than pyrolysis process. Higher degree of crystallization, therefore, explained lower P availability (formic P and water soluble P) from those with higher production temperatures and combustion process. Both incubation and pot experiments clearly demonstrated high P availability in soils amended with thermally treated bones. The P fertilization effect of thermally treated bone was, however, more pronounced in lower pH soil. Furthermore, our findings revealed that pyrolysis resulted in better P fertilizing effect compared with combustion, particularly at higher production temperatures. The finding suggested processing bone below temperatures of 700^{o}C resulted in less crystallinity, thus higher P solubility. The P-fertilizing value of thermally treated bones was more pronounced in lower pH soil.

CHAPTER THREE

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

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3.1. INTRODUCTION

Modern agriculture relies on mineral phosphorus (P) fertilizer produced from rock phosphate (RP), a non-renewable and quickly depleting resource (Klinglmair et al., 2015). As demand for P is expected to double by 2050, there is mounting evidence that the world's supply of RP is under threat (Chowdhury et al., 2017). In recent years, P scarcity has been identified as a bottleneck in the sustainability of agricultural systems (Cordell 2011, Chowdhury et al., 2018). 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 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 (Simons et al., 2014).

Various researches have shown that pyrolyzed animal bone (i.e. bone-char) could be used as a phosphorous fertilizer and thereby could reduce the dependency on chemical fertilizer in a sustainable way (Christel et al., 2014; Glæsner et al., 2019; Ahmed et al., 2021). 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 (Glæsner et al., 2019; Ahmed et al., 2021). 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 phosphorous solubilizing microorganisms (PSMs). Co-pyrolysis of different lignocellulose agricultural waste with animal bone can significantly reduce Ca-P crystallinity during pyrolysis (Zwetsloot et al. 2014) 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 (Tarafdar et al. 1995; Richardson 2001). 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 study to improve the fertilizer value of animal bone waste through combining co-pyrolysis with bio-augmentation. This study was therefore aimed to determine whether coupling of co-pyrolysis with bio-augmentation influence the fertilizer value of slaughterhouse waste.

3.2. MATERIALS AND METHODS

3.2.1. 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 chars-based P fertilizers were prepared through pyrolysis process at a temperature of 450°C and 850ºC with a heating rate of 5°C per minute and two hours 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).

3.2.2. Basic characterization of the different bone chars-based P fertilizers

Total C and N were measured using a CHN analyzer (2400 series II, Perkin Elmer) as described by Yeomans and Bremner (1988). The pH and electrical conductivity (EC) were measured using auto-electrical pH and EC meter (Seven Easy S20, Mettler Toledo). Total P and Ca (Enders et al., 2017), 2% formic acid extractable P (Rajan et al., 1992), 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. (2018). 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.

3.2.3. Bio-augmentation as a strategy to increase P solubility

3.2.3.1. Phosphorous solubilizing strains and inoculum preparation

Four different *Penicillium* strains: *Penicillium bilaiae* JCM22749, *Penicillium glanrum* 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 (Raymond et. al 2018). Spore concentrations in the suspension were adjusted with milliQ water after determining the spore concentration with hemacytometer.

3.2.3.2. 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) (Nautiyal, 1999). 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 mL of spore suspension (10^6 spores mL^{-1}) of each strain were 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 acids 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. (2014). 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^{o}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).

3.2.4. Bone char-P solubilization by PSM augmentation under soil condition

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} (Tigist et al., 2020). 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 (Zwetsloot et al., 2014). The soil was classified as Nitisol (IUSS, 2014) 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, passed through a

2 mm sieve, sterilized, and visible root biomass was removed by hand. Fifteen grams of dried soil were 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 nutrients 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; $ZnSO_4{\cdot}7H_2O$, 1.2 Zn; $CuSO_4{\cdot}5H_2O$, 0.2 Cu (Nautiyal, 1999).

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) was added to the soil. The moisture content was kept at 60% water holding capacity (WHC) and incubated in the dark for six 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. Destructive 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) (Trevors and Cook, 1992). In brief, 3 g of fresh composite soil sample were 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 were 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.

3.2.5. 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.

3.3. RESULTS

3.3.1. 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 3-1). 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. 3-1).

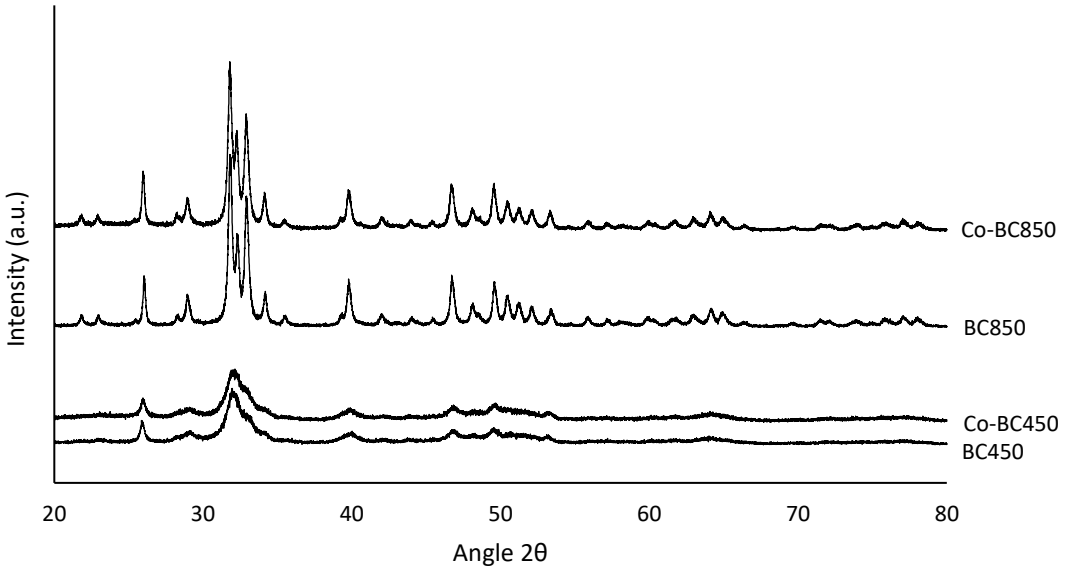

Figure 3-1. X-ray powder diffraction spectra from different bone chars produced at 450°C and 850°C

3.3.2. In vitro experiment

3.3.2.1. Phosphorous 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 450ºC (BC450 and Co-BC450) had higher P solubility than bone chars produced at 850ºC (BC850 and Co-BC850) (Fig. 3-2). For instance, *P. bilaiae* increased P solubility by 65%- 99% from

Table 3-1. Basic chemical characteristics of bone char types used in this experiment

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 pyrolysed at 450ºC; Co-BC450: biomass co-pyrolyzed bone char at 450ºC; BC850: bone char pyrolyzed at 850ºC; Co-BC850: biomass co-pyrolyzed bone char at 850ºC

TC: total carbon; TN: total nitrogen; WEOC: water-extractable organic C; C/N: carbon to nitrogen ratio and Ca/P: calcium to phosphorous ratio.

bone-char produced at a 450 ºC and by more than tenfold from bone-char produced at 850 ºC (Fig. 3-2C). 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. 3-2).

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% (Figs. 3-2A and 3-2B, respectively). Similarly, at higher pyrolysis temperature, combining co-pyrolysis with bio-augmentation increased P solubility by 10- to 45-fold (Figs. 3-2C and 3-2D, respectively), 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. 3-2A and 3-2B). The pH remained same in BC450 whereas it continued to decrease in Co-BC450 throughout the incubation period (Fig. 3-2A and 3-2B). The pH 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. 3-2D). However, in BC850, only *P. bilaiae*, augmentation resulted in reduced pH (Fig. 3-2C).

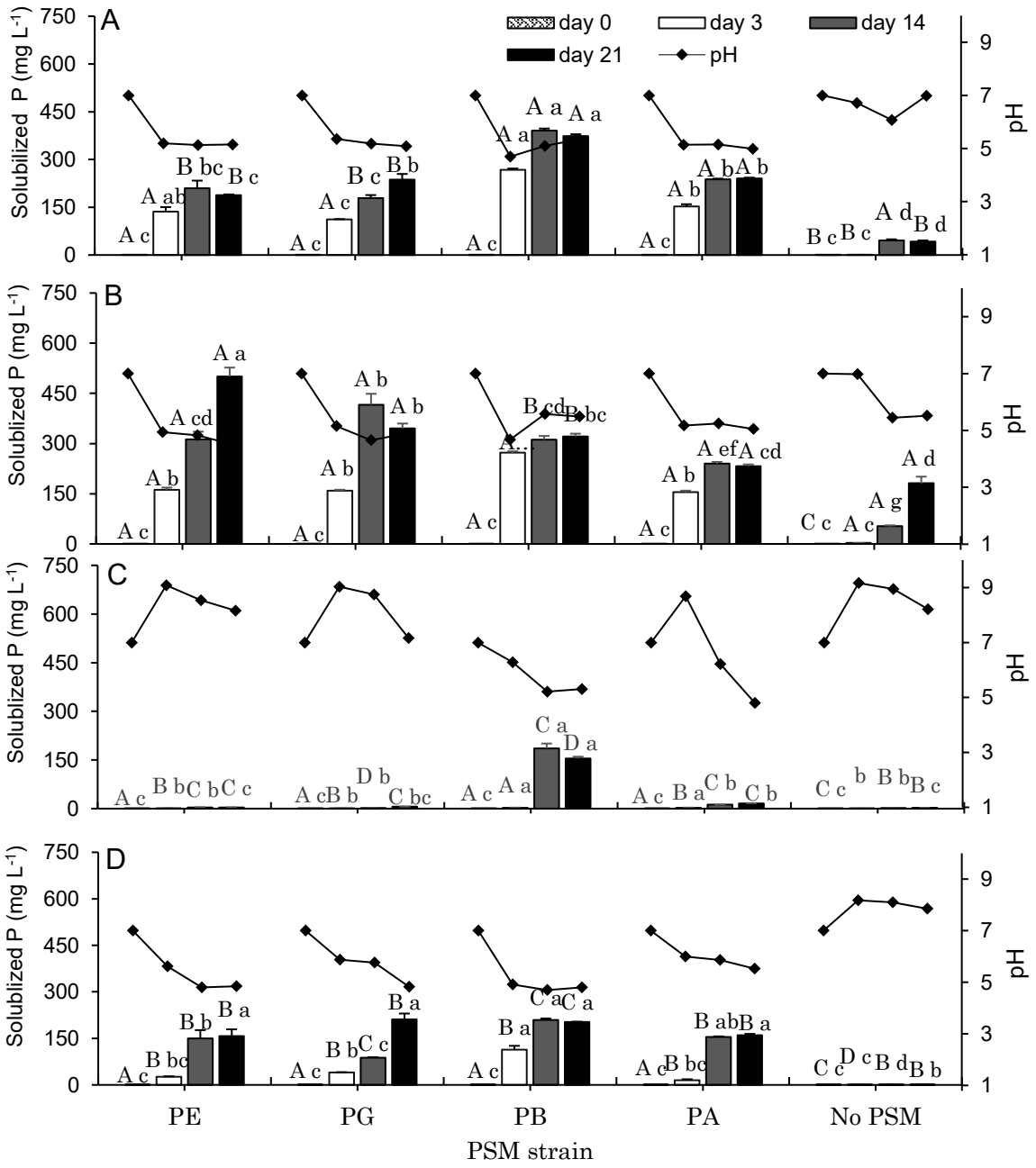

Figure 3-2. Change in solubilized phosphorous and pH in different bone chars (A) BC450, (B) Co-BC450, (C) BC850, (D) Co-BC850 by inoculation of different PSM strains (PE, PG, PB, PA and No PSM) sampled at different days of incubation. Bars represent mean of four replicates ±standard error. Uppercase 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

3.3.2.2. 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. 3-3). Lactic, pyruvic, acetic, butyric, and formic acids were the predominant organic acids produced but in different amounts (Fig. 3-4). Higher concentration of organic acids production was recorded from bone char produced at 450ºC (Figs. 3-3A and 3-3B) than bone chars produced at 850ºC (Figs. 3-3C and 3-3D). Very low organic acid production was recorded on BC850, however, it increased very significantly when co-pyrolysis was combined with bio-augmentation (Figs. 3-3C and 3-3D). Pyruvic, acetic and formic acids were produced in large amounts from BC450 and Co-BC450 treatments by all of the PSMs (Figs. 3-4A and 3-4B). 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 (Figs. 3-4C and 3-4D).

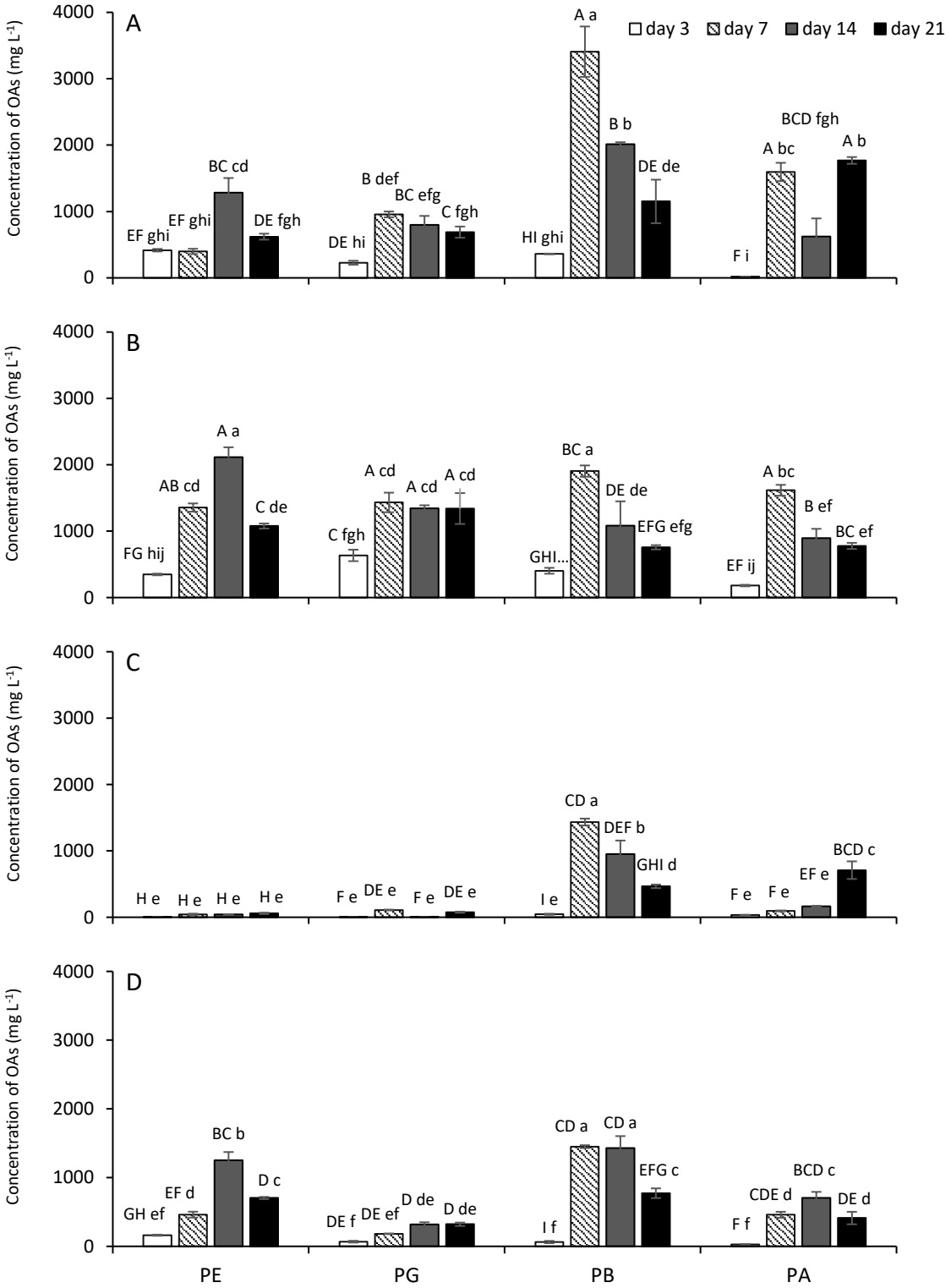

Figure 3-3. Concentration of low molecular weight organic acids produced with different bone chars (A) BC450, (B) Co-BC450 (C) BC850, (D) Co-BC850 augmented with different PSM strains (PE: *Penicillium expansum*; PG: *Penicillium glanrum*; PB: *Penicillium bilaiae*; and PA: *Penicillium aculeatum*. Bars 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

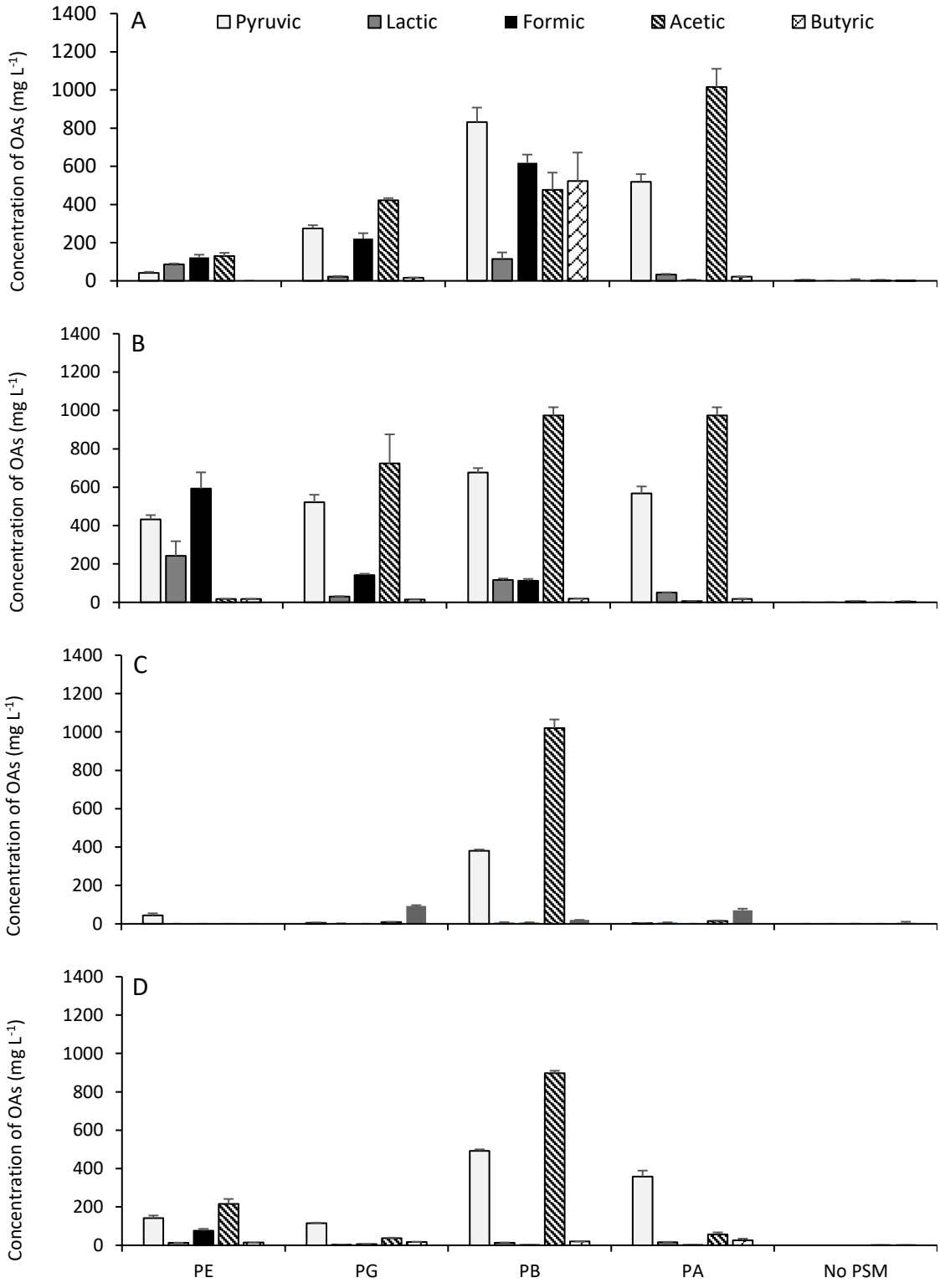

Figure 3-4. Concentration of carboxylic groups of low molecular weight organic acids produced by the strains at the different bone char types (A) BC450, (B) Co-BC450 (C) BC850, (D) Co-BC850 (only Day 14 data are presented)

3.3.2.3. 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 3-5). All the inoculated strains were able to grow and heavily colonize all the bone chars produced at 450°C with some regions densely covered with hyphae and spores (Figs. 3-5A, 3-5B and 3-5C). *P. bilaiae* was the only strain that was able to grow on BC850 (Fig. 3-5D) compared to the rest of the strains where no hyphae growth was detected (Fig 3-5E). However, increased growth and colonization of all the strains was observed on the biomass co-pyrolyzed bone char (Co-BC850) (Fig. 3-5F).

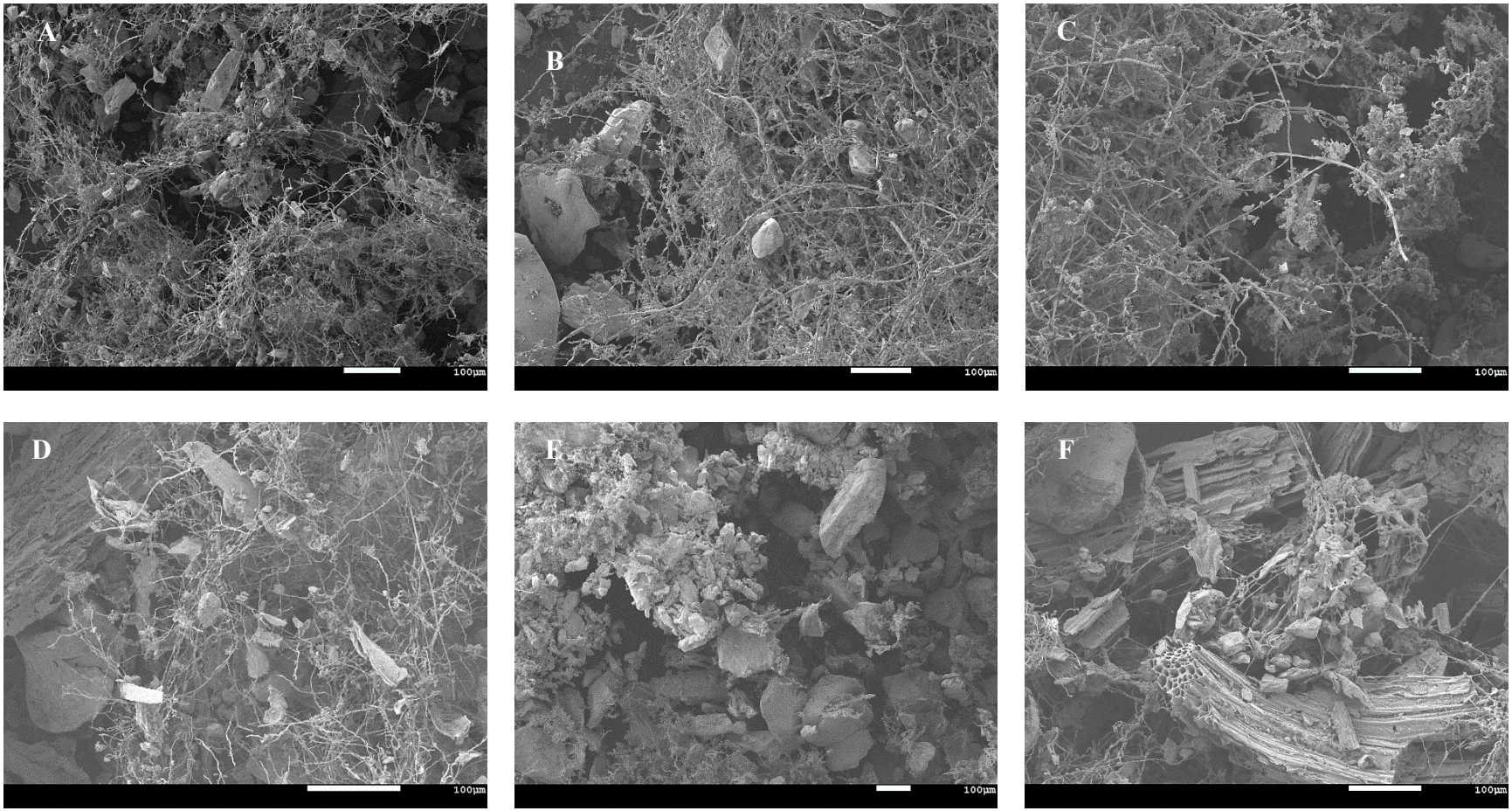

Figure 3-5. Scanning electron microscopy (SEM) images of the surface morphology of different bone char materials: (A) *P. bilaiae* growing on BC450, (B) *P. aculeatum* growing on BC450, (C) *P. expansum* growing on BC450, (D) *P. bilaiae* growing on BC850, (E) *P. expansum* on BC850, and (F) *P. expansum* growing on Co-BC850

3.3.2.4. 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. 3-6). 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. 3-6A). 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. 3-6C).

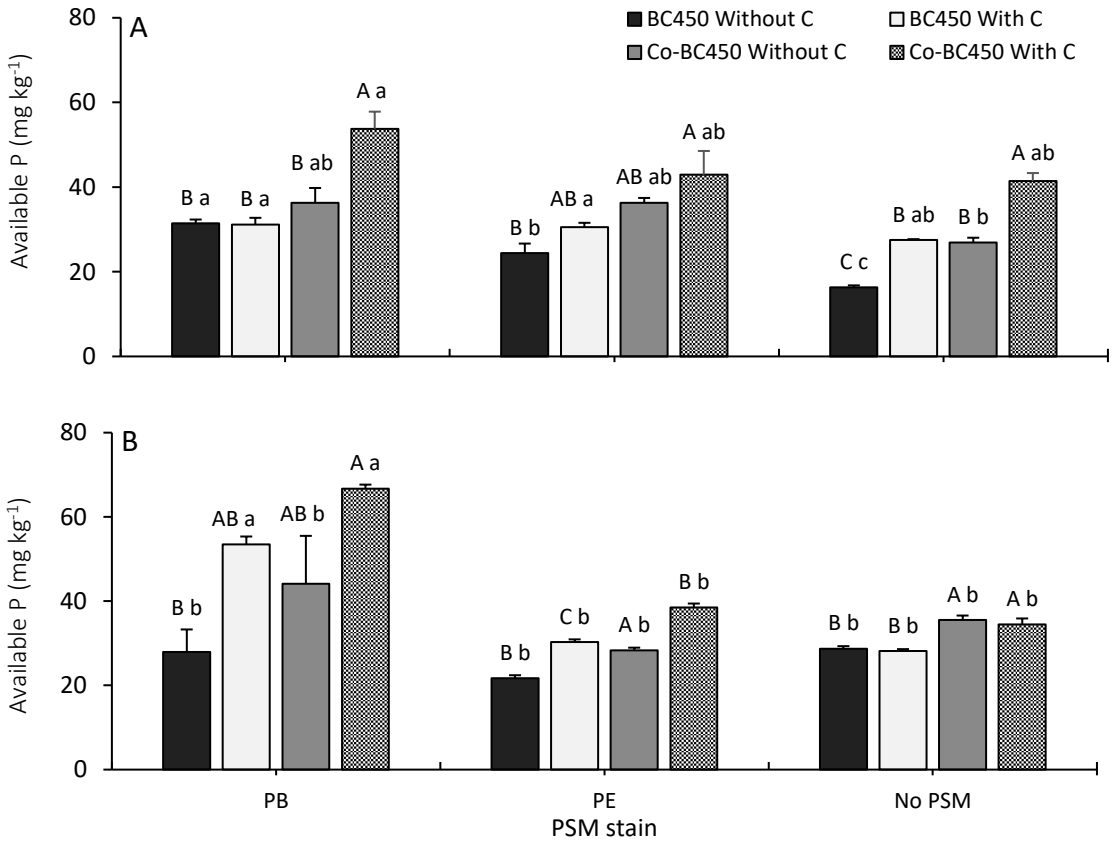

Figure 3-6. P release from different bone chars as affected by C addition to soil and PSM inoculation over time. Available P after (A) 7, (B) 35 days of incubation. Bars represent

the mean of four replicates ±standard error. Uppercase letters indicate differences across different bone chars (BC450 and Co-BC450) without or with C 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 C addition.

3.3.2.5. PSM survival rate in soil

Ensuring 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 more suitable environmental condition was created for the PSM through co-pyrolysis of biomass with animal bone (Table 3-2). 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*.

Table 3-2. Survival rate of *Penicillium bilaiae* and *Penicillium expansum* added to different bone chars as affected by OC addition with sucrose or co-pyrolysis with sugarcane bagasse (Co-BC450) after 35 days of soil incubation

PSM strain	Bone char	CFU g^{-1} dry soil	
		Without C addition	With C addition
P. bilaiae	BC450	151 x 10^4	571 x 10^4
	Co-BC450	199 x 10^4	820 x 10^4
P. expansum	BC450	46.6 x 10^4	59.7 x 10^4
	Co-BC450	88.6 x 10^4	213 x 10^4

3.3.3. Effect of different PSM strains on growth and phosphorus uptake of plant grown in different carbon content soils (pot experiment)

The average plant P uptake, plant dry weight, and plant available P were affected by different PSM strains inoculated without and with OC addition to soil (Fig. 3-7). Overall, higher plant P uptake was obtained with addition of the strains with OC addition compared to without OC addition (Fig. 3-7A). Compared to the control, *P. bilaiae* and *P. expansum* addition to BC450, with OC addition increased plant P uptake by 30% and 55%, respectively. Statistical analysis revealed no significant differences in plant dry weight and plant available P with or without PSM inoculation. Similarly, no significant increases were observed with inoculation of PSM compared to non-inoculated soil (Figs. 3-7B and 3-7C).

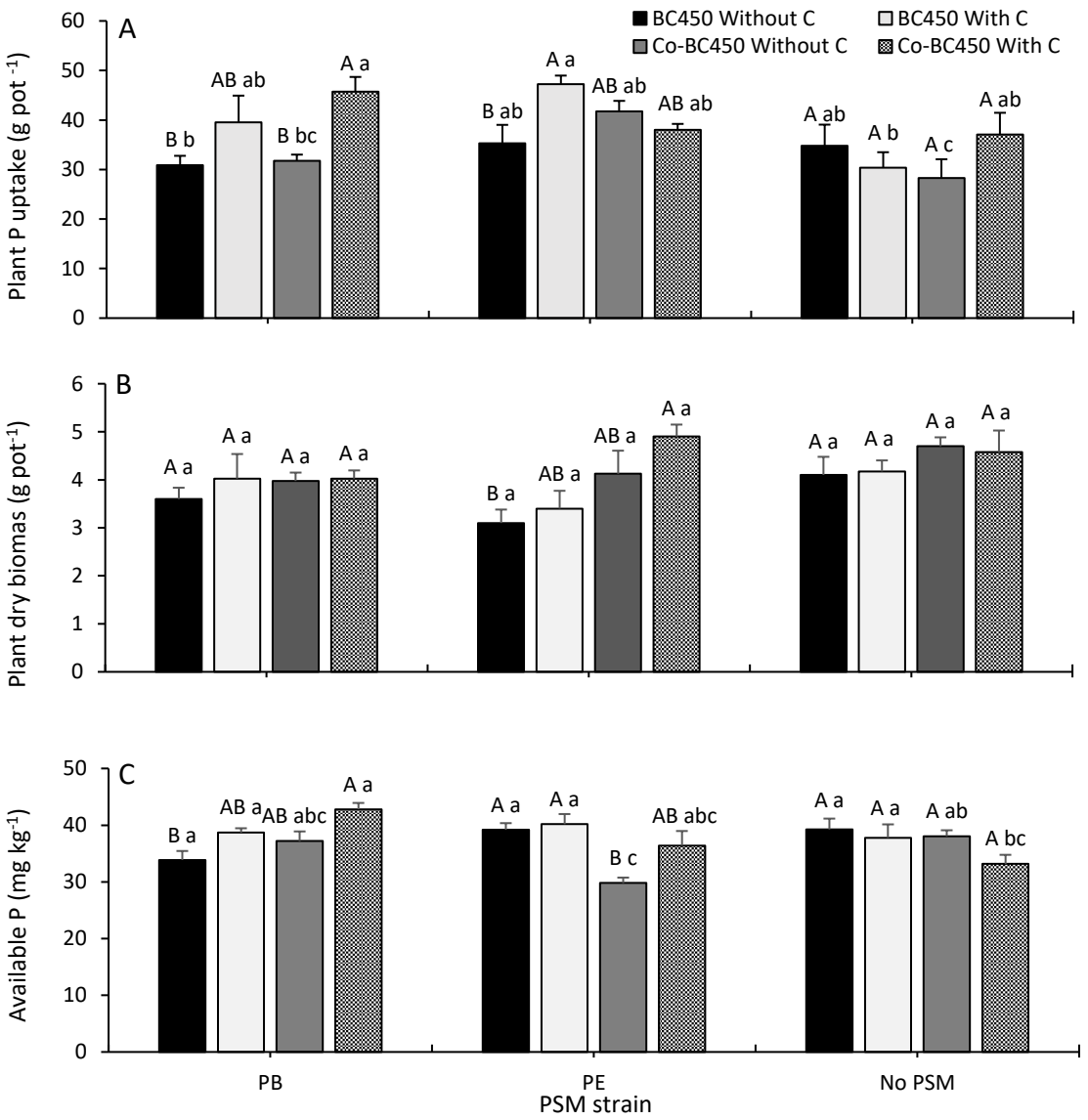

Figure 3-7. Plant P uptake (A), plant dry biomass and (B) and soil available P (C) in response to C addition and PSM inoculation. Bars represent the mean of four replicates ±standard error. Uppercase letters indicate differences across different bone chars (BC450 and Co-BC450) without or with C addition within the same PSM inoculation levels, whereas lowercase letters indicate differences across different PSM inoculation levels within the same bone chars without and with C addition

3.4. DISCUSSION

3.4.1. Increased P solubilization by PSM 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. 3-2). 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. 3-1). 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 (Zwetsloot et al., 2014). 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 (Tingting et al. 2019). 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. (2019) 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 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 (Bashan et al., 2013; Mendes et al., 2014). As the phosphate compounds 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.

3.4.2. Increased P solubilization by PSM in bone chars co-pyrolyzed with biomass

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 3-1). 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 (Scervino et al., 2011, Jacoby et al. 2017). 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 (Stefanoni Rubio et al., 2016; Rymond et al., 2018). 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.

3.4.3. 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. 3-3) and biomass co-pyrolyzed bone char. Availability of C and N 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 bioavailable organic and inorganic C and N pools and leading to increased organic acid production. Apart from this pyrolysis temperature and pyrolysis condition (co-pyrolysis) could cause structural difference in P form (Zwetsloot et al. 2014) and it was well documented that such structural difference in P form affect the nature and amount of organic acids produced by PSM (Scervino et al., 2011; Mendes et al., 2014; Stefanoni Rubio et al., 2016).

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 3-3). The higher concentrations of organic acid (Figs. 3-3A and 3-3B) and solubilized P (Figs. 3-2A and 3-2B) 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.

Table 3-3. 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***

*** $p < 0.001$.

3.4.4. 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 3-2). 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 3-2), 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 (Lehmann et al., 2011; Noyce et al., 2015; Zhang et al., 2018).

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 3-2). 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 (Rymond et al., 2018), who 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.

3.5. 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 450ºC coupled with PSM augmentation increased the P solubility of bone char up to 50% of the total P. P solubility by bio-augmentation pure bone char produced at 850ºC 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, 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 in to valuable products.

CHAPTER FOUR

4. GENERAL DISCUSSION

4.1. Introduction

Global P fertilizer demand will continue to rise over the next half century, as the food demand will increase following the increasing population. However, the total dependency of modern agriculture on a non-renewable resource and the limited future availability of P is likely to limit the productivity. Recycling P from P-rich organic wastes such as animal bone, through pyrolysis, can be a sustainable strategy to reduce the dependency on rock phosphate. However, P contained in this product is often poorly available to plant, resulting in rather low uptake efficiency when applied back to agriculture. As a fertilizer, the availability of P is very critical for its application. Even though, bone char as a sustainable P fertilizer has received more scientific attention over the past few years, factors affecting P solubility and bioavailability during thermal treatments have not been fully understood. Moreover, biological treatment to enhance P solubility of thermally treated bones has rarely studied. Hence this dissertation aimed to elucidate different factors affecting P solubility and bioavailability (chapter 2), and investigate effects of biological treatment (i.e., PSM) on P solubility (chapter 3) from thermally treated bones.

4.2. The findings and potential application

This dissertation clearly demonstrated how processing method (pyrolysis vs. combustion) and processing temperature, chemical and structural difference in different animal bones, and soil pH affects P solubility and bioavailability from processed bone. The result of this study suggested that bone char produced at a temperature less than 700ºC is an efficient P fertilizer than expensive inorganic fertilizer (TSP) in acidic soil. Furthermore, it was also demonstrated that P solubility from bone char can be enhanced using phosphorous solubilizing microorganisms.

The finding from the current studies could play significant role in the effort to enhance P solubility from animal bone which have great potential application in providing bio fertilizer for modern agriculture and reduce the dependency on phosphate rock. The P release from bone char is not as rapid as commercially available fertilizers (i.e. highly soluble chemical fertilizers) thus, less prone to leaching, which is, therefore, advantageous in reducing environmental risks, especially water contamination.

Furthermore, developing countries that lack mineable phosphorus reserves and manage soil under sever phosphorous deficiency could achieve greater autonomy by recycling the phosphorus contained in animal bone. The huge amount of phosphate fertilizer presented in developing countries in the form of organic waste products would allow these countries to secure a significant fraction of their phosphorus demands, therefore saving money and foreign currency.

4.3. Future research directions

Efforts have been made throughout this dissertation to illustrate the possible factors that affect P solubility and bioavailability from thermally treated animal bone; and to increase P solubility by using PSM. The findings from chapter 2 demonstrated the effect of bone type, thermal processing method, production temperature, and soil pH on P availability from thermally treated animal bone. Plant bioavailability was tested with pot experiment however, further studies are recommended to determine the P fertilizing value under field condition and in diverse cropping system. The finding from chapter 3 provided insights on the release and transformation of bone char P mediated by PSM and revealed the effects of pyrolysis temperature, biomass co-pyrolysis, and soil C content on P solubilizing potential and survival of PSM strains in soil and plant P uptake. However, as

microbial P solubilization is a complex phenomenon which depends on many factors such as inherent characteristics of PSM strains used, soil condition, nutrient availability, as well as plant type grown, more studies are still required to understand how the above-mentioned factors influence the efficacy of PSM to solubilize P from bone char. Based on the results of this dissertation, the following research areas are recommended:

1. Clarify whether pre-treatment of bones (i.e. boiling and extraction of fat) has effects on crystal formation and P availability
2. Investigate how P availability and the form in which P exist may be significantly affected by the surface area or particle size of the bone char
3. Investigate the long-term effects of bone char amendment on diverse cropping system and soil type
4. Study the effects of PSM under different soil property and plant types and non-sterile condition
5. Screen several PSMs for their ability to solubilize bone char P and develop an optimum condition for their survival and efficacy
6. Investigate the effect of single or combined inoculation of different PSM on P solubilization
7. Establish how soil organic carbon content and organic carbon composition/type influence PSM P solubilization efficiency and survival rate
8. Investigate the long-term effects of soil OC and root turnover on PSM inoculation

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