Use of Penicillium bilaiae to improve phosphorus bioavailability of thermally treated sewage sludge

A potential novel type biofertiliser

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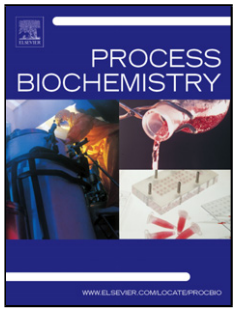

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Use of *Penicillium bilaiae* to improve P-bioavailability of thermally treated sewage sludge - a potential novel biofertiliser

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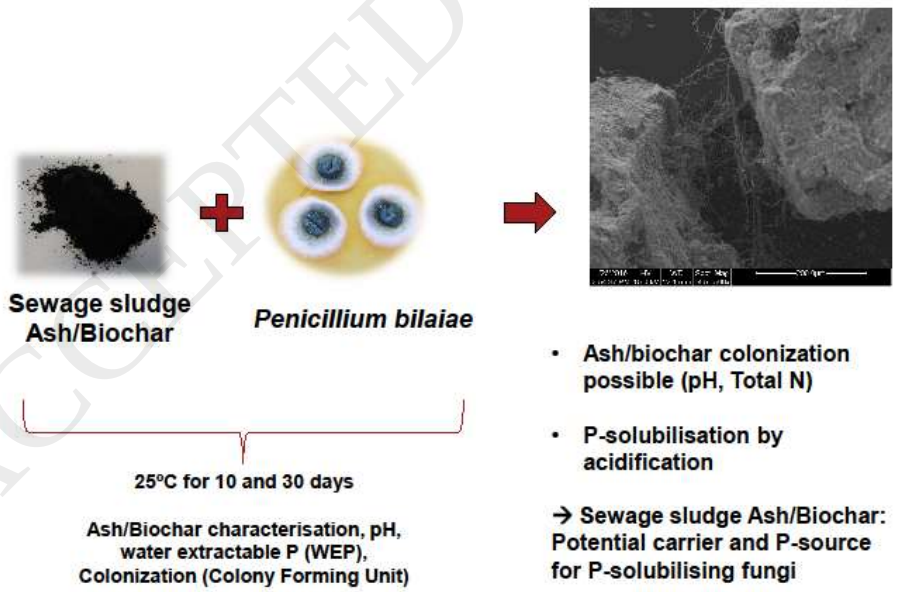
Graphical abstract



Highlights

- Biochars and ashes from sewage sludge could serve as a growth carrier for the phosphate(P) solubilising fungus, *P.bilaiae.*
- The amount of glucose added, the pH and the total nitrogen content of the ashes and biochars were the characteristics mainly influencing *P.bilaiae* colonization.
- On three of the tested ashes and biochars, P.bilaiae increased P-availability by acidification

Abstract

This study explored the potential of different phosphorus (P)-rich sewage sludge biochars and ashes to be colonized and be used as a P sources for the phosphate-solubilising fungus, *Penicillium bilaiae*. *P.bilaiae* was inoculated on five different biochars and ashes supplemented with nutrient solution. Fungal colonization, pH and water-extractable P (WEP) in the materials were determined after incubation.

P.bilaiae colonized at similar rates on all materials tested, but colonization was affected by glucose level, pH and total N content in the material. A pH decline, accompanied by an increase in WEP concentration, was observed in three materials. The amount of soluble P was significantly greater at the high glucose level and showed the largest relative increase in incineration ash (>100-fold after 10 days). The results show a potential to use P-solubilising microorganisms to solubilise P from thermally converted sewage sludge, but the approach has to be further investigated regarding its effects in a soil/plant system.

Key words: Bioproducts; *Penicillium*; Biofertiliser; P-solubilisation; Biochar;colonization; Sludge

Highlights

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- The amount of glucose added, the pH and the total nitrogen content of the ashes and biochars were the characteristics mainly influencing P.bilaiae colonization.
- On three of the tested ashes and biochars, P.bilaiae increased P-availability by acidification

1. Introduction

The world's population is increasing and the sustainability of food production is being challenged. Phosphorus (P) fertiliser is one of the key factors in increasing food security, as there is no substitute for P in agricultural production [1]. The quantities of remaining P reserves have been intensely debated in the past decade [2-6]. However, there is general agreement that, regardless of the future degree of P resource limitation, it can be aggravated by supplier monopoly, potentially threatening global P and food security [7, 8].

Concurrently, the quantities of P in solid organic waste and municipal wastewater are rising due to rapid urbanisation and industrialisation [5], thus offering possibilities for increased P recycling.

Thermal conversion of sewage sludge by incineration, gasification or pyrolysis can be used to combine removal of organic contaminants with additional benefits such as high energy output [9, 10]. Depending on the thermal conversion method used, a residual ash or biochar product that is free of pathogen contamination, reduced in weight and volume and high in P concentration compared with the raw sludge is obtained [10]. However, the P contained in these products is often poorly available to plants, resulting in rather low uptake efficiency when recycled back to agriculture [11-14].

Microorganisms such as fungi of the genus *Penicillium* that are able to solubilise unavailable inorganic P forms have been intensively studied and show promising solubilisation activity on different P sources *in vitro* [15-17]. Acidification of the environment and secretion of organic anions are known to be key mechanisms behind this P solubilisation. However, despite the success encountered *in vitro*, phosphate-solubilising microorganisms (PSM) and many other biofertiliser products have been found to be much less successful in increasing P availability and plant P uptake when applied to soil [18-20]. There are many reasons for this, including poor survival of the inoculated strain in the soil. Competition with indigenous soil

microorganisms and adverse abiotic conditions such as lack of water or nutrients can greatly harm the success of inoculation. A suitable carrier providing a protective environment for the inoculant is therefore key to ensuring its efficacy [21]. Biochar and ash materials possess characteristics such as high porosity, water-holding capacity and carbon content, which makes them potentially interesting for use as microbe carriers [22-24].

Available studies on biochar as a carrier for bacterial strains show positive effects of the biochar on the inoculated microorganisms [22, 25, 26]. For example, Hale, Luth, Kenney and Crowley [22] used pinewood biochar as an inoculant carrier for a plant growth-promoting rhizobacteria (PGPR) strain (*Enterobacter cloacae* UW5) in a pot trial and observed an improvement in survival of the bacteria compared with conventional inoculation with a microbial suspension.

If biochar or ash could be used as a P source for plants and at the same time as an inoculant carrier for PSM applied to increase P availability, this would be a highly interesting bio-based fertiliser product, enhancing the recycling options for these residuals. The aim of this study was therefore to explore the ability of a phosphate-solubilising fungus, *Penicillium bilaiae*, to colonize and solubilise P from different P-rich biochar and ash products deriving from sewage sludge, in order to evaluate their potential as inoculant carriers and P sources. The *P. bilaiae* strain used in this study colonises plant roots [17] and is known to be capable of *in vitro* solubilisation of different forms of P such as calcium, iron and aluminium phosphate and rock phosphate [16, 27]. However, little is known about its ability to colonize and solubilise P from more chemically and physically complex sources such as biochar and ash [28]. The hypotheses tested were that: (1) *P. bilaiae* grows better on residues with greater surface area and more labile forms of carbon (C), (2) P availability from biochar and ash is improved when used by *P. bilaiae* as a growth substrate and (3) addition of a easily degradable C source results in greater *P. bilaiae* colonization and P solubilisation from biochar and ash.

2. Material and Methods

2.1. Sewage sludge biochar and ash products

Sewage sludge from Bjergmarken wastewater treatment plant (WWTP) (Roskilde, Denmark) was the feedstock used for production of the biochar and ash substrates tested. The sludge was anaerobically digested and then dried at the WWTP. The dried sewage sludge was processed by different thermal conversion processes as a part of the Cross Platform Sludge Experiment (CPSE) described in detail by Thomsen [29]. In this study, we used the residual material from five different thermal conversion processes: slow pyrolysis (SP) (~2 h, 600 °C), fast pyrolysis (FP), two-stage downdraft gasification (TS-G) (950 °C), low temperature circulating fluidised bed gasification (LT-CFBG) (750 °C) and fluid-bed incineration (FB-I) (850 °C).

Chemical and physical characteristics of the different materials were determined after crushing and sieving (<1 mm) and sterilisation by autoclaving for 20 min at 121 °C. pH was assessed in a suspension of 1 g material in 25 mL milliQ water. Electrical conductivity (EC) was measured with a conductivity meter (CDM 210, MeterLab, Radiometer Analytical, France) in a suspension of 1 g material in 20 mL milliQ water after filtration through Whatman® filter paper no. 42. Total P content was measured by ICP-OES (Agilent 5100, Agilent Technologies, Manchester, UK) in 25 mg material oxidised with 2.5 mL 70% nitric acid and 1 mL 30% hydrogen peroxide by digestion in a microwave (Multiwave 3000, Anton Paar GmbH, Graz, Austria), mixed with 500 µL fluoric acid and left to stand overnight before ICP-OES measurement. Water-extractable P (WEP) was chosen as an indicator of plant-available P [30]. In brief, 0.5 g material was suspended in 30 mL milliQ water and shaken at 150 rpm for 16 h. After filtration through Whatman® filter paper no. 42, the molybdate blue-ascorbic acid assay was performed as

described in Murphy and Riley [31] and Watanabe and Olsen [32]. Water-holding capacity of the different materials was measured according to ISO 14238:2012(E), modified for biochar analysis. In brief, ash/biochar was soaked in water for 24 h, drained for 2 h on quartz sand and the wet weight was recorded. The material was then dried in an oven at 40 °C to constant weight.

Surface area was determined by the Brunauer-Emmett-Teller (BET) method [33] (Gemini VII 2390, Micrometrics Instrument Corp. Norcross, GA, USA), which calculates the surface area of a material by measuring nitrogen (N) gas molecules adsorbed onto its solid surfaces.

To characterise the carbon forms present in the materials, surface functional groups of the different materials were investigated by Fourier transform mid-infrared photoacoustic spectroscopy (FTIR-PAS) [34, 35], using a Nicolet 6700 FTIR spectrometer (Thermo Scientific, USA) combined with a PA301 Photoacoustic (PAS) detector with a cantilever microphone (Gasera Ltd., Finland). The ash samples were packed in 10 mm diameter ring cups and inserted in the PAS detector, with helium as purge gas to remove noise arising from ambient moisture and CO_2 and to improve detector sensitivity. For each sample, spectra were recorded as an average of 128 scans and with a resolution of 4 cm^{-1} in the range 4000–400 cm^{-1}.

2.2. Phosphate-solubilising strain and inoculum preparation

Penicillium bilaiae ATCC 20851 [15] was provided by Novozymes (Denmark) as a spore stock stored at -80 °C. Spores from the stock were propagated on potato dextrose agar (PDA) and grown for approximately 2 weeks, followed by sub-culturing for another 2 weeks on a new PDA plate. Spores from the second generation were collected by washing the plate with sterile milliQ water. The spore suspension was then filtered through sterile glass wool (Miracloth, EMD Millipore Corporation, Billerica, MA 01821 USA) to remove the hyphae. The filtrate was centrifuged at 4000 rpm and 4 °C for 10 min. Spore counting was carried out with a

haemacytometer (Improved Neubauer, Brand, Germany) and the desired spore concentration was prepared using sterile milliQ water.

2.3. Incubation study

All biochars and ashes were supplemented with nutrient solution containing: sodium nitrate (2 g L^{-1}), magnesium sulphate (0.5 g L^{-1}), potassium chloride (0.5 g L^{-1}) and ferrous sulphate (0.019 g L^{-1}). Two different levels of glucose were also added (30 g L^{-1} and 60 g L^{-1}). Biochars, ashes and nutrient solutions were sterilised separately by autoclaving for 20 min at 121 °C and then 14.5 g of each material was placed in a 5.5 cm diameter Petri dish and supplemented with the nutrient broth in order to add 12.5 mg (low) or 25 mg (high) glucose g^{-1} material. The spore suspension was added at a concentration of 1.10^6 spores g^{-1} of material. The water content of the materials was adjusted to approximately 45% (of wet weight) with sterile milliQ water. The Petri dishes were incubated at 25 °C (± 1 °C) in the dark for 10 or 30 days. For each treatment, a non-inoculated control was also included and each treatment had three replicates.

2.4. pH, P solubilisation, colony-forming unit (CFU) enumeration and determination of glucose concentration

The pH and WEP of the materials were assessed as described above at after 10 days and 30 days of inoculation. Each measurement was made in duplicate. For determination of *P. bilaiae* viability and colonization, one sub-sample of 1 g (DW) was shaken in 9 mL sterile 0.05% Tween-80 solution for 20 min, serially diluted and 100 µL suspension of two different dilutions was spread on PDA plates. Each dilution was plated in triplicate. Plates were incubated for about 3 days at 25 °C and macroscopically visible colonies were counted. Plates with 20-200 colonies were used to determine CFU.

Glucose concentration in the ash materials was determined using the WEP extract. The extract was filtered through a 0.22 µm syringe filter and analysed using anion chromatography Dionex ICS-5000 (Thermo-Scientific) by injecting 2.5 µL in a solvent mix of 30 mM NaOH and 1M NaOAc and using a retention time of 20 min.

2.5. Scanning electron microscopy

The growth phenotype of *P. bilaiae* on the surface of the different materials was investigated using scanning electron microscopy (SEM). Around 1 g of material from the incubation experiment was placed in a 10 mL glass vial, submerged in Karnovsky's fixation solution and kept under vacuum for 1 h. The vials were then placed in a circular rotator for 20 min. After discarding the fixation solution, two series of 0.1 M sodium cacodylate buffer were applied to the samples for 20 min on the rotator. After washing with milliQ water, a series of dehydration steps with acetone was applied to the material (10%, 20%, 30%, 50%, 70%, 90%, 90% and 100% acetone). The samples were then submerged in 100% hexamethyldisilizane for 20 min in the rotator. Finally, the samples were dried overnight on filter paper under the hood.

Samples were placed onto specimen stubs and sputter-coated (Polaron SC7640 Sputter Coater, Quorum Technologies Ltd, Sussex, UK) with gold and palladium before examination under a FEI Quanta 200 microscope (FEI Company, USA). Images presented in this study were taken at x500 and x1500 magnification.

2.6. Statistical analysis

Statistical analysis was performed using RStudio (R i386 3.3.2, GNU Project) in the RStudio development environment. For each biochar or ash material, differences in CFU count, WEP and pH after incubation were tested in a simple multivariate linear model including

interactions followed by Tukey's HSD post hoc test (agricolae package; significance level set to $p<0.05$).

Hierarchical regression analysis was performed for variables predicting *P. bilaiae* colonization (LogCFU) and P solubilisation after incubation, in one set of analyses including the physical characteristics of all the biochars and ashes and in another only those of biochars and ashes where *P. bilaiae* had a significant effect on P availability, using a multiple linear regression model (significance level set to $p<0.01$).

Regressions between WEP and pH were generated with SigmaPlot 13 (Systat Software, Inc., San Jose, CA, USA), using a non-linear regression model (exponential decay single, two parameters, significance level set to $p<0.05$).

3. Results

3.1. Characteristics of ashes and biochars

The biochars and ashes had higher pH than the raw sludge, with values varying from 8.1 to 11.6 (Table 1). The EC varied greatly between the materials, with FB-I showing the highest value and TS-G the lowest. The ash products contained only small amounts of total C and N [9, 10], whereas there were considerable amounts of C and some N present in the two biochars. Accordingly, total P content was higher in the ashes compared with the biochars. Water-extractable P was in general very low in all the materials.

Scanning electron microscopy revealed similar surface morphology for the ashes and biochars, as shown in Fig. 1 for TS-G ash, FB-I ash and SP biochar. Surface structures showed sags and crests, but few pores.

The FTIR-PAS spectra of the different biochars and ashes showed common peaks for the materials, but at different abundances (Fig. 2). The FP biochar displayed higher peak intensities

in O-H, C-H aliphatic (3000-2800 cm^{-1}), COOH carboxylic (1710 cm^{-1}), aromatic (1600 cm^{-1}) and C-H (1440 cm^{-1}) vibrations compared with the other materials. The SP biochar also displayed carboxylic, aromatic and C-H liaisons, but less than FP. Interestingly, FB-I ash also displayed O-H and C-H aliphatic liaisons and contained the highest amount of Si-O liaisons (1200-1000 cm^{-1}).

3.2. *P. bilaiae* colonization on biochar and ash materials

Overall, the number of CFU increased on average by 100-fold on the different materials during the incubation (Fig. 3). The number of CFU counted was dependent on the growth substrate, the amount of glucose added and incubation duration ($p<0.01$). The FP biochar, combined with the higher amount of glucose (25 mg g^{-1} ash), showed the highest CFU count after both 10 and 30 days of incubation, while LT-CFBG ash at the high glucose level showed the lowest CFU count after 10 days of inoculation.

Colonization on SP biochar and LT-CFBG ash was affected by both the glucose concentration and incubation duration ($p<0.01$). For those two materials, increasing the glucose concentration did not increase the CFU count, but rather tended to decrease it in the first 10 days of incubation. The CFU count on FB-I ash was only influenced by incubation duration ($p<0.01$), with an increasing number after 30 days of incubation compared with after 10 days. On the other hand, FP biochar was only influenced by glucose concentration ($p<0.01$), with increasing concentration generating a higher CFU count. Colonization of the fungal strain on TS-G ash was not affected by glucose concentration or incubation duration ($p>0.05$)

The SEM surface images of the different materials inoculated with *P. bilaiae* and with different glucose concentrations showed no visible differences after 30 days of incubation (Fig. 1). The fungus grew in a similar way on all the materials, with some regions densely covered with

hyphae and spores (Fig. 1D) and other areas with only a few hyphae (Fig. 1E). Hyphal connection of particles was also observed (Fig. 1F).

According to the multiple linear regression model, initial pH of the materials ($p<0.0001$), total N ($p<0.0001$), and glucose concentration and duration of inoculation ($p<0.01$) significantly influenced the colonization of *P. bilaiae* (LogCFU) (Table 2).

3.3. Water-extractable P concentration and pH

Incubation of biochar and ash materials from sewage sludge significantly altered WEP concentration and pH over the incubation period (Fig. 4). WEP increased over time in both inoculated and non-inoculated treatments and was positively and significantly influenced by the glucose concentration, *P. bilaiae* inoculation and incubation duration.

The WEP concentration in the non-inoculated treatments increased in all materials after 10 days of incubation. After a 30-day incubation period, WEP in the non-inoculated SP biochar and LT-CFBG ash generally increased further, while it decreased in FP biochar and remained stable in TS-G and FB-I ash, irrespective of the amount of glucose added. In the *P. bilaiae*-inoculated SP biochar, LT-CFBG ash and FB-I ash, the WEP concentration increased after 10 days of incubation compared with the non-inoculated controls and the increase was larger with the high glucose level. FB-I ash inoculated with *P. bilaiae* and the high level of glucose showed the largest increase in WEP over the 10-day incubation period, with a 100-fold increase compared with the WEP concentration at the beginning of the experiment. In contrast, FP biochar and TS-G ash did not show any increase in WEP concentration after 10 days of inoculation with the fungal strain. After 30 days of incubation, the WEP concentration tended to remain stable in the inoculated treatments except at the high glucose level, where WEP tended to decrease in some of the materials, such as SP biochar and FB-I ash.

The pH of the materials was also strongly dependent on the glucose concentration, inoculation with the fungal strain and incubation duration (Fig. 4). For SP biochar and all three ash products, a sharp drop in pH was observed after 10 days of incubation. For all the materials except TS-G ash, the lowest pH values were observed for *P. bilaiae* inoculation combined with the high glucose level. FP biochar, which was initially less alkaline, showed a less distinct drop in pH after the first period of incubation. After 30 days of incubation, in most treatments the pH remained stable or continued to decrease, but to a lower extent than in the first 10 days of incubation. The only exception was FB-I ash, for which high glucose level combined with *P. bilaiae* inoculation gave a slight pH increase.

For all biochar and ash materials, significant negative correlations between WEP concentration and pH were found (Fig. 4). The correlation coefficient was highest (r^2= 0.94) for FB-I ash and lowest (r^2 = 0.33) for TS-G ash.

Colonization (LogCFU) and WEP concentration after incubation did not show any correlation ($p>0.05$).

In the biochar and ash materials where *P. bilaiae* had a significant effect on available P, multiple linear regression analysis revealed that only the glucose concentration influenced the amount of P solubilised by *P. bilaiae* (r^2 = 0.13; $p<0.0001$).

3.4. Glucose consumption and recovery

The total amount of glucose added was consumed in all treatments inoculated with *P. bilaiae* already after 10 days of incubation (Table 3). In the control treatments, glucose was still present at that time except in the FP biochar treatment with the high glucose level. Glucose recovery rate varied between the materials, with LT-CFBG and FB-I ashes showing the highest and FP biochar and TS-G ash the lowest recoveries.

4. Discussion

4.1. Sewage sludge biochars and ashes as a growth substrate for *P. bilaiae*

Establishment of the inoculum on a substrate is critical for successful targeted microbial actions such as P solubilisation. To the best of our knowledge, this is the first study to examine the possibility of using biochar and ash products from sewage sludge as a growth and inoculation carrier, although some previous studies have investigated the option of using biochar from acacia wood, coconut shell or pine wood as a storage medium or inoculant carrier for beneficial microorganisms [22, 24, 25, 36]. In the present study, the focus was on different thermal residues from a single batch of sewage sludge, resulting in some contrasting features arising from the processing that may have caused some of the variation in *P. bilaiae* colonization and performance. However, SEM revealed a similar phenotypic fungal colonization pattern on all materials except FP biochar, which had some more porous particles. These were presumably small cereal straw/straw char particles that probably originated from improperly cleaned parts of the pyrolysis unit [10].

Biochars and ashes often have low nutrient availability and *P. bilaiae* was therefore provided with an easily available initial pool of carbon and nutrients. Overall, the amount of glucose added and incubation duration had a dominant positive effect on fungal colonization. In previous studies using submerged fermentation to test different strains for solubilisation of different P sources, the nutrients have been provided in larger amounts than in our solid-state culture test. For example, Whitelaw et al., [37] added the equivalent of 30 g sucrose per 1000 mg P, whereas we only used the equivalent of 8.6 and 17.2 mg glucose per 1000 mg P (low and high level, respectively). The slow colonization observed between day 10 and day 30 of

inoculation may be the result of lack of carbon source, as all the glucose had been consumed in the inoculated treatments after 10 days.

However, we also observed a strong negative correlation between fungal colonization and initial pH of the material. All materials tested had an alkaline pH, although at varying levels, a characteristic commonly observed after thermal conversion of sewage sludge with a high ash content. Alkalinity of the medium is often reported as a potential obstacle to colonization and performance of microbial inoculants, e.g. Scervino et al., [38] demonstrated that *Penicillium purpurogenom* was able to colonize and perform P solubilisation under alkaline conditions, but less well than at pH 6.5.

Colonization of *P. bilaiae* was also positively correlated with total N concentration in the material. Total N content was higher in the biochars than in the ashes and the highest colonization rate of the fungus was observed on FP biochar, which had a high N content combined with a comparatively low pH. However, we cannot exclude the possibility that feedstock contamination with straw contributed to the increased fungal colonization on this material. Furthermore, it has been shown that biochar from fast pyrolysis processes may contain a fraction of labile C that is easily degradable for microorganisms, in contrast to materials originating from slow pyrolysis [39]. This was evident from the FTIR-PAS spectra of the materials tested here, with C forms that showed common peaks but with varying intensities. The O-H stretch peak was more intense for FP biochar than for all other materials and was completely absent for the gasification ashes. The C-H aliphatic peak was also seen only in the biochar spectra, which is in agreement with results for switchgrass converted in different thermal processes reported by Brewer et al., [40], who attributed it to more labile C in the respective products. FP and SP biochars also showed higher –COOH, COO/C=C and C-H stretch peaks, which may likewise be related to the amount of C present in the materials.

Characteristics such as specific surface area and water-holding capacity did not display differences discriminating any colonization preferences, and therefore the hypothesis that the physical properties of the materials have a beneficial effect on fungal colonization was rejected. The use of other feedstocks in the thermal processes would have allowed more varying characteristics and could perhaps have distinguished other colonization preferences. For the sewage sludge biochar and ash materials tested, the pH and most probably the forms of C in the material proved to be major determinants of *P. bilaiae* colonization.

4.2. P solubilisation on the different materials

After 10 days of incubation, WEP had increased in all the treatments. An increase was also observed in the non-inoculated treatments, where the alkalinity of the materials may have led to absorption of atmospheric CO_2, thereby causing a natural, gradual pH decline and potentially promoting abiotic P solubilisation. Furthermore, oxidation reactions may have occurred, hydrolysing the glucose and creating acidity [41]. FP biochar and TS-G ash showed the highest increase in WEP for the non-inoculated treatments and recovered the lowest amount of glucose after incubation, confirming that glucose may have acted as an abiotic factor for P solubilisation. Although the fungus could colonize on the materials, no further P solubilisation was observed, probably because P solubilisation was not needed as the environment contained enough available P to sustain *P. bilaiae* colonization.

The addition of *P. bilaiae* further increased WEP concentration in the three other materials tested (SP biochar and LT-CFBG and FB-I ashes), confirming our second hypothesis. The WEP concentration resulting from fungal P solubilisation activity was enhanced when more glucose was added, confirming our third hypothesis. Enhanced fungal P solubilisation with increasing C source concentration has been demonstrated previously in other works using *Penicillium purpurogenum* with calcium phosphate in a liquid culture [38] or *Aspergillus niger* FS1,

Penicillium canescens FS23, *Eupenicillium ludwigii* FS27 and *Penicillium islandicum* FS30 with rock phosphate in solid state fermentation [42]. As mentioned earlier, even the high level of glucose added in this study may have been too low to achieve optimal fungal colonization and P-solubilisation activity and further work is needed to determine the glucose concentration that ensures the highest P-solubilisation efficiency. Growth of the inoculant is frequently correlated to P-solubilisation performance of the fungal strain [37, 38].

The increasing amount of WEP occurred together with acidification in all materials. The acidification effect associated with the activity of P-solubilising microorganisms is known to be caused by proton and organic anion (OA) excretion [38, 43, 44]. Takeda and Knight [16] and Cunningham and Kuiack [27] concluded that *P. bilaiae* secretes mainly citric and oxalic acids to solubilise P. Efthymiou et al.,[28], who investigated P solubilisation from slow pyrolysis sewage sludge biochar by the same *P. bilaiae* strain as used in our study, reached similar conclusions. The production of OA by P-solubilising microorganisms depends on many factors, but in particular on the availability, type and concentration of the C source [45-47]. In the present study we used glucose as the main source of C, but other studies have shown that different *Penicillium* strains show higher P-solubilisation activity with other C sources, depending on the type of P to be solubilised and the pH of the growth medium [47]. Alkaline pH may have a negative effect on OA excretion and therefore on P-solubilisation activity [38]. Further investigations are necessary to identify C sources that result in greater P solubilisation in alkaline sewage sludge biochar and ash materials combined with *P. bilaiae*.

It has also been found that the N source and concentration interact with the P-solubilisation ability of phosphate-solubilising microorganisms [47]. In this study nitrate was used as the N source, but it has been reported that the use of ammonium N causes greater P solubilisation [37] due to the acidification generated by ammonium uptake into fungal cells. However, Reyes

et al., [48] found that the P-solubilisation efficiency of *Penicillium rugulosum* was inhibited by ammonium as the N source on different insoluble P sources, due to poor assimilation and low metabolic performance of the ammonium. De Oliveira Mendes et al., [43] found that some fungal strains are more efficient at solubilising specific forms of P, e.g. *Aspergillus niger* FS1 solubilised a large amount of P from aluminium phosphate and calcium phosphate, whereas *Penicillium islandicum* FS30 solubilised calcium phosphate, but only very small amounts of aluminium phosphate and iron phosphate. Those authors attributed the P solubilisation from different insoluble P forms to acidification of the growth medium and production of OA. Biochar and ash products from sewage sludge are rather complicated materials as regard P speciation, but several studies show the presence of complex P forms such as apatite and iron-oxide P [49, 50]. Mackay et al.,[11] used a sequential extraction method to compare the P availability of the biochars and ashes used in the present study and found that the P solubility differed between the materials. FB-I ash was more acid-soluble than the other materials, indicating that the main P form may be calcium-P, which would also explain the high solubilisation from FB-I ash after acidification by *P.bilaiae*. In contrast, Efthymiou et al., [28] concluded that slow pyrolysis biochar P is solubilised by chelation with citrate, rather than by acidification, and also found that *P. bilaiae* was very efficient in solubilising calcium-P, but less efficient in solubilising iron-P. Therefore, the varying efficiency of *P.bilaiae* in solubilising P from the different materials tested here and the different correlation coefficients between WEP concentration and pH could also be related to different dominant P species in the biochars and ashes.

After 30 days of incubation, in some of the *P. bilaiae*-inoculated treatments (SP biochar and FB-I ash with high glucose) we observed a decrease in the amount of WEP. Possible reasons are that (1) *P.bilaiae* consumed some the solubilised P and immobilised it and/or (2) the soluble P was re-sorbed onto the materials. Sorption of P by different biochars has been

described and attributed to the presence of adsorption sites on surfaces (e.g. colloidal and nano-sized MgO particles) that can absorb P in solution or on biochar and ash surfaces [51, 52].

Conclusion

We were able to grow the P-solubilising fungus *P. bilaiae* on different biochar and ash materials from sewage sludge in a solid state fermentation system, but to different extents depending on the characteristics of the material. Amount of glucose added, initial pH and total N content in the material were the most influential characteristics for fungal colonization on biochars and ashes from sewage sludge. An increase in soluble P in slow pyrolysis (SP) biochar and two types of ash (low temperature circulating fluidized bed gasification (LT-CFBG) and fluid-bed incineration (FB-I)) was observed after inoculation, suggesting that these materials can be used as a inoculant carrier and P source to be solubilised by *P. bilaiae.* The P-solubilisation activity was only influenced by the amount of glucose added, emphasising the importance of carbon availability for the process of P solubilisation. Further work is needed to optimise the P-solubilisation activity by investigating the effect of specific C and N sources at different concentrations. The possibility to grow *P. bilaiae* on sewage sludge biochars and ashes, with increased P availability in some cases, proved that this combination could be an interesting biofertiliser when applied to soil.

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Conflict of interest:

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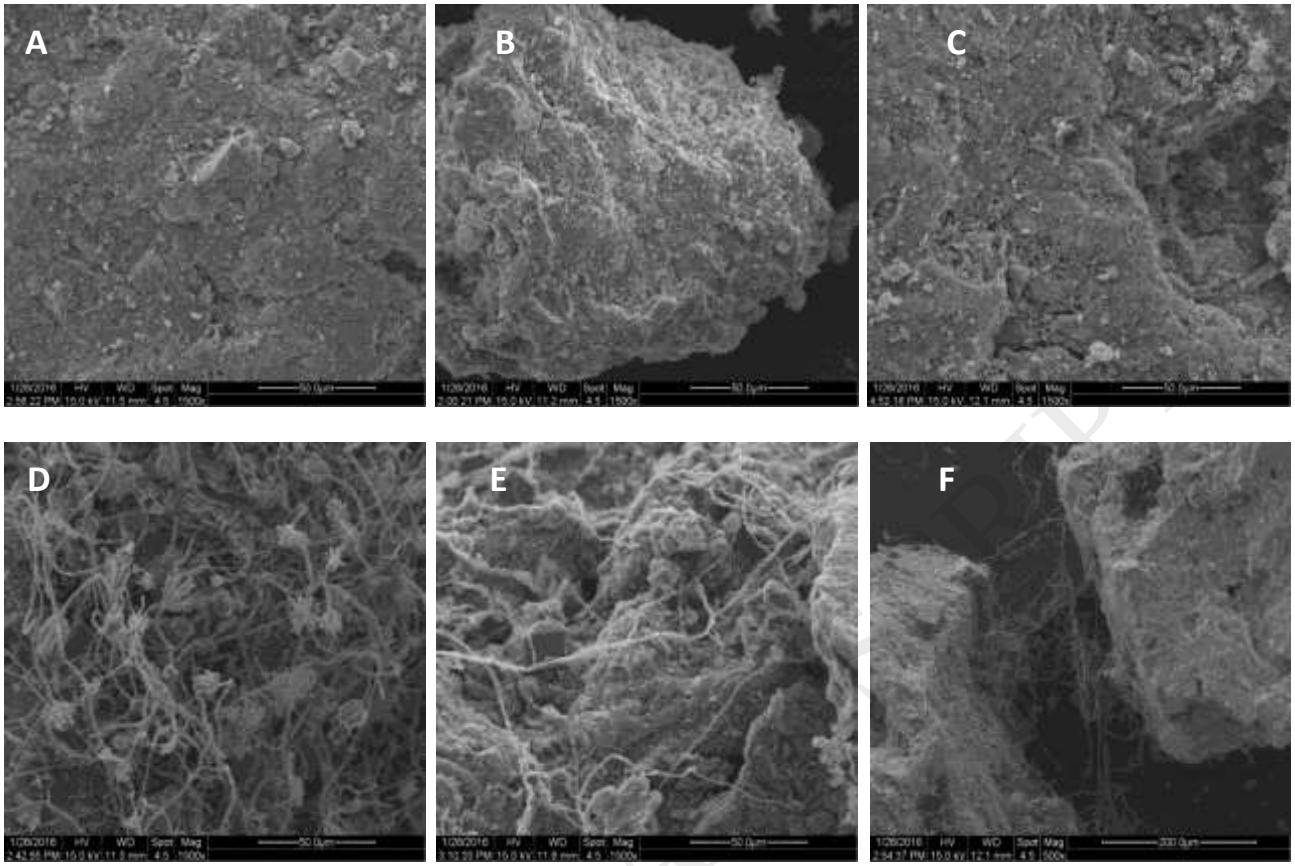

Figure 1: Scanning electron microscopy (SEM) images of the surface morphology of different materials (magnification 1500x and 500x): A: Two-stage downdraft gasification (TS-G) ash, B: fluid-bed incineration (FB-I) ash and C: slow pyrolysis (SP) biochar, D: slow pyrolysis (SP) biochar with growing *Penicillium bilaiae* with dense fungal colonisation, E: slow pyrolysis (SP) biochar with growing *Penicillium bilaiae* with less dense fungal colonisation and F: slow pyrolysis (SP) biochar with growing *Penicillium bilaiae* with fungal hyphae growing between the biochar particles (lower magnification).

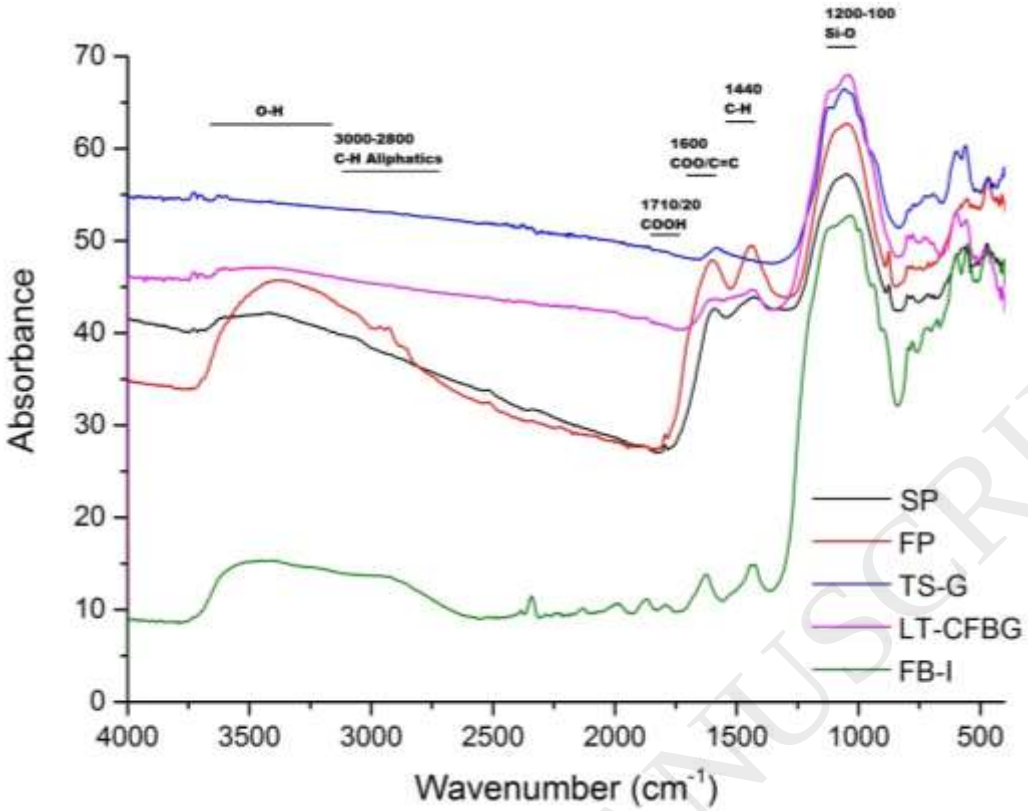

Figure 2: Comparison of the absorption spectra of the ash and biochar materials. SP: slow pyrolysis biochar; FP: fast pyrolysis biochar; TS-G: two-stage downdraft gasification ash; LT-CFBG: low temperature circulating fluidised bed gasification ash; FB-I: fluid-bed incineration ash.

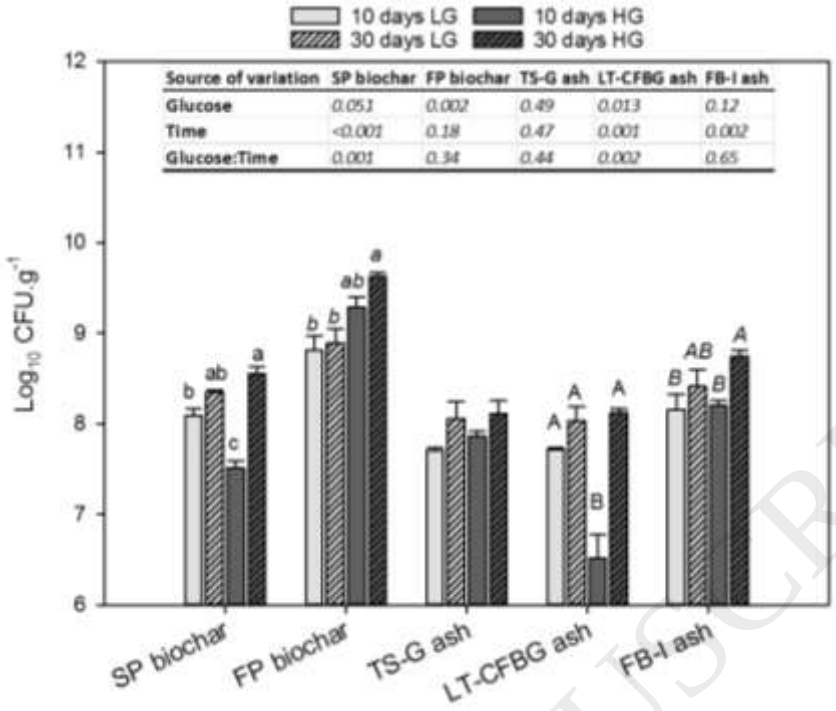

Figure 3: Log_{10} colony-forming units (CFU) count per g of the different ash and biochar materials after 10 and 30 days of incubation and Anova p-values for testing the effect of glucose concentration (Glucose) and incubation time (Time) on fungal colonisation. Name abbreviations of the biochars and ashes are specified in Table 1. Letters above the bars indicate significant differences between the treatments at different incubation times ($p<0.05$). Values are the mean (n = 3) ± S.E. (bars).

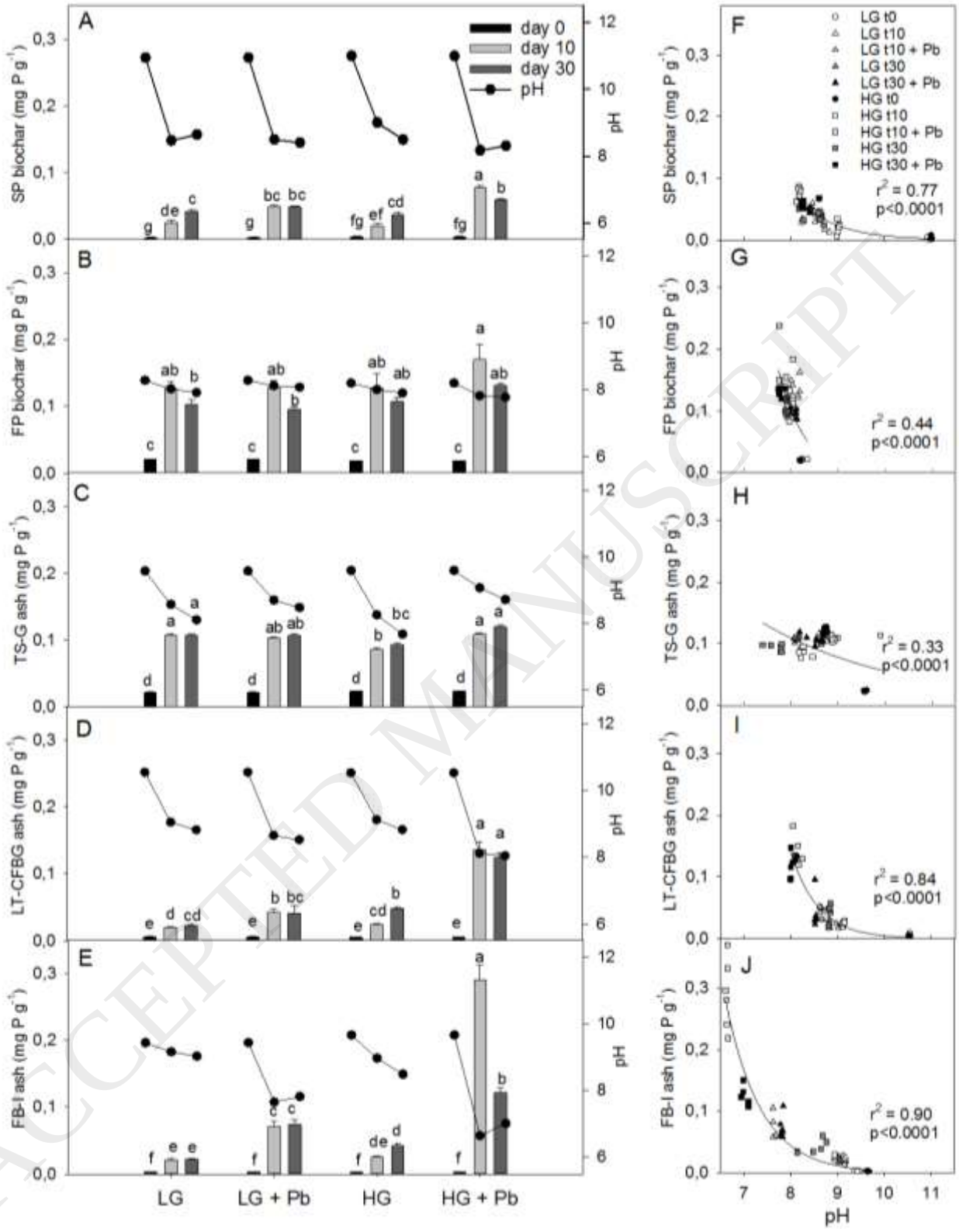

Figure 4: Change in concentration of water-extractable P (WEP) and in pH in the different biochar and ash treatments at different sampling times (A-E) and non-linear correlations (exponential decay) between pH and WEP (F-J). "LG" refers to low glucose and "HG" to high glucose. Name abbreviations of the biochars and ashes are specified in Table 1. Letters above the bars indicate significant differences between the treatments at different incubation times ($p<0.05$). Values are the mean ($n = 6$) ± S.E. (bars).

List of the Tables:

Table 1: Selected chemical and physical properties of the ash and biochar materials produced from sewage sludge. SP: slow pyrolysis; FP: fast pyrolysis; TS-G: two-stage downdraft gasification; LT-CFBG: low temperature circulating fluidised bed gasification; FB-I: fluid-bed incineration. Letters indicates significant differences between materials characteristics according to a HSD test ($p < 0.05$). Values are the mean ($n = 3$) ± S.E.

Properties	Raw sludge	SP biochar	FP biochar	TS-G ash	LT-CFBG ash	FB-I ash
pH	7.2 (f)	11.6 (a)	8.1 (e)	9.9 (c)	10.7 (b)	9.2 (d)
Electrical conductivity (µS cm^{-1} (25°C))	ND	419.7 ± 6.7 (c)	478.7 ± 25.7 (b)	76.7 ± 6.4 (e)	321.7 ± 22.2 (d)	945.7 ± 32.0 (a)
Total C (mg g^{-1} (DM))[1]	267	226	22	58	72	5
Total N (mg g^{-1} (DM))[1]	37	22	21	10	5	-
C:N ratio	7.21	10.27	10.81	58	14.4	-
Total P (mg g^{-1} (DM))	38.3	76.0 (c)	67.5 (d)	103.3 (a)	101.5 (a)	95.2 (b)
Water-extractable P (% of total P)	1.91 (a)	0.002 (c)	0.024 (c)	0.105 (b)	0.004 (c)	0.008 (c)
Water-holding capacity (% wt)	ND	24 ± 1 (c)	29 ± 2 (abc)	25 ± 1 (bc)	32± 4 (a)	29 ± 1 (ab)
BET[2] surface area (m^2 g^{-1})	ND	24.46 ± 0.35	22.16 ± 0.30	73.04 ± 0.92	24.88 ± 0.33	5.08 ± 0.06

[1]From (Thomsen et al. (2015), Thomsen et al. (2017))

[2]Brunauer-Emmett-Teller method

Table 2: Summary of hierarchical regression analysis for variables predicting *P.bilaiae* colonisation (log_{10} CFU) applied with a strict value criterion of p<0.01 for variables to stay in the model

	Δr^2	B	SE B	β	p
Constant	*0.73*	*11.21*	*0.41*		*<0.0001*
Duration (30 days)		*0.26*	*0.13*	*0.19*	*>0.01*
Glucose (high)		*-0.25*	*0.14*	*-0.19*	*>0.01*
pH		*-0.35*	*0.04*	*-0.61*	*<0.0001*
Total N		*0.30*	*0.05*	*0.43*	*<0.0001*
Duration (30 days): Glucose (high)		*0.54*	*0.19*	*0.40*	*<0. 01*

Where Δr^2: indicating that the cross validity of the model; B: indicating the relationship between *P. bilaiae* colonization and each predictor; SE B: B associated standard error; β: standardized beta estimates; p: p-value

Table 3: Anova p-values for testing the effect of *P.bilaiae* inoculation (Pb), glucose concentration (Glucose) and incubation time (Time) on biochars and ashes water extractable P (WEP) and pH. Name abbreviations of the biochars and ashes are specified in Table 1.

Source of variation	SP biochar		FP biochar		TS-G ash		LT-CFBG ash		FB-I ash	
	WEP	pH	WEP	pH	WEP	pH	WEP	pH	WEP	pH
Pb	*<0.001*	*<0.001*	*<0.001*	*0.18*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Glucose	*0.002*	*0.92*	*<0.001*	*<0.001*	*0.03*	*0.58*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Time	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
***Pb*:Glucose**	*<0.001*	*<0.001*	*0.026*	*<0.001*	*< .001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
***Pb*:Time**	*<0.001*	*<0.001*	*0.48*	*0.18*	*0.005*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Glucose:Time	*0.034*	*0.007*	*<0.001*	*0.052*	*0.002*	*0.41*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
***Pb*:Glucose:Time**	*0.003*	*<0.001*	*0.52*	*<0.001*	*<0.001*	*0.01*	*0.041*	*<0.001*	*<0.001*	*<0.001*

Table 2: Glucose recovery after the incubation periods in the different char and ash treatments. Values are the mean (n = 6) ± S.D.

Glucose treatment	Day	Glucose recovered (% of initial amount added)				
		SP biochar	FP biochar	TS-G ash	LT-CFBG ash	FB-I ash
12.5 mg g^{-1} ash control	10	24 ± 16	7 ± 16	17 ± 8	91 ± 37	81 ± 10
	30	54 ± 34	4 ± 8	8 ± 0.4	43 ± 5	83 ± 4
12.5 mg g^{-1} ash + Pb	10	0	0	0	0	0
	30	0	0	0	0	0
25 mg g^{-1} ash control	10	61 ± 5	0	40 ± 33	49 ± 28	90 ± 3
	30	72 ± 26	0	12 ± 8	62 ± 31	46 ± 25
25 mg g^{-1} ash + Pb	10	0	0	0	0	0
	30	0	0	0	0	0