Article

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Calcium-Based Biochar Loaded with *Paenibacillus mucilaginosus* Acted as an Eco-Friendly Strategy to Enhance Soil Nutrients and Plant Biomass

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Abstract: The loose and porous structure and abundant pores of biochar provide conditions for being a suitable fertilizer carrier. Biochar loaded with beneficial microorganisms will greatly promote plant growth and soil improvement. In this work, an environmentally friendly *Paenibacillus mucilaginosus* loaded biochar-based fertilizer (B@PM) with high nutrient content, good slow-release effect and increased biological activity was prepared. The released mechanism of P-K-Ca from B@PM and the synergistic effect on the soil nutrients and plant growth were also analyzed. The experimental results showed that the release behavior of P, K and Ca from B@PM was diffusive and dissolved, which conforms to the Power function and Parabolic diffusion models, respectively. The potting experiment showed that the root length (30.77%), leaf width (71.67%), and plant height (81.08%) of Chinese cabbage were enhanced after the application of B@PM, and the organic matter, available phosphorus, available potassium, and total calcium in the soil were also substantially enhanced. In addition, the abundance of the *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* in the in the rhizosphere soil was increased, as well as the activities of catalase (82.84%) and alkaline phosphatase (39.64%). The results of RDA analyses showed that B@PM increased the content of the environmental factors, such as OM, URE, EC, TCa, AK, CAT, AP and ALP, thus improving soil quality and plant length.

Keywords: *Paenibacillus mucilaginosus*; biochar; release mechanism; RDA analyses; microbial diversity

1. Introduction

In traditional agriculture, chemical fertilizers are usually applied in large quantities to increase crop yields and raise food production. However, long-term reliance on chemical fertilizers to increase production has affected not only the ecological environment but also the quality of agricultural products to a great extent[1]. Although chemical fertilizers can provide a large amount of nutrients for a short period, they can also disrupt the soil pH balance, reduce the fertility of the soil itself and

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seriously affect the survival of beneficial bacteria and soil organisms[2,3]. Numerous studies have shown a positive correlation between the amount of applied fertilizer and the amount of nitrates in vegetables, the combination of nitrites and amines can form potent carcinogens that are hazardous to human health [1]. The development of new efficient and environmentally friendly fertilizers is urgent.

Paenibacillus mucilaginosus belongs to a genus of silicate bacteria *Bacillus* [4]. The *Bacillus* can decompose soil minerals such as phosphorus, potassium, silicate, aluminates and mica [5], releasing the phosphorus, potassium and other elements that can be absorbed and utilized by crops [6]. According to the characteristics of the strains, many researchers have prepared some of the dominant strains into microbial fertilizers and produced high application results [7–9]. Microbial fertilizer is a new type of fertilizer in agricultural production, which is a product containing specific microbial organisms and nutrient matrices [10]. After applying fertilizer, beneficial microorganisms activate soil fertility through their life activities, promote plant nutrient uptake, regulate plant growth, increase crop yields and prevent diseases[11]. Liu et al. investigated the effects of complex microbial fertilizer on the growth conditions and soil nutrients of tomatoes, and the results showed that microbial fertilizer would substantially increase the root length, plant height, and leaf area of tomatoes [12]. Similarly, Koryagin has demonstrated that microbial fertilizers boost crop growth and yield [13]. However, the population and survival time of microorganisms in bio-fertilizers is still a huge challenge [14]. Therefore, preparation of environmentally friendly and suitable microbial carriers becomes an essential solution.

The selected carrier should be insoluble, non-toxic, inexpensive, readily available, stable and renewable [15,16]. In addition, the carrier must have an excellent binding capacity to the microorganisms, and promote their functional properties [6,17]. As a new type of porous carrier with high surface area, porous structure, cation exchange capacity and easy functionalization, biochar shows great potential for application in microbial loading [18]. Wolna-Maruwka et al. explored the effects of biofertilizer that was derived from biochar loaded with microorganisms (algae, mycorrhizal fungi) on the soil and the environment [19]. The results of the two-year study showed that charcoal-based microbial fertilizers have a positive effect on both soil physicochemical properties and microorganisms under certain conditions. Song et al. applied bacterial-laden biochar to annual fir trees and showed that bacterial-laden biochar increased soil nutrients and promoted the diversity of soil microbial communities [15]. Although biochar has better loading properties for microorganisms, there are a lots of problems to common carriers (peat, clay, minerals) [20,21]: (a) the carrier has a single nutrient structure and fails to provide the nutrients required for microbial growth, which does not ensure microbial viability; (b) it can only be used to load with microorganisms and does not deliver nutrients to plant in a sustained manner.

Currently, biochar was usually used to load microorganisms or fertilizers [22–26], and few studies have investigated the loading of nutrient-enriched biochar on microorganisms, as well as the nutrient release pattern and soil improvement effect. In this study, an environmentally friendly biochar-based amendments (named B@PM) in alkaline soil with high nutrient content, good slow release and high bioactivity was prepared by loading *Paenibacillus mucilaginosus* on biochar, which was prepared by co-pyrolysis of eggshells (ES), potassium dihydrogen phosphate (KH_2PO_4), and phosphoric acid (H_3PO_4). The effects of nutrient-enriched biochar immobilized *Paenibacillus mucilaginosus* on soil nutrients, enzyme activities, and microbial community structure in rhizosphere soil were further explored. In practical application, the synergy mechanism of B@PM on soil nutrient-microbe-plant growth was also analyzed. This experiment provided new ideas for microbial fertilizer carriers and promoted the application of environmentally friendly fertilizers.

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2. Result and Discussion

2.1. Characteristics of B@PM & BCEKH

2.1.1. SEM and EDS Analysis of BCEKH& B@PM

The SEM scanning electron micrograph (Figure 1a) shows that many needle-like and massive crystals were generated on the surface and inside the biochar, partially combined with the biochar matrix. EDS analysis showed that the BCEKH biochar surface was enriched with high C, O, Ca, P and K elements and with small amounts of Mg and Si elements. In the process of pyrolysis, the reaction of KH_2PO_4 and H_3PO_4 with calcium carbonate ($CaCO_3$) in eggshells forms crystalline agglomerates [27]. These agglomerates preserve significant nutrients, including phosphorus, potassium, and calcium [28]. Both ICP-OES and EDS analyses provide evidence of the presence of these nutrients. By incorporating chemicals rich in phosphorus and potassium into maize stover, we can use the unique structure of biochar to conserve these nutrients[29]. The nutrients stored in biochar can nourish phosphorus-loving and potassium-soluble bacteria, supporting their survival [25].

Figure 1b allows visual confirmation that BCEKH has been inoculated with *Paenibacillus mucilaginosus*. The SEM images showed that most of the bacteria grew in the tubular structure and lateral folds of biochar. The culture of *Paenibacillus mucilaginosus* requires high amounts of phosphorus, potassium, calcium, and silicon. The potential of BCEKH as a suitable inoculum carrier was indirectly demonstrated through SEM and EDS images. BCEKH can provide survival nutrients and space for *Paenibacillus mucilaginosus* while reducing competition from other bacteria. This would increase the activity and function of the phosphoric and potassic bacteria microbial community, as evidenced by the findings of previous experiments[8]. This will improve the activity and function of the phosphate-potassium-solubilizing microbial community, which is also supported by the conclusions of previous experiments.

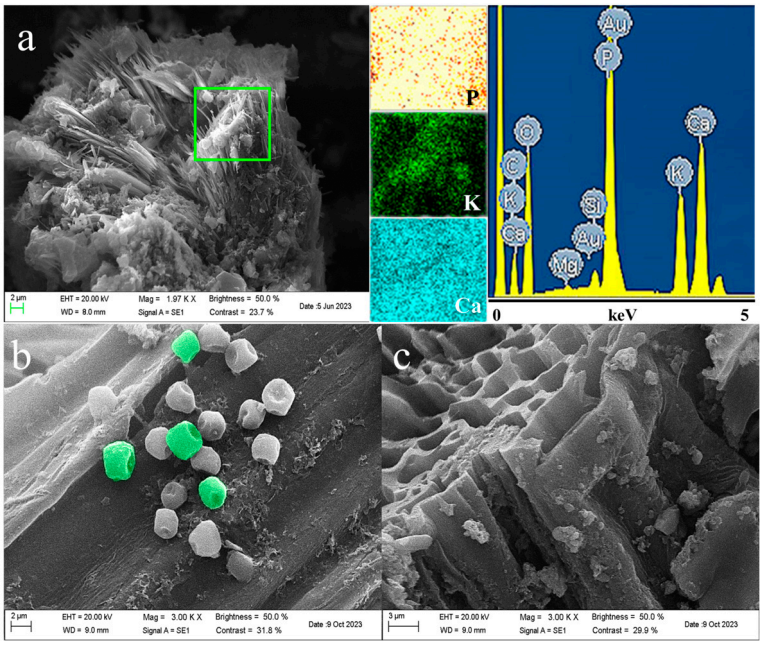

Figure 1. (a) SEM-EDS figures of BCEKH; (b) *Paenibacillus mucilaginosus* loading images on BCEKH; (c) SEM image of B@PM after 35d.

2.1.2. XRD and FTIR Analysis of BCEKH& B@PM

Figure 2a shows that BCEKH exhibits a strong peak of $K_2CaP_2O_7$ at 2θ (18.745°, 20.543°, 28.429°, 31.693°, 38.017, 41.504°)[30]. This result indicates that the $CaCO_3$ in ES reacted successfully with KH_2PO_4. This reaction retains the elements of phosphorus, potassium, and calcium in the feedstock. As a result, it increases the nutrient content in BCEKH[31]. The X-ray diffraction pattern showed that

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$Ca_2P_2O_7$ peaks appeared at 2θ of 20.18°, 23.893°, 26.652°, 27.674°, 29.867°, 35.285°, indicating that a part of $CaCO_3$ reacted with H_3PO_4 during pyrolytic heating process [32]. Previous studies have shown that phosphorus-modified biochar can increase the number and thickness of biofilms, further increasing beneficial microbial survival[24]. The crystals produced by BCEKH can provide *Paenibacillus mucilaginosus* with P, K, Ca, and other required elements. These crystals can effectively provide the nutrients required by the microorganisms in a continuous way. This proves that BCEKH is an excellent carrier for loading *Paenibacillus mucilaginosus*. Numerous studies have demonstrated that incorporating biochar with metallic or non-metallic materials as a carrier substrate can improve electron mobility and increase adsorption sites[17,33,34]. We believe that BCEKH provides more adsorption sites for microorganisms and improves their toxicity tolerance.

The peak at 1037 cm^{-1} in the FTIR pattern is attributed to the C-O stretching vibration of the aromatic ring (Figure 2b), the absorption peak at 1093 cm^{-1} is the C-O stretching vibration of the alcohol and ether groups, the peak at 1130 cm^{-1} may represent the P-O stretching vibration and the fluctuation at 719 cm^{-1} represents the P-O-P stretching[35]. Based on the analysis of the XRD patterns, the peaks at 908, 719, and 550 cm-1 may be due to $Ca_2P_2O_7$ and $K_2CaP_2O_7$. The surface functional groups of B@PM showed no significant changes compared to BCEKH, except in absorption peaks at 3743 cm^{-1}, 1633 cm^{-1}, and 1457 cm^{-1}. The peak observed at 3743 cm^{-1} is ascribed to the stretching vibration of -OH due to intermolecular hydrogen bonding binding alcohols and phenols[36]. The peak at 1633 cm^{-1} is due to the stretching vibration of C＝C, C＝O, and asymmetric stretching vibration peaks of COO- in the aromatic ring[7]. This indicates that the biochar has a stable aromatic skeleton, which is conducive to the adhesion and growth of bacteria and their propagation[37]. The peak at 1457 cm^{-1} shows a COO- bending vibration[38]. This indicated that electrostatic adsorption may play an important role in enhancing the loading of *Paenibacillus mucilaginosus* on BCEKH. In addition, it was deduced that the microorganisms use some of these alkaline groups on the biochar surface to complete their physical loading. The infrared spectra showed that *Paenibacillus mucilaginosus* was successfully loaded on modified biochar, and the functional groups of BCEKH biochar and the bacterium were combined to improve the bacterium's stability and the loading effect[39]. Both SEM and FTIR analyses revealed that the rough and porous surface of BCEKH provides a habitat for microorganisms to live and bind appropriately.

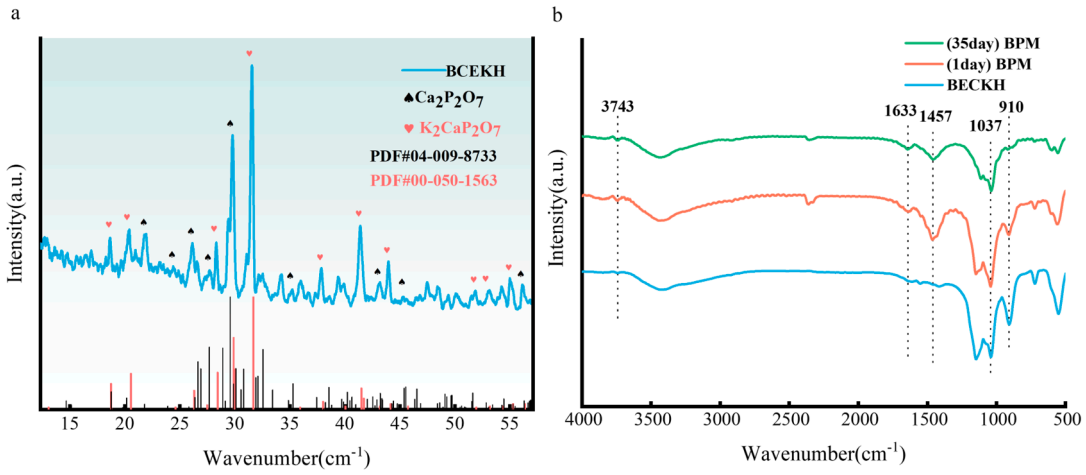

Figure 2. (a) XRD map of BCEKH; (b) IR maps of BCEKH, day 1 B@PM and day 35 B@PM.

2.2. Effective Number of Live Bacteria Loaded by B@PM

Effective bacteria counts were determined after storing BCEKH-loaded *Paenibacillus mucilaginosus* in a 4°C fridge for 1, 7, 14, 21, 28, and 35 days. This allows further assessment of BCEKH loading on *Paenibacillus mucilaginosus*. Since it was prepared through a relatively simple process and stored in a 4°C refrigerator, B@PM had a lesser effect on bacterial activity [40]. It can be seen from Figure 3 that after 35 days, the number of effective viable bacteria decreased from 4.84×10^8 to 1.4×10^7CFU/g. As per the Chinese compound microbial fertilizer standard (NY/T 798-2015), the solid-

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type dosage form must contain a minimum of 0.2×10^8 CFU·g^{-1} of live bacteria. B@PM had higher viable bacterial values within one month than the composite microbial fertilizer standard. These findings suggest that BCEKH should be an excellent agricultural inoculant carrier for *Paenibacillus mucilaginosus*.

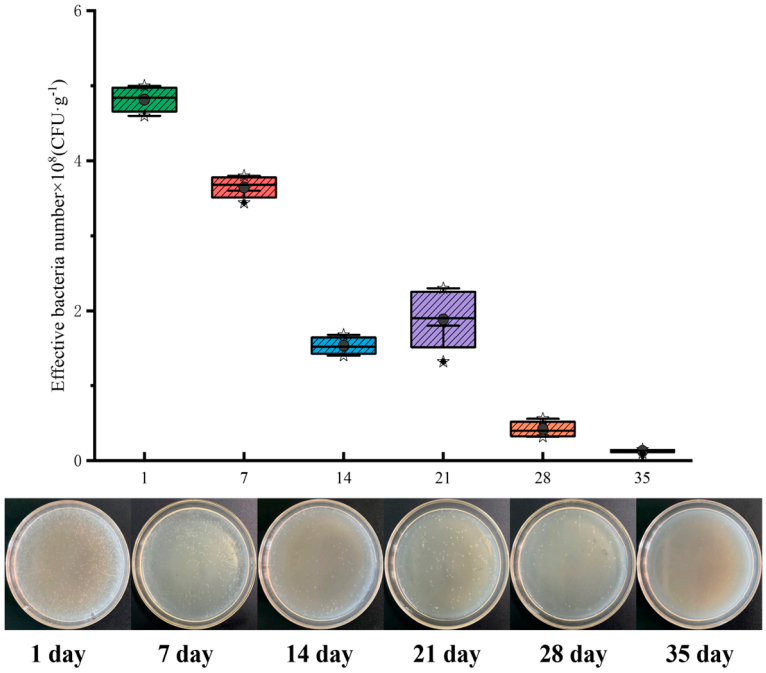

Figure 3. Effective number of viable bacteria for B@PM loads.

2.3. Slow-Release Behavior of the B@PM

Soil column leaching experiment was conducted followed by the potting experiment to investigate the release of nutrients from BCEKH and B@PM during their practical utilization in soil. Throughout the experiment, the temperature was kept constant within the range of 23°C to 28°C, while the humidity remained within the range of 35% to 47%. The results in Figure 4 indicated that the elements P, K, and Ca of the CS group exhibited similar release pattern, with the total release of 0.897mg·ml $^{-1}$, 1.122mg·ml $^{-1}$, and 1.091mg·ml $^{-1}$, respectively, after 35 days. The release of P and K was considerably higher in the J group than in the CS group, and the cumulative release was about eight times higher than in the CS group. This statement indirectly proves that *Paenibacillus mucilaginosus* can break down insoluble phosphorus and potassium elements in the soil. Additionally, it can break down potassium minerals (such as feldspar and mica) and phosphorus-containing minerals (such as apatite) in the soil, transforming them into water-soluble phosphorus and potassium[41]. This process is favourable for the uptake of water-soluble phosphorus and potassium by crops. The concentration of nutrients in the BCEKH's drench solution increased continuously from day 1 to day 14. However, it decreased significantly after 4 times of leaching. Cumulatively, 36.80%, 57.16%, and 31.71% of their P, K, and Ca were released in the soil within 35 days. It is worth noting that BCEKH shows better Ca release performance than CS. The slow release of nutrients by B@PM (10TJ, 20TJ, 30TJ) was significantly higher than BCEKH. The release increased with the concentration of the bacterial solution (30TJ > 20TJ > 10TJ). This may be due to the decomposition of insoluble salt crystals ($Ca_2P_2O_7$ and $K_2CaP_2O_7$) from BCEKH into water-soluble nutrients by *Paenibacillus mucilaginosus*.

The fitted slope parameters (K1) and R2 of the six sets of release kinetic equations are shown in Table (S3, S4, S5). The release of P, K and Ca by BCEKH and B@PM was consistent with the Power function and Parabolic diffusion models, with R^2 greater than 0.99. Based on the Power function, it has been observed that the release of P and Ca by BCEKH and B@PM increases with time. The initial release of the 30TJ group is the highest. Furthermore, P and Ca releases are more aligned with the

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Power function as indicated by the parameters c and an in the Power function. Kinetic equations have rarely been used in previous studies to assess the release of modified biochar-loaded bacteria in soil, and the kinetics of modified biochar on P in static water generally conforms to the Power function model and Parabolic diffusion model[32,42]. In this case, the release of K is more in line with the Parabolic diffusion model (R^2 up to 0.99 in both cases), which proves that the release of K is more likely to be diffuse. The A-constant of the Parabolic diffusion model determines the rate at which a substance is released[43]. The A-values of BCEKH and B@PM (10TJ, 20TJ, 30TJ) were 8.166, 8.952, 9.257, and 9.344, respectively. This means that the K release rate was faster in the B@PM (30TJ) group compared to the other groups. Both the Power function model and the Parabolic diffusion model are good fits for the release of K, with similar R^2 values. When the R^2 values are similar, a consistent process may be responsible for long-term releases[44]. Phosphorus release can be controlled by a combination of diffusion and dissolution rather than merely diffusion.

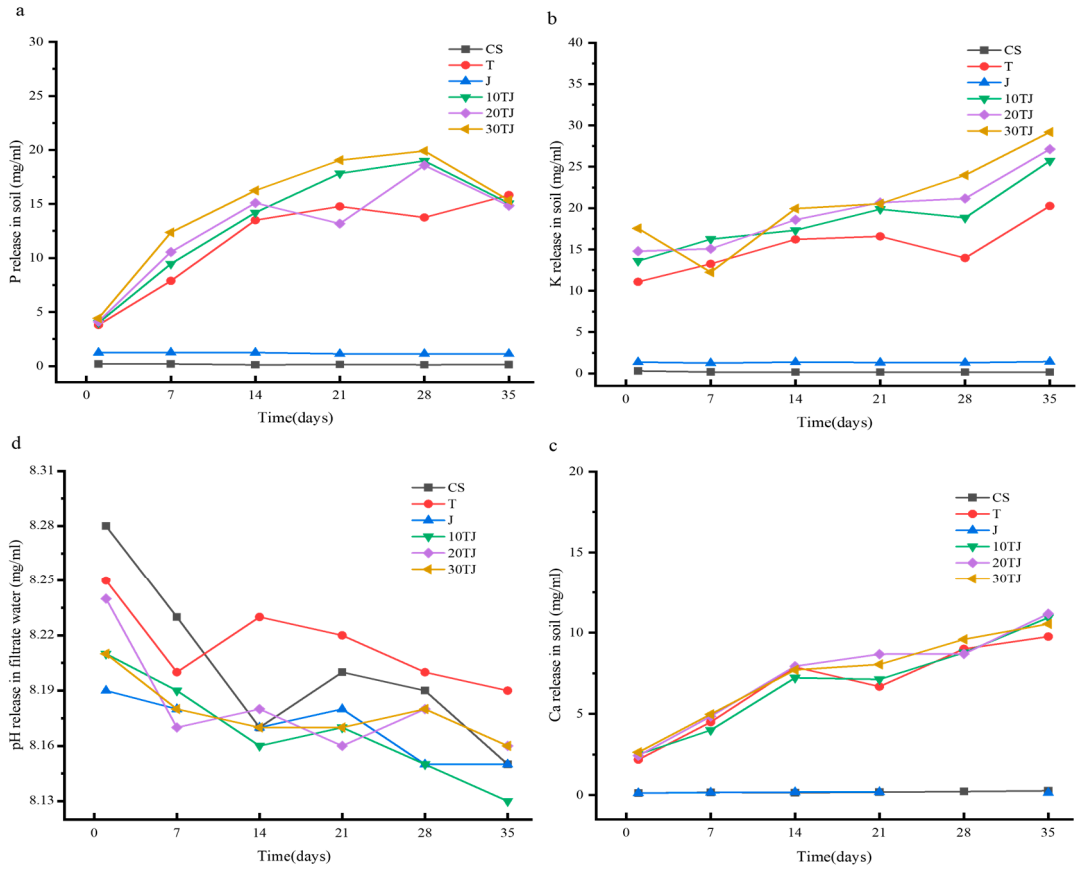

Figure 4. P-K-Ca leaching column experiments for each group (CS, T, J, 10TJ, 20TJ, and 30TJ) in potting experiments with (a) slow-release curves for P in soil for each group; (b) slow-release curves for Ca release in soil for each group; (c) slow-release curves for Ca release in soil for each group; and (d) changes in the pH of the leaching solution during the slow-release process.

2.4. Effect of B@PM on Soil Physico-Chemical Properties and Enzyme Activities

The physicochemical properties of the soil in each group before and after planting are shown in Figure 5. After 35 days of planting, the pH of the soil decreased in all groups, which is likely to be due to the secretion of organic acids by the root system of the plant during the growth process. Further observation shows that the groups had less effect on the pH of the soil (Figure 5a). This is due to the soil itself has a specific buffering capacity. Application of quantitative amount of biochar does not change the pH of the soil evidently, which corresponds to the previous conclusion [45,46]. The EC values of the B@PM group (10TJ, 20TJ, and 30TJ) were greater than those of CS, T, and J. The EC values gradually increased as the concentration of the B@PM load *Paenibacillus mucilaginosus* increased (Figure 5b). This may be due to the accelerated decomposition of nutrients in BCEKH by

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Paenibacillus mucilaginosus, and the decomposition of $Ca_2P_2O_7$ and $K_2CaP_2O_7$ increases the ionic concentration in the soil. This increased the EC, the effective phosphorus (AP), effective potassium (AK), and total calcium (TCa) content of the soil (Figure 5d, e, f). Nutrients were significantly higher in all groups after 35 days of planting. AP, AK and TCa of group T were increased by 123.15%, 38.24% and 46.67%, respectively, in soil as compared to CS. The levels of AP, AK and TCa were increased in the B@PM group by 22.25% to 54.69%, 34.12% to 93.58% and 56.81% to 73.86%, respectively, on compared with T. In addition, the metabolites of *Paenibacillus mucilaginosus* contain phytohormones and organic acids, which can promote the growth of apple seedlings and the accumulation of potassium in apple plants [47].It is well-known that organic matter (OM) indicates soil fertility. In Figure 5c, the OM content of the 20TJ group was 28.11%, which was 38.00% higher than the CS group. Previous studies have demonstrated thatcombination of *Bacillus subtilis* SL-44 and apple wood biochar also significantly enhanced the organic matter content, available phosphorus (AP), and total nitrogen (TN)[24]. In summary, the content of quick nutrients and organic matter of soil in the B@PM group was much higher than that of the CS, T and J groups. The Soil physicochemical properties of B@PM group are arranged as follows: 20TJ > 10TJ > 30TJ. Since it is more important to improve the physical and chemical properties of the alkaline soil with suitable microbial fertilizer [48], the co-application of BCEKH with *Paenibacillus mucilaginosus* is a sustainable method to increase soil organic matter and effective plant nutrients.

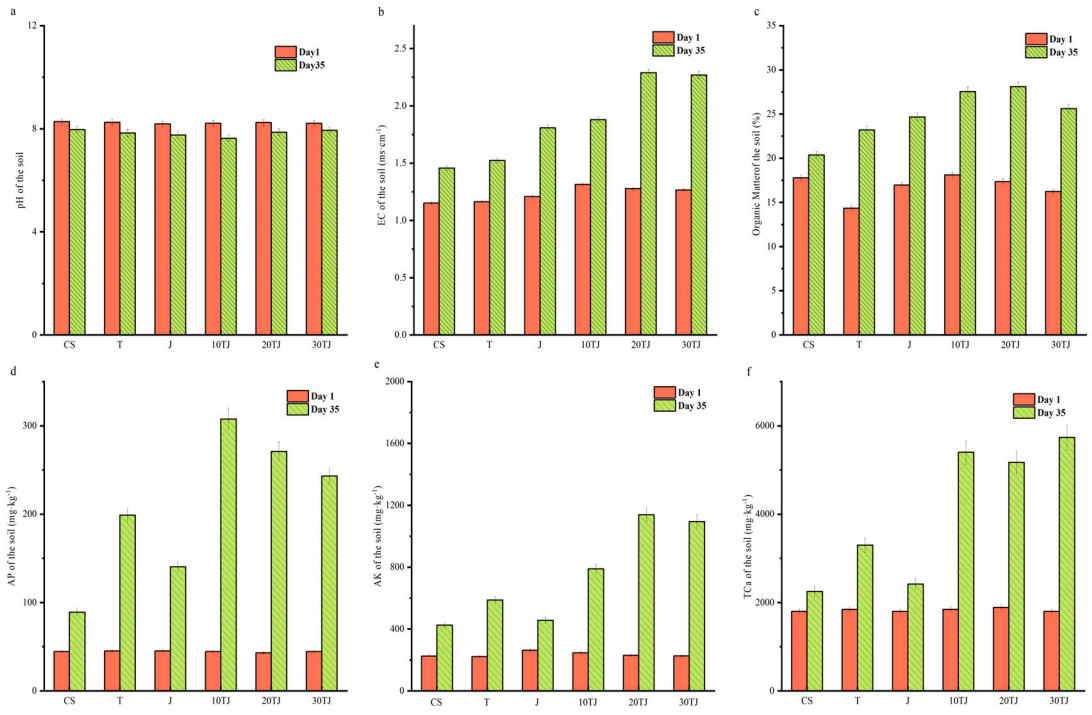

Figure 5. (a) the pH of the soils in each group after 1 and 35 days of planting; (b) EC of the soils in each group; (c) organic matter content of the soils in each group; (d) effective phosphorus content of the soils in each group; (e) effective potassium content of the soils in each group; (f) total calcium content of the soils in each group.

2.5. Effect of B@PM on Plant Growth Conditions

According to Figure 6e, the growth sizes of Chinese cabbage are followed as: 10TJ > 20TJ > 30TJ > J > T > CS. As can be seen from Figure 6a, the root weight and single-leaf weight of Chinese cabbage in the 10TJ treatment reached the maximum, which were 4.21 and 1.93 times higher than that of the blank control group (CS), respectively. The growth status of the edible tissues of cabbage is shown in Figure 6b. The B@PM group (10TJ, 20TJ, and 30TJ) grew 1.81, 1.55, and 1.45 times higher than the CS group. Leaf width and root length of the T, J and B@PM groups were higher than the CS group.

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Compared with the control, it was noted that the root length and leaf width increased nearly by30.77% and 71.67%, respectively. The study found that the treatment of 10 TJ resulted in the highest growth condition and biomass of potted plants. However, it was also observed that an increase in the concentration of carrier bacteria led to a gradual decrease in the growth condition and biomass of chinese cabbage. It was demonstrated that low dose of BCEKH loaded with *Paenibacillus mucilaginosus* promotes plant growth and increases cabbage yield. This was consistent with the previous studies [9,24–26,44].

It was noted that the soil urease activity fluctuated to some extent with the nitrogen level in the soil, and there was no significant difference in initial soil urease (UR) between the groups (Figure 6c). No nitrogen fertilizer was applied in all of the groups during the planting. After 35 days of planting, as shown in Figure 6d, there was an increasing trend in urease for all groups. However, the growing trend in the CS and T groups was insignificant and similar to the initial values. J and B@PM groups had a more significant effect on urease activity in the soil, which proves that *Paenibacillus mucilaginosu* may have some nitrogen fixation effect during cabbage cultivation[49,50]. It was suggested *Paenibacillus mucilaginosu* has a great potential to mineralize organic nitrogen to ammonium nitrogen during plant growth and fix ammonium nitrogen in combination with biochar in the soil. The catalase activity of T group in the soil was 14.92% higher than that of CS. This is due to not only the protective effect of the porous structure of the biochar, but also the nutrient contained in biochar contribute to the growth of microorganisms, which could promote the cycling of carbon and nitrogen.

After 35 days of planting, the catalase activity (CAT) level increased by 37.11% for the treatment of 10 TJ, and 82.84% for 30TJ on compared with the initial data in soil. Compared with the blank control group B@PM group increased by 82.84%(30TJ).~37.11%(10TJ). This is possiblely related with the fact that *Paenibacillus mucilaginosus* promotes the decomposition of insoluble phosphorus and potassium. In fact, the urease and catalase activities were also increased while applying maize biochar loaded with Pseudomonas sp. NT-2 in the soil during 75 days of potting experiment [7]. This suggested that the synergy of biochar and microorganisms is important for soil improvement. Conversion of organic phosphorus into readily absorbed inorganic phosphorus is strongly correlated with phosphatase. Higher alkaline phosphatase (ALK) levels in the rhizosphere soil mainly promote plant root growth and increase plant phosphorus uptake[51]. Figure 6 shows a noticeable increase in enzyme activities across all groups after 35 days of growth in Chinese cabbage. The activity of alkaline phosphatase in the rhizosphere soil for the 10TJ treatment increased greatly by 39.64%. In general, the content of available nutrient and enzyme activities for all of the treatments of BCEKH, *Paenibacillus mucilaginosus* and B@PM increased, especially the low dose (10%) of complex.

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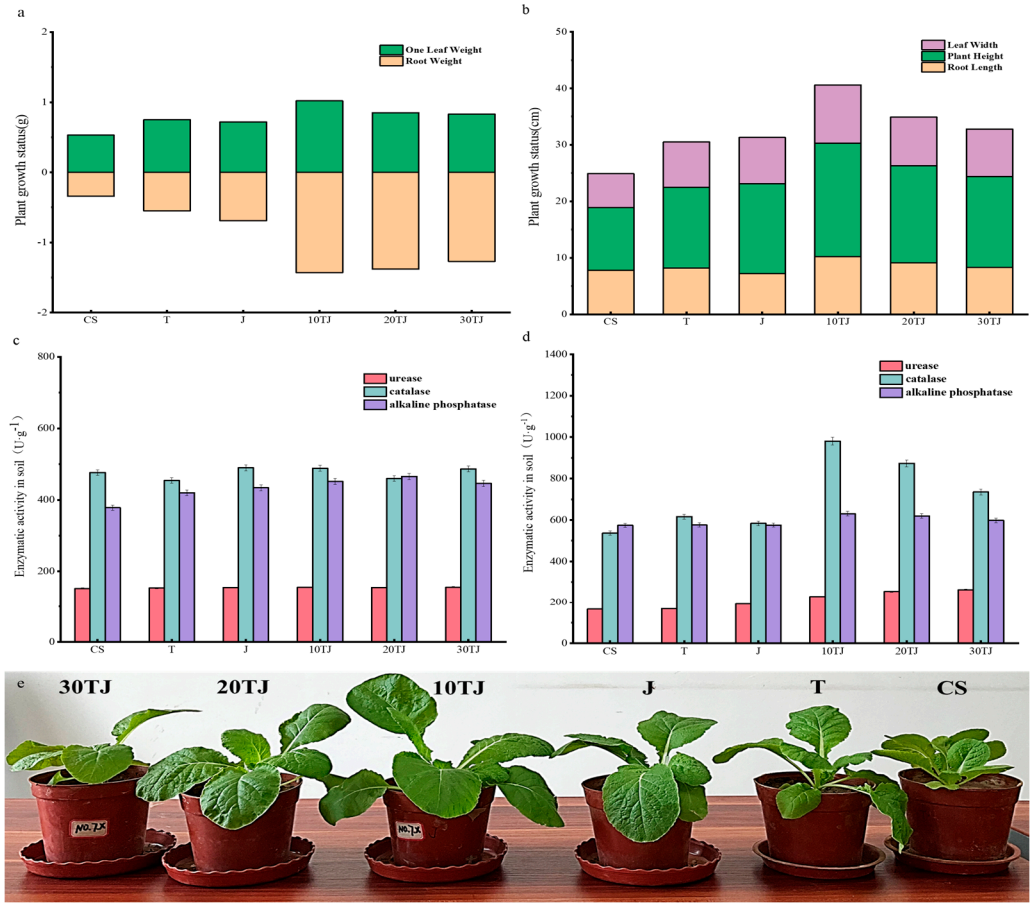

Figure 6. (a) Cabbage plant height, root length and leaf width in each group; (b) Cabbage leaf weight and root weight; (c) Initial soil enzyme activity in each group; (d) Soil enzyme activity in each group after 35 days. (e) Plant growth after 35 days.

2.6. Effect of B@PM on Microbial Communities in Soil

2.6.1. Microbial Community Structure

After harvesting cabbage, the effect of B@PM on soil bacterial community structure was analyzed using high-throughput sequencing technology. The bar graph in Figure 7a shows that *Proteobacteria* was the most abundant species before planting, followed by *Acidobacteria*. The addition of BCEKH and *Paenibacillus mucilaginosu* increased the abundance of *Proteobacteria*, *Cyanobacteria-Chloroplast*, *Bacteroidetes*, and *Firmicutes* while decreasing the abundance of *Verrucomicrobia*, *Planctomycetes*, and *Gemmatimonadetes* on compared with CS. At the time of crop harvest (Figure 7b), the abundance decreased from 42.61% to 14.51%, whereas it still remained at a high level for Firmicutes branch, which also included the *Paenibacillus mucilaginosus*. Compared with CS, the application of BCEKH, *Paenibacillus mucilaginosus*, and B@PM increased the abundance of *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. Treatment of B@PM significantly reduced the relative abundance of *Verrucomicrobia*, *Nitrospirae*, and *Cyanobacteria-Chloroplast*. It was found a negative correlation between the *Verrucomicrobia* gate and the nutrient content of the soil, which corresponds to previous studies [52]. *Nitrospirae* is commonly associated with the initial stage of nitrification in soil [53], during which ammonium ions oxidized to nitrite. Therefore, it was suggested that excessive amount of *Nitrospirae* has a detrimental impact on the overall health of environment.

The above study found that application of *Paenibacillus mucilaginosus* increased not only the soil content of fast-acting phosphorus and fast-acting potassium, but also the activities of UR, ALK, and CAT. On the other hand, many nitrogen-fixing communities are associated with the above species, including *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*[54], from which we infer that B@PM has a nitrogen-fixing effect on soil. It has been shown that applying biochar will significantly increase the

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abundance of *Proteobacteria* and *Actinobacteria* [26,55]. This study found that B@PM led to an increase in the abundance of the Bacteroidetes group, which is essential for carbon and nitrogen cycling in soil, as well as ecological stability [56]. In this case, it was believed that Bacteroidetes also influence the activities of UR, ALK, and CAT. The experimental results showed that BCEKH, a carrier of *Paenibacillus mucilaginosus*, increased the abundance of soil microbial and activities of enzymes in rhizosphere soil. The reason is ascribed to not only the nutrients (C, N, P, K, Ca) that provided by the BCEKH for the growth of *Paenibacillus mucilaginosus*, but also a suitable living space for bacteria, protecting them from soil drought and offering a moisture zone. As beneficial microorganisms in agriculture, most of the *Firmicutes* species can help plants access essential resources, such as nitrogen, phosphorus, calcium, potassium, iron, and other vital minerals, and inhibit the growth of plant pathogens [57]. Thus, it was believed that utilization of the biochar carrier could greatly increase the survival probabilities of the *Firmicutes* gate.

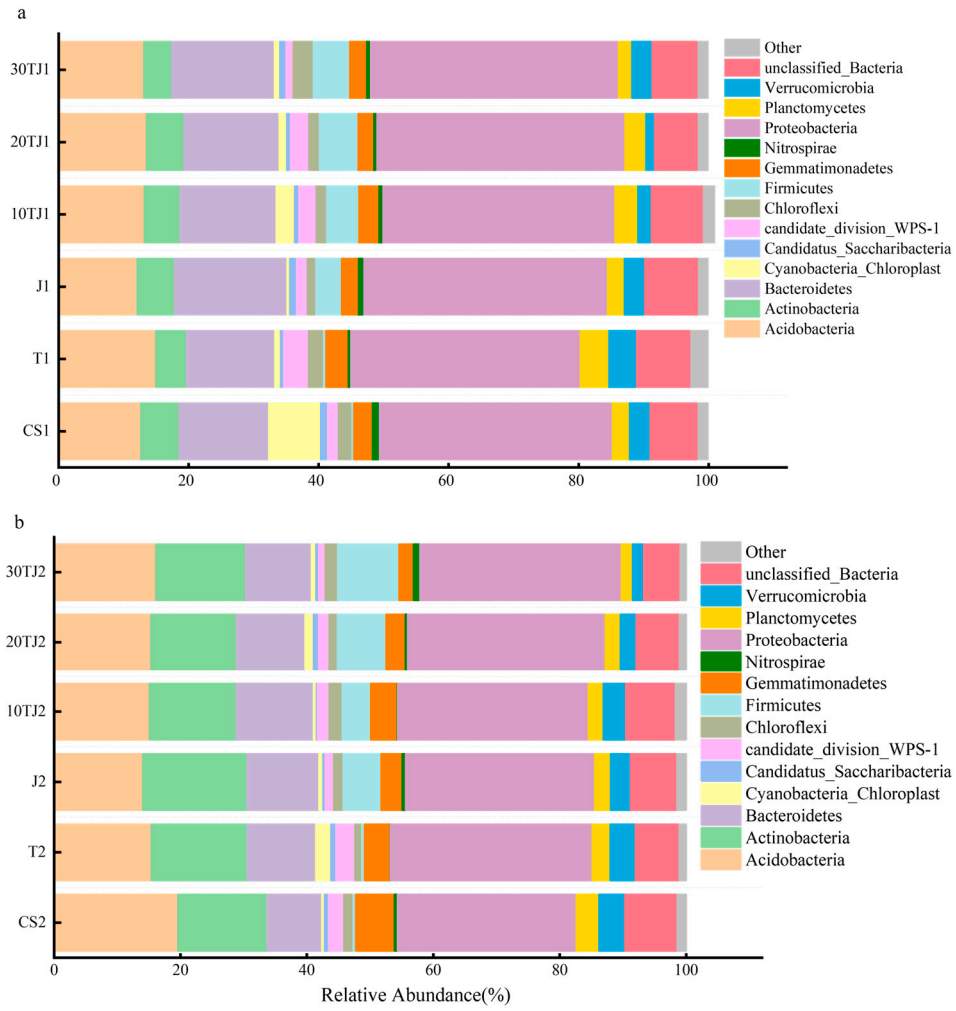

Figure 7. (a) Bacterial abundance of soil 16SrRNA at the gate level on the first day of planting; (b) Bacterial abundance of soil 16SrRNA at the gate level after planting.

2.6.2. RDA Analyses between Samples and Environmental Factors and Species

In this text, redundancy analysis (RDA) was used to determine the main environmental factors that contributed to variations in soil microbial communities. The soil microbial community structure indicators were used as the response variables, while the soil physicochemical properties and enzyme activities were acted as the critical indicators. Figure 8 shows that the horizontal and vertical axes totally explained 83.28% of the variation in microbial community composition of the soil samples. The analysis found that the species composition of treatments of 30TJ, 20TJ, 10TJ, and J was more

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similar to each other except the T group at the end of the planting period. However, it was observed that the species composition of T and J groups was more similar to each other at the initial stage of planting. This proved that the modified role of microbe loaded biochar is much more significant than that the biochar, which may just provide some nutrients rather than change the dominant bacterial species [22]. Soil enzyme activities and nutrients strongly correlated with *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospirae*, and *Firmicutes*, which was considered as the dominant species. The correlation between the dominant bacterial species and environmental factors after harvest was ranked as follows: OM > URE > EC > TCa > AK > CAT > AP > ALP > pH. There is a strong correlation between soil ALP and AP with *Proteobacteria* and *Bacteroidetes*. The amount of *Proteobacteria* and *Bacteroidetes* in soil increases with the levels of ALP and AP. *Acidobacteria, Actinobacteria, Gemmatimonadetes,* and *Verrucomicrobia* were strongly associated with pH. Similarly, previous studies have found a correlation between *Acidobacteria* and *Verrucomicrobia* and soil pH [52,58]. Based on the vertical distance between the samples and the environmental factors, it can be observed that the OM and URE activities were higher for 30TJ and 20TJ treatments than the 10TJ group. However, the AP, ALP, and CAT of the 10TJ group were greatly increased, which was consistent with the previous report [24]. Compared with groups T, J, and CS, the B@PM groups were more strongly associated with other environmental factors besides PH. It is reasonable to assume that *Paenibacillus mucilaginosus*, a BCEKH load, is an excellent fertilizer based on biochar. Therefore, application of B@PM improved significantly the microbial community structure in the rhizosphere soi, nutrients, and enzyme activities.

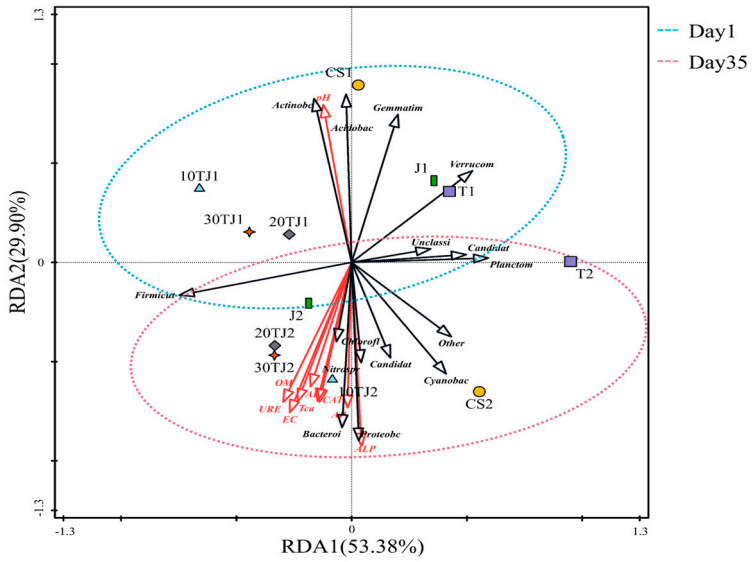

Figure 8. Plot of RDA analysis between samples and environmental factors and species bacterial communities, with red arrows representing soil environmental factors, black arrows representing bacterial communities at the gate level, and different shapes and colours of samples representing other treatment groups.

3. Materials and Methods

3.1. Materials

The straw (CS) used in this experiment was obtained from farmland around Yuzhong County, Lanzhou City, Gansu Province, China, and the eggshells (ES) used were obtained from the canteen of Northwest University for Nationalities. KH_2PO_4 (AR), H_3PO_4 (AR, 85%, China) has been purchased from Shanghai Experimental Reagent Co. All solutions were prepared using deionized water (16.37 MΩ).The research team collected the experimental soil from the surrounding areas of Yuzhong County, Lanzhou City (35.929°N-35.933°N; 104.179°E-104.181°E). Remove stones and plant roots from the soil and pass through a 10-mesh sieve. The physico-chemical properties of the test soils were

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shown in Table S1, the low nutrient content was due to the poor alkaline soil collected from Northwest of China.

Biochar (BCEKH) was mass-produced from CS, ES, KH_2PO, and H_3PO_4 in a molar ratio of P:Ca=1:2 in a mullite sagger (160 cm×160 cm×70 cm) and a box-type resistance furnace (SX-4-10, Beijing Kewei Yongxing Instrument Co., Ltd.) at 800 ℃. The pyrolysis process utilizes sand (SiO_2) and purges nitrogen to achieve an oxygen-limited state, with the unit heating up at approximately 15-20℃/min. BCEKH was ground and sieved through a 100-mesh sieve, washed with deionized water, and then dried in an electrically heated blast drying oven (DHG-9245A, Shanghai Yiheng Scientific Instrument Co., Ltd.) at 85℃ for 3 hours. The bacterial strain *Paenibacillus mucilaginosus* used in this experiment was obtained from the BeNa Culture Collection (BNCC). The culture conditions were 28℃, aerobic, silicate bacterial medium, 24 h-48 h. Silicate bacterial medium was prepared with sucrose 5.0 g, disodium hydrogen phosphate 2.0 g, magnesium sulphate 0.5 g, ferric chloride 0.005 g, calcium carbonate 0.1 g, and bauxite 0.5 g (pH 7.1±0.2). To prepare the biochar-bacterial material (B@PM), 0.5 g of biochar material (BCEKH) was firstly mixed with the bacterial broth of *Paenibacillus mucilaginosus* (OD_{600}=0.53) in the ratio of 1:200 (m: V), and oscillated for 24 h at 28℃ and 120 rpm. The biochar was incubated in a constant temperature incubator at 28℃ for 12 hours after centrifugation of the suspension at low temperature to obtain B@PM and then placed in a refrigerator at 4℃ for further study.

3.2. Characterization of Modified Biochar

The micro-morphology of BCEKH and B@PM were observed by scanning electron microscopy (SEM, EVO18, Carl Zeiss Nanomaterials GmbH, with X-MAX detector, Oxford Instruments), and the surface elements of BCEKH were analyzed by X-ray energy spectroscopy. Surface functional groups of BCEKH and B@PM were analyzed by Fourier transform infrared spectroscopy (FTIR，Nicolet is5 ，Thermo). X-ray diffraction (XRD，X pert PRO，PANalytical B.V.) was measured in the range of 4° to 80° (2θ), and the resulting data were analyzed with the ICDD-PDF-4+ database.

3.3. Determination of Effective Bacterial Count

The effective number of viable bacteria of B@PM stored in a 4℃ refrigerator was determined by plate dilution coating method on days 1, 7, 14, 21, 28 and 35, respectively, and this experiment can further evaluate the loading effect of BCEKH on *Paenibacillus mucilaginous* [59]. The number of viable bacteria loaded on BCEKH was calculated by plate colony counting after shaking B@PM in sterile water and diluting to 10^{-5}g/ml with deionized water, coated and incubated in a 28℃ constant temperature and humidity chamber (LHS-150HC-I, Shanghai Yiheng Scientific Instrument Co.) for 24 h. The plate colony counting method calculated the number of viable bacteria loaded on BCEKH [60].

3.4. Potting Experiment

The potting experiment was conducted in the Yuzhong Campus of Northwest University for Nationalities (35.999°N, 104.24429°E). The experiment was conducted from 5 July 2023 to 10 August 2023 with a sunshine duration of about 14/6 h and a daytime temperature of 28℃. Firstly, cabbage seeds of uniform size were sterilized by soaking them in 70% alcohol (30 seconds), and then the seedlings were raised and planted. Afterwards, the plants were planted in plastic pots (8 cm × 8 cm × 10 cm) that contained 500 g of soil with water content maintained at about 35%. The experiment consisted of 6 separate groups, with 3 parallel groups. The control group (CS) was the group without fertilizer, (T) the group with BCEKH applied (Biochar mass ratio of 0.2% of soil mass), (J) the group with *Paenibacillus mucilaginosus* applied (Bacterial solution 10% of biochar mass), (10TJ) the group with B@PM loaded with a low percentage of bacterial solution (Bacterial solution 10% of biochar mass), (20TJ) the group with B@PM loaded with a medium percentage of bacterial solution (20% of biochar mass), and (30TJ) the group with B@PM loaded with a high percentage of bacterial solution

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(30% of biochar mass). Plants and the rhizosphere soil samples were harvested separately after 35 days of potting.

3.5. Kinetics of Nutrient Release from Biochar in Soil

The slow release behavior of different groups (CS, T, J, 10TJ, 20TJ, 30TJ) in the potting experiment was further investigated using the soil column leaching experiment with three replications for each treatment group [61]. In the experiment, the leaching solution was collected at 1, 7, 14, 21, 28 and 35d. After passing through a 0.45 μm nylon filter, the P, K and Ca in solution was analyzed by using ICP-OES(5110，Agilent) and fitted according to the following equations:

The zero order (linear)

$$q_t = a_0 + k_0 t \quad (1)$$

Pseudo-first order

$$\mathrm{Ln}(q_e - q_t) = \mathrm{Ln}q_e - k_1 t \quad (2)$$

Pseudo-second order

$$\frac{t}{q_t} = \frac{1}{k_1 q_e^2} + \frac{t}{q_e} \quad (3)$$

Elovich equation

$$q_t = \frac{1}{\beta}\mathrm{Ln}\alpha\beta + \frac{1}{\beta}\mathrm{Ln}t \quad (4)$$

Parabolic diffusion model

$$q_t = A + R \cdot t^{\frac{1}{2}} \quad (5)$$

Power function

$$\mathrm{Ln}q_t = \mathrm{Ln}\, a + c\mathrm{Ln}\, t \quad (6)$$

Where, q_t is the cumulative nutrient released at time t; q_e is the maximum nutrient released. a_0 is a constant; α is the initial release constant; β is the release constant; k_0 and k_1 are rate constants; R is the intraparticle diffusion rate; and A is a constant proportional to the boundary layer thickness.

3.6. Analysis of Plant Growth

At the time of harvesting the cabbages on 10 August, the plant height, leaf width and root length of cabbages were measured three times for each group, and the averaged results were obtained. Three representative leaves and intact roots of Brassica napus were taken from each group and placed in a refrigerator at 4℃ for freshness, which was used to determine the fresh weight of single leaves and roots; after determining the fresh weight, the roots and leaves were placed in an oven at 75℃ and dried to a constant weight for determining their dry weights. The averaging method was used to determine the fresh and dry weights.

3.7. Analysis of the Soil Physicochemical Properties

After removing large particles from the collected fresh soil, 2.5 g of soil was added to 25 mL of deionized water, stirred to disperse the soil particles nicely, and left to stand for half an hour to determine the pH and electrical conductivity (EC) values. The electrical conductivity (EC) and pH values were obtained in triplicate. The pH was determined in a pH meter (PHS-3C, Shanghai Yidian Scientific Instrument Co.) and the EC in a conductivity meter (DDS-307, Shanghai Yidian Scientific Instrument Co.), calibrated prior to measurement. In this experiment, soil organic matter (OM), adequate phosphorus (AP), quick-acting potassium (AK) and total calcium (TCa) concentrations were all measured using a soil nutrient tachometer (YT-TRB, Shandong Yuntang Intelligent Technology Co.). The activities of soil urease (URE), catalase (CAT) and alkaline phosphatase (ALP)

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were determined using soil enzyme activity kits (A121-1-1\T005\T010, Nanjing Jiancheng Bioengineering Research Institute) [26].

After 35 days of planting, the soil between the roots of each group of plants was collected and frozen with liquid nitrogen. The frozen soil was then stored in a refrigerator at -80℃. The bacterial communities in the soil were detected by amplifying DNA fragments using polymerase chain reaction (PCR) and then subjecting them to high-throughput gene sequencing of 16SrRNA [62]. To extract the DNA, individual soil samples were processed using the E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA，M5635-02). The first round of PCR amplification was carried out with the bacterial V3-V4 region primer 341F-805R (CCTACGGGGNGGCWGCAG) using a PCR instrument (ETC 811, Beijing Dongsheng Innovation Biotechnology Co.). A bridge PCR was performed with the Illumina platform in the second round of PCR (Bridge amplification)[9]. Library size was detected by 2% agarose gel electrophoresis (FR-1000, Shanghai Fuzzy Technology Co. Ltd.), and library concentration was determined using a Qubit 3.0 fluorescence quantifier (Q33226，ThermoFisher), with all samples mixed in 1:1 aliquot. The composition of communities in each set of soil samples was determined using four databases (RDP 16S, Silva 16S, NCBI 16S, and GTDB).

3.8. Statistical Analysis

Data was processed by using Origin Pro 2021, MDI Jade9, Canoco 5, Cinema 4D 2021, and Microsoft Office Student Edition 2021. The statistical test Student-Newman-Keuls method is used to compare multiple sample means. Differences are considered statistically significant if the p-value is less than or equal to 0.05. Shannon and Simpson's community diversity index was utilized to estimate the microbial diversity index in the samples. The diversity index is commonly used to reflect the alpha diversity index [63]. Multivariate data were analyzed using PLS-DA to differentiate observations between groups and identify influenced variables.

4. Conclusion

Biochar (BCEKH) prepared by maize stover, eggshells, potassium dihydrogen phosphate, and phosphoric acid was used as a carrier for *Paenibacillus mucilaginosus* in this experiment. The loading effect of *Paenibacillus mucilaginosus* on BCEKH was investigated during the experiment, and the synergistic mechanism between them was also analyzed. The rich nutrients, large specific surface area and pore size of BCEKH can provide a good place for loading *Paenibacillus mucilaginosus*. In this experiment, we studied the way of nutrients, such as phosphorus (P), potassium (K), and calcium (Ca), released from B@PM in alkaline soil. We also examined how this fertilizer affects soil properties and the activity of soil microbes and enzymes. Finally, the practical application of this fertilizer in soil shows that it can improve the soil quality. The experimental results showed nutrient from BCEKH and B@PM are constantly released, where K was diffused and P and Ca were diffused and solubilised. Compared with CS, application of B@PM can substantially increase not only AP, AK, and TCa but also EC and OM in the soil. In the potting experiment, B@PM similarly promoted Chinese cabbage growth, especially the 10TJ group. Applying B@PM promoted the abundance of dominant species in the rhizosphere soil, such as *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. RDA analysis revealed strong associations between B@PM and *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospirae*, and *Firmicutes*. Eight environmental factors, OM, URE, EC, TCa, AK, CAT, AP and ALP, had a serious impact on the species in the soil. Therefore, the production of biochar-based fertilizer loaded with *Firmicutes phylum* was considered as good soil remediation agent with significant practical value.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Data Availability Statement: All available data are contained within the article.

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