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Microbial-mediated coal gangue reclamation: enhancing alfalfa growth with potassium and phosphorus-solubilizing bacteria

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Abstract: Large accumulations of coal gangue (CG) pose significant environmental challenges, including land occupation, soil degradation, water contamination, and air pollution. Therefore, the ecological and sustainable utilization of CG is urgently needed. This study aimed to isolate microorganisms from various environmental samples that are capable of solubilizing potassium and phosphorus in CG. The bacteria exhibiting the greatest solubilization potential were identified and characterized, and their effects on alfalfa growth were evaluated in pot experiments using soil mixtures of CG and sandy soil. The findings indicated that *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens* significantly improved alfalfa germination rates, whereas *Citrobacter freundii* and *Bacillus velezensis* were effective in enhancing its growth. These results suggest a promising approach for the clean and sustainable utilization of CG through the application of these microorganisms.

Introduction

Coal gangue (CG), a byproduct of coal mining, encompasses various waste materials, including tunnel gangue from excavation, waste extracted from the coal seam, and material from washing processes (Yuguo et al. 2019; (Yuxin et al. 2019). This waste is complex, primarily composed of inorganic substances such as silicon dioxide and aluminum oxide, with minor organic components (Haobo et al. 2022). It also contains trace elements, including iron, calcium, magnesium, sodium, potassium, and phosphorus, as well as rare metal oxides such as gallium, vanadium, titanium, and cobalt (Bin et al. 2023; (Yuting et al. 2021). The presence of heavy metals in CG is a concern, as prolonged exposure to rainwater can lead to the leaching of these harmful elements into the soil and groundwater, causing environmental pollution (Zhenqi et al. 2020). In extreme cases, this can lead to water body acidification, degradation of water quality, and adverse effects on the environment and aquatic life (Junmeng et al. 2024). Additionally, CG contamination can negatively impact agricultural productivity and groundwater safety. Therefore, addressing the environmental issues associated with CG is imperative.

Phosphate-solubilizing bacteria (PSB) play a crucial role in converting insoluble phosphorus in soil into forms that plants can absorb (Jinge et al. 2021). This conversion is necessary because plants struggle to absorb insoluble phosphorus forms. In agricultural practices, PSB are commonly used to produce microbial fertilizers, which are combined with organic or inorganic materials and fermented by efficient PSB. This fermentation process releases soluble phosphorus, enhancing plant root absorption and promoting plant growth (Xiaohu et al. 2016). Studies have shown that PSB are vital for maintaining and enriching the soil phosphorus cycle (Jieliang et al. 2020). Their activity improves soil quality and facilitates the uptake of essential trace elements, such as calcium and copper, by plants (Chandrima et al. 2017). Furthermore, PSB proliferation can inhibit pathogenic microorganisms, thereby increasing crop resistance to diseases (Chaonan et al. 2023). For instance, *Bacillus* species not only facilitate phosphorus decomposition for plant uptake but also produce antibacterial compounds that suppress various plant pathogenic bacteria. This dual functionality reduces crop disease risks and minimizes reliance on chemical pesticides (Zhan et al. 2021).

Potassium-solubilizing bacteria (KSB), alternatively referred to as potassium-solubilizing microorganisms or

silicate bacteria, are capable of decomposing aluminosilicate minerals, particularly potassium feldspar (Yifan et al. 2022). This decomposition releases essential nutrients such as silicon, phosphorus, and potassium, which are crucial for plant growth (Madhumonti et al. 2016). Both phosphorus (P) and potassium (K) play vital roles in influencing crop yield and quality (Can et al. 2022). Research indicates that microbial activity can convert insoluble phosphorus and potassium in CG into soluble forms through their metabolic processes (Juan et al. 2021; (Wang et al. 2024). This transformation enhances nutrient availability, fosters plant growth, and mitigates the ecological issues related to CG accumulation (Zhu et al. 2022). However, studies on enhancing the sustainable use of CG in land reclamation using phosphate-and potassium-solubilizing remain limited.

This study aims to isolate and characterize bacteria from diverse environmental samples that can solubilize phosphorus and potassium using selective media. These bacteria were then employed to enhance the solubilization of nutrient elements such as P and K from CG. Subsequently, the AP and AK concentrations were measured after CG degradation to identify the most effective phosphorus-and potassium-solubilizing bacterial strains. Finally, pot experiments were conducted to assess the impact of these bacteria on alfalfa growth in soil matrices composed of CG and sandy soils.

Materials and Methods

Coal gangue and samples for bacterial screening

Coal gangue samples were collected from the Dahaize Coal Mine in Shenmu County, Shaanxi Province, Western China (38.14°N, 109.55°E). The samples were immediately sealed in zip-lock bags and transported to the laboratory for analysis. Concurrently, soil samples were collected from the rhizosphere of plants growing near coal gangue dumps at a depth of approximately 10 cm. These samples were immediately placed in sterile zip-lock bags and stored at -80°C to preserve microbial integrity. Water samples were obtained from mine drainage at the Hancheng Coal Mine (35.28°N, 110.27°E), Shaanxi Province, China. They were transported to the laboratory in pre-sterilized polyethylene bottles to prevent contamination. Additionally, sludge samples were collected from the Sewage Treatment Station at the Lintong Campus of Xi'an University of Science and Technology. All sampling sites were selected based on their direct exposure to coal gangue or coal-associated environments, ensuring that the isolated bacterial strains were naturally adapted to coal gangue biosolubilization. Strict sterile protocols were followed during sample collection, transportation, and processing to minimize cross-contamination risks.

Bacterial culture

The Aleksandrov medium was composed of glucose (5g), $(NH_4)_2SO_4$ (0.5g), $MgSO_4$ (0.3g), Na_2HPO_4 (2g), $FeSO_4$ (0.03g), $MnSO_4$ (0.03g), feldspar (2g), yeast extract (0.5g), and agar (15g) in 1 liter of deionized water (Diyan et al. 2021). The Pikovskaya (PVK) medium contained glucose (10g), $(NH_4)_2SO_4$ (0.5g), yeast extract (0.5g), $MgSO_4$ (0.3g), $FeSO_4$ (0.03g), $MnSO_4$ (0.03g), NaCl (0.3g), KCl (0.3g), $Ca_3(PO_4)_2$ (5g), and agar (15g) in 1 liter of deionized water. The Aleksandrov and Pikovskaya media were used to isolate potassium-solubilizing bacteria and phosphorus-solubilizing microorganisms, respectively (Xiaoli et al. 2016). Isolated bacteria were further purified using Luria-Bertani (LB) medium, which consists of peptone (10g), sodium chloride (10g), and yeast extract (5g) in 1 liter of deionized water. The medium's pH was adjusted to 7.2 prior to sterilization at 121°C for 15 minutes.

Isolation and purification of microorganisms

A 10 g portion of the collected soil samples was transferred into a sterilized Erlenmeyer flask, followed by the addition of 90 mL of sterile water. The flask was placed on a constant-temperature shaker set to 30°C at 160 rpm for 30 minutes. After incubation, the samples underwent serial dilution ranging from 10^{-1} to 10^{-6}. A 100 µL aliquot of the supernatant was spread onto PVK and Aleksandrov media and incubated at 30°C for one week. During isolation, bacterial strains exhibiting clear halo zones around their colonies on agar plates were identified as potential phosphate- or potassium-solubilizing candidates. The solubilization index (SI), a quantitative measure of bacterial solubilization capacity, was calculated as the ratio of the halo zone diameter to the colony diameter. To ensure robust activity, only strains with an SI exceeding 1.5 were selected and further purified through at least three generations. Identical procedures were applied for bacterial screening from mine water and sludge samples.

Analysis of the solubilization capability of bacteria

The selected bacterial strains were initially cultured overnight in LB medium before being inoculated into 90 mL of PVK liquid medium, where $Ca_3(PO_4)_2$ was replaced with 2 grams of CG. This culture was incubated at a constant temperature of 30°C for four days. After incubation, the available AP concentration in the supernatant was measured using the molybdenum-antimony resistance colorimetry method (Rui-Qing et al. 2022). This involved mixing 10 mL of centrifuged supernatant with 20 mL of distilled water and 2,4-dinitrophenol. The mixture was decolorized with sulfuric acid (2 mol/L), followed by the addition of 0.75 mL of ascorbic acid and 5 mL of molybdate. The mixture was then diluted to 50 mL with distilled water and incubated at room temperature for 30 minutes. The absorbance was measured at 700 nm using a UV–Vis spectrophotometer (Puxi TU-1900, Beijing, China). The available AK concentration was determined using Flame Atomic Absorption Spectrophotometry (FP640, Shanghai, China), following the manufacturer's guidelines. A standard curve was generated by correlating light intensity emitted with potassium concentration in KCl standards of known concentrations. A 2 mL aliquot of the centrifuged supernatant was used for light emission measurement, and AK concentration was derived from the standard curve (Yaping et al. 2022).

Identification of isolated bacterial strain

An optical microscope (EX30, Ningbo, China) was used to analyze the morphological characteristics of the isolated bacteria. Subsequently, a genomic DNA extraction kit (Solarbio Science & Technology Co, Ltd, Beijing, China) was used to isolate the genomic DNA, and the 16S rRNA gene fragment was PCR-amplified using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). The PCR products

Table 1. The main chemical elements of raw coal gangue.

Component	SiO_2	Al_2O_3	Fe_2O_3	CaO	K_2O	SO_3	MgO	TiO_2
Content (wt. %)	49.51	19.32	6.84	0.05	3.45	5.27	0.88	1.88

Table 2. The content of heavy metals in raw coal gangue

Elements	Cr	Hg	As	Pb	Cd	Cu	Ni	Zn
Content (mg/kg)	0.0889	0.0693	ND	31.2	0.07	ND	ND	93.6
Standard (mg/kg)	150-250	1.3-3.4	25-40	70-170	0.3-0.6	50-100	60-190	200-300

ND means not detected.

were sequenced by Shenggong Biotechnology (Shanghai, China), and the sequences were analyzed using the GenBank BLAST tool on the National Center for Biotechnology Information (NCBI) website. A phylogenetic tree was constructed using the neighbor-joining method in MEGA version 6.0. The bootstrap method with 1000 resamplings was used to evaluate the reliability of internal branches.

Pot experiment

Pot experiments were carried out using a soil mixture of CG and sandy soil, collected from Yulin City, northern Shaanxi Province (38.29°N, 109.73°E). Each plastic pot (15.5 cm in diameter, 10.9 cm in height) was filled with 500 cm^3 of a 1:1 C- to-sandy-soil mixture. On August 20, 2024, alfalfa seeds were sown at a rate of 30 seeds per pot, and the pots were maintained at room temperature. Bacterial strains with high available phosphorus and potassium solubilization were selected to evaluate their plant growth-promoting effects. A 6 mL aliquot of overnight bacterial culture (OD_{600} 1.0) was introduced into each pot's soil matrix. Control pots received an equivalent volume of sterile culture medium. Each treatment was replicated three times. The plants were regularly watered throughout the growth cycle, and growth parameters were monitored and recorded. After one month of growth, plants were harvested, and key parameters, including germination rate, root length, above-ground plant height, and fresh weight, were measured.

Statistical analysis

All figure results are expressed as means ± standard error (SE). Statistical analysis was performed using IBM SPSS version 26.0, employing a two-factor analysis of variance followed by the least significant difference (LSD) test. A 5% significance level ($p<0.05$) was used to assess the bacterial impact on plant growth promotion.

Table 3. The contents of P and K nutrient elements in raw coal gangue.

Elements	P	K
Total content (mg/kg)	386	28351

Results and Discussion

Raw coal gangue characterization

The chemical composition of CG is summarized in Table 1. Silica (49.51%) and aluminum (19.32%) are the predominant components, with iron oxide (6.84%) and potassium oxide (3.45%) also present. CG is particularly rich in silicon and contains significant amounts of essential nutrients, including iron (Fe), potassium (K), phosphorus (P), calcium (Ca), and magnesium (Mg). Table 2 shows that heavy metal concentrations in CG are below the thresholds set by the "Agricultural Soil Pollution Risk Control Standard" (GB15618-2018), suggesting that CG can be safely used as a mineral fertilizer. The initial phosphorus and potassium concentrations in CG are detailed in Table 3. In raw form, potassium and phosphorus mainly exist as annite and monetite, respectively (Xu et al. 2017), which are not readily absorbed by plants. Therefore, the use of potassium- and phosphate-solubilizing bacteria may enhance nutrient solubility, making them more accessible for plant uptake (Shiming et al. 2021).

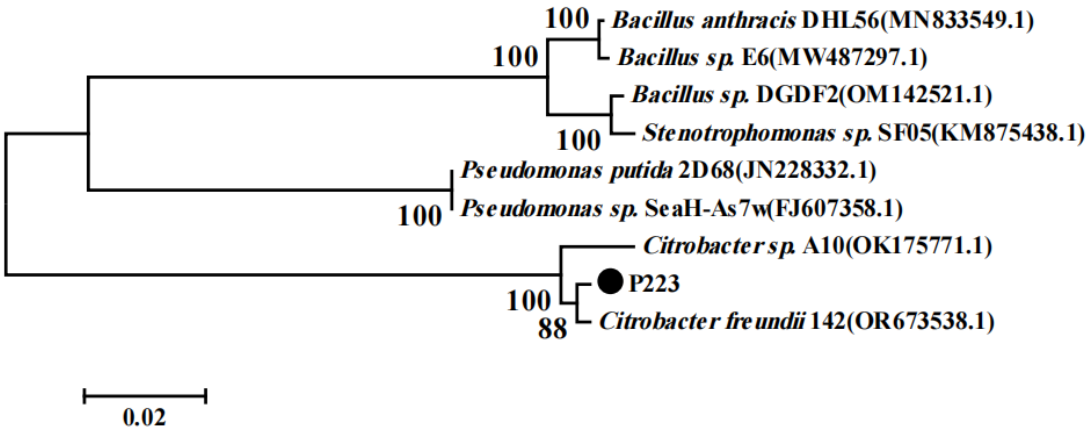
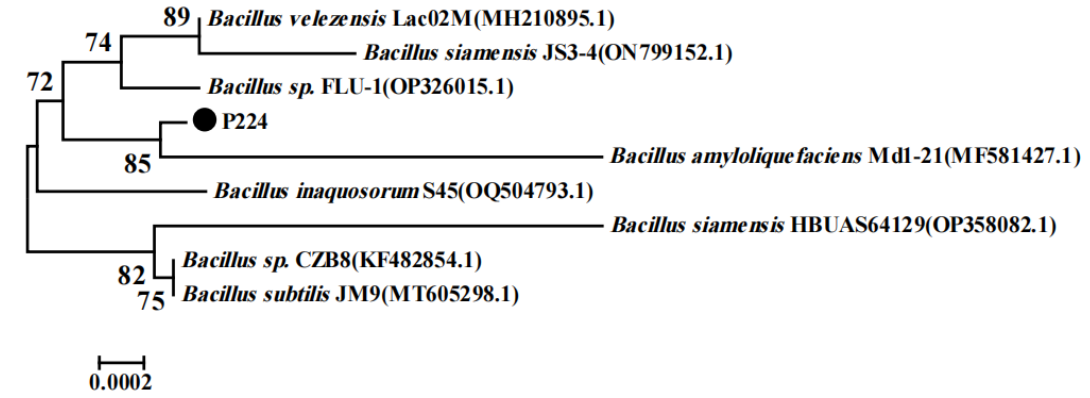
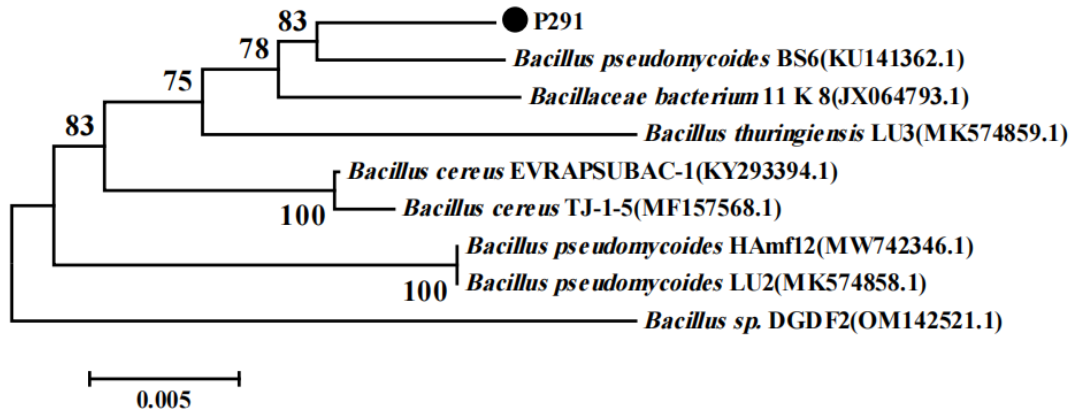
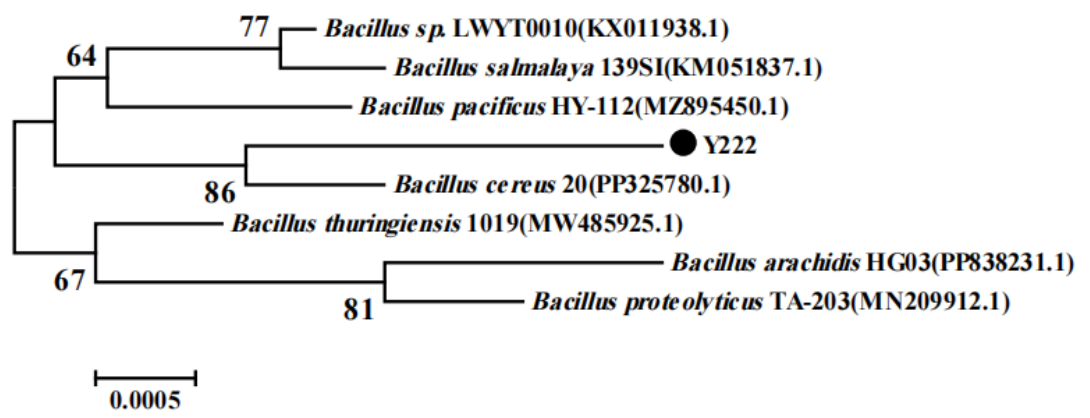
Bacterial screening from different samples

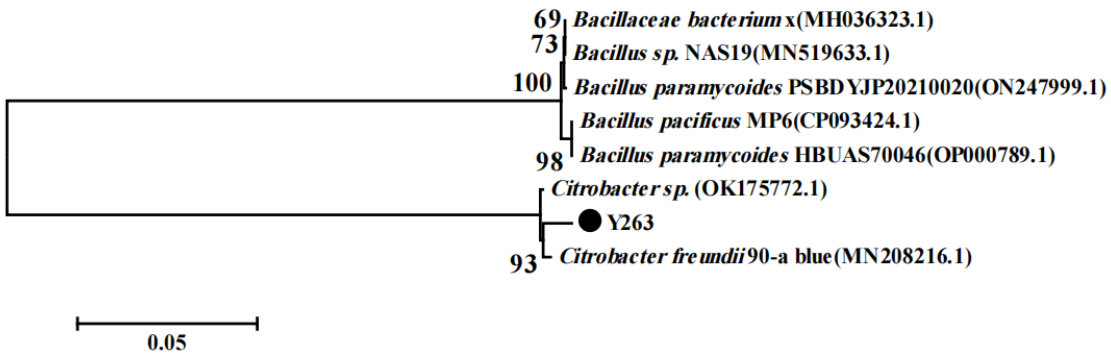
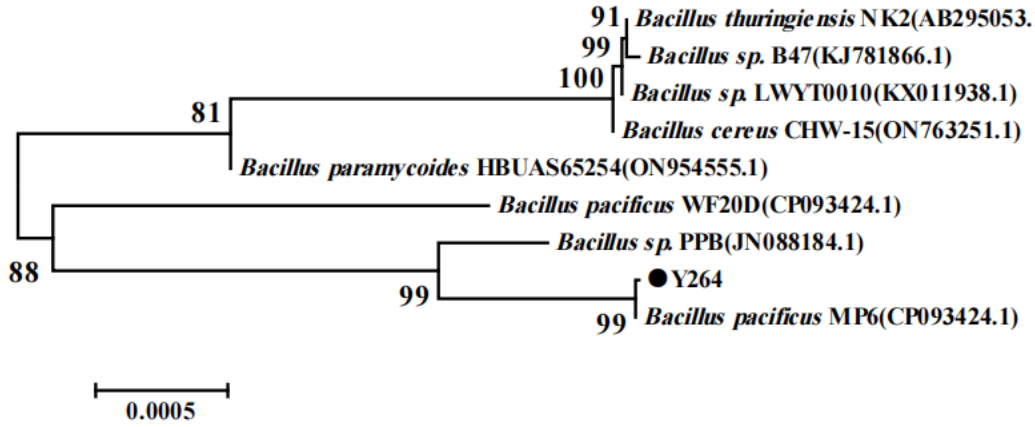
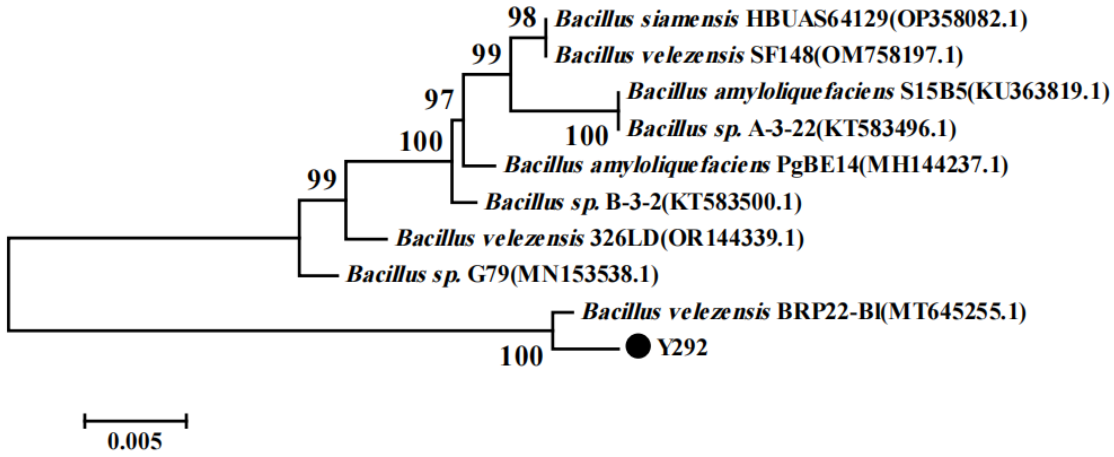
Bacteria were isolated from soil, mine water, and sludge samples, subjected to stepwise dilution, and evenly spread on both Aleksandrov and Pikovskaya (PVK) solid media. Incubation was carried out in a constant-temperature culture chamber to promote bacterial growth. Colonies with clear halo zones were selected,

Table 4. Source and nomenclature of the isolated bacterial strains.

Source	Alexander solid medium	Pikovskaya solid medium
sludge	Y291、Y292	P291、P292
soil	Y261、Y262、Y263、Y264	P111、P112、P113、P114
mine water	Y181、Y182、Y183、Y221、Y222、Y223	P221、P222、P223、P224、P225、P226

Table 5. Phylogenetic trees of bacterial strains

Type	Phylogenetic trees of bacterial strains
P223	
P224	
P291	
Y222	

Type	Phylogenetic trees of bacterial strains
Y263	
Y264	
Y292	

cultured, and archived for subsequent CG biodegradation studies. A total of twenty-four bacterial strains were isolated, with most phosphate- and potassium-solubilizing bacteria identified as Gram-positive (Nurul et al. 2022). The bacteria identified from different samples are detailed in Table 4.

Comparison of the isolated bacteria's capacity for solubilizing AP and AK

To confirm the solubilization ability of the selected bacteria, they were applied to enhance CG nutrient release. The AP concentration was measured using the molybdenum-antimony colorimetric method. As shown in Figure 1, strains P223, P291, P224, Y264, and Y222 displayed high AP levels, indicating their effective phosphorus solubilization capabilities. Similarly, AK levels for these bacteria were determined using the ammonium acetate extraction-flame photometry method. As shown in Figure 2, strains Y292, Y261, Y263, P223, and P114 exhibited high AK levels, confirming their potent potassium-solubilizing abilities and efficacy in degrading potassium-containing minerals in CG.

Identification of highly efficient bacteria for the degradation of CG

DNA extraction and phylogenetic tree construction were carried out for seven bacteria that were highly efficient in solubilizing phosphorus (P223, P291, P224) and potassium (Y292, Y261, Y263, P223). The resulting phylogenetic trees are presented in Table 5. The BLAST tool was applied to evaluate the sequence similarity of these bacteria. Notably, the 16S rRNA gene

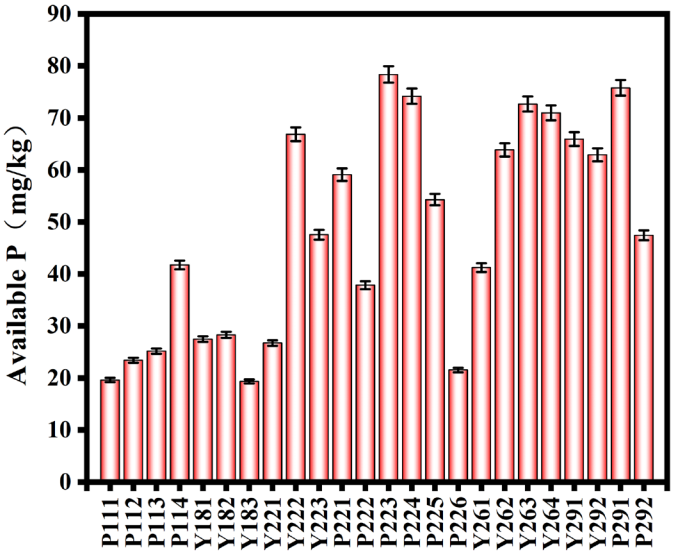


Figure 1. Determination of AP content by the selected bacteria during CG biodegradation.

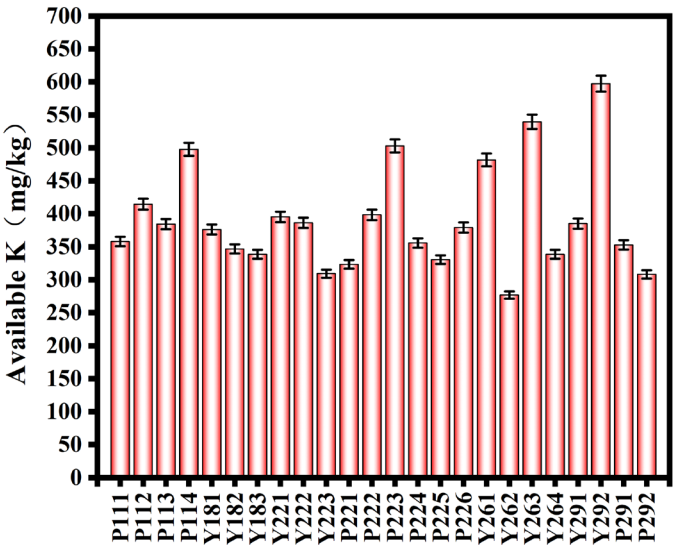


Figure 2. Determination of AK content by the selected bacteria during CG biodegradation.

sequence of P223 showed 98.73% homology with *Citrobacter freundii*, suggesting that P223 is highly likely a member of this species. Similarly, the 16S rRNA gene sequence of Y263 exhibited 98.92% homology with *Citrobacter freundii*. Although both Y263 and P223 were identified as *Citrobacter freundii*, our phylogenetic analysis suggests they may represent distinct subtypes within the species. *Citrobacter freundii* is a member of the *Enterobacteriaceae* family, found in diverse environments and often residing in the intestines of humans and animals (Nityanand et al. 2021). While recognized as an opportunistic pathogen capable of causing infections, it also plays a beneficial role in the environment by converting nitrate to nitrite (Muhammad Salihu et al. 2021).

P224 was identified as *Bacillus amyloliquefaciens*, a bacterium known for producing a variety of antibacterial substances that are effective against fungi and other bacteria. This organism is commonly used as a biocontrol agent (Dajun et al. 2022). P291 was classified as *Bacillus pseudomycoides*, a gram-positive bacterium with straight, rod-shaped cells, typically found in pairs or chains with rounded or square ends. Studies have shown that *Bacillus pseudomycoides* has a direct antagonistic effect on tomato plants (Ahmed et al. 2023). Research suggests that *Bacillus pseudomycoides* can remove copper from the soil, thus preventing its uptake by plants (Raimonda et al. 2022). Additionally, it has been demonstrated that *Bacillus pseudomycoides* can promote growth and enhance drought stress tolerance in wheat by suppressing the MYB gene (Paul et al. 2022). Y222 was categorized as *Bacillus cereus*, a bacterium that provides significant ecological benefits by producing antibacterial compounds that inhibit the proliferation of harmful microorganisms (Kulkova et al. 2023). Furthermore, it has been reported that *Bacillus cereus* BCS1, a heavy metal-tolerant strain, can degrade pyrethroids at various concentrations while maintaining high degradation efficiency even under heavy metal (Pb, Cr, and Cd) stress (Yanfeng et al. 2024). Y264 was classified as *Bacillus pacificus*, an arsenic-resistant bacterium that promotes the growth of rice seedlings (Kabiraj et al. 2023). Y292 was identified as *Bacillus velezensis*, a spore-forming, gram-positive bacterium that fosters plant growth (Rabbee et al. 2019). Moreover, *Bacillus velezensis* produces a variety of biologically active secondary metabolites that inhibit plant pathogenic microflora (Jung-Ae et al. 2022). This bacterium also generates a broad range of enzymes, with potential applications in enzyme production, fermented foods, pollutant degradation, and bioenergy (Chao et al. 2021; (Su et al. 2024). The findings discussed above indicate that the bacterial strains isolated in this study have considerable potential to promote plant growth. Consequently, their growth-promoting effects on alfalfa were further investigated in subsequent experiments. Our results confirm that these isolated potassium- and phosphorus-solubilizing microorganisms can improve alfalfa germination and growth in soil matrices composed of CG and sandy soils.

Growth-promoting effects of CG and bacteria on alfalfa

This study evaluated the potential of identified phosphate- and potassium-solubilizing bacteria to enhance alfalfa growth. The selected bacterial strains were introduced into a soil matrix, which was a mixture of CG and sandy soils in equal proportions. After a two-week growth period, the germination percentage of alfalfa was measured (Figures 3 and 4). The findings showed a substantial increase in alfalfa germination rate with bacterial treatment compared to the control. Germination rates were 81.42%, 79.98%, and 74.88% with the introduction of P291, P224, and Y292 bacteria, respectively. These results suggest that these bacteria significantly improve alfalfa seed germination in a soil matrix containing CG and sandy soils. The data suggest that the bacteria may promote alfalfa seed germination by facilitating the degradation of CG.

After a growth period of one and a half months, plant samples were harvested to measure growth parameters, including root length, above-ground height, and fresh weight. The data presented in Figures 4 and 5 demonstrate that alfalfa growth in the experimental groups, to which bacteria were added, was significantly better than in the control group that received no bacterial treatment. Notably, the Y292 and P223 strains exhibited strong growth-promoting effects, while the

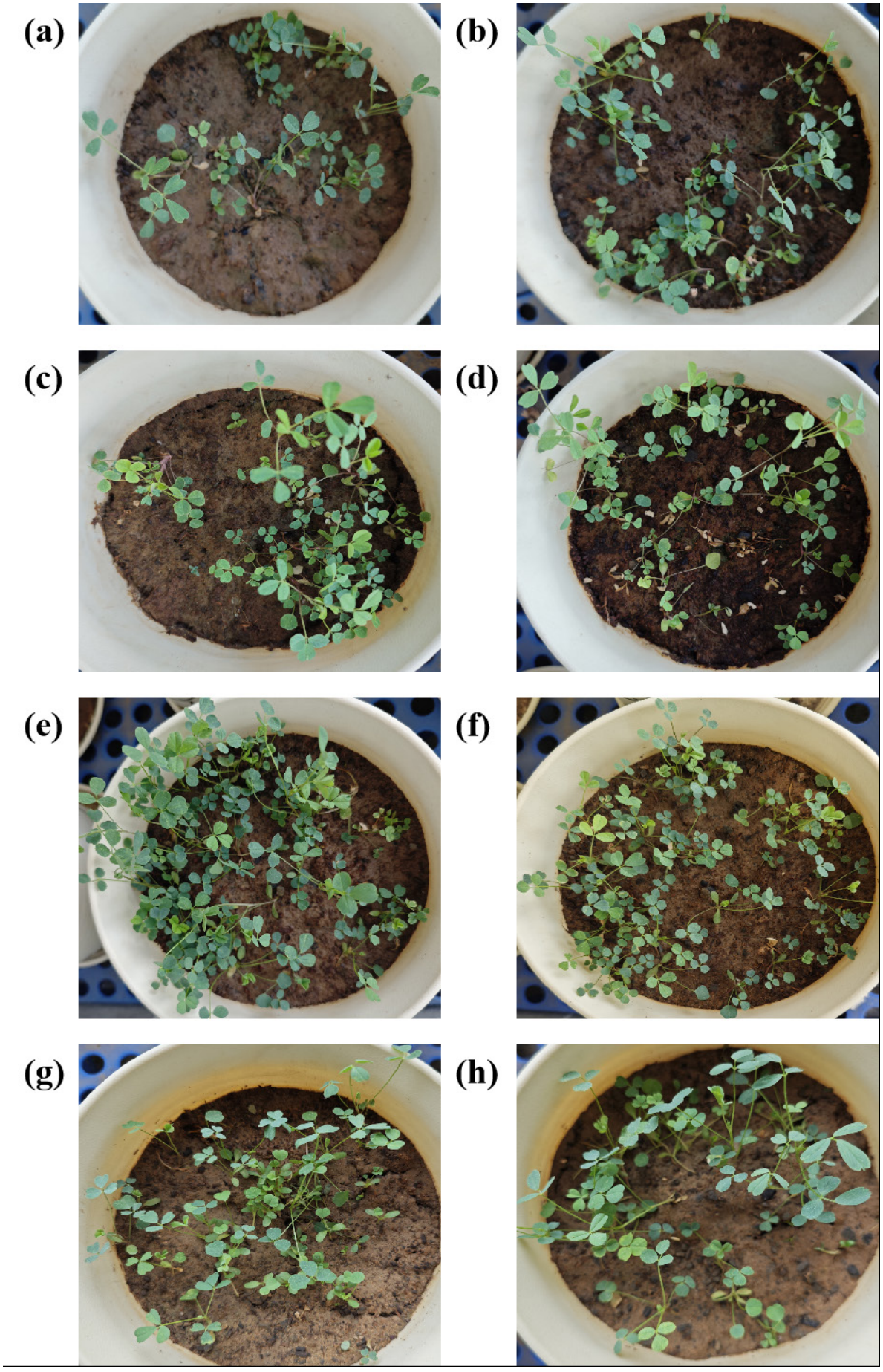


Figure 3. Growth-promoting effects on alfalfa with the supplementation of the isolated bacteria. (a) control (without bacteria addition), (b) Y263, (c) Y222, (d) Y264, (e) P291, (f) P224, (g) P223, (h) Y292.

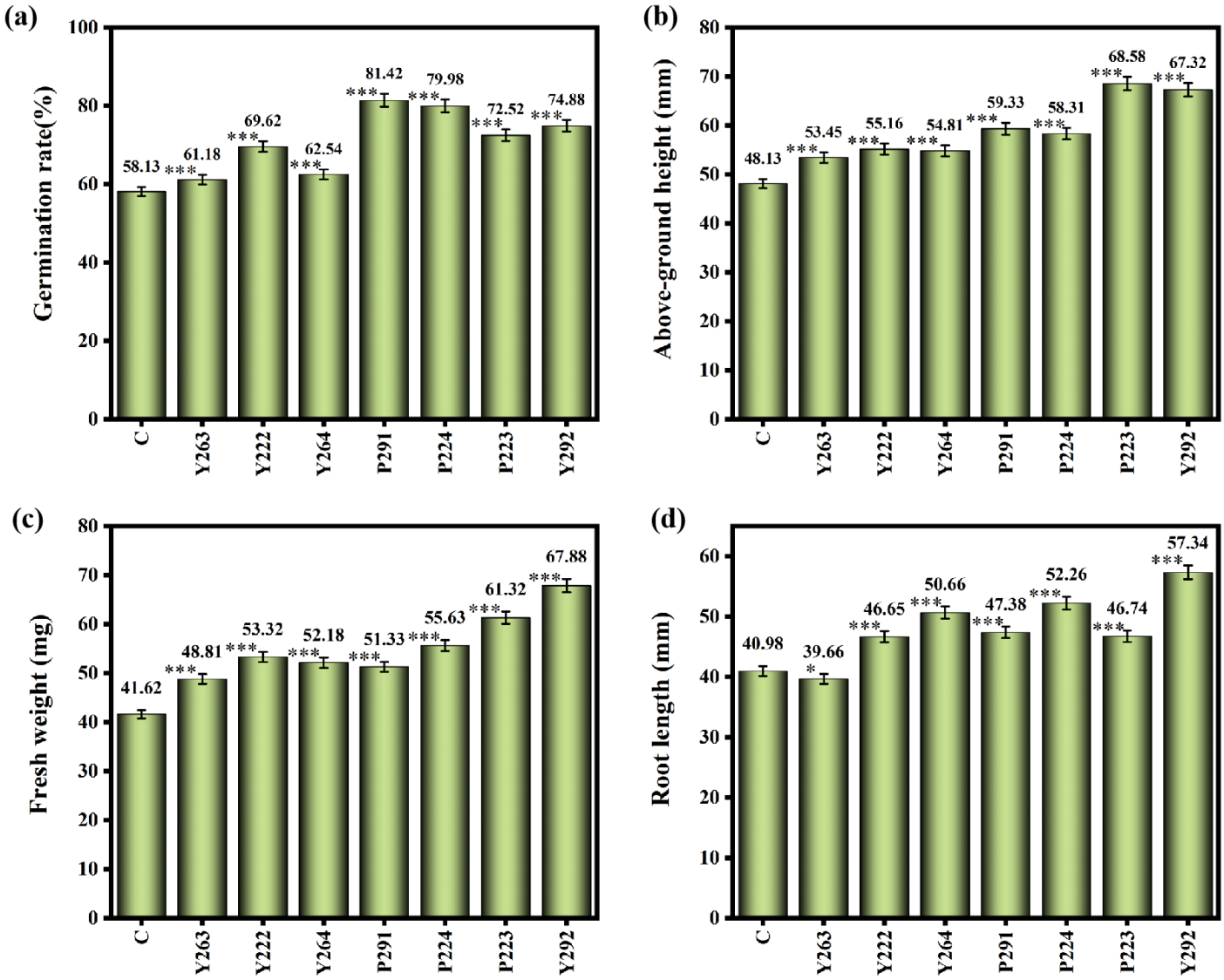


Figure 4. Growth parameters of alfalfa cultivation. (a) germination percentage, (b) above-ground height, (c) fresh weight, (d) root length. (***, $p < 0.001$; *, $p < 0.05$).

P291 strain was particularly effective in enhancing germination rates. The bacterial treatments led to substantial improvements in root length, germination rate, fresh weight, and above-ground height. These findings indicate that the isolated bacteria, capable of solubilizing phosphorus and potassium, positively affect plant growth in soil matrices containing CG and sandy soils. In summary, plants treated with these bacteria developed longer root systems and showed more robust growth compared to the control group (Raimonda et al. 2022).

The isolated strains, which have demonstrated effective phosphorus and potassium solubilization capabilities, hold significant theoretical and practical implications for environmental ecology and the development of microbial fertilizers for the sustainable utilization of CG. It is hypothesized that these bacteria may enhance alfalfa growth by solubilizing nutrients from CG, and this hypothesis will be investigated in future studies. In conclusion, the application of these microorganisms could potentially reduce reliance on chemical fertilizers, mitigate environmental pollution associated with CG, and foster sustainable agricultural practices.

Conclusions

The results of this study significantly contribute to our understanding of microbial biotechnology applications in CG waste management and land reclamation. The isolated potassium-solubilizing bacteria and phosphorus-solubilizing bacteria demonstrated notable effectiveness in enhancing the release of potassium and phosphorus from CG, thereby facilitating nutrient availability and promoting alfalfa growth. Among the bacterial strains, *Citrobacter freundii*, *Bacillus pseudomycoides*, and *Bacillus amyloliquefaciens* were particularly effective in phosphorus solubilization, while *Bacillus velezensis* and *Citrobacter freundii* demonstrated strong potassium solubilization capabilities. Notably, *Bacillus pseudomycoides* and *Bacillus amyloliquefaciens* significantly improved alfalfa germination rates, whereas *Citrobacter freundii* and *Bacillus velezensis* played a crucial role in enhancing plant growth. These results underscore the potential of KSB and PSB in the sustainable utilization of CG for land reclamation. In summary, this study highlights the great

Figure 5. Alfalfa root growth in the control group and the bacteria supplementation group.

potential of microbial-based strategies as eco-friendly solutions for CG waste treatment and resource recovery, supporting the goals of sustainable environmental management.

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