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Bacillus Strains with Catalase Enzyme Improve the Physiology and Growth of Rice (*Oryza sativa* L.)

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Abstract: Catalase can decompose hydrogen peroxide in plants under controlled and stressed conditions. In a stressed environment, an increase in hydrogen peroxide production makes catalase activity a major defense system for plant growth. The current study was conducted to evaluate the catalase activity of the pre-isolated and identified bacterial strains *Bacillus aryabhattai* (AN30), *Bacillus megaterium* (AN24), *Bacillus megaterium* (AN31) and *Bacillus megaterium* (AN35) and their potential for rice seedling growth promotion. These strains were characterized for quantitative catalase, urease, siderophore and exopolysaccharide production using LB media. Subsequently, the effectiveness of these strains was checked by quantifying the catalase activity in the rhizosphere, roots and shoots of rice seedlings. The secretion of organic and phenolic compounds produced by the tested strains in liquid culture was also investigated. Plant growth parameters were also studied in a growth room trial. Our results showed that the strain AN24 showed the maximum catalase activity (1.36 mol cm^{-1}), urease activity (1.35 mol cm^{-1}) and exopolysaccharide (4.20 µg mL^{-1}) and siderophore (2.32%) production in LB media. All tested strains showed significantly higher catalase activity in soil compared to the control. Among sole applications, strain AN24 showed better results; however, the consortium application of strains AN24 + AN30 + AN35 + AN31 showed the maximum improvement in dry biomass, shoot and root length, and increase in catalase activity of rice seedlings. The results showed that a consortium of these *Bacillus* strains with catalase activity has greater potential to enhance the antioxidant defense system and growth promotion of rice seedlings. However, further experimentation under natural conditions is required before using these strains as potential bioinoculants for improving rice growth and yield.

Keywords: antioxidative enzymes; *Bacillus* spp.; catalase activity; exopolysaccharide production; reactive oxygen species

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1. Introduction

Enzymes are mainly derived from crops, animals and microbes covalently attached, linked and incorporated inside soil debris microcapsules [1]. In plants, reactive species formation is a by-product of normal metabolic processes [2]. Hydrogen peroxide is the most active reactive species, formed in peroxisomes under stress conditions [3] and also during metabolic processes [4]. At optimum levels, hydrogen peroxide acts as a signaling molecule for plant growth [5], while its higher concentration damages plant biomolecules and ultimately causes the death of plants [2].

Catalase is an important intracellular and heme-containing enzyme found in aerobic and facultative anaerobic microorganisms [6]. It belongs to the oxidoreductase family, a widely used industrial enzyme with multiple diagnostic and therapeutic applications [7], and maintains the balance of redox homeostasis [8,9]. Its function is to remove hydrogen peroxide in plants by breaking it down into water and oxygen and also to protect cells and serve as a defense molecule for plants and help them grow [10,11]. It is also used to detoxify the hydrogen peroxide produced in various processes such as the electron transport chain, photorespiratory oxidation and fatty acid oxidation [3].

Rice is a staple crop that follows the C_3 photosynthetic pathway. Chloroplasts and stromules occupy 95 percent of the cell periphery in rice cells, while peroxisomes and mitochondria, which are important for photorespiration in the cell, are lined up along the chloroplast wall [12]. In addition to photorespiration, PSI generates hydrogen peroxide under high light stress in plants, which can affect crop growth, yield and physiology while the catalase enzyme's presence is important to protect them against certain stresses [13]. In addition, it has been studied in dicotyledonous plants [14], but there are few studies on monocotyledonous plants such as rice [15]. During the rice germination process, the catalase enzyme is crucial for the hydrolysis of the seed's endosperm, which allows its development, providing the necessary energy for root and shoot development. The presence of catalase enzyme in the rice mesophyll cells plays a most important role in photorespiration and CO_2 refixation. Therefore, catalase production can improve the plant establishment and growth of the rice plant [16]. Moreover, urease production improves the optimum concentration of nitrogen for plant growth by converting the natural or added urea into ammonia and carbonate. This ammonia is further converted into nitrite and then nitrate so that plants can use it easily for their growth. Urease activity enhances the utilization rate of nitrogenous fertilizers [17]. The catalase enzyme activity is also directly related to the production of organic acid that lowers the pH and improves the availability of nutrients [18]. Moreover, in one study, the application of bacteria that produce citric acid significantly increased catalase activity in rice [18]. Citric acid, as a supplement, increases catalase enzyme activity, which reduces the damaging effect of reactive oxygen species. According to a recent study, exogenic utilization of citric acid increases the activity of antioxidant enzymes, demonstrating the plant's defense mechanism against oxidative stress [19].

Microbial enzymes can be used to overcome problems caused by various environmental conditions that can damage the physiology of plants; therefore, microbial enzymes have replaced plant enzymes because of genetic operation [20]. For resisting pathogen attack, plant-growth-promoting rhizobacteria (PGPR)-mediated immune systemic resistance (ISR) provides a defense mechanism through enzyme production (i.e., catalase) and release of allelopathic compounds [21]. Bacteria have two types of catalase enzymes, one which catalyzes only H_2O_2 and another that also has peroxidase activity. Catalase catalyzes the transformation of H_2O_2 to water and oxygen in bacteria [6]. It is also the defensive mechanism for bacteria against stress [6]. It may also contribute to various cellular processes such as cell expansion, variation and metabolite production [22]. However, bacteria are a diverse group of microorganisms that can grow under harsh environmental conditions. Therefore, bacterial strains with catalase activity can be helpful to improve plant growth under changing climatic conditions [23]. They are mostly favored due to their higher yield, economic viability, product optimization and alteration, and fast growth of microbes in an inexpensive medium. Under changing climate conditions, the role of rhizobacteria with the ability to produce stress enzymes, hormones, exopolysaccharides and siderophores becomes crucial, as they can play an important role in solubilizing fixed organic and inorganic nutrient sources [24].

Plants need an optimum level of hydrogen peroxide for their growth and therefore produce a specific amount of catalase for their protection. Various strategies are used to eliminate hydrogen peroxide in plants. One of them is catalase production by microbes that are able to maintain the level of hydrogen peroxide in plants. Keeping in view the importance of catalase, rice and rhizobacterial *Bacillus* strains, we hypothesized that the

catalase production ability of *Bacillus* strains can support plants and bacteria and maintain the growth and physiology of rice under normal conditions. The objective of this study was to evaluate the potential of the pre-isolated and identified *Bacillus* strains AN24 (*B. megaterium*), AN35 (*B. megaterium*), AN30 (*B. aryabhattai*) and AN31 (*B. megaterium*) in sole and consortium application for quantitative catalase, urease and exopolysaccharide and siderophore production ability. The effectiveness of these strains was also evaluated for the promotion of rice seedlings' growth under controlled conditions in a growth room trial.

2. Results

2.1. Biochemical Characterization of Bacillus Strains

The biochemical characterization of *Bacillus* strains AN24 (*B. megaterium*), AN35 (*B. megaterium*), AN30 (*B. aryabhattai*) and AN31 (*B. megaterium*) is presented in Figure 1. All the tested strains show different behavior in terms of catalase activity, urease activity, and siderophore and exopolysaccharide (EPS) production in broth culture. Statistical analysis revealed that catalase (Figure 1a) and urease (Figure 1b) activity were similar in the catalase-producing *Bacillus* strains AN31 and AN35. Moreover, EPS (Figure 1c) and siderophore (Figure 1d) production were statistically different in all tested strains. However, strain AN24 showed the maximum catalase activity (1.36 mole cm^{-1}), urease activity (1.35 mole cm^{-1}), EPS production (13.5 µg mL^{-1}), and siderophore production (23.0 µg mL^{-1}) compared to the other strains.

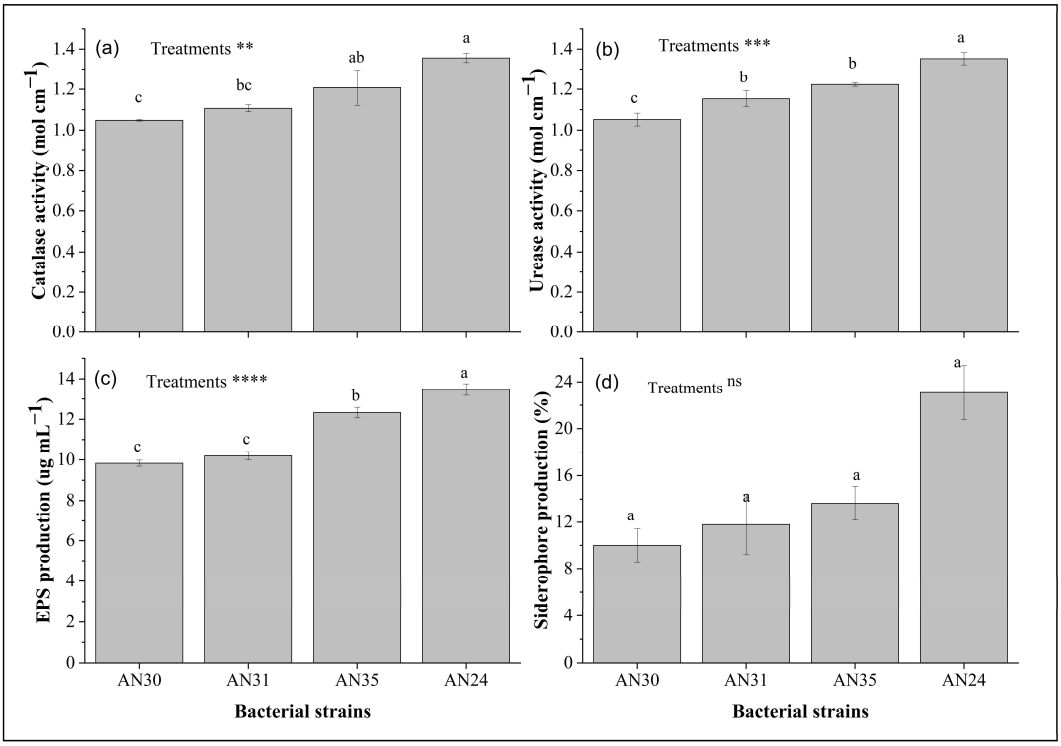

Figure 1. Biochemical characterization of *Bacillus* strains for catalase (**a**), urease (**b**), exopolysaccharide (EPS) (**c**) and siderophore (**d**) production. Bars with the same letter(s) do not differ from one another statistically at $p \leq 0.05$.

2.2. Effect of Catalase-Producing Bacillus Strains to Improve Catalase Activity in Soil and Plant

The efficacy of *Bacillus* strains with catalase activity was evaluated for improvement in catalase activity in soil and rice plants (shoot and root). The tested strains significantly improved catalase activity in the soil and plants (Figure 2). In sole inoculation, the *Bacillus* strain AN24 gave the maximum increase in catalase activity in soil (24%), shoot (27%) and root (30%) compared to the uninoculated control. However, co-inoculation yielded better results than sole inoculation. Consortium application of AN24 + AN35 + AN31+ AN30

resulted in the maximum increase in catalase activity of 43, 51 and 55% in the soil, shoot and root, respectively.

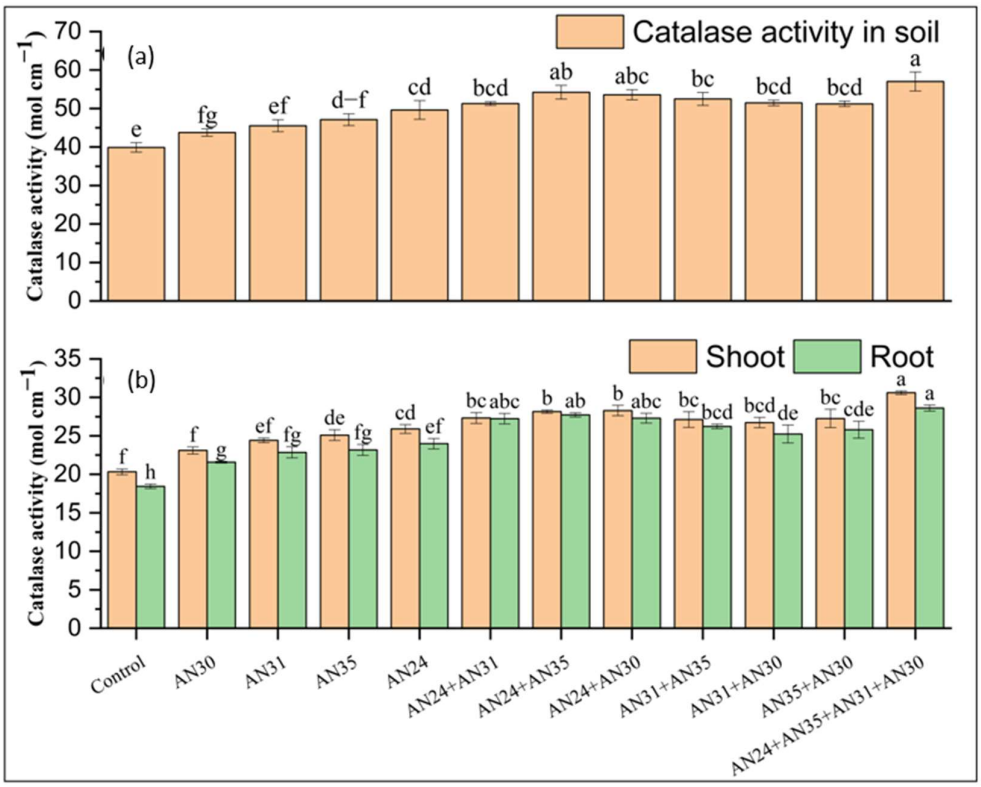

Figure 2. Effect of sole and combined application of *Bacillus* strains with catalase activity to improve catalase activity in soil (**a**), shoot and root (**b**). Bars with the same letter(s) do not differ from one another statistically at $p \leq 0.05$.

2.3. Organic and Phenolics Production in Bacterial Culture

High-performance liquid chromatography (HPLC) analyses depicted the production of organic acids by the tested strains in liquid culture (Figure 3). Production of citric acid, pyruvic acid and oxalic acid was observed for all tested strains. The maximum pyruvic acid (43 µg mL^{-1}) was produced by strain AN30. Strain AN24 produced 10 and 125 µg mL^{-1} of melonic acid and succinic acid, respectively, whereas the other tested strains did not produce malonic acid and succinic acid. Fumaric acid was observed for strains AN31 (64 µg mL^{-1}) and AN35 (120 µg mL^{-1}). Bacterial cultures of the tested strains were analyzed for phenolic compounds. The HPLC analysis revealed that the only strain that produced gallic acid (0.61 µg mL^{-1}) was AN24, whereas quercetin was produced by all the tested strains. However, the maximum (2.24 µg mL^{-1}) quercetin was produced by strain AN35, followed by strain AN31 with 2.01 µg mL^{-1}.

2.4. Plant Growth Promotion by the Application of Catalase-Producing Bacillus Strains

The application of *Bacillus* strains with catalase activity significantly improved the growth and root morphology of rice seedlings (Table 1). Among sole inoculation, the maximum 22% increase in shoot length was observed for strain AN24 compared to the control, followed by strain AN35, which showed a 19% increase in plant shoot length. Strain AN24 showed a maximum increase of 25 and 35% in root length and seedling dry weight, respectively, compared to the other sole treatments, where up to 20% improvement was observed for sole inoculation of strains AN30, AN31 and AN35. However, the consortium application (AN24 + AN35 + AN31 + AN30) showed even better results compared to the sole applications. The consortium application showed the maximum increase in shoot length (46%), root length (55%) and seedling dry weight (56%) compared to the

uninoculated control. Similarly, maximum root surface area was observed for AN24, with a 16% increase compared to strain AN35 with a 13% increase, whereas a minimum 4% increase in root surface area was observed for strain AN30 compared to the uninoculated control. Moreover, the maximum root surface area, root diameter and root volume were observed for the consortium application, which showed 33, 62 and 46% increases in the above-mentioned parameters, respectively.

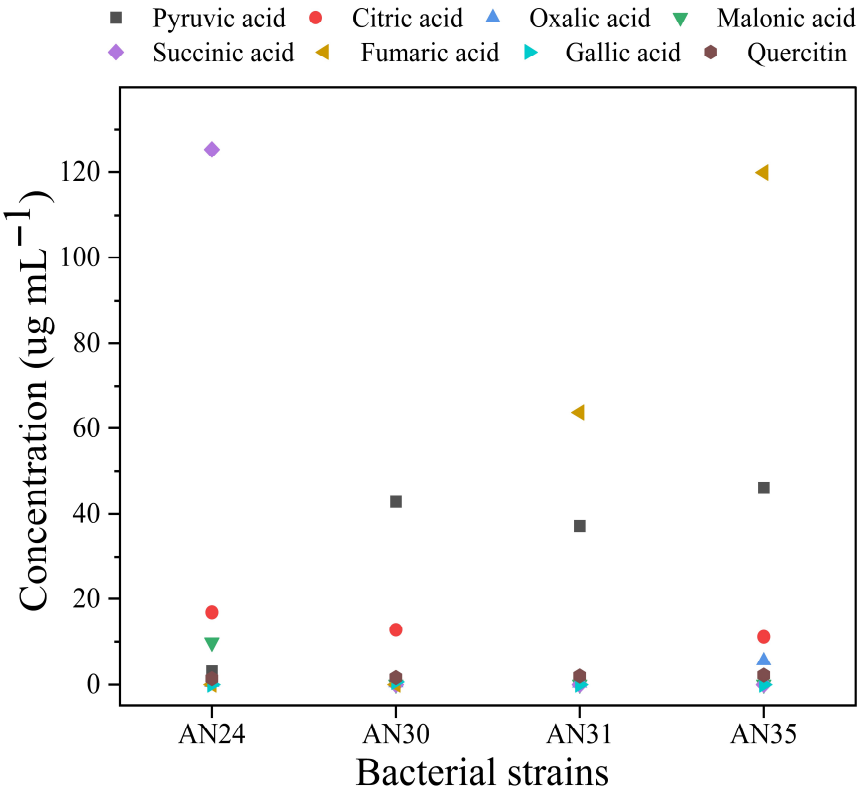

Figure 3. Scatter diagram showing the variation in the secretion of organic acids by the tested strains in liquid culture.

Table 1. Effectiveness of *Bacillus* strains with catalase enzyme to improve growth and root morphology of rice seedlings.

Treatment	Shoot Length (cm)	Root Length (cm)	Dry Weight (g Seedling^{-1})	Root Surface Area (cm^2)	Root Diameter (mm)	Root Volume (cm^3)
Control	6.5 ± 0.08 ^{f}	4.3 ± 0.12 ^{f}	0.51 ± 0.01 ^{f}	9.8 ± 0.06 ^{d}	0.53 ± 0.03 ^{e}	0.37 ± 0.09 ^{c}
AN30	7.1 ± 0.18 ^{e}	4.9 ± 0.11 ^{e}	0.59 ± 0.02 ^{de}	10.1 ± 0.15 ^{d}	0.57 ± 0.03 ^{de}	0.39 ± 0.05 ^{ac}
AN31	7.9 ± 0.16 ^{de}	5.2 ± 0.16 ^{de}	0.61 ± 0.01 ^{de}	10.9 ± 0.06 ^{cd}	0.60 ± 0.06 ^{de}	0.40 ± 0.06 ^{bc}
AN35	8.0 ± 0.32 ^{de}	5.5 ± 0.10 ^{de}	0.58 ± 0.01 ^{e}	11.3 ± 0.25 ^{cd}	0.63 ± 0.03 ^{cde}	0.43 ± 0.03 ^{ac}
AN24	8.3 ± 0.23 ^{cd}	5.7 ± 0.05 ^{cd}	0.64 ± 0.01 ^{cd}	11.8 ± 0.09 ^{bc}	0.63 ± 0.03 ^{cde}	0.44 ± 0.04 ^{ac}
AN24 + AN31	8.8 ± 0.09 ^{b}	6.1 ± 0.10 ^{b}	0.67 ± 0.03 ^{bc}	12.5 ± 0.83 ^{b}	0.77 ± 0.03 ^{abc}	0.48 ± 0.02 ^{ac}
AN24 + AN35	8.8 ± 0.16 ^{b}	6.1 ± 0.16 ^{b}	0.71 ± 0.01 ^{b}	12.4 ± 0.27 ^{b}	0.67 ± 0.03 ^{cde}	0.47 ± 0.03 ^{ac}
AN24 + AN30	8.7 ± 0.05 ^{bc}	5.8 ± 0.09 ^{bc}	0.68 ± 0.00 ^{bc}	12.3 ± 0.24 ^{b}	0.80 ± 0.06 ^{ab}	0.47 ± 0.03 ^{ac}
AN31 + AN35	8.5 ± 0.09 ^{bc}	5.8 ± 0.09 ^{bc}	0.68 ± 0.01 ^{bc}	12.0 ± 0.94 ^{bc}	0.63 ± 0.03 ^{de}	0.47 ± 0.03 ^{ac}
AN31 + AN30	8.8 ± 0.08 ^{bc}	5.9 ± 0.08 ^{bc}	0.66 ± 0.03 ^{bc}	11.9 ± 0.73 ^{bc}	0.70 ± 0.06 ^{bcd}	0.51 ± 0.01 ^{ab}
AN35 + AN30	8.4 ± 0.16 ^{bc}	5.8 ± 0.09 ^{bc}	0.67 ± 0.02 ^{bc}	12.0 ± 0.58 ^{bc}	0.70 ± 0.06 ^{bcd}	0.47 ± 0.03 ^{ac}
AN24 + AN35 + AN31 + AN30	9.4 ± 0.26 ^{a}	6.6 ± 0.10 ^{a}	0.80 ± 0.02 ^{a}	13.0 ± 0.25 ^{a}	0.87 ± 0.12 ^{a}	0.54 ± 0.07 ^{a}
LSD ($p \leq 0.05$)	0.5161	0.3056	0.0562	1.3950	0.1589	0.1317

Means with the same letter(s) do not differ from each other statistically at $p \leq 0.05$.

2.5. Effect of Bacillus Strains with Catalase Activity on Soil Biological Properties

The catalase-producing *Bacillus* strains' effect on soil biological properties is explained in Figure 4. All the applied treatments showed a significant increase in bacterial population in rhizospheric soil; however, co-inoculation and consortium application performed better than sole inoculation (Figure 4a). Among sole inoculation, the maximum increase was observed (24%) in the bacterial population for strain AN24. The highest increase (32%) was observed for the consortium application of AN24 + AN35 + AN31 + AN30, followed by co-inoculation of AN24 + AN35 and AN24 + AN31, which showed a 29.3% increase in bacterial population in rhizospheric soil. Microbial biomass carbon (MBC) (Figure 4c) and microbial biomass nitrogen (MBN) (Figure 4d) were significantly different in the applied treatments in comparison with uninoculated control, whereas sole and co-inoculation showed results that were not statistically significantly different from each other. However, the consortium application of AN24 + AN35 + AN31 + AN30 showed significantly higher results than all applied treatments, where a 26 and 30% increase in microbial biomass carbon and nitrogen, respectively, was observed. Regression and correlation between bacterial population and microbial biomass carbon and nitrogen showed a straight line, which indicated the direct relation between the tested parameters (Figure 4b). A positive relation between bacterial population and microbial biomass carbon was recorded, with a Pearson correlation coefficient of R = 0.94 and a linear regression coefficient of R^2 = 0.88. Furthermore, bacterial population and microbial biomass carbon showed a direct relation with R = 0.89 and R^2 = 0.77, respectively.

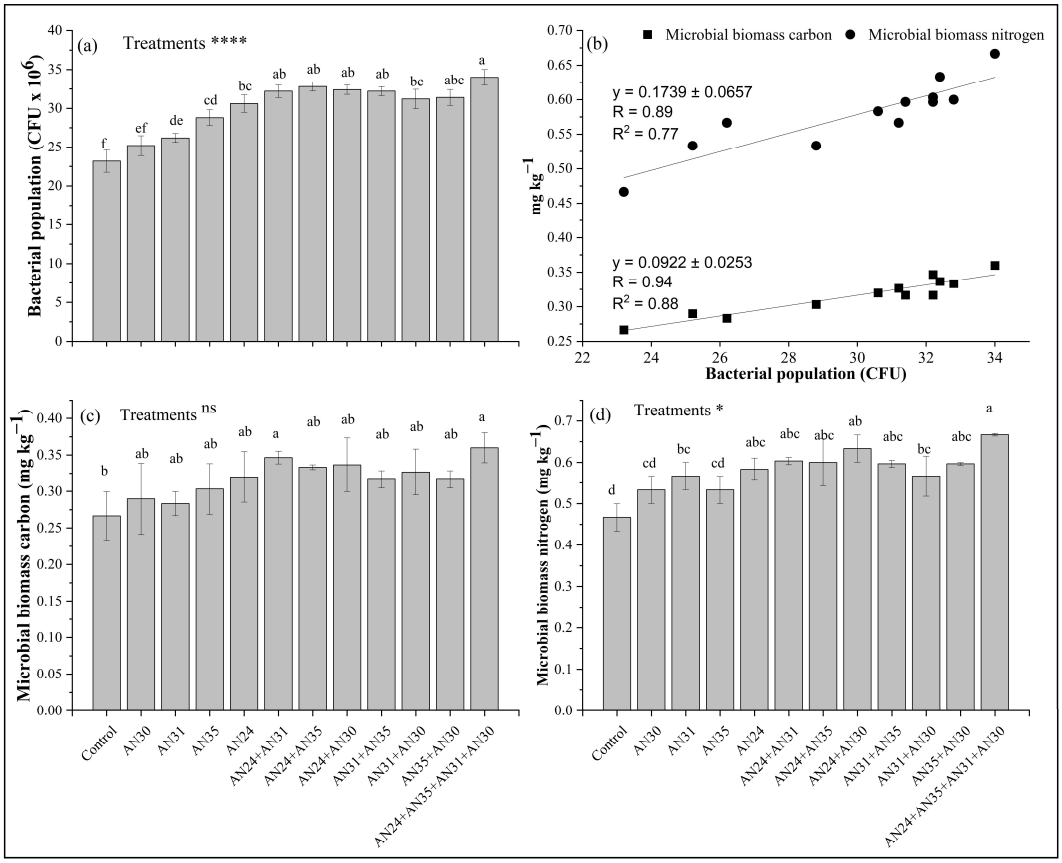

Figure 4. Effect of *Bacillus* strains with catalase activity on soil biological properties: (**a**) bacterial population; (**b**) correlation between microbial biomass carbon/microbial biomass nitrogen and bacterial population; (**c**) microbial biomass carbon; (**d**) microbial biomass nitrogen. Bars with the same letter(s) do not differ from one another statistically at $p \leq 0.05$.

3. Discussion

The growth of plants stimulated by use of microbial inoculants leads to healthier food and improves sustainability and food supply [25]. Bacteria produce siderophores and EPS, which are high-molecular-weight polymers and iron scavengers that help bind soil particles and improve soil aggregation. Catalase and urease enzymes are essential elements for the hydrolysis of endosperm in seed and boost the energy level of root and shoot development. Catalytic rhizobacterial strains play an important role in several microbial processes independent of oxidative stress and remove hydrogen peroxide (H_2O_2) from the cells; therefore, catalase production by *Bacillus* strains has proved to be significant for hydrogen peroxide detoxification in plants or in microbes for their survival [26]. In the present study, the *Bacillus* strains showed positive results for the production of catalase with high catalytic activity and the removal of hydrogen peroxide (H_2O_2) [27]. Microbes with catalase production have different mechanisms: breakdown of organic matter, promotion of plant growth and protection against reactive oxygen species [27].

The identified rhizobacterial strains showed a significant increase in urease activity in rice seedlings, suggesting that these *Bacillus* strains enhance nitrogen utilization in plants and promote root growth [28]. It has been reported that rhizobacterial strains such as *B. pasteurii* and *B. megaterium* potentially enhance the hydrolysis of urea and stimulate urease activity [29]. In addition to nutrient solubility, *Bacillus* strains increase the amount of siderophore and have a high affinity to iron. Microbial siderophores are iron-scavenging, low-molecular-weight ligands that are produced in the case of iron deficiency [30]. Moreover, the *Bacillus* strains exhibited variable growth with increasing EPS concentration and improved root and shoot growth with root adhering ability in soil. Our results also correlate with various *Bacillus* strains that produce EPS, such as *B. licheniformis* and *B. subtilis*. These strains have higher-molecular-weight polymers such as proteins, carbohydrates and other organic compounds [31]. The EPS is thus released by bacteria to trigger the plant and is associated with the formation of an adaptive mechanism under stressful conditions.

Plant-growth-promoting rhizobacterial *Bacillus* strains with catalase activity also showed organic acid production. In the soil, organic acid production increased the hydrogen ion concentration by solubilizing nutrients, which improved plant growth [32]. The results were similar to those in the literature [19]. Citric acid and catalase activity plays a protective role in plants to minimize oxidative stress. In our study, all *Bacillus* strains produced organic acids like succinic acid, pyruvic acid, citric acid, gallic acid, oxalic acid, fumaric acid and quercetin. Similarly, our study was also in line with Mumtaz et al. [33], who reported that *Bacillus* sp. strain ZM20, *B. cereus* and *B. subtilis* strain ZM63 produced citric acid, acetic acid, formic acid, lactic acid, isovaleric acid, succinic acid, formic acid and isobutyric acid.

The identified *Bacillus* strains were inoculated with rice seeds and tested for their effects on plant growth promotion and root development. The inoculation with rhizobacterial *Bacillus* strains improved root colonization, root surface area, root diameter, dry biomass and root/shoot length in the current study. The strains showed different responses and maximum growth with the inoculation of catalase-producing Bacillus strains in rice seedlings. However, a consortium of *Bacillus* strains (AN24+ AN35+ AN30 +AN31) showed the maximum growth promotion. Our study was also consistent with previous research that showed these rhizobacterial bacillus strains improve the length of plant roots, thus increasing water and nutrient uptake, growth and seedling physiology [34]. Another study also proved there was catalase production by *Bacillus* strains and its possible influence on plant growth [35]. This percentage increase in plant growth parameters is due to the inoculated rice seedlings stimulating the growth-promoting abilities of the bacterial isolates. Our study showed that the microbial catalytic activity significantly improved the catalase activity in both soil and plant. Maximum catalase activity was observed for the consortium application of AN24+ AN35+ AN30 +AN31. The catalase enzyme is closely linked with the rhizosphere of rice seedlings and enzymes released by roots, its expansion and tolerance mechanisms [36]. Soil enzymes can positively influence plant growth through catalase,

involving various biochemical reactions that improve soil health and plant growth [37]. Furthermore, the present study demonstrated that the use of catalytic *Bacillus* strains enhanced soil chemical and biological properties. The microbial biomass carbon, microbial population in the rhizosphere and microbial biomass nitrogen significantly improved with the inoculation of the catalytic strains. It is well documented that seed inoculation significantly increases bacterial population [38]. The bacterial population improves the amounts of nitrate–nitrogen, ammonium–nitrogen and microbial biomass carbon [39]. This was also reported by Iqbal et al. [40]. The treatments applied in our study showed a remarkable improvement in microbial carbon biomass and acted as a substrate for nutrient availability in soils, hence improving their nutrient availability and organic matter.

4. Materials and Methods

4.1. Quantitative Characterization of Bacillus Strains

In the present study, the following pre-isolated and identified *Bacillus* strains were used, provided by the Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, the Islamia University of Bahawalpur: AN24 (*B. megaterium*), AN35 (*B. megaterium*), AN30 (*B. aryabhattai*) and AN31 (*B. megaterium*). These strains have plant-growth-promoting traits and are well characterized for this purpose, and the ability to produce indole-3-acetic acid (IAA), catalase, urease, exopolysaccharides and siderophores has been positively reported [32]. The current study was planned for the quantitative characterization of *Bacillus* strains for their catalase activity and role in plant growth promotion.

The *Bacillus* strains were assessed for catalase activity in broth culture as studied by Beers et al. [41] and Hildebrandt et al. [42]. A nutrient broth medium (1 L of distilled water containing 3 g yeast extract, 10 g peptone and 1 g NaCl, (pH 7.2)) was used. The overnight-grown bacterial strains with 0.6 OD600 were further inoculated in a nutrient broth medium and incubated in a rotary shaker at 30 °C for 24 h. To obtain crude extracts, cultures were centrifuged at 4500 rpm for 10 min. Then, 1.5 mL of a 30 mM H_2O_2 solution was added to 0.1 mL of extract in 2.7 mL of buffer (50 mM Na_2HPO_4/NaH_2PO_4). To calculate the quantitative catalase production, absorbance was recorded using a spectrophotometer (Model Carry 60, Agilent Technologies Santa Clara, CA, USA) at 240 nm.

4.2. Biochemical Quantification of Strains

The *Bacillus* strains were tested for their urease-producing ability for growth promotion by following the method of Burbank et al. [43]. The overnight-grown bacterial isolate was added to Christensen's urea broth [44] and placed in incubate at 30 ± 1 °C in a rotary shaker for 24 h. Urease buffer was prepared by adding 1 mg EDTA, 50 mg HEPES and 20 g urea/L, cooled at 4 °C before use. Then, 2 µL of Christensen's urea bacterial culture was added to 1800 µL urease buffer (1 liter buffer prepared by adding 1 mg EDTA, 50 mg HEPES and 20 g urea and cooling at 4 °C before use) in microtubes placed in an ice bath, followed by the addition of sodium hypochlorite solution (17.5 g NaOH, 59.45 g Na_2HPO_4 and 200 mL bleach per liter solution) and 0.2 mL of phenol nitroprusside (70 g phenol and 0.34 g nitroprusside per liter). The mixture was gently shaken by inverting the tubes, and they were then incubated at 30 ± 1 °C for 20 min. Ammonia standards, i.e., 1, 2, 3, 4, 5, 10, 15 and 20 µg mL^{-1}, were prepared by using ammonium chloride (NH_4Cl). The urease buffer solution was also added to the standards. Urease activity in standard solutions and samples was measured at 640 nm using a spectrophotometer (Model Carry 60, Agilent Technologies Santa Clara, CA). The concentration of urease in the samples was calculated through comparison with a standard curve.

The bacterial isolates were further tested for siderophore production in Chrome Azurol S (CAS) media [45]. For this purpose, 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) and 10 mg of $FeCl_3.6H_2O$ were dissolved in 40 mL distilled water, while 60.5 mg of CAS was dissolved in 50 mL distilled water in a separate tube. To prepare CAS media, both solutions were mixed and made up to a volume of 100 mL with distilled water. The overnight-grown bacterial strains (0.1 mL) with 0.6 OD_{600} were inoculated in sterilized

King's B broth (99.9 mL) and incubated for 48 h at 30 °C. After this, the culture was centrifuged at 4500 rpm for 15 min and the supernatant was collected. By mixing 0.5 mL supernatant with 0.5 mL CAS reagent, the color was developed, and after 20 min of incubation, the optical density was measured on a spectrophotometer (Model Carry 60, Agilent Technologies Santa Clara, CA, USA) at 630 nm. Siderophore was calculated as percent siderophore unit (psu) [46].

The bioassay developed by Ashraf et al. [47] was used to determine the synthesis of exopolysaccharides. For the EPS bioassay, bacterial culture was added to RCV broth medium, which was then incubated for 72 h at ±30 °C under shaking (100 rpm). The culture was centrifuged for 20 min at 1000 rpm to separate the supernatant in a tube. In order to form EPS crystals, the extracted supernatant was combined (1:3) with cooled acetone and left overnight at 4 °C. The solution was centrifuged at 4500 rpm for 15 min, and the supernatant was discarded. To remove water, the remaining EPS pellets were lyophilized and then dissolved in a 1:5 solution of phenol and sulfuric acid. To measure the absorbance at 640 nm, a spectrophotometer (Model Carry-60, Agilent Technologies Santa Clara, CA, USA) was used. Through comparison with a standard curve prepared with glucose standards with concentrations of 1, 2, 3, 4, 5, 10, 15 and 20 g mL^{-1}, the EPS concentration was determined [48].

4.3. Determination of Organic Acids

Organic acids produced in bacterial culture were determined as studied by Butsat et al. [49]. The bacterial isolates (100 µL) were inoculated in test tubes that contained DF minimal broth (14.9 mL) and incubated for 72 h at 28 °C. After this, the cultures were centrifuged at 10,000 rpm, and the supernatant was mixed with HPLC-grade methanol in a 1:1 ratio. By using HPLC, the organic acids were measured along with standards (Shimadzu, Kyoto, Japan).

4.4. In Vitro Rice Growth Promotion Characterization of Catalase-Producing Bacillus Strains

A loop full of the respective bacterial strains (AN24, AN30, AN31 and AN35) was dipped in 100 mL of sterilized Luria Bertani (LB) liquid medium separately [50] and incubated at 100 rpm at 30 ± 2 °C in a shaking incubator (S19R-2, Sheldon Manufacturing, Cornelius, OR, USA). After 48 h of incubation, a bacterial broth culture with 1.2 OD600 was used as the inoculum. The PK 386 variety rice seeds were disinfected by dipping them in 0.2% solution of $HgCL_2$ for 30 s followed by one-minute dipping in ethanol (95%) and thoroughly rinsing 5 times with sterilized water. Then, disinfected seeds were dipped in respective bacterial cultures for 30 min before sowing. For co-inoculation, an equal volume of both cultures was mixed, and rice seeds were dipped in this mixture. Ten inoculated rice seeds were sown in plastic jars filled with 600 g of sterilized sandy loam soil that was characterized before placing it in the jars (Table 2). Five replications of each treatment were arranged in a completely randomized design (CRD) and placed in a growth room that was set to 12 h light (1000 flux) with 35 ± 1 °C temperature and 12 h dark with 25 ± 2 °C temperature. Humidity was also adjusted to 60–70% throughout the experiment. After germination, the plant population was maintained at five plants per jar. Half-strength Hoagland solution (20 mL) was used on alternate days to fulfill the water requirement. Thirty-five days after sowing, the rice seedlings were uprooted and growth parameters (dry biomass of shoots and roots, length of shoots and roots, root volume, root surface area and root diameter) were measured. Plant samples were analyzed for root colonization and catalase activity in roots and shoots.

4.5. Postharvest Soil Analysis

After harvesting the rice seedlings, soil samples were collected and tested for microbial biomass nitrogen and carbon as well as for catalase activity. Through the use of the serial dilution and pour plate technique, the bacterial population was identified [51].

Table 2. Physio-chemical characteristics of soil.

Analysis	Unit	Values
Textural class		Sandy loam
EC_e	dS m^{-1}	1.59 ± 0.0233
pH		7.91 ± 0.0581
Saturation percentage	%	31.73 ± 0.2333
Organic matter	%	0.58 ± 0.0088
Phosphorus	mg kg^{-1}	3.02 ± 0.0722
Potassium	mg kg^{-1}	75.50 ± 1.0408
Nitrogen	%	0.03 ± 0.0006

Data are presented as the means of three replicates with standard errors.

The catalase activity in soil was determined through potassium permanganate titration [52,53]. For this purpose, two grams of freshly collected soil samples was dissolved in 40 mL of sterilized distilled cooled water followed by adding 1.5 mL of H_2O_2 (3%) and mixing by shaking for 30 min on an orbital shaker. Soil particles and impurities were removed through filtration of the solution using sterile Whatman filter paper number 1. After that, a 25 mL filtrate was titrated against a 0.1 M potassium permanganate solution until the endpoint was colorless. The units of catalase activity are represented by the volume of the 0.1 M potassium permanganate solution used for the titration. The breakdown of 0.1 moles of H_2O_2 in one minute is represented by one unit (1 U) of catalase activity.

4.6. Statistical Analysis

Five replications were maintained in all experiments for better comparison of results. A linear-model completely randomized design (CRD) was used for statistical analysis by using one-way analysis of variance. Statistics 8.1 (Informer Technologies, Inc., Los Angeles, CA, USA) was used to perform multiple comparisons of means at a par 5% probability level. Linear regression and correlation analysis between bacterial population in soil and microbial biomass carbon and nitrogen was performed using Origin 2018. Scatter diagrams were plotted for comparisons of organic acids released by the tested strains.

5. Conclusions

Catalase-producing *Bacillus* strains, i.e., *B. megaterium* AN35, *B. megaterium* AN24, *B. megaterium* AN31 and *B. aryabhattai*, have several plant-growth-promoting characteristics in terms of urease, exopolysaccharide and siderophore production. The sole and co-inoculation of catalase-producing *Bacillus* strains improved the growth of rice seedlings. The outcome showed that a consortium of *Bacillus* strains with catalase activity has more potential to promote an antioxidant defense system in plants and promote the growth of rice seedlings. Thus, a consortium of catalytic bacterial strains with plant growth promotion traits can be used as a bioinoculant to improve the growth and physiology of crops.

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Data Availability Statement: The raw data presented in this study are available and will be available on editor's request.

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References

1. Thangaraj, B.; Solomon, P.R. Immobilization of lipases–A review. Part I. Enzyme Immobilization. *ChemBioEng. Rev.* **2019**, *6*, 157–166. [CrossRef]
2. Nandi, A.; Yan, L.J.; Jana, C.K.; Das, N. Role of Catalase in Oxidative Stress- and Age- Associated Degenerative Diseases. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 9613090. [CrossRef]
3. Wang, X.; Li, S.; Liu, Y.; Ma, C. Redox regulated peroxisome homeostasis. *Redox Biol.* **2015**, *4*, 104–108. [CrossRef] [PubMed]
4. Simanjuntak, E.; Zulham. Superoxide dismutase (SOD) and free radical. *J. Keperawatan Dan Fisioter. JKF* **2020**, *2*, 124–129. [CrossRef]
5. Afzal, S.; Chaudhary, N.; Singh, N.K. Role of soluble sugars in metabolism and sensing under abiotic stress. In *Plant Growth Regulators*; Springer: Cham, Switzerland, 2021; pp. 305–334.
6. Johnson, L.A.; Hug, L.A. Distribution of reactive oxygen species defense mechanisms across domain bacteria. *Free Radic. Biol. Med.* **2019**, *140*, 93–102. [CrossRef] [PubMed]
7. Foyer, C.H.; Noctor, G. Stress-triggered redox signaling: What's in pROSpect? *Plant Cell Environ.* **2016**, *39*, 951–964. [CrossRef] [PubMed]
8. Gupta, A.; Tiwari, A.; Ghosh, P.; Arora, K.; Sharma, S. Enhanced lignin degradation of paddy straw and pine needle biomass by combinatorial approach of chemical treatment and fungal enzymes for pulp making. *Bioresour. Technol.* **2023**, *368*, 128314. [CrossRef]
9. Chen, C.H. *Xenobiotic Metabolic Enzymes: Bioactivation and Antioxidant Defense*; Springer: Cham, Switzerland, 2020; pp. 221–234.
10. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alex. J. Med.* **2018**, *54*, 287–293. [CrossRef]
11. Falade, A.O.; Mabinya, L.V.; Okoh, A.I.; Nwdo, U.U. Studies on peroxidase production and detection of Sporotrichum thermophile-like catalase-peroxidase gene in a Bacillus species isolated from Hogsback Forest reserve, South Africa. *Heliyon* **2019**, *5*, e03012. [CrossRef]
12. Schlüter, U.; Bouvier, J.W.; Guerreiro, R.; Malisic, M.; Kontny, C.; Westhoff, P.; Stich, B.; Weber, A.P.M. Brassicaceae display diverse photorespiratory carbon recapturing mechanisms. *bioRxiv* **2022**. [CrossRef]
13. Eisenhut, M.; Roell, M.S.; Weber, A.P. Mechanistic understanding of photorespiration paves the way to a new green revolution. *New Phytol.* **2019**, *223*, 1762–1769. [CrossRef] [PubMed]
14. Fernie, A.R.; Bauwe, H. Wasteful, essential, evolutionary stepping stone? The multiple personalities of the photorespiratory pathway. *Plant J.* **2020**, *102*, 666–677. [CrossRef] [PubMed]
15. Garcia, C.P.; Filippis, D.L.; Gul, A.; Hasanuzzaman, M.M.; Ozturk, V.; Altay, M.; Lao, T. Oxidative stress and antioxidant metabolism under adverse environmental conditions: A review. *Bot. Rev.* **2021**, *87*, 421–466. [CrossRef]
16. Busch, F.A. Photorespiration in the context of Rubisco biochemistry, CO_2 diffusion and metabolism. *Plant J.* **2020**, *101*, 919–939. [CrossRef]
17. Nosheen, A.; Bano, A. Potential of plant growth promoting rhizobacteria and chemical fertilizers on soil enzymes and plant growth. *Pak. J. Bot.* **2014**, *46*, 1521–1530.
18. Khatun, M.R.; Mukta, R.H.; Islam, M.A.; Hud, A.N. Insight into Citric Acid-Induced Chromium Detoxification in Rice (*Oryza sativa* L). *Int. J. Phytoremediation* **2019**, *21*, 1234–1240. [CrossRef] [PubMed]
19. Tahjib, U.A.M.; Zahan, M.I.; Karim, M.M.; Imran, S.; Hunter, C.T.; Islam, M.S.; Murata, Y. Citric acid-mediated abiotic stress tolerance in plants. *Int. J. Mol. Sci.* **2021**, *22*, 7235. [CrossRef] [PubMed]
20. Choudhary, D.K.; Kasotia, A.; Jain, S.; Vaishany, A.; Kumari, S.; Sharma, K.P.; Varma, A. Bacterial-mediated tolerance and resistance to plants under abiotic and biotic stresses. *J. Plant Grow Regul.* **2016**, *35*, 276–300. [CrossRef]
21. Hussain, S.; Khan, F.; Cao, W.; Wu, L.; Geng, M. Seed priming alters the production and detoxifcation of reactive oxygen intermediates in rice seedlings grown under sub-optimal temperature and nutrient supply. *Front. Plant Sci.* **2016**, *7*, 439. [CrossRef]
22. Bibián, M.E.; Pérez-Sánchez, A.; Mejía, A.; Barrios, G.J. Penicillin and cephalosporin biosyntheses are also regulated by reactive oxygen species. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 1773–1783. [CrossRef]
23. Taniguchi, I.; Yoshida, S.; Hiraga, K.; Miyamoto, K.; Kimura, Y.; Oda, K. Biodegradation of PET. Current status and application aspects. *Acs Catal.* **2019**, *9*, 4089–4105. [CrossRef]
24. Chauhan, A.; Siani, R.; Sharma, J.C. Plant growth promoting rhizobacteria and their biological properties for soil enrichment and growth promotion. *J. Plant Nutr.* **2021**, *45*, 273–299. [CrossRef]

25. Harman, G.; Khadka, R.; Doni, F.; Uphoff, N. Benefits to plant health and productivity from enhancing plant microbial symbionts. *Front. Plant Sci.* **2021**, *11*, 610065. [CrossRef]
26. Babiker, B.M.; Ahmed, A.E.; Ibrahim, H.M. Isolation & identification of catalase producing *Bacillus* spp: A comparative study. *Int. J. Adv. Res.* **2017**, *4*, 1206–1211.
27. Philibert, T.; Rao, Z.; Yang, T.; Zhou, J.; Huang, G.; Irene, K.; Samuel, N. Heterologous expression and characterization of a new heme-catalase in *Bacillus subtilis* 168. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 729–740. [CrossRef] [PubMed]
28. Sun, B.O.; Bai, Z.; Bao, L.; Xue, L.; Zhang, S.; Wei, Y. Bacillus subtilis biofertilizer mitigating agricultural ammonia emission and shifting soil nitrogen cycling microbiomes. *Environ. Int.* **2020**, *144*, 105989. [CrossRef]
29. Anbu, P.; Kang, C.H.; Shin, Y.J.; So, J.S. Formations of calcium carbonate minerals by bacteria and its multiple applications. *Springer Plus* **2016**, *5*, 1–26. [CrossRef]
30. Dar, A.A.; Pan, B.; Qin, J.; Zhu, Q.; Lichtfouse, E.; Usman, M.; Wang, C. A review on sustainable ferrate oxidation: Reaction chemistry, mechanisms and applications to eliminate micro pollutant (pharmaceuticals) in wastewater. *Environ. Pollut.* **2021**, *290*, 117957. [CrossRef]
31. Morcillo, R.; Manzanera, M. The Effects of Plant-Associated Bacterial Exopolysaccharides on Plant Abiotic Stress Tolerance. *Metabolites* **2021**, *11*, 337. [CrossRef] [PubMed]
32. Naseer, I.; Ahmad, M.; Hussain, A.; Jamil, M. Potential of zinc solubilizing *Bacillus* strains to improve rice growth under axenic conditions. *Pak. J. Agric. Sci.* **2020**, *57*, 1057–1071.
33. Mumtaz, M.Z.; Barrya, K.M.; Bakera, A.L.; Nichols, D.S.; Ahmad, M.; Zahir, Z.A.; Britza, M.L. Production of lactic and acetic acids by *Bacillus* sp. ZM20 and *Bacillus cereus* following exposure to zinc oxide: A possible mechanism for Zn solubilization. *Rhizosphere* **2019**, *12*, 100170. [CrossRef]
34. Timmusk, S.; Behers, L.; Muthoni, J.; Muraya, A.; Aronsson, A.C. Perspectives and challenges of microbial application for crop improvement. *Front. Plant. Sci.* **2017**, *8*, 49.
35. Trivedi, P.; Leach, J.E.; Tringe, S.G.; Singh, B.K. Plant-microbiome interactions: From community to plant health. *Nat. Rev. Microbial.* **2020**, *18*, 607–621. [CrossRef] [PubMed]
36. Liu, H.Q.; Lu, X.B.; Li, Z.H.; Tian, C.Y.; Song, J. The role of root-associated microbes in growth stimulation of plants under saline conditions. *Land Degrad. Dev.* **2021**, *32*, 3471–3486. [CrossRef]
37. Jian, S.; Li, J.; Chen, J.I.; Wang, G.; Mayes, M.A.; Dzantor, K.E.; Hui, D.; Luo, Y. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. *Soil Biol. Biochem.* **2016**, *101*, 32–43. [CrossRef]
38. Hussain, A.; Zahir, Z.A.; Ditta, A.; Tahir, M.U.; Ahmad, M.; Mumtaz, M.Z.; Hayat, K.; Hussain, S. Production and implication of bio-activated organic fertilizer enriched with zinc-solubilizing bacteria to boost up maize (*Zea mays* L.) production and biofortification under two cropping seasons. *Agronomy* **2020**, *10*, 39. [CrossRef]
39. Lukashe, N.S.; Mupambwa, H.A.; Green, E.; Mnkeni, P.N.S. Inoculation of fy ash amended vermicompost with phosphate solubilizing bacteria (Pseudomonas fuorescens) and its influence on vermi-degradation, nutrient release and biological activity. *Waste Manag.* **2019**, *84*, 14–22. [CrossRef]
40. Iqbal, Z.; Bushra; Hussain, A.; Dar, A.; Ahmad, M.; Wang, X.; Brtnicky, M.; Mustafa, A. Combined Use of Novel Endophytic and Rhizobacterial Strains Upregulates Antioxidant Enzyme Systems and Mineral Accumulation in Wheat. *Agronomy* **2022**, *12*, 551. [CrossRef]
41. Beers. R., F.; Sizer, I.W. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **1952**, *195*, 133–140. [CrossRef]
42. Hildebrandt, G.; Roots, I. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. *Arch. Biochem. Biophy.* **1975**, *171*, 385–397. [CrossRef]
43. Burbank, M.B.; Weaver, T.J.; Williams, B.C.; Crawford, R.L. Urease activity of ureolytic bacteria isolation from six soils in which calcite was precipitated by indigenous bacteria. *Geomicrobiol. J.* **2012**, *29*, 389–395. [CrossRef]
44. Cappuccino, J.G.; Sherman, N. Microbiology. In *Laboratory Manual*, 8th ed.; Pearson: London, UK, 2002; Volume 13, ISBN 978-0805325782.
45. Schwyn, B.; Neilands, J.B. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* **1987**, *160*, 47–56. [CrossRef]
46. Payne, S.M. Iron acquisition in microbial pathogenesis. *Trends Microbiol.* **1993**, *1*, 66–69. [CrossRef]
47. Ashraf, M.; Hasnain, S.; Berge, O.; Mahmood, T. Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. *Biol. Fertil. Soils* **2004**, *40*, 157–162. [CrossRef]
48. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
49. Butsat, N.; Weerapreeyakul, N.; Siriamornpun, S. Change in the phenolic acids and antioxidant activity in Thai rice husk at fie growth stages during grain development. *J. Agric. Food Chem.* **2009**, *57*, 4566–4571. [CrossRef] [PubMed]
50. Bertani, G. Studies on Lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* **1952**, *62*, 293–300. [CrossRef] [PubMed]
51. Wollum, A.G., II. Cultural methods for soil microorganisms. In *Methods of Soil Analysis: Chemical and Microbial Properties*; Page, A.L., Miller, R.H., Keeney, D.R., Eds.; ASA and SSSA Publication: Madison, WI, USA, 1982; pp. 718–802.

52. Cao, J.; Ji, D.; Wang, C. Interaction between earthworms and arbuscular mycorrhizal fungi on the degradation of oxytetracycline in soils. *Soil Biol. Biochem.* **2015**, *90*, 283–292. [CrossRef]
53. Li, Q.; Liang, J.H.; He, Y.Y.; Hu, Q.J.; Yu, S. Effect of land use on soil enzyme activities at karst area in Nanchuan, Chongqing Southwest China. *Plant Soil Environ.* **2014**, *60*, 15–20. [CrossRef]