**Running Title:** *Bacillus* as Biofertilizer and Biocontrol

**The Potential of Several *Bacillus* Rhizobacteria as Biofertilizer and Biocontrol**

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**Novelty**

Various rhizobacteria currently used have a single function, including N fixation, nutrient solvents (P and K), or biocontrol. We found that several *Bacillus* Rhizobacteria have multifunctional potential as biofertilizers and biocontrol.

**Abstract**

Using biofertilizers and biocontrol agents can reduce the reliance on chemical inputs and be environmentally friendly in agricultural production. The Bacillus rhizobacteria are a group of microorganisms that can serve as both biofertilizers and biocontrol agents. This study explores the potential of several *Bacillus* strains as biofertilizers and biocontrol agents. A total of 17 *Bacillus* isolates were collected from various rhizospheres of plants and different soil types in South Sulawesi, Indonesia. The isolates were molecularly identified at the strain level through 16S rRNA gene sequencing. These isolates were evaluated for their biofertilizer potential (ability to fix N, solubilize P and K, thermotolerance, exudation of GA3 and IAA) and biocontrol potential (proteolytic, chitinolytic, cellulolytic). The results indicate that *Bacillus* rhizobacteria can be isolated from various plant rhizospheres and soil types and exhibit multifunctionality. The ability of each *Bacillus* isolates differs, even at the same strain. Isolates LSi-3 (*B. albus* strain MCCC 12605), JSi-4 (*B. cereus* IAM 12605), PGa-1.2 (*B. albus* strain MCCC 1A02146), KW0-2.2 (*B. cereus* strain SJ37), KWo-2.1 (*B. proteolyticus* strain MCCC 1A00365), BMBe-3 (*Bacillus albus* strain VIT-RPJ) dan BGa-2.1(*Bacillus proteolyticus* strain MCCC 1A00365) are highly promising for use as biofertilizers and biocontrol agents, either individually or in the consortium. Isolates BGa-2.2 (*B. cereus* strain IAM 12605) and JBe-4 (*B. tropicus* strain AOA-CPS1) have the potential to be used in consortia with other rhizobacteria to enhance the exudation of IAA by biofertilizers.

Keywords: Rhizobacteria*,* N-fixation, P-solubilisation, K-solubilisation, phytohormone, environmentally friendly

**Introduction**

The increase in population must be balanced with an increase in food production. However, most of the increase in food production is achieved through intensive farming systems that rely heavily on synthetic chemicals, such as fertilizers and pesticides, to boost plant growth and suppress pests and diseases. The long-term use of chemical fertilizers for agricultural production negatively impacts the environment. Excessive use of inorganic fertilizers is significantly correlated with a reduction in biodiversity (Mozumder *and* Berrens, 2006). Thus, there is a need to develop alternative fertilizers that are inexpensive, effective, and environmentally friendly and work in harmony with nature (Pirttilä *et al.* 2021).

The use of chemical pesticides poses significant risks to the environment and non-target organisms, i.e. beneficial soil microorganisms, insects, plants, fish, and birds, as well as causing pathogen resistance (Pirttilä *et al.* 2021; Sharma and Singhvi, 2017). Furthermore, intensive use of chemical pesticides has a negative impact on soil environments, as pesticide residues persist in the soil for a considerable amount of time. Most pesticides negatively affect microorganisms' biological function, diversity, composition, and biochemical processes (Meena *et al.* 2020).

Efforts to reduce chemical usage and promote environmentally friendly agriculture include the utilization of rhizobacteria as biofertilizers and biocontrol. Rhizobacteria are bacteria that colonize plant roots and have the ability to fix nitrogen, solubilize nutrients, produce plant growth hormones, produce fungal growth inhibitors, and control plant pathogens without disrupting plant and other ecosystem components (Kang *et al.* 2015; Mahanty *et al.* 2016; Mendes *et al.* 2020; Li *et al.* 2017; Poria *et al.* 2021).

One commonly used rhizobacterium for biofertilization and biocontrol is *Bacillus*. The genus Bacillus has multifunctionality, acting as a biofertilizer that promotes plant growth through nutrient availability and phytohormone production. Additionally, *Bacillus* can be a biocontrol with antagonistic activity by releasing extracellular metabolites such as antibiotics, cell wall hydrolases, and siderophores (Ma *et al.* 2018; Miljakovi'c *et al.* 2020). Various plants have shown positive effects from the application of *Bacillus spp*. on growth and yield, including tomato, potato, cucumber, maize, beans, soybean, sunflower, wheat, pepper, rice, and many others (Akinrinlola *et al.* 2018; Husna and Pratiwi, 2020; Khan *et al.* 2022). Each strain has a different potential as a biofertilizer and biocontrol.

The aim of this study is to investigate the potential of several *Bacillus* strains as biofertilizers and biocontrol.

**Materials and Methods**

**Isolation and identification of *Bacillus* from the plant rhizosphere**

Rhizobacteria isolates were obtained from various plant rhizospheres, including maize, taro, cocoa, soybean, pine, bamboo, carrot, pepper, and grass, in several locations in the province of South Sulawesi, Indonesia. The bacterial isolation was conducted using the serial dilution method up to 108. One gram of soil was dissolved in 10 ml of distilled water and shaken for 30 minutes. Then, 1 ml of the soil suspension was added into a test tube containing 9 ml of distilled water. Subsequently, 0.1 ml of the final suspension was cultured on Nutrient Agar (NA) medium in a Petri dish and incubated at 28°C for 24 hours.

The identification of selected *Bacillus* rhizobacteria isolates at the strain level was conducted through molecular methods, specifically sequencing of the 16S rRNA gene. Bacterial DNA was isolated and purified using the Quik-DNA TM fungal/bacterial Miniprep Kit (D6005). The isolated DNA was then amplified using a pair of primers: 27F (5' AGAGTTTGATCCTGGCTAG 3') and 1492R (5' TACGGYTACCTTGACGACTT 3'). The PCR product was electrophoresed using MyTag HS Red Mix (Bioline) and visualized under a UV transilluminator. The DNA amplification of the selected isolate resulted in a 1400 bp product. The sequencing results were used to search for homologous 16S rRNA sequence matches in the DNA database (GenBank) using the BLAST program by the National Centre for Biotechnology Information (NCBI).

**Determine the Ability of Bacteria as a Biofertilizer**

**Nitrogen fixation and phosphate and potassium solubilization**

The ability of the bacterial isolates to fix nitrogen was tested using Ashby's Mannitol Agar medium (composed of 20 g mannitol, 0.2 g K2HPO4, 0.1 g NaCl, 0.1 g K2SO4, 0.2 g MgSO4.7H2O, 5 g CaCO3, 20 g agarose, and distilled water to make 1L). Each isolate was streaked onto the surface of the Ashby's Mannitol agar medium and incubated at room temperature for seven days. The presence of a clear zone around the bacterial colonies indicates the ability to fix nitrogen.

The ability of bacterial isolates to solubilize phosphate was tested using Pikovskaya's agar medium with the following composition: 10 g glucose, 5 g Ca3(PO4)2 (TCP), 0.5 g yeast extract, 0.5 g (NH4)2SO4, 0.2 g KCl, 0.2 g NaCl, 0.1 g MgSO4.7H2O, a small amount of MnSO4 and FeSO4, 20 g agarose, and 1 L distilled water. Each isolate was streaked onto the surface of Pikovskaya's agar medium and incubated for seven days at room temperature. The presence of clear zones around the bacteria indicated their ability to solubilize phosphate.

The potassium solubilization ability of bacterial isolates was tested using Alexandrov agar medium (containing 5 g glucose, 0.5 g MgSO4.7H2O, 0.006 g FeCl3, 0.1 g CaCO3, 2 g Ca3PO4, 3 g K2HPO4, 20 g agar, and 1000 mL distilled water). Each Bacillus isolate was streaked onto the surface of Alexandrov agar medium and incubated at room temperature for seven days. Clear zones around bacterial colonies indicated the ability to solubilize potassium.

**Phytohormones (Indole Acetic Acid and Gibberellin Acid) production**

The ability of bacteria to produce IAA was tested by growing them in Nutrient Broth (NB) supplemented with L-tryptophan at 0.1 g/L, at a temperature of 28°C in the dark for seven days. The supernatant was then centrifuged, and 1 mL was transferred to a reaction tube containing 1 mL of Salkowski's reagent (composed of 150 mL H2SO4, 250 mL sterile aquades, and 7.5 mL FeCl36H2O 0.5 M), and stored at room temperature in the dark for 24 hours. A pink colour change in the culture indicates the presence of IAA production. The concentration of IAA was measured using a spectrophotometer at a wavelength of 535 nm with an IAA standard.

To determine the ability of bacteria to produce Gibberellic Acid (GA3), they were grown on Nutrient Broth (NB) medium. A 1 ml bacteria isolate was inoculated into the NB medium and incubated at room temperature for 7 days, followed by centrifugation for 15 minutes. Next, 15 ml of the supernatant was mixed with 2 ml of zinc acetate, and then 2 ml of potassium ferrocyanide was added after 2 minutes, followed by centrifugation for 10 minutes. Then, 5 ml of the mixture was added to a test tube containing 30% hydro colloidal acid and incubated for 75 minutes at 28°C. GA3 was measured using a spectrophotometer at a wavelength of 254nm with a GA3 standard.

**Thermotolerant Bacteria**

 The purpose of the thermotolerance test is to obtain bacteria that are resistant to high temperatures. Heat-resistant bacterial isolates are tested by incubating the bacterial isolate suspension at 50°C. 100 µl of the isolate is added to 10 ml of NB medium and then incubated for 72 hours. After incubation, the bacterial isolate suspension is regrown on Nutrient Agar, and its growth is observed after 24 hours. The growth of the isolate indicates that it is resistant to the temperature tested, and the population of living bacterial colony isolates is calculated. The number of colonies that grow is then converted into cfu/ml units using the formula:

X

Bacterial population. = -------------

p x v

Information:

X = The number of colonies that grew on the petri dish with a dilution factor of (cfu).

p = dilution factor of

v = The suspension volume spread on the petri dish (mm).

**Determine the ability of bacteria as a biocontrol**

**The activity of chitinase, cellulase and protease of isolates**

The chitinolytic testing that is commercial chitin colloid and crab shell chitin was prepared by adding 20 g (of commercial and crab shell chitin) into 300 ml of concentrated HCl and homogenizing the mixture. The solution was then incubated in a refrigerated cabinet for 24 hours, and 200 mL of cold distilled water was added and left to stand overnight at 4°C. The solution was then filtered using glass wool, and the filtrate was neutralized with 12 N NaOH to pH 7. The solution was then centrifuged at 4,000 rpm for 10 minutes, and the resulting precipitate was washed with sterile distilled water and centrifuged again at 4,000 rpm for 10 minutes. The bacterial isolates were streaked onto chitin agar medium (0.05% MgSO4.7H2O, 0.07% K2HPO4, 0.1% yeast extract, 0.5% crab shell chitin colloid, and 1.5% agar) and incubated at room temperature for 48-120 hours. The diameter of the halo zone was then observed.

The protease production was determined by the procedures i.e. skim Milk Agar medium is prepared by dissolving 10 grams of skim milk in 100 ml of distilled water, heating the solution on a hot plate until dissolved, and sterilizing it at 110°C for 15 minutes. Furthermore, 18 grams of Nutrient Agar (NA) is dissolved in 900 ml of distilled water, boiled on a hot plate, homogenized using a magnetic stirrer, and sterilized at 121°C for 15 minutes. The NA medium is mixed homogeneously with the skim milk medium while hot. The resulting medium is then poured into 9 cm diameter Petri dishes. One colony of bacteria is inoculated onto the medium and incubated at room temperature for 24 hours. The presence of a halo zone surrounding the bacterial colony indicates proteolytic activity.

Cellulolytic test using the procedure i.e., rhizobacteria isolate was cultivated on Carboxymethyl cellulose (CMC) medium, which was composed of MgSO4.7H2O (0.05 g/100 ml), Na2HPO4.2H2O (0.5 g/100 ml), NaCl (0.23 g/100 ml), yeast extract (0.2 g/100 ml), CMC (1 g/100 ml), and agar (2.5 g/100 ml). A single colony was inoculated and incubated for 24 hours at 35°C. The colony was then stained with 0.1% Congo red and incubated for 30 minutes, then rinsed with a 1% NaCl solution. A halo zone around the colony was observed and measured for its diameter.

**Data Analysis**

The ability to fix N and solubilize P and K are classified into four categories, namely: (-) negative indicating the absence of halo zones, (+) halo zone <2 cm (low), (++) halo zone 2-3 cm (moderate), and (+++) halo zone >3 cm (high).

Comparison of the data on IAA, GA, thermotolerance, chitinolytic, and proteolytic activity, were analysed using the following equations.

Xi = ̅X$\pm $ Std, ------ X >0

Xi = comparison data

 ̅X = Average value of all data

Std= standard deviation from all data

Criteria: if X= 0 (no value), X>X i ( ̅X + Std) is high, X=X i = value between ( ̅X - Std) and ( ̅X + Std) is medium, and X < Xi ( ̅X - Std) is low

Determining rhizobacteria that are superior to other bacteria is done by scoring each bacterium with a value of 0 – 3, namely 0 = no value, 1 = low, 2 = medium, and 3 = high. The highest total value is considered superior bacteria.

**Results**

**Isolated sources**

A collection of rhizobacteria was obtained from various locations in South Sulawesi, Indonesia. The rhizobacterial isolates were taken from the rhizosphere of maize, taro, cacao, shallot, soybean, pine, bamboo, carrot, pepper, and grass plants. These plants were grown in environments ranging from lowlands (30m above sea level) to highlands (1602m above sea level), on acidic, calcareous, and saline soils with pH values ranging from 5.2 to 8.2 (as shown in Table 1).

Table 1. Isolate code, Plant Rhizosphere, and soil properties of rhizobacteria collection in

South Sulawesi, Indonesia

| No |  Isolate code | Plant rhizosphere | Coordinates and latitude | Soil properties |
| --- | --- | --- | --- | --- |
| 1 | JJMs-3 | Maize | 4054’33.8’’S119051’49.7’’E306 m asl (metre above the sea level) |  Calcareous soil, pH 8.2 |
| 2 | TMs-4 | Taro | 5007’27.0’’S119036’48.0’’E91 m asl | Aciid soil, pH 5.2 |
| 3 | CBe-2.2 | Cacao | 4031’25.7’’S120007’58.5’’E251 m asl |  Calcareous soil, pH 7,6 |
| 4 | BMBe-3 | Shallot | 4035’42.3’’S120015’46.6’’E100 m asl | Calcareous soil, pH 8 |
| 5 | JBe-4 | Maize | 4014’34.1’’S120013’52.4’’E102 m asl |  Calcareous soil, pH 7.5 |
| 6 | KWo-2.1 | Soybean | 4°03'00.4"S 120°01'11.4"E30 m asl | Normal pH 7 |
| 7 | KWo-2.2 | Soybean | 4°03'00.4"S 120°01'11.4"E30 m asl | Normal soil, pH 7 |
| 8 | PGa-1.2 | Pinus | 5014’22.1’’S119038’20.3’’E139 m asl | Normal soil, pH 6.7 |
| 9 | PGa-1.3 | Pinus | 5014’22.1’’S119038’20.3’’E139 m asl | Normal soil, pH 6.7 |
| 10 | BGa-2.1 | Bamboo | 5016’47.8’’S119045’49.7’’E407m asl | Normal soil, pH 7 |
| 11 | BGa-2.2 | Bamboo | 5016’47.8’’S119045’49.7’’E407 m asl | Normal soil, pH 7 |
| 12 | WGa-3 | Carrot | 5015’08.5’’S119055’17.7’’E1602 m asl |  Normal soil, pH 6.7 |
| 13 | WGa-3.1 | Carrot | 5015’08.5’’S119055’17.7’’E1602 m asl |  Normal soil, pH 6.7 |
| 14 | JSi-1 | Maize | 5014’18.1’’S119059’51.1’’E 1021 m asl | Normal soil, pH 6.9 |
| 15 | LSi-3 | Pepper | 5013’19.1’’S120008’16.5’’E220 m asl |  Acid soil, pH 5.8 |
| 16 | JSi-4 |  Maize | 5013’59.3’’S120008’27.3’’E356 m asl |  Normal soil, pH 6.9 |
| 17 | RBg-1 | types of grass | 5035’12.9’’S120005’08.8’’E5 m asl | Salin soil, pH 8.5 |

**Morphology of bacterial isolates**

The isolated rhizobacteria were identified as Bacillus sp. based on their Gram-positive reaction. The colony morphology exhibited variations, ranging from irregularly circular, irregularly circular with rough margins, to undulated circular with elevated and flat edges. The colonies were white and pale yellow (Table 2).

Table 2. Morphological Characterization of Bacterial Isolates

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| No | Isolate Code | Colony Form | Edge of the colony | Colony Elevation | Colony colour | Gram reaction |
|   |   |   |   |   |   |   |
| 1 | JMs-3 | Round | Choppy | Appear | White | (+) |
| 2 | TMs-4 | Round | Complete | Appear | Yellowish white | (+) |
| 3 | CBe-2.2 | Round | Choppy | Appear | White | (+) |
| 4 | BMBe-3 | Round | Choppy | Appear | White slightly translucent | (+) |
| 5 | JBe-4 | Round | Complete | Appear | White | (+) |
| 6 | KWo-2.1 | Round | Choppy | Appear | White | (+) |
| 7 | KWo-2.2 | Round | Choppy | Appear | Clear white | (+) |
| 8 | PGa-1.2 | Round | Choppy | Appear | White | (+) |
| 9 | PGa-1.3 | Round | Choppy | Appear | White | (+) |
| 10 | BGa-2.1 | Round | Choppy | Flat | White | (+) |
| 11 | BGa-2.2 | Round | Choppy | Appear | White | (+) |
| 12 | WGa-3 | Round | Choppy | Appear | Beige | (+) |
| 13 | WGa-3.1 | Round | Choppy | Appear | White | (+) |
| 14 | JSi-1 | Round | Complete | Appear | White | (+) |
| 15 | LSi-3 | Round | Jagged | Flat | White | (+) |
| 16 | JSi-4 | Irregular | Choppy | Flat | White | (+) |
| 17 | RBg-1 | Round | Jagged | Appear | White | (+) |

**Strain identification of using 16s rRNA gene partial sequences**

After the identification of isolates using 16S rRNA gene sequence homology analysis with GenBank database, 17 isolates were identified as *Bacillus*, with the following strains obtained: 1) *B. proteolyticus* MCCC 1A00365, which were isolated from maize, soybean, bamboo, and carrot plants (isolates JMs-3, W0-2.1, BGa-2.1, and WGa-3.1, respectively), 2) *B. cereus* XS.7-1 (TMs-4), which was isolated from taro plants, 3) *B. cereus* IAM 12605, which were isolated from bamboo and maize plants (isolates BGa-2.2 and JSi-4, respectively), 4) *B. cereus* SJ37 (KWo-2.2), which was isolated from soybean plants, 5) *B. cereus* B.30 (PGa.1-3), which was isolated from pine plants, 6) B. cereus BXC15 (Isolate JSi-1), which was isolated from maize plants, 7) *B. parantracis* MN1F (CBe-2.2), which was isolated from cacao plants, 8) *B. albus* VIT-RPJ (BMBe-3), which was isolated from shallot plants, 9) *B. albus* strain MCCC 1A02146, which were isolated from pine, chili, and rice plants (isolates PGa-1.2, LSi-3, and RBg-1, respectively), 10) *B. tropicus* AOA-CPS1 (JBe-4), which were isolated from maize, pepper, and grass plants, 11) *B. paramycoides* MCC1A04098 (WGa-3), which was isolated from carrot plants. The level of similarity between the isolates was 99.65% to 100% (Table 3).

**Table 3. DNA Sequencing result of several Bacillus sp**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| No | Isolate | Bacillus strain | % Similarity | Assessment No. |
| 1 | JMs-3 | *Bacillus proteolyticus* Strain MCCC 1A00365 | 99.93 | NR157735.1 |
| 2 | TMs-4 | *Bacillus cereus* strain XS.7-1 | 100 | MT1000007.1 |
| 3 | CBe-2.2 | *Bacillus paranthaccis strain* MN1F | 99.93% | CP046887.1 |
| 4 | BMBe-3 |  *Bacillus albus* strain VIT-RPJ | 99.86% | KJ437475 |
| 5 | JBe-4 | *Bacillus tropicus strain* AOA-CPS1 | 100 % | CP0491 |
| 6 | KWo-2.1 | *Bacillus proteolyticus* Strain MCCC 1A00365 | 99.93% | NR157735 |
| 7 | KWo-2.2 | *Bacillus cereus* strain SJ37 | 100% | NT103054 |
| 8 | PGa-1.2 | *Bacillus albus* MCCC 1A02146 | 99.33% | NR157729 |
| 9 | PGa-1.3 | *Bacillus cereus strain B.30*  | 99.74% | LN890206.1 |
| 10 | BGa-2.1 | *Bacillus proteolyticus* strain MCCC 1A00365 | 99.79% | NR157735 |
| 11 | BGa-2.2 | *Bacillus cereus* IAM 12605 | 99.86% | NR115526 |
| 12 | WGa-3 | *Bacillus paramycoides* MCCC 1A04098 | 99.81% | NR157734.1 |
| 13 | WGa-3.1 | *Bacillus proteolyticus* MCCC 1A00365 | 99.86% | NR157735 |
| 14 | JSi-1 | *Bacillus cereus* BXC15 | 100% | MN227492.1 |
| 15 | LSi-3 | *Bacillus albus J*MCCC 1A02146 | 100% | NR157729 |
| 16 | JSi-4 | *Bacillus cereus* IAM 12605 | 99.65% | NR115526 |
| 17 | RBg-1 | *Bacillus albus* strain 1A02146 | 99.74% | NR157729 |

**Potential as biofertilizer**

**Nitrogen fixation, Phosphate and Potassium solubility**

The results of this study indicated that of the 17 isolates of Bacillus rhizobacteria (Table 4) which showed the formation of a halo zone on Ashby's Mannitol Agar media, two isolates were revealed that could fix N, namely Kwo-21 (*B. proteolyticus* strain MCCC 1A00365 ) and PGa-1.2 (*B. albus* strain MCCC 1A02146). P solubility using Pikovskaya media were isolates KWo-2.1 and BGa-2.1, both of which were *B. proteolyticus* strain MCCC 1A00365, as well as isolates PGa-1.2 (*B. albus* strain MCCC 1A02146) and PGa-1.3 (*B. cereus* strain B.30), while solubility of K using Alexandrov media showed that all tested Bacillus isolates could dissolve K, except for isolates Kwo-2.1 and BGa-2.2. The ability to dissolve K. BGa-2.1 (*B. proteolyticus* strain MCCC 1A00365), JSi-4 (*B. cereus* IAM 12605), and RBg-1 (*B. albus* strain 1A02146) has a relatively higher ability to solubilize K than other isolates.

Table 4. N-Fixation, P-Solubility, and K-Solubility of Bacterial Isolates from Various Plant Rhizospheres after 7 Days of Incubation

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| No | Isolate Code | N- Fixation  | P-solubility  | K- solubility |
| 1 | JMs-3 | - | - |  ++ |
| 2 | TMs-4 | - | - | + |
| 3 | CBe-2.2 | - | - | + |
| 4 | BMBe-3 | - | - | + |
| 5 | JBe-4 | - | - |  ++ |
| 6 | KWo-2.1 | ++ | +++ | - |
| 7 | KWo-2.2 | - | - | + |
| 8 | PGa-1.2 | + |  ++ | - |
| 9 | PGa-1.3 | - |  + | + |
| 10 | BGa-2.1 | - |  ++ | +++ |
| 11 | BGa-2.2 | - | - | ++ |
| 12 | WGa-3 | - | - | + |
| 13 | WGa-3.1 | - | - | + |
| 14 | JSi-1 | - | - |  ++ |
| 15 | LSi-3 | - | - | ++ |
| 16 | JSi-4 | - | - | +++ |
| 17 | RBg-1 | - | - | +++ |

Description: (-) no halo zone, (+) halo zone <2 cm (low), (++) halo zone 2-3 cm (medium), and (+++) halo zone >3 cm (high).

**Exudation of phytohormones**

The results of this study indicate that all tested *Bacillus* isolates can produce Indole Acetic Acid (IAA) ranges between 13.29-101.28 ppm (Table 5). Isolates JBe-4 (*B. tropicus* strain AOA-CPS1) and BGa-2.2 (B. cereus strain IAM 12605) produce higher amounts of IAA than other isolates, with 101.28 and 90.17 ppm, respectively. Isolates JBe-4 (*B. proteolyticu*s strain MCCC 1A00365) and BGa-2.2 (*B. cereus* strain IAM 12605) also produce GA3 with 19.12 and 12.94 ppm, respectively.

All tested isolates could produce GA3 between 12.94 to 28.08 ppm. Isolate LSi-3 (B. albus strain MCC 1A04098) and JSi-4 (*B. cereus* strain IAM 12605) produced higher levels of GA3 than other isolates, at 25.79 ppm and 28.06 ppm, respectively. These two isolates also produced IAA at 64.14 ppm and 62.25 ppm, respectively (Table 5).

**Thermotolerant bacteria**

Testing thermotolerance on 17 bacterial isolates showed a reduction in population size with increasing temperature. However, all tested isolates could grow up to a temperature of 50°C, with populations ranging from 8.8 x 106 – 266.4 x 106 cfu/ml. Isolates PGa-1.2 (*B. proteolyticus* strain MCCC 1A00365), BGa-2.2 (*B. cereus* strain IAM 12605), RBg-1 (*B. albu*s strain MCCC 1A02146), BGa-2.1 (*B. proteolyticus* strain MCCC 1A00365), and WGa.-3.1 (*B*. *proteolyticus* strain MCCC 1A00365) were classified as thermotolerant at a temperature of 50°C, with their populations showing a significant increase, surpassing 194.69 x 106 cfu/ml (Table 5).

Table 5. Hormone and Thermotolerant (bacterial testing at 50 °C that can grow on culture media).

|  |  |  |  |
| --- | --- | --- | --- |
| No | Isolate Code | Hormone (ppm) | Thermotolerant/Bacterial Population at 50 °C (cfu/ml) |
| **IAA**  | **GA3**  |
| 1 | JMs-3 | 83,46 | 19,17 | 83,2 x 106 |
| 2 | TMs-4 | 44,44 | 16,89 | 96,6 x 106 |
| 3 | Be-2 | 53,50 | 16,73 | 9,6 x 106 |
| 4 | BMBe-3 | 79,05 | 19,89 | 25,9 x 106 |
| 5 | JBe-4 | 101,28 | 19,12 | 17,4 x 106 |
| 6 | KWo-2.1 | 59,31 | 14,79 | 29 x 106 |
| 7 | KWo-2.2 | 66,87 | 17,58 | 140 x 106 |
| 8 | PGa-1.2 | 55,20 | 17,01 | 266,4 x 106 |
| 9 | PGa-1.3 | 13,29 | 13,53 | 159,2 x 106 |
| 10 | BGa-2.1 | 71,66 | 19,19 | 227,6 x 106 |
| 11 | BGa-2.2 | 90,17 | 12,94 | 244,7 x 106 |
| 12 | WGa-3 | 65,49 | 14,79 | 11,7 x 106 |
| 13 | WGa-3.1 | 78,98 | 14,13 | 203,2 x 106 |
| 14 | JSi-1 | 76,06 | 16,08 | 8,8 x 106 |
| 15 | LSi-3 | 64,14 | 25,79 | 22,3 x 106 |
| 16 | JSi-4 | 62,25 | 28,06 | 13,7 x 106 |
| 17 | RBg-1 | 54,96 | 19,74 | 241,8 x 106 |
|  | Average | 65.89 | 17,97 | 101,95 |
|  | Std | 19.26 | 3,92 | 92,74 |
|  | Height | >85.15 | >21,894 | >194,69 |
|  | Medium | 46.67-85.15 | 13.59-21.89 | 9,2 - 194,69 |
|  | Low | <46.67 | <13,59 | <9,2 |

**The potential as biocontrol**

b

**Chitinolytic, proteolytic, and cellulolytic activity**

In this study, several Rhizobacteria bacteria could produce hydrolytic enzymes such as chitinase, proteinase, and cellulose, as indicated by the formation of clear zones around bacterial colonies. The chitinolytic index ranged from 0.13 to 0.73, the proteolytic index ranged from 0.13 to 1.71, and the cellulolytic index ranged from 0.04 to 1.65 (Table 6). The isolates KWo-2.2 (B*. cereus* strain SJ37) and WGa-3 (*B. paramycoides* strain MCCC1A04098) exhibited high chitinolytic activity, with respective chitinolytic indices of 0.46 and 0.73. Isolates TMs-4 (*B. cereus* XS.7-1), Wo-22, and PGa-1.2 exhibited high proteolytic indices, ranging from 0.92 to 1.71, compared to the other isolates. Meanwhile, isolates WGa-3 and BMBe-3 (*B. albus* strain VIT-RPJ) exhibited high cellulolytic indices, with respective indices of 0.63 and 1.63 (Table 6).

Table 6. Mean values of chitinolytic, proteolytic, and cellulolytic activity indices of bacterial isolates after 24-hour incubation.

|  |  |  |
| --- | --- | --- |
| No | Isolate Code | Activity Index |
| Chitinolytic | Proteolytic | Cellulolytic |
| 1 | JMs-3 | 0.23 | 0.13 | 0.44 |
| 2 | TMs-4 | 0 | 0.92 | 0.08 |
| 3 | Be-2 | 0 | 0 | 0.04 |
| 4 | BMBe-3 | 0.27 | 0.26 | 1.65 |
| 5 | JBe-4 | 0.31 | 0.48 | 0 |
| 6 | KWo-2.1 | 0.19 | 0.15 | 0 |
| 7 | KWo-2.2 | 0.46 | 1.03 | 0,4 |
| 8 | PGa-1.2 | 0.17 | 1.71 | 0.11 |
| 9 | PGa-1.3 | 0.16 | 0.68 | 0 |
| 10 | BGa-2.1 | 0.17 | 0.30 | 0 |
| 11 | BGa-2.2 | 0 | 0.22 | 0 |
| 12 | WGa-3 | 0.73 | 0.58 | 0.63 |
| 13 | WGa-3.1 | 0 | 0.44 | 0 |
| 14 | JSi-1 | 0.13 | 0.45 | 0 |
| 15 | LSi-3 | 0.45 | 0.27 | 0.48 |
| 16 | JSi-4 | 0.31 | 0.53 | 0.47 |
| 17 | RBg-1 | 0.37 | 0 | 0 |
|  |  Average | 0,30 | 0,54 | 0,55 |
|  |  Std | 0,16 | 0,40 | 0,49 |
|  | Height | >0,46 | >0,94 | >1.04 |
|  | Medium | 0.14 - 0.46 | 0.14 - 0.94 | 0.06 - 1.04 |
|  | Low | <0,14 | <0,.14 | <0.06 |

**Discussion**

Bacillus rhizobacteria could live around the roots of plants that grow in acid-alkaline soils. Notably, *Bacillus sp*. has been reported to have a broad pH range for optimal growth, from pH 5.0 to 9.0 (Parab *et al.* 2020). The differences in the plant rhizosphere and the growing environment caused morphology, population, rhizobacteria strains, and physiology variations. The diversity of soil conditions and plant rhizosphere is responsible for the diverse rhizobacterial isolates that can be obtained (Oo *et al.* 2020; Hasra and Pratiwi, 2013; Liu *et al.* 2016).The level of similarity of isolate between the isolates was 99.65% to 100% (Table 3). This level of similarity is high because that a using 16S rRNA marker, the identity is considered similar at the species level when the "percentage identity" value is >97.5% and at the genus level when the "percentage identity" value is >95% (Stackebrandt and Goebel, 1994).

The results of this study indicate that some *Bacillus* have a dual function in the availability of plant nutrients as indicated by the presence of N fixation and solubilization of P and K. Isolates KWo-2.1 (*B. proteolyticus* strain MCCC 1A00365) and PGa-1.2 (*B. albus* strain MCCC 1A02146) are good at fixing N and solubilizing P but are unable to solubilize K. Isolates PGa-1.3 and BGa-2.1 can solubilize P and K but cannot fix N. The JMS-3, TMs-4, CBe-2.2, BMBe-3, JBe-4, KWo-2.2, BGa-2.2, WGa-3, WGa-3.1, JSi-1, LSi-3, JSi-4, and RBg-1 isolates can only solubilize K, meanwhile, Ga-2 isolate can only solubilize P. In similar research, *Bacillus sp*. can fix N and solubilize P (Husna and Pratiwi 2020; Sahin *et al.* 2004). The enhancement of nutrient availability and plant growth by inoculation with N-fixation, P or K-solubilizing rhizobacteria has been extensively studied and documented by many researchers (Han and Lee 2006; Zhu *et al.* 2011; Pilar *et al.* 2013; Sheng and He 2014; Liu *et al.* 2016; Zhang *et al.* 2017; Husna *and* Pratiwi, 2020; Singh *et al.* 2020; Sembiring *et al.* 2021; Gupta *et al.* 2021)

All *Bacillus* isolates can produce Indole Acetic Acid (IAA) and Gibrellin Acid (GA3). Similarly, the research findings of Gusmiaty *et al.* (2019) and Mendes *et al.* (2020) indicate that more than 80% of bacteria isolated from the rhizosphere can produce IAA and Gibberellin. The production of IAA by bacteria varies depending on species and strain. Differences in morphological characteristics of rhizobacterial colonies support the variation of bacterial strains in producing IAA (Lestari *et al.* 2017; Kumar *et al.* 2014). IAA produced by *Bacillus* isolates significantly improves vigour of rice and corn seeds when inoculated with the bacteria (Lestari *et al.* 2015; Lwin *et al.* 2012 (Pakhtunkhwa *et al.* 2017; Chandra *et al.* 2018). the use of bacillus rhizobacteria which can exudate the hormone gibberellin can improve plant growth (Kang *et al.* 2014; Desai, 2017; Kang *et al.* 2019). The production of IAA and GA3 by rhizobacteria suggests using these bacteria as a potential source of biofertilizers to enhance plant growth and productivity, especially under adverse environmental conditions. Thus, identifying rhizobacteria with IAA and GA3-producing ability and their subsequent use as biofertilizers could have important implications for sustainable agriculture.

The use of rhizobacteria as a biocidal agent has gained increasing attention due to its potential as an eco-friendly alternative to pesticide use, which is non-resistant to target organisms and has a stimulating effect on plant growth. One of the mechanisms by which bacterial strains become biocidal agents is by degrading the cell wall of pathogenic microorganisms through the production of hydrolytic enzymes (Roca-couso and Flores-f, 2021; Khan *et al.* 2022). Testing the chitinolytic, proteolytic, and cellulolytic activities of bacteria aims to determine the presence of chitinase, protease, and cellulase enzymes produced by the bacteria. These enzymes play an important role in controlling plant pests/diseases by degrading the cell walls of phytopathogens (Akinrinlola *et al.* 2018; Oo *et al.* 2020). In this study, several rhizobacteria bacteria could produce hydrolytic enzymes such as chitinase, proteinase, and cellulose, as indicated by the formation of clear zones around bacterial colonies. *Bacillus* sp can produce hydrolytic enzymes such as chitinase, gluconase, cellulase, lipase, and protease, which can hydrolyse the main components of fungal cell walls and suppress the development of plant pathogens (Bonaterra *et al.* 2022). Enzyme-producing bacteria capable of hydrolyzing protein, cellulose, and chitin on test media have the ability to inhibit the growth of *Fusarium sp*  (Meliah *et al.* 2020). The antagonistic properties of hydrolytic enzymes against various phytopathogens play a significant role in biocidal activity (Jadhav *et al.* 2017).

 The results of this study demonstrate that *Bacillus* spp. has significant potential as an alternative to inorganic/chemical fertilizers and pesticides to promote plant growth and production. Each isolate possesses multifunctional activity and can serve as both a biofertilizer (with the ability to nitrogen fixation, solubilize phosphorus and potassium, and produce plant hormones such as IAA and GA3) and a biocontrol agent (with the ability to produce chitinase, protease, cellulase, and inhibit fungal growth), whether used as single rhizobacterial isolates or as a consortium of rhizobacterial isolates. Each isolate exhibits distinct capabilities, even within the same strain level. Therefore, to select the most effective isolate as a biofertilizer and biocontrol agent, it is necessary to score the potential of each isolate. The top-scoring rhizobacterial isolates for use as biofertilizers or biocontrol agents are those with a total value ranking between 1-3 (Table 7). Based on these criteria, the selected isolate are LSi-3 (B. albus strain MCCC 12605), JSi-4 (*B. cereus* IAM 12605), SWo-2.2 (*B. cereus* strain SJ37), KWo-2.1 (*B. proteolyticus* strain MCCC 1A00365), PGa-1.2 (*B. albus* strain MCCC 1A02146), BMBe-3 (*Bacillus albus* strain VIT-RPJ) dan BGa-2.1(*Bacillus proteolyticus* strain MCCC 1A00365) . Although each of the selected isolates can be used as a biofertilizer and biocontrol agent on their own, each isolate has its own strengths and weaknesses, therefore, it is recommended to create a consortium by combining the strengths of each isolate to overcome their respective weaknesses. Even isolates BGa-2.2 (*B. cereus* strain IAM 12605) and JBe-4 (*B. tropicus* strain AOA-CPS1), whose total values do not fall within the top three ranks, are worthy of inclusion in a consortium as they have the ability to produce higher levels of IAA than other isolates. Furthermore, consortiums of rhizobacteria have been shown to improve nutrient availability and N, P, and K uptake and increase rice yields more than single rhizobacterial strains (Gupta *et al.* 2021).

**Conclusion**

*Bacillus* rhizobacteria exhibit multifunctional properties and possess great potential as biofertilizers and biocontrol due to their ability to fix nitrogen, solubilize phosphorus and potassium, exhibit thermotolerance, and secrete plant growth regulators such as GA3 and IAA. Additionally, they possess promising bioprotective properties, including proteolytic, chitinolytic, and cellulolytic activities. However, each *Bacillus* strain exhibits its unique advantages, and to fully utilize these advantages, a group of strains is recommended for use as biofertilizers and bioprotectants.

Isolates LSi-3 (*B. albus* strain MCCC 12605), JSi-4 (*B. cereus* IAM 12605), PGa-1.2 (*B. albus* strain MCCC 1A02146), KW0-2.2 (*B. cereus* strain SJ37), KWo-2.1 (*B. proteolyticus* strain MCCC 1A00365), BMBe-3 (*Bacillus albus* strain VIT-RPJ) dan BGa-2.1(*Bacillus proteolyticus* strain MCCC 1A00365) are highly promising for use as biofertilizers and biocontrol agents, either individually or in the consortium. Isolates BGa-2.2 (*B. cereus* strain IAM 12605) and JBe-4 (*B. tropicus* strain AOA-CPS1) have the potential to be used in consortia with other rhizobacteria to enhance the exudation of IAA by biofertilizers.

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| --- |
| Table 7. Assessment and ranking for their ability to function of isolate |
| No |  Isolate | N- Fixation  | P-solubility  | K- solubility | IAA | GA3 | Thermo-tolerant | Chitinolytic | proteolytic  | Cellulolytik | Total value | Rank |
| 1 | JMs-3 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 13 | 4 |
| 2 | TMs-4 | 0 | 0 | 1 | 1 | 2 | 2 | 0 | 2 | 2 | 10 | 7 |
| 3 | Be-2 | 0 | 0 | 1 | 2 | 2 | 1 | 0 | 0 | 1 | 7 | 10 |
| 4 | BMBe-3 | 0 | 0 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 14 | 3 |
| 5 | JBe-4 | 0 | 0 | 2 | 3 | 2 | 2 | 2 | 2 | 0 | 13 | 4 |
| 6 | KWo-2.1 | 2 | 3 | 0 | 2 | 2 | 2 | 1 | 2 | 0 | 14 | 3 |
| 7 | KWo-2.2 | 0 | 0 | 1 | 2 | 2 | 2 | 3 | 3 | 1 | 14 | 3 |
| 8 | PGa-1.2 | 1 | 2 | 0 | 2 | 2 | 3 | 1 | 3 | 2 | 16 | 1 |
| 9 | PGa-1.3 | 0 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 0 | 9 | 8 |
| 10 | BGa-2.1 | 0 | 2 | 3 | 2 | 2 | 3 | 1 | 2 | 0 | 15 | 2 |
| 11 | BGa-2.2 | 0 | 0 | 2 | 3 | 1 | 3 | 0 | 2 | 0 | 11 | 6 |
| 12 | WGa-3 | 0 | 0 | 1 | 2 | 2 | 1 | 3 | 2 | 2 | 13 | 4 |
| 13 | WGa-3.1 | 0 | 0 | 1 | 2 | 1 | 2 | 0 | 2 | 0 | 8 | 9 |
| 14 | JSi-1 | 0 | 0 | 2 | 2 | 2 | 1 | 1 | 2 | 0 | 10 | 7 |
| 15 | LSi-3 | 0 | 0 | 2 | 2 | 3 | 3 | 2 | 2 | 2 | 16 | 1 |
| 16 | JSi-4 | 0 | 0 | 3 | 2 | 3 | 2 | 2 | 2 | 2 | 16 | 1 |
| 17 | RBg-1 | 0 | 0 | 3 | 2 | 2 | 3 | 2 | 0 | 0 | 12 | 5 |

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