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# Screening of Plant Growth-Promoting Halotolerant Bacteria Isolated from Weeds Rhizosphere Grown in Saline Soil

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**ABSTRACT**

This study aimed to screen and characterize halotolerant bacterial isolates, which could enhance plant growth performance in saline soil. Halotolerant bacteria was isolated from weeds rhizosphere grown in saline soil of coastal agricultural land located at Brondong District, Lamongan Regency, East Java Province, Indonesia. This research was conducted from June to September 2018. Seven bacterial isolates can grow in a Nutrient Agar medium containing 10% of NaCl, suggesting that these bacteria were halotolerant. Furthermore, all bacterial isolates were shown to produce indol acetic acid (IAA) and do not induce a hypersensitive response when infiltrated into tobacco leaves. These results explain that these bacteria had potency as plant growth-promoting rhizobacteria (PGPR) and were not tend to be the plant pathogen. The growth of seedlings when inoculated in cucumber seed grown in saline media were higher than those in control. This result suggests that the halotolerant bacteria can enhance the development of cucumber seedlings in saline stress conditions. Three potential halotolerant bacteria i.e., SN22, SN23, SN26 were selected and molecularly identified as *Bacillus megaterium*, *Bacillus* sp., and *B. megaterium*, respectively.

## INTRODUCTION

Indonesia has 106,000 km of coastline with more than one million hectares of land potential for agriculture (Arifin, Herawati, Mujiyo, & Widijanto, 2021). However, agricultural land near the coastline is potentially exposed to salinity caused by tidal waves and seawater intrusion (Gopalakrishnan, Hasan, Sanaul Haque, Jayasinghe, & Kumar, 2019). One of the efforts to overcome soil high salinity is by using tolerant crops to high salinity conditions. However, several studies report that few cultivars of tomato, cucumber, corn, and rice were shown susceptible to salinity stress (Baghbani, Forghani, & Kadkhodaie, 2013; Hoffmann, Berni, Hausman, & Guerriero, 2020; Reddy, Kim, Yoon, Kim, & Kwon, 2017; Shtereva, Vassilevska-Ivanova, & Karceva, 2015). Efforts to address the high salinity problem in soil are not sufficient only with tolerant plant cultivars since limited plant cultivars were easy against salinity stress (Shrivastava & Kumar, 2015). Therefore, improving soil fertility in saline soils through the seasonal planting arrangements and using microbial technology to improve plant growth in a high saline environment were promised.

Microbial technology has been widely used to enhance soil fertility and reduce synthetic chemicals by using microbes to function as biofertilizers (Nosheen, Ajmal, & Song, 2021; Ortiz & Sansinenea, 2022). Microbial technology to manage salinity in soil can be done by utilizing microbes, especially soil inhabitant bacteria that tolerate growing in soil with high salinity levels called halotolerant bacteria (Shrivastava & Kumar, 2015). Several studies have reported the finding of several types of halotolerant bacteria which can help plants to overcome salinity stress and grow better on saline (Egamberdieva, Shurigin, Gopalakrishnan, & Sharma, 2014; Iqbal, Khalid, Zahir, & Ahmad, 2016;

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Zafar-ul-Hye, Farooq, Zahir, Hussain, & Hussain, 2014). Some of these bacteria were even capable of producing plant growth regulators, defined as plant growth-promoting bacteria (PGPB) (Islam et al., 2016; Paul & Lade, 2014). PGPB can improve nutrient acquisition and soil fertility in saline soil and enhance crop productivity (Shilev, 2020; Teo et al., 2022). These bacteria used to live and grow naturally (indigenous) by colonizing the rhizosphere of plants on soil that contains high salinity (Shrivastava & Kumar, 2015). Isolation and utilization of such indigenous bacteria improve crop resistance against salinity stress and enhance microbial biodiversity in saline soils (Etesami & Beattie, 2018).

Information on the utilization of indigenous halotolerant rhizobacteria of saline soil, particularly those isolated from weeds inhabited in saline soil, is still limited. Hence more studies are needed to obtain high potential halotolerant bacteria. Therefore, this study aimed to obtain halotolerant bacteria recovered from the rhizosphere of weeds grown in saline soil of farmland located in the coastal area in Lamongan District, East Java which has the potency of plant growth-promoting bacteria (PGPB).

## MATERIALS AND METHODS

### Halotolerant Bacteria Sampling and Isolation

The sampling location was conducted in the coastal farmland in Brondong District, Lamongan Regency, East Java, from June to September 2018. Isolation of halotolerant bacteria originating from the rhizosphere of weeds grown in saline soil was performed by the method described by Munif, Hallmann, & Sikora (2013) with minor modification. The samples used in this study were the rhizosphere, i.e., roots and surrounding soil roots of weeds grown in saline soil of agricultural land. A total of 10 g of the sample were suspended in 90 ml of sterile aqua dest and then subjected to serial dilution. The suspension in each dilution series (100 µl) was spread over nutrient agar (NA) plates, and then the culture was incubated for 24 hours at 27-28°C. The growing bacterial colonies were then purified on a fresh NA plate and then subjected to further screening by growing them in NA plates containing NaCl with a concentration of 10%. The purified bacteria recovered from colonies grown on the agar plates containing 10% NaCl were determined as halotolerant bacteria and stored for the next experimental stage.

### Characterization of Halotolerant Bacteria

Bacterial morphological characters such as shape, color, and edges of the colony were obtained from observations of single colonies grown on the NA plate. In addition, gram reaction, cell shape, and spore production were observed microscopically after Gram and spore staining by a method according to Schaad, Jones, & Chun (2001). The halotolerant bacterial isolates were also tested on a 1-month-old tobacco plant by infiltrating the bacterial suspension on tobacco leaves to determine hypersensitivity reactions (HR).

### Selection of Halotolerant Bacteria in Production of Indole-3-Acetic Acid (IAA)

Halotolerant bacterial isolates were quantitatively tested for their capability to excrete IAA. The assay was performed using selective media containing L-tryptophan (Dawwam, Elbeltagy, Emara, Abbas, & Hassan, 2013). The bacterial isolates were cultivated in a liquid Nutrient Broth (NB) containing tryptophan (0.1 g/l) for three days at a temperature of 28°C in the dark. The bacterial culture was then centrifuged (10,000 rpm for 10 minutes). Next, two ml of supernatant were incorporated with 4 ml of Salkowski reagent (7.5 ml FeCl3.7H2O 0.5 M; 150 ml concentrated H2SO4; 250 ml sterile aqua dest), homogenized, and then left for 15 minutes. When the solution changed its color, the absorbance was measured using a spectrophotometer (wavelength of 530 nm). Finally, the absorbance was calculated into the standard curve equation to determine the IAA concentration.

### The Effect of Halotolerant Bacteria Inoculation on the Growth of Cucumber Seedling

Cucumber seeds were surface sterilized by soaking with either 95% alcohol for 5 minutes and then 5% NaClO solution for 1 minute. The seeds were then rinsed three times using sterile distilled water. They were then dipped in 48 hours of halotolerant bacterial culture suspension for 15 minutes. About 25 cucumber seeds were put into seed paper and placed on a Petri dish. The seed paper was moistened with NaCl solution (EC value of 5 dS/m). The cucumber seeds were grown for two weeks and subjected to the observation of growth parameters, i.e., the percentage of germination, root length, and hypocotyl length.

**Molecular Identification of Halotolerant Bacteria**

The molecular identification of the halotolerant bacteria selected as PGPR was performed by sequencing the 16S rRNA gene. Genomic DNA from halotolerant bacteria was isolated using PrestoTM Mini gDNA Bacteria Kit (Gene aid). The 16S rRNA gene in genomic DNA was amplified by PCR reaction using KOD FX Neo (Toyobo, Japan). DNA amplification was performed on a PCR machine with the following reaction stages: heating at 95°C for 1 minute, annealing at 55°C for 1 minute, and elongating at 72°C for 5 minutes. The PCR results were then subjected to gel electrophoresis with 1% agarose in the TBE buffer (Green and Sambrook, 2012). The amplicon of the PCR result was then purified and sequenced. The sequence of the 16S rRNA gene was used for the search for a homologous series of 16S rRNA sequences in the DNA database (GenBank) using the BLASTN software available on the NCBI website. The phylogenetic tree was constructed using MEGA version 7 (Kumar, Stecher, & Tamura, 2016).

**Data Analysis**

The effect of halotolerant bacteria inoculation on cucumber seedlings was analyzed with ANOVA. If the results were significantly different among the treatments, a posthoc test was used with the Duncan Multiple Range Test (DMRT) at an error level of 5%. Data were analyzed using SPSS 22.0, and visualized by ggplot2 in RStudio.

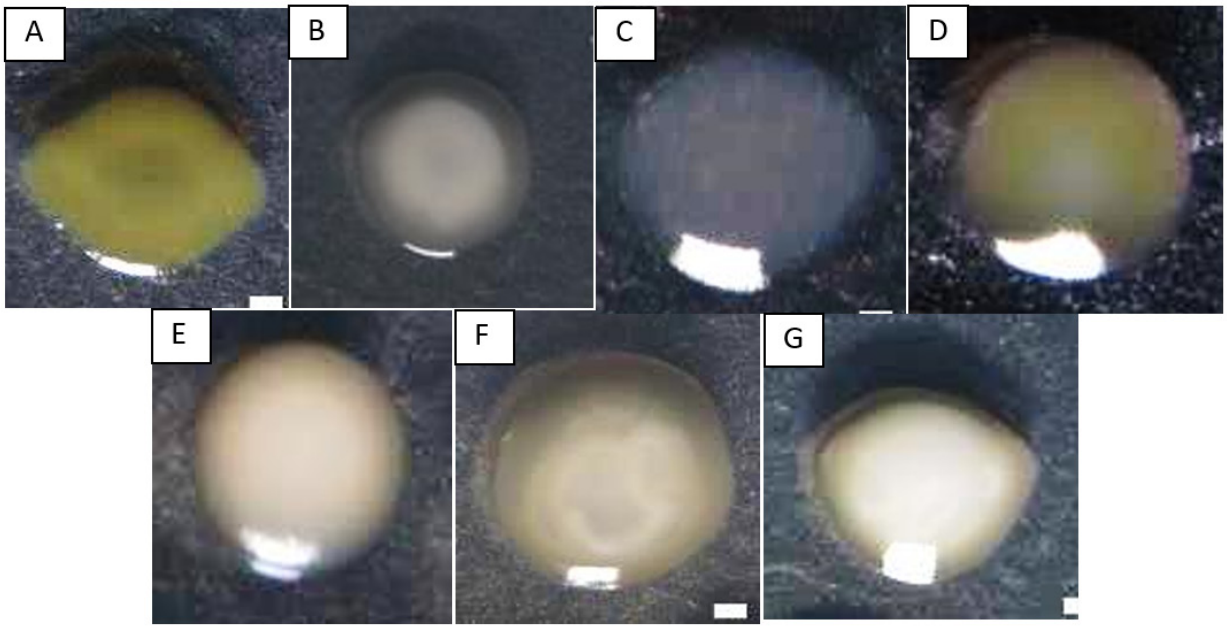


**Fig. 1.** Colony morphology of halotolerant bacterial isolates: A. SN1, B. SN2, C. SN6, D. SN15, E. SN22, F. SN23, and G. SN26

**Table 1.** Morphology, cell morphology, and the hypersensitive reaction of halotolerant bacterial isolates

| Isolates | Colony Morphology | | Cell Morphology | | | | Hypersensitive Reaction |
|---|---|---|---|---|---|---|---|
| | Form | Color | Margin | Gram | Shape | Spore Production | |
| SN1 | Round | White | - | + | bacilli | - | - |
| SN2 | Round | White, translucent | - | + | bacilli | - | - |
| SN6 | Round | White, opaque | - | + | bacilli | + | - |
| SN15 | Round | Yellow | - | + | bacilli | - | - |
| SN22 | Round | White, opaque | - | + | bacilli | + | - |
| SN23 | Round | White | - | + | bacilli | + | - |
| SN26 | Round | White, opaque | - | + | bacilli | + | - |

## RESULTS AND DISCUSSION

### Characterization of Halotolerant Bacteria

Twenty-three bacteria have been isolated from the rhizosphere of weeds inhabited in saline farmland. Seven bacterial isolates could be grown on an NA medium containing 10% NaCl. Hence it could be determined that the seven bacterial isolates were halotolerant bacteria. Seven isolates have different colony morphology (Fig. 1). They can not produce a hypersensitive reaction when the bacterial cells were infiltrated on tobacco leaves, indicating that they tend to had no potential as plant pathogens. All isolates were Gram-positive bacilli but differ in spore-forming ability (Table 1). Only four halotolerant bacterial isolates, i.e., SN6, SN22, SN23, and SN26, produce spores that are the main character of the genus of *Bacillus*. Several bacterial species that have been found as halotolerant are reported to be Gram-positive *Bacillus* (Obeidat, 2017; Ruginescu et al., 2020). Berrada et al. (2012) also reveal that 102 of 124 bacteria isolated from marsh and saltern ecosystem in Morocco are Gram-positive halotolerant bacteria dominated by the genus of *Bacillus*, *Jeotgalibacillus*, *Planococcus*, *Staphylococcus*, and *Thalobacillus*.

### IAA Producing Halotolerant Bacteria

The screening for IAA-producing bacteria shows that all bacterial isolates can excrete IAA with a concentration of 1.15 to 10.08 ppm (Table 2). These results indicate that all bacterial isolates were potential PGPR since IAA functions as a plant hormone to promote plant growth. It has been widely described that certain types of bacteria that originated from the plant rhizosphere were capable of producing IAA. For example, Chaiharn & Lumyong (2011) found that 18.05% of 216 bacteria isolated from rice plants in Thailand can produce IAA. Mohite (2013) reports that 10 bacteria isolated from banana, wheat, and maize rhizosphere were IAA producers and identified as *Lactobacillus acidophilus, L. casei, B. megaterium*, *B. subtilis*, and *B. cereus*. Mike-Anosike, Braide, & Adeleye (2018) also reported several bacteria isolated from *Panicum maximum* rhizosphere identified as *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp., and *Pseudomonas* were able to produce IAA ranging 4.0 mg/l to 10.0 mg/l.

**Table 2.** Production of IAA by halotolerant bacteria

| Isolate | IAA concentration (ppm) |
|---|---|
| SN1 | 3.10 |
| SN2 | 2.50 |
| SN6 | 1.15 |
| SN15 | 5.24 |
| SN22 | 10.08 |
| SN23 | 2.10 |
| SN26 | 1.35 |

### The Effect of Halotolerant Bacteria Inoculation on Cucumber Seedlings in Salinity Stress Condition

Inoculation of halotolerant bacterial isolates affects the cucumber seedlings' growth during the germination stage under the salinity stress conditions. The seed germination percentage in the inoculation of five halotolerant bacterial isolates, i.e., SN6, SN15, SN22, SN23, and SN26, were higher than control (Fig. 2A). SN26 and SN23 show the highest percentage of seed germination. However, the percentage of seed germination in the inoculation of two halotolerant bacterial isolates, i.e., SN1 and SN2, did not differ compared to that of control. These results suggested that the injection of five halotolerant bacterial isolates, i.e., SN6, SN15, SN22, SN23, and SN26, enhanced the seed germination in cucumber seedlings under the salinity stress condition. Ramadoss, Lakkineni, Bose, Ali, & Annapurna (2013) also reported that wheat seed germination was enhanced by more than 50% through inoculation with halotolerant bacteria strain compared to an uninoculated seed. A higher percentage of germination due to inoculation of halotolerant bacteria was also reported on alfalfa and sugar beet (Ansari, Shekari, Mohammadi, Biró, & Végvári, 2017; Zhou, Zhao, & Tian, 2017).

Cucumber seedlings with halotolerant bacterial isolates show longer roots than uninoculated seedlings (Fig. 2B). The length of hypocotyl shows higher in the cucumber seedling inoculated with all halotolerant bacterial isolates than that of the uninoculated seedling (Fig. 2C). These results suggest that the inoculation of the halotolerant bacteria enhances the growth of root and hypocotyl of cucumber seedlings when grown in salinity stress conditions. Yañez-Yazlle, Romano-Armada, Acreche, Rajal, & Irazusta (2021) report

that halotolerant bacteria isolated under saline stress can induce root length of chia (*Salvia hispanica* L.) and quinoa (*Chenopodium quinoa* Willd.) from 60% to 92%. Albdaiwi, Khyami-Horani, Ayad, Alananbeh, & Al-Sayaydeh (2019) also report six halotolerant bacterial strains as PGPR increased survival on inoculated plants on high levels salinity conditions as indicated on seedling root and hypocotyl growth and when compared to uninoculated plants.

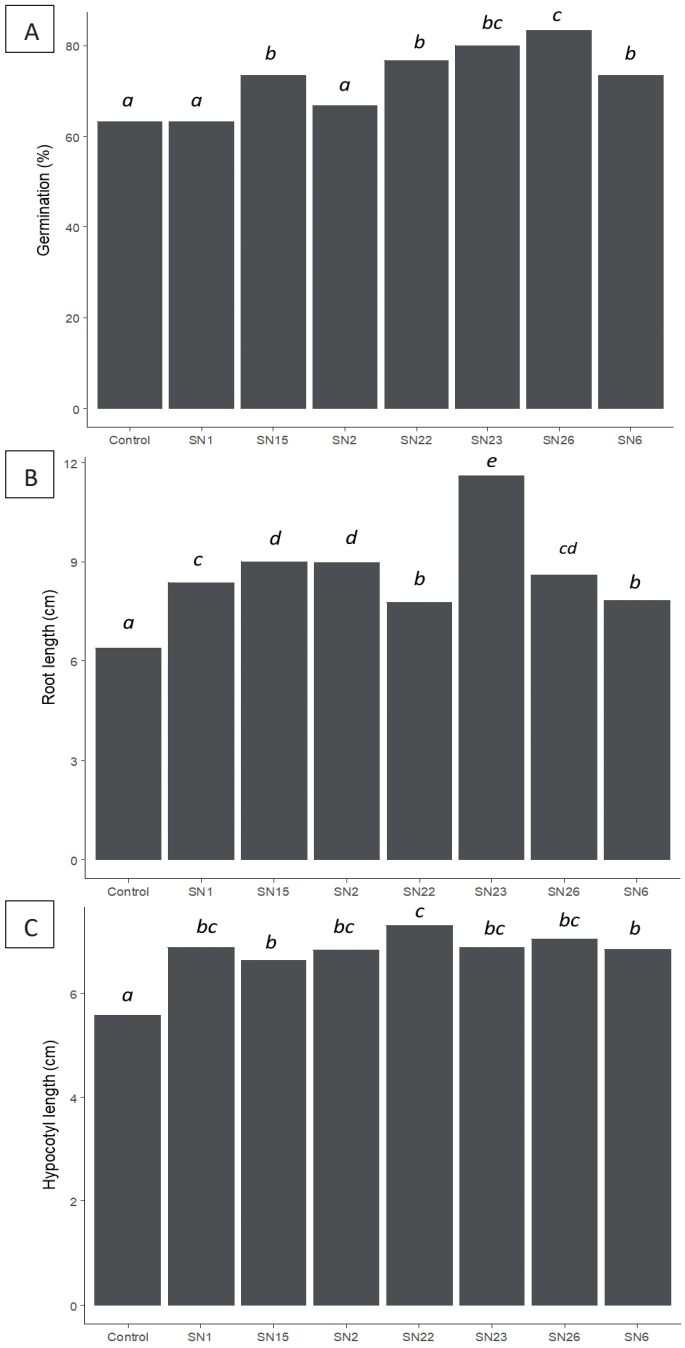


**Fig. 2.** The effect of halotolerant bacteria inoculation on cucumber seedlings based on (A) the percentage of seed germination, (B) root length, and (C) hypocotyl lenght. Bar with different letters is significantly different between isolates, at $p < 0.05$, according to DMRT.

**Molecular Identification of Potential Halotolerant Bacteria as PGPR**

In this study, three isolates of halotolerant bacteria had high potential as PGPR, i.e., SN 22, SN 23, and SN 26, based on their ability to produce IAA and enhance cucumber seedlings under the high salinity condition. Based on the results of BLAST-N using the DNA sequence of the gene encoding 16s rRNA, the halotolerant bacterial isolate of SN23 has 99.93% similarity to *Bacillus* sp. strain NE3RP2, SN26 had 99.58% similarity to *B. magaterium* strain MUGA128, and SN 22 had 99.93% similarity to *B. magaterium* strain WSH-002 (Table 3). Furthermore, the phylogenetic tree developed using maximum likelihood showed that each halotolerant bacterial isolate was clustered following the result of affinity. For example, the phylogenetic tree analysis using 16S rDNA showed SN23 and *B. magaterium* NBRI16I in one clade, and the affinity of strains in trees showed 72% (Fig. 3).

These results suggest that the molecular identification was consistent with the results of morphological and physiological characterizations. All the three halotolerant bacteria were from the genus of *Bacillus*, indicating that the genus of *Bacillus* used to had the capacity to grow under salinity stress. Several species of *Bacillus* were reported can improve the crop growth under salinity stress, such as *B. megaterium* on cucumber (Nadeem et al., 2016), *B. subtilis* on rice (Jha & Subramanian, 2014; Jha, Subramanian, & Patel, 2011), *B. safensis* and *B. cereus* on wheat (Saxena, Kumar, Chakdar, Anuroopa, & Bagyaraj, 2020), *B. subtilis* on maize (Ferreira et al., 2018), *B. subtilis* on radish plant (Sedki & El-Mohamedy, 2012), and *B. subtilis* on Indian bassia (Abeer et al., 2015).

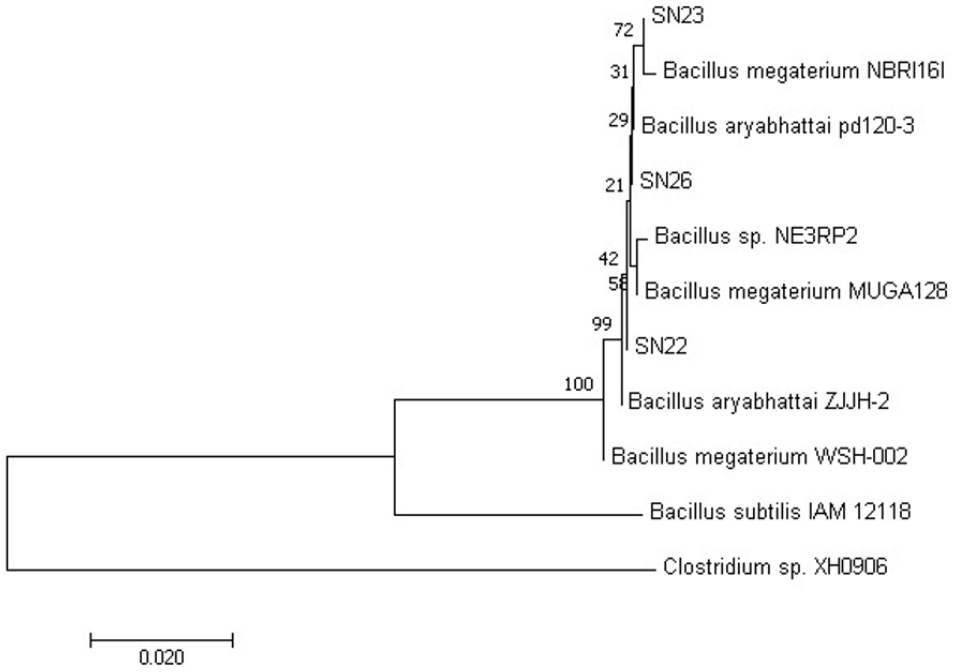


**Fig. 3.** Maximum likelihood tree, numbers above branches indicate branch lengths. The accession numbers on the right are identified from sequences from GenBank, and other sequences are obtained from the BOLD public database.

**Table 3.** The results of halotolerant bacteria identification potential as PGPR by similarity index of 16S rRNA gene sequence

| Isolate | Max. Score | E. Value | Ident | Accession Code | Species Name |
|---|---|---|---|---|---|
| SN 22 | 2531 | 0.0 | 99.93% | CP003017.1 | *Bacilus megaterium* |
| SN 23 | 2509 | 0.0 | 99.93% | KT900606.1 | *Bacilus* sp. |
| SN 26 | 2498 | 0.0 | 99.58% | KT719649.1 | *Bacilus megaterium* |

The identification of potential endophytic bacteria as biostimulants showed the dominance of *Bacillus* sp. Several *Bacillus* species can survive in high temperatures (thermotolerant) and high salinity (halotolerant) (Farace et al., 2015; Ji et al., 2022).

Based on the results, it has been suggested that the halotolerant bacteria enhanced the growth of cucumber seedlings higher than that in control without bacteria. Bacteria isolated from saline farmland have been reported to be a biostimulant in enhancing plant growth. One of the mechanisms of soil microorganisms in helping plant growth is their ability to produce phytohormones (de Souza, Ambrosini, & Passaglia, 2015). Phytohormones can be produced by certain microorganisms or plants that affect plants' physiological processes (Egamberdieva, Wirth, Alqarawi, Abd_Allah, & Hashem, 2017). Bacteria that can grow in extreme conditions will enhance the production of plant hormones. This hormone accelerates growth so that the yield of plants obtained enhances. Some soil microorganisms are known to have the ability to produce IAA phytohormones in halophilic plant areas (Orhan, 2016). The finding of plant growth-promoting halotolerant bacteria recovered from weeds rhizosphere is relatively new. It can be used as a novel approach to obtain the halotolerant bacteria that also play a role as PGPR.

## CONCLUSION

Seven bacterial isolates were halotolerant bacteria. All isolates were Gram-positive bacilli but differed in spore-forming ability (SN 1, SN 2, SN6, SN 15, SN 22, SN23, SN 26). Bacterial isolates that produce the highest IAA were SN22, and SN23 and SN26 isolates can enhance the germination of cucumber plants. The molecular identification has shown that SN22, SN23, and SN26 isolates were identified as *B. megaterium*, *Bacillus* sp., and *B. megaterium,* respectively.

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