



# Multifarious Potential of Biopolymer-Producing *Bacillus subtilis* NJ14 for Plant Growth Promotion and Stress Tolerance in *Solanum lycopersicum* L. and *Cicer arietinum* L: A Way Toward Sustainable Agriculture

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## Abstract

Diverse practices implementing biopolymer-producing bacteria have been examined in various domains lately. PHAs are among the major biopolymers whose relevance of PHA-producing bacteria in the field of crop improvement is one of the radical unexplored aspects in the field of agriculture. Prolonging shelf life is one serious issue hindering the establishment of biofertilizers. Studies support that PHA can help bacteria survive stressed conditions by providing energy. Therefore, PHA-producing bacteria with Plant Growth-Promoting ability can alter the existing problem of short shelf life in biofertilizers. In the present study, *Bacillus subtilis* NJ14 was isolated from the soil. It was explored to understand the ability of the strain to produce PHA and augment growth in *Solanum lycopersicum* and *Cicer arietinum*. NJ14 strain improved the root and shoot length of both plants significantly. The root and shoot length of *S. lycopersicum* was increased by 3.49 and 0.41 cm, respectively. Similarly, *C. arietinum* showed a 9.55 and 8.24 cm increase in root and shoot length, respectively. The strain also exhibited halotolerant activity (up to 10%), metal tolerance to lead (up to 1000 µg/mL) and mercury (up to 100 µg/mL), indicating that the NJ14 strain can be an ideal candidate for a potent biofertilizer.

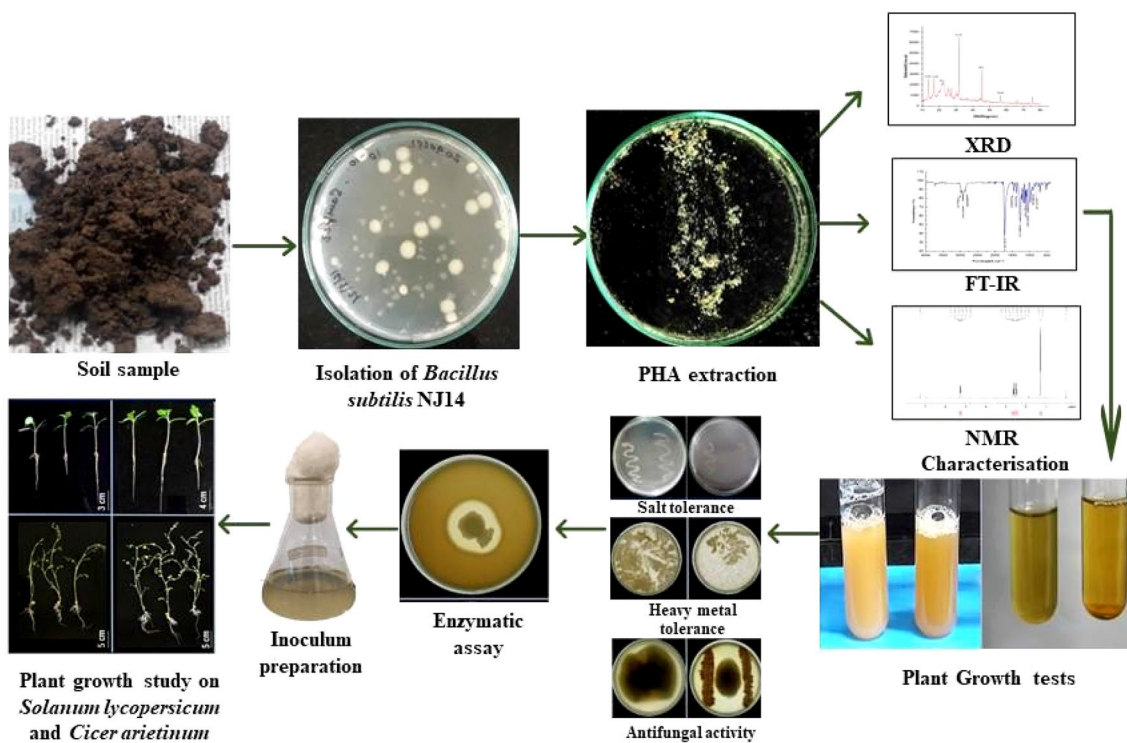
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## Graphical Abstract



**Keywords** Biopolymer · Polyhydroxyalkanoates · Biofertilizer · Plant growth promotion · Halotolerance

## Introduction

Biofertilizers containing one or more microorganisms can accelerate plant growth by transforming unavailable nutrients into useful forms for plant growth. Scientists currently favor and are attempting to ameliorate biofertilizers due to the negative consequences of chemical fertilizers [1]. The shelf life of biofertilizers is one of the significant challenges hindering their establishment. The bacteria themselves, as well as the carrier used for formulation, determine how long biofertilizers last. Polyhydroxyalkanoates (PHA) synthesizing bacteria have been enhancing their chances of survival under challenging circumstances [2]. Hence, PHA-producing bacteria are promising candidates for refining the quality of biofertilizers. While under stress, different prokaryotes tend to mass other biopolymers as energy sources. One such polymer is the polyesters of hydroxyalkanoates, commonly known as PHAs. This biopolymer is built and accumulated as granules when the prokaryotes are subjected to stress by restricting the intake of any necessary nutrients, such as nitrogen, with excess carbon. These PHA granules are also known to develop the organisms' resistance to various stresses, such as maintaining cell integrity throughout an osmotic shock by acting as an intracellular scaffold in

bacterial cells. In contrast, osmotic pressure is present, protecting the cells from severe plasmolysis that could harm the cell membrane [3]. PHA granules also protect against temperature fluctuations; bacterial cells start mobilizing PHA to boost the intracellular concentration of monomers and take advantage of their shielding function to get protection during temperature fluctuations. PHA has also been proven to combat elevated pH, pressure fluctuations, and oxygen pressure [4]. Among the PHAs, the mainly studied class is poly-beta-hydroxybutyric acid or polyhydroxybutyrate (PHB), derived primarily from prokaryotes [5]. The exact role and significance of PHB in enhancing the plant growth ability of rhizobacteria are not studied extensively. Comparing PHA-producing bacteria to mutants defective in PHA production is one way to recognize their role in colonization with plant roots. When *Herbaspirillum seropedicae* was studied for plant growth promotion of *Setaria viridis*, the study proved that *H. seropedicae* SmR1 (PHB producing) appeared to improve total and lateral root area in *Setaria viridis* compared to mutants (incapable of PHB production) [6].

The biosynthesis of PHA can follow different pathways depending on the substrate types employed. The substrates utilized are sugars, fatty acids, and alkanes/alkenes. Three genes are responsible for converting sugar to PHA: A, B,

and C. Acetyl-coenzyme A is generated through pyrolysis, which condenses to yield acetoacetyl-CoA through the catalysis of PHA A gene encoded  $\beta$ -ketoacetyl-CoA thiolase. The NADPH-dependent acetoacetyl-CoA dehydrogenase, ciphered by the pha B gene, reduces the acetoacetyl-CoA to (R)-3-hydroxybutyrate monomer. Later, PHA C-encoded PHA synthase enzymes polymerize the monomer units through ester linkage. PHB is a potential candidate for replacing synthetic polyethylene due to its versatile properties [7].

Stress is one of the major reasons that lead to reduced yield in plants. Various types of stresses like temperature, excess of light, pathogens, pests, lack or excess of nutrients, salinity, water, and mechanical stresses can deteriorate normal growth in plants therefore affecting their yield. The harmful effect of chemical fertilizers and moreover the efficiency of biological agents have drawn attention toward Plant Growth-Promoting Bacteria (PGPR) as a sustainable remedy to manage stress tolerance in plants. PGPR is one of the most focused areas of research and is the current trend in agriculture which is capable of replacing chemical fertilizers. Under stress, PGPR produces PHA in the soil, and therefore, application of biopolymer-producing bacteria as a plant growth-promoting agent is the major objective of this study.

Since these PHA granules boost the organisms' forbearance to a variety of challenges by improving survival, stress tolerance, and cell division, which help extend shelf life, this study focuses on identifying an able PHA-producing applicant that has Plant Growth-Promoting (PGP) activities and on testing its capability to improve growth parameters on a leguminous crop plant *Cicer arietinum* and a non-leguminous plant *Solanum lycopersicum*.

## Methods

### Isolation of *Bacillus subtilis* NJ14

Soil from the rhizosphere of healthy *Coffea arabica* plants was sampled from Kodagu district, Karnataka, India. The collected sample was utilized for isolation by adding soil (1 g) to 100 mL of nutrient broth (peptone-3 g, NaCl-5 g, yeast extract-3 g, 1 L distilled water, pH 7), incubated at  $37 \pm 2$  °C, 24 h, for enrichment. Later, serial dilution of the enriched culture was done up to  $10^{-6}$  and plated onto nutrient agar plates (peptone-3 g, NaCl-5 g, yeast extract-3 g, agar-1.5% (w/v), 1 L distilled water, pH 7). The plates were then placed in a 37 °C incubator for 24 h, and with morphological differences were pure cultured onto fresh nutrient agar plates for maintenance. By evaluating each isolate in terms of their PHA-producing and PGP ability, the potential isolate was determined for further involvement in the study [8].ss

## PHA Staining and Extraction

### Nile Blue Staining

The Nile blue test was a primary method to find PHA-yielding bacteria among the isolates. 0.003 g of Nile blue dye was added in 10 mL of DMSO. 2 mL of this solution was mixed with 100 mL of nutrient agar media (peptone-3 g, NaCl-5 g, yeast extract-3 g, agar-1.5% (w/v), 1 L distilled water, pH 7). The isolates were inoculated on the plates and were incubated (37 °C) for a day. After incubation, the culture on the plates was viewed under a UV chamber for fluorescence. The fluorescence intensity determined the isolates' PHA-producing potentiality [9].

### Sudan Black Staining

The Sudan black test was done to further confirm the presence of PHA granules in the isolates [10]. 0.003% (w/v) of Sudan black solution was prepared using ethanol. The isolates to be tested were smeared on glass slides, and 2 drops of Sudan black solution were added to the slides and placed aside for 10 min for staining. The stain was removed, and the smear was counter-stained using safranin. After 1 min, the smear was gently washed with distilled water to remove excess safranin. The slide was then observed under a Leica microscope (DMi8). PHA granules in bacteria-stained black and vegetative parts stained red indicated positive results.

### Extraction of PHA

Overnight culture (1 mL) of the isolate was added to 100 mL of media for extraction of PHA (NaCl-10 g,  $\text{Na}_2\text{HPO}_4$ -3.7 g,  $\text{KH}_2\text{PO}_4$ -1 g,  $(\text{NH}_4)_2\text{HPO}_4$ -0.5 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.2 g, Glycerol-2%(v/v), tryptone-5 g, yeast extract-0.5 g in 1 L of distilled water,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ , and glycerol were autoclaved separately and added to media) and incubated at  $37 \pm 2$  °C. After 72 h of incubation, the culture was transferred to pre-weighed centrifuge tubes and centrifuged at 6500 rpm for 10 min to collect the pellets. The tubes were placed at 50 °C for 2 h to air dry the pellets completely. The centrifuge tubes with pellets were weighed to get the cell dry weight (g/100 mL). The pellets were treated with 4% (w/v) sodium hypochlorite at  $37 \pm 2$  °C for 1 h to digest all the cell components except PHA. The mixture was spun at 10,000 rpm for 10 min to collect the pellet, which was washed twice with distilled water followed by a wash with a 1:1:1 ratio of acetone: diethyl ether: methanol. Boiling chloroform was added to the centrifuge tubes after washing to dissolve the pellet and poured into a pre-weighed petri dish, and the chloroform

was evaporated. The weight of the petri dish with PHA was noted as a dry weight of PHA (g/100 mL) [11].

## Characterization of Extracted PHA

### Fourier-Transform Infrared (FTIR) Characterization of PHA

FTIR is a routinely used technique to study the functional groups in the extracted polymer by exposing it to IR rays that create distinctive peaks as a function of the chemical groups present in the polymer. FTIR analysis of the extracted polymer was performed by spreading it (100 mg) on to KBr disc, followed by measuring the spectrum at a wavelength range between 400 and 4000  $\text{cm}^{-1}$  using an FTIR spectrometer (Shimadzu IR Spirit-L) [12].

### X-Ray Diffraction (XRD) Analysis of PHA

The crystalline nature of the extracted polymer was assessed through XRD analysis (Philips' Xpert PRO instrument) by scanning at a  $2\theta$  range between 10 and 80° [13].

### Nuclear Magnetic Resonance (NMR) Analysis of PHA

The precise monomeric structure of the extracted PHA was studied using  $^1\text{H}$  NMR analysis. This relies on the characteristic magnetic spin of the particles present in the polymer to deduce their monomeric composition. The extracted polymer (5 mg) was dissolved in deuterated chloroform ( $\text{CDCl}_3$ ), and  $^1\text{H}$  NMR was recorded (Bruker Avance III spectrometer) at 400 MHz using tetramethyl silane as internal standard [14].

## Plant Growth-Promoting Traits

The isolate that confirmed PHA production and was employed for PHA extraction was further subjected to different PGP assays to verify its ability as a significant candidate for bioinoculant.

### ACC Deaminase Test

ACC-deaminase activity of the isolate was checked using DF salts minimal media ( $\text{KH}_2\text{PO}_4$ -4 g,  $\text{Na}_2\text{HPO}_4$ -6 g,  $\text{H}_3\text{BO}_3$ -10  $\mu\text{g}$ ,  $\text{MnSO}_4$ -10  $\mu\text{g}$ ,  $\text{ZnSO}_4$ -70  $\mu\text{g}$ ,  $\text{CuSO}_4$ -50  $\mu\text{g}$ ,  $\text{MoO}_3$ -10  $\mu\text{g}$ ,  $\text{C}_6\text{H}_{12}\text{O}_6$ -2 g,  $\text{FeSO}_4 \cdot 0.7\text{H}_2\text{O}$ -1 mg,  $\text{C}_6\text{H}_{12}\text{O}_7$ -2 g,  $\text{C}_6\text{H}_8\text{O}_7$ -2 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.2 g, agar-1.5 g ( $\text{NH}_4$ ) $_2\text{SO}_4$ -0.3033 g in 1 L distilled water). The isolate was inoculated on media and incubated at  $37 \pm 2$  °C. After 120 h, the plates were checked for growth which indicated positive results [15, 16].

## Phosphate Solubilization Test

The capacity to solubilize phosphate was discerned by inoculating the isolate onto Pikovskaya media (yeast extract-0.5 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.1 g, dextrose-10 g,  $\text{Ca}_3(\text{PO}_4)_2$ -5 g,  $\text{FeSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.0001 g,  $(\text{NH}_4)_2\text{SO}_4$ -0.5 g,  $\text{KCl}$ -0.2 g,  $\text{MnSO}_4$ -0.0001 g, agar-15 g, distilled water-1 L). After inoculation, the plates were incubated at  $28 \pm 2$  °C for 7 days, and the clearance zone was checked on the seventh day [17, 18]. The phosphate Solubilization Index (PSI) of the organism was calculated using the following equation [19, 20]:

$$\text{PSI} = \text{Diameter of colony} + \text{halo} / \text{diameter of colony}$$

## Zinc Solubilization Test

Pikovskaya media altered with  $\text{ZnCl}_2$  (yeast extract-0.5 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.1 g, dextrose-10 g,  $\text{FeSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.0001 g,  $\text{Ca}_3(\text{PO}_4)_2$ -5 g,  $(\text{NH}_4)_2\text{SO}_4$ -0.5 g,  $\text{KCl}$ -0.2 g,  $\text{MnSO}_4$ -0.0001 g, agar-15 g,  $\text{ZnCl}_2$ -2 g distilled water-1 L) were used to test Zn solubilization capacity of the isolate. The media were inoculated with isolate and left at  $28 \pm 2$  °C for a week. The incubated plates were checked for halo framing of the colonies. The Zinc Solubilization Index (ZSI) was obtained by using the following equation [19, 21]:

$$\text{ZSI} = \text{Diameter of colony} + \text{halo} / \text{diameter of colony}$$

## Indole Acetic Acid Test

Nutrient broth (5 mL) was altered with 1% (w/v) tryptophan and added with 1 mL of overnight isolate culture. The broth was placed at  $28 \pm 2$  °C incubator for 48 h and was spun at 10,000 rpm at 4 °C (10 min). 99.9% (w/v) orthophosphoric acid (2 drops) followed by Salkowski reagent, 2 mL (0.5 M  $\text{FeCl}_3$ -1.5 mL, distilled water-50 mL, conc.  $\text{H}_2\text{SO}_4$ -30 mL) was added to 2 mL of supernatant. A blank was prepared by mixing the reagents with 2 mL deionized water instead of the supernatant. IAA was utilized to prepare the standard, with 10  $\mu\text{g}/\text{mL}$  to 500  $\mu\text{g}/\text{mL}$  concentrations. The mixtures were incubated for half an hour (in the dark), and absorbance was measured at 535 nm. Pink coloration in the sample tube denoted the successful production of IAA [8, 22].

## Siderophore Test

The presence of siderophores (small molecular iron chelators) was determined using  $\text{FeCl}_3$ . The overnight culture of

the isolate was spun for 10 min (10,000 rpm), and 2 mL of 2% (w/v)  $\text{FeCl}_3$  was added into 5 mL of the supernatant. The presence of brown color indicated positive results [23, 24].

### Ammonia Test

The isolate was tested for ammonia production using Nessler's reagent. 1 mL of overnight culture was added into 5 mL peptone water (peptone-50 g, NaCl-5 g, distilled water-1 L, pH 7.2) and was placed in a  $28 \pm 2$  °C incubator. After 3 days, the tube was mixed with Nessler's reagent (2 mL). The change in color to light brown from yellow showed positive results [25, 26].

### Nitrogen Fixation Test

The nitrogen-fixation ability of the isolate was tested by inoculating onto Jensen's agar media (Sucrose-20 g,  $\text{K}_2\text{HPO}_4$ -1 g,  $\text{CaCO}_3$ -0.1 g, NaCl-0.1 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.5 g,  $\text{FeSO}_4 \cdot 0.7\text{H}_2\text{O}$ -0.1 g,  $\text{Na}_2\text{MoO}_4 \cdot 0.2\text{H}_2\text{O}$ -0.005 g, agar-1.5% (w/v) distilled water-1 L). The inoculated media were incubated in a  $28 \pm 2$  °C incubator for 3 days, and bacterial growth was checked [27, 28].

### HCN Test

King's B media (Peptone-16 g,  $\text{K}_2\text{HPO}_4$ -1.6 g,  $\text{MgSO}_4 \cdot 0.7\text{H}_2\text{O}$ -1.6 g, glycerol-0.1 L, agar 20 g, distilled water-0.99 L) modified with 4.4 g/L glycine were inoculated with the isolate. A sterile Whatman filter paper (no.1) soaked with picric acid solution (1.5% (w/v)-dissolved a picric acid-2.5 g (w/v) and  $\text{Na}_2\text{CO}_3$ -12.5 g (w/v), in 1 L of distilled water) was stuck on the upper plate of the petri dish. The inoculated plates were incubated for 3 days in a  $28 \pm 2$  °C incubator. Positive HCN production was confirmed when yellow filter paper changed its color to brown [8, 29].

### Enzymatic Assay

The breakdown of substrates and product generation detect the production of different enzymes. The yield of enzymes by the isolate can increase their potential in industrial applications.

Starch agar (Starch-20 g, peptone-20 g, agar 20 g, distilled water-1 L) was used for checking the amylolytic enzyme production by the isolate. The isolate was inoculated on Sterile starch agar plates, which were incubated at  $37 \pm 2$  °C for a day. After incubation, 20 mL of 1% iodine solution was poured onto the media to observe the hydrolysis zone [30].

Cellulase production of the isolate was tested by inoculating on Carboxymethylcellulose (CMC) agar (yeast extract-2 g,  $\text{KH}_2\text{PO}_4$ -1 g,  $\text{MgSO}_4$ -5 g, CMC (w/v)-5 g, agar-15 g in 1 L of distilled water). The inoculated plate with isolate was incubated at  $37 \pm 2$  °C for a day, and post-incubation, the plate was flooded with 15 mL 0.1% Congo red, which was washed out with 20 mL 1 M NaCl solution. Afterward, the plates were checked for positive results by observing a hydrolysis zone around the colony [31].

Skimmed milk agar (yeast extract-2.5 g, tryptone-5 g,  $\text{C}_6\text{H}_{12}\text{O}_6$ -1 g, skim milk powder-28 g, agar-15 g, distilled water-1 L) was used for the screening of protease production. The isolate was inoculated and placed in a  $37 \pm 2$  °C incubator for 48 h. A zone of hydrolysis was observed, framing the colony as a positive result [31].

The production of catalase enzymes can be tested using hydrogen peroxide. 6%  $\text{H}_2\text{O}_2$  (w/v) was dropped on a clean glass slide. From the overnight nutrient agar plate inoculated with the isolate, a single colony was taken using an inoculation loop and mixed with the drop of  $\text{H}_2\text{O}_2$ . The positive result was indicated by the formation of air bubbles [32].

### Halotolerance Test

The halotolerant test investigates the quality of microorganisms flourishing in varying concentrations of NaCl. Nutrient agar plates with different concentrations of NaCl (2%, 4%, 6%, 8%, 10%, and 15% (w/v)) were inoculated with the isolate. The inoculated plates were incubated at  $37 \pm 2$  °C for 24 h, and the percentage of halotolerant was determined by visually observing the bacterial growth on the plates [33].

### Metal Tolerance Test

Metal tolerance can determine a bacterial strain's capacity to mitigate and thrive in an environment with high concentrations of heavy metals. Nutrient agar plates were amended separately with 50, 100, 250, 500, and 1000  $\mu\text{g}/\text{mL}$  of Hg, Cd, and Pb heavy metals. The isolate was inoculated on the plates and incubated at  $37 \pm 2$  °C for 24 h. After visual observation of the bacterial growth, the most promising isolate was selected for further research [34].

### Antifungal Assay

Four fungal plant pathogens, *Talaromyces albobiverticillius*, *Cladosporium tenuissimum*, *Aspergillus niger*, and *Fusarium solani*, were employed to check the antifungal activity of the isolate. Fungal discs were prepared by inoculating the fungi onto Sabouraud's Dextrose Agar (dextrose-4 g, peptone-1 g, agar-1.5 g, in 100 mL of distilled water) with filter paper discs. All four fungi were obtained from soil, and these molecularly identified fungi were

preserved in the Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, Karnataka, India. The plates were incubated at room temperature for 5 days for mycelial growth. Meanwhile, SDA plates were prepared by inoculating the bacterial isolate by streaking two parallel lines 6 cm apart. These plates were placed in a  $37 \pm 2$  °C incubator for a day for bacterial growth. Later the prepared fungal discs were placed on the center of SDA plates with the isolate streak and incubated for 7 days at room temperature. SDA plates without the isolate were also prepared and inoculated with the same fungi to serve as a control. Fungal growth was observed for all the fungi, and the positive results were regarded as restricted fungal growth in the presence of the isolate [35].

### Biochemical Molecular Characterization [36, 37].

Preliminary characterization, like gram staining and biochemical tests, was performed for the plant growth-promoting bacterial isolate capable of producing PHA before enzyme assays and molecular characterizations. Later 16S rRNA gene sequencing was done at Barcode Biosciences, Bangalore. After the genomic DNA isolation from the culture, it was resolved using agarose gel electrophoresis (1% agarose gel) followed by amplifying the DNA fragment of 16S rRNA gene through PCR with 16SrRNA-F and 16SrRNA-R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl genetic analyzer. Aligner sequence software was used to deduce the consensus sequence. BLAST was performed with the ‘nr’ NCBI GenBank database, scrutinizing the first 10 sequences based on the maximum identity score. Multiple sequence alignment was done using Clustal  $\omega$  software. Distance matrix and phylogenetic tree were constructed using MEGA 10 software [36, 37].

### Statistical Optimization of Two-Factor Interaction Model for the Bacterial Biomass

To optimize the measurable output (response) bacterial biomass (g/L) of the isolate, a DOE-based two-level, Reduced Quartic model was set for the multivariate study using Design Expert 13 software. The input parameters were A: Incubation period (h), B: Inoculum level (%), and C: NaCl (g/L). A total of 20 runs with different combinations of input parameters were performed. The run details are shown in Table 2. Finally, the validation of optimized parameters in triplicates was performed within the range of the established design space, as adopted as per ICH Q8 guideline (<https://www.ich.org/page/quality-guidelines>) and our previous studies [38, 39].

## Seed Germination Test of Tomato and Chickpea

### Microbial Bioinoculant Preparation

*B. subtilis* NJ14 were cultured in nutrient broth (100 mL). After 24 h, bacteria were collected from the media by centrifuging the culture at 6000 rpm for 10 min at 4 °C. The cells were procured carefully without cell loss and washed twice with Sterile distilled water (SDW) to remove the media components. The washed cells were resuspended in SDW (100 mL) to acquire  $\cong 10^4$  CFU mL<sup>-1</sup> rhizobacterial density.

### Seed Treatment

The seeds of *S. lycopersicum* (Arka-Samrat variety from IIHR, Bangalore, Karnataka, India) and *C. arietinum* (MNK-1 variety from UAS, Raichur, Karnataka, India) were utilized for the study. The seeds were coated with 1% (w/v) CMC adhesive after being surface sterilized using 0.1% (w/v) HgCl<sub>2</sub>. These seeds were then immersed in bioinoculant prepared and were soaked for 30 min in a shaker incubator at 120 rpm. Seeds soaked in SDW without the bioinoculant were maintained as control. The seeds were then planted in a seedling tray with sterilized soil to monitor seed germination and growth over 27 days [19].

### Growth Parameters

The seed germination was calculated based on the observed germination of the total number of seeds sown. To calculate the Total Germination Percentage (TGP), the following formula was utilized:

Other than germination percentage, root length and shoot lengths were observed 4 weeks after sowing. These parameters were used to compare the difference in the growth rate of the sample plant against the control.

### Statistical Analysis

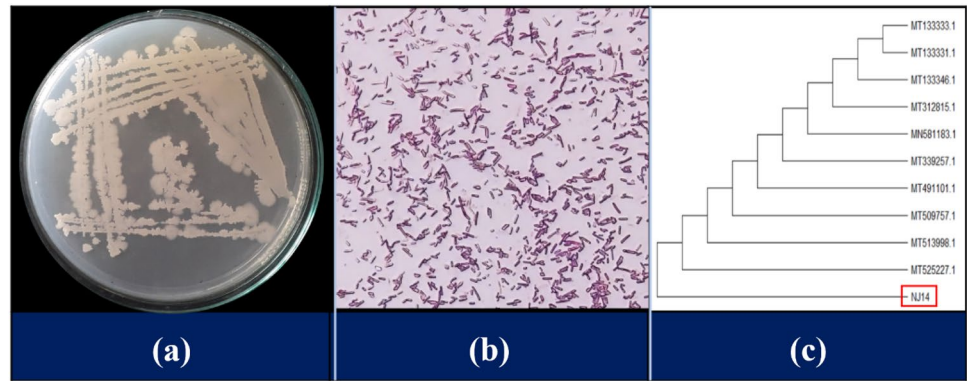
The significant difference ( $p < 0.05$ ) was determined by One-way ANOVA operating DMRT in IBM SPSS Statistics 21. All the values were expressed as Mean  $\pm$  SE.

## Results and Discussion

### Isolation and Identification of *B. subtilis* NJ14

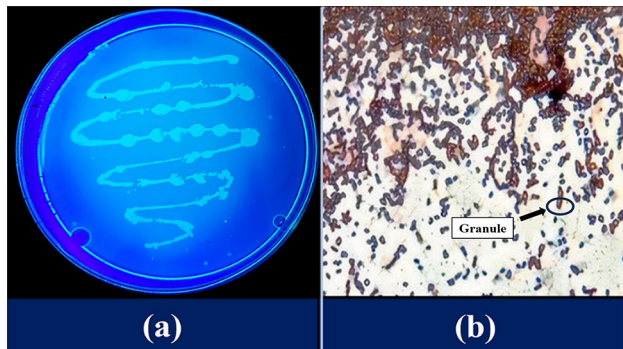
The rhizospheric soil collected from the rhizosphere of *Coffea arabica* was used for isolation, and 14 isolates were obtained. The obtained isolates were subjected to preliminary PHA production tests and PGP tests. The most promising isolate NJ14 (Fig. 1a, 1b) which had both traits was

**Fig. 1** **a** Isolate NJ14 in nutrient agar plate **b** Gram-positive rods of NJ14 isolate **c** Phylogenetic tree showing *Bacillus subtilis* NJ14 strain



**Table 1** Biochemical characterization of NJ14 isolate

Sl. no	Biochemical test	NJ14
1	Indole production test	+
2	Methyl red test	+
3	Voges-Proskauer test	–
4	Citrate test	–
5	Carbohydrate fermentation test	–
6	Oxidase test	+
7	Urease test	–



**Fig. 2** **a** Nile blue staining on plate **b** Sudan black stain showing black PHA granules (inset) in red vegetative cells

biochemically characterized (Table 1) and later molecularly characterized for identification. The isolate was identified as *B. subtilis* after constructing a phylogenetic tree using Clustal  $\omega$  and MEGA 6 software. The accession number OM780222 was received for the strain after submitting the sequence to GenBank.

## PHA Staining and Extraction

### Nile Blue Staining

Nile blue stain is a lipophilic dye used to stain PHA in bacteria. In a work, *B. subtilis* has been studied to show fluorescence in

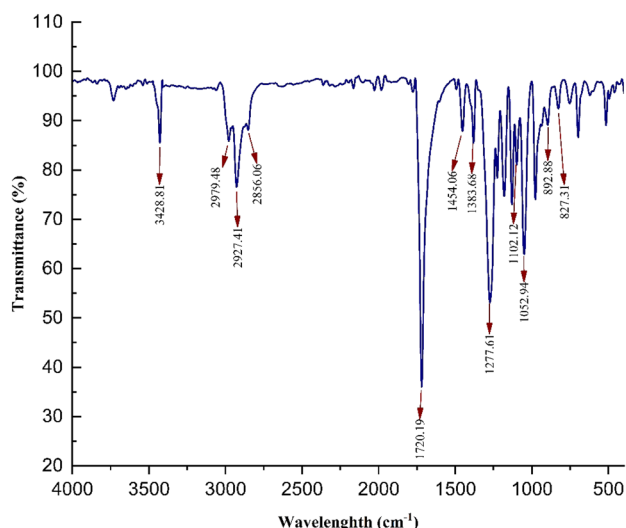
Nile blue staining, confirming the presence of PHA granules [40]. Another work recommended that *B. subtilis* NRR-B-941 exhibited PHA production when the Nile blue staining method was applied [41]. When the plates with isolates were placed under a UV chamber, isolate NJ14 with PHA production displayed fluorescence indicating the presence of PHA granules (Fig. 2a).

### Sudan Black Staining

Sudan black staining was performed to confirm further the existence of PHA granules in the bacterial cells. A work reported PHA granules in *B. subtilis* by Sudan black staining method [42]. Another work utilized Sudan black staining and confirmed that *B. subtilis* could build and use PHA as a carbon source [43]. In this study, the isolate that exhibited fluorescence in Nile blue staining confirmed the presence of PHA by showing black-stained PHA granules (Fig. 2b).

### Extraction of PHA

Extraction of PHA is accomplished in 3 steps conventionally: Cell digestion, separation, and recovery. In this study, an alkali salt, 4% sodium hypochlorite, was used for cell digestion, and PHA was separated from the lysed cells by solvent extraction method using acetone: diethyl ether: methanol in a 1:1:1 ratio. And the PHA was dissolved and recovered using boiling chloroform. In a work, *B. subtilis* yielded 3.09 g/L of PHB at pH 7.0, 37 °C with 10 g/L of glucose [13]. In another work, *B. subtilis* was reported to produce 2.09 g/100 mL of PHA when 10% sugarcane molasses was employed as carbon substrate [44]. A study reported that *B. subtilis* produced 22.98 g/L of PHA from 36.98 g/L cell dry mass [42]. In the present study, *B. subtilis* NJ14 yielded 2.32 g/L of PHA from 9.74 g/L of dry cell mass.



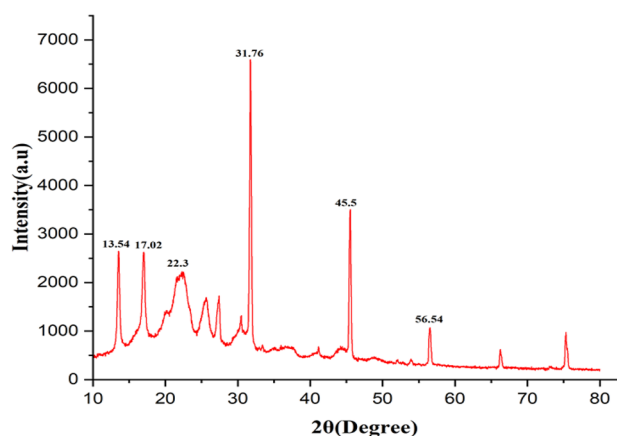
**Fig. 3** FT-IR spectrum of PHA extracted from the isolate

## Characterization of PHA

### Fourier-Transform Infrared (FTIR) Characterization of PHA

The FTIR spectrum of the extracted polymer gives a precise understanding of the various functional groups present in the molecule as a function of their wave number. The nature and intensity of the peaks in the FTIR spectrum are used to have a preliminary identification of the nature of biopolymers. The FTIR spectrum of the extracted polymer in this study is depicted in Fig. 3.

The central peak at  $2927\text{ cm}^{-1}$  corresponds to aliphatic stretching contributed by the alkane group and the peak at  $3428\text{ cm}^{-1}$  is contributed by the  $\text{-OH}$  group. A sharp rise at  $1720\text{ cm}^{-1}$  signifies weak  $\text{C=O}$  stretching contributed by the conjugated carbonyl group, and  $1277\text{ cm}^{-1}$  corresponds to symmetric and asymmetric stretch vibration of  $\text{C-O-C}$ . The peak at  $1052\text{ cm}^{-1}$  reflects the presence of alkyl halides in the extracted polymer. The peaks obtained in the FTIR spectrum of the extracted polymer confirmed the polymer to be PHA due to the peak similarity with available literature. In a study dealing with PHA production from *Bacillus subtilis* RS1, major peaks obtained were at  $2924\text{ cm}^{-1}$  ( $\text{C-H}$  stretching of bonds of methyl group),  $1722\text{ cm}^{-1}$  ( $\text{C=O}$  stretching of ester groups), and  $1278\text{ cm}^{-1}$  ( $\text{C-O-C}$  stretching) which is similar to the peaks obtained in this study [45]. In another study with *Bacillus cereus* SH-02, characteristic peaks reflecting PHA were obtained at  $2933\text{ cm}^{-1}$  and  $1724\text{ cm}^{-1}$ , which strongly correlates with the result obtained in this study [46]. PHA production study using distillery effluent with *Bacillus subtilis* NCDC 0671 revealed characteristic peaks at  $2920\text{ cm}^{-1}$  corresponding to  $\text{C-H}$  vibrations of the methyl group,  $1724\text{ cm}^{-1}$  signifying carbonyl groups  $\text{C=O}$



**Fig. 4** XRD spectrum of PHA extracted from the isolate

from ester and carboxyl groups, and peaks at  $1033\text{ cm}^{-1}$  suggesting  $\text{C-O-C}$  stretching [12]. This correlates with the peaks obtained in this study.

### X-Ray Diffraction (XRD) Analysis of PHA

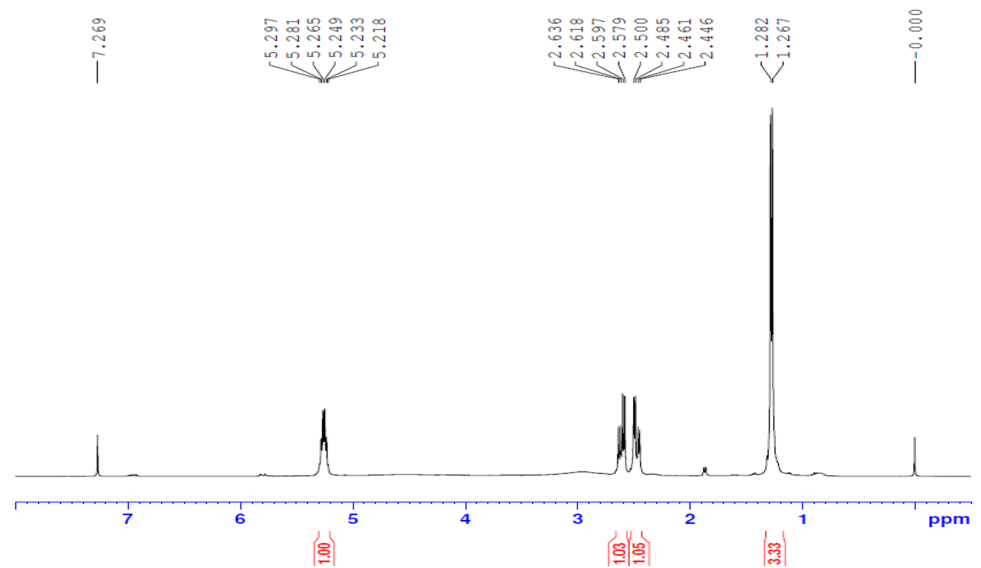
The crystalline nature of the PHA can be deduced from the pattern of XRD peaks and is thus a routine method for polymer characterization and identification [47]. In the present study, the XRD spectrum of the extracted biopolymer showed characteristic diffraction ( $2\theta$ ) peaks at 13.54, 17.02, 22.03, 31.76, 45.5, and 56.54 (Fig. 4). These peaks were in correlation with the XRD peaks of PHA reported in other studies and reflected the semi-crystalline nature of PHA. A research study on PHA film preparation using acetic acid-based solvent casting methods obtained characteristic XRD peaks ( $2\theta$ ) at 13.5, 16.85, 21.4, 27.2, and 44. These peaks were similar to those reported in this study [48]. In another study reporting the production of PHB using fish solid waste hydrolysate as a cost-effective medium, characteristic XRD peaks ( $2\theta$ ) were obtained at 13.34, 16.96, 21.95, 25.92, 27.29, 31.77, 45.70, and 56.39 which reflects the crystalline lattice of the biopolymer [13].

### Nuclear Magnetic Resonance (NMR) Analysis of PHA

$^1\text{H}$  NMR analysis is a susceptible technique to understand the precise monomeric units and functional groups present in the biopolymers. The  $^1\text{H}$  NMR spectrum of the biopolymer extracted in this study revealed characteristic peaks suggesting the polymer to be PHB (the most widely studied class of PHA). In the  $^1\text{H}$  NMR spectrum, a doublet peak at 1.2 ppm indicates the presence of a methyl group ( $\text{-CH}_3$ ) of the hydroxybutyrate (HB). Similarly, the cliffs at 2.4 ppm, 2.5 ppm, and 2.6 ppm denote the methylene group ( $\text{-CH}_2$ ) in HB, and the multiple peaks



**Fig. 5** NMR spectrum of PHA extracted from the isolate



at 5.2 ppm indicate the methine group ( $-\text{CH}$ ) of HB. The sharp peak at 7.2 ppm is contributed by the deuteriochloroform ( $\text{CDCl}_3$ ) used for sample preparation (Fig. 5).

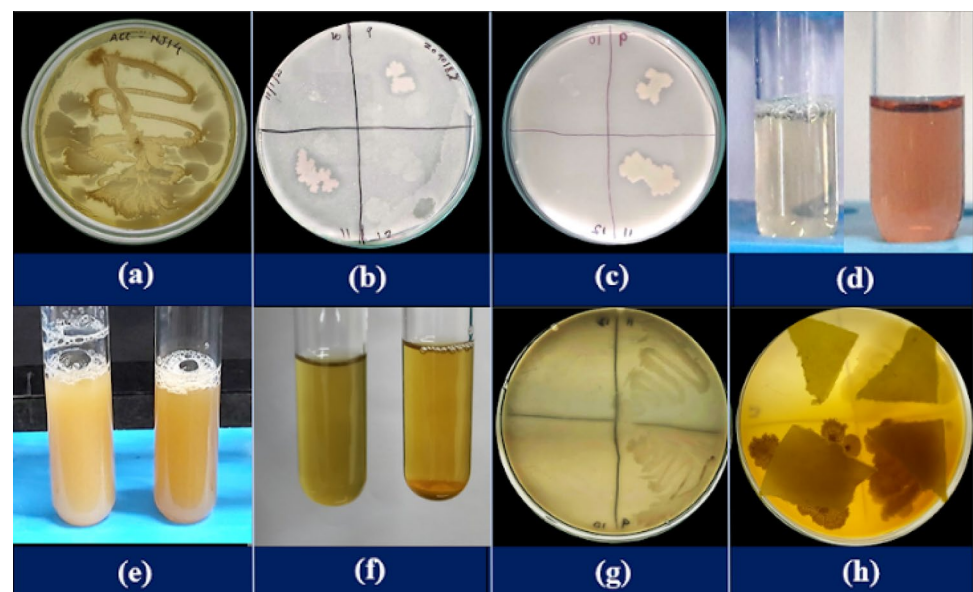
$^1\text{H}$  NMR spectrum of PHB synthesized from *Bacillus* sp. NII2 using damaged wheat grains (DWG) revealed characteristic peaks at 1.268–1.281 ppm, 2.453–2.630 ppm, and 5.239–5.302 ppm [49]. These results were in agreement with the  $^1\text{H}$  NMR spectrum reported in this study. The  $^1\text{H}$  NMR spectrum of PHB extracted from *Bacillus cereus* strain 4N revealed characteristic peaks at 1.24 ppm, 2.48 ppm, 2.5 ppm, and 5.45 ppm, similar to that reported in this study [50].

## Plant Growth-Promoting Tests

### ACC Deaminase Test

Production of ACC-deaminase is a mechanism of Plant Growth-Promoting Rhizobacteria (PGPR) that elevates plant growth in stress conditions. According to a study, tomatoes' capability to withstand drought stress has been boosted by *B. subtilis* Rhizo SF, which induces ACC-deaminase [51]. Another study indicated that *B. subtilis* (NBRI 28B), *B. subtilis* (NBRI 33 N), and *B. safensis* (NBRI 12 M) yielded ACC-deaminase and helped mitigate salt stress in *Zea mays* [52]. A study reported that ACC-deaminase producing *B. subtilis* BERA 71 appeared to stimulate salt stress in

**Fig. 6** **a** ACC- deaminase test **b** Phosphate solubilization test **c** Zinc solubilization test **d** Indole Acetic acid test **e** Siderophore test **f** Ammonia test **g** Nitrogen fixation test **h** HCN test



Chickpeas [53]. In this study, *B. subtilis* NJ14 has shown ACC-deaminase-production indicating its potential to boost plant growth in stress conditions (Fig. 6a).

### Phosphate Solubilization Test

Plants utilize P only in 2 forms,  $\text{HPO}_4$  (monobasic) or as  $\text{H}_2\text{PO}_4$  (dibasic) ions; [54]. A study by [55] *B. megaterium* mj1212 has proved to have phosphate solubilization and enhanced carbohydrates and amino acids in mustard plants. Another work by [56] dealing with thermotolerant *B. subtilis* strain displayed phosphate solubilization. Another work by [57] revealed that *B. velezensis* strain Ag75 appeared to have phosphate solubilization and promoted growth in maize and soybean crops. In this study, *B. subtilis* NJ14 has shown phosphate solubilization and had  $1.431 \pm 0.12$  cm of PSI (Fig. 6b).

### Zinc Solubilization Test

Zn insufficiency is considered a significant global risk factor for human and plant health [58]. The test for Zinc solubilizing bacteria was performed with Pikovskaya media containing insoluble  $\text{ZnCl}_2$ . A work reported a zone of  $4.3 \pm 0.3$  cm in CDK25 species of *B. megaterium* [59]. Another study has suggested that *Bacillus* spp. IA16 exhibited Zn solubilization to enhance cotton growth [60]. Another work involving *B. subtilis* RH5 showed that the strain could solubilize Zn and protect rice from Sheath blight disease from *Rhizoctonia solani* [61]. In the current study, *B. subtilis* NJ14 showed Zn solubilization and expressed ZSI of  $1.223 \pm 0.31$  cm (Fig. 6c).

### Indole Acetic Acid Test

IAA is also appreciated for its involvement in inducing flowering and fruiting in plants. In a study *B. subtilis* was inferred to produce  $4 \pm 0.2$   $\mu\text{g/mL}$  IAA [62]. In another study it was observed that, after 24 h of incubation, *B. subtilis* (Mt3b) expressed  $322.6$   $\mu\text{g/mL}$ , and *B. cereus* (So3II) displayed  $241.6$   $\mu\text{g/mL}$  auxin concentrations [63]. In the present study, *B. subtilis* NJ14 yielded  $30.55$   $\mu\text{g/mL}$  of IAA, thus can help in root elongation (Fig. 6d).

### Siderophore Test

Ferric ions play an essential role in monitoring oxygen during ATP synthesis, heme formation, and deteriorating ribotide precursors of DNA [64]. Siderophores yielding bacteria residing near the roots of plants help in this process [65]. Research has studied bacillibactin siderophores from *B. subtilis* and applied them in plant growth and oil production from sesame [66]. In another study, *B. subtilis* MF497446

having siderophores production has shown to control *Cephalosporium maydis* in Maize plants [67]. Another work has shown that *B. subtilis* CAS15-inducing siderophores helped control *Fusarium* wilt and elevated plant growth in pepper [68]. In this study, *B. subtilis* NJ14 produced siderophores and thus can help in the chelation of Fe ions (Fig. 6e).

### Ammonia Test

The influential repression caused due to the increased pH in soil by the production of ammonia (pH 9–9.5) keeps away certain harmful fungi and other Nitrobacter and undermines spore formation in many fungi. A study has indicated ammonia production in 95% of *Bacillus* spp [69]. Another study explored ammonia production of ammonia in *B. subtilis* as a volatile compound with antifungal activity against *Rhizoctonia solani* and *Pythium ultimum* [70]. Another research showed *B. cereus* CUAMS116 has shown ammonia yielding and supporting the growth of *Phaseolus vulgaris* L [71]. In this study, *B. subtilis* NJ14 produced ammonia as another promising PGP trait (Fig. 6f).

### Nitrogen Fixation Test

The Diazotrophy pathway is mediated by diazotrophs which are prokaryotic. A work describing *Bacillus* spp. that was isolated from Egyptian soil has reported nitrogen-fixing activity [72]. *Bacillus* spp., which can fix nitrogen, has been isolated from a tropical estuary and studied by [73]. Another work has concluded that *B. subtilis* is a nitrogen-fixing agent [74]. In the current study, *B. subtilis* NJ14 has grown on Jensen's nitrogen-free media confirming its nitrogen-fixing ability (Fig. 6g).

### HCN Test

HCN production in bacteria is majorly correlated to its antagonistic trait, thereby can hinder the growth of other phytopathogens as the producers are resistant [75]. Trace amounts of HCN near the plant roots are considerably less harmful to the plants. In research they investigated *B. subtilis* for HCN production and used it to stimulate sesame plant growth and oil production [66]. In another work, *Bacillus* spp. is studied and reported to induce HCN as a trait of plant growth promotion [76]. In another work *B. subtilis* PF1 has shown HCN production [77]. In the current study, *B. subtilis* NJ14 exhibited HCN production, contributing to its antagonistic trait (Fig. 6h).

### Enzymatic Assay

Protease and cellulase production can help in plant growth promotion by cell wall degradation of pathogenic fungi

[78]. Amylase helps plant growth by degrading organic compounds like starch in the soil [79]. Bacteria produce catalase to protect themselves from hydrogen peroxide, which is poisonous to bacteria and plant roots [80]. Cellulase production from *B. licheniformis* has been reported [81]. The effect of the C:N ratio on alpha-amylase production has been studied [82]. Other studies have shown the production of alpha-amylase from *B. licheniformis* using pearl millet as a medium [5]. Catalase activity has been reported in *Bacillus* spp. in a work [83]. In the current study, *B. subtilis* NJ14 showed positive results in all four lytic enzyme assays (Fig. 7).

### Halotolerance Test

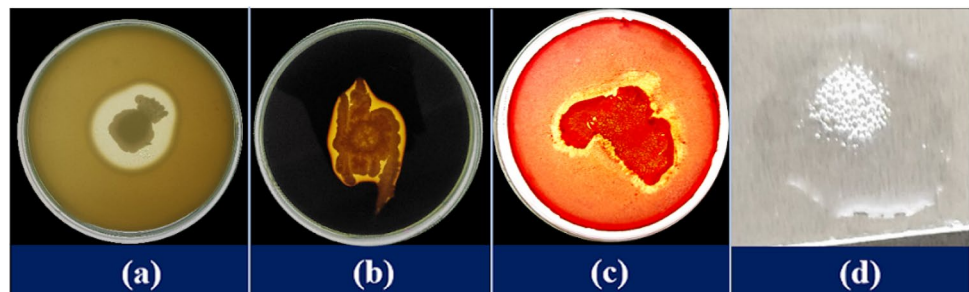
Halotolerant PGPR ameliorates plant growth by activating enzymes such as peroxidase, catalase, and superoxide dismutase which are part of the antioxidant defense in plants. The cumulation of hazardous  $\text{Na}^+$  ions is reduced by boosting the selective ingestion of  $\text{K}^+$  ions that maintains the  $\text{K}^+/\text{Na}^+$  ratio level. Exopolysaccharide (EPS) evacuated by bacteria can bind  $\text{Na}^+$  ions and increase soil macropores, which meliorates water retention near the roots. Many PGPR

genera, including *Bacillus*, have been inferred to produce EPS and accelerate biofilm formation. A work proved that *B. subtilis* HG-15 displayed halotolerant (NaCl) up to 30% [84]. Another work dealing with *B. subtilis* BBK-1 showed NaCl tolerance of up to 16% and production of biosurfactants up to 8% in NaCl [85]. Another report concluded that halotolerant *B. subtilis* helped mitigate salt stress in hydroponically grown soybeans [86]. In the current study, *B. subtilis* NJ14 could tolerate NaCl up to 10% and be utilized in salt-stressed soil (Fig. 8).

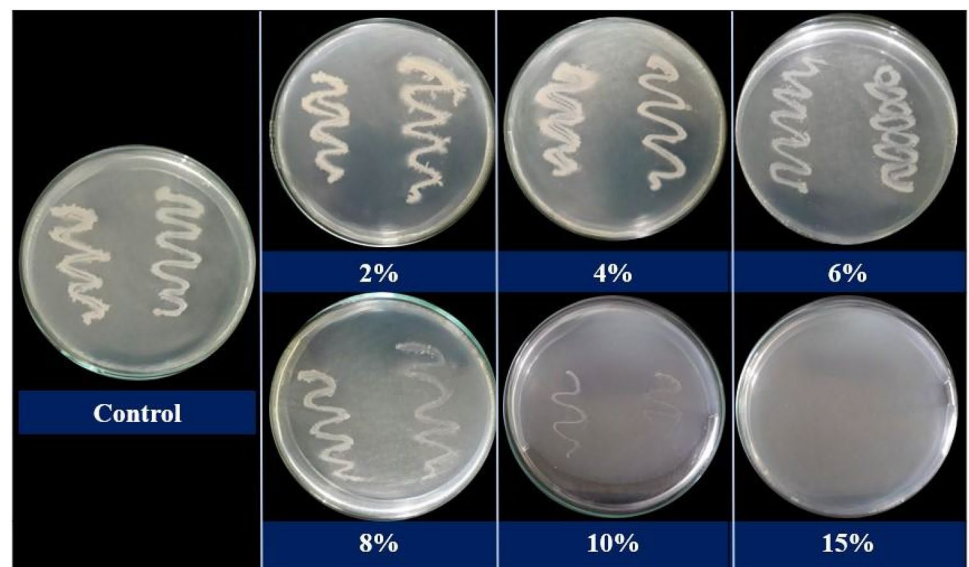
### Metal Tolerance Test

Metal tolerance in bacteria helps them thrive in metal-contaminated soil and makes them a promising applicant for heavy metal removal. A work has reported a novel *Bacillus* spp. with Pb resistance [87]. Another work concluded that *B. anthracis* and *B. subtilis* showed tolerance to Cr, Cu, and Hg [88]. Biosorption of Pb has been studied using *B. subtilis* MTCC 2423 [89]. In the present study, *B. subtilis* NJ14 could tolerate Pb up to 1000  $\mu\text{g}/\text{mL}$ , Hg up to 100  $\mu\text{g}/\text{mL}$ , and did not display any tolerance to Cd (Fig. 9).

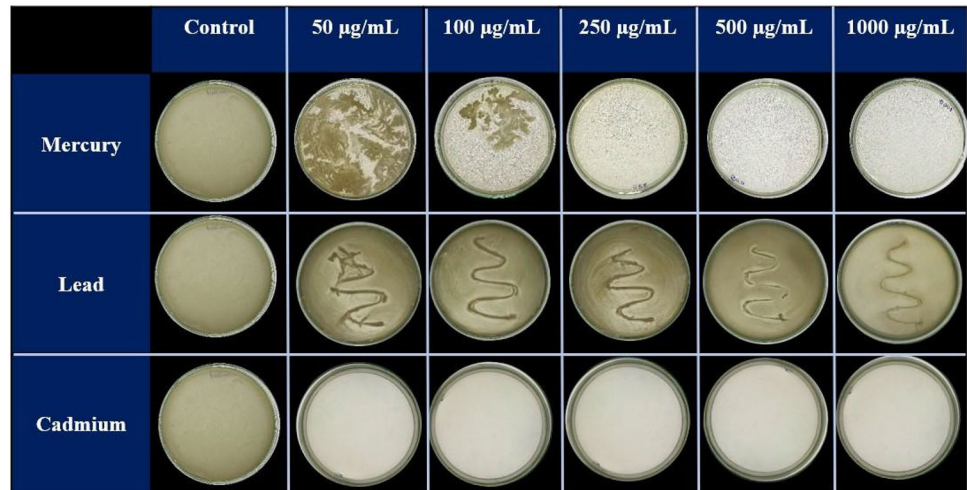
**Fig. 7** a Protease test b Amylase test c Cellulase test d Catalase test



**Fig. 8** Halotolerance test for *B. subtilis* NJ14



**Fig. 9** Heavy metal tolerance test for *B. subtilis* NJ14

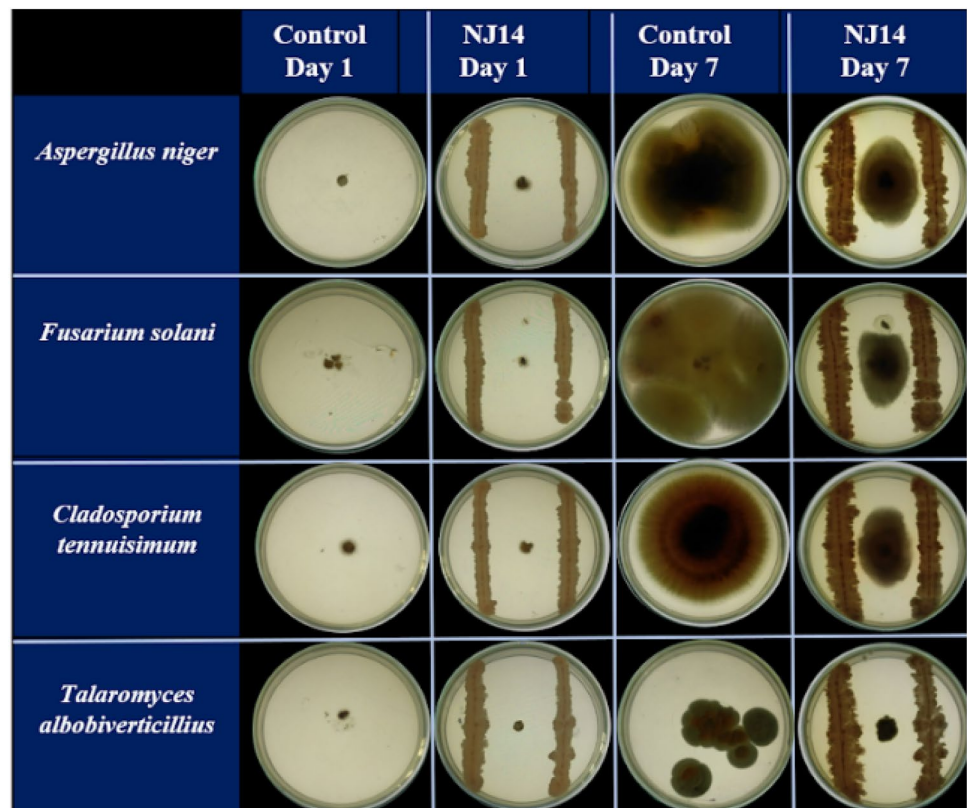


### Antifungal Assay

Antagonism against pathogenic fungi is one of the vital traits of a PGPR. *Fusarium* spp. is a threatening pathogen to many plants, including tomatoes, that causes *Fusarium* wilt disease [90]. Some strains of *Cladosporium* are also known as saprophytic plant pathogens and affect many plant hosts [91]. An antifungal compost has been developed from the *B. licheniformis* KJ-9 strain in research [92]. *Aspergillus flavus* is an opportunistic pathogen affecting

maize plants weakened by drought or heat stress [93]. *Talaromyces* are generally non-pathogenic to plants and are studied as an applicant for biofertilizers. A work has inferred that *B. subtilis* GM5 expressed antagonism against *Fusarium oxysporum* [94]. Another work revealed that *B. subtilis* and *B. amyloliquefaciens* antagonize against *Aspergillus parasiticus* [95]. *Talaromyces trachyspermus* has shown antibacterial activity against *B. subtilis*, confirming antifungal activity [96]. In the current study, *B. subtilis* NJ14 exhibited antagonism against all four

**Fig. 10** Antagonistic test of *B. subtilis* NJ14 against fungi



**Table 2** Result of two-factor reduced Quartic model

Run	A: Incubation period (h)	B: Inoculum level (%)	C: NaCl (g/L)	Bacterial biomass (g/L)
1	62.27	1.81	2.82	0.63
2	48	3	5.5	0.96
3	48	3	1	0.66
4	33.73	1.81	2.82	0.9
5	62.27	4.19	8.18	0.68
6	33.73	4.19	8.18	0.61
7	48	3	5.5	0.99
8	62.27	1.81	8.18	0.67
9	33.73	4.19	2.82	1.03
10	33.73	1.81	8.18	0.64
11	48	3	5.5	0.95
12	48	3	5.5	0.99
13	62.27	4.19	2.82	0.9
14	48	3	10	0.69
15	48	3	5.5	0.97
16	72	3	5.5	0.78
17	48	1	5.5	0.69
18	48	5	5.5	1.86
19	24	3	5.5	0.68
20	48	3	5.5	0.95

fungi recommending its ability to control phytopathogens (Fig. 10).

### Statistical Optimization of Two-Factor Interaction Model for the Bacterial Biomass

The data in Table 2 were utilized to construct a two-factor interaction model representing the response variable (bacterial biomass g/L) of *B. subtilis* NJ14. An empirical equation was obtained from the data; the Y response (bacterial

biomass (g/L) defined as a function of A: Incubation period (h), B: Inoculum level (%), and C: NaCl (g/L). The proposed equation is,

$$Y = 0.968333 + 0.0297302A + 0.347843B + 0.00891905C + 0.0224858AB + 0.0624022AC - 0.0523812BC - 0.0842636A^2 + 0.108423B^2 - 0.103709C^2 - 0.300395A^2B - 0.116255A^2C - 0.0671419AB^2 - 0.130936A^2B^2$$

$$Y = 0.968333 + 0.0297302A + 0.347843B + 0.00891905C + 0.0224858AB + 0.0624022AC - 0.0523812BC - 0.0842636A^2 + 0.108423B^2 - 0.103709C^2 - 0.300395A^2B - 0.116255A^2C - 0.0671419AB^2 - 0.130936A^2B^2$$

The input variables, namely Incubation period (A), Inoculum level (B), AB, AC, and BC, were observed to be statistically significant ( $p \leq 0.05$ ) for the bacterial biomass (g/L) (Table 3). F value for the model was noted as 231.31. The F value 231.31 showed that there was only a 0.01% chance that the result could occur due to noise. The regression coefficient of the selected factorial model, 99.8%, indicated adequate precision in the response prediction.

### Establishment of the Design Space

Following the guideline outlined by ICH Q8 (<https://www.ich.org/page/quality-guidelines>) and according to our previous works [38, 39], an overlaid plot also known as design space that decides a standard range of interacting input variables for specific responses for the optimized process in terms of the response bacterial biomass was established.

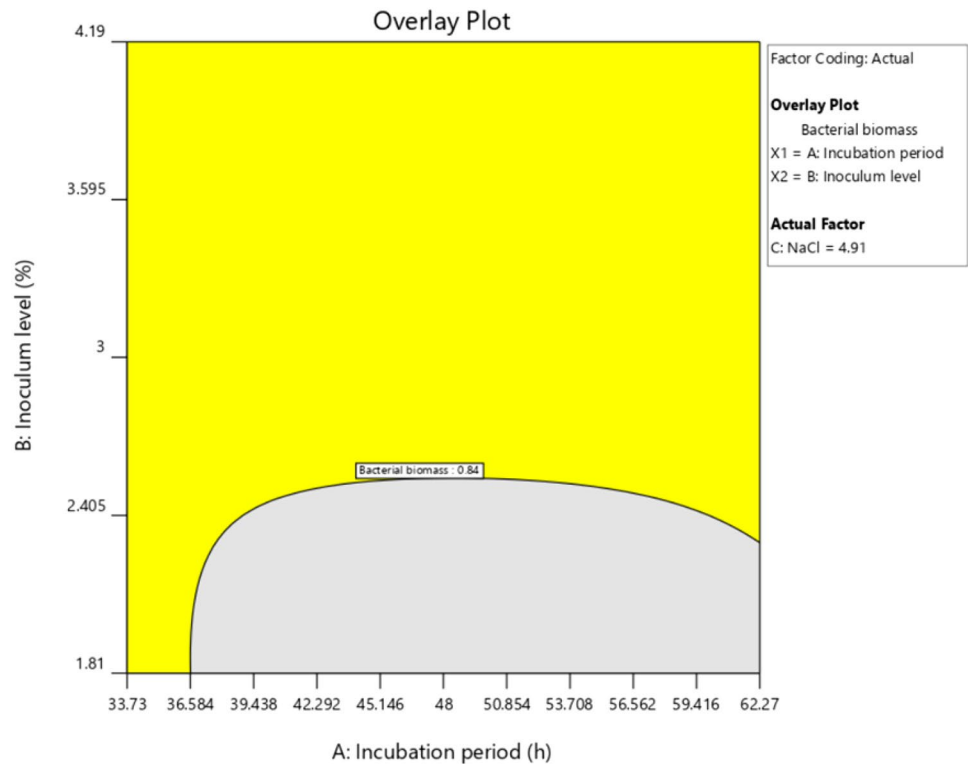
In the present study, interacting variables Incubation period (h) and Inoculum level (%) (AB) were selected as the x ( $\times 1$ ) and y ( $\times 2$ ) axis of the plot, respectively (Fig. 11).

**Table 3** Two-way ANOVA table with respect to bacterial biomass (g/L)

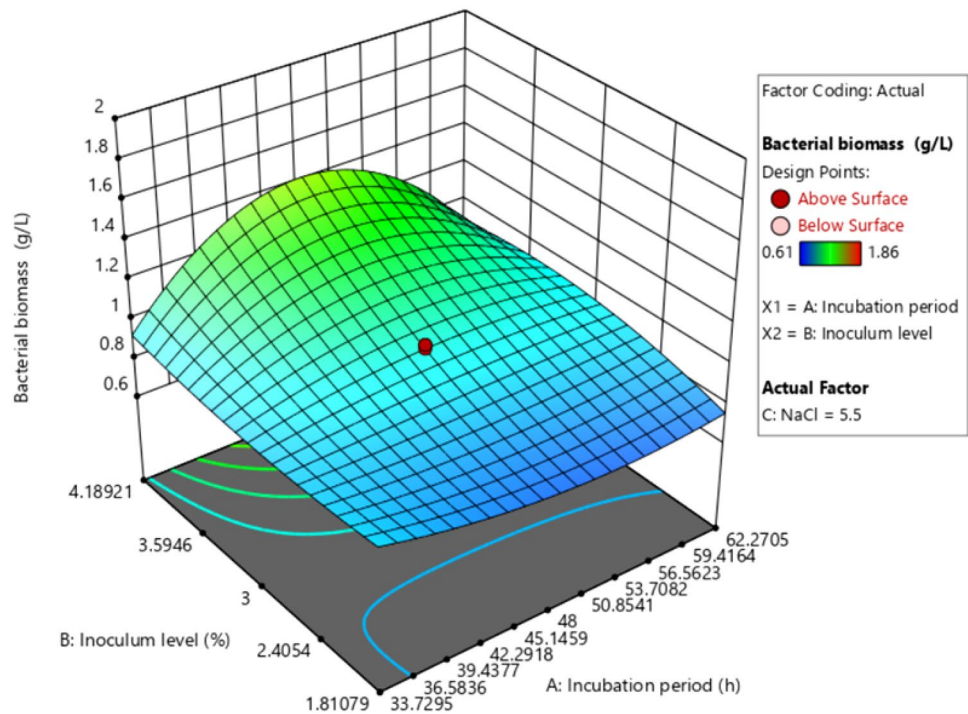
Source	Sum of Squares	df	Mean Square	F value	p value
Model	1.47	13	0.1131	231.31	<0.0001*
A-Incubation period	0.0050	1	0.0050	10.23	0.0186*
B-Inoculum level	0.6845	1	0.6845	1400.01	<0.0001*
C-NaCl	0.0004	1	0.0004	0.9205	0.3744
AB	0.0040	1	0.0040	8.28	0.0281*
AC	0.0312	1	0.0312	63.92	0.0002*
BC	0.0220	1	0.0220	45.10	0.0005*
Residual	0.0029	6	0.0005		
Lack of Fit	0.0013	1	0.0013	3.71	0.1119
Pure Error	0.0017	5	0.0003		
Cor Total	1.47	19			

\*Statistically significant ( $p \leq 0.05$ )

**Fig. 11** Established design space (overlay plot), Yellow space showing as  $\geq 0.84$  g/L bacterial biomass



**Fig. 12** Three-dimensional contour plot of interacting parameters (Incubation period X Inoculum level)



Another variable NaCl (g/L) was maintained as a constant parameter. The yellow space of the plot represented by the incubation period within the range of 33.73–62.27 h, the inoculum level within the range of 1.81–4.19 g/L, and NaCl 4.91 g/L could provide the best

possible optimized solution as  $\geq 0.84$  g/L bacterial biomass. The interaction plot revealed that an increase in the Incubation period (h) and Inoculum level (%) could jointly enhance the bacterial biomass. This result was further appraised by a 3D contour plot (Fig. 12).

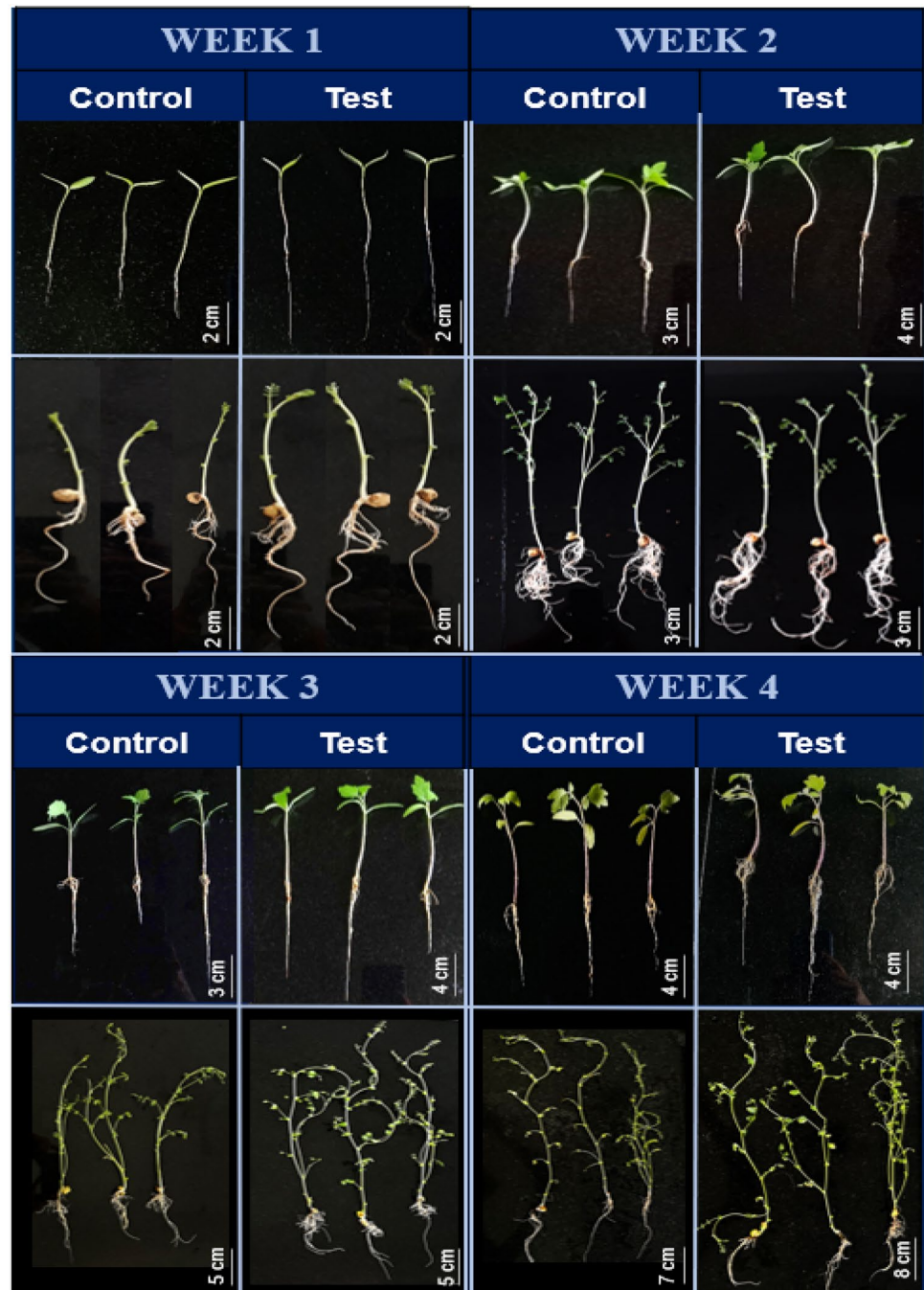
## Design Space Validation

The design space was validated by selecting random values from the optimized variable ranges. Selected values were 58.8 h during the Incubation period, 3.73% Inoculum level, and 4.9 g/L NaCl. The validation experiments showed the bacterial biomass as  $1.05 \pm 0.02$  g/L. The value fitted well within the prediction interval range with 95% confidence as provided by the software (1.01 to 1.09 g/L).

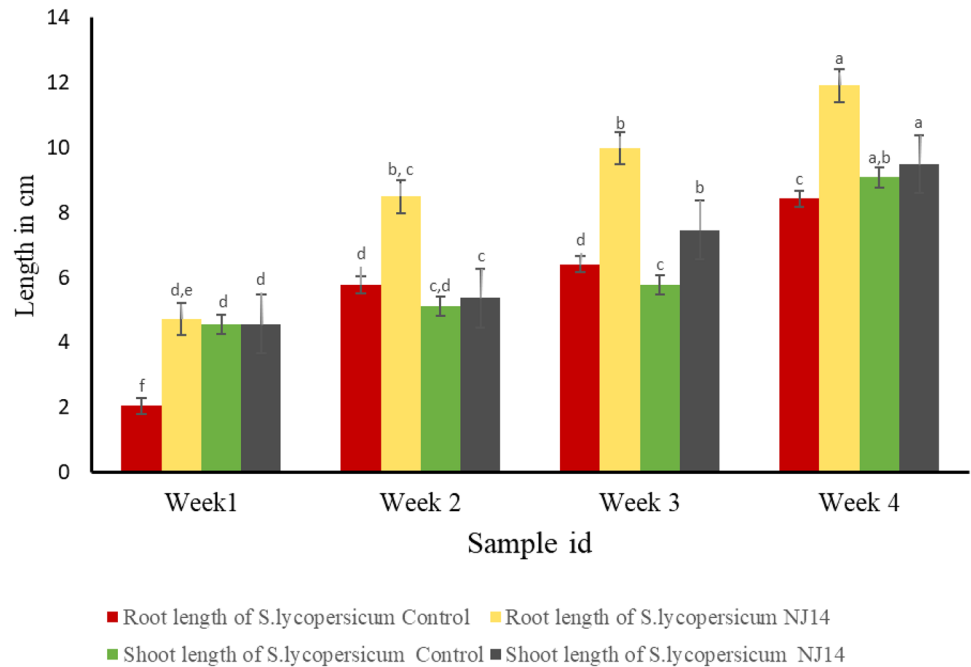
## Plant Growth Study on *S. lycopersicum* and *C. arietinum*

After confirming PHA production and PGP traits in *B. subtilis* NJ14, a Plant Growth study was carried out using the dip method [33] on a leguminous plant *C. arietinum* (MNK-1 variety from UAS, Raichur) and a non-leguminous plant *S. lycopersicum* (Arka-Samrat variety collected from IIHR, Bangalore), and the study was carried out for 27 days. The results are presented in Fig. 13. The plants were healthy and free from fungal attack throughout the

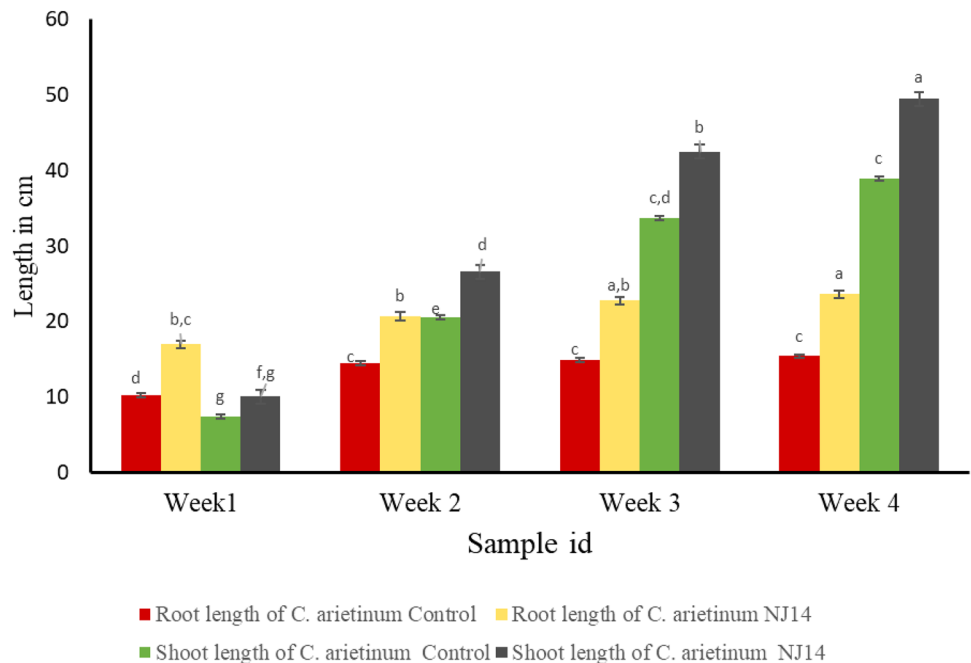
**Fig. 13** Plant growth study on *S. lycopersicum* and *C. arietinum* using *B. subtilis* NJ14 as biofertilizer



**Fig. 14** Comparison of growth parameters in *S. lycopersicum*



**Fig. 15** Comparison of growth parameters in *C. arietinum*



study, possibly due to the antagonistic trait of *B. subtilis* NJ14 against fungi. The total Germination Percentage (TGP) was the same for both the control and sample in *S. lycopersicum*, which was 80.56%. But in *C. arietinum*, the sample expressed higher TGP than the control, 72, and 68%, respectively. There was a significant difference in root length of the control and sample of *S. lycopersicum* which were  $8.42 \pm 0.53$  and  $11.91 \pm 0.7$  cm, respectively,

and  $15.42 \pm 0.65$  and  $23.66 \pm 0.81$  cm for *C. arietinum*, respectively (Figs. 14, 15). Shoot length did not display a notable significant difference in *S. lycopersicum* which were  $9.08 \pm 0.21$  and  $9.49 \pm 0.33$  cm in control and sample, respectively. However, the sample showed improved shoot growth compared to the control in *C. arietinum* which were  $38.94 \pm 0.51$  and  $48.49 \pm 0.93$  cm, respectively.



## Conclusion

*Bacillus subtilis* NJ14 is a significant applicant for fabricating a reliable biofertilizer that can enhance the growth of *S. lycopersicum* and *C. arietinum*. The ability of *B. subtilis* NJ14 to produce PHA assures augmenting the shelf life of biofertilizer. Plant Growth-Promoting traits like the production of ACC-deaminase, Indole Acetic Acid, HCN, Siderophores, Ammonia, lytic enzymes (Protease, Catalase, Cellulase, and Amylase); Nitrogen fixation; Phosphate and Zinc solubilization; and antagonism against fungi make *B. subtilis* NJ14 a definitive PGPR that can ameliorate plant growth. This work can be extended by implementing *B. subtilis* NJ14 in formulating a considerable biofertilizer with remarkable shelf life by utilizing a suitable carrier. A product of *B. subtilis* NJ14 can also be used in salt-stressed land (up to 10%) and lead-stressed land due to its halotolerant and metal tolerance properties, respectively. The PHA-producing ability of *B. subtilis* NJ14 can also be employed in various other applications in the field of agriculture, like the preparation of mulch. Therefore, PHA-producing PGPR *B. subtilis* NJ14 is an ideal candidate for biofertilizers that can replace chemical fertilizers in the market.

**Funding** Not Applicable.

**Data Availability** The original data related to this research work will be available on special request to the authors.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Ethical Approval** This research work does not involve the use of any human and/ or animal studies and thus ethical approval is not applicable.

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