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Plant growth promotion activities of *Bacillus* spp. isolated from Jakrem hot water spring of Meghalaya, North East India

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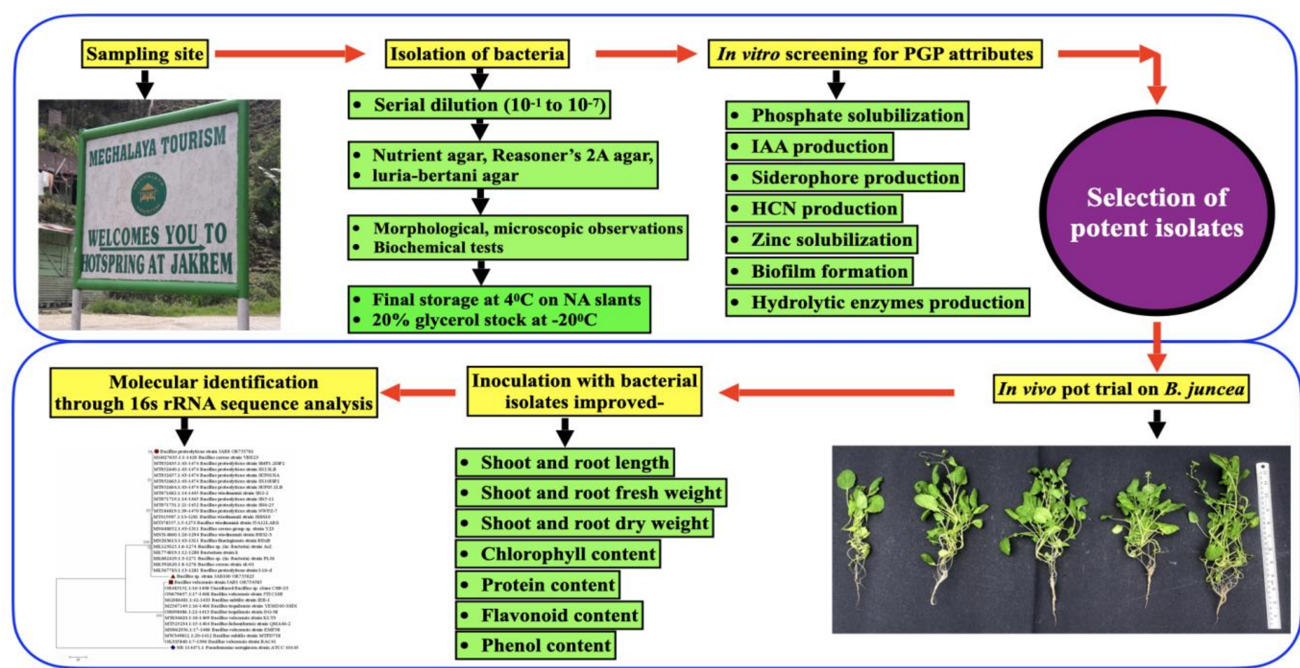
GRAPHICAL ABSTRACT



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ABSTRACT

The study aims to investigate plant growth promotion (PGP) activities of thermophilic bacteria isolated from the Jakrem hot spring in Meghalaya, North-East India, and determine their effect on *Brassica juncea's* growth. The bacteria were isolated by a culture-dependent approach following a serial dilution method in a nutrient agar medium. All the isolates were determined for PGP attributes such as indole acetic acid, phosphate solubilization, hydrolytic enzymes, and siderophore production. The potent bacterial isolates were characterized by 16S rDNA sequencing and phylogenetic analysis. Altogether, 53 bacterial isolates were obtained, most belonging to the genus *Bacillus*. Of the total isolates, 37.7% exhibited both PGP and hydrolytic enzyme activities. Three isolates, namely JAB1, JAB8, and JAB100, showed promising PGP and were identified as *Bacillus velezensis*, *B. proteolyticus*, and *Bacillus* sp., respectively. The PGP attributes of these isolates were determined *in vivo* on *B. juncea,* and their effects were measured in terms of shoot and root length biomass and biochemical contents. It was observed that combined inoculation of all three isolates significantly enhanced the growth and development of *B. juncea,* evident by increased shoot and root length, fresh and dry weight, and higher levels of protein, phenol, flavonoid, and chlorophyll content compared to the control. In conclusion, the study highlights the potential application of thermophilic *Bacillus* spp. from hot springs as bioinoculants to enhance crop productivity in sustainable agricultural practices.

1 Introduction

Substantial demand for food has increased in recent years due to limited land resources and the rise of the global population. Food production has increased significantly through chemical fertilizers in conventional agricultural systems to feed the growing population (Mishra and Dash 2014). However, indiscriminate and excessive use of chemical fertilizers to increase crop productivity has severely adverse effects on living organisms and the environment. Therefore, a sustainable agricultural system is urgently needed to overcome this problem and achieve food security for the growing global population (Glick 2018). Organic farming using beneficial microorganisms has gained tremendous attention as an alternative to agrochemicals. These microbes are used as bioinoculants to increase soil fertility and improve plant growth and soil health by increasing the supply of readily available nutrients or protecting them from biotic and abiotic stresses (Lugtenberg and Kamilova 2009).

Hot springs represent a unique ecological niche for microorganisms thriving under extreme environmental conditions. Recently, microbes, especially bacterial communities isolated from such environments, appeared to be of utmost importance due to producing industrially significant enzymes and secondary metabolites with multiple applications (Verma et al. 2018). Bacterial genera belonging to such extreme conditions have been reported to possess higher metabolic rates and stability when compared with their mesophilic counterpart (Verma et al. 2018). Moreover, enzymes produced by various bacterial genera, such as *Bacillus* sp. isolated from hot springs, remained stable and active at higher temperatures (Panda et al. 2013). Due to this property, thermophilic microorganisms have gained considerable attention for large-scale production of enzymes, sugars, and a wide array of secondary metabolites (Satyanarayana et al. 2005; Lele and Deshmukh 2016; Mohammad et al. 2017). Besides, thermophilic microorganisms have also shown their ability to increase plant growth, resistance to salinity in crops of agricultural interest, and biocontrol against phytopathogens (Verma et al. 2018; Shilev 2020). Many instances have demonstrated that hot springs microorganisms, especially *Bacillus* spp., are potent agents for inducing the growth of plants through various plant growth-promoting attributes (Saharan and Verma, 2014; Verma et al. 2018). Production of different phytohormones viz. indole-3-acetic acid (IAA), cytokinins, gibberellins along with phosphate solubilization, iron chelation, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and nitrogen-fixing properties that aid in plant growth promotion had also been previously reported from different species of *Bacillus* isolated from extreme environmental condition (Stein 2005; Yang et al. 2009; Arguelles-Arias et al. 2009; Rahman et al. 2016; Kaki et al. 2017; Verma et al. 2018; Kumar et al. 2021). Therefore, isolating beneficial microorganisms, particularly bacteria, from pristine environments, such as hot springs, would be paramount for bioinoculants for plant growth and health (Podile and Kishore 2006; Saharan and Nehra 2011).

Meghalaya is situated in India's North-Eastern Region (NER) and is rich in various natural resources. The state is endowed with several high-altitude lakes and hot springs. One such hot spring is Jakrem, which is less explored and located in one of the mega biodiversity-rich zones of the world in the Himalayan geothermal belt (Rakshak et al. 2013; Panda et al. 2016). Preliminary studies on microbial diversity of this hot spring by culture-independent approach had revealed a rich diversity of

microorganisms dominated by Firmicutes, Cloroflexi, unclassified bacteria, and a large number of sequences reads from bacterial taxa Arthronema which may represent novel species (Panda et al. 2016). However, a culture-dependent approach has not yet been undertaken to investigate the microbes for biotechnological applications. Therefore, the present study aimed to isolate and characterize the bacterial species from this hot spring by a culture-dependent approach, evaluate the isolates for plant growth promotion potential, and observe their effect on the growth and development of *B. juncea*, a commonly used leafy vegetable of North East India.

2 Materials and Methods

2.1 Study site and collection of sample

The hot spring of Jakrem is located in the West Khasi hill district of the Meghalaya state, which is situated at 25°24.463′ N and 91°32.409 ′E and an altitude of 1439 m above mean sea level (MSL). Geographical positioning data and temperature of the water sample were recorded at the sampling site using a GPS locator system (Garmin eTrex 10) and portable thermometer (HICKS, Oval) respectively. The pH of the collected samples was recorded in the laboratory using a digital pH meter (Cyberscan, EUTECH). For collection of water samples, plastic containers (500 ml) were first cleaned with 20% sodium hypochlorite solution, washed thoroughly with several steps of sterile distilled water, and autoclaved at 121°C for 15 min. The containers were dried in a hot air oven at 50°C for 1 h and sterilized further under UV light in a laminar airflow cabinet for 1 h. Water samples were randomly collected in triplicates from hot springs in these plastic containers, stored in a dry ice box, and transported immediately to the laboratory (Kambura et al. 2016).

2.2 Isolation and identification of bacteria

Bacteria were isolated following serial dilution technique (10^{-1} to 10^{-7}) using three different growth mediums viz. nutrient agar (NA), Reasoner's 2A agar (R2A), and Luria-Bertani agar (LBA) medium. Briefly, 100 µl aliquot of each dilution was spread on growth media and sealed with paraffin strips. These plates were then incubated at 44±1^{0}C for 72-96 h. After the incubation period, the dilution factor, at which the number of colonies ranged between 30-300, was further selected to calculate the colony format unit (CFU)/ml of the water sample. Distinct colonies on the growth medium were purified by repeated sub-culturing, and finally, purified bacterial isolates were stored at 4^{0}C on NA slants and 20% glycerol stock at -20^{0}C (Fasina et al. 2020; Kumar et al. 2021). Microscopic examinations were carried out at 1000X magnification using immersion oil in the compound microscope (LYNX, Lawrence & Mayo), and Cell morphology, viz. shape, size, elevation, pigmentation, and colour were recorded. Different biochemical tests viz. catalase, oxidase, gram staining, and citrate utilization were carried out for identification up to genus level (Brenner et al. 2005; Yazdani et al. 2009; Kumar et al. 2012; Islam et al. 2017; Fasina et al. 2020; Tripathi and Sapra 2021).

2.2.1 Thermo-tolerance profile of isolated bacterial strains

The thermo-tolerant property of the bacterial isolates was determined by growing the isolates in freshly prepared NA medium and then incubating them at different temperatures: 30±1°C, 40±1°C, 50±1°C and 60±1°C for a period of 24 to 72 hours. The visible appearance of bacterial growth at specific thermal intervals was recorded as positive and was classified according to binary code viz. 0 (no growth) and 1 (appearance of growth on the medium), as suggested by Moreno et al. (2021) and López et al. (2021).

2.3 Physico-chemical analysis of water sample

Samples were analyzed for turbidity, Iron, chloride, fluoride, total hardness, calcium, magnesium, sulphate, nitrate, lead, nitrite, alkalinity, sodium, potassium, sulphide, cadmium, chromium, phosphorous, total organic carbon, total nitrogen, total suspended solids (TSS), volatile suspended solids (VSS) and total dissolved solids (TDS) as per the standard methodology (Indian Standard "IS 3025" 2009; APHA 2017; Kalsait et al. 2018; Agarwal et al. 2019).

2.4 Screening of the isolated bacterial isolates for plant growth-promoting traits

2.4.1 Solubilization of phosphate

The isolated bacterial isolates were screened for their inorganic phosphate solubilization potential using Pikovskaya's agar medium. A loopful of bacterial culture was spot inoculated onto the agar plates and incubated at 30±1°C for 72-96 hours. The ability of the bacteria to solubilize inorganic phosphate was determined by forming a clear halo zone surrounding the bacterial colony, indicating phosphate solubilization activity (Syiemiong and Jha 2019). The diameter of the halo zone was measured to quantify the solubilization efficiency of each isolate. Phosphate solubilization was quantified in Pikovskaya's broth medium, which contained 0.5% tri-calcium phosphate as a sole source of inorganic phosphorous. The broth medium was inoculated with 100 µl of overnight grown bacterial strains (10^6 cells/ml) and incubated in continuous shaking (120 rpm) for 144 h at 30±1^{0}C. After incubation, a cell-free supernatant was obtained by centrifugation for 5 minutes at 10000 rpm. The obtained cell-free supernatant (0.5 ml) was mixed with 10% trichloroacetic acid (0.5 ml) and color reagent (4 ml) [1 (3M H_2SO_4): 1 (2.5% ammonium molybdate): 1 (10% ascorbic acid): 2 (distilled water)] (Syiemiong and Jha 2019). The resulting mixture was incubated at 30±1^{0}C for 15 min, and absorbance of the resulting mixture was read at 820 nm

wavelength against control using a spectrophotometer. Solutions of KH_2PO_4 was used at different concentrations to prepare the standard curve, and the amount of phosphorous solubilized by the bacterial isolates was calculated and expressed as µg/ml (Syiemiong and Jha 2019).

2.4.2 Indole-3-acetic acid (IAA) production

IAA production was quantified by supplementing L-tryptophan (0.2%) as the precursor for IAA synthesis in the Luria Bertani broth medium. The broth medium was inoculated with overnight grown bacterial strains (100 µl; 10^6 cells/ml) and incubated at 30 ± 1^0C for 96 h in shaking condition (120 rpm). Following incubation, cell-free supernatant (1 ml) was obtained by centrifugation at 10000 rpm for 10 min and mixed with Salkowsky reagent (3 ml). The reaction mixture was incubated in the dark for 15 min, and the absorbance of the developing color was read at 530 nm wavelength using a spectrophotometer against control (3 ml of Salkowskyreagent + 1 ml Luria-Bertani broth medium supplemented with 0.2% L-tryptophan). For estimation of IAA produced by bacterial strains, a standard curve was prepared from different concentrations viz. 5, 10, 20, 50 and 100 µg/ml of pure IAA was used and expressed as µg/ml (Syiemiong and Jha 2019).

2.4.3 Siderophore production

A qualitative assay for Iron chelating activity by the bacterial isolates was performed on CAS (Chrome azurol S) agar medium. For this assay, solution A (60.5 mg of CAS in 50 ml of sterile distilled water was mixed with 10 ml of 1 mM $FeCl_3.6H_2O$ prepared in 10 mM HCl) was mixed with solution B (72.9 mg of hexadecyl tri-methyl ammonium bromide in 40 ml of sterile distilled water) (Kumar et al. 2021). A solid agar medium (Kings medium B base; 42.23 gm/l) was prepared separately, and both the stock solution and the agar medium were autoclaved and allowed to cool inside the laminar airflow cabinet. When the temperature lowered to 40–50^0C, both the solutions were mixed and poured onto petri plates and allowed to solidify. Bacterial strains were spot-inoculated onto petri plates and incubated for 96 h at 30 ± 1^0C. The development of the orange-yellow zone surrounding the bacterial colony was recorded as positive for siderophore production (Kumar et al. 2021).

2.4.4 Hydrogen cyanide (HCN) production

A qualitative assay for HCN production was carried out on Tryptic soy broth (30 g/l) amended with glycine (4.4 g/l). Briefly, overnight grown bacterial strains (100 µl, 10^6 cells/ml) were inoculated onto broth medium, and sterilized filter paper strips of uniform size (5 cm×0.5 cm) soaked into a solution of 0.5 % picric acid in 2% Na_2CO_3 was placed inside the tube in hanging position. The tubes were air-tight with parafilm and incubated at 30 ± 1^0C for 96 h. Following incubation, the filter paper was observed for color change from yellow to orange-brown for positive HCN production by the bacterial strains (Wani and Khan 2013).

2.4.5 Zinc solubilization

Zinc solubilization was estimated qualitatively using zinc oxide (0.1%) amended in a minimal agar medium (MM9). A loopfull culture of bacterial isolate was spot inoculated onto the agar plates and incubated at 30 ± 1^0C for 48 h. Following the incubation period, the development of a clear zone surrounding the colony was recorded as positive for zinc solubilization (Goteti et al. 2013).

2.4.6 Biofilm formation

Quantitative detection of biofilm activity was carried out by inoculating bacterial strains (100 µl, 10^6 cells/ml) in a 5 ml broth medium. Following inoculation, the broth medium was incubated for 72 h at 30 ± 1^0C under shaking conditions (120 rpm). After an incubation period, the cultured medium containing the bacterial cells was discarded completely and washed with phosphate buffer saline thrice (1X PBS pH 7.2) to remove adherent cells. The tubes were dried inside the laminar air flow cabinet and stained with crystal violet solution (0.2%) for 5 min. The tubes were again rinsed with phosphate buffer saline to remove excess stain, followed by air-drying. The absorbance of the stain adhered was read at 630nm wavelength by adding 2 ml of 95% ethanol in each tube against blank (Lotfi et al. 2014). The degree of adhesión was recorded as:

$ODs < ODc$: non-adherent

$ODc < ODs < 2XODc$: weakly-adherent

$2x\ ODc < ODs < 4XODc$: moderately-adherent

$4xODc < ODs$: strongly-adherent

Where ODc- control; ODs-sample, respectively.

2.5 Screening for the production of extracellular hydrolytic enzymes

2.5.1 Cellulase

For this assay, bacterial isolates were spot inoculated onto solid agar plates amended with 0.1% carboxymethyl cellulose (CMC) as substrate. Following incubation, for 48 h at 30 ± 1^0C, the solid agar plates were flooded with 0.1% congo red solution for 5 min and decanted. The plates were again flooded with 1M sodium chloride for 15 min. The development of a clear zone surrounding the bacterial colony was recorded as positive for extracellular cellulase activity (Kumar et al. 2021).

2.5.2 Amylase

For this assay, bacterial isolates were spot inoculated onto solid agar plates amended with 1% soluble starch as substrate. Following incubation, for 48 h at 30±1^{0}C, the plates were flooded with grams iodine solution for 5 min and decanted. Development of the clear zone surrounding the bacterial colony was recorded as positive for extracellular amylase activity (Kumar et al. 2021).

2.5.3 Lipase

For this assay, a loopfull culture of bacterial strain was spot inoculated onto Tributyrin agar (without Tributyrin) supplemented with Tributyrin (10 ml/ 1000 ml) as substrate and incubated at 30±1^{0}C for 48 h. Following incubation, the development of the clear zone surrounding the bacterial colony was recorded as positive for extracellular lipase activity (Berg 2009).

2.5.4 Protease

For the hydrolysis of protease, 10% skim milk was used as a substrate onto a solid agar medium (MM9). This medium was autoclaved and cooled to 40–50°C, then poured onto petri plates to solidify. Bacterial isolates were then spot-inoculated onto agar plates, following incubation at 30±1^{0}C for 48 h. The development of a clear zone surrounding the bacterial colony was recorded as positive for extracellular protease activity (Kumar et al. 2021).

2.5.5 Laccase

A solid agar medium was amended with 0.1% guaiacol as a substrate for laccase activity. Bacterial isolates were spot inoculated onto agar plates and incubated at 30±1^{0}C for 96 h. Following incubation, grams iodine was flooded onto petri plates for 5 min. Development of the clear zone surrounding the bacterial colony was recorded as positive for laccase activity (Kumar et al. 2021).

2.6 Mass multiplication of bacterial isolates

Based on the results from various *in-vitro* evaluations for plant growth promotion activities, the potent bacterial isolates were selected for *in-vivo* pot experimental trials. Mass multiplication of bacterial isolates was carried out by inoculating a loopfull culture of bacterial isolates in a 150 ml nutrient broth medium and incubating under continuous shaking conditions for 24 h at 30±1^{0}C. Following incubation, cells were harvested by centrifugation at 10000 rpm for 10 min and suspended in sterile distilled water. Bacterial density was further adjusted to 10^6 cells/ml using a haemocytometer count and stored at 4^{0}C for field application (Kumar et al. 2021).

2.7 Pot experimental trial of potent bacterial isolates

A pot experimental trial on *B. juncea* evaluated the in-vivo plant growth-promoting potential of the potent bacterial isolates. Briefly, seeds of *B. juncea* were obtained from the National Bureau of Plant Genetic Resources (NBPGR), Umiam, Meghalaya, with cultivar number-IC 597866 and surface sterilized with a solution of sodium hypochlorite (2.5%) for 10 min. After surface sterilization, seeds were thoroughly washed with several steps of sterile distilled water (until traces of sodium hypochlorite was removed). The surface sterilized seeds were dried on sterile filter paper inside a laminar airflow cabinet (Gupta et al. 2020; Goswami and Deka 2020; Kumar et al. 2021). Ten seeds were transferred to each pot containing 200 g of double autoclaved sterilized soil and 20 ml of bacterial cell suspension (10^6 cells/ml) was inoculated in pot with following sets of treatment: T1- Control (seeds without bacterial inoculation); T2 - seeds inoculated with bacterial isolate JAB1; T3 seeds inoculated with isolate JAB8; T4- seeds inoculated with isolate JAB100; T5- seeds inoculated with isolates JAB1+ JAB8; T6- seeds inoculated with isolates JAB8+ JAB100; T7- seeds inoculated with isolates JAB1+ JAB100; T8- seeds inoculated with isolates JAB1+ JAB8+ JAB100.

2.8 Growth and biochemical parameters studied

2.8.1 Evaluation of length (shoot and root), fresh weight and dry weight

After 45 days of seed sowing, different growth parameters, like shoot and root length, were determined using a standard scale. Fresh weight (root and shoot) was determined by harvesting the mustard plant and then washing it with running water to remove adhering particles. Excess moisture was removed using filter papers, and the fresh weight (root and shoot) was measured using an electronic weighing balance. Plant samples were then dried in an oven at 70^{0}C and measured consecutively after 24 h until constant weight was attained for dry weight determination (Huang et al. 2017).

2.8.2 Chlorophyll content

The chlorophyll content was estimated in mustard leaves with 80% acetone. Briefly, mustard leaves (0.5 g) were finely ground using a mortar and pestle in 20 ml of acetone (80%). Supernatant from the resulting mixture was collected following centrifugation at 10000 rpm for 10 min, and the absorbance was recorded at 645 nm and 663 nm wavelength in a spectrophotometer against blank (only 80% acetone). Chlorophyll content was recorded as milligrams (mg) per gram (g) of fresh weight (FW) using the following formula (Arnon 1949; Sarkar and Kalita 2022).

Chlorophyll a (mg/g FW): {12.7× (Absorbance at 663nm)- 2.69× (Absorbance at 645nm)} ×V/1000×W

Chlorophyll b(mg/g FW): {22.9 × (Absorbance at 645nm)- 4.68× (Absorbance at 663nm)} ×V/1000×W

Total chlorophyll (mg/g FW): {20.2× (Absorbance at 645nm) +8.02× (Absorbance at 663nm)} ×V/1000×W

Where, V: Final volume of 80% acetone; W: Sample weight

2.8.3 Protein content

Estimating total protein content in leaves was conducted per the methodology described by Lowry et al. (1951) with some modifications. Briefly, fresh leaves (0.2 g) were crushed in 10 ml of 0.1M phosphate buffer using mortar and pestle. The resulting mixture was centrifuged for 10 min at 10000 rpm, and the obtained supernatant (1ml) was mixed with alkaline copper reagent (5 ml) [48 ml of 2% Na_2CO_3 prepared in 0.1N NaOH (48 ml)+ 1% $KNaC_4H_4O_6{\cdot}4H_2O$ (1 ml)+ 0.5% $CuSO_4$ (1 ml)]. The final mixture was incubated for 15 min, and then 2N freshly prepared Folin-Ciocalteau reagent (1 ml) [Folin-Ciocalteau reagent (1): water (1)] was further added, followed by additional incubation in the dark for 30 min. After incubation, the absorbance of the developing mixture was read at 660 nm wavelength against control using a spectrophotometer. The amount of soluble protein was calculated from a bovine serum albumin (BSA) standard and expressed as mg BSAE/ g of fresh weight (Sarkar et al. 2020).

2.8.4 Phenol content

Total phenolic content was estimated according to the methodology described by Sarkar and Kalita (2022) with some modifications. Dried leaves (0.5g) were crushed using a mortar and pestle in chilled methanol and centrifuged at 10000 rpm for 10 min. Estimation of total phenol was carried out by adding 2.5 ml of Folin- Ciocalteau reagent (10% in water) and 2.5 ml of 7.5% Na_2CO_3 to 500 µl of the obtained supernatant, followed by incubation in the dark for 40 min at 45 ± 1^0C. The absorbance of the mixture was read at 765 nm wavelength using a spectrophotometer. The total phenol content in the sample was expressed as mg of gallic acid equivalent (GAE) g^{-1} DW using gallic acid as the standard.

2.8.5 Flavonoid content

Flavonoid content was estimated as per the methodology described by Sarkar and Kalita (2022). Briefly, supernatant extracted in methanol (100 µl) was mixed with distilled water (400 µl) and 5% $NaNO_2$ (30 µl) and incubated for 5 min at 30 ± 1^0C. 10% AlCl3 (30 µl) was added to the mixture and incubated for 6 min at 30 ± 1^0C. After incubation, 1M NaOH (200 µl) was added to the mixture, and the final volume was adjusted to 1 ml with distilled water. The mixture was vortexed and incubated again for 5 min at 30 ± 1^0C. After incubation, the absorbance of the sample was read at 510 nm wavelength using a spectrophotometer and expressed as mg of quercetin equivalent (QE) g^{-1} FW of the sample using quercetin as standard.

2.9 Molecular identification of potent bacterial isolates

Genomic DNA of the potent bacterial isolates was extracted using a Genomic DNA extraction kit as per manufacturer instructions. The extracted DNA was subjected to PCR amplification using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) in a thermal cycler. The reaction mixture contained dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), and dTTP (0.2 mM), 27F (1 µM), 1492R (1 µM), $MgCl_2$ (2 mM), Taq DNA polymerase (1.25 U), genomic DNA (1 µl) with following reaction condition, 95^0C (2 min), followed by 35 cycles of 95^0C (30 s), 53^0C (30 s) and 72^0C (1.30 min) and final extension at 72^0C (7 min). The amplified products were resolved through electrophoresis containing 1.2% agarose and were imaged in a gel documentation system. Purification and 16S rRNA gene sequencing of the amplified PCR products were performed at Unigenome (Unipath Specialty Laboratory). Using 16S rRNA gene sequences of the isolates, the phylogenetic tree was constructed using the neighbour-joining method to know their phylogenetic affiliations. The sequences were submitted to the GenBank NCBI database, and accession numbers were obtained (Agarwal et al. 2020; Kumar et al. 2021).

2.9.1 Statistical analysis

All the experiments were conducted in triplicates and expressed as a mean of three replicates with standard deviation. For data analysis of plant growth, ANOVA was conducted using *R* software (Version-1.2.1335) and significant differences were determined using the least significant differences test (LSD) at a significance level of $P \leq 0.05$*.

3 Results and Discussion

3.1 Bacteria isolated from hot water spring

The pH of water samples obtained from the Jakrem hot spring was alkaline (8.54±0.02) with a recorded temperature of 44.96 ± 0.2^0C at the sampling site. The temperature range of the hot water spring of Jakrem was classified as moderate thermophilic, and in many instances, the temperature of hot water springs located in different parts of India has also been reported to be moderately thermophilic (Yadav et al. 2015; Poddar and Das 2018; Narsing Rao et al. 2021). Our result corroborates with many previous studies where the pH of hot water springs was reportedly alkaline, and the temperature was often higher than 40^0C (Mohammad et al. 2017; Sahay et al. 2017). Following serial dilutions, 53 distinct bacterial isolates were isolated in different media compositions. The water samples' colony forming unit (CFU) was 1.36 x 10^{-1} CFU/ml.

Based on morphological, microscopic and biochemical characterization, the isolates were rod-shaped and gram-positive bacteria belonging to the genus *Bacillus.* Worldwide geothermal sites are favourable habitats for thermophilic bacteria, which have developed unique survival strategies to adapt to such an environment. The dominant isolated bacteria belong to the genus Bacillus in the present study. The prevalence of the genus *Bacillus* and related genera as the dominant members have been previously reported from different hot springs (Adiguzel et al. 2009; Yadav et al. 2015; Priya et al. 2016). The occurrence of *Bacillus* species in different hot springs worldwide may be attributed to their high GC content and endospore formation, which might have favoured better adaptability for surviving in such extreme environmental conditions (Panda et al. 2013).

3.1.1 Thermo-tolerance profile of bacterial isolates

In the present study, thermo-tolerance profiles of the 53 bacterial isolates were determined at 4 different thermal intervals viz. 30, 40, 50 and 60°C. The results showed a score of 0 (no growth) for only 2 bacterial isolates at 30°C and 1 (visible growth) for 51 bacterial isolates at 30°C. In the present study, all 53 bacterial isolates were recorded positive and showed visible growth on NA medium with a score of 1 at 40 and 50°C. However, when the incubation temperature was increased to 60°C, only 29 bacterial isolates were recorded positive with a score of 1 (visible growth); therefore, these isolates can be considered thermophilic. The three potent bacterial isolates identified as *B. velezensis*, *B. proteolyticus*, and *Bacillus* sp. were thermophilic. Depending on the temperature range required for growth, bacteria can be classified as mesophilic, psychrophilic, thermophilic or thermotolerant (Moreno et al. 2021). Microorganisms that grow in wide temperature ranges offer better adaptability for survival in extreme environments (Kumar et al. 2013; Pandey et al. 2015; Khan et al. 2020). Isolation of thermo-tolerant strains of *Bacillus* and subsequent application as plant growth-promoting organic amendment have been cited in numerous literature (Kumar et al. 2013; Pandey et al. 2015; Khan et al. 2020). In this study, combined inoculation of the selected Bacillus species enhanced the growth and development of *B. juncea*. A similar result was observed in which inoculation with thermotolerant *Bacillus* sp. increased plant growth and development in soybean (Khan et al. 2020).

3.2 Physico-chemical properties of the water sample

The collected water sample was subjected to a comprehensive physico-chemical analysis. The physicochemical analysis of the water samples recorded 14 different mineral elements (Table 1).

The major elements consisted of sodium (20.76±1.45 mg/l), sulphate (14.53±0.35 mg/l) and chloride (14.47±0.67 mg/l). The chemical composition of hot water is predominantly influenced by the interactions it undergoes with reservoir rocks and the minerals present in those rocks during its ascent, potentially leading to the spring water exhibiting either acidity or alkalinity (Chan et al. 2017). Notably, sodium content was found to be considerably low, which was agreed upon compared to a similar study on Jakrem conducted by Panda et al. (2016). Such low sodium content is often a characteristic of medium-low temperature geothermal systems (Mao et al. 2022). The physicochemical properties of the water samples followed the result obtained by Singh et al. (2018), who studied water samples from 9 different hot springs with increased concentrations of these elements. In this study, the sulphur content of the water sample was found to be high. Poddar and Das (2018) have also reported high sulphur content in water

Table 1 Physico-chemical properties of water samples from Jakrem hot spring

Parameter	Values
Temperature	44.96±0.2
pH	8.54±0.02
Turbidity (NTU)	0.49±0.37
Iron (mg/l)	0.02±0.005
Chloride (mg/l)	14.47±0.67
Fluoride (mg/l)	0.06±0.01
Calcium (mg/l)	4.73±1.44
Magnesium (mg/l)	0.51±0.16
Sulphate (mg/l)	14.53±0.35
Nitrate (mg/l)	10.73±0.15
Lead (mg/l)	0.003±0.001
Nitrite (mg/l)	0.001±0.0005
Alkalinity (mg/l)	8.56±1.25
Sodium (mg/l)	20.76±1.45
Potassium (mg/l)	2.83±0.65
Sulphide (mg/l)	0.004±0.001
Chromium (mg/l)	0.001±0.0005
Cadmium (mg/l)	Not Detected
Phosphorous (mg/l)	0.27±0.21
Total Organic Carbon (TOC) (mg/l)	0.42±0.03
Total nitrogen (mg/l)	0.04±0.011
Total hardness (mg/l)	16.9±6.45
Total Dissolved Solids (TDS) (mg/l)	141±27.22
Total Suspended Solids (TSS) (mg/l)	0.44±0.02
Volatile Suspended Solids (VSS) (mg/l)	0.85±0.54

Values are the mean of 3 replicates (n-3); ± standard deviation

samples of different hot springs in India. As suggested by earlier studies, the abundance of sulfate-reducing bacterial forms in the water sample could be the reason for this observation (Panda et al. 2016). In addition, trace amounts of heavy metals, specifically chromium (0.001±0.0005 mg/l) and lead (0.003±0.001 mg/l), were also detected, with their low concentrations unlikely to be toxic to the microbial population. Tekere et al. (2012) have also reported the presence of trace amounts of chromium and lead in water samples collected from different hot springs. Gram-positive bacteria identified in this study indicate a potential tolerance to the existing concentrations of these heavy metals, as they are generally more resilient to chromium than gram-negative counterparts (Fathima and Rao 2018). The sample also revealed 141±27.22 mg/l of total dissolved solids (TDS), 0.44±0.02 mg/l of total suspended solids (TSS) and 0.85±0.54 mg/l of volatile suspended solids (VSS). A lower TDS level usually favors microbial growth, unlike a high TDS level, which can create a hostile environment for certain microbes, potentially affecting their survival and growth (Hanson et al. 2019). Similarly, the total dissolved solids (TDS) were in accordance with the water samples of different hot springs reported by many previous workers (Tekere et al. 2011; Kumar and Sharma 2019). Additionally, total organic carbon (TOC) and total nitrogen were found to be 0.42±0.03 mg/l and 0.04±0.011 mg/l, respectively, providing insights into the water's organic matter and nutrient content (Badhai et al. 2015). It was interesting to note that the local communities used the water sample of Jakrem hot spring for their curative properties against various skin ailments. This may be because sulphur from such natural hot springs has been known to alleviate the symptoms and have curative properties of an array of diseases, such as skin, high blood pressure and arthritis (Das et al. 2016; Jena et al. 2018). These results contributed to our understanding of the physico-chemical characteristics of Jakrem hot spring water and highlighted the potential implications for microbial communities in similar environments.

3.3 Plant growth promotion properties of the bacterial isolates

3.3.1 production of extracellular hydrolytic enzymes

In the present study, out of 53 bacterial isolates, qualitative analysis revealed that isolate JAB100 showed 19 bacterial isolates positive for proteolytic activity and maximum activity. Similarly, 19 isolates showed positive results for cellulolytic activity and amongst the positive isolates, maximum halozone formation was shown by the isolate JAB1. Again, 20 isolates showed positive results for lipolytic activity with maximum halozone formation, as shown by the isolate JAK9. For amylolytic activity, 16 bacterial isolates showed positive results and maximum halozone formation was observed in the isolate JAK17. Screening for laccase enzyme also revealed 19 isolates with positive activity, and maximum halozone formation was shown by the isolate JK54 (Table 2). The result indicated that thermophilic bacterial isolates could produce different hydrolytic enzymes. Similarly, Lele and Deshmukh (2016) reported that bacterial isolates isolated from Indian hot springs produced considerable hydrolytic enzymes. Production of different hydrolytic enzymes is considered an important attribute towards induction of plant growth promotion and biocontrol against numerous phytopathogens (Villarreal-Delgado et al. 2018; Morales-Cedeno et al. 2021). In addition, enzymes produced by thermophilic bacteria have an inherent ability to remain active at higher temperatures and can withstand harsh environments; therefore, they have potential industrial applications (Sahay et al. 2017). Since the isolates producing these enzymes are *Bacillus* species, the findings corroborate with Aanniz et al. (2015), who reported cellulase, amylase and protease enzyme production by thermophilic *Bacillus* spp. These enzymes have been known to play an important role in the biological transformation of wastes. Therefore, it can be assumed that producing hydrolytic enzymes by the thermophilic bacterial isolates might help decompose organic matter, thereby determining the physical and chemical properties of the hot springs water.

3.3.2 Indole-3-acetic acid (IAA) production

Altogether, 20 isolates showed positive results for IAA activity in qualitative analysis both in the presence and absence of L-tryptophan (Table 3). Quantitative analysis revealed that the bacterial isolate JAB100 showed the highest IAA production. The isolate produced 2.751±0.078 µg/ml of IAA in the presence of L-tryptophan, followed by the isolates JAB1 and JAB8, which produced 2.717±0.138 µg/ml and 1.916±0.047 µg/ml of IAA respectively. However, in the absence of L-tryptophan, the highest IAA production was shown by the isolate JAB1, which produced 1.849±0.048 µg/ml of IAA, followed by the isolates JAB8 and JAB100 with IAA production of 1.458±0.074 µg/ml and 1.097±0.054 µg/ml respectively. Similarly, Verma et al. (2018) have also reported the production of IAA by thermophilic *Bacillus* strains isolated from the hot springs of the Leh and Ladakh regions of India. However, very few reports on the IAA production by thermophilic bacteria isolated from hot springs water are available. In recent years, global warming and climate change have severely affected the agricultural system, threatening food security. In this context, the production of IAA by thermophilic bacteria is of great significance, and such isolates are applicable as bioinoculants that withstand drought and abiotic stresses. Among various phytohormones, IAA is pivotal in enhancing cell division, yield, and plant growth (Verma et al. 2018). It was observed that the isolates could produce considerable IAA in the growth medium. However, adding L-tryptophan in the growth medium significantly enhanced IAA production. Ahmad et al. (2005) obtained a similar result and observed that *Azotobacter* and *Pseudomonas* enhanced IAA production when the medium was added with L-tryptophan as a precursor.

Table 2 Extracellular enzyme activities of the bacterial isolates isolated from Jakrem hot spring

Bacterial isolates	Enzymes activity (in mm)				
	Cellulase	Amylase	Lipase	Protease	Laccase
JAK1	9.2±0.36	7.32±0.3	20.79±1.7	25.13±0.82	10.26±0.58
JAK3	5.2±0.26	12.44±0.41	28.63±0.43	16.35±1.15	6.15±0.59
JAK6	19.98±1.25	11.68±0.69	14.16±0.3	11.33±0.55	7.64±1.08
JAK7	11.44±0.64	13.79±0.82	20.18±1.25	26.74±1.91	7.82±0.31
JAK9	26.26±1.15	14.61±0.53	39.92±1.66	22.08±0.91	10.02±0.72
JAK10	-	-	21.08±1.69	-	-
JAK17	16.77±1.81	15.72±0.43	27.3±1.95	26.42±1.33	8.55±0.26
JAK18	17.91±0.84	9.29±0.52	21.59±1.59	29.79±1.6	5.09±0.94
JAK22	13.2±1.4	10.42±0.56	28.22±1.91	26.35±1.18	8.34±0.46
JK24	27.13±1.66	18.06±0.3	16.18±1.29	23.52±1.12	15.03±0.16
JAK31	10.99±1.07	8.66±0.57	10.64±0.56	8.57±0.5	2.79±0.46
JK54	9.72±1.39	12.16±0.28	18.03±0.21	31.23±1.18	15.39±1.13
JAK251	16.71±0.43	7.45±0.38	21.04±1.74	27.39±0.91	13.84±0.95
JAW1	10.78±1.03	8.65±0.29	5.99±0.27	9.3±0.51	10.03±0.09
JAB1	38.63±0.88	-	14.91±0.5	20.21±1.01	8.91±0.18
JAB8	9.06±0.83	10.06±0.13	15.17±0.48	15.56±0.6	13.11±0.86
JAB9	11.81±0.81	15.31±0.48	13.16±0.99	20.64±1.49	10.16±0.2
JAB11	10.57±0.38	-	8.9±0.2	4.01±0.5	9.59±0.28
JAB12	12.96±0.76	4.88±0.45	16.09±0.87	9.51±0.69	3.66±0.38
JAB100	11.04±1.67	-	9.32±0.29	36.6±0.89	7.56±0.37

Values are the mean of 3 replicates (n-3); ± standard deviation

3.3.3 Solubilization of phosphate

The positive isolates for IAA production were further determined for qualitative and quantitative phosphate solubilization. Qualitative analysis showed 18 bacterial isolates as potential phosphate solubilizers that formed distinct halo zones surrounding bacterial colonies on Pikovskaya's agar medium (Table 3). The quantitative analysis of the strains revealed that the isolate JAB100 showed the highest Solubilization of inorganic phosphorous with a solubilization value of 19.4±0.73 µg/ml**.** The other isolates that showed considerable phosphate solubilization were JAB1 and JAB8, with a solubilization value of 14.28±0.65 µg/ml and 11.23±0.23 µg/ml, respectively. Amongst macro elements, phosphorus is essential for plant growth and health. However, this element is considered a limiting factor in plants as most available phosphorus is insoluble. In this context, the Solubilization of inorganic phosphorous by phosphate solubilizing bacteria would be beneficial in improving plant growth and soil health and the maintenance of regular biogeochemical cycles (Gamalero and Glick 2011). In the present study, most bacterial isolates showed good phosphate solubilization activity. Similarly, different bacterial genera such as *Pseudomonas*, *Rhizobium*, *Burkholderia*, *Enterobacter* and several *Bacillus* species have been reported with phosphate solubilization activity increasing productivity in agricultural crops (Erman et al. 2010; Rajput et al. 2013; Pereira and Castro 2014). Studies conducted by Rodriguez and Fraga (1999) believed that applying phosphate-solubilizing bacteria could effectively reduce the application of synthetic phosphorous fertilizers by 50% without affecting crop productivity.

3.3.4 Siderophore, hydrogen cyanide, zinc solubilization and biofilm activities

The isolates that showed positive for phosphate solubilization were further determined to produce siderophore, hydrogen cyanide (HCN), Solubilization of zinc and biofilm activities. Out of the 18

Table 3 *In vitro* plant growth promoting (PGP) activities of bacterial isolates of Jakrem hot spring

Bacterial isolates	IAA production with Trp (µg/ml)	IAA production without Trp (µg/ml)	Phosphate solubilization (µg/ml)	Siderophore production	Zinc solubilization
JAK1	1.394±0.054	0.745±0.037	4.29±0.07	-	-
JAK3	0.575±0.032	0.494±0.013	-	-	-
JAK6	0.85±0.029	0.775±0.026	4.38±0.14	-	-
JAK7	1.319±0.054	0.618±0.040	-	-	-
JAK9	1.488±0.051	0.55±0.016	6.33±0.15	-	-
JAK10	0.672±0.009	0.485±0.036	3.27±0.09	-	+
JAK17	0.698±0.032	0.683±0.017	3.14±0.11	-	-
JAK18	1.222±0.064	0.605±0.023	7.98±0.15	-	+
JAK22	0.913±0.036	0.88±0.037	7.41±0.08	-	-
JK24	1.147±0.061	0.292±0.013	2.73±0.02	+	+
JAK31	1.336±0.016	0.941±0.038	4.005±0.21	-	+
JK54	0.709±0.042	0.532±0.048	2.73±0.02	+	+
JAK251	1.241±0.003	1.04±0.019	5.94±0.17	-	+
JAW1	0.457±0.006	0.253±0.013	5.03±0.08	-	-
JAB1	2.717±0.138	1.849±0.048	14.28±0.65	+	+
JAB8	1.916±0.047	1.458±0.074	11.23±0.23	+	+
JAB9	0.502±0.038	0.406±0.030	4.94±0.14	+	+
JAB11	0.715±0.019	0.588±0.026	1.18±0.08	-	-
JAB12	0.999±0.023	0.61±0.019	4.83±0.05	-	-
JAB100	2.751±0.078	1.097±0.054	19.4±0.73	+	+

Values are mean of 3 replicates (n=3); ± standard deviation; + indicate positive activity; - indicate no activity; Trp- Tryptophan

bacterial isolates, 6 were recorded positive for siderophore production, which was indicated by forming a distinct orange-yellow halo zone surrounding the bacterial colony on CAS blue agar medium. However, qualitative analysis revealed that all the bacterial isolates were negative for HCN production. Again, 10 bacterial isolates were recorded positive for the Solubilization of zinc and formed distinct halo zones surrounding the bacterial colonies under qualitative analysis. Only 11 bacterial isolates were recorded positive for biofilm-forming activity (Table 3). Plants require Iron as an important micronutrient for different metabolic processes, such as nitrogen fixation, respiration, photosynthesis, etc. (Slatni et al. 2008). However, the availability of Iron in the soil is generally much lower than required for the normal functioning of plants. Numerous bacteria of the genus *Bacillus* and *Pseudomonas* have been reported to maintain the bioavailability of Iron through the production of low molecular weight siderophore molecules, which chelate Fe^{3+} with high affinity and transport the labelled Iron, i.e. siderophore-Fe^{3+}complex directly to the plants (Sudisha et al. 2006; Beneduzi et al. 2012; Gupta et al. 2015). In the present study, the bacterial isolates have shown siderophore activity that might be useful for promoting plant growth and health. Yu et al. (2011) reported the biocontrol efficiency of siderophore-producing *B. subtilis* CAS15 on fusarium wilt disease. Further, increased plant growth with reduced disease intensity has been reported from rhizospheric bacteria *B. megaterium* mediated through siderophore production (Sivasakthi et al. 2014). Zinc is also an essential micronutrient for plant growth and health. Its deficiency affects various metabolic processes like nitrogen metabolism, photosynthesis, flowering, fruit formation and maturity, reducing crop plants' nutritional status and overall productivity (Kushwaha et al. 2021). In the present study, some bacterial isolates also showed zinc solubilization activity. Similarly, various workers have reported zinc solubilization and plant growth promotion of *Bacillus* spp. in numerous agricultural crops (Ramesh et al. 2014; Hussain et al. 2015; Khande et al. 2017; Zaheer et al. 2019). The bacterial isolates have also been reported to form biofilm in *in-vitro* studies. Biofilm formation enables the bacteria to survive in adverse environmental conditions mediated through quorum sensing by different bacterial species (Camele et al. 2019).

Table 4 Effect of potent bacterial isolates on growth and development of *B. juncea* under different treatments

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)
Control	18.8±0.65 ^{h}	8.46±0.15 ^{g}	5.56±0.43 ^{f}	0.542±0.03 ^{fg}	0.053±0.008 ^{efg}	0.035±0.003 ^{de}
JAB1	22.13±1.05 ^{fg}	10.9±0.2 ^{ef}	6.58±0.32 ^{e}	0.621±0.02 ^{def}	0.068±0.004 ^{d}	0.04±0.002 ^{cd}
JAB8	23.7±1.22 ^{def}	10.46±0.66 ^{f}	6.16±0.06 ^{e}	0.681±0.06 ^{de}	0.081±0.007 ^{b}	0.042±0.003 ^{bc}
JAB100	23.8±1.15 ^{def}	11.1±0.26 ^{def}	7.15±0.24 ^{d}	0.803±0.08 ^{c}	0.077±0.006 ^{c}	0.042±0.003 ^{bc}
JAB1+JAB8	25.3±1.32 ^{bcd}	11.63±0.61 ^{cde}	8.08±0.2 ^{c}	0.936±0.06 ^{b}	0.083±0.006 ^{bc}	0.062±0.001 ^{a}
JAB8+JAB100	26.16±0.9 ^{bc}	12.26±0.81 ^{bc}	8.15±0.21 ^{c}	0.822±0.03 ^{c}	0.09±0.009 ^{bc}	0.025±0.005 ^{gh}
JAB1+JAB100	24.23±1.59 ^{cde}	11.76±0.61 ^{cd}	8.83±0.2 ^{b}	0.938±0.06 ^{b}	0.084±0.006 ^{bc}	0.047±0.004 ^{b}
JAB1+JAB8+JAB100	31.3±1.8 ^{a}	13.76±0.45 ^{a}	10.87±0.47 ^{a}	1.131±0.12 ^{a}	0.116±0.007 ^{a}	0.064±0.005 ^{a}

Data were calculated after 45 days of inoculation and are mean of 3 replicates ± standard deviation; Different letters indicate significantly different values; *≤0.05; LSD test

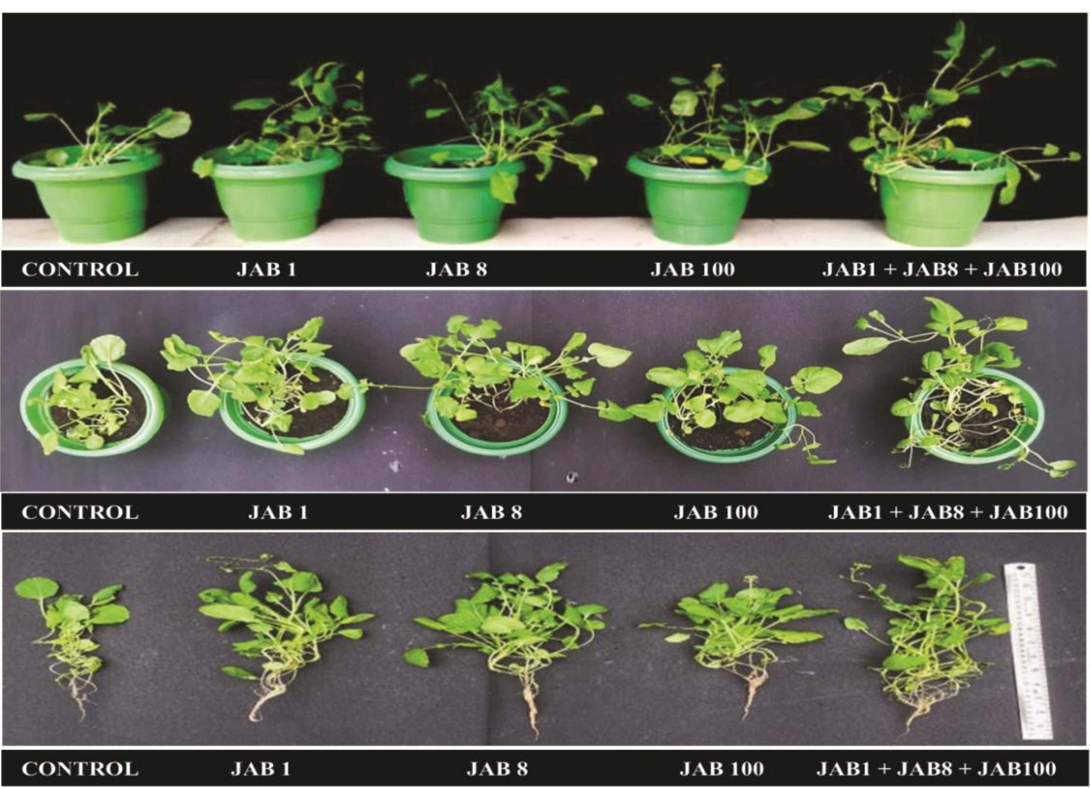

Figure 1 Root and shoot length of *B. juncea* after different treatments using potent bacterial isolates

3.4 Effect of bacterial isolates on growth promotion of *B. juncea*

3.4.1Plant length (shoot and root) and weight (fresh and dry)

Based on *in vitro* plant growth-promoting activities, 3 bacterial isolates, JAB1, JAB8 and JAB 100 were selected for *in vivo* pot experimental trials. The experiment was evaluated following inoculation of the bacterial isolate alone and in combination with each other. After that, the growth promotion of the experimental plant was determined in terms of length, fresh weight, and dry weight. The result revealed that the highest shoots and root length were observed in the treatment sets where all three bacterial isolates (JAB1, JAB8 and JAB100) were co-inoculated in the rhizosphere region (Table 4, Figure 1). It was observed that the shoot length increased by 66.48%, whereas the root length increased by 62.64% as compared to the control (without bacterial inoculation). Similarly, fresh weight and dry weight were also

recorded as being highest in the same treatment set where shoot fresh weight increased by 95.5% and root fresh weight increased by 118.86% compared to control. Again, it was observed that the shoot dry weight increased by 108.67%, and the root dry weight increased by 82.85%**.** A similar study conducted by Sun et al. (2016) reported increased height, fresh weight, and dry weight of *B. napus* when co-inoculated with two strains of *B. subtilis* (LHS11 + FX2). In another study, the tomato's increased height and dry weight were observed after co-inoculation with *B. subtilis* (Singh et al. 2012). Therefore, our present study corroborates with many previous studies which showed that co-inoculation of bacterial isolates with plant growth-promoting activity indicated better enhancement in plant growth-promotion abilities, and such strains can be used as bioinoculants for application in crop productivity without the requirement of any chemicals.

3.4.2 Chlorophyll and protein content

Highest chlorophyll a, b, total chlorophyll and protein content were recorded in the treatment set with co-inoculation of all the selected bacterial isolates (JAB1+JAB8+JAB100) in the rhizospheric region (Table 5). It was observed that the treatment produced 2.13±0.003 mg/g FW of chlorophyll a, 0.917±0.006mg/g FW of chlorophyll b, and 3.04±0.006mg/g FW of total chlorophyll. There was also a gradual increase in total soluble protein content, which was 76.62±4.11 µg/ml, as compared to the control, which showed only 33.54±1.44µg/ml of total soluble protein. Our study was similar to the findings of Cui et al. (2019), who reported increased chlorophyll a and b content in maize plants after treatment with *B. amyloliquefaciens* T B9601-Y2. The bio-based application of *Bacillus* for promoting plant growth with increased biochemical constituents has been cited in numerous literature (Goswami and Deka 2020; Kumar et al. 2021). The exogenous application of *B. methylotrophicus* KE2 as a plant growth promoter has been reported to increase protein content in lettuce leaves (Radhakrishnan and Lee 2016).

3.4.3 Phenol and flavonoid contents

The total phenolic (TPC) and flavonoid content were recorded highest again in the treatment set, which was co-inoculated with all the potent bacterial isolates (JAB1+JAB8+JAB100) (Table 5). The result showed that such a treatment set recorded 1.25±0.005 mgGAE/gram dried plant tissue compared to the control, which recorded 0.71±0.048 mg GAE/gram dried plant tissue. The flavonoid content was observed to be 0.486±0.016 QE/gm plant tissue in the combined inoculated set compared to the control, which showed 0.265±0.009 QE/gm plant tissue. These phenolic derivatives act as a precursor for lignin biosynthesis, and the fungi's toxic nature mediates the defense responses induction in plants and inhibits the growth of the fungal pathogen (Nakkeeran et al. 2006; Dutta et al. 2008). Increased flavonoid content after applying *B. subtilis* CBR05 has been reported in tomatoes (Chandrasekaran et al. 2019). Similarly, inoculation of maize plants with *B. thuringiensis* PM25 under salt stress has reported a 9.24% increase in total flavonoid content (Ali et al. 2022). Thus, the present study agreed with many previous reports on increased phenolic content in plant tissue upon inoculation with plant growth-promoting bacteria.

3.5 Molecular identification of potent bacterial isolates

The molecular identification of all three potent bacterial isolates (JAB1, JAB8 and JAB100) was confirmed by 16S rRNA gene sequences analysis (Figure 2). Based on the NCBI BLAST search, the isolate JAB1 showed the closest homology to *B. velezensis* (similarity 100%) with accession number ON679657, and the isolate JAB8 showed the closest homology with *B. proteolyticus* (similarity 100%) with accession number MT184819. The isolate JAB100 also showed homology with *Bacillus* sp.; however, the percentage of nucleotide sequence similarity was found to be less than 97%, which is below the minimum percentage similarity required for species-level identification (Drancourt et al. 2000).

Table 5 Effect of potent bacterial isolates on biochemical parameters of *Brassica juncea* under different treatments

Treatment	Protein content (µg/ml)	Phenol content (mg/gm DW)	Flavonoid content (mg/gm FW)	Chlorophyll a (mg/gm FW)	Chlorophyll b (mg/gm FW)	Total chlorophyll (mg/gm FW)
Control	33.54±1.44 [j]	0.71±0.048 [k]	0.265±0.009 [cd]	1.36±0.007 [k]	0.591±0.004 [i]	1.95±0.003 [m]
JAB1	58.46±3.25 [de]	0.9±0.009 [ij]	0.297±0.009 [c]	1.89±0.011 [c]	0.781±0.007 [d]	2.67±0.008 [d]
JAB8	53.79±0.94 [ef]	0.91±0.015 [ij]	0.281±0.009 [cd]	1.81±0.015 [f]	0.671±0.008 [f]	2.48±0.011 [h]
JAB100	59.79±0.38 [d]	0.93±0.006 [hi]	0.303±0.024 [c]	1.45±0.006 [j]	0.586±0.006 [i]	2.03±0.013 [l]
JAB1+JAB8	66.37±0.14 [bc]	0.95±0.005 [h]	0.297±0.008 [c]	1.68±0.025 [e]	0.84±0.005 [b]	2.52±0.027 [g]
JAB8+JAB100	70.37±4.75 [b]	1.18±0.04 [f]	0.382±0.022 [b]	1.85±0.006 [d]	0.741±0.004 [e]	2.59±0.003 [f]
JAB1+JAB100	62.46±8.59 [cd]	0.89±0.01 [j]	0.378±0.018 [b]	1.89±0.008 [c]	0.812±0.009 [c]	2.7±0.019 [c]
JAB1+JAB8+JAB100	76.62±4.11 [a]	1.25±0.005 [e]	0.486±0.016 [a]	2.13±0.003 [a]	0.917±0.006 [a]	3.04±0.006 [a]

Data were calculated after 45 days of inoculation and are mean of 3 replicates ± standard deviation. Different letters indicate significantly different values; *≤0.05; LSD test).

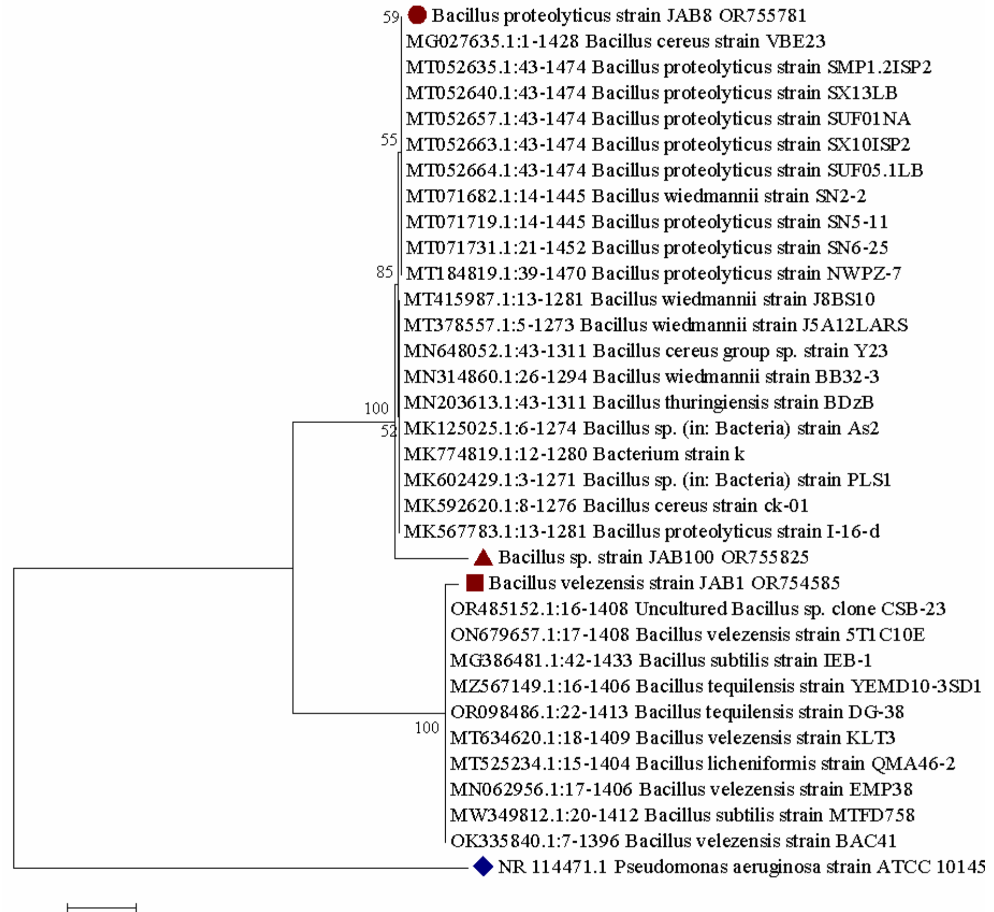

Figure 2 Phylogenetic trees showing the evolutionary relationship of three potent bacterial isolates (JAB1, JAB8 and JAB100) inferred using the Neighbour-joining method considering 16S rDNA sequences.

Therefore, JAB100 could not be conclusively identified at the species level and requires further analysis for accurate taxonomic classification. The phylogenetic tree generated by the neighbor-joining method also validated the identity of the bacterial isolates. The sequences of the isolates JAB1, JAB8 and JAB100 were deposited in NCBI, GenBank database with accession numbers OR754585, OR755781 and OR755825, respectively. Many previous workers have reported the occurrence and dominance of Bacillus species in various hot springs (Adiguzel et al. 2009; Nazari and Mehrabi 2019). The isolation of *B. velezensis* strain MRC 5958f has recently been reported from the Bakra hot spring (Sarangthem et al. 2023). Such findings indicated that members of the genus *Bacillus* might be common inhabitants and well-adapted to survive in extreme environments like hot springs.

Conclusion

The study revealed that Jakrem hot water springs are a rich source of thermophilic bacteria that are dominant by *Bacillus* spp., capable of producing hydrolytic enzymes, and possess plant growth-promoting potential. Characterization of the potent *Bacillus* isolates by morphological, biochemical and 16S rRNA sequencing identified the isolates as *B. velezensis*, *B. proteolyticus*, and *Bacillus* sp. Combined inoculation of these *Bacillus* strains demonstrated significant improvements in plant growth parameters and biochemical constituents of *B. juncea* in *in-vivo* experiments. The findings highlight the ecological significance and biotechnological potential of hot spring bacteria for application as bioinoculants for sustainable agriculture. Harnessing their capabilities can offer promising solutions to mitigate agricultural challenges, promote soil health, and contribute to enhanced crop productivity in the face of evolving climate change and environmental degradation.

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Authors contribution

All the authors contributed to research conception, data analysis and interpretation. AK Collected the sample, performed the experiment and drafting. JR-Participated in the drafting and critical revisions of the article. KT- Writing, review, editing, supervision and validation. All the authors consented and approved to submit the work in the present journal.

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Conflict of interest

The authors have no conflict of interest.

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