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Assessing *Grewia optiva* Rhizobacteria's Potential as a Bioformulation to Stimulate Plant Development

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) involves the utilization of large array of soil bacteria to improve yield and plant growth. The bio-fertilizer with plant growth promoting rhizobacteria (PGPR) is reported to influence the growth, yield, quality and nutrient uptake by a variety of mechanisms. PGPR are reported to synthesize phyto-hormones such as indole-3-acetic acid (IAA), volatile organic compounds, increased mineral nutrient solubilization, nitrogen fixation, making nutrients available for the plant, repression of soil borne pathogens by the production of hydrogen cyanide and siderophore. India is an agriculture dominated country having largest livestock population in the world. Apart from this, livestock rearing is one of the major occupations in India that provides manure, forms important source of food and cash income to million. This domestication requires fodder which comes from local land areas, nurseries or habitat around the domestication. Providing a good fodder value, quality is necessary for livelihood. One such tree is *Grewia optiva* which is a multipurpose tree from tiliaceae family widely used as fodder in Norther Himalayan regions. Various studies have documented the increased health and productivity of different plant species by the application of plant growth promoting rhizobacteria under both normal and stressed conditions. The plant-beneficial rhizobacteria may decrease the global dependence on hazardous agricultural chemicals which destabilize the agro-ecosystem. The present investigation was conducted with aim of isolating and identifying the bacterial isolates from rhizopheric soil of *Grewia optiva* on the basis of different agroclimatic zone of Himachal Pradesh and evaluate them for PGPR traits to increase fodder quality. A total of 99 bacterial isolates were isolated and screened out of which 23 isolates were positive for PGPR traits. The isolates which showed maximum activity were further tested for quantitative estimation of growth promoting traits. Among the 23 isolates two isolates HKD1 and HSD8 demonstrated best results. HKD1 showed maximum P- solubilization (74.40 µg/ml), siderophore (71.50 µg/ml) and IAA (68.76 µg/ml) whereas HSD8 showed maximum P-solubilization (70.60 µg/ml), siderophore (66.56 µg/ml) and IAA (65.76 µg/ml). These isolates were further identified on the basis of 16S rDNA gene sequence. The two isolates were identified as *Leclercia adecarboxylata* and *Enterobacter ludwigii*. These isolates were further screened for biocontrol activity against *Fusarium oxysporum* and *Rhizoctonia solani* and were able to inhibit their growth. On the basis of plant growth promoting traits, two isolates HKD1 and HSD8 were selected and applied as biofertilizers for growth promotion of *G. optiva*. The biofertilizer with 40% RDF significantly increased the shoot and root parameters as well as fodder parameters. The available NPK content of the soil was also improved with application of biofertilizer.

Keywords: Biofertilizer; bioinoculant; PGPR; rhizosphere; sustainable agriculture.

1. INTRODUCTION

“India is an agriculture dominated country having largest livestock population in the world. Apart from this, livestock rearing is one of the major occupations in India that provides manure, forms important source of food and cash income to millions. This domestication requires fodder which comes from local land areas, nurseries or habitat around the domestication” (Lau, et al., 2020). Providing a good fodder quality is necessary for livelihood and is key for enhancing performance of animals and develop a normal rumen of ruminants. Fodder production is seeing a decrease as farmers are more interested in utilizing their land for cash crop production to meet the needs of ever-growing human population. Also, in the hills farmers cannot afford to allocate their lands for fodder crops due to small land holdings. Due to these reasons livestock are being supplemented with feed that are low quality and are given in minimum amount (Mahanty et al. 2017).

Tree fodder can be a viable option of enhancing fodder quality for livestock. There are many fodder trees with the potential of producing green fodder which is nutritious and cheaper than agriculture crops. They are rich in proteins, energy, vitamins and minerals and have high levels of calcium, sodium and sulphur along with iron and zinc (Dhillion et al. 2023).

“Among various trees used in agro forestry, *Grewia optiva*, locally called as “Beul” is an important multipurpose tree. It belongs to family *Tiliaceae* and is one of the most important fodder tree of north western and central Himalayas. It is distributed throughout the sub- Himalayan tract found up to an altitude of 1800 m” (Brandis

1972). “Common names for it in Hindi are Dhaman, Biung, Biul, Bihul, Bhimal, Bhengal, Bewal, and behal; in Nepali, they are Shayalphusro, phusre, ghotli, and Bhimal” (Kumar et al. 2022). “It is sparingly found in forest area and mostly raised along agriculture fields and heavily lopped for palatable fodder. It is very important among farmers for feeding their cattle during winter period when no other green fodder is available. *Grewia optiva* is one of the most important tree species used as fodder in Himachal Pradesh. This multifunctional tree has many other uses that include usage as fuelwood as the dried barks burns readily without release of smoke. Beul trees are considered sub-tropical trees since they are found on the foothills of the Western Himalaya, ranging in elevation from 500–2500 m above mean sea level, spanning from J&K to Nepal. Although the tree can be planted in a variety of soil types, it grows best in sandy loam soil that receives enough precipitation” (Kumar et al. 2022). Due to its semi hard nature and elasticity it is utilised for making axe handles, oar-shafts, bows and shoulder poles. The fibre obtained from *Grewia optiva* is used for making ropes, baskets, bags, wall hangings that requires strength and elasticity (Bhagta et al., 2019; Mariappan 2007). The bark of tree is also utilised as shampoo as it contains saponin and is considered good treatment for dandruff and graying hair. Medicinal properties are also associated with this plant and is used as traditional medicine for treating cough, dysentery, diarrhoea, small pox, malaria, typhoid, rheumatism and eczema and are also reported to have Anti-bacterial and Anti-malarial activity (Uddin et al. 2013).

“The quality of planting material could be significantly improved by changing nursery practices. Also, there is need to increase the forage yield and quality to meet the demand of livestock. The use of chemical fertilizers to achieve high yield though desirable decreases soil health in long run” (Thakur et al., 2022). The inappropriate use of these chemical fertilizers alters but soil pH affects the soil crust, enhances soil acidification and increases pests attack which ultimately results in decreased soil organic carbon and micro and macro-organisms (Mahanty et al. 2017). “A decrease in soil health affects also growth and yield of other crops. Use of effective biofertilizers to enhance and increase fodder quality is a cheap and effective way for quality production of fodder crops” (Bhagta et al., 2019; Kumar et al. 2022).

Plant growth promoting rhizobacteria (PGPR), which act as biofertilizers or biostimulants, offer a viable alternative for sustainable and environmentally friendly agricultural production, seeking to reduce the usage of chemical fertilizers and pesticides. The researchers emphasised the need of continuing to investigate these beneficial bacteria in the rhizosphere, including their interactions and symbiosis with plants (Vocciante et al., 2022; Agbodjato & Babalola 2024). “Understanding these interactions is critical for evaluating the potential of PGPR for crop development and determining how to maximize their usefulness in agricultural production. This continuous research is critical for encouraging sustainable agriculture practices and improving food security. The biofertilizer with plant growth promoting rhizobacteria (PGPR) are reported to influence the growth, yield, quality and nutrient uptake by an array of mechanisms” (Singh et al., 2023; Bakki et al., 2024). “PGPR are the soil bacteria inhabiting the root surface as a population that competitively colonizes plant root and increases their growth and also reduces plant diseases. The use of plant growth promoting rhizobacteria as biofertilizers provides a potential alternative to harmful chemical fertilizers and pesticides” (Kloepper and Adesemaye 2009). This type of potential biofertilizer had significantly influenced the soil fertility and increase the plant growth by mobilizing nutrients in soils, producing plant growth regulators, protecting plants from various fungal pathogen and also protect the environment by bio-remediating the polluted soils through sequestering toxic heavy metal species and degrading xenobiotic compounds (like pesticides) (Bhattacharyya and Jha 2012). These rhizobacteria acts as an efficient biofertilizers due to presence of different mechanisms that increase crop nutrient uptake. These rhizobacteria surrounding the roots fix atmospheric nitrogen and make it available to plants and iron is made available by production of siderophore by these rhizobacteria (Vocciante et al., 2022). Sulfur-oxidizing bacteria oxidize elemental sulfur making it available to plants. Similarly, phosphate solubilizing bacteria convert phosphorus to available forms for uptake. The rhizobacteria are also known to produce certain other metabolites such as Indole Acetic Acid (IAA) and ACC deaminase, chitinase, β- 1, 3-glucanase, antibiotics and HCN which enhance the quality and yield of the crops (Oo et al., 2020). “The use of commercial biofertilizers containing best PGPR strains is increasing rapidly, and for this reason, the importance for

the search of PGPRs and their mode of action is increasing day by day. There are varying degrees of specificities in the requirement of tree species for effective rhizobacterial strains. There are even differences among provenances in the effectiveness of particular strains" (Wickramasinghe et al., 2021; Dhiman et al., 2022; Enebak et al., 2023).

"The microbial inoculants can be applied by various methods to plants, soil and seeds to augment various benefits to the plants. Seed coating with carrier-based inoculants is most common method of application; however, the liquid formulations are now in demand. A major purpose of bacterial inoculant formulation is to offer suitable micro-habitat for survival in the soil ecosystem" (Dhiman et al. 2019). A lot of work has been done on selection of suitable biofertilizers for agricultural species. However, very little work has been reported on the forest tree species. Therefore the present work was carried out with following objective.

1.1 Objectives

1. Isolation and screening of plant growth promoting rhizobacteria from rhizosphere soil of *Grewia optiva*.
2. Characterization, optimization and molecular identification of selected isolates.

2. MATERIALS AND METHODS

2.1 Site Sampling and Assessment of Strawberry Soil Physicochemical Properties

The rhizospheric soil samples of *Grewia optiva* tree were collected during dry period mid - April to May on the basis of agroclimatic zone from six different district of Himachal Pradesh India, as shown in Fig. 1 (Table 1).

Soil physicochemical parameters, such as pH, electrical conductivity (based on the soil-to- water suspension method of Jackson (1973) and organic carbon (based on the chromic acid titration method of Walkley and Black (1934) were measured. The moisture content was determined by subtracting the fresh weight from the dry weight of the soil and dividing it by the dry weight as a percentage.

2.2 Isolation and Enumeration of Culturable Rhizobacterial Diversity

The soil samples were serially diluted and spread on nutrient-agar Petri plates; furthermore, the enumeration of bacterial colonies was performed. Morphologically distinct colonies were picked for the isolation of bacterial cultures by subculturing and purifying numerous single colonies, which were maintained for further use.

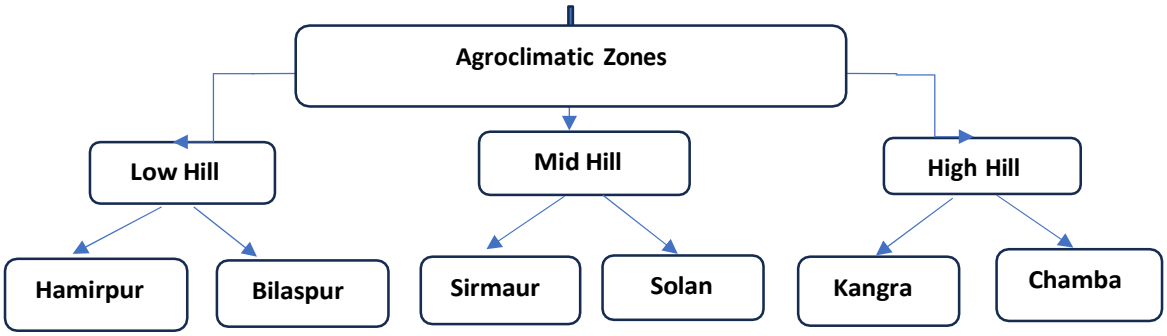


Chart 1. Isolation and enumeration of culturable rhizobacterial diversity

Table 1. Location of different sample collection sites of Himachal Pradesh

Himachal Pradesh				
Districts	**Locations**	**Altitude**	**Latitude**	**Longitude**
Hamirpur	Bharari	780	31º65'01757"	76˚64'71336"
Bilaspur	Ghumarwin	700	31°26'29.98"	76°42'54.32"
Sirmaur	Deothal	932	30° 50' 23"	77° 9' 59"
Solan	Kasauli	1800	30°53'60.00"	76°57'36.00"
Chamba	Shahu	1006	32º55'33673"	76º12'58043"
Kangra	Dharamshala	1457	32˚13'8.5584"	76˚19'24.247"

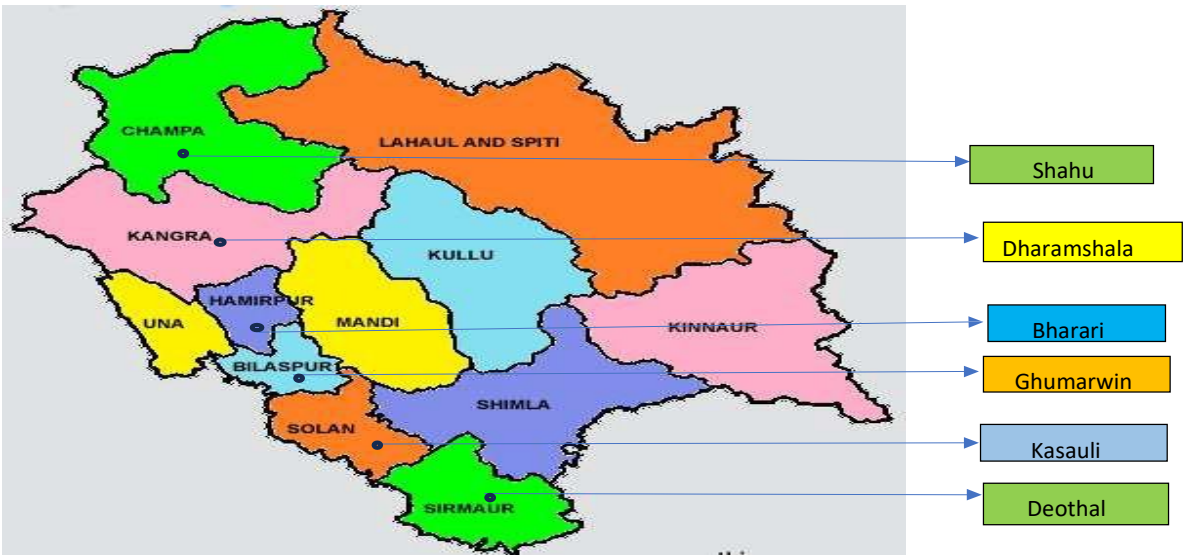


Fig. 1. Map showing sample collection sites for *Grewia optiva* from Himachal Pradesh

2.3 Assessment of the Plant Growth-Promoting Traits of Rhizobacteria

2.3.1 Phosphate solubilization efficacy

Tricalcium phosphate (TCP) and other inorganic P forms are solubilized by chelation of ions due to organic acids and reduced pH by bacteria. Bacterial solubilization of TCP as an inorganic source of phosphate was determined using Pikovskaya agar media (Pikovaskya 1948). The PVK media plates were spot inoculated with rhizobacterial isolates for 72 h at 30 ± 2 ◦C, after which the halo zones (>0.1 mm in diameter) were evaluated.

The qualitative estimation of the soluble phosphorous content in PVK with 0.5% TCP was determined by inoculating 1% of 24 h rhizobacterial cultures and incubating them at 30 ± 2 ◦C for 72 h on a 100- rpm rotating shaker. Subsequently, the cultures were centrifuged for 20 min at 15,000 rpm at 4 ◦C. The amount of soluble phosphorous in the culture supernatant was estimated as per procedure (Bray and Kutrz, 1945).

2.3.2 Siderophore production

The chrome-azurol sulfonate assay uses a strong ligand, such as a siderophore produced by bacteria, which results in a chelation effect with iron supplemented within the CAS dye. The siderophore iron complex releases free dye, resulting in a color change. King's B media was supplemented with a separately prepared solution of CAS dye consisting of 60.5 mg/50 ml CAS, 1 mM $FeCl3{\cdot}6H2O$ in 10 mM HCl, and 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) in 40 ml deionized water. The rhizobacterial isolates spot-inoculated on the media were observed for halo zone formation (Schwyn and Neilands 1987).

The siderophore concentration was estimated quantitatively with an aliquot of 0.1 ml culture supernatant mixed in 0.5 ml of CAS solution. After incubation for 10 min, the absorbance of the mixture (Am) was read at 630 nm, considering minimal media as a blank and CAS with minimal media as a reference (Ar) for determining% siderophore units as *[(Ar - Am)/Ar] × 100*.

2.3.3 Indole 3-acetic acid (IAA) production

Rhizobacterial cultures were amended with 5 mM L-tryptophan under shaking incubator. The cultures were centrifuged at 15,000 rpm for 20 min. Supernatant was treated with orthophosphoric acid and Salkowski reagent and incubated for half an hour in darkness. Pink coloration intensity was quantified at 530 nm spectrophotometrically. To determine the concentration of IAA, a calibration curve was constructed using IAA as a standard (ranging from 10 to 100 µg mL^{-1}) (Gorden and Palleg 1957).

2.3.4 Nitrogen fixing activity

Jensen's N-free medium was used to test the nitrogen-fixing ability of rhizobacterial isolates

(Aryal et al. 2015). The exact percentages of the ingredients in Jensen's medium were as follows: 20.0% sucrose, 1.0% potassium bicarbonate (KH_2PO_4), 0.5% magnesium sulfate ($MgSO_4$), 0.5% sodium chloride (NaCl), 0.1% sulfur dioxide (SO_4), 0.005% sodium molybdate ($NaMoO_4$), 2.0% calcium carbonate ($CaCO_3$), and 20.0% agar (g L^{-1}). The bacterial cultures were spread out in a loop on the plate using sterile toothpicks and then incubated for 48 h at 30 ±2 ◦C. The nitrogen-fixing potential of the PGPR isolates was represented by a halo zone around the colonies.

2.3.5 HCN production

Rhizobacterial isolates were screened for hydrogen cyanide (HCN) generation. Bacterial cultures were streaked on prepoured plates of King's B media supplemented with 1.4 g/l glycine. Each petri plate lid was lined with Whatman No. 1 filter paper strips soaked in 0.5% picric acid in 2% sodium carbonate. The samples were incubated at 30 ±2 ◦C for 1–4 days on parafilm-sealed plates. To compare the results, an uninoculated control was used. The plates were inspected for a change in filter paper color from yellow to orange brown (Bakker and Schippers 1987).

2.4 Phenotypic and Biochemical Characterization Rhizobacterial Isolates

Based on screening tests performed for growth-promoting traits, rhizobacterial isolates with better potential were further tested for phenotypic and biochemical characteristics and were optimized for growth before further identification.

2.5 Phenotypic and Biochemical Characterization

The colony characteristics of the rhizobacterial isolates HKD-1and HSD-8 were examined for characteristic features in the culture medium. Bacterial identification was performed using Gram's staining, which is the most commonly used differential staining technique, under light microscopy. The biochemical characteristics of the tested rhizobacterial isolates were investigated using Bergey's Manual of Determinative Bacteriology.

2.6 *In-vitro* Growth Optimization for pH, Temperature, and Growth Media Incubation

The initial pH of the growth medium was adjusted from 5 to 9 to observe the effect of pH. Similarly, the growth medium was incubated for cell growth at temperatures ranging from 25 ◦C to 45 ◦C to determine the optimum growth temperature. The viability of the rhizobacterial isolates was observed for 72 h while maintaining the conditions at the optimum temperature. The Log CFU $ml^{-}1$ growth was recorded after incubation for 48 h at each observation concurrently with incubation with broth at the same dilution.

2.7 Antagonistic Activity of Bacterial Isolates against Test Fungus

All the isolates were test for their antifungal activities against *Fusarium oxysporum* and *Rhizoctonia solani* on potato dextrose agar medium. The isolates were streaked on the surface of the plate 2cm away from the fungal disc. The antagonistic activity was observed after incubation at 28 ± 1°C for 7 days and per cent growth inhibition was calculated as described by Vincent (1947).

$$I = \frac{C-T}{C} \times 100$$

Where,

I = Percent growth inhibition
C = Growth of fungus in control
T = Growth of fungus in treatment

2.8 Molecular Identification and Phylogenetic Analysis of Drought Stress- Tolerant Rhizobacterial Isolates

The potential rhizobacterial isolates were identified at the species level via sequence analysis at the The sequencing was performed from Biokart Genomic lab, Bangalore, India, using both forward and reverse primers. The sequence alignment was done using the Clustal W (Thompson et al. 1994) and manually edited using the Bioedit package (Hall 1999). The cladograms were constructed by neighbor-joining method (Saitou and Nei 1987) with Kimura 2 – parameter model (Kimura 1980) and were bootstrapped using the software programs

in the MEGA 6.0 package (Tamura et al. 2013). Subsequently, the gene sequences were submitted into GenBank, where they were assigned unique accession numbers.

2.9 Statistical Analysis

Data collection on biochemical analysis and physico-chemical properties of different PGPR were subjected to statistical analysis by adopting method of "Analysis of Variance". The significance of treatment effect was judged with the help of "F" test (Variance ratio). The difference of the treatment mean was test using critical difference (C.) at 5 per cent level of probability (Gomez and Gomez 1984). If the variance ratio of test was found significant 0.05 per cent level of significance, the standard error of the mean (SEM ±) and critical difference (CD)were calculated for further treatment comparisons.

3. RESULTS AND DISCUSSION

3.1 Physio-Chemical Properties of Soil Sample Collected from Different Districts of Himachal Pradesh

The rhizospheric soil samples for the present study were collected from diverse *Grewia optiva growing sites*. It was evident from the data that all the parameters showed significant variations (Table 2). Difference in moisture content might be due to water holding capacity of soil. The electrical conductivity was ranged between 0.27 to 0.31 dsm^{-1} and maximum value for EC (0.31 dsm^{-1}) was recorded in Dharamshala site of Kangra district. The variations may be due to the different climatic conditions of various locations. Tadesse et al. (2012) observed that organic matter increases the holding capacity because of its affinity towards water thereby increasing moisture content of soil. "Most of the uppermost soil in the middle to lower Himalayan region is of the alluvial type, which is rich in organic matter content that, upon degradation, also releases other micronutrients. This soil type nourishes microbes in the plant rhizosphere in the presence of organic matter" (Arunachalam et al., 1999; Saini et al., 2021).

3.2 Isolation and Enumeration of Bacteria Associated with Grewia Optiva Rhizosphere

The rhizospheric soil consists of bacteria surrounding the plants in soil habitat. The enumeration of the soil samples observed the difference in population of rhizosphere bacteria. A significant variation was recorded for bacterial count in the rhizospheric soil samples of six sites (Table 2). The bacterial count ranged between 3.00 to 4.30×10^6 log cfu/g in the rhizospheric soil samples collected from six districts of Himachal Pradesh. Variations in bacterial populations observed among different locations might be due to varied climatic conditions and soil physico-chemical properties Vacheron et al. (2013). Total of ninety-nine bacterial isolates were isolated from soil samples of different districts of Himachal Pradesh (Table 3). These bacterial isolates were screened for various plant growth-promoting traits.

3.3 Screening of Bacterial Isolates for Growth Promoting Traits

All the 99 bacterial isolates were selected and then screened for plant growth promoting traits *viz*., phosphate solubilization, and nitrogen fixing ability, IAA production, HCN production and siderophore production (Table 4). The data presented in Fig. 2 showed that out of 99 bacterial isolates 41 bacterial isolates showed positive results for phosphate solubilization (41.4 per cent), 86 isolates were nitrogen fixers (86.8 per cent), 37 bacterial were able to produce IAA (37.3 per cent), 23 (23.23 per cent) isolates

Table 2. Physio- chemical properties of soil samples collected from different sites of Himachal Pradesh

Districts	Locations	pH	EC (dSm^{-1})	Organic Carbon (%)	Moisture content (per cent)
Hamirpur	**Bharari**	6.9	0.29	1.31	19.90
Bilaspur	**Ghumarwin**	6.7	0.28	1.31	19.82
Solan	**Kasauli**	6.7	0.28	1.30	19.51
Sirmaur	**Deothal**	7.0	0.30	1.32	20.52
Chamba	**Shahu**	6.6	0.27	1.30	19.52
Kangra	**Dharamshala**	7.1	0.31	1.33	21.11
CD (0.05)		**0.18**	**0.02**	**0.02**	**0.13**

Table 3. Isolation and enumeration of total bacterial population

Districts	Locations	Bacterial count (cfu×10^6 cfu/g)	Name of isolates	Number of isolates
Hamirpur	Bharari	3.9	HHB	17
Bilaspur	Ghumarwin	3.8	HBG	16
Solan	Kasauli	3.6	HSK	16
Sirmaur	Deothal	4.1	HSD	17
Chamba	Shahu	3.0	HCS	10
Kangra	Dharamshala	4.3	HKD	23
CD (0.05)		**0.17**		
Total				**99**

showed positive results for HCN production and 29 isolates were siderophore producers (29.29 per cent). These findings are consistent with other research of Attar et al. (2018); Devi and Thakur (2018); Jhilta (2018); Dhiman (2018) and Oo et al. (2020) who isolated bacteria inhabiting roots and a few isolates showed positive results for P-solubilization, siderophore, HCN and IAA production.

Table 4. Screening of bacterial isolates for growth promoting traits

Sr. No	Bacterial Isolates	P Solubilization	N- free Medium	IAA Production	HCN Production	Siderophore Production
1	HHB-1	-	+	-	-	-
2	HHB-2	+	++	++	+	+
3	HHB-3	-	+	-	-	-
4	HHB-4	++	++	++	++	+
5	HHB-5	++	++	++	++	+
6	HHB-6	-	+	-	-	-
7	HHB-7	++	++	++	+	+
8	HHB-8	-	+	-	-	-
9	HHB-9	+	+	-	-	-
10	HHB-10	-	-	+	-	-
11	HHB-11	+	+	-	-	-
12	HHB-12	+	-	+	-	-
13	HHB-13	-	+	-	-	-
14	HHB-14	+	+	-	-	-
15	HHB-15	-	+	-	-	-
16	HHB-16	+	+	-	-	-
17	HHB-17	-	-	+	-	-
18	HBG-1	-	+	-	-	-
19	HBG-2	-	+	-	-	-
20	HBG-3	++	++	++	+	+
21	HBG-4	-	+	-	-	-
22	HBG-5	+	++	++	+	+
23	HBG-6	+	++	++	++	+
24	HBG-7	-	-	+	+	-
25	HBG-8	+	+	-	-	-
26	HBG-9		+	+	-	+
27	HBG-10	+	+	-	-	-
28	HBG-11	-	+	+	-	-
29	HBG-12	-	+	+	-	-
30	HBG-13	+	+	-	-	+
31	HBG-14	-	+	-	-	-
32	HBG-15	-	+	+	-	-
33	HBG-16	+	+	-	-	-
34	HSK-1	-	+	-	-	-

Sr. No	Bacterial Isolates	P Solubilization	N- free Medium	IAA Production	HCN Production	Siderophore Production
35	HSK-2	-	+	-	-	-
36	HSK-3	++	++	+	+	+
37	HSK-4	-	+	-	-	+
38	HSK-5	-	+	-	-	-
39	HSK-6	++	++	+	+	-
40	HSK-7	-	+	+	-	-
41	HSK-8	-	+	-	-	-
42	HSK-9	+	+	+	-	-
43	HSK-10	-	+	-	-	-
44	HSK-11	-	+	-	-	-
45	HSK-12	-	+	-	-	-
46	HSK-13	+	+	-	-	-
47	HSK-14	-	+	-	-	-
48	HSK-15	+	+	-	-	-
49	HSK-16	-	-	+	-	-
50	HSD-1	-	+	-	-	-
51	HSD-2	++	+++	++	++	+
52	HSD-3	++	++	++	++	+
53	HSD-4	-	+	-	-	-
54	HSD-5	++	+++	++	++	++
55	HSD-6	-	+	-	-	-
56	HSD-7	-	+	-	-	-
57	HSD-8	+++	+++	++	+++	+++
58	HSD-9	++	++	++	++	++
59	HSD-10	-	-	+	-	-
60	HSD-11	-	+	-	-	-
61	HSD-12	-	+	-	-	-
62	HSD-13	+	+	-	-	-
63	HSD-14	-	+	-	-	-
64	HSD-15	+	-	+	-	-
65	HSD-16	-	+	-	-	-
66	HSD-17		-	+	-	+
67	HCS-1	+	+	-	-	+
68	HCS-2	-	+	-	-	-
69	HCS-3	-	+	-	-	-
70	HCS-4	-	-	-	-	-
71	HCS-5	+	+	-	+	+
72	HCS-6	+	-	+	-	-
73	HCS-7	-	+	-	-	-
74	HCS-8	-	+	-	-	-
75	HCS-9	-	+	-	-	-
76	HCS-10	-	+	-	-	-
77	HKD-1	+++	+++	+++	+++	+++
78	HKD-2	+	+	-	-	+
79	HKD-3	+	-	-	-	-
80	HKD-4		+	-	-	-
81	HKD-5	++	++	++	++	++
82	HKD-6	-	-	+	-	-
83	HKD-7	+	+	-	-	+
84	HKD-8	-	+	-	-	-
85	HKD-9	-	+	+	-	+
86	HKD-10	-	+	-	-	+
87	HKD-11	-	+	-	-	-
88	HKD-12	-		-	-	-
89	HKD-13	-	+	-	-	-

Sr. No	Bacterial Isolates	P Solubilization	N- free Medium	IAA Production	HCN Production	Siderophore Production
90	HKD-14	++	++	++	++	++
91	HKD-15	-	++	-	-	-
92	HKD-16	++	++	++	+++	++
93	HKD-17	++	+++	++	++	++
94	HKD-18	-	+	-	+	-
95	HKD-19	++	++	++	++	++
96	HKD-20	-	+	-	-	-
97	HKD-21	-	+	-	-	+
98	HKD-22	-		-	-	-
99	HKD-23	+	+	-	-	-

Highest efficiency= (+++), Moderate efficiency = (++), Low efficiency = (+), No efficiency= (-)

It was found that out of 99 isolates, 23 isolates showed high plant growth promoting traits. The isolate HKD-1 and HSD-8 showed high plant growth promoting traits out of which HKD-1 exhibited maximum plant growth promoting traits (Table 4).

3.4 Quantitative Estimation of Selected Bacterial Isolates for Growth Promoting Traits

Among 99 bacterial isolates 23 isolates were selected and screened for quantitative assessment of phosphate solubilization, siderophore production and IAA production.

3.4.1 Screening of rhizobacteria isolates for phosphate solubilization

All 23 bacterial isolates can solubilize phosphate ranged between 19.73 µg/ml to 74.40 µg/ml (Table 5). A significant variation was recorded for all the isolates under study with respect to phosphate solubilization. Bacterial isolate HKD-1 had the highest P-solubilization efficiency (85.33 per cent).

"Phosphorous is one of the necessary plant nutrients needed by plants for photosynthesis, energy, signal transduction, macromolecular biosynthesis, respiration, growth and metabolism" (Khan et al. (2010) and Zhang et al. 2017) Bulk of P in soil is fixed but plant accessible P is rarely available against the riches of both inorganic and organic P forms in the soils Rashid et al. (2022). A group of soil microorganisms are able to transforming insoluble P into soluble and plant available forms called as phosphate-solubilizing microorganisms (PSM). Several PSBs can convert insoluble phosphate into soluble forms ($H_2PO_4^-$ and HPO_4^{2-}) that are accessible via acidification, chelation, and organic acid generation Lebrazi et al. (2020); Uzma et al. (2022).

3.4.2 Screening of bacterial isolates for siderophore production

The siderophore generation efficiency of selected bacterial isolates was quantitatively assessed using the brilliant zone on CAS medium. The data in Table 5 revealed that all 23 selected bacterial isolates from the rhizosphere of *Grewia optiva* showed a substantial variance in per cent siderophore efficiency and siderophore synthesis in liquid medium. Iron is an essential metal for practically all living creatures to carry out metabolic functions. Among all the isolates, the isolates HDK1 (71.50 µg/ml) produced a highest siderophore units after quantification (Table 5).

Siderophores are ferric ion-specific chelating agents with a low molecular weight generated by bacteria and fungi under low iron stress. Siderophore synthesis can potentially serve as a biocontrol strategy, depriving fungus of iron Mazhar et al. (2016), Pahari and Mishra (2017) and Murali et al. (2021).

3.4.3 Indole-3-acetic acid (IAA) production by selected bacterial isolates

The rhizobacterial isolates cultured in tryptophan-supplemented medium were assayed from the supernatant for their ability to produce auxin. IAA production differed significantly among the culture supernatants, ranged between 20.06 µg/ml to 68.76 µg/ml. The bacterial isolates HKD-1 produced maximum (68.76 µg/ml) amount of IAA followed by HSD-8 (65.76 µg/ml) (Table 5).

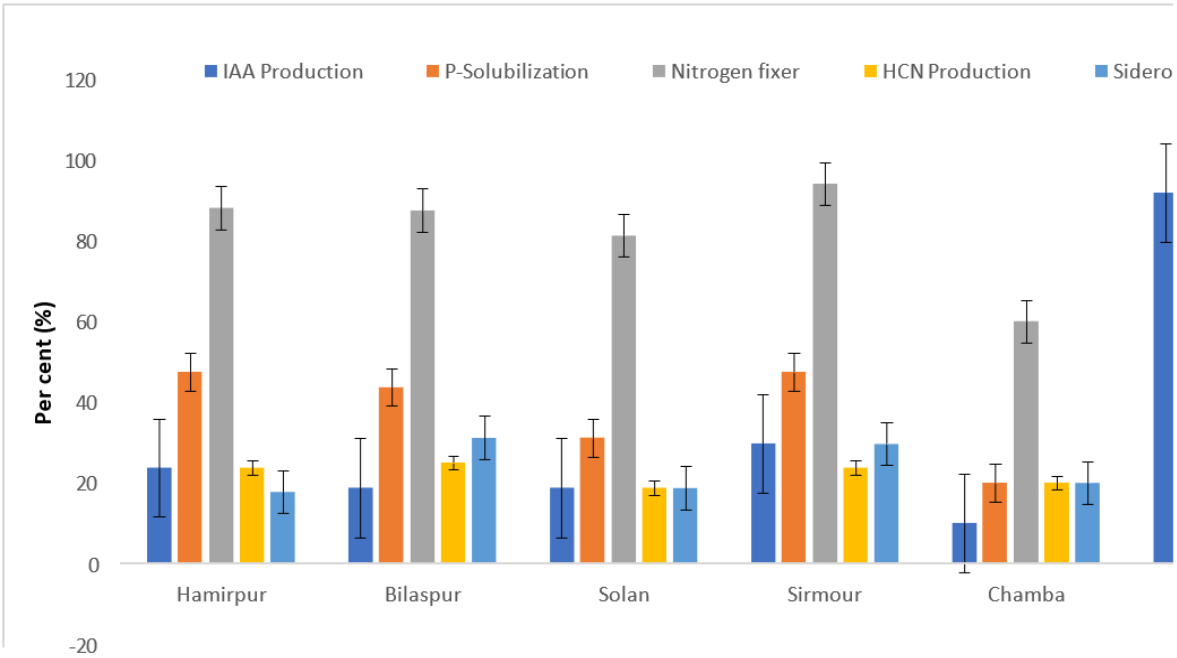


Fig. 2. Percentage of isolates exhibiting growth promoting traits

Table 5. *In- vitro* PGPR traits of selected bacterial isolates from Grewia optiva

Sr. No	Bacterial Isolates	P-Solubilization in Liquid medium (µg/ml)	Siderophore production in liquid medium (µg/ml)	IAA production by bacterial isolates (µg/ml)
1	HHB-2	33.70	29.53	33.03
2	HHB-4	47.06	41.50	35.76
3	HHB-5	49.70	42.56	37.86
4	HHB-7	31.50	28.73	43.63
5	HBG-3	34.66	29.56	41.90
6	HBG-5	31.60	28.66	42.63
7	HBG-6	30.30	27.63	46.65
8	HSK-3	29.43	26.66	39.76
9	HSK-6	37.03	30.60	37.90
10	HSK-9	50.23	45.33	37.23
11	HCS-1	19.73	26.40	20.06
12	HCS-5	28.33	18.50	21.80
13	HSD-2	37.63	31.53	51.30
14	HSD-3	47.60	41.63	53.73
15	HSD-5	51.63	45.60	55.73
16	HSD-8	70.60	66.56	65.76
17	HSD-9	64.16	46.40	53.73
18	HKD-1	74.40	71.50	68.76
19	HKD-5	55.06	47.40	50.46
20	HKD-14	56.63	46.60	54.90
21	HKD-16	63.53	50.60	54.46
22	HKD-17	63.43	52.60	55.90
23	HKD-19	39.63	49.76	57.60
	CD(0.05)	**1.79**	**0.25**	**0.80**

Indole-3-acetic acid (IAA) synthesis is a major feature of rhizosphere bacteria, which stimulates and facilitates growth. IAA is an important phytohormone secreted by plants for their growth and development (Baggam et al., 2017). Plant growth promoting rhizobacteria (PGPR)

have ability to synthesize IAA naturally. It promotes cambial activity, inhibits or delays abscission of leaves, induces flowering and fruiting (Zhao 2010).

The above findings are consistent with the research conducted by Kumar (2018). Similar results were reported by Baggam et al. (2017) for 2 strains with highest IAA production of 27.1µg/ml and 33.5µg/ml respectively. Lebrazi et al. (2020) isolated four rhizospheric bacteria (*Phyllobacterium spp., Bacillus spp., Agrobacterium spp., and Rhizobium spp.*) from surface sterilized *Acacia cyanophylla* root nodules and tested their ability to solubilize inorganic phosphate and produce IAA.

3.4.4 Antifungal activity against fungal pathogens by selected bacterial isolates

The data presented in Table 6 represents the percent growth inhibition by selected bacterial isolates against two fungal pathogens *Fusarium oxysporum* and *Rhizoctonia solani.* All the bacterial isolates showed significant inhibition of growth of the two tested fungi. Among the selected 23 bacterial isolates, HKD-1 bacterial isolate showed maximum (75.20 per cent) and (61.40 per cent) growth inhibition against *Fusarium oxysporum* and *Rhizoctonia solani,* respectively and isolate HSD-8 also showed 61.40 per cent inhibition against *Fusarium oxysporum* and 55.80 per cent against *Rhizoctonia solani*.

In their study Shoebitz et al. (2009) isolated strain of *Enterobacter ludwigii* from *Lolium perenne* rhizosphere and reported inhibition of mycelial gowth of *F. solani.* The results are also supported with the work of Dhiman et al. (2018) they isolated rhizobia from the root nodules of *Dalbergia sissoo* DBD-1 which demonstrated antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani* with per cent growth of inhibition of 68.89 per cent and 66.67 per cent, respectively. Similar results were obtained by Meenakshi (2019) and Rashid et al. (2022).

Table 6. Antifungal activity against fungal pathogens by selected bacterial isolates

Sr. No	Bacterial isolates	Per cent inhibition	
		Fusarium oxysporum	***Rhizochtonia solani***
1	HHB-2	-	-
2	HHB-4	52.26	-
3	HHB-5	56.20	-
4	HHB-7	49.16	-
5	HBG-3	-	16.40
6	HBG-5	-	-
7	HBG-6	46.06	-
8	HSK-3	-	-
9	HSK-6	39.20	-
10	HSK-9	-	-
11	HCS-1	-	15.50
12	HCS-5	-	-
13	HSD-2	53.06	-
14	HSD-3	-	17.40
15	HSD-5	**-**	**-**
16	HSD-8	71.30	55.80
17	HSD-9	64.20	35.36
18	HKD-1	75.20	61.40
19	HKD-5	64.13	-
20	HKD-14	-	33.50
21	HKD-16	57.01	-
22	HKD-17	61.26	-
23	HKD-19	66.13	20.33
CD (0.05)		**0.18**	**0.11**

***% Growth inhibition = C-T× 100, Where, C= Growth of fungus in control; T= Growth of fungus in test*
*** No inhibition = (-)*

3.5 Selection of Growth Promoting Bacterial Isolates

The Data on plant growth promoting traits on the basis of quantitative assay were subjected to cluster analysis. The Dendrogram was constructed using average linkage (Between groups) in IBM SPSS software. A dendrogram depicts the hierarchical relationship between clusters, displaying order in which clusters are merged aiding in visualizing the data and providing insights into relationship between clusters (Fig. 3).

The dendrogram divides 23 bacterial isolates into two main clusters. The upper cluster is divided into two main cluster having 13 isolates. One sub cluster having two isolates (HSD- 8 and HKD-1) and another having 11 isolates (HSD-9, HKD-19, HKD-5, HKD-16, HKD-17, HHB-7, HBG-6, HSK-6, HHB-4, HHB-5, HSD-2). The lower cluster is also divided into main cluster having 10 isolates. One sub cluster having 8 isolates (HSD-3, HKD-14, HSD-5, HBG- 3, HBG-5, HSK-3, HSK-9, HHB-2) and another having two isolates HCS--1 and HCS5.Among all the bacterial isolates two bacterial isolates HKD-1 and HSD-8 exhibit positive result for all the multifarious growth promoting traits such as phosphate solubilization, siderophore production and IAA production. Therefore, these two bacterial isolates HKD-1 isolated from district Kangra and HSD-8 isolated from district Sirmaur were selected on the basis of maximum PGPR traits.

3.6 Morphological, Biochemical and Physiological Characteristics of Selected Bacterial Isolates

The bacterial isolates HKD-1 and HSD-8 were selected on the basis of result obtained from plant growth promoting traits and were further examined for morphological, physiological and biochemical characteristics.

3.6.1 Morphological characterization of selected isolates

The two bacterial isolates HKD-1 and HSD-8 were characterized according to their morphological characteristics. Gram staining was performed to identify shape and gram reaction of the selected bacterial isolates beneath light microscope. The results revealed that both the bacterial isolate HKD-1 and HSD-8 was gram negative. The bacterial isolates, HKD1 was circular form, raised elevation, entire margin, smooth surface and slimy texture. However, bacterial isolate HSD-8 was irregular form, rod shaped, raised elevation, smooth margin, smooth surface and slimy texture.

The results are supported by Dhiman (2018) and Meenakshi et al (2019). They isolated bacteria from the rhizosphere of *Delbergia sissoo* and *Albizia procera,* respectively and reported them to be gram negative.

3.6.2 Biochemical characterization of selected bacterial isolates

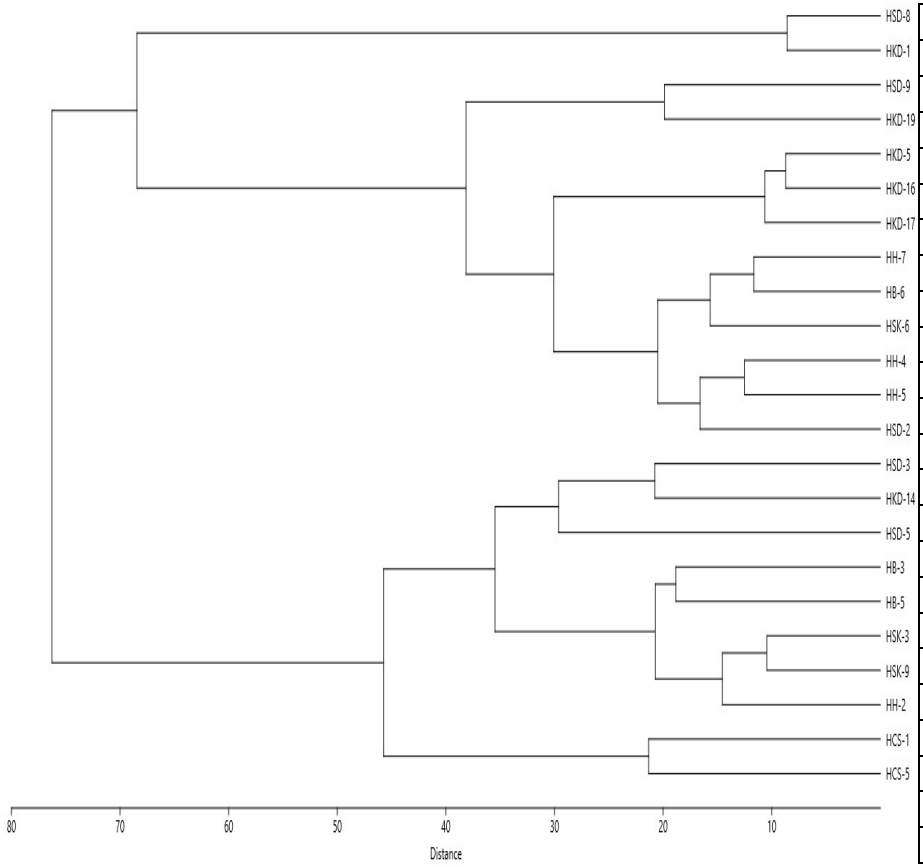
Two selected bacterial isolates namely HKD-1 from Kangra and HSD-8 from Sirmaur district were characterized by a series of biochemical test and results are presented in Table 8. Present results revealed that both bacterial isolates were positive for the Indole test, Methyl red test, Catalase test, Citrate utilisation, Urease test, Carbohydrate test, Glucose, Xylulose, Fructose, Sucrose, Galactose, Maltose, and Mannitol, but negative for Voges Proskauer test, Hydrogen sulphide production and Casein test. In terms of carbon utilisation, both isolates were capable of fermenting xylulose, sucrose, galactose, maltose, mannitol, and fructose with the exception of lactose. According to the Bergey's manual of systematic bacteriology the results on morphological and biochemical tests of selected bacterial isolates in the present study revealed that bacterial isolates were gram negative in reaction and rods in shape so these selected bacterial isolates may belong to the *Leclercia and Enterobacter spp.*

The present results are supported by Dhiman et al. (2019) and Singh et al. (2020) who characterized the bacterial isolate for biochemical testing and reported that it was positive for catalase, urease, and ammonia synthesis, but negative for indole, Voges Proskauer, H_2S, citrate, and casein.

3.7 Physiological Characterization of Selected Bacterial Isolates

3.7.1 Effect of incubation period for growth of selected bacterial isolates

The results presented in Fig. 4 shows the growth of isolates HKD-1 and HSD-8 at different incubation period (12-72 hours). The result showed that maximum growth of selected bacterial isolate HKD-1and HSD-8 was obtained



PGPR traits

Isolates	P-sol.	Sid.	IAA	HCN	*F. oxi*	*Rhizoc*
HHB2	+	+	+	-	-	-
HHB4	++	+	+	-	+	-
HHB5	++	+	+	+	+	-
HHB7	++	+	+	+	+	-
HBG3	+	+	+	+	-	+
HBG5	+	+	+	+	-	-
HBG6	++	+	+	+	+	-
HSK3	++	+	+	-	-	-
HSK6	+	+	+	-	+	-
HSK9	+	+	+	+	-	-
HCS1	-	+	+	+	-	-
HCS5	+	+	+	+	-	+
HSD2	++	+	+	-	+	-
HSD3	+	+	+	+	-	+
HSD5	++	+	++	-	-	-
HSD8	+++	+++	+++	+++	++	++
HSD9	++	+	+	+	+	+
HKD1	+++	+++	+++	+++	+++	++
HKD5	++	++	+	+	+	-
HKD14	++	+	+	-	-	+
HKD16	++	+	+	-	+	-
HKD17	++	++	++	+	+	-
HKD19	++	++	++	+	+	+

Fig. 3. Dendrogram of based on Plant growth promoting traits by bacterial isolates

Table 7. Morphological characterization of selected bacterial isolates

Bacterial Isolates	Form	Elevation	Margin	Surface	Texture	Shape	Arrangement	Grams Reaction
HKD-1	Circular	Raised	Entire	Smooth	Slimy	Rod-shaped	Cluster	-ve
HSD-8	Irregular	Raised	Smooth	Smooth	Slimy	Rod shaped	Cluster	-ve

Table 8. Biochemical characterization of selected bacterial isolates

Sr. No	Bacterial isolates	HKD-1	HSD-8
1	Indole Test	+	+
2	Methyl red test	+	+
3	Voges Proskauer test	-	-
4	H_2S production	-	-
5	Catalase test	+	+
6	Citrate utilization	+	+
7	Gelatin liquification	-	+
8	Starch Hydrolysis	-	-
9	Casein hydrolysis	-	-
10	Urease test	+	+
11	Oxidase test	-	-
12	Carbohydrate test	+	+
13	Glucose	+	+
14	Xylulose	+	+
15	Fructose	+	+
16	Sucrose	+	+
17	Galactose	+	+
18	Maltose	+	+
19	Mannitol	+	+
20	Lactose	-	-
21	Sorbitol	-	+
22	Dextrose	+	+

Positive = (+), Negative = (-)

at incubation period of 48 hrs. Both isolates showed minimum growth between 0 to12 hours. However, after 48 hours of incubation, the selected bacterial isolates' growth and development were significantly reduced. Fig. 6 clearly demonstrates that the two isolates showed an increase in growth after 12 hours of incubation and a decrease in growth after 60 hours of incubation.

Our result is supported by Dipta (2017), Jhilta (2018) and Dhiman (2018) also observed growth of Rhizobium on YEMA medium at pH 7.0 after incubation for 48-72 hrs at 35ºC.

3.7.2 Effect of temperature on growth of selected bacterial isolates

Temperature plays a vital role in the growth and development of bacteria. Temperature changes have the greatest impact on enzymes and their activity, with an optimal temperature resulting in fastest metabolism and subsequent growth rate. Temperatures below optimum cause a decrease in enzyme function and slower metabolism, but higher temperatures can denature proteins like enzymes and carrier proteins, resulting in cell death.

Fig. 5 showed bacterial growth at different temperature ranging from 25-45°C. The maximum growth of selected bacterial isolates was recorded at the temperature of 35°C with viable count of 45×10^6 cfu/ml for HKD-1 isolate and 42×10^6 cfu/ml for HSD-8 isolate. Both the isolates were able to grow at the temperature range 25 ºC- 35º C. It was clearly seen that none of the tested bacteria were able to grow after 45ºC.

The findings of current study are similar with the study conducted by Dinesh et al. (2015) who also reported effect of temperature on different rhizo-bacterial isolates and found *Enterobacter spp. and Bacillus spp.* showed ability to growth at 28 ºC to 40 ºC. Similarly, Zahid et al. (2015)

reported isolates were able to grow in temperature range of 20 to 45° C, and optimum temperature of 35 ° C was best for growth of the bacteria.

3.7.3 Effect of pH on the growth of selected bacterial isolates

The data in Table 9 revealed that different pH levels have a substantial effect on the growth of selected bacterial isolates. The results indicated that both selected bacterial isolates were able to grow at pH range of 5-9.0. However, the selected bacterial isolates exhibited limited growth below pH 5 and after pH 9.0. At pH 7.0, selected bacterial isolates HKD-1 and HSD-8 had maximal growth rates of 48 $\times 10^6$ cfu/ml for HKD-1 and 45 $\times 10^6$ cfu/ml for HSD-8. Table 9 shows that the two isolates' greatest development occurred at pH 7.0, which declined below and above the optimum level.

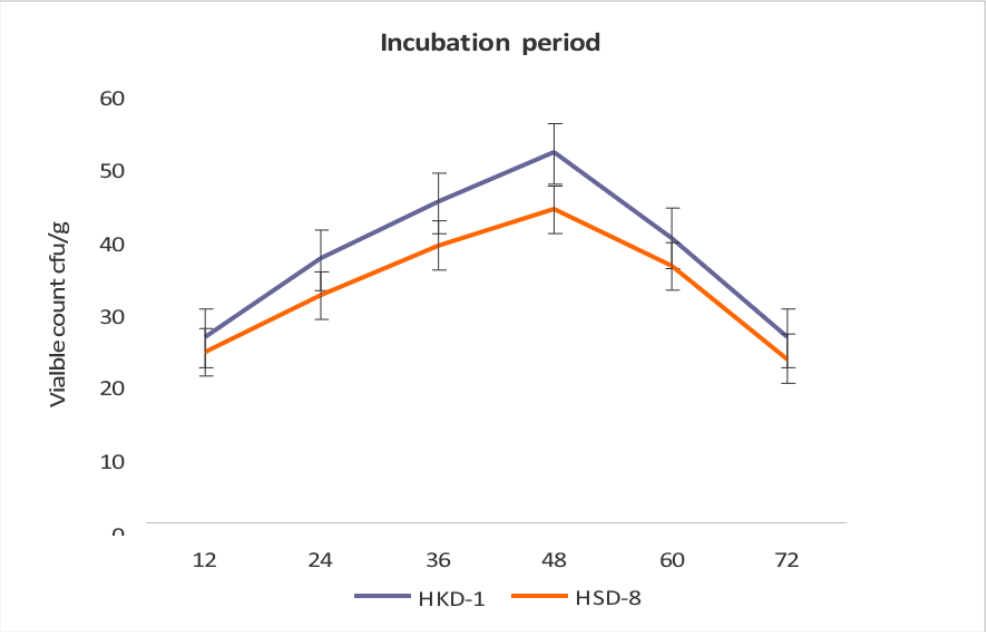


Fig. 4. Effect of different incubation period on growth on the bacterial isolates

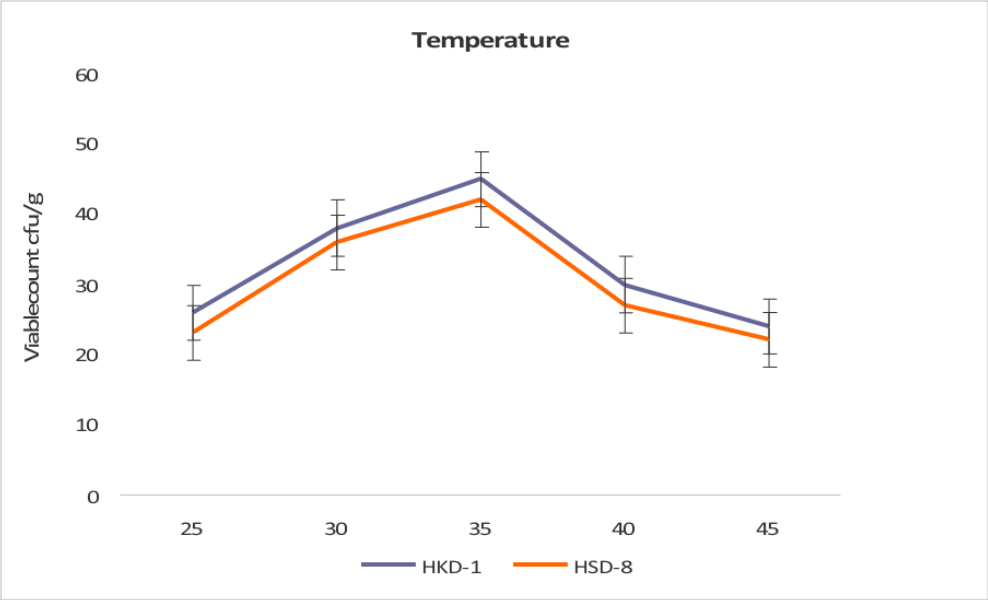


Fig. 5. Effect of temperature on growth of selected bacterial isolates

Table 9. Effect of different pH on growth of selected bacterial isolates

pH			Viable count (× 10^6 cfu/ml)	
	HKD-1	HSD-8	HKD-1	HSD-8
5.0	**+**	**+**	29	27
6.0	**++**	**++**	39	38
7.0	**+++**	**+++**	48	45
8.0	**++**	**++**	30	27
9.0	**+**	**+**	25	22
CD (0.05)			**0.24**	**0.34**

Highest efficiency= (+++), Moderate efficiency = (++), Low efficiency = (+), No efficiency= (-)

The findings of current study are similar with the study conducted by Dinesh et al. (2015) reported that *Bacillus* and *Pseudomonas spp.* thrive at pH 7.0. Dhiman et al. (2019) observed that the isolated bacteria could grow in the pH range of 4.0 to 9.0, however the greatest results were achieved at pH 7.0.

3.8 Molecular Identification of Selected Bacterial Isolates

The most efficient plant growth promoting two bacterial isolates viz., HKD1 and HSD8 were selected for molecular identification based on 16S rDNA sequencing. These bacterial isolates exhibited all multifarious plant growth promoting traits. The DNA was extracted by conventional method Sambrook et al. (1989) and purified DNA was amplified using universal bacterial primers. The amplicon of expected size i.e. (1400 bp) was obtained. The PCR product was purified using PCR extraction Kit and was further sequenced (Biokart India Pvt. Ltd.) (Fig. 6).

"The sequence data of the 16S rDNA of selected isolates were subjected to BLAST analysis. As 16S rDNA sequence provide accurate grouping of organism even at subspecies level it is considered as a powerful tool for the rapid identification of bacterial species"

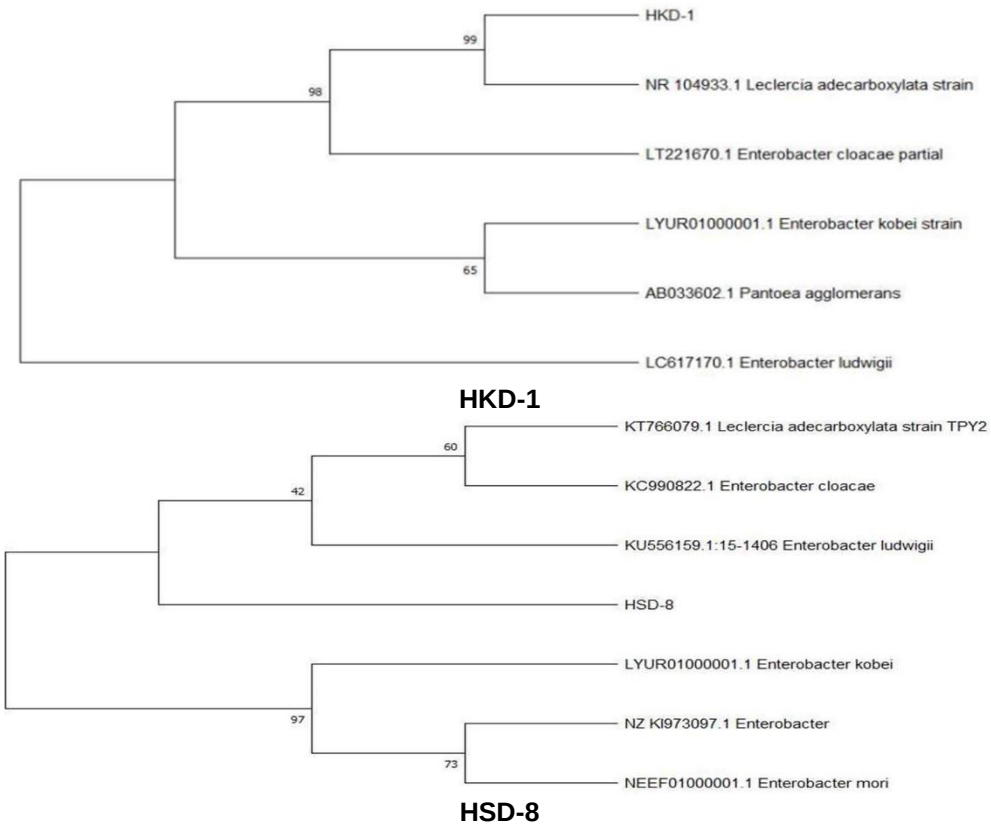


Fig. 6. Neighbour joining tree based on 16S rDNA sequences showing the phylogenetic relationship of bacterial isolates HKD-1 and HSD-8

(Jill and Clarridge, 2004). The sequence analysis of 16S rDNA revealed that strain (PGPR1) (Accession No. PP195922) showed maximum similarity of 98.16 per cent with *Leclercia adecarboxylata* and strain PGPR2 (Accession No. PP195925) showed maximum similarity of 98.73 per cent with *Enterobacter ludwigii*. The phylogenetic analysis of 16S rDNA sequence of the isolates along with the sequence retrieved from the NCBI was carried out with MEGA X using the neighbor joining method with 1,000 bootstrap replicates.

4. CONCLUSION

The studies were conducted to select best plant growth promoting rhizobacteria from rhizosphere of *Grewia optiva.* The bacterial count of soil collected from rhizosphere of *Grewia optiva* from six different sites of Himachal Pradesh ranged between 3.0 to 4.3×10^6 cfu/g of soil. The maximum (4.3×10^6 cfu/g) bacterial count was recorded from site Dharamshala of district Kangra. A total of 99 bacterial isolates were obtained from *Grewia* rhizospheres of six different locations. Bacterial isolate HKD-1 from Himachal Pradesh districts Kangra produced a significantly higher amount of IAA (68.76 µg/ml), showed maximum growth inhibition against *Fusarium oxysporum* (75.20 per cent), and *Rhizoctonia soloni* (61.40 per cent). On the basis of exhibiting maximum PGPR traits viz., P-solubilization, siderophore production, IAA production, N- fixing activity and HCN production, two best isolates, i.e., HKD-1and HSD-8 were selected for further study. Based on morphological, biochemical and molecular investigation of two bacterial isolates, HKD-1 (PGPR-1) was identified as *Leclercia adecarboxylata* (Accession No. PP195922) and HSD-8 (PGPR-2) was identified as *Enterobacter ludwigii* (Accession No. PP195925).

By focusing on key plant growth-promoting traits and validating their efficacy through well-designed experiments, researchers can develop sustainable bioformulations that not only enhance plant development but also reduce reliance on chemical fertilizers. Continued research in this area promises to contribute significantly to sustainable agriculture and environmental conservation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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