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Integrated screening and bioformulation of plant growth–promoting bacteria for sustainable Agriculture

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Abstract Plant growth-promoting microbes are isolated and screened for a total of ten representative isolates as the potential for ammonia, acetoin siderophore production, nitrogen fixation, and indole-3-acetic acid synthesis and phosphate solubilization. These isolates are identified as *Streptomyces albid*o flavus KA18, *Streptomyces spectabilis* NBB36, *Streptomyces nigra* SAN20, *Streptomyces violaceus* SAN35, *Brevibacillus borstelensis* VERB6, *Streptomyces rochei* VCIB11, *Bacillus licheniformis* VCIB2, *Paracoccus sanguinis* VCOB5, *Brevundimonas subvibrioides* VCOB6, and *Neotabrizicola shimadae* VCOB8. All tested isolates exhibited the multiple actions which found to be strongly enhanced available plant nutrients for the growth of plants. These strains are found to be tolerance to potassium dichromate up to 1000 ppm which applicability under the contaminated soil conditions and abiotic stress. *S. rochei* VCIB11, *S. spectabilis* NBB36, *S. violaceus* SAN35, *S. albidoflavus* KA18 expressed antimicrobial activity against plant pathogen (*Ralstonia solanacearum*). Moreover, all selected strains are prove to be synergistic interaction with no antagonistic effects. It is supported their suitability for consortium-based formulations. The highlighted result is proved for the multifunctional potential of these strains to be an eco-friendly biofertilizer and bio-bacteriocide to decrease the toxic chemical contaminants in soil and water incorporated for decreasing heavy metals to revitalize soil and bioremediation.

Keywords: Sustainable agriculture, Plant growth-promoting bacteria, Actinobacteria, Biofertilizer, Biocontrol, Bacterial consortium

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Introduction

Agriculture for Indian economy is recommended to continue a role of national development. India is possed 2.3% of the world's land and 4.2% of global freshwater resources which can be supported about 17% of the world population (Chand *et al*., 2022). The major crops as wheat, rice, sugarcane, cotton, tea, vegetables and fruits are leading producer which the agricultural sector is provided livelihoods over 60% of the population while contributing about 17% to the national GDP. Agriculture in India is faced the challenges of climate changes, soil health declining, scarcity of water leading to low productivity with invading with insect pests and phytopathogens. The estimated yield in India become 30–50% in 2022 which lower than many emerging economies. The insect pests and phytopathogens are affected the quantity and quality crop yield which they had estimated approximately 20–30% of crop losses from Indian agricultural research institutions and contributed to annual economic losses (Shukla *et al*., 2022). The critical limitation are particularly faced of global food security. Food and Agriculture Organization (FAO) and allied United Nations agencies indicate that reducing hunger, hundreds of millions population will be still faced food insecurity by 2030 which the urgent need to improve agricultural productivity for sustainable agriculture.

The extensive application of chemical fertilizers and pesticides has proved for short-term to improve the yield which degradation the environment and agroecosystem into decrease soil fertility and adverse effects on human health. Sustainable Development Goal 2 (Zero Hunger) would be achieved increasing the environmentally protection for sustainable agriculture which include the acceptability of biofertilizers, biopesticides, and other microbial-based scientific investigation to revitalize agricultural soil and enhance crop productivity (Kunhachan *et al*., 2024; Boubekri *et al*., 2022a). With this, the research findings on plant growth-promoting bacteria (PGPB) including actinobacteria are reported by several scientists to improve available plant nutrients, releasing phytohormones, eradicating phyto pathogens, and increase tolerance stress in crop production.

Actinobacteria, in particular, are recognized as prolific producers of bioactive secondary metabolites and are estimated to contribute nearly 45% of known microbial bioactive compounds (Barka *et al*., 2016). These characteristics make them promising candidates for sustainable agriculture applications, including biofertilizer and biocontrol formulations. The present study aimed to isolate and characterize bacterial strains from forest and insect-associated environments and to evaluate their plant growth-promoting traits, heavy metal tolerance, antimicrobial activity, and synergistic interactions. The objective was to identify compatible bacterial consortia with potential application as eco-friendly bioinoculants for sustainable agriculture.

Materials and methods

Collection of samples

Samples were collected from multiple locations in Kanyakumari and Kanchipuram districts of Tamil Nadu, India. Forest sediment samples were obtained from Vallichunai 8.26° N, 77.36° E and Vellimalai 8.1436° N, 77.1381° E. In addition, insect-associated materials, including insect nest casts, were collected from Kanchipuram 12° 50' 3.0264" N and 79° 42' 13.1184" E. The collected samples were dried and powdery in appearance. All samples were air-dried in sterile petri dish at room temperature for 24 h and pre-treated by heating at 60°C for 10 min to reduce the growth of fast-growing, non-target microorganisms (Radhakrishnan *et al*., 2007).

Isolation and screening of bacteria for PGP traits

Bacterial isolation was carried out using the spread plate method on Starch Casein Agar (SCA), Nutrient Agar (NA), and Actinomycetes Isolation Agar (AIA). Five grams of pre-treated sample were suspended in 50 mL of sterile distilled water and agitated for 30 min. A tenfold serial dilution (10^{-1} to 10^{-6}) was prepared, and 100 µL from dilutions 10^{-3} to 10^{-5} were spread onto agar plates. Plates were incubated at 37°C for five days, and morphologically distinct colonies were selected and purified (Vasconcellos *et al*., 2010).

Qualitative assessment of phosphate solubilization potential

Phosphate solubilization was assessed on Pikovskaya's agar medium. Bacterial isolates were spot-inoculated and incubated at 37°C for 5–7 days. The formation of clear halo zones around colonies was considered indicative of phosphate solubilization ability (Netsawang *et al*., 2023).

Qualitative detection of siderophore biosynthesis

Siderophore production was evaluated using Chrome Azurol S (CAS) agar medium. Isolates were spot-inoculated and incubated at 37°C for 5–7 days. The development of yellow or orange halos around colonies indicated positive siderophore production (Schwyn and Neilands, 1987; *)*.

Qualitative detection of nitrogen-free medium

Nitrogen-fixing ability was assessed using Jensen's nitrogen-free agar medium. Growth of isolates on this medium after incubation at 37°C for 5–7 days indicated the potential for atmospheric nitrogen fixation (Boddey and

Knowles 1987). Ammonia production was evaluated by inoculating isolates into peptone broth and incubating at 37°C for 5–7 days. Following incubation, Nessler's reagent was added, and the development of yellow to brown coloration was recorded as a positive result.

Qualitative detection of acetoin production

Acetoin production was assessed using Voges–Proskauer (VP) broth. After incubation, Barritt's reagents A and B were added. The development of pink to red coloration indicated positive acetoin production (Monteiro *et al*., 2020).

Qualitative detection of indole-3-acetic acid synthesis

IAA production was assessed in Luria–Bertani broth supplemented with 0.2% L-tryptophan. After incubation, cultures were centrifuged and the supernatant was mixed with Salkowski's reagent. The appearance of pink to red coloration indicated IAA production (Etesami *et al*., 2015a).

Heavy metal tolerance assay

Heavy metal tolerance was evaluated using ISP2 medium supplemented with potassium dichromate ($K_2Cr_2O_7$) at concentrations of 50, 100, 250, 500, 750, and 1000 ppm. Isolates were incubated at 37°C for 5–7 days, and growth at higher concentrations was considered indicative of heavy metal tolerance (Muniswamy *et al*., 2016).

In vitro antagonism assay against Ralstonia solanacearum

Antimicrobial activity was evaluated against the plant pathogen *Ralstonia solanacearum* by using the agar plug and agar diffusion methods on Mueller–Hinton agar. Zones of inhibition were measured after 24 h of incubation at 37°C (Ortlieb *et al*., 2021).

Evaluation of interstrain synergy and consortium compatibility

Synergistic interactions among ten selected strains (VCIB11, VCIB2, VCOB5, VCOB6, VCOB8, NBB36, KA18, SAN20, SAN35, and VERB10) were evaluated by co-spotting on nutrient agar plates and observing enhanced growth or compatibility. For consortium evaluation, equal volumes of each strain were mixed, incubated with shaking, and plated to assess collective growth behavior (Bhattacharyya and Jha, 2012).

Effect of microbial combination on growth of lettuce

Preliminary test for plant growth was with five replications and treatments were as follows:- T1 was non-treated control and T2 was treated with biosimulant (strain combination of *Streptomyces albid*oflavus KA18, *Streptomyces spectabilis* NBB36, *Streptomyces nigra* SAN20, *Streptomyces violaceus* SAN35, *Brevibacillus borstelensis* VERB6, *Streptomyces rochei* VCIB11, *Bacillus licheniformis* VCIB2, *Paracoccus sanguinis* VCOB5, *Brevundimonas subvibrioides* VCOB6, and *Neotabrizicola shimadae* VCOB8) at 5 ml per 1 liter of water every 7 days.

Results

Isolation and screening of bacteria for PGP Traits

A diverse range of bacterial colonies exhibiting distinct pigmentation, colony texture, margin characteristics, and morphological variations were obtained from forest- -associated samples during the isolation process (Figure 1). The actinobacterial strains were screened on ISP2 medium that proved purification and effective revitalization (Figure 2). The serial dilutions were done using the 10^{-3} dilution proved to be higher colony diversity on Starch Casein Agar which indicated the medium was appropriated to isolate and culture actinobacteria. With this,158 bacterial cultures were found from sampling locations in different sources and bacterial colonies were discovered from varied sampling locations with Vallai Chuni stream inside (VCIB) to distinguish 26 morphologically colonies. Velli Malai rock bacterial sample (VERB) is comprised 25 colonies**,** Nest bee cast bacteria (NBB) found to be the highest colony count of 35 colonies**,** Vallai Chuni stream outside (VCOB) recorded 26 colonies, Sathyabama ant cast (SAN) accounted for 26 colonies, and Kancheepuram ant cast (KA) showed 20 colonies. The comparison showed the higher number of isolates with NBB that revealed a greater bacterial abundance in ecological heterogeneity from sampling site, whereas the other sites were demonstrated substantial bacterial richness which showed the biological diversity in forest associated with bacterial microbial communities.

Pure cultures of 158 strains were identified and characterized colony morphology, pigmentation, gram reaction, cell morphology, and spore-forming ability. All strains were screened for multiple plant growth-promoting (PGP) for siderophore, nitrogen fixation, indole-3-acetic acid (IAA), ammonia, phosphate solubilization and pesticide degradation. Results found that ten isolates are exhibited superior PGP performance which a good efficiency of biodegradation, then proved species level by molecular phylogenic identification. These isolates are proved to be VCIB11 (*Streptomyces rochei*), VCIB2 (*Bacillus licheniformis*), VCOB5 (*Paracoccus*

sanguinis), VCOB6 (*Brevundimonas subvibrioides*), VCOB8 (*Neotabrizicola shimadae*), NBB36 (*Streptomyces spectabilis*), KA18 (*Streptomyces albidoflavus*), SAN20 (*Streptomyces nigra*), SAN35 (*Streptomyces violaceus*), and VERB6 (*Brevibacillus borstelensis*). Morphological and molecular phylogenic identification of these strains are shown in Table 1. Molecular phylogeny was performed by sequencing 16S rRNA gene for taxonomic confirmation through phylogenetic analysis using the Neighbor-Joining method with 1,000 bootstrap replications to construct the phylogenetic trees (Figure 3A–J). It showed that the strains are closely clustered with respective type or reference strains which retrieved from the NCBI GenBank database to confirm taxonomic identities.

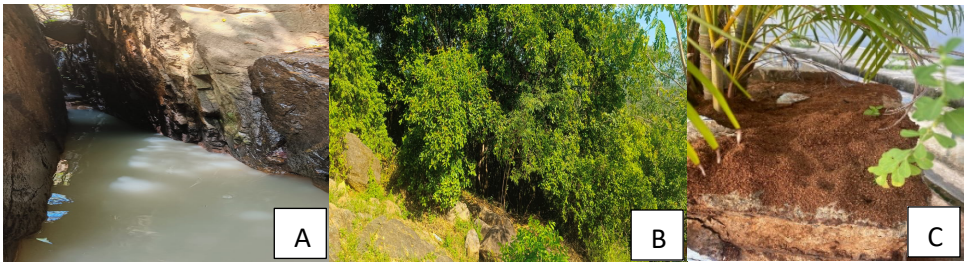

Figure 1. Sampling site showing Forest sediment soil collected from A. Vallichunai, B. Vellaimalai forest regions and C. Ant cast for bacterial isolation

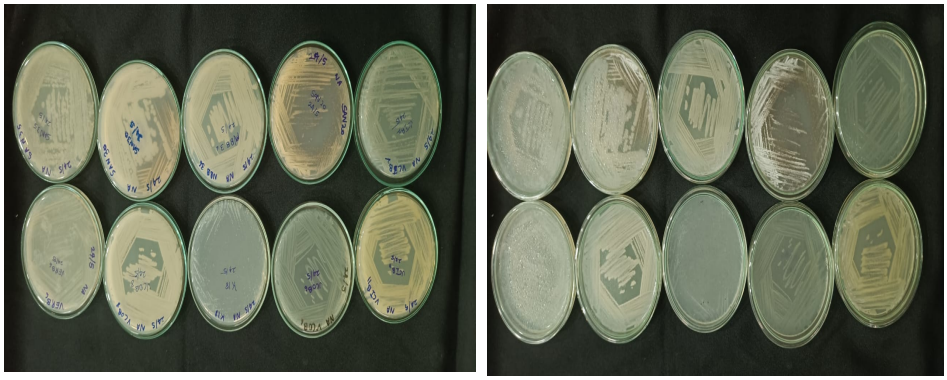

Figure 2. Pure culture isolation of bacterial strains VCIB11, VCIB2, VCOB5, VCOB6, VCOB8, NBB36, KA18, SAN20, SAN35, and VERB10 on ISP2 agar medium following incubation at 28–30 °C for 48–72 h

Assessment of siderophore, nitrogen fixation, ammonia and IAA production and phosphate solubilization

*Streptomyces albid*oflavus KA18, *S. spectabilis* NBB36, *S. nigra* SAN20, *S. violaceus* SAN35, *B. borstelensis* VERB6, *S. rochei* VCIB11, *B. licheniformis* VCIB2, *P. sanguinis* VCOB5, *B. subvibrioides* VCOB6, and

N. shimadae VCOB8 were expressed the multiple plant growth-promoting functions of siderophore production, Indole-3-acetic acid synthesis, nitrogen fixation, phosphate solubilization, ammonia and acetoin production. Siderophore production and phosphate solubilization are presented in Table 2. It proved that which indicated that *S. spectabilis* NBB36 and *S. nigra* SAN20 expressed a strong activity more than *S. rochei* VCIB11, *B. licheniformis* VCIB2, *P. sanguinis* VCOB5, *B. subvibrioides* VCOB6, *N. shimadae* VCOB8, *S. albid*oflavus KA18, *S. violaceus* SAN35, and *B. borstelensis* VERB6. Nitrogen fixation ability found those strains can be expressed higher under nitrogen-free conditions (Table 3). Synthesis of Indole-3-acetic acid (IAA) expressed positive rection of auxin production in all bacterial strains (Table 4). The multiple PGP of all tested bacterial strains are proved to be simultaneous expression for a broad functional activities. Colorimetric biochemical changes were resulted for functional assays that confirmed active metabolic responses (Figure 5).

Heavy metal tolerance assay

Streptomyces albidoflavus KA18, *S. spectabilis* NBB36, *S. nigra* SAN20, *S. violaceus* SAN35, *B. borstelensis* VERB6, *S. rochei* VCIB11, *B. licheniformis* VCIB2, *P. sanguinis* VCOB5, *B. subvibrioides* VCOB6, and *N. shimadae* VCOB8 were tested by increasing concentrations of potassium dichromate. The tolerance of heavy metal revealed that all strains are tolerated to chromium at concentrations ranged from 50 to 1000 ppm. With this, *S. spectabilis* NBB36 and *S. violaceus* SAN35 expressed better growth intensity to all tested concentrations (Table 5).

In vitro antagonism assay against Ralstonia solanacearum

S. rochei VCIB11, *S. spectabilis* NBB36, *S. violaceus* SAN35, and *S. albidoflavus* KA18 found to be significantly inhibited *Ralstonia solanacearum* causing bacterial wilt (Table 6). Among them, *S. rochei* VCIB11 and *S. albidoflavus* KA18 were higher inhibited the bacterial wilt than the other strains.

Evaluation of Interstrain synergistic and consortium compatibility

Synergistic interaction expressed that *Streptomyces albidoflavus* KA18, *S. spectabilis* NBB36, *S. nigra* SAN20, *S. violaceus* SAN35, *B. borstelensis* VERB6, *S. rochei* VCIB11, *B. licheniformis* VCIB2, *P. sanguinis* VCOB5, *B. subvibrioides* VCOB6, and *N. shimadae* VCOB8 expressed a strong synergistic reaction with no signs of antagonistic effects (Figure 6).

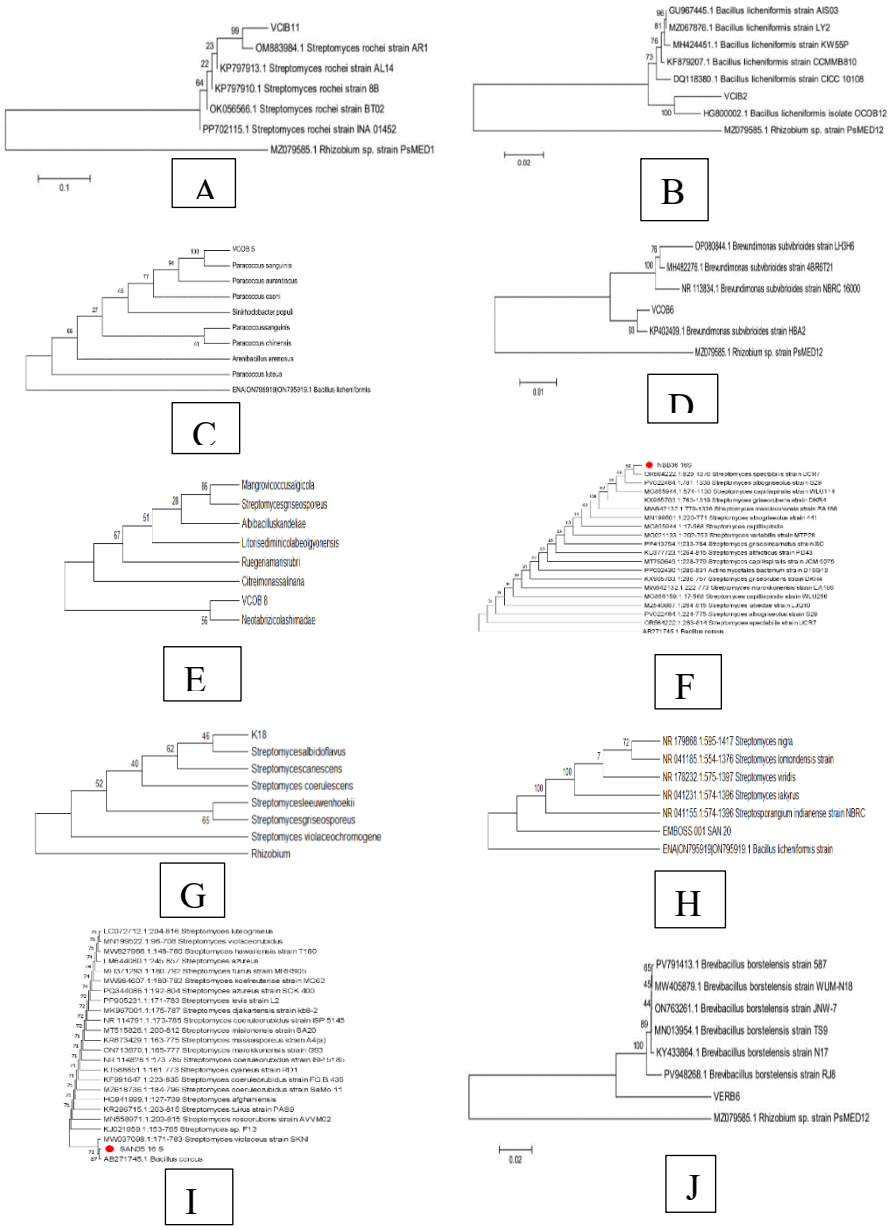

Figure 3. Phylogenetic analysis of the ten selected bacterial isolates based on 16S rRNA gene sequences: The phylogenetic trees were constructed using the **Neighbor-Joining (NJ)** method in **MEGA X** with **1,000 bootstrap replicates**. Bootstrap values are indicated at the branch nodes, and the scale bar represents the number of nucleotide substitutions per site. The phylogenetic trees confirm the taxonomic identities of the selected isolates by clustering them with their closest reference strains retrieved from the NCBI GenBank database. **(A)** VCIB11 (*Streptomyces rochei*), **(B)** VCIB2 (*Bacillus licheniformis*), **(C)** VCOB5 (*Paracoccus sanguinis*), **(D)** VCOB6 (*Brevundimonas subvibrioides*), **(E)** VCOB8 (*Neotabrizicola shimadae*), **(F)** NBB36 (*Streptomyces spectabilis*), **(G)** KA18 *(Streptomyces albidoflavus*), **(H)** SAN20 (*Streptomyces nigra*), **(I)** SAN35 (*Streptomyces violaceus*), and **(J)** VERB6 (*Brevibacillus borstelensis*)

Table 1. Morphological characteristics, pigmentation, spore-forming ability, and molecular identification of the ten bacterial isolates selected based on preliminary screening for plant growth-promoting (PGP) traits and pesticide biodegradation potential

S. No	Isolate	Identified species	Colony morphology	Pigmentation	Spore formation
1	VCIB11	*Streptomyces rochei*	Dry, powdery, irregular colony with aerial mycelium	grey	Aerial spores present
2	VCIB2	*Bacillus licheniformis*	Large, smooth, circular colony	Cream	Endospore forming
3	VCOB5	*Paracoccus sanguinis*	Smooth, circular, convex colony	pale yellow	Absent
4	VCOB6	*Brevundimonas subvibrioides*	Smooth, circular colony	Cream	Absent
5	VCOB8	*Neotabrizicola shimadae*	Smooth, circular colony with entire margin	Pale yellow	Absent
6	NBB36	*Streptomyces spectabilis*	Dry, powdery, irregular colony with aerial mycelium	Grey	Aerial spores present
7	KA18	*Streptomyces albidoflavus*	Dry, powdery colony	Grey	Aerial spores present
8	SAN20	*Streptomyces nigra*	Dry, rough colony with aerial mycelium	Greyish white	Aerial spores present
9	SAN35	*Streptomyces violaceus*	Dry, chalky colony	Grey	Aerial spores present
10	VERB6	*Brevibacillus borstelensis*	Large, opaque, irregular colony	Orange	Endospore forming

Table 2. Siderophore production was assessed using Chrome Azurol S (CAS) agar medium

S. No.	Strain name	Siderophore production	Phosphate Solubilization
1	VCIB11	++	++
2	VCIB2	+	+
3	VCOB5	+	+
4	VCOB6	++	++
5	VCOB8	+	+
6	NBB36	+++	+++
7	KA18	+	+
8	SAN20	+	+
9	SAN35	+++	+++
10	VERB6	+	+

Activity was recorded based on the intensity of halo zone formation surrounding bacterial colonies Growth response intensity is represented using the following symbols: **+++ (very strong response),** ++ **(strong response)**, + **(moderate response),** and – **(no observable response)**

Table 3. Nitrogen fixation ability of selected bacterial strains Nitrogen fixation was evaluated on Jensen's nitrogen-free medium

S. No.	Strain name	Nitrogen fixation
1	VCIB11	++
2	VCIB2	+
3	VCOB5	+
4	VCOB6	++
5	VCOB8	+
6	NBB36	+++
7	KA18	+
8	SAN20	+
9	SAN35	+++
10	VERB6	+

Growth intensity was used as an indicator of nitrogen-fixing potential Growth response intensity is represented using the following symbols: +++ **(very strong response)**, ++ **(strong response)**, + **(moderate response)**, and – **(no observable response).**

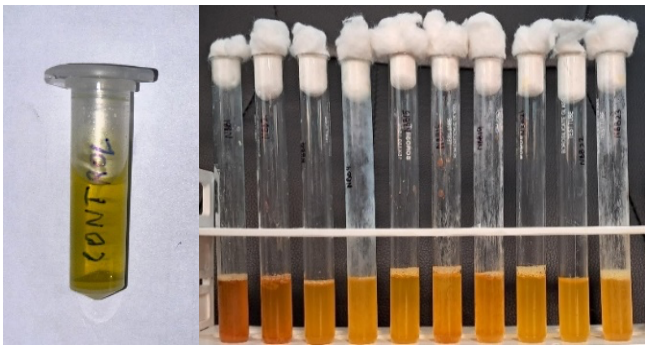

Figure 4. Pigment production by selected bacterial isolates showing the emergence of subtle yellow to deep brown coloration on ISP2 agar plates after incubation at 28–30 °C for 72h

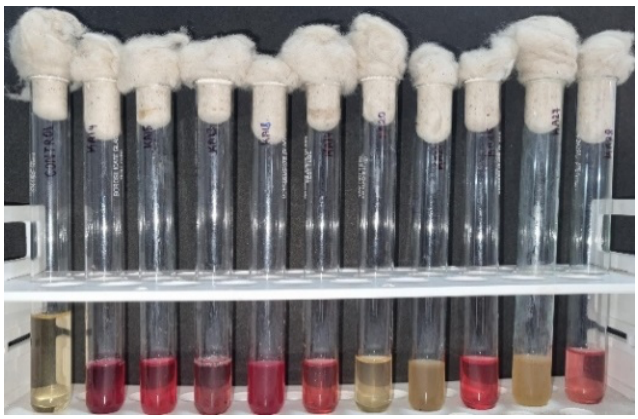

Figure 5. Colorimetric changes observed in bacterial cultures indicating a transition of hue from yellow to pink or red, suggesting metabolic and biochemical activity during incubation at 30 ± 2 °C for 48–72 h

Table 4. Indole-3-acetic acid (IAA) production by selected bacterial strains

S. No.	Strain name	IAA production
1	VCIB11	+
2	VCIB2	+
3	VCOB5	+
4	VCOB6	+
5	VCOB8	+
6	NBB36	+
7	KA18	+
8	SAN20	+
9	SAN35	+
10	VERB6	+

IAA production was determined using Salkowski's reagent following growth in L-tryptophan-supplemented medium. Colour intensity was used as a qualitative indicator of IAA synthesis Growth response intensity is represented using the following symbols: **+++ (very strong response), ++ (strong response), + (moderate response), and – (no observable response).**

Table 5. Heavy metal tolerance of selected bacterial strains

S. No.	Strain name	Heavy metal tolerance
1	VCIB11	++
2	VCIB2	+
3	VCOB5	+
4	VCOB6	++
5	VCOB8	+
6	NBB36	+++
7	KA18	+
8	SAN20	+
9	SAN35	+++
10	VERB6	+

Heavy metal tolerance was evaluated on ISP2 medium supplemented with potassium dichromate ($K_2Cr_2O_7$). Growth intensity reflects tolerance level at increasing concentrations (50–1000 ppm) Growth response intensity is represented using the following symbols: **+++ (very strong response), ++ (strong response), + (moderate response), and – (no observable response)**

Table 6. Antimicrobial activity of selected bacterial strains against *Ralstonia solanacearum*

S. No.	Strain name	Antimicrobial activity
1	VCIB11	++
2	VCIB2	-
3	VCOB5	-
4	VCOB6	-
5	VCOB8	-
6	NBB36	+
7	KA18	++
8	SAN20	-
9	SAN35	+
10	VERB6	-

Antibacterial activity was evaluated using the agar diffusion method. Antagonistic potential was recorded based on zone of inhibition intensity Growth response intensity is represented using the following symbols: **+++ (very strong response), ++ (strong response), + (moderate response), and – (no observable response)**

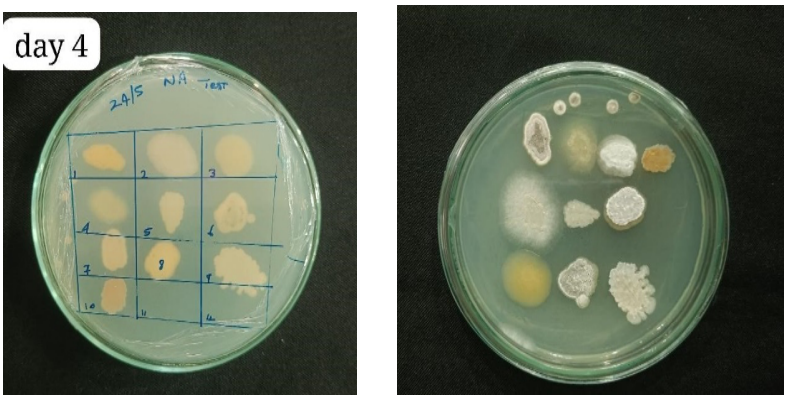

Figure 6. Compatibility (spotting) assay showing the absence of antagonistic or inhibitory interactions among the selected bacterial strains on Nutrient Agar plates after incubation at 30 ± 2 °C for 4 days

Effect of bacterial combination on plant growth

The bacterial combination of *Streptomyces albidoflavus* KA18, *S. spectabilis* NBB36, *S. nigra* SAN20, *S. violaceus* SAN35, *B. borstelensis* VERB6, *S. rochei* VCIB11, *B. licheniformis* VCIB2, *P. sanguinis* VCOB5, *B. subvibrioides* VCOB6, and *N. shimadae* VCOB8 are formulated as biostimulant which evaluated for the growth of lettuce **(***Lactuca sativa***)** in pot experiment. T1 was nontreated control and T2 was treated with biostimulant with the sterilized mixed loamy soil. The results showed the plant height, and fresh weight in biostimulant treatment increased greater than growth the non-treated control.

Discussion

The recovery of 158 microbial cultures from forest- and insect-associated samples are confirmed that these habitats are rich sources of metabolically diverse bacteria with potential agricultural importance. Variation in colony morphology and pigmentation indicated broad physiological diversity, while Nest bee cast bacteria (NBB) recorded the highest number of isolates, suggesting that insect-associated substrates provide nutrient-rich microenvironments favorable for microbial abundance. Similar higher microbial recovery from biologically active substrates such as earthworm casts has been reported previously, where organic-rich habitats supported increased microbial diversity and activity (Kumar *et al*., 2012). Qualitative screening further showed that selected isolates possessed multiple plant growth-promoting traits, with NBB36 and SAN35 exhibiting stronger phosphate solubilization and siderophore production than VCIB11, VCIB2,

VCOB5, VCOB6, VCOB8, KA18, SAN20, and VERB6. These strains also showed stronger growth under nitrogen-free conditions, while all isolates produced indole-3-acetic acid, indicating broad nutrient mobilization and plant growth-promoting potential, which agrees with earlier reports describing multifunctional plant growth-promoting bacteria in sustainable agriculture (Etesami *et al*., 2015b; Glick, 2012).

All selected strains tolerated chromium concentrations up to 1000 ppm, with NBB36 and SAN35 showing comparatively stronger growth, indicating enhanced heavy metal tolerance. Similar stress adaptation has been reported in environmentally resilient actinobacteria capable of surviving under adverse conditions and contributing to stress mitigation (Barka *et al*., 2016). Antagonistic activity against *Ralstonia solanacearum* was observed in VCIB11, NBB36, SAN35, and KA18, with VCIB11 and KA18 showing stronger inhibition, supporting previous reports that beneficial bacteria suppress phytopathogens through antimicrobial metabolite production (Compant *et al*., 2005). In addition, all strains were compatible under consortium testing, and consortium-treated plants showed greater growth than controls, reaching 9.70 ± 0.67 cm by Day 20 compared with 6.00 ± 0.47 cm, supporting the effectiveness of compatible microbial consortia for sustainable crop improvement (Boubekri *et al*., 2022 b).

It is concluded that *Streptomyces albid*oflavus KA18, *Streptomyces spectabilis* NBB36, *Streptomyces nigra* SAN20, *Streptomyces violaceus* SAN35, *Brevibacillus borstelensis* VERB6, *Streptomyces rochei* VCIB11, *Bacillus licheniformis* VCIB2, *Paracoccus sanguinis* VCOB5, *Brevundimonas subvibrioides* VCOB6, and *Neotabrizicola shimadae* VCOB8 are promising potential to develop as multifunction bio-stimulant to improve soil and promote plant growth.

Conflicts of interest

The authors declare no conflict of interest.

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