

Characterization of Indigenous *Bacillus thuringiensis* Strains from the Western Ghats of Karnataka

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Received : July 2025

Accepted : July 2025

ABSTRACT

The Western Ghats of Karnataka, a globally recognized biodiversity hotspot, harbour a rich diversity of soil microorganisms with potential agricultural applications. However, systematic exploration of native *Bacillus thuringiensis* strains from this region remains limited, particularly those exhibiting both insecticidal and plant growth-promoting traits. In the present study, five indigenous *B. thuringiensis* strains were isolated from soil samples collected at ten ecologically distinct locations across the Western Ghats. Initial screening based on colony morphology, Gram staining and parasporal crystal formation confirmed the presence of *B. thuringiensis* isolates. Molecular identification using 16S rRNA gene sequencing revealed over 99 per cent similarity with reference *B. thuringiensis* strains and phylogenetic analysis placed them within the *B. thuringiensis* lineage. The strains were further assessed for their enzyme production and plant growth-promoting potential. All the five strains demonstrated protease activity, with variable production of lipase, lecithinase and chitinase, indicating potential entomopathogenic capabilities. Plant growth-promoting traits, including indole-3-acetic acid production, ammonia production and siderophore synthesis, were detected in most of the strains. Notably, strain NBAIR Bt191 exhibited all four enzymatic and three PGPR traits, indicating high multifunctional ability. These findings highlight the functional potential of native *B. thuringiensis* strains from the Western Ghats for dual application in pest control and sustainable crop production. The study emphasizes the importance of bioprospecting unexplored ecosystems for microbial resources and identifies strain NBAIR Bt191 as a promising candidate for future *in vivo* validation and bio formulation development to support nutrient and pest management systems in agriculture.

Keywords : Biocontrol, Chitinase, Lipase, Protease, Plant growth promotion, Western Ghats

BIOLOGICAL control forms an integral part of Integrated Pest Management (IPM), offering eco-friendly alternatives to chemical pesticides through the use of beneficial organisms such as parasitoids, predators and microbial agents (Rani *et al.*, 2021). Among microbial biopesticides, *Bacillus thuringiensis* stands out as the most extensively utilized

and successful bioagent for insect pest control (Verma *et al.*, 2024). *Bacillus thuringiensis* is a Gram-positive, rod-shaped, aerobic, spore-forming soil bacterium that produces proteinaceous insecticidal crystalline inclusions during the stationary phase of growth, commonly referred to as Cry and Cyt toxins or δ -endotoxins (Domínguez-Arrizabalaga *et al.*, 2020 and Venu *et al.*, 2024).

These crystal proteins, encoded by various *cry* and *cyt* genes, display a broad range of toxicities and morphologies, including bipyramidal, cuboidal, amorphous and spherical forms, which are associated with specific insect orders such as Lepidoptera, Coleoptera, and Diptera (Mukhija & Khanna, 2018 and Khorramnejad *et al.*, 2020). The mode of action involves toxin activation by midgut proteases and binding to specific receptors in the insect midgut epithelium, leading to pore formation, osmotic lysis, and death of the insect (Prabhulliger & Narasimha, 2017 and Manjunatha *et al.*, 2023). Due to their high specificity and safety, *B. thuringiensis* toxins do not affect non-target organisms, including humans, animals and beneficial insects (Wu *et al.*, 2021).

The ecological distribution of *B. thuringiensis* is extensive. It is considered as a ubiquitous microorganism found in soil, phylloplane, compost, aquatic habitats, insect cadavers, grain storage sites and herbivore feces (Garcia-Suarez *et al.*, 2017). Through the ability to form resistant spores, *B. thuringiensis* can survive harsh environmental conditions and persist in diverse ecological niches. Its ease of cultivation, target specificity and environmental safety have made *B. thuringiensis*, the keystone of many commercial biopesticide formulations globally (Ragasruthi *et al.*, 2024).

In addition to its entomopathogenic properties, several *B. thuringiensis* strains have been recognized for their plant growth-promoting attributes, such as the production of phytohormones like indole acetic acid (IAA), ammonia and siderophores, as well as enzymes like protease, chitinase and lipase (Qi *et al.*, 2016 and Azizoglu *et al.*, 2019). These multifunctional traits not only enhance plant health and productivity but also suppress phytopathogens and improve nutrient availability in the rhizosphere, indicating their potential as biofertilizers and biostimulants.

The Western Ghats of Karnataka, a UNESCO-designated biodiversity hotspot, harbours unique microbial diversity owing to its varied altitudes, vegetation types and climatic conditions (Ankitha

et al., 2023). However, systematic exploration of *B. thuringiensis* strains from this region remains limited. The current study builds upon this by focusing on the Western Ghats region, aiming to tap into its unexplored microbial reservoir. The present study aimed to isolate and characterize native *B. thuringiensis* strains from the Western Ghats of Karnataka. The isolates were identified morphologically and molecularly and screened for enzyme production and plant growth-promoting traits. The findings of this study aim to identify multifunctional *B. thuringiensis* strains that can contribute to eco-friendly pest management and sustainable agriculture.

MATERIAL AND METHODS

Sample Collection and Isolation of *Bacillus thuringiensis*

Soil samples were collected from ten ecologically distinct locations within the Western Ghats of Karnataka, covering regions such as Hassan, Kodagu, Chikkamagaluru, Shivamogga, Dakshina Kannada and Udupi districts (Table 1 & Fig. 1). From each site, soil was collected from a 5-10 cm depth using sterile spatulas and stored in sterile containers at 4°C until processing.

Isolation of *B. thuringiensis* was done following a heat-shock and selective enrichment method. Briefly, 1 gram of soil was suspended in 9 mL of sterile distilled water, vigorously mixed and heated at 80°C for 10 minutes to eliminate non-spore-forming and Gram-negative organisms. The suspension was serially diluted and plated on nutrient agar. Plates were incubated at 30°C for 24-48 hours and colonies with typical *Bacillus* morphology (white or cream, flat or raised colonies with a rough surface) were selected for further study. This selective enrichment approach was adapted and modified from the protocol of Travers *et al.* (1987).

Morphological and Microscopic Characterization

The purified bacterial isolates were cultured on Luria Bertani (LB) agar plates and observed for various morphological features, including colony shape, colour, surface texture, elevation and margin. The

TABLE 1
Geographical coordinates of soil samples collected across the Western Ghats of Karnataka for isolation of *Bacillus thuringiensis*

Isolate name	Locations	Geographical coordinates
WGB1	BisleGhat, Hassan	12.7000° N, 75.6500° E
WGB2	Kudremukh, Chikkamagaluru	13.1350° N, 75.2500° E
WGB3	Agumbe, Shivamogga	13.5090° N, 75.0886° E
WGB4	Pushpagiri Wildlife Sanctuary, Kodagu	12.6050° N, 75.6800° E
WGB5	Nagarhole National Park, Kodagu	12.0150° N, 76.2670° E
WGB6	Brahmagiri Wildlife Sanctuary, Kodagu	12.3870° N, 75.4910° E
WGB7	Talacauvery, Kodagu	12.4167° N, 75.5333° E
WGB8	Baba Budangiri, Chikkamagaluru	13.4229° N, 75.7704° E
WGB9	CharmadiGhat, Dakshina Kannada	13.0500° N, 75.4000° E
WGB10	Someshwara Wildlife Sanctuary, Udupi	13.5000° N, 74.8000° E

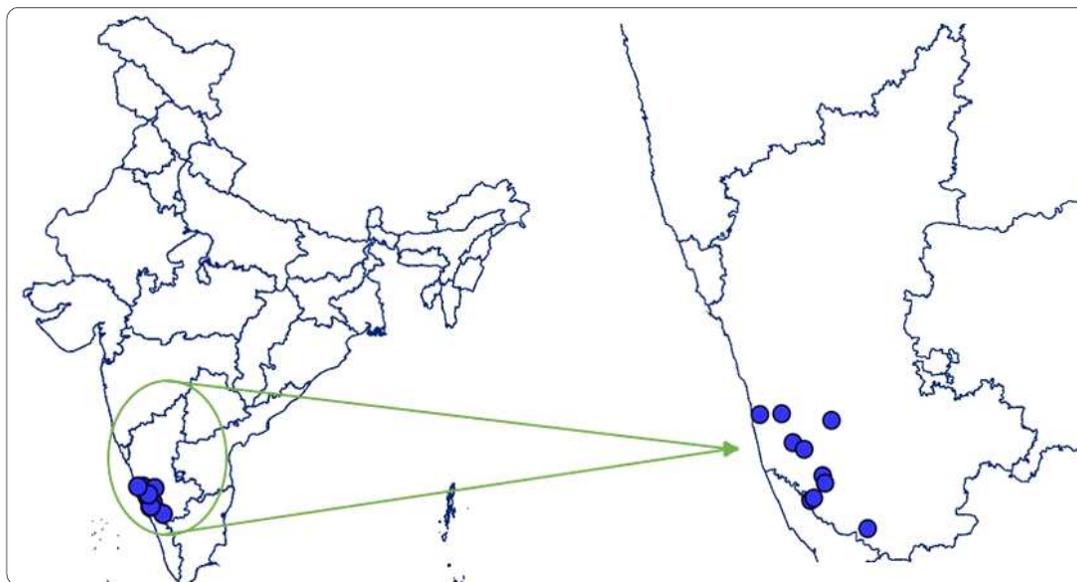


Fig. 1 : Geographical mapping of the different sample collection sites from the Western Ghats of Karnataka

cells from individual colonies were examined under a compound microscope (Olympus BX41, Pennsylvania, USA) for cellular structure, Gram reaction and spore formation following the method outlined by Padole *et al.* (2017). Isolates exhibiting *B. thuringiensis*-like morphology were further cultured in T3 broth (containing tryptone, tryptose, yeast extract, sodium phosphate buffer pH 6.8 and manganese chloride) and incubated at 30/°C for 5-6 days to induce sporulation and crystal formation. To confirm crystal protein production, a 10 μ L aliquot

culture was taken, heat-fixed on a clean glass slide, stained with crystal violet and viewed under 100 \times oil immersion. Isolates showing crystal inclusions were recorded as crystalliferous and evaluated for crystal morphology.

Molecular Characterization and Phylogenetic Analysis

Genomic DNA was isolated from the confirmed *B. thuringiensis* isolates using the DNeasy Blood and Tissue Kit (Qiagen, Germany) as per the

manufacturer's instructions. Amplification of the 16S rRNA gene was carried out using the universal primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' -TACGGYTAC CTTGTTACGA CTT-3') as described by Dos Santos *et al.* (2019). Thermocycling began with an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute (denaturation), annealing at 57°C for 1 minute and extension at 72°C for 90 sec with final extension at 72°C for 10 min (Aarthi *et al.*, 2024). The amplified products were run on a 1.5 per cent agarose gel and visualized under a gel documentation system (Gel Doc Go, Biorad, USA). Sanger sequencing was used to determine the nucleotide sequences, and sequence homology was verified using NCBI's BLASTn tool. The validated sequences were submitted to the NCBI GenBank database to obtain accession numbers. Phylogenetic relationships were constructed using MEGA version 11.0 (Kumar *et al.*, 2018) and evolutionary relationships were analyzed using the neighbor-joining method with 1000 bootstrap replications (Felsenstein, 1985).

Enzymatic Characterization of *Bacillus thuringiensis* Strains

The *B. thuringiensis* strains were screened for their ability to produce extracellular enzymes associated with biocontrol activity. All assays were performed in triplicate using substrate-specific agar plate methods. Protease activity was assessed on skim milk agar; the presence of a clear zone around colonies indicated positive casein degradation (Aneja, 2014). Lipase production was evaluated using tributyrin agar plates. A 10 µL aliquot of 24-hour broth culture was spot-inoculated, and halo formation after 48 hours at 30°C indicated lipid hydrolysis. Lecithinase activity was determined using egg yolk agar plates. An opaque precipitation zone around colonies after 48 hours of incubation was recorded as positive (El-Kersh *et al.*, 2016). Chitinase activity was tested on agar plates containing 4 per cent crude chitin as the sole carbon source. Zones of clearance around colonies indicated chitin degradation (Kuddus and Ahmad, 2013).

Screening for Plant Growth-Promoting (PGP) Traits of *Bacillus thuringiensis* Strains

Bacillus thuringiensis strains were evaluated for key plant growth-promoting attributes through qualitative methods. Indole-3-acetic acid (IAA) production was tested using Salkowski's reagent, where the development of a pink coloration indicated positive IAA synthesis by the strain (Gordon and Weber, 1951). Ammonia production was determined by growing the strains in peptone water, followed by the addition of Nessler's reagent; the appearance of a brown to yellow colour was considered a positive result (Cappuccino and Sherman, 1992). Siderophore production was evaluated on Chrome Azurol S (CAS) agar plates, where the appearance of orange or yellow zones surrounding the colonies indicated siderophore activity (Schwyn and Neilands, 1987).

RESULTS AND DISCUSSION

Isolation and Morphological Identification of *Bacillus thuringiensis* Strains

A total of ten bacterial isolates were obtained from ten soil samples collected across diverse ecological sites in the Western Ghats of Karnataka, through heat treatment and serial dilution plating. Among these, five isolates (WGB2, WGB3, WGB5, WGB6 and WGB8) were selected based on their colony morphology and staining characteristics similar to *B. thuringiensis*. These colonies exhibited typical features such as flat or raised elevation, dry surface texture, irregular to undulate margins and a creamy to white appearance on LB agar (Table 2, Plate 1a). Similar colony traits have been previously reported in *B. thuringiensis* strains derived from agro ecosystems and forest soils by Manoj and Tamilvendan (2025).

Microscopic examination confirmed that all five isolates were Gram-positive, rod-shaped and spore-forming, consistent with the defining traits of the genus *Bacillus* (Plate 1b). Further confirmation of *B. thuringiensis* identity was achieved by crystal staining, which revealed distinct parasporal crystalline inclusions adjacent to the spores in each

TABLE 2
Morphological and microscopic characterization of *Bacillus* sp. and screening for *Bacillus thuringiensis* isolates through crystal staining

Isolate name	Colony shape	Elevation	Colour	Margin	Texture	Gram staining	Endospore staining	Crystal staining
WGB1	Circular	Flat	White	Entire	Rough	+	+	-
WGB2	Circular	Flat	Cream	Undulated	Rough	+	+	+
WGB3	Circular	Flat	White	Entire	Rough	+	+	+
WGB4	Circular	Flat	White	Entire	Rough	+	+	-
WGB5	Irregular	Flat	White	Entire	Rough	+	+	+
WGB6	Circular	Flat	Cream	Undulated	Rough	+	+	+
WGB7	Circular	Raised	White	Entire	Rough	+	+	-
WGB8	Circular	Flat	White	Entire	Rough	+	+	+
WGB9	Circular	Flat	White	Entire	Rough	+	+	-
WGB10	Circular	Flat	White	Entire	Rough	+	+	-

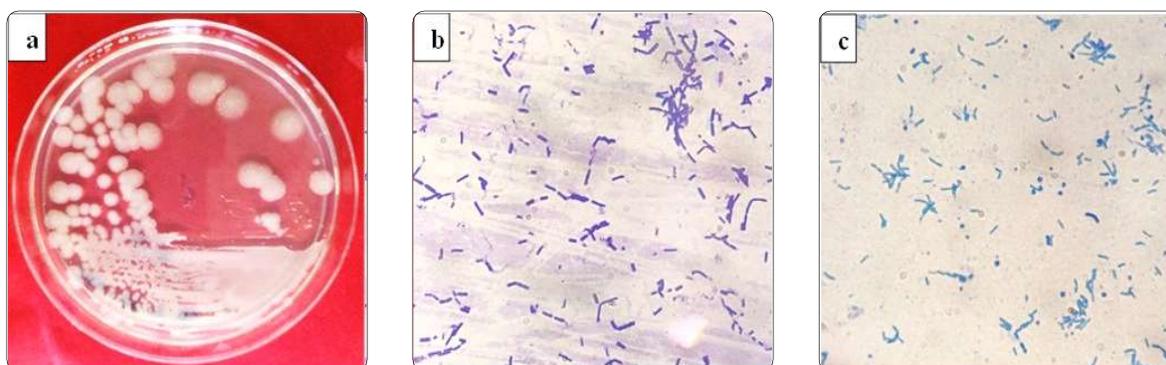


Plate 1 : Morphological and physiological characterization of *Bacillus thuringiensis* strain a) Pure culture showing white coloured, round and raised colonies, b) Gram staining showing Gram-positive rod-shaped bacteria, c) Crystal staining showing crystals and vegetative cells

isolate (Plate 1c). The shape of these crystals varied, with some appearing spherical while others were more irregular. This crystal diversity was also observed in previous studies (Padole *et al.*, 2017) and often reflects variation in *cry* gene composition, which influences insecticidal specificity. The presence of crystal proteins is the symbol of *B. thuringiensis* and their successful detection validates the morphological identity of the isolates.

Molecular Characterization and Phylogenetic Confirmation

All the five crystal-positive isolates were subjected to 16S rRNA gene sequencing to confirm their

molecular identity. PCR amplification produced single, distinct amplicons of approximately 1.5 kb in size (Plate 2), as expected for the universal 16S region. The sequences were analyzed using NCBI BLASTn and all isolates showed more than 99 per cent similarity with reference strains of *B. thuringiensis*. The sequences were submitted to the NCBI database and accession numbers were obtained (Table 3). Although the 16S rRNA gene alone cannot differentiate closely related *Bacillus* species with complete confidence, high identity values combined with parasporal crystal confirmation provide robust taxonomic evidence.

TABLE 3
Molecular characterization of crystalliferous *Bacillus thuringiensis* strains with GenBank accession numbers for 16S rRNA gene

Isolate name	Strain name	Accession number
WGB3	<i>Bacillus thuringiensis</i> strain NBAIR Bt188	PV815644
WGB4	<i>Bacillus thuringiensis</i> strain NBAIR Bt189	PV815687
WGB5	<i>Bacillus thuringiensis</i> strain NBAIR Bt190	PV815686
WGB6	<i>Bacillus thuringiensis</i> strain NBAIR Bt191	PV815703
WGB8	<i>Bacillus thuringiensis</i> strain NBAIR Bt192	PV815702

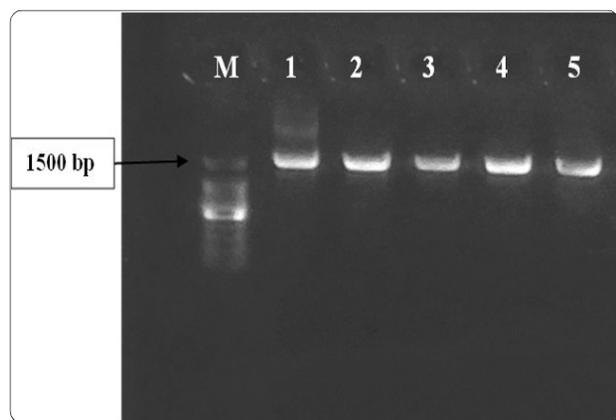


Plate 2 : Agarose gel electrophoresis of a PCR amplification of the 16S rRNA gene from *Bacillus thuringiensis* strains. Lane 1-5: *Bacillus thuringiensis* NBAIR Bt188-192. Lane M- ladder (100bp)

To establish phylogenetic relationships, a neighbor-joining tree was constructed using MEGA version 11.0 with 1000 bootstrap replicates (Fig. 2). The resulting circular dendrogram delineated all five strains within the *B. cereus* sensu lato (s.l.) group, which includes closely related species such as *B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. wiedmannii* and *B. toyonensis*. This clustering pattern is consistent with the taxonomic complexity of the *B. cereus* group, wherein 16S rRNA sequences often show high similarity across species, but phylogenetic grouping combined with phenotypic traits such as crystal protein production helps distinguish *B. thuringiensis* (Guinebretière *et al.*, 2008). Thus, the integration of molecular data with

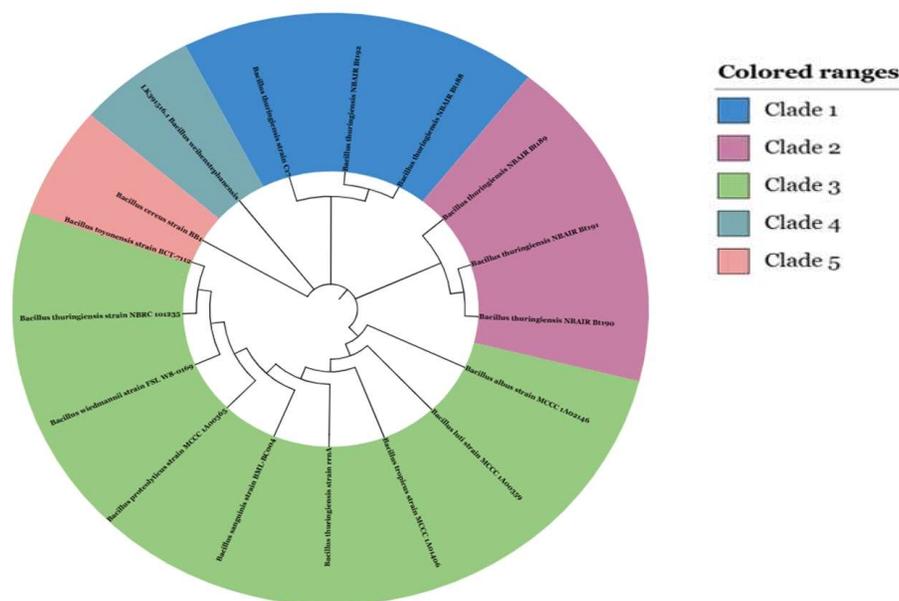


Fig. 2 : Phylogenetic tree based on 16S rRNA gene sequences of *Bacillus thuringiensis* strains. The tree illustrates the clustering of the strains with known *Bacillus* species

TABLE 4
Details of plant growth-promoting and enzyme production in *Bacillus thuringiensis* strains

Strain name	IAA production	Ammonia production	Siderophore production	Protease	Lipase	Lecithinase	Chitinase
<i>Bacillus thuringiensis</i> strain NBAIR Bt188	+	+	-	+	+	+	+
<i>Bacillus thuringiensis</i> strain NBAIR Bt189	+	+	-	+	+	+	-
<i>Bacillus thuringiensis</i> strain NBAIR Bt190	+	+	-	-	+	-	-
<i>Bacillus thuringiensis</i> strain NBAIR Bt191	+	+	+	+	+	+	+
<i>Bacillus thuringiensis</i> strain NBAIR Bt192	+	+	-	+	-	-	+

morphological and biochemical evidence provides a reliable basis for confirming the identity of the strain as *B. thuringiensis* (Dos Santos *et al.*, 2019).

Enzymatic Activity of *Bacillus thuringiensis* Strains

All five *B. thuringiensis* strains were screened for their ability to produce extracellular enzymes involved in biocontrol, particularly those targeting insect cuticles and cell membranes. The results revealed distinct variations among the strains (Table 4). Protease activity, indicative of casein hydrolysis and protein degradation potential, was evident in all the strains. Clear zones of hydrolysis surrounding the colonies on skim milk agar confirmed strong proteolytic capabilities (Plate 3a). Proteases are known to synergize with crystal proteins by weakening insect gut membranes, thereby enhancing toxin penetration (Vachon *et al.*, 2012). Their presence in all strains indicates a conserved trait among these native *B. thuringiensis* strains. Lipase production was observed in strains NBAIR Bt188, NBAIR Bt189,

NBAIR Bt190 and NBAIR Bt191 (Plate 3b), while Bt192 lacked visible lipolytic zones. Lipases facilitate the breakdown of lipid components in insect tissues and may assist in the early stages of infection (Stehr *et al.*, 2003). The differences in lipase activity among the strains may reflect genetic variation or ecological adaptation to distinct soil environments. Previous studies, including those by El-Kersh *et al.* (2016), have also noted such strain-dependent variation in lipase activity among environmental *B. thuringiensis* strains.

Lecithinase activity, which reflects phospholipid degradation potential, was detected in strains NBAIR Bt188, NBAIR Bt189 and NBAIR Bt191. Opaque zones formed around the colonies on egg yolk agar, confirming lecithin hydrolysis (Plate 3c). Lecithinase-producing *B. thuringiensis* strains are considered to possess stronger virulence, as this enzyme can disrupt cellular membranes of both insect pests and phytopathogenic fungi (Ahsan *et al.*, 2024). Chitinase

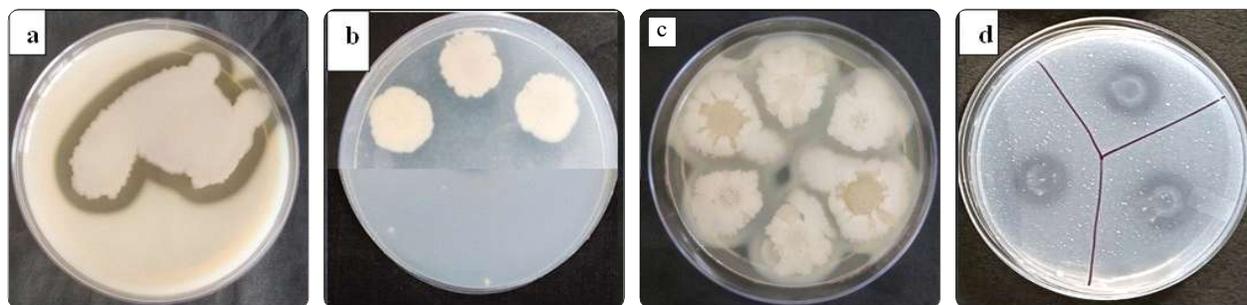


Plate 3 : Biochemical characterization showing enzyme production of *Bacillus thuringiensis* strains. a) Protease test showing clear zone around the colony as positive in starch casein media, b) Lipase test showing hallow zone around the colony as positive in tributyrin media, c) Lecithinase test showing opaque zone around the colony as positive in egg yolk agar media, d) Chitinase test showing hallow zone around the colony as positive in colloidal chitin agar media

production, essential for degrading chitin-rich structures such as insect exoskeletons and fungal cell walls, was observed in strains NBAIR Bt188, NBAIR Bt191 and NBAIR Bt192. Chitinase-positive strains produced clear halos on chitin agar plates containing 4 per cent crude chitin as the sole carbon source (Plate 3d). These enzymes are crucial for *B. thuringiensis* entomopathogenic function and are frequently cited as key indicators of biocontrol efficacy (Patel and Goyal 2017). Interestingly, strain NBAIR Bt191 showed positive activity for all four enzymes, suggesting it might be the most potent biocontrol candidate among the five strains.

Evaluation of Plant Growth-Promoting Activities of *Bacillus thuringiensis* Strains

In addition to biocontrol potential, the selected *B. thuringiensis* strains were evaluated for their ability to support plant growth through key PGP mechanisms. The strains displayed varied profiles in IAA production, ammonia production and siderophore production (Table 4). All five strains produced IAA, as evidenced by the development of pink to red coloration in the presence of Salkowski's reagent (Plate 4a). IAA is a vital auxin that promotes root elongation, lateral root formation and overall plant vigour. *Bacillus thuringiensis* strains producing IAA have been previously reported to enhance seed

germination and plant biomass in several crops, including rice and maize (Ranjan *et al.*, 2024). Ammonia production was also recorded in all strains. The colour changes from pale yellow to brown upon the addition of Nessler's reagent, confirming the release of ammonia in peptone water broth (Plate 4b). Ammonia contributes to nitrogen availability and can act as an antimicrobial compound against competing microbes in the rhizosphere (Richardson *et al.*, 2009).

Siderophore production, assessed on CAS agar, was confirmed only in strain NBAIR Bt191, which produced a distinct orange halozone (Plate 4c). Siderophores chelate iron from the environment, enhancing iron availability to plants while simultaneously restricting its access to pathogenic microbes (Chaabouni *et al.*, 2012; Ahmed & Holmstrom, 2014 and Kukreti *et al.*, 2024). The ability to produce siderophores underlines the competitiveness of *B. thuringiensis* strains like NBAIR Bt191 in nutrient-limited rhizosphere environments. Taken together, the data demonstrate that while all five *B. thuringiensis* strains possessed some level of PGP activity, NBAIR Bt191 again emerged as the most multifunctional strain. Its ability to produce IAA, ammonia and secrete siderophores suggests high potential as a biofertilizer. These results align with studies by Aguilar *et al.* (2014), who characterized similar native *B. thuringiensis* strains with growth-promoting effects in cereals.

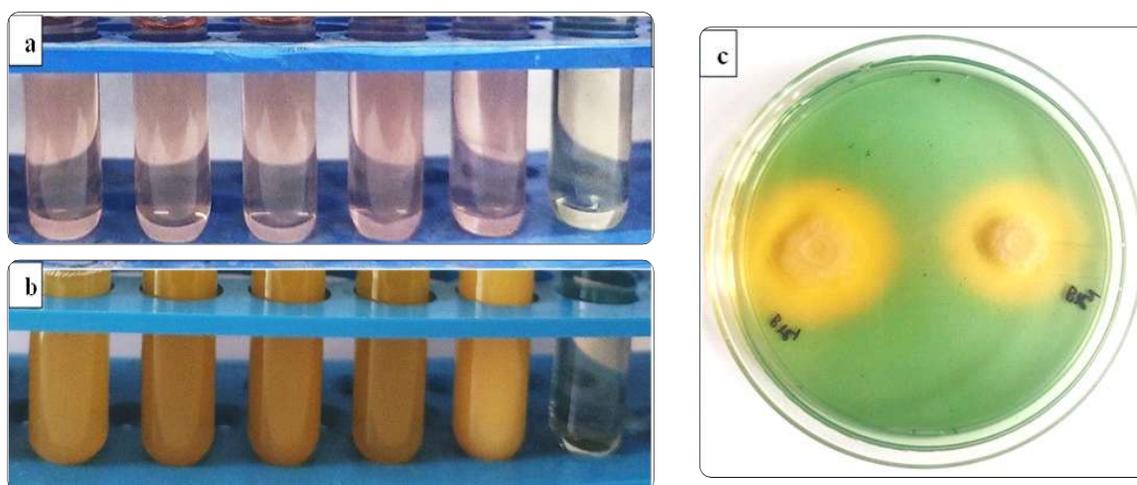


Plate 4 : Plant growth-promoting activities of *Bacillus thuringiensis* strains a) IAA production test showing pink-orange colour as positive, C- control, b) Ammonia production test showing Yellow-brown colour as positive, C- control, c) Siderophore production showing yellow zone around the colony as positive in CAS agar

The multi-trait assessment of the five *B. thuringiensis* strains obtained from the Western Ghats shows their functional diversity and agricultural relevance. Each strain demonstrated key characteristics associated with effective biocontrol, including crystal protein formation, sporulation and at least one or more extracellular enzymes and plant growth-promoting traits. Among these, strain NBAIR Bt191 showed the most promising multifunctional traits, exhibiting all four tested enzymatic activities as well as positive results for IAA production, ammonia production and siderophore production. Such versatility underscores its potential as a dual-purpose bioinoculant that can simultaneously manage soil-dwelling insect pests and promote crop growth.

The ecological richness and microbial diversity of the Western Ghats region offer a valuable reservoir for the discovery of novel, native microbial strains with unique functional attributes. The present findings support the premise that indigenous *B. thuringiensis* strains, adapted to specific local soil and climatic conditions, can be effective candidates for sustainable agricultural applications. Continued exploration and characterization of such microbial resources are essential for the development of region-specific biocontrol formulations and biofertilizers that align with the principles of eco-friendly and integrated farming systems.

Acknowledgements : The authors extend their gratitude to The Karnataka Science and Technology Promotion Society (KSTePS), Department of Science and Technology, Govt. of Karnataka for providing the fellowship to conduct the research successfully

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