5 DISCUSSIONS CHAPTER 5

In this study, endophytic bacteria were isolated from the roots, stems and leaves of three medicinal plant of Kokrajhar, Assam, India. For the isolation of endophytic bacteria, the proper surface sterilisation of tissue is the most cruicial step. Surface sterilisation, or the removal of epiphytic microorganisms from plant tissues, is the first step in the isolation of endophytic bacteria. It is important to use the appropriate sterilant solution, concentration, and exposure time to ensure proper surface sterilisation with the least amount of damage to the endophytic variety. Commonly used surface sterilants include ethanol (70%-90%), sodium hypochlorite (2%-10%), mercuric chloride (0.1%), and formaldehyde (40%) (Sahu et al., 2022). Treatments were different for different plants and plant tissues (Das & Das, 2024). Endophytic bacteria were previously isolated from common bean leaves using 70% alcohol for one minute, 2.5% sodium hypochlorite for 4 minutes, ethanol for 30 seconds, and finally three rinses in sterilised distilled water (Emanuel, 2012). To isolate, endophytic bacteria from the roots of the medicinal plant *Alkanna tinctorial*, 70% ethanol was used for 5 minutes, 1.4% NaOCl for 20 minutes, and 2% Na2S2O3 for 10 minutes (Rat et al., 2021). In this study for the surface sterilisation, 70% ethanol and different concentration of sodium hypochlorite was used. For the isolation of endophytic bacteria from *G. pentaphylla*, plant tissues were first treated with 70% ethanol for 3 minutes and then 2% sodium hypochlorite 7-10 mins for leaves, 10-12 minutes for stems, 12-14 minutes for roots. For *P. thyrsiformis*, plant tissues were at first treated with 70% ethanol for 1 minute and then 1% sodium hypochlorite 7-10 mins for leaves, 1.5% sodium hypochlorite for 10-12 mins for stems, 2% sodium hypochlorite for 10-12 mins. And for *H. auriculata*, tissues were treated with 70% ethanol for 1 minute and then 0.9% sodium hypochlorite 8-10 mins for leaves, 1% sodium hypochlorite for 12-14 mins for stems, 2% sodium hypochlorite for 10 to 12 mins for roots.

Endophytic bacteria were isolated, characterised, and identified from the leaves, stems, and roots of three medicinal plants that were grown in various locations around the Kokrajhar district of Assam. A total of 16 endophytic bacteria were isolated from the various tissues of *H. auriculata*, *P. thyrsiformis*, and *G. pentaphylla*. Both *H. auriculata* and *P. thyrsiformis* belongs to Acanthaceae family whereas *G. pentaphylla* belongs to Rutaceae family. Previously, endophytic bacteria were isolated and identified from

leaves, stems, and roots of *Hygrophila spinosa* that were collected from Serampore College's medicinal plant garden in Hooghly, West Bengal. Tissues like leaf, stem and root yielded 6, 3 and 2 isolates, respectively. Isolated EB as belonging to the bacterial genera *Acidomonas*, *Staphylococcus*, *Ralstonia*, *Pseudomonas*, *Paenibacillus*, and *Bacillus* (Pal, 2014). The location of plants, plant age, genotypes, precipitation, seasonal change, and soil characteristics all affect the endophytic genera' spatial distribution (Rosenblueth & Martínez-Romero, 2006). In this study, a total of 5 endophytic bacteria were isolated from *H. auriculata*: 2 from leaves; 2 from stems; and 1 from roots. These isolates belong to diverse bacterial genera and phyla. Primarily, isolates belong to the Proteobacteria and Firmicutes phyla and the *Pseudomonas*, *Proteus*, *Agrobacterium*, *Stenotrophomonas* and *Lysinibacillus* genera. Previously, endophytic bacteria belonging to the Proteobacteria and Firmicutes phyla were also isolated from the leaves of *Phaseolus vulgaris* (Emanuel, 2012). Endophytic bacterial isolation of *P. thyrsiformis* from additional regions has not yet been reported. In 2024, we reported two isolates from the leaves of *P. thyrsiformis*: *Solibacillus silvestris* DDBU6 and *Kocuria assamensis* DDBU9 (Das et al., 2024). A total of 6 EBs were isolated from *P. thyrsiformis*: 2 from leaves, 2 from stems and 2 from roots. Isolates belong to different phylla and genera. Isolates belong to Firmicutes, Actinobacteria and Proteobacteria phyla and the *Prescottella*, *Alkalicoccobacillus*, *Kocuria*, *Solibacillus*, *Agrobacterium* and *Pseudomonas* genera. The isolation of endophytic bacteria from *G. pentaphylla* was not reported previously. In the year 2022, we have reported 3 endophytic bacteria from the leaves of *G. pentaphylla* collected from Kokrajhar district of Northeast India (Das & Das, 2022). A total of 5 EBs were isolated from *G. pentaphylla*: 3 from leaves; 1 from stems and 1 from roots. All the isolates belong to Firmicutes phylum and diverse genera like *Alkalicoccobacillus* and *Bacillus*. From this study it was noted that isolation of endophytic bacteria from firmicutes phylum is common in all three plants.

The isolates showed distinct morphological and biochemical characteristics. The isolates' morphologically observable characteristics, such as their shape, colour, and appearance, were observed in order to perform morphological characterisation. Catalase, oxidase, indole, citrate, methyl red, and Voges-Proskauer assays were used for biochemical characterisation. Scanning electron microscopy analysis and gram staining were performed for microscopic examination.

Different medicinal plants were previously used to isolate endophytic bacteria. To characterise, those EB, most of them were found to show different plant growth promotion activities like IAA production, phosphate solubilisation, nitrogen fixation etc. Some of strains also showed to have the ability of tolerance against abiotic and biotic stress.

The total of 16 endophytic bacteria were identified from three medicinal plants are as follows: *Pseudomonas oryzihabitans* strain HAL1DD, *Proteus mirabilis* strain HAL2DD, *Stenotrophomonas geniculata* strain HAS3DD, *Agrobacterium cavarae* strain HAS4DD, *Lysinibacillus macrolides* strain HARDD from *H. auriculata*; *Alkalicoccobacillus gibsonii* strain GPDD1, *Bacillus cereus* strain GP2DD, *Bacillus subtilis* strain GP3DD, *Bacillus cereus* strain GP4DD, *Bacillus australimaris* strain GPRDD from *G. pentaphylla*; *Pseudomonas aeruginosa* strain DD3, *Agrobacterium larrymoorei* strain DDBU3, *Solibacillus silvestris* strain DDBU6, *Kocuria assamensis* strain DDBU9, *Alkalicoccobacillus gibsonii* strain PTR1DD, *Prescottella equi* strain PTR2DD from *P. thyrsiformis*.

The plant-growth-promoting (PGP) capability of *Pseudomonas oryzihabitans* GDW1 was previously assessed in tomato plants in a pot experiment following a month of inoculation using *P. oryzihabitans* GDW1 isolated from a healthy pine tree. The outcome shown that, in comparison to untreated plants, *P. oryzihabitans* might increase tomato plant development. In comparison to the control, the fresh weight, dry weight, shoot length, and root length of tomato plants treated with *P. oryzihabitans* increased by 44%, 38%, 54%, and 59%, respectively (Ahmed et al., 2025). Previously, *P. oryzihabitans* has also been isolated from *Hibiscus rosa-sinensis* (Bhagat et al., 2016). In this study, *P. oryzihabitans* HAL1DD isolated from the leaves of *H. auriculata*. This isolate showed positive for ammonia and IAA production and negative for phosphate solubilisation. It was able to produce 121 ± 1.63 µg/ml concentration of IAA. The isolate was able to tolerate up to 6% NaCl and showed antimicrobial activity against pathogenic bacteria *B. subtilis* by forming inhibition zone 8.2 ± 0.71 mm. In extracellular enzyme production activity, the isolate showed positive for amylase, protease, lipase, cellulase and pectinase production.

Proteus mirabilis strain HAL2DD was isolated from the leaves of *H. auriculata* and showed several different characteristics. However, according to the review, following surface sterilisation with 70% ethanol and 1% sodium hypochlorite, the plant-associated *P. mirabilis* was isolated from the roots, stems, and leaves of agricultural tomato crops. Different types of PGP activities were shown by different varieties of *P. mirabilis* like for phosphate solubilisation, some showed positive results and some showed negative results whereas all isolates showed positive for IAA production and all showed antimicrobial activity against *Fusarium oxysporum*. For extracellular enzyme activity, some showed positive for protease production whereas some other showed positive for amylase and cellulase production (Amaresan et al., 2021). Here it showed positive for protease, amylase and lipase activity and for antibacterial activity it showed inhibitory activity against pathogenic bacteria *B. subtilis* and formed 14.16 ± 0.78 mm inhibition zone, while testing against 6 pathogenic bacteria which includes, *S. aureus*, *E. coli*, *B. subtilis*, *E. aerogenes*, *K. pneumonia* and *P. aeruginosa*. It was able to tolerate up to 5% NaCl in salt tolerance activity. In plant growth promotion activities, the isolate showed positive for ammonia production and negative for IAA production and phosphate solubilisation.

Arisaematis rhizoma was previously used to isolate the endophytic bacterium *Stenotrophomonas geniculata* KJ-6. This strain can suppress three Lanzhou lily postharvest pathogenic fungus which includes *Trichoderma lixii* F-2, *Talaromyces tumuli* F-3, and Fusarium *annulatum* F-6 (Ling et al., 2024). In the year 2022, from cowpea tissue, *Stenotrophomonas geniculata* NWUBe21 was identified. This isolate was shown to have several characteristics that promote plant growth, such as the ability to solubilise phosphates and produce auxin, siderophore, hydrogen cyanide, exopolysaccharide, ammonia, and 1-aminocyclopropane-1-carboxylic acids (Omomowo & Babalola, 2022). In this study, *S. geniculate* strain HAS3DD was isolated from stem of the *H. auriculata* and it has shown several PGP activities which includes phosphate solubilisation, ammonia production, having high salt tolerance ability (7% NaCl). In extracellular enzyme production ability, it has shown positive results for amylase and pectinase production. In antimicrobial activity test, the isolate showed anti-bacterial activity against *K. pneumonia*. The isolate was able to form 14.6 ±0.48 mm inhibition zone against *K. pneumonia*.

Previously, from a *Zea mays* L. root that was gathered in Spain, *Agrobacterium cavarae* sp. nov. was isolated (Flores-Félix et al., 2020). Whereas the PGP activities and antimicrobial activities test of the isolate was reported in this study. *A. cavarae* strain HAS4DD was isolated from the stem of *H. auriculata*. The isolate showed positive for ammonia production and negative for phosphate solubilisation and IAA production while PGP activities were determined. It was able to tolerate 3% NaCl in salt tolerance ability test. For extracellular enzyme production test, *A. cavarae* HAS4DD showed positive for protease, cellulase and xylanase activities.

Lysinibacillus macrolides, which was isolated from *Zingiber officinale*, demonstrated distinct capacities for supporting plant development and producing extracellular enzymes. The isolate demonstrated the capacity to produce lipase, protease, and esterase. In plant growth promotion activity, isolate showed positive for IAA production whereas negative for phosphate solubilisation. Between two *L. macrolides*, one isolate showed positive for nitrogen fixation another one showed negative for nitrogen fixation (Bódalo et al., 2023). In this study, *L. macrolides* was isolated from the root of *H. auriculata*. In plant growth promotion activities, the isolate showed positive for ammonia and IAA production and was able to produce 258.6 ± 2.05 µg/ml of IAA and negative for phosphate solubilisation. The isolate was able to tolerate high salt concentration which is 6% NaCl. For extracellular enzyme production, *L. macrolides* showed positive activities for protease, lipase, pectinase and xylanase production. The isolate also showed antibacterial activity against pathogenic bacteria *E. coli* and it was able to form 11.16 ± 0.86 mm inhibition zone.

According to the review, *Alkalicoccobacillus gibsonii* is not mentioned as an endophytic bacterium. Whereas, *Alkalihalobacillus gibsonii* 2H2 was previously isolated from halophytes. It also revealed that the isolate possessed characteristics that mitigated the effects of salt stress (Sahu et al., 2023). In this study, *Alkalicoccobacillus gibsonii* GPDD1 was isolated from the leaves of *G. pentaphylla* plant. The isolate showed high salt tolerance ability which is 7% NaCl. In plant growth promotion activities, the isolate showed positive for ammonia and IAA production whereas negative for phosphate solubilisation. The isolate was able to produce 76.33 ± 1.24 µg/ml of IAA. In extracellular enzyme production, it showed positive for protease, lipase, pectinase, and xylanase

activities. The isolate was also able to inhibit the growth of pathogenic bacteria *E. coli* and *E. aerogenes* by forming 15.8 ± 0.8 mm and 10.1 ± 0.29 mm inhibitory zone. It was evident from multiple earlier studies that the same endophytic bacteria may be isolated from the same or different plant tissue of the same or different plants. Different strain of *A. gibsonii* was isolated from the roots of *P. thyrsiformis*. *A. gibsonii* strain PTR1DD showed positive result for phosphate solubilisation ability whereas negative for ammonia and IAA production. It showed 5% NaCl tolerance ability in salt tolerance activity test. In extracellular enzyme activity, the isolate showed positive for amylase and protease production.

In the past, *B. cereus* YN917, which was isolated from a rice leaf, had exceptional antifungal activity against *Magnaporthe oryzae*. According to earlier research, *B. cereus* YN917 can encourage seed germination and the growth of seedling plants. According to genome study of the isolate, gene clusters for the manufacture of antifungal and plant-promoting substances such phenazine, tryptophan, siderophores, and IAA were found (Zhou et al., 2021). Previously, *B. cereus* strain T4S was identified from sunflower plants. The T4S genome contained secondary metabolites that support the effectiveness of bacterial biocontrol against phytopathogens, including petrobactin, bacillibactin, bacitracin, molybdenum factor, zwittermicin, and fengycin. Using strain T4S for seed and root inoculation increased sunflower production in greenhouse tests (Adeleke et al., 2021). In this study, *B. cereus* has been isolated from both leaf and stem tissue of *G. pentaphylla*. *B. cereus* strain GP2DD isolated from leaf showed positive results for ammonia and IAA production. The isolate (GPL-2) which was obtained from leaf able to produce 75.83 ± 1.31 µg/ml of IAA whereas the isolate (GPS-4) obtained from stem, *B. cereus* strain GP4DD showed production of 76.66 ± 2.49 µg/ml of IAA. GPL-2 was showed tolerance to 7% NaCl whereas GPS-4 showed tolerance unto 8% NaCl. For extracellular enzyme production activities, GPL-2 showed positive for protease, lipase, cellulase and pectinase production whereas GPS-4 showed positive for protease, lipase and xylanase production. Whereas, for antimicrobial activity both GPL-2 and GPS-4 showed anti-bacterial activities against *S. aureus* and *E. coli*.

Previously there was a report on IAA that was produced by *B. subtilis* strains that were isolated from *Zea mays* (Bolivar-Anillo et al., 2021). In addition to producing

protease, amylase, pectinase, cellulase, IAA, ammonia, catalase, and ACC deaminase, *B. subtilis* CB2 isolated from wheat seed demonstrated the capacity to solubilise phosphate (Taheri et al., 2023). In this study, *B. subtilis* strain GP3DD isolated from the leaves of *G. pentaphylla*. The isolate showed positive for amylase, protease and cellulase production. In plant growth promotion activities, *B. subtilis* GP3DD showed positive for phosphate solubilisation, ammonia and IAA production in plant growth promotion activities. It was able to produce 80.16 ± 2.89 µg/ml of IAA. The isolate was able to tolerate 7% NaCl. In anti-microbial activity, the isolate showed antagonistic activity against pathogenic *B. subtilis* (MTCC 441) by forming 16.1 ± 0.62 mm inhibition zone.

Bacillus australimaris BLR41 was previously isolated from the root of the therapeutic herb *Barleria lupulina*. It was positive for lipase, amylase, citrate, and protease. *B. australimaris* BLR41 isolates were found to solubilise zinc (N. Kumar & Dubey, 2022). In this study, *B. australimaris* strain GPRDD isolated from the roots of medicinal plant, *G. pentaphylla*. For plant growth promotion activity, the isolate showed positive for phosphate solubilisation, ammonia and IAA production. It showed the ability to produce 31.66 ± 2.49 µg/ml of IAA. *B. australimaris* GPRDD was showed ability to tolerate 5% NaCl concentration. In extracellular enzyme production activity, it showed positive for protease, cellulase and xylanase production. In antimicrobial activity test, the isolate showed anti-bacterial activity against *E. aerogenes* (MTCC 2822) and *K. pneumonia* (MTCC 109) with zone of inhibition 14.2 ± 0.52 mm and 10.5 ± 0.77 respectively.

Pseudomonas aeruginosa strain DD3 was isolated from the stems of *P. thyrsiformis*. In this study, isolate showed negative for ammonia production and positive for phosphate solubilisation and IAA production. It showed 15 ± 0.81 µg/ml of IAA. In salt tolerance ability, the isolate showed tolerance to 4% NaCl concentration. In extracellular enzyme activity, the isolate showed positive for protease, cellulase, pectinase and xylanase production. Excellent antimicrobial activity against five pathogenic microorganisms was demonstrated by the isolate. The isolate was able to show zone of inhibition against 5 pathogenic bacteria, *S. aureus* (MTCC 737), *E. coli* (MTCC 443), *B. subtilis* (MTCC 441), *E. aerogenes* (MTCC 2822), and *P. aeruginosa* (MTCC 1688) with zone of inhibition 14 ± 0.81 mm, 12.6 ± 0.5 mm, 12.3 ± 0.4 mm, 15.6 ± 0.43

mm, and 11.16 ± 0.86 mm respectively. Previously, *P. aeruginosa* isolated from the leaves of *Achyranthes aspera* L. exhibited substantial role in the production of healthier and antioxidant-rich A. aspera plants (Devi et al., 2017). *P. aeruginosa* also isolated from the bulbs of *Lilium davidii* showed many plant growth promotion activities (Khan et al., 2022). *P. aeruginosa* strain FG106 was previously isolated from the rhizosphere of tomato plants and demonstrated a variety of plant growth promotion activities, such as phosphate solubilisation, the production of siderophores, ammonia, indole acetic acid (IAA), and hydrogen cyanide (HCN), as well as the formation of biofilms that facilitate biocontrol and promote plant growth (Ghadamgahi et al., 2022).

In this study, *Agrobacterium larrymoorei* strain DDBU3 showed positive for all three plant growth promotion activities which includes ammonia, IAA production and phosphate solubilisation. It was isolated from the stems of *P. thyrsiformis*. It showed production of 67 ± 1.24 µg/ml of IAA. Isolate DDBU6 showed high salt tolerance ability, it was able to tolerate 10% NaCl. In extracellular enzyme activities, the isolate showed positive for protease, pectinase and xylanase production. It also showed anti-bacterial activity against pathogenic *E. coli*, *B. subtilis* and *P. aeruginosa* and formed inhibition zone, 20.2 ± 0.58 mm, 9.2 ± 0.61 mm and 10.76 ± 0.5 mm respectively. Previously, *A. larrymoorei* was isolated from *Zea mays* L. plant showed ability to solubilise nutrients (Marag & Suman, 2018).

S. silvestris, an endophytic bacterium, has shown promise as a multi-stress reliever, bioremediation agent, and crop growth stimulator in important crops (Kaur & Karnwal, 2023c). IAA production was previously demonstrated by *Solibacillus silvestris* DL3R2, which was isolated from *Hemerocallis fulva* roots (Kaur & Karnwal, 2023c). Here, *S. silvestris* strain DDBU6 was isolated from the leaves of *P. thyrsiformis* showed positive for protease, ammonia and IAA production whereas negative for phosphate solubilisation. In salt tolerance ability, it showed tolerance to 5% NaCl concentration. In anti-microbial activity test, the isolate showed antagonistic activity against *S. aureus* (MTCC 737), *B. subtilis* (MTCC 441), *P. aeruginosa* (MTCC 1688) by forming zone of inhibition 10.73 ± 0.5 mm, 16.13 ± 0.98 mm, 14.06 ± 0.29 mm respectively. In extracellular enzyme production, the isolates showed positive activity for protease production.

Kocuria assamensis strain DDBU9 has been identified as an endophytic bacterium for the first time in this investigation. *K. assamensis* sp. nov was discovered in Assam, India, from a water sample collected from the Brahmaputra River (Kaur et al., 2011). A number of *Kocuria* species, including *K. arsenatis* and *K. palustris*, have previously been discovered to be endophytic bacteria in a variety of plant species. (Román-Ponce et al., 2016); (Zacaria Vital et al., 2019). In this study, *K. assamensis* was isolated from the leaves of the leaves of *P. thyrsiformis*. In plant growth promotion activities, it showed positive for ammonia and IAA production. The isolate was able to produce 45 ± 0.47 µg/ml of IAA. In salt tolerance, the isolate was able to tolerate 5% NaCl concentration. In extracellular enzyme activities, the isolate showed positive for amylase, lipase and xylanase production. It showed anti-microbial activity against *K. pneumonia* (MTCC 109) by forming inhibition zone 16.13 ± 0.65 mm.

Prescottella equi has never before been reported as an endophytic bacterium. In this study, *P. equi* strain PTR2DD isolated from the root of the *P. thyrsiformis* plant. It is a Gram +ve, rod shaped bacteria which showed positive for catalase production in biochemical test. The isolate also showed positive for ammonia production and phosphate solubilisation in plant growth promotion activity. It was able to tolerate 6% NaCl concentration in salt tolerance test. In extracellular enzyme activities it showed positive for protease, lipase, cellulase, pectinase, and xylanase production. It also showed anti-bacterial activity against *K. pneumonia* (MTCC 109) and formed 9.9 ± 0.73 mm inhibition zone.