A STUDY ON ISOLATION, CHARACTERISATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM SOME MEDICINAL PLANTS OF KOKRAJHAR DISTRICT, ASSAM

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6 SUMMARY CHAPTER 6

Endophytic bacteria are those which live inside the plant tissues without harming the plants. Nearly any plant and plant tissue can be used to isolate these bacteria. Many of these microorganisms have previously demonstrated a variety of direct or indirect plant growth promoting activities- directly, by fixing atmospheric nitrogen, producing growth hormone, ammonia, and phosphate solubilisation, among other processes. It also indirectly aided in the plant's growth and development by enhancing its ability to withstand biotic and abiotic stressors. These bacteria also showed antagonistic activities against human as well as plant pathogens. Several bioactive compounds can also be extracted from these bacteria which make them use in pharmaceutical industries. These microorganisms shown the capacity to produce extracellular enzymes, such as lipase, protease, cellulase, pectinase, amylase etc, which are also utilised in a variety of other industries, including the textile, leather, and food and beverage sectors. With so many uses, these bacteria are still not completely understood in a wide variety of plants. Furthermore, the endophytic bacteria isolated from medicinal plants got so much of importance, as these plants themselves are the storehouse of several important bioactive compounds. However, there are a number of therapeutic plants that have not yet been investigated for endophytic bacterial isolation. The present study was conducted to isolate, characterise and identify endophytic bacteria present in some medicinal plants of Kokrajhar, Assam. With the aforementioned concerns in mind, the current study has accomplished the following goals:

1. To isolate the endophytic bacteria associated with the leaf, stem, and root of medicinal plants like *Glycosmis pentaphylla*, *Hygrophila auriculata*, and *Phlogacanthus thyrsiformis*.

Under this objective, endophytic bacteria were successfully isolated from the leaves, stems, and roots of medicinal plants like *G. pentaphylla*, *H. auriculata*, and *P. thyrsiformis* collected from different locations of Kokrajhar, Assam. At first, sample plants were collected and authenticated from Bodoland University Botanical Herbarium, Department of Botany, Bodoland University. The voucher specimen was deposited in BUBH for accession number. The most important step for isolation of endophytic bacteria is the surface sterilisation of plant tissues, which includes the use of different sterilants to

remove contaminants from the tissue. Here, in this study, 70% ethanol, different concentrations of sodium hypochlorite (0.9 to 2%) for different durations are used for surface sterilisation of the tissues. Both LB and NA medium were utilised to isolate endophytic bacteria from various tissues. In contrast, LB performed comparably well in isolation and subculture as compared to NA media. Thus, LB media was employed throughout the entire study to conduct various tests. Following their successful isolation, sixteen endophytic bacteria were kept at 4°C to complete the remaining tests.

2. To study the morphological, microscopic and biochemical characterisation of the isolated endophytic bacteria.

Here, morphological characterisation was done by observing their morphology of the colony like shape, colour, and appearance on the media. Microscopic characterisation was done by performing both gram staining and SEM. Ten of the sixteen isolates had Gram-positive characteristics, whereas six had Gram-negative characteristics. The majority of the isolates are rod-shaped. For biochemical characterisation, catalase, oxidase, indole, citrate, methyl red and voges-proskauer tests were performed by following standard protocol. All the isolates showed positive for the catalase test; ten isolates showed positive for the oxidase test; almost all isolates showed positive for the citrate test except PTR-6; all the isolates showed negative for the indole test; all the isolates showed negative for the MR test except HAL-2, which showed positive for the MR test; most of the isolates showed positive for VP test except PTS-1, PTL-4, PTR-6, HAL-1 and HAL-2. Antibiotic sensitivity tests were also performed for the isolates against 5 standard antibiotics which includes, ampicillin, gentamicin, penicillin, ciprofloxacin, cefotaxime was used. Isolates PTS-1, PTR-5, GPL-2, GPS-4, GPR-5, HAL-1, HAL-2, HAS-3, HAS-4 and HAR-5 showed resistant to ampicillin, whereas PTS-2, PTL-3, PTL-4, PTR-6 and GPL-1 showed susceptible to ampicillin. All the isolates showed resistance to penicillin. For gentamicin, almost all isolates showed susceptible except GPR-5 and HAS-3. Except for GPR-5, all isolates showed susceptible to ciprofloxacin. For cefotaxime, PTS-1, GPL-2, GPS-4, GPR-5, HAL-1, HAL-2, HAS-3, HAS-4, and HAR-5 showed resistant; PTR-6 showed intermediate resistant, whereas isolates PTS-2, PTL-3, PTL-4, PTR-5, GPL-1, and GPL-3 showed susceptible to cefotaxime.

3. Molecular identification of the isolates.

For molecular identification, genomic DNA of each endophytic bacteria were isolated using CTAB method. Then, PCR amplification of 16S rDNA gene was done using universal primer, 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT). After successful amplification, 1500 bp PCR amplicon was purified and sequencing was performed using Sanger sequencing method. BLAST was performed using the NCBI Genbank database and the 16S rDNA gene sequence. The first ten sequences were chosen based on the maximum identity score, and they were aligned using the multiple alignment software Clustal W. MEGA 11 was used to produce the distance matrix and construct the phylogenetic tree. The total of 16 endophytic bacteria were identified from three medicinal plants are as follows: *Pseudomonas oryzihabitans* strain HAL1DD (HAL 1), *Proteus mirabilis* strain HAL2DD (HAL 2), *Stenotrophomonas geniculata* strain HAS3DD (HAS 3), *Agrobacterium cavarae* strain HAS4DD (HAS 4), *Lysinibacillus macrolides* strain HARDD (HAR 5) from *H. auriculata*; *Alkalicoccobacillus gibsonii* strain GPDD1 (GPL 1), *Bacillus cereus* strain GP2DD (GPL 2), *Bacillus subtilis* strain GP3DD (GPL 3), *Bacillus cereus* strain GP4DD (GPS 4), *Bacillus australimaris* strain GPRDD (GPR 5) from *G. pentaphylla*; *Pseudomonas aeruginosa* strain DD3 (PTS 1), *Agrobacterium larrymoorei* strain DDBU3 (PTS 2), *Solibacillus silvestris* strain DDBU6 (PTL 3), *Kocuria assamensis* strain DDBU9 (PTL 4), *Alkalicoccobacillus gibsonii* strain PTR1DD (PTR 5), *Prescottella equi* strain PTR2DD (PTR 6) from *P. thyrsiformis*. In this study, *K. assamensis*, *P. equi* and *A. gibsonii* are identified for the first time as endophytic bacteria. All the sequences were submitted in NCBI GenBank for accession number.

4. To study a number of plant growth promotion activities of the isolated endophytic bacteria.

Isolates plant growth promotion activities were determined using standard protocol which includes, phosphate solubilisation, IAA and ammonia production. For phosphate solubilisation activity test, isolates were inoculated on pikovskaya's agar medium and incubated for 5 to 7 days at 28℃. Clearing zone formation around the colony indicates positive result. In this study, out of sixteen isolates, seven (PTS-1, PTS-2, PTR-5, PTR-6, GPL-3, GPR-5, and HAS-3) demonstrated positive results in phosphate solubilisation

activity. Solubilisation index was determined from diameter of solubilization zone and colony diameter. PTS-1 isolates had the highest solubilisation index which is 4.07, followed by PTR-6 (3.17), GPR-5 (3.09), PTR-5 (2.94), GPL-3 (2.60), PTS-2 (2.34), and HAS-3 (2.19). For ammonia production, isolates were inoculated in peptone water and incubated at 30℃ for 5 days. After proper incubation 1 ml of Nessler's reagent was mixed in each tube if the color changes to faint to deep yellow indicates positive results. Here, almost all isolates showed positive results except PTS-1 and PTR-5. In IAA production test, isolates grow in LB broth supplemented with 400 µg/ml of L-tryptophane and incubated at 25 ± 2 °C for 72 h. The supernatant was then mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski reagent and incubate for 1 hour at dark place at normal temperature. After incubation, the production of a pink colour indicates positive results. The optical density was measured at 530 nm using spectrophotometer. To determine the amount of IAA produced by the isolates which showed positive results, a standard curve for pure IAA was used. In this study, 11 isolates showed positive for IAA production activity. Highest production was observed in HA-5 followed by HAL-1, PTL-3, GPL-3, GPS-4, GPL-1, GPL-2, PTS-2, PTL-4, GPR-5, and PTS-1. In plant growth promotion activities salt tolerance ability of the isolated endophytic bacteria was also determined. Bacteria isolates were inoculated on the LB media containing 1 to 10% NaCl. Here almost all the isolates showed a good salt tolerance ability with minimum tolerance to 3% NaCl. NaCl tolerance varied among the isolates: PTS-2 could withstand up to 10%, GP-4 up to 8%, GP-1, GP-2, GP-3, and HA-3 up to 7%, HA-1 and PTR-6 up to 6%, GP-5, HA-2, PTR-5, PTL-4, and PTL-3 up to 5%, PTS-1 up to 4%, and HA-4 up to 3%.

5. To screen the ability of isolated endophytic bacteria to produce extracellular enzymes.

The ability of isolated endophytic bacteria to produce different extracellular enzyme was also evaluated. To carried out the test's different standardised protocol was followed. The activities of amylase, protease, lipase, cellulase, pectinase, and xylanase were assessed in 16 endophytic bacterial isolates in this investigation. Amylase was the least common enzyme, discovered in only six isolates, whereas protease was the most often synthesised, found in thirteen. For amylase activity, isolates, PTL-4, PTR-5, GPL-3 and HAS-3

showed positive result. Maximum number of isolates showed positive for protease activity except PTL-4 and HAS-3. For lipase activity, PTL-4, PTR-6, GPL-1, GPL-2, GPS-4, HAL-1, HAL-2, and HAR-5 showed positive results. Whereas, PTS-1, PTR-6, GPL-2, GPS-3, GPR-5, HAL-1, and HAS-4 showed positive for cellulase activity. For pectinase activity, PTS-1, PTS-2, PTR-6, GPL-1, GPL-2, HAL-1, HAS-3, and HAR-5 showed positive results.

6. To evaluate anti-microbial activities of isolated endophytic bacteria.

Bacterial crude extract 400 mg/ml concentration was used to carry out the antimicrobial activities against six pathogenic microbes, namely, *S. aureus* (MTCC 737), *E. coli* (MTCC 443), *B. subtilis* (MTCC 441), *E. aerogenes* (MTCC 2822), *P. aeruginosa* (MTCC 1688), and *K. pneumonia* (MTCC 109). The excellent activity was observed in PTS-1 isolate which was obtained from the stem of *P. thyrsiformis*. This isolate showed inhibitory activity against 5 pathogenic bacteria, and formed 14 ± 0.81 mm against *S. aureus*, 12.6 ± 0.5 mm against *E. coli*, 12.3 ± 0.4 mm against *B. subtilis*, 15.6 ± 0.43 mm against *E. aerogenes*, and 11.16 ± 0.86 mm for *P. aeruginosa*. Following PTS 1, PTS-2 and PTL-3 exhibited antibacterial activity against three of six pathogenic bacteria. Then, GPL-1, GPL-2, GPS-4 and GPR-5 showed inhibitory activity against two of six pathogenic bacteria. Antimicrobial activity against at least one pathogenic bacterium was demonstrated by PTL-4, PTR-6, GPL-3, HAL-1, HAL-2, HAS-3, and HAR-5.

CONCLUSIONS CHAPTER-7

Endophytic bacteria can be a solution to various issues in the health sector, such as medication resistance, and in the agriculture sector, such as increased environmental pollution and health risks for both humans and animals due to the use of chemical fertilisers. Several endophytes that were isolated from various plants previously demonstrated antimicrobial and anti-cancer properties in addition to their ability to promote plant development. Since numerous endophytic bacteria have been shown to be able to produce extracellular enzymes including amylases, pectinases, proteases, and lipases, among others, they can also be used in other industries, such as the food and beverage, textile, and leather sectors. A wide variety of economically significant endophytic bacteria are found in medicinal plants. Although, many medicinal plants have not yet been thoroughly investigated, even though endophytic bacteria are present in almost all plants. In this study, endophytic bacteria were isolated, characterised and identified from three different medicinal plants, including *G. pentaphylla*, *H. auriculata* and *P. thyrsiformis* from different locations of the Kokrajhar district, Assam. Surface sterilisation is crucial for the isolation of endophytic bacteria as it ensures both the preservation of endophytic bacteria and the elimination of epiphytic bacteria. Here, endophytic bacteria from three medicinal plants have been successfully isolated using the optimised surface sterilisation approach, where the different concentrations of sodium hypochlorite are used for different durations along with 70% ethanol. Following isolation, these endophytic bacteria were characterised through morphological features, Gram staining, SEM analysis, antibiotic sensitivity to some common antibiotics, and some biochemical analysis. In this study, gram-positive bacteria are more common than gram-negative bacteria. 10 gram-positive isolates and 6 gram-negative isolates were found among the 16 endophytic bacteria. Almost all isolates are rod shaped except PTL-4 which is circular in shape.

A total of 16 endophytic bacterial strains were identified, belonging to diverse phyla, including Firmicutes, Proteobacteria, and Actinobacteria, and genera such as *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Solibacillus*, *Lysinibacillus*, and *Kocuria*. The findings of this study contribute to the identification of endophytic bacteria – *Kocuria assamensis*, *Prescottella equi* and *Alkalicoccobacillus gibsonii* – among 16 that have been

reported for the first time as endophytic bacteria. Also, this is first report on the isolation of endophytic bacteria from the tissues of *G. pentaphylla* as well as *P. thyrsiformis*.

The isolated endophytic bacteria exhibited diverse plant growth-promoting (PGP) activities, including the production of IAA, ammonia, and phosphate solubilisation. Out of sixteen, eleven isolates showed positive results for the IAA production ability. Highest production was observed in HAR-5 (258.6 ± 2.05 µg/ml) followed by HAL-1 (121 ± 1.63 µg/ml), PTL-3 (81 ± 1.24 µg/ml), GPL-3 (80.16 ± 2.89 µg/ml), GPS-4 (76.66 ± 2.49 µg/ml), GPL-1 (76.33 ± 1.24 µg/ml), GPL-2 (75.83 ± 1.31 µg/ml), PTS-2 (67 ± 1.24 µg/ml), PTL-4 (45 ± 0.47 µg/ml), GPR-5 (31.66 ± 2.49 µg/ml), PTS-1 (15 ± 0.81 µg/ml) in presence of 400 µg/ml of L-tryptophane. For ammonia almost, all isolates showed ability to produce ammonia except PTS-1 and PTR-5. In this study, seven isolates showed the ability to solubilise calcium phosphate. Highest clearing zone was observed for PTS-1 (3.50 ± 0.11 mm) isolates followed by PTR-6 (2.66 ± 0.28 mm), PTR-5 (2.1 ± 0.12 mm), PTS-2 (2 ± 0.05 mm), GPR-5 (1.6 ± 0.12 mm), HAS-3 (1.59 ± 0.06 mm) and GPL-3 (1.4 ± 0.08 mm) when growing in Pikovskaya's agar medium containing calcium phosphate. The diverse plant growth-promoting traits of the isolated endophytic bacteria suggest their potential as biofertilisers in sustainable agriculture. Their ability to produce IAA, ammonia, and solubilise phosphate can enhance plant growth, improve nutrient availability, and reduce dependence on chemical fertilizers. With a minimum tolerance of 3% NaCl and a maximum tolerance of 10% NaCl, all isolates demonstrated remarkable resistance to high salt concentrations. The isolates exhibited varying degrees of NaCl tolerance: PTS-2 tolerated up to 10%, GPS-4 up to 8%, GPL-1, GPL-2, GPL-3, and HAS-3 up to 7%, HAL-1 and PTR-6 up to 6%, GPR-5, HAL-2, PTR-5, PTL-4, and PTL-3 up to 5%, PTS-1 up to 4%, and HAS-4 up to 3%. In agriculture, these isolates can be used to boost plant growth when salt stress is present. In antibiotic sensitivity test, some isolates, such as PTS-1, PTR-5, GPL-2, GPS-4, GPR-5, HAL-1, HAL-2, HAS-3, HAS-4, and HAR-5 exhibited resistant (R) to multiple antibiotics, suggesting a strong ability to withstand certain treatments. Others, like PTS-2, PTL-3, PTL-4, PTR-6, GPL-1, and GPS-3 demonstrated high sensitivity to multiple antibiotics, as evidenced by large inhibition zone diameters across several antibiotics. Intermediate responses were also observed in some cases, indicating partial susceptibility. The isolates which showed resistant to multiple antibiotics can be used for bioremediation treatment to reduce

environmental pollution. These isolates are also crucial for biotechnological research because these isolates can use in extraction of antibiotic-resistant genes.

The hydrolytic enzyme-producing abilities of 16 endophytic bacterial isolates were evaluated for amylase, protease, lipase, cellulase, pectinase, and xylanase activities. Protease was the most commonly produced enzyme, detected in 13 isolates, while amylase was the least common, found in only six isolates. Some isolates, like PTR-6 and HAL-1, exhibited broad enzyme production, whereas others, such as PTL-3 and HAL-2, showed limited enzymatic activity. These isolates can be used in the food and beverage, textile, leather, and pharmaceutical industries for mass production of such enzymes.

Among the isolates, PTS-1 exhibited the strongest antimicrobial activity against five pathogenic bacteria: *S. aureus* (MTCC 737), *E. coli* (MTCC 443), *B. subtilis* (MTCC 441), *E. aerogenes* (MTCC 2822), and *P. aeruginosa* (MTCC 1688). Following PTS 1, PTS-2 and PTL-3 exhibited antibacterial activity against three of six pathogenic bacteria. Then, GPL-1, GPL-2, GPS-4 and GPR-5 showed inhibitory activity against two of six pathogenic bacteria. Antimicrobial activity against at least one pathogenic bacterium was demonstrated by PTL-4, PTR-6, GPL-3, HAL-1, HAL-2, HAS-3, and HAR-5. These isolates can be used in the production of antimicrobial drugs whereas thorough study is needed.

In conclusion, the work makes a substantial contribution to the development of a biofertiliser that can be used in agriculture in place of chemical fertilisers. Also, the isolates can be used in agriculture to fight abiotic stress like salt stress, as the isolates can help the plant to grow in the soil which contains high salt concentration. The findings also contributed to introducing *K. assamensis*, *P. equi* and *A. gibsonii* as endophytic bacteria for the first time.