4 RESULTS CHAPTER 4

4.1 Isolation of Endophytic bacteria

For the successful isolation of endophytic bacteria surface sterilisation (SS) of the tissues plays an important role. SS removes all the contaminants and epiphytes from the surface of the tissue. In this study, 70% ethanol and sodium hypochlorite are used in different concentrations as well as for different durations for different tissues. Proper optimization of SS was done. Standardised methods were shown in Table 4.

Table 4: Standardised surface sterilisation method for isolation of endophytic bacteria

Sl. No.	Tissues	Medicinal plants	70% ethanol duration	% of sodium hypochlorite with duration	10% sodium bicarbonate duration
1	Leaves	*G. pentaphylla*	3 minutes	2% sodium hypochlorite 7-10 mins	10 minutes
2	Stems			2% sodium hypochlorite 10-12 minutes	10 minutes
3	Roots			2% sodium hypochlorite 12-14 minutes	10 minutes
4	Leaves	*P. thyrsiformis*	1 minute	1% sodium hypochlorite 7-10 mins	10 minutes
5	Stems			1.5% sodium hypochlorite for 10-12 mins	10 minutes
6	Roots			2% sodium hypochlorite for 10-12 mins	10 minutes

7	Leaves	*H. auriculata*	1 minute	0.9% sodium hypochlorite 8-10 mins	10 minutes
8	Stems			1% sodium hypochlorite for 12-14 mins	10 minutes
9	Roots			2% sodium hypochlorite for 10 to 12 mins	10 minutes

The conformation of isolation of endophytic bacteria was done by inoculating last sample washed water in both LB agar and NA media. And incubated at 30℃ for 7 to 10 days. No microbial growth was observed which means the isolated microorganisms were endophytic bacteria.

After successful SS, endophytic bacterial growth was observed from all the tissues, like leaves, stems, and roots, of three medicinal plants: *G. pentaphylla*, *H. auriculata*, and *P. thyrsiformis* (Plate 2, 3 & 4). Following the successful isolation, sub-culturing was done for multiple times to obtain pure culture of each isolate. Pure culture was obtained for each isolate and then the isolates were stored at 4℃ for performing rest of the process. Samples were inoculated in both LB and nutrient media. During sub-culturing, the best isolate growth was observed in LB media. As some of isolates were unable to grow properly in nutrient media. So, for the rest of the process, LB media was used. A total of 16 endophytic bacteria were isolated from roots, stems and leaves of the three medicinal plant. From *P. thyrsiformis*, 2 isolates from roots; 2 isolates from leaves; 2 isolates from stems. From *H. auriculata*, 2 isolates from leaves; 2 from stems; 1 from roots. From *G. pentaphylla*, 3 from leaves; 1 from stems; 1 from roots.

4.2.1 Morphological and microscopic (SEM) characterisation:

From the roots, stems, and leaves of three therapeutic plants, 16 endophytic bacteria were identified. Ten of the sixteen had gram-positive characteristics, whereas six had gram-negative characteristics. The morphological features of these microorganisms

were distinct (Table 5), such as isolates of *P. thyrsiformis* such as PTS-1 is green color, irregular in shape and raised colony; PTS-2 is white color, small, circular, raised colony; PTL-3 is creamy color, irregular, raised; PTL-4 is yellow color, small, circular, raised colony; PTR-5 is creamy white, small, circular, slightly raised colony; PTR-6 is pale orange, small, circular, flat colony. Colony morphology of isolates of *H. auriculata*, such as HAL-1 is yellow, irregular, medium size, raised colony; HAL-2 is white, irregular, large size, raised colony; HAS-3 is yellow color, circular, medium size, slightly raised to convex colony; HAS-4 is creamy color, circular, medium size, slightly raised colony; HAR-5 is white, circular, large sizes slightly raised colony. Colony morphology of isolates of *G. pentaphylla*, such as GPL-1 is creamy color, small round, raised colony; GPL-2 is white creamy color, medium size, raised colony; GPL-3 is light pink, white filamentous, irregular, slightly raised colony; GPS-4 is white color, round, medium size, raised colony; GPR-5 is white color, small, round, raised colony (Plate 5, 6, 7).

For microscopic analysis bacterial cells were observed under SEM to examine their microscopic morphology. The majority of the isolates (15 out of 16) displayed rod shapes with the exception of PTL-4, which displayed circular under SEM. The images were taken in different magnifications (Plate 8.1, 8.2 & 8.3). The sizes of the rods were varied which ranges from 1 µm to 4 µm.

PLATE 2

Isolation of endophytic bacteria from different tissues of *G. pentaphylla*: (A, B) isolation from leaves; (C) plate used for sterility test; (D) isolation of endophytic bacteria from roots; (F) plate used for sterility test

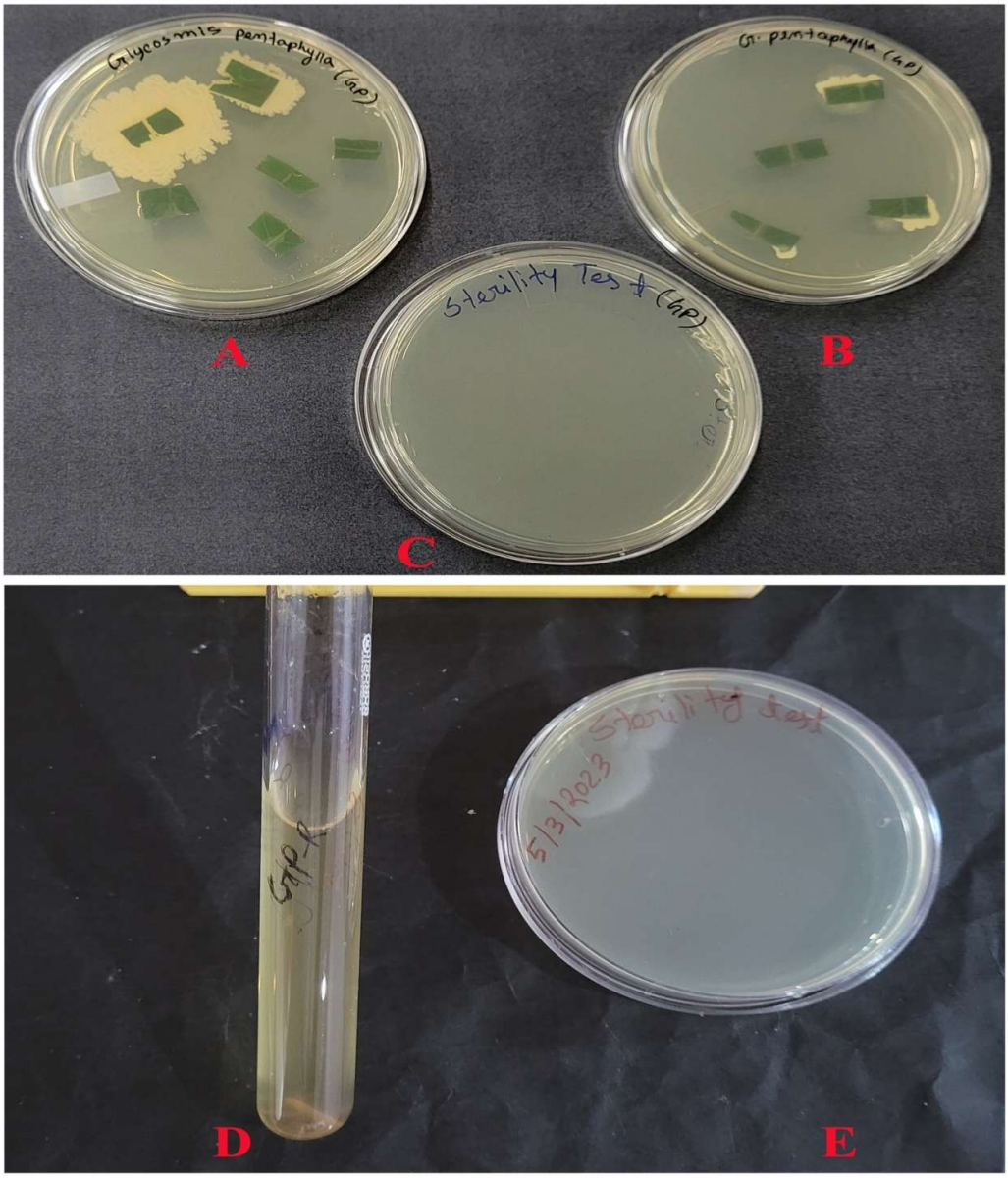


PLATE 3

Isolation of endophytic bacteria from different tissues of *P. thyrsiformis*, (A) Isolation of endophytic bacteria from leaves; (B) plate used for sterility test; (C) isolation of endophytic bacteria from roots; (D) isolation of endophytic bacteria from roots in broth media

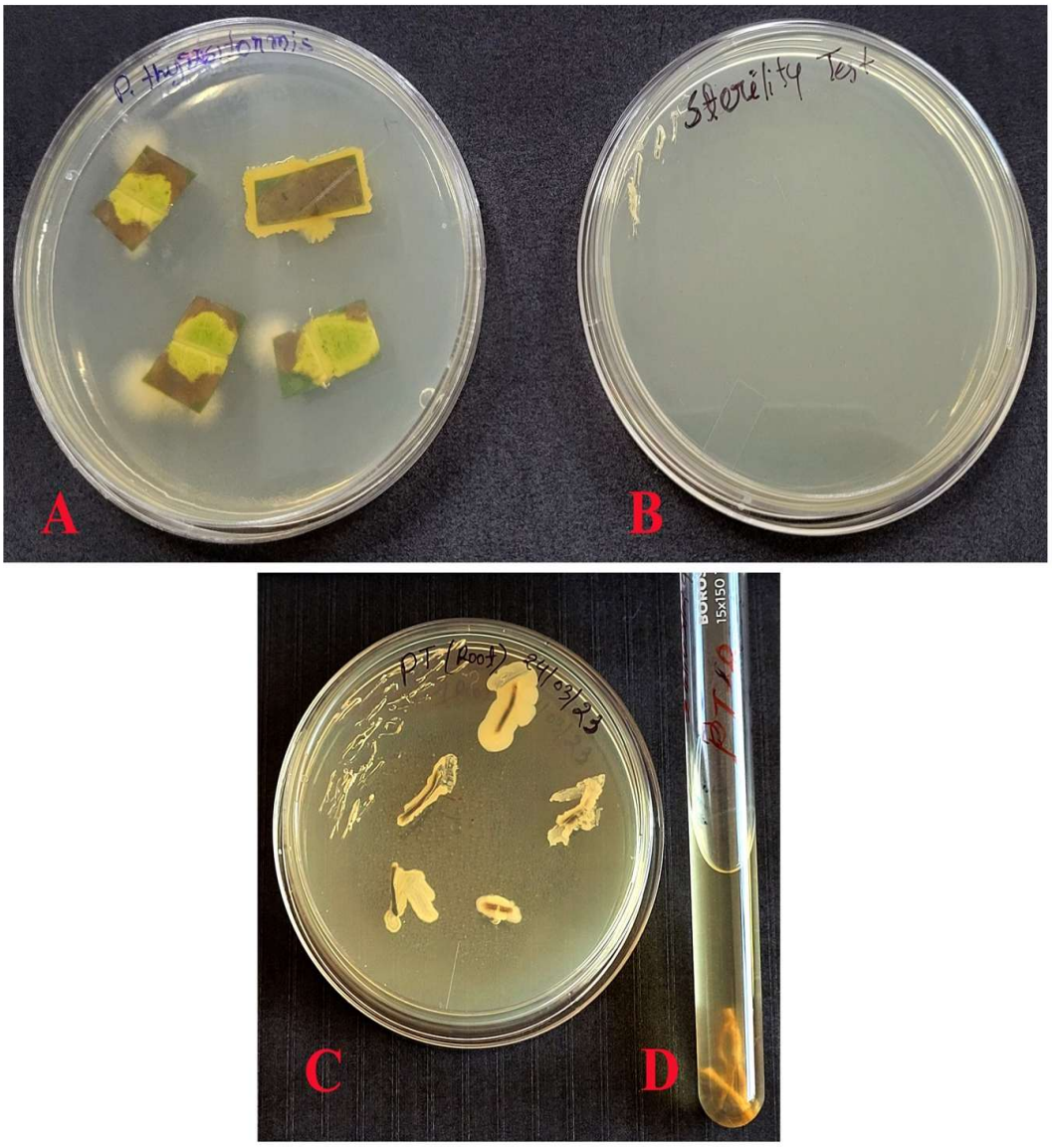


PLATE 4

Isolation of endophytic bacteria from different tissues of *H. auriculata*; (A) Plate used in sterility test; (B) isolation of endophytic bacteria from leaves; (C) isolation of endophytic bacteria from roots; (D) isolation of endophytic bacteria from roots in broth; (E) plate used for sterility test

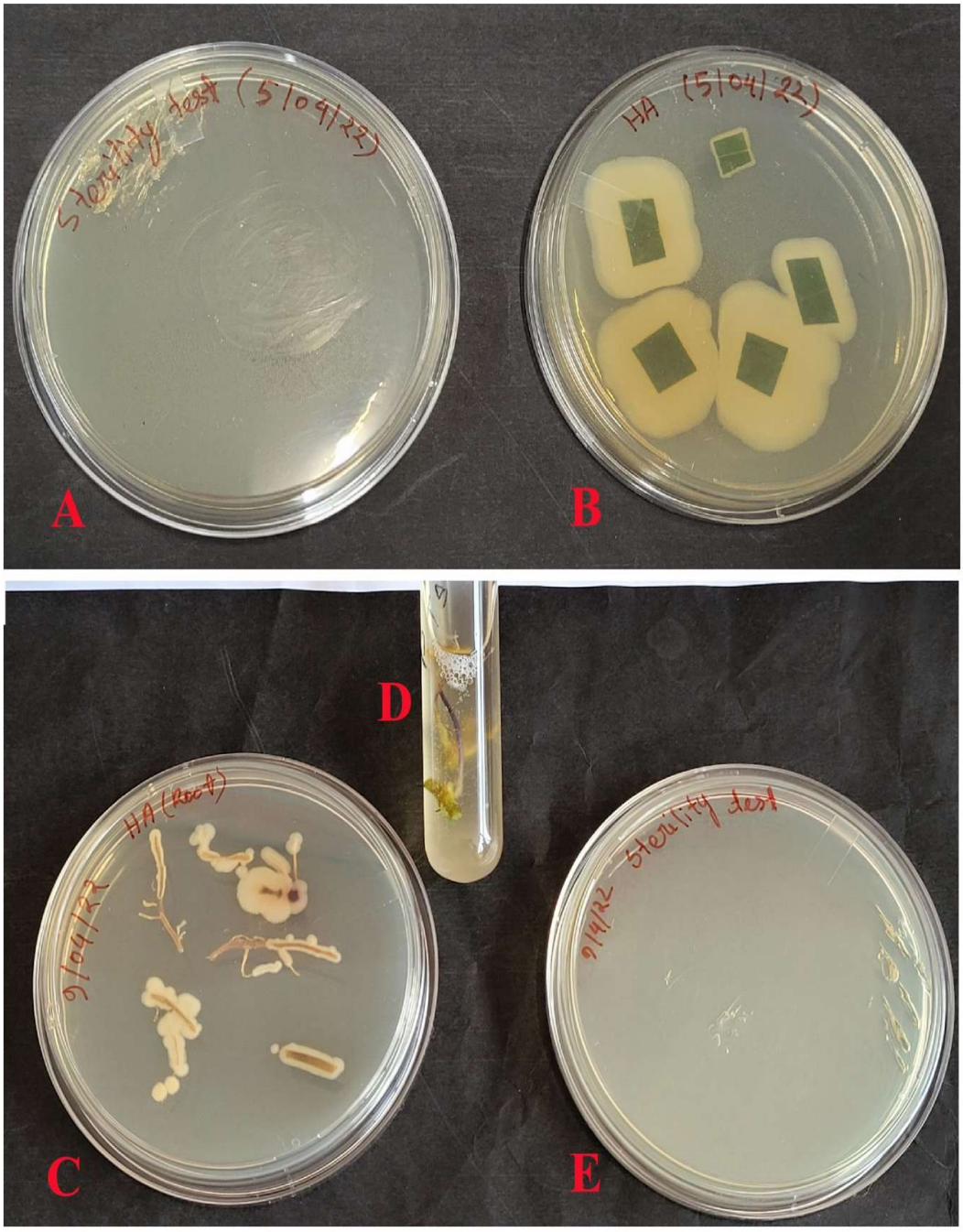


PLATE 5

Isolates of *G. pentaphylla*, (A) GPL-1; (B) GPL-2 (upper one), GPL-3 (lower one); (C) GPS-4; (D) GPR-5

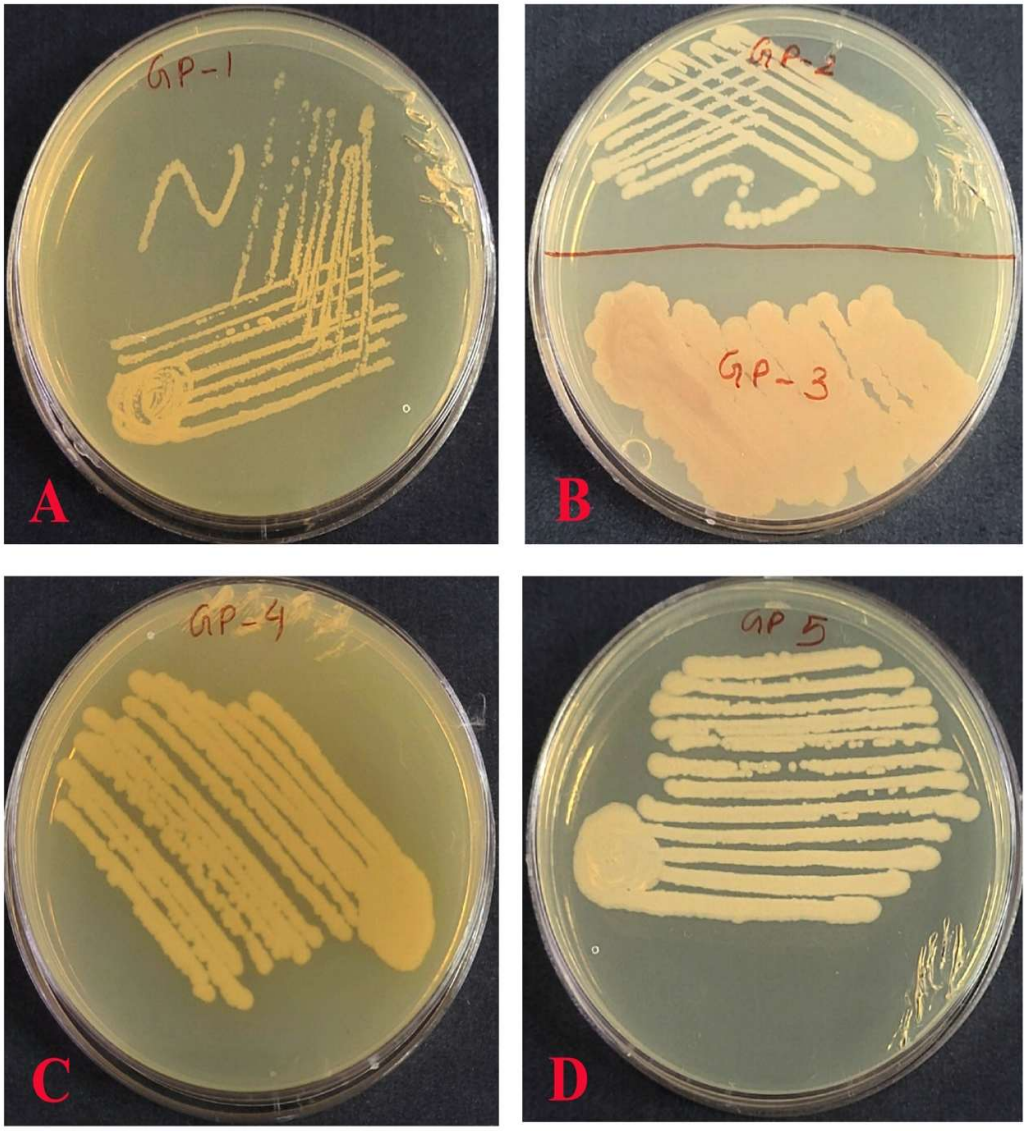


PLATE 6

Isolates of *P. thyrsiformis*, (A) PTS-1; (B) PTS-2; (C) PTL-3; (D) PTL-4; (E) PTR-5; (F) PTR-6

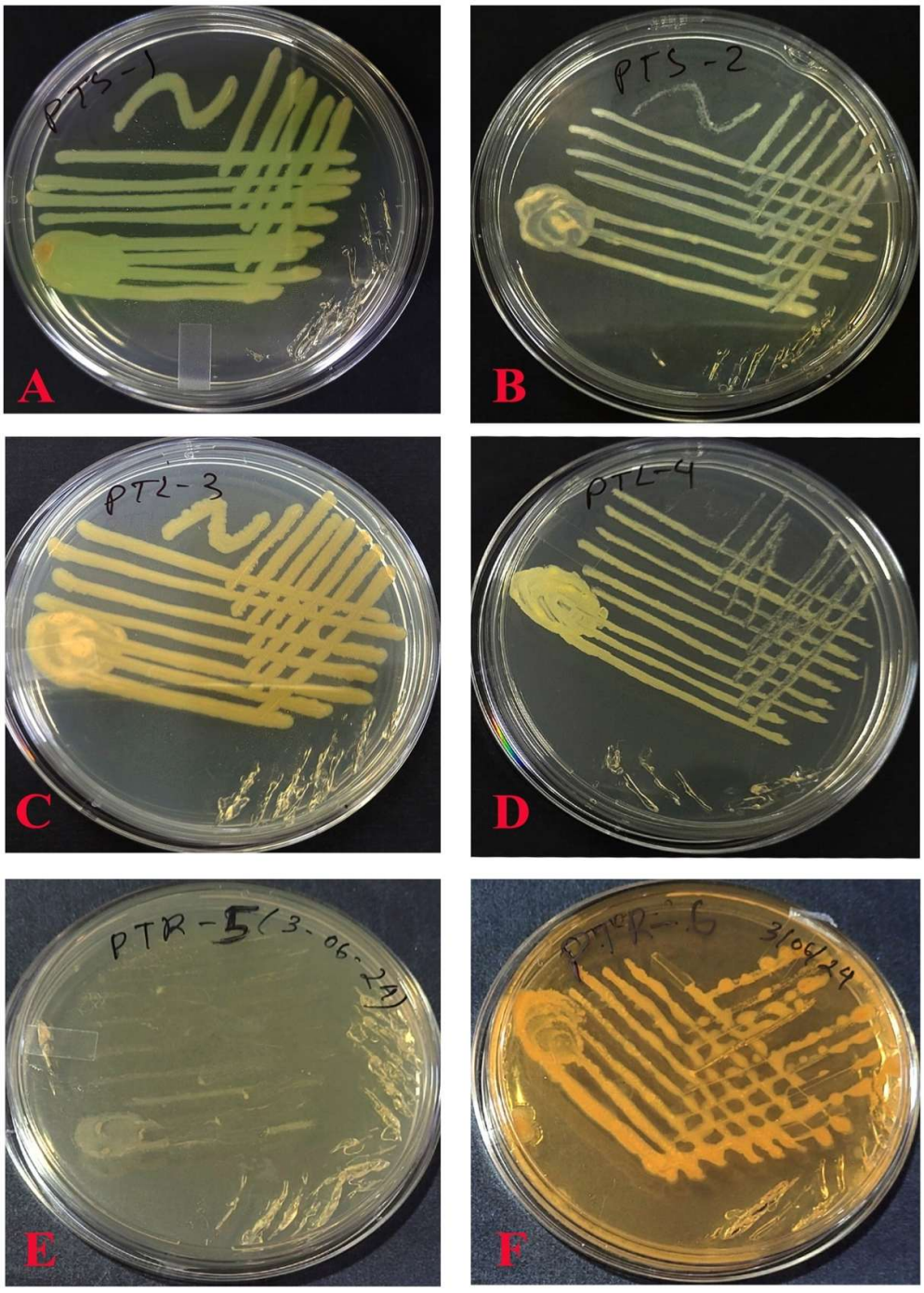


PLATE 7

Isolates of *H. auriculata*, (A) HAS-4; (B) HAL-1; (C) HAL-2; (D) HAS-3; (E) HAR-5

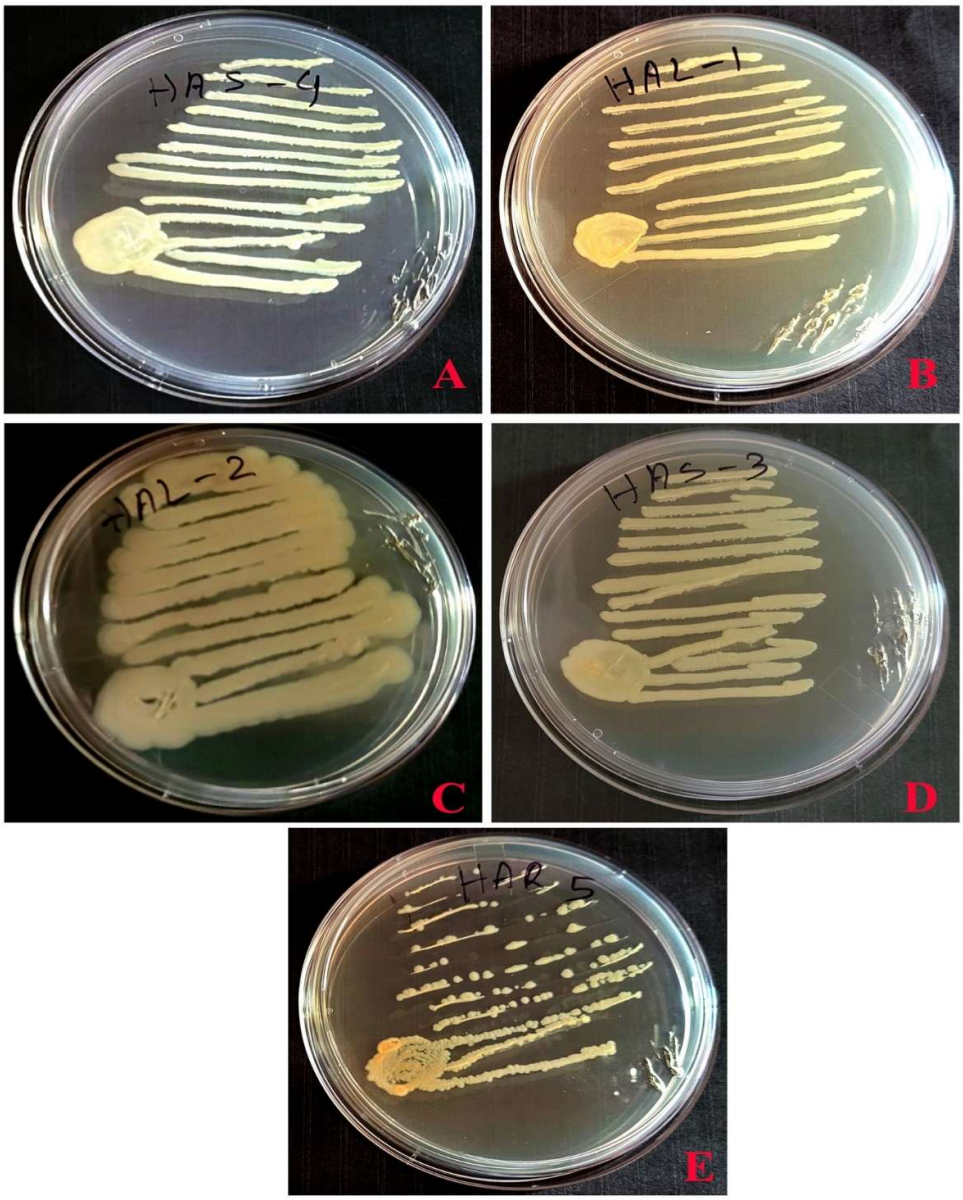


Table 5: Morphological characterisation of isolated endophytic bacteria

Sl. no	Isolates	Gram nature	Colony morphology		
			Color	Shape	Appearance
1	PTS-1	Gram -ve, rod shaped	Green color	Irregular	Raised
2	PTS-2	Gram -ve, rod shaped	White color	Small, circular	Raised,
3	PTL-3	Gram +ve, rod shaped	Creamy color colony	Irregular,	Raised
4	PTL-4	Gram +ve, cocci	Yellow color colony	Small, circular	Raised
5	PTR-5	Gram +ve, rod shaped	Creamy white	Small, circular	Slightly raised
6	PTR-6	Gram +ve, rod shaped	Pale orange	Small, circular	Flat
7	GPL-1	Gram +ve, rod shaped	Creamy color	Small round	raised
8	GPL-2	Gram +ve, rod shaped	White creamy color	Medium size, round	raised
9	GPL-3	Gram +ve, rod shaped	Light pink, white filamentous,	Irregular	Slightly raised
10	GPS-4	Gram +ve, rod shaped	White color	Round, medium size	Raised
11	GPR-5	Gram +ve, rod shaped	White color	Small, round	Raised
12	HAL-1	Gram -ve, rod shaped	Yellow	Irregular, medium size	Raised
13	HAL-2	Gram -ve, rod shaped	White	Irregular, large size	Raised

14	HAS-3	Gram -ve, rod shaped	Yellow color	Circular, medium size	Slightly raised to convex.
15	HAS-4	Gram -ve, rod shaped	Creamy color	Circular, medium size	Slightly raised
16	HAR-5	Gram +ve, rod shaped	White	Circular, large size	Slightly raised

PLATE 8.1

Images of isolates under SEM, (A) GPL-1; (B) GPL-2; (C) GPL-3; (D) GPS-4; (E) GPR-5; (F) HAL-1

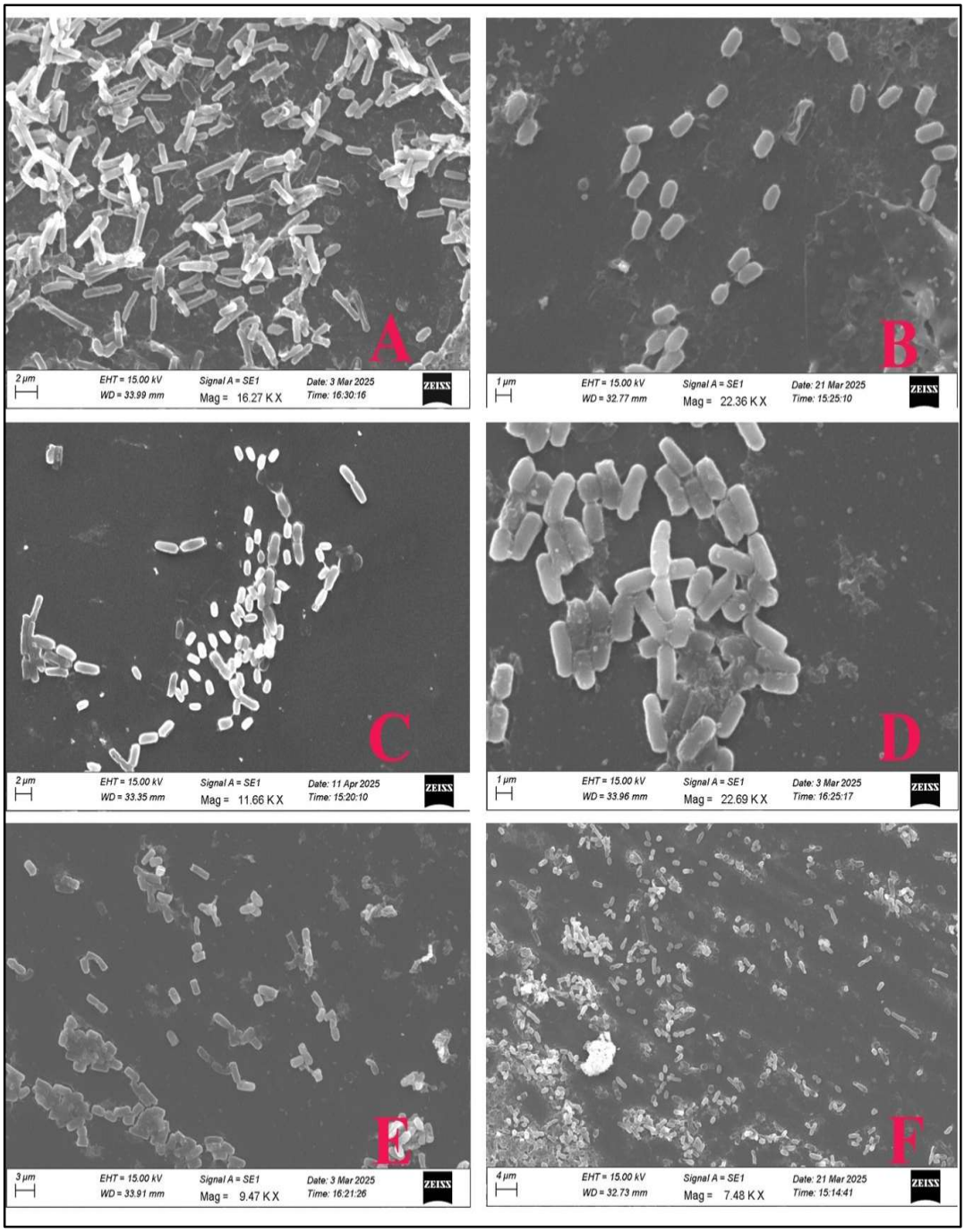


PLATE 8.2

Images of isolates under SEM, (G) HAL-2; (H) PTL-4; (I) HAS-3; (J) HAS-4; (K) PTL-3; (L) HAR-5

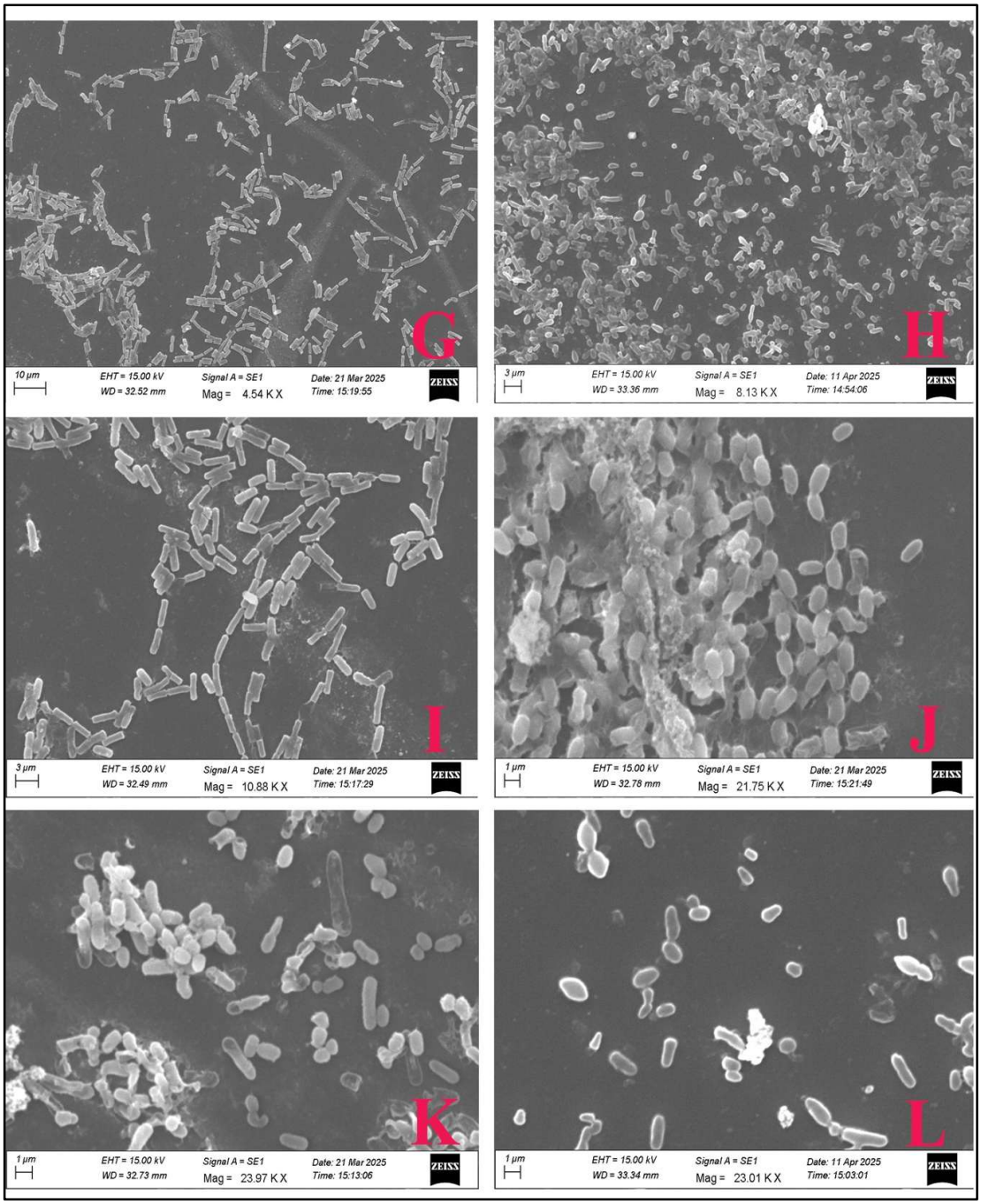
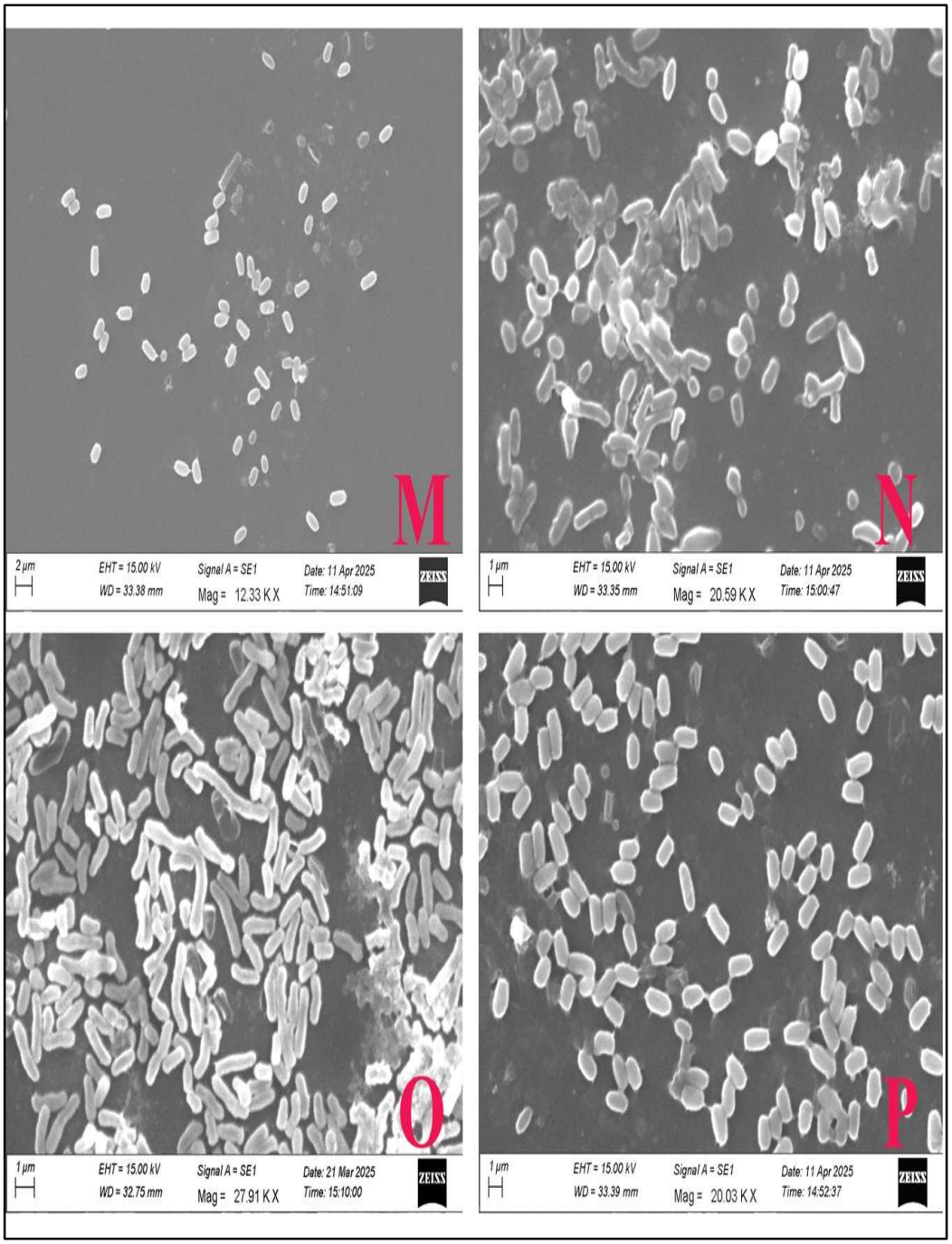


PLATE 8.3

Images of isolates under SEM, (M) PTR-5; (N) PTR-6; (O) PTS-1; (P) PTS-2



4.2.2 Biochemical characterisation of the isolates:

In biochemical characterisation almost all isolates showed positive for catalase test and negative for indole test. Almost all isolates showed positive for oxidase test except PTL-3, PTL-4, PTR-6, HAL-2, HAS-3 and HAR-5. All the isolates showed positive for citrate test except PTR-6. In methyl red test, all isolates showed negative results except HAL-2 which showed positive result. Out of sixteen, eleven isolates showed positive and five showed negative for voges-proskauer test (Table 6).

Table 6: Biochemical characterization of the isolated endophytic bacteria

SL NO	Isolates	Catalase	Oxidase	Citrate	Indole	Methyl red	Voges proskauer
1	PTS-1	Positive	Positive	Positive	Negative	Negative	Negative
2	PTS-2	Positive	Positive	Positive	Negative	Negative	Positive
3	PTL-3	Positive	Negative	Positive	Negative	Negative	Positive
4	PTL-4	Positive	Negative	Positive	Negative	Negative	Negative
5	PTR-5	Positive	Positive	Positive	Negative	Negative	Positive
6	PTR-6	Positive	Negative	Negative	Negative	Negative	Negative
7	HAL-1	Positive	Positive	Positive	Negative	Negative	Negative
8	HAL-2	Positive	Negative	Positive	Negative	Positive	Negative
9	HAS-3	Positive	Negative	Positive	Negative	Negative	Positive
10	HAS-4	Positive	Positive	Positive	Negative	Negative	Positive
11	HAR-5	Positive	Negative	Positive	Negative	Negative	Positive
12	GPL-1	Positive	Positive	Positive	Negative	Negative	Positive

13	GPL-2	Positive	Positive	Positive	Negative	Negative	Positive
14	GPL-3	Positive	Positive	Positive	Negative	Negative	Positive
15	GPS-4	Positive	Positive	Positive	Negative	Negative	Positive
16	GPR-5	Positive	Positive	Positive	Negative	Negative	Positive

4.2.3 Antibiotic sensitivity test:

Isolates were tested for antibiotic sensitivity against 5 standard antibiotics. An antibiotic sensitivity test was performed by inoculating the isolates on the MHA media and placing the 5 discs of the standard antibiotics over it. Then, the plates were incubated at 30 ℃ for 24 h. After 24 h of incubation, the zone of inhibition was measured (Plate 9.1 & 9.2), and the isolates were divided into three categories, like resistant, intermediate, and susceptible. Zones of inhibition were measured, and detailed results were included in Table 7. 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.

PLATE: 9.1

Antibiotic Sensitivity Test Results of (A) HAS-3 (B) HAL-2, (C) PTS-1, (D) PTS-2, (E) PTL-3, (F) PTL-4

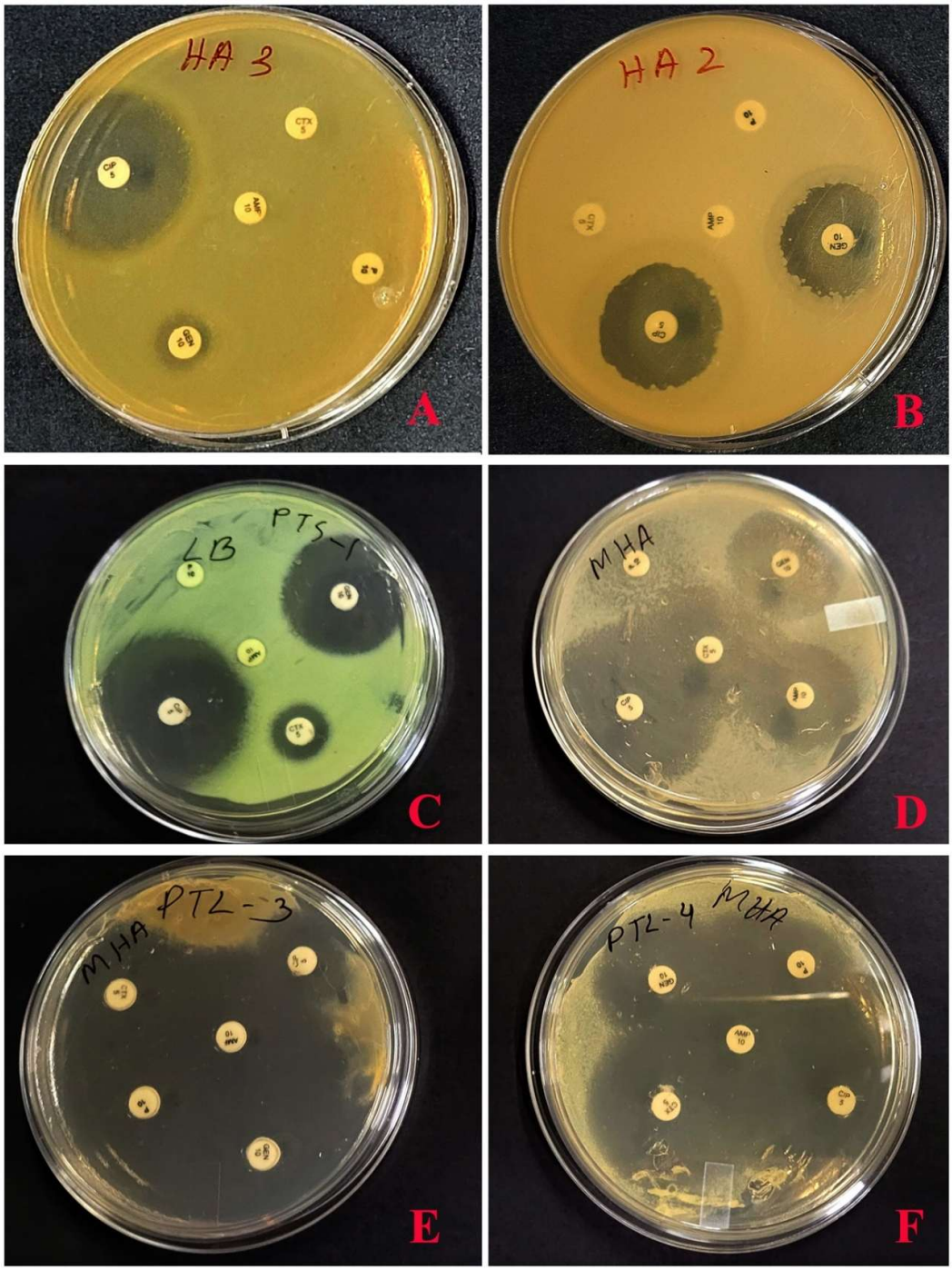


PLATE 9.2

Antibiotic sensitivity test results of (A) GPS-4; (B) GPR-5; (C) GPL-3; (D) GPL-2; (E) GPL-1; (F) HAL-1

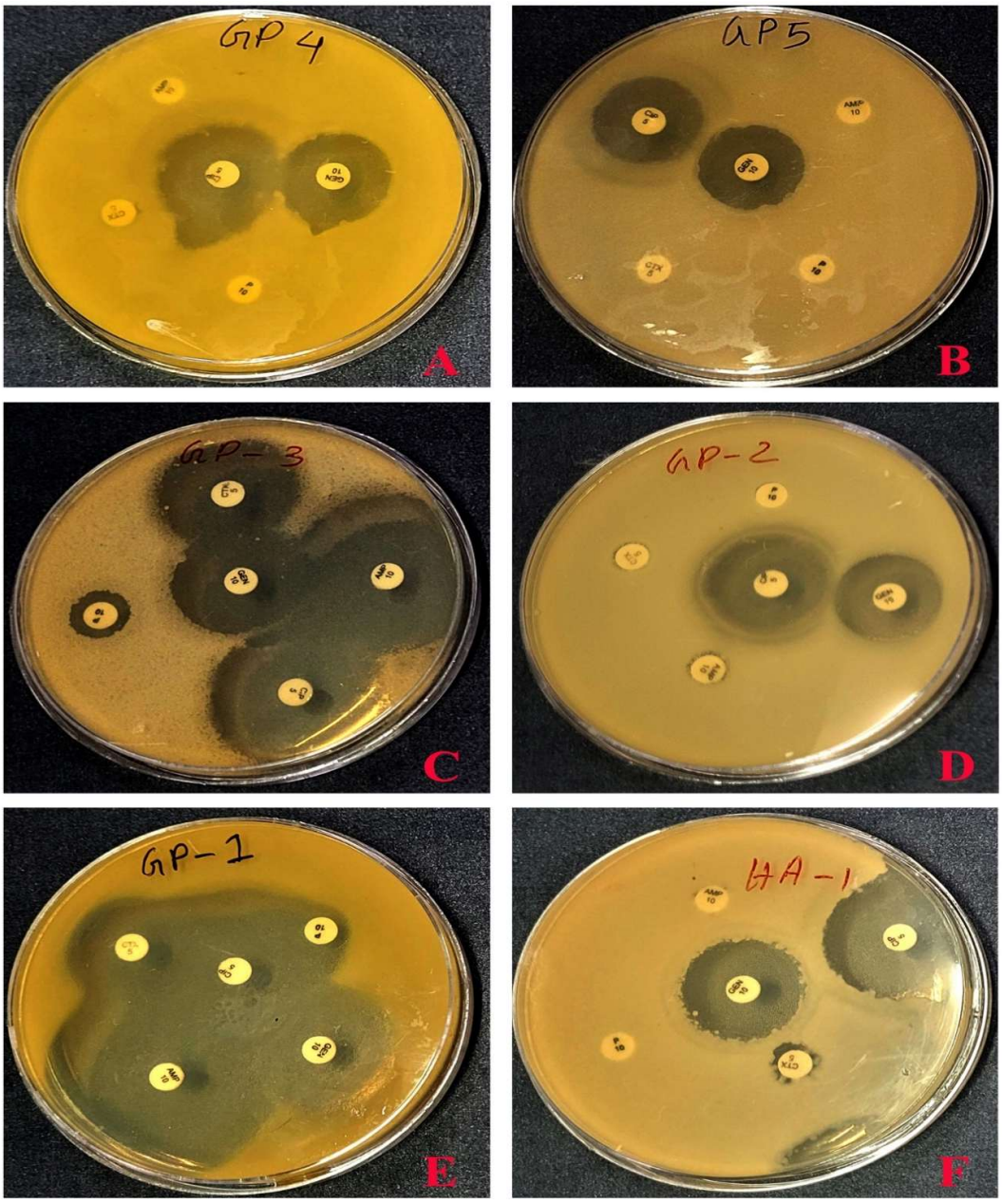


Table 7: Antibiotic sensitivity test results of the isolated endophytic bacteria

Isolates	Antibiotics with different concentrations				
	Amp 10	**P 10**	**GEN 10**	**CIP 5**	**CTX 5**
PTS-1	**R**	**R**	23.5 ± 0.40 mm (S)	28.16 ± 0.62 mm (S)	10.16 ± 0.84 mm **(R)**
PTS-2	19 ± 0.81 mm (S)	**R**	22.16 ± 0.62 mm (S)	33.66 ± 0.62 mm (S)	25.16 ± 0.84 mm (S)
PTL-3	38.9 ± 0.53 mm (S)	10.6 ± 0.43 mm **(R)**	26.73 ± 0.52 mm (S)	25.23 ± 0.71 mm (S)	29.8 ± 0.58 mm (S)
PTL-4	40.5 ± 1.08 mm (S)	19.9 ± 0.29 mm **(R)**	29 ± 0.16 mm (S)	32 ± 0.81 mm (S)	38.5 ± 0.40 mm (S)
PTR-5	**R**	**R**	19.1 ± 0.29 mm (S)	27.5 ± 0.40 mm (S)	21.43 ± 0.49 mm (S)
PTR-6	28.63 ± 0.38 mm (S)	**R**	24.03 ± 0.20 mm (S)	21.76 ± 0.20 mm (S)	**18.9 ± 0.29 mm (I)**
GPL-1	49.06 ± 0.41 mm (S)	20.66 ± 0.49 mm **(R)**	30.5 ± 0.40 mm (S)	39.93 ± 0.73 mm (S)	29.76 ± 0.20 mm (S)
GPL-2	**R**	**R**	20.73 ± 0.52 mm (S)	25.36 ± 0.26 mm (S)	**R**
GPL-3	40.16 ± 0.62 mm (S)	15 ± 0.40 mm **(R)**	26.53 ± 0.41 mm (S)	35.53 ± 0.44 mm (S)	30.6 ± 0.43 mm (S)

GPS-4	**R**	**R**	23.33 ± 0.47 mm (S)	24.53 ± 0.41 mm (S)	**R**
GPR-5	**R**	**R**	2.2 ± 0.21 mm **(R)**	**19.03 ± 0.12 mm (I)**	**R**
HAL-1	**R**	**R**	25 ± 0.16 mm (S)	30.7 ± 0.53 mm (S)	10.23 ± 0.20 mm **(R)**
HAL-2	**R**	**R**	20.5 ± 0.40 mm (S)	24.86 ± 0.26 mm (S)	**R**
HAS-3	**R**	**R**	10.23 ± 0.20 mm **(R)**	34.93 ± 0.32 mm (S)	**R**
HAS-4	**R**	**R**	28.76 ± 0.20 mm (S)	31.5 ± 0.40 mm (S)	11.9 ± 0.29 mm **(R)**
HAR-5	**R**	**R**	24.86 ± 0.28 mm (S)	25.56 ± 0.32 mm (S)	**R**

Here, R= Resistant; I= Intermediate; S= Susceptible.

4.3 Molecular identification:

After successful DNA isolation from the endophytic bacterial colony, PCR was done to amplify the 16S r DNA gene using universal primers (27F and 1492R) primers. A 1% agarose gel was used to assess its quality, and a single band of high molecular weight DNA was seen (Fig 6). The PCR amplicon was resolved on an Agarose gel and showed a single distinct band of 1500 bp. With the use of aligner software and forward and reverse sequence data, consensus sequences for the 16S rDNA gene were produced. All the sequences were purified using BioEdit 7.2 software. After that BLAST was performed for 16S rDNA gene sequences, with the NCBI GenBank databases. After observing the maximum identity score, the first ten sequences were chosen and aligned using Clustal

W, a multiple alignment software application. MEGA 11 software was used to create phylogenetic trees using the maximum likelihood method after the distance matrix was generated (Plate 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7 & 10.8). PTS-1 was identified as *Pseudomonas aeruginosa* strain DD3; PTS- 2 as *Agrobacterium larrymoorei* strain DDBU3; PTL-3 as *Solibacillus silvestris* strain DDBU6; PTL-4 as *Kocuria assamensis* strain DDBU9; PTR-5 as *Alkalicoccobacillus gibsonii* strain PTR1DD; PTR-6 as *Prescottella equi* strain PTR2DD; HA-1 as *Pseudomonas oryzihabitans* strain HAL1DD; HA-2 as *Proteus mirabilis* strain HAL2DD; HA-3 as *Stenotrophomonas geniculata* strain HAS3DD; HA-4 as *Agrobacterium cavarae* strain HAS4DD; HA-5 as *Lysinibacillus macrolides* strain HARDD; GP-1 as *Alkalicoccobacillus gibsonii* strain GPDD1; GP-2 as *Bacillus cereus* strain GP2DD; GP-3 as *Bacillus subtilis* strain GP3DD; GP-4 as *Bacillus cereus* strain GP4DD; GP-5 as *Bacillus australimaris* strain GPRDD. The identified sequences were submitted to NCBI for accession number which were included in Table 8.

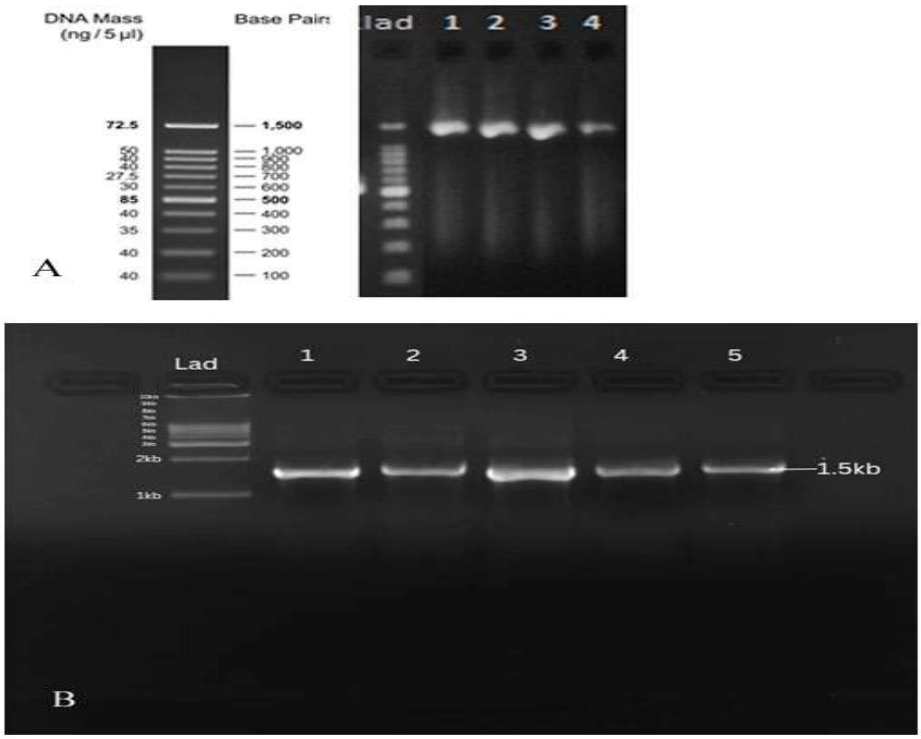


Fig 6: Gel electrophoresis photos of 16S r DNA gene amplification of isolates (A) Lad (ladder), (1) PTS-1, (2) PTS-2, (3) PTL-3, & (4) PTL-4; (B) (Lad) 1kb ladder, (1) GPL-1, (2) GPL-2, (3) GPL-3, (4) GPS-4, (5) GPR-5

PLATE 10.1

Phylogenetic tree of (A) GPL-1 (*Alkalicoccobacillus gibsonii* strain GPDD1); (B) GPL-2 (*Bacillus cereus* strain GP2DD)

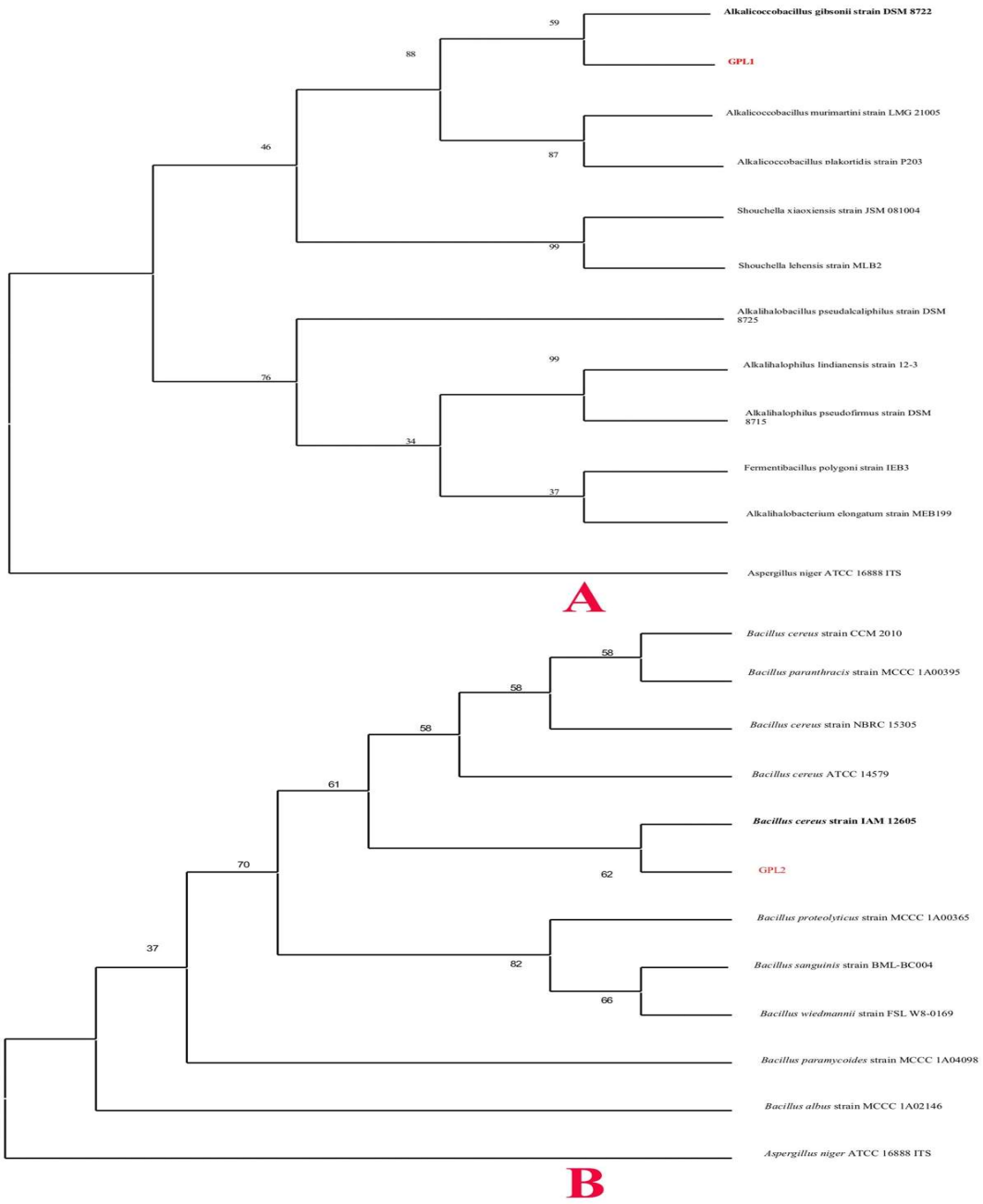


PLATE 10.2

Phylogenetic tree of (C) GPL-3 (*Bacillus subtilis* strain GP3DD) & (D) GPS-4 (*Bacillus cereus* strain GP4DD)

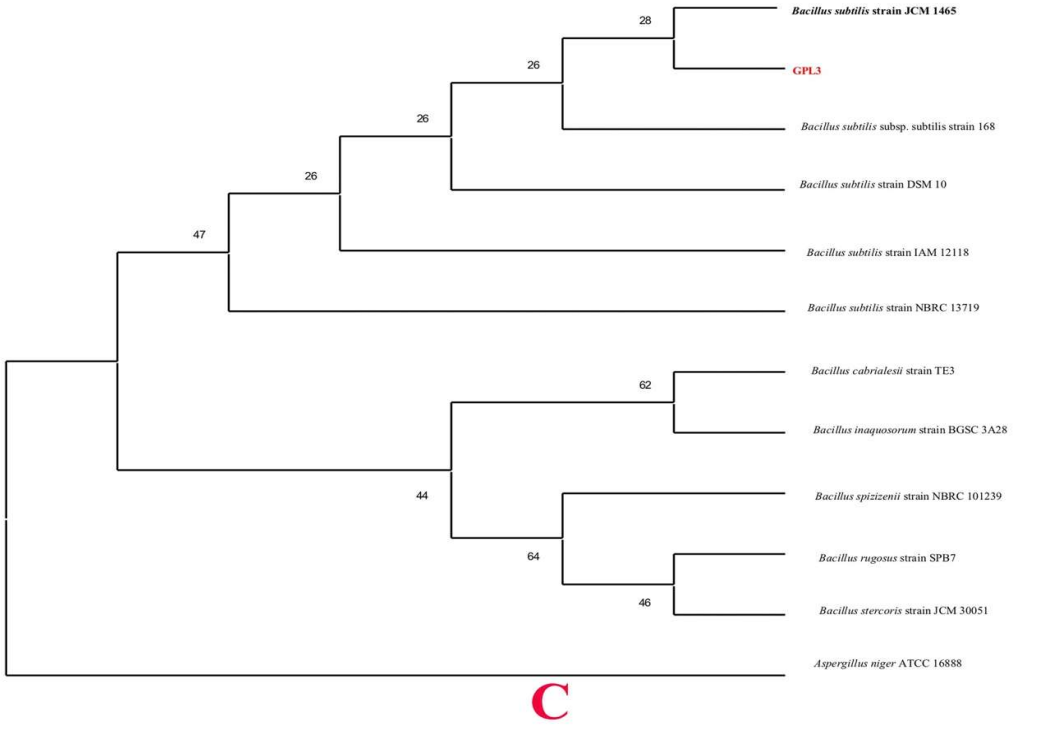
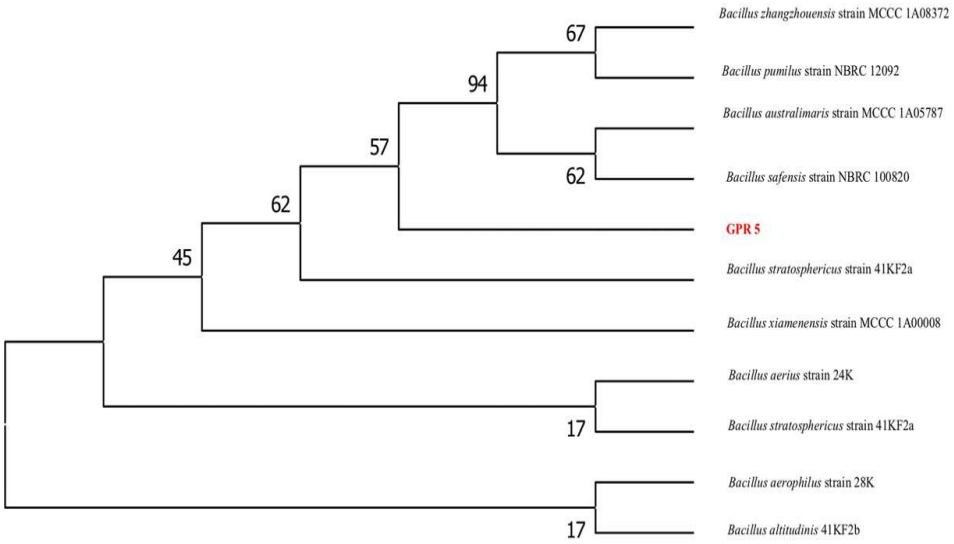
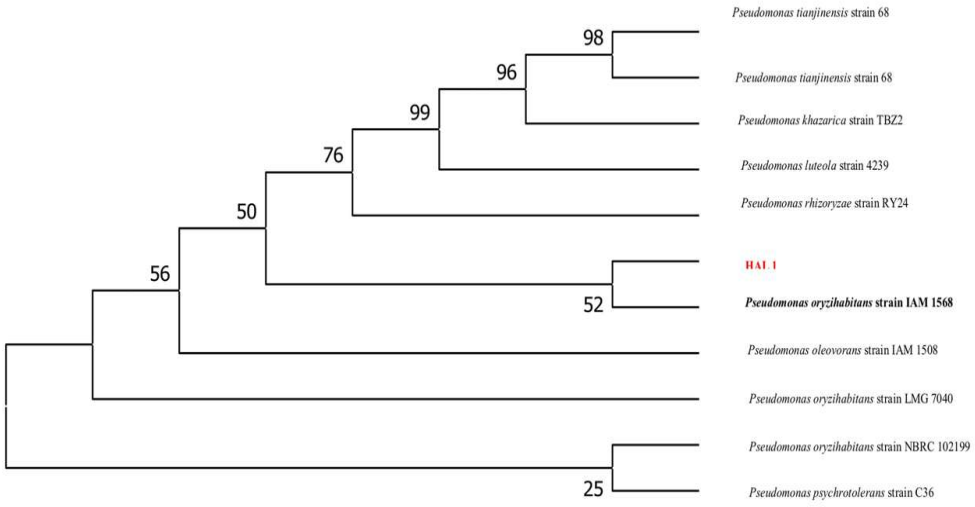


PLATE 10.3

Phylogenetic tree of (E) GPR-5 (*Bacillus australimaris* strain GPRDD) & (F) HAL-1 (*Pseudomonas oryzihabitans* strain HAL1DD)



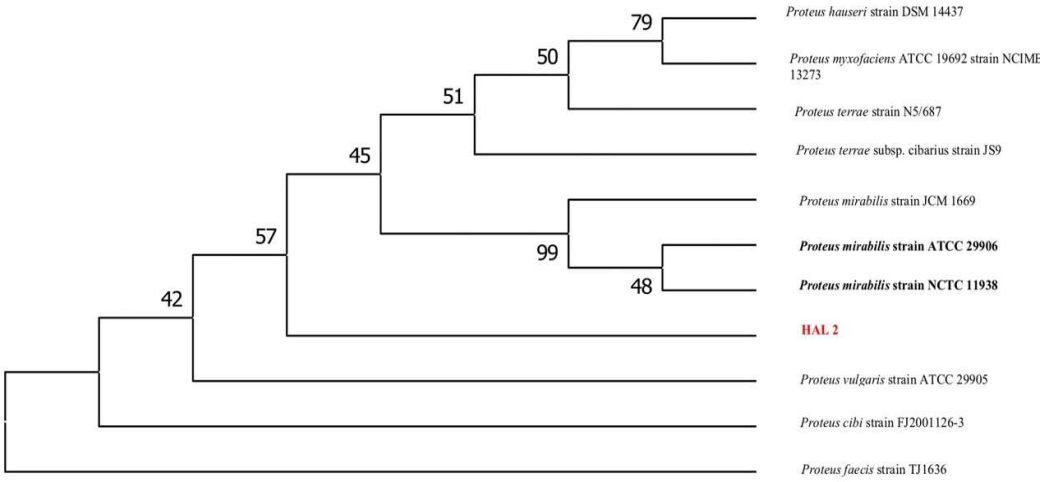
E



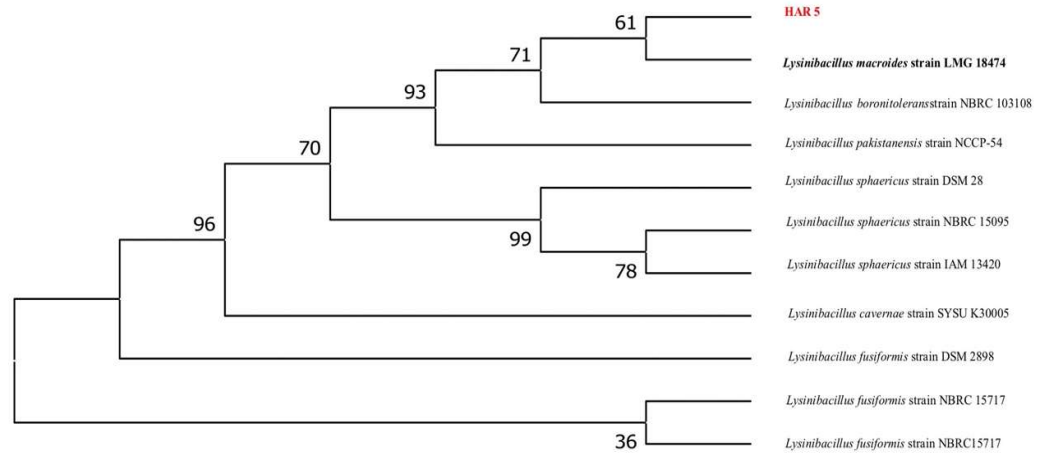
F

PLATE 10.4

Phylogenetic tree of (G) HAL-2 (*Proteus mirabilis* strain HAL2DD) & (F) HAR-5 (*Lysinibacillus macrolides* strain HARDD)



G



H

PLATE 10.5

Phylogenetic tree of (I) HAS-3 (*Stenotrophomonas geniculata* strain HAS3DD) & (J) HAS-4 (*Agrobacterium cavarae* strain HAS4DD)

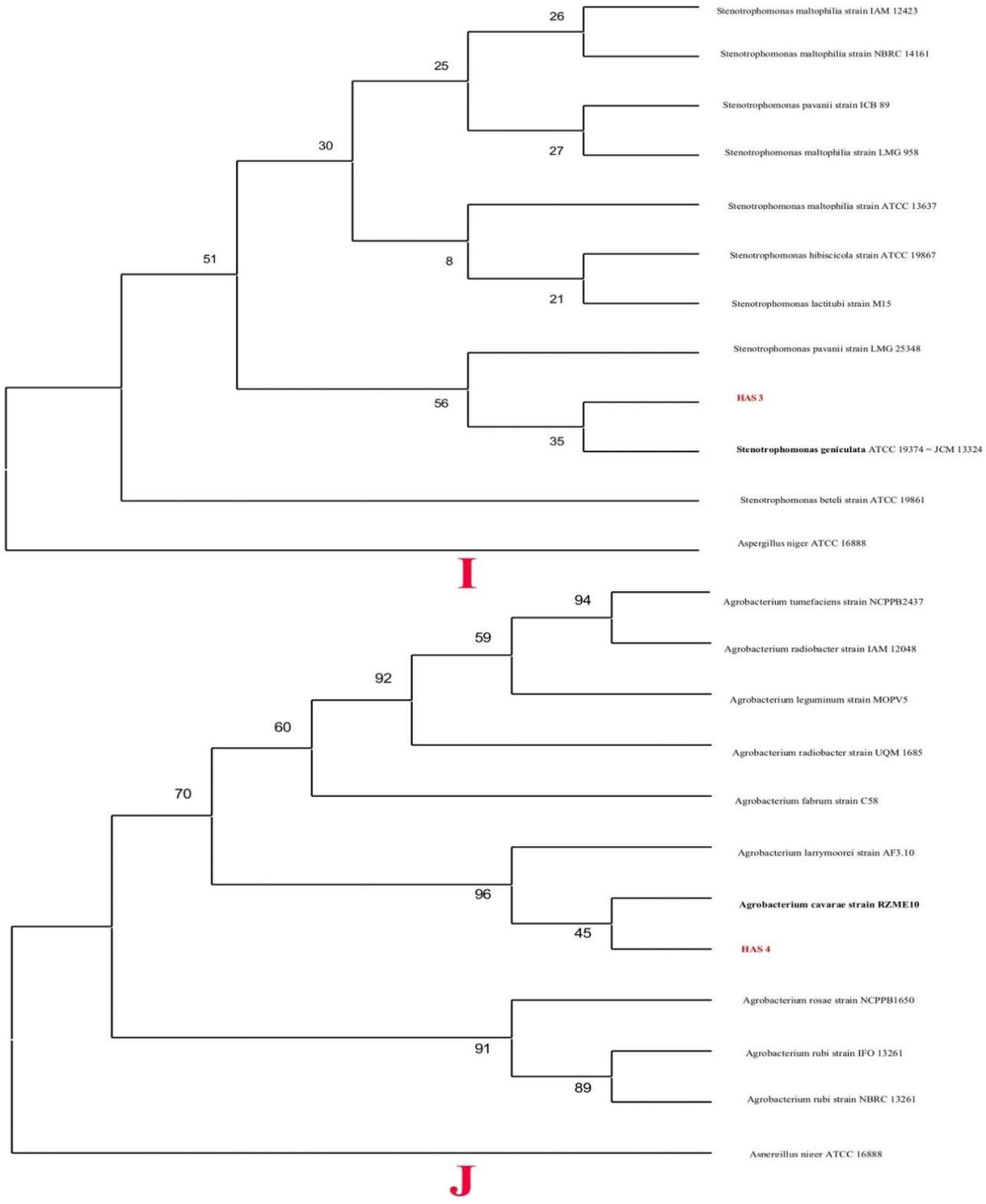


PLATE 10.6

Phylogenetic tree of (K) PTL-3 (*Solibacillus silvestris* strain DDBU6) & (L) PTL-4 (*Kocuria assamensis* strain DDBU9)

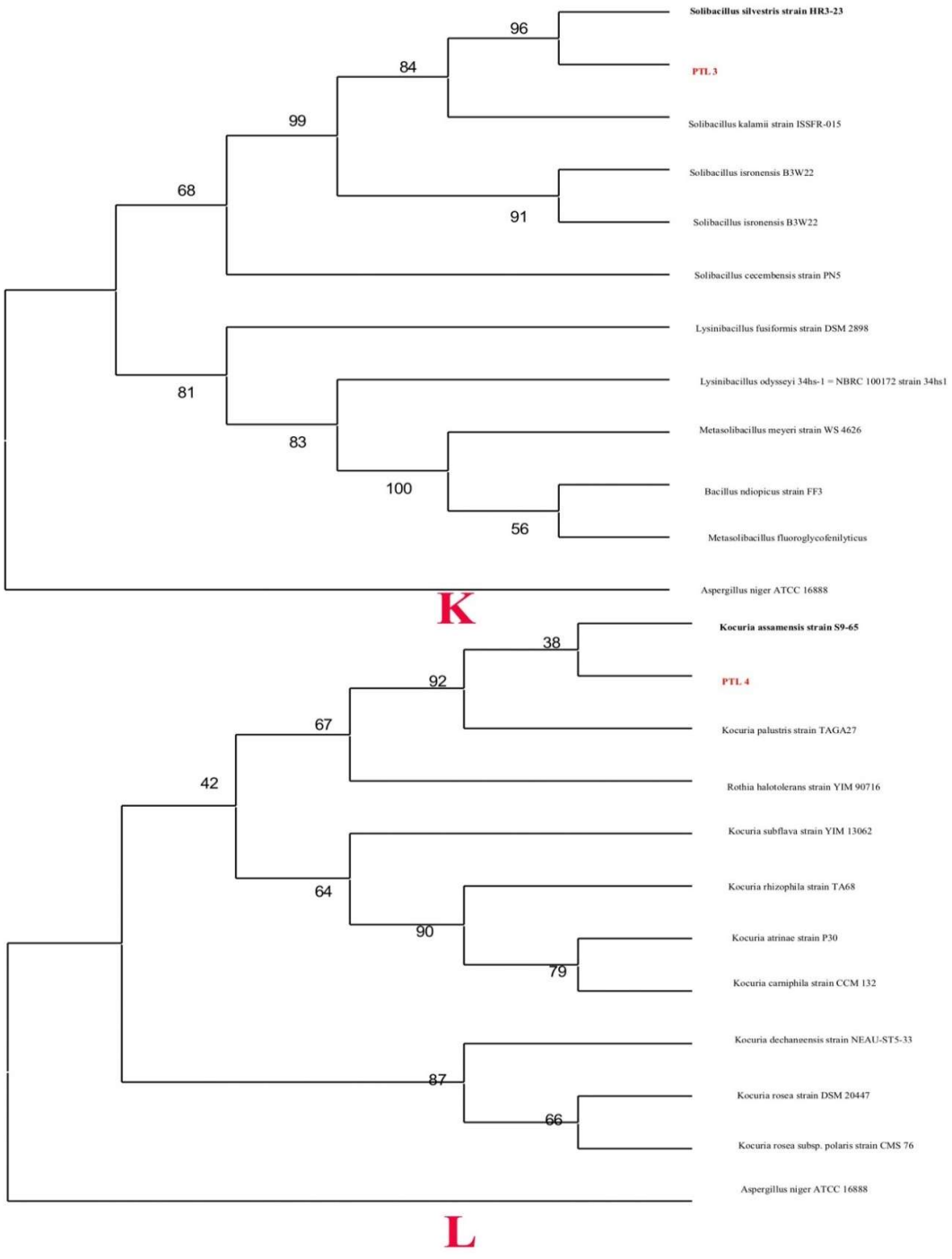
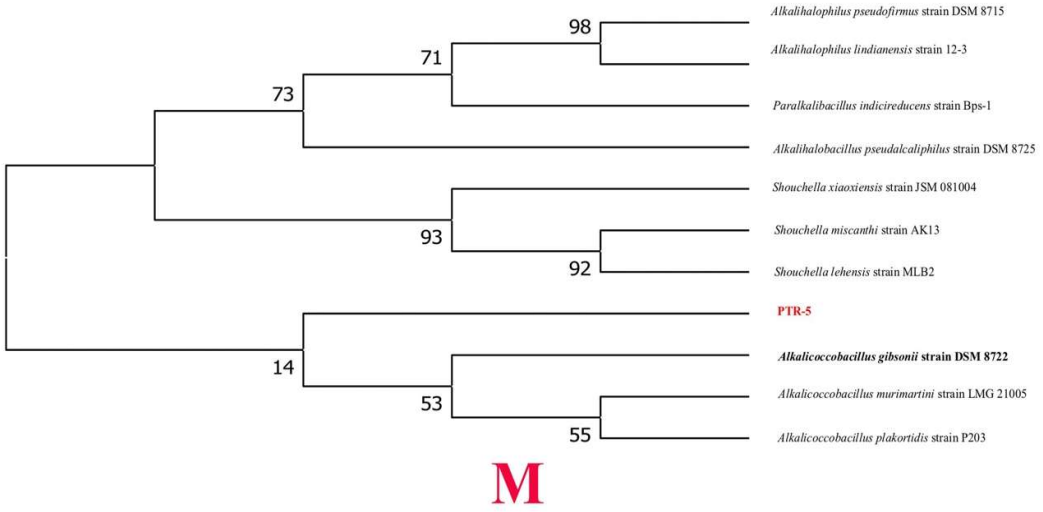
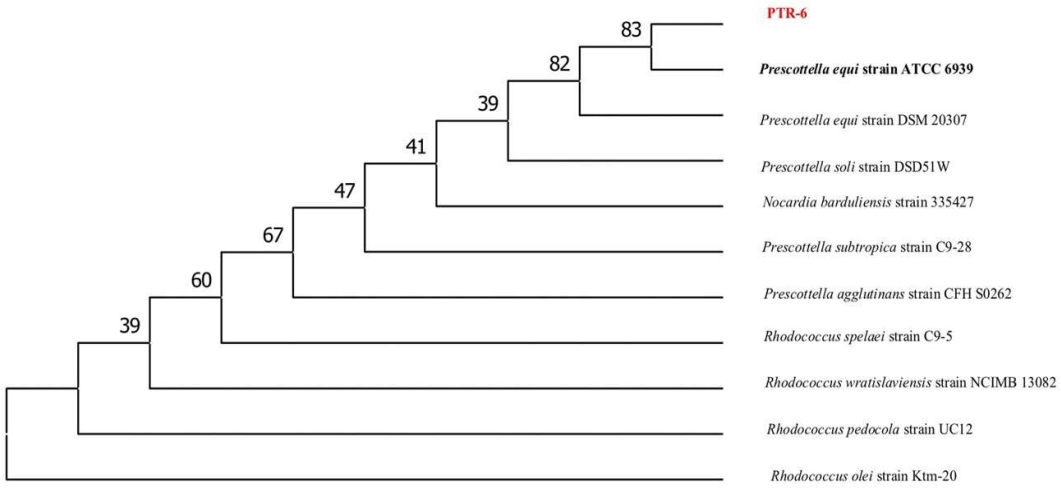


PLATE 10.7

Phylogenetic tree of (M) PTR-5 (*Alkalicoccobacillus gibsonii* strain PTR1DD) & (N) PTR-6 (*Prescottella equi* strain PTR2DD)



M



N

PLATE 10.8

Phylogenetic tree of (O) PTS-1 (*Pseudomonas aeruginosa* strain DD3) & (P) PTS-2 (*Agrobacterium larrymoorei* strain DDBU3)

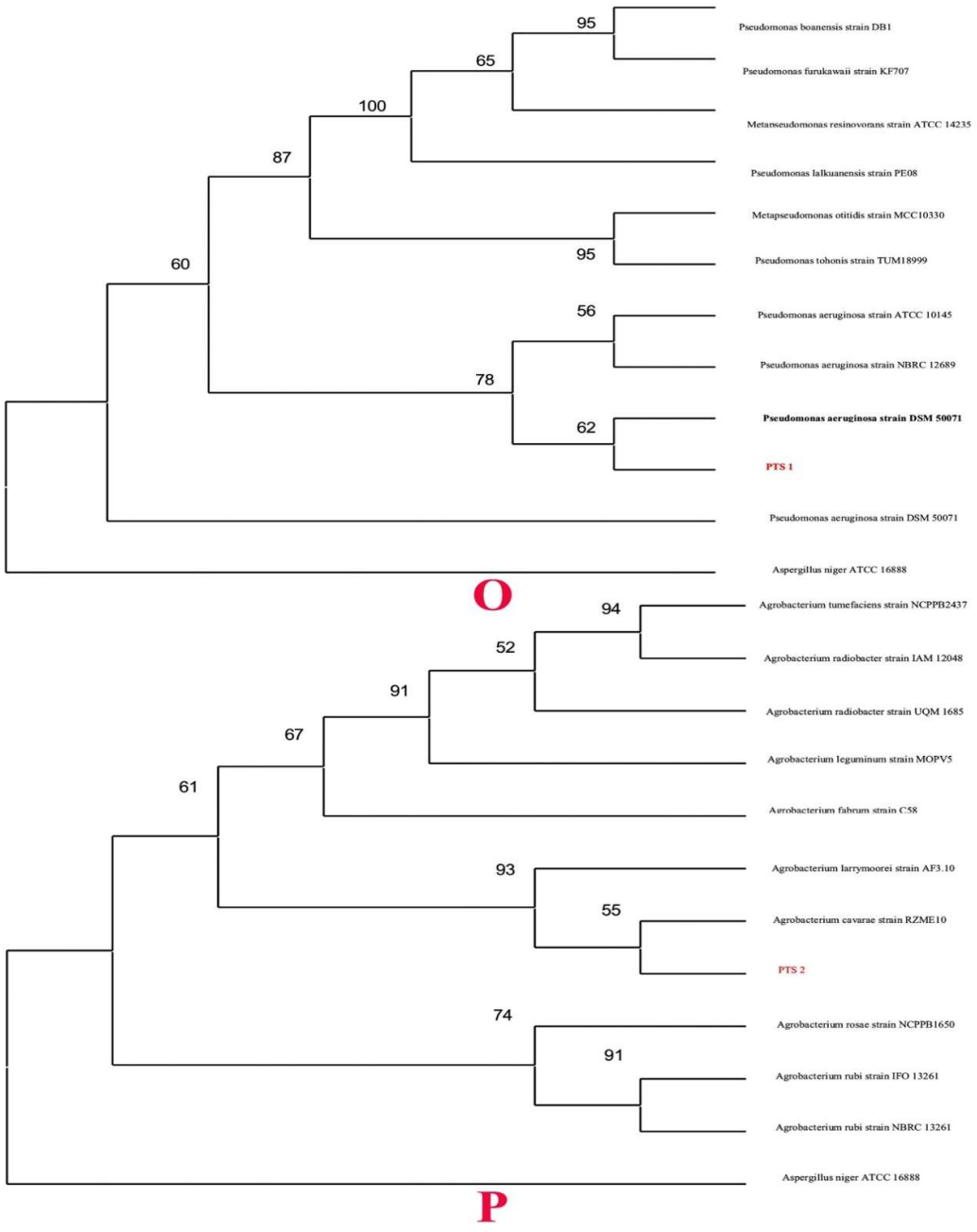


Table 8: Identified endophytic bacteria isolated from different parts of three medicinal plants

Sl. No	Isolates	Identified organism	Accession no.	Tissue used in Isolation	Host plant
1	PTS-1	*Pseudomonas aeruginosa* strain DD3	PP413726	Stem	*Phlogacanthus thyrsiformis*
2	PTS-2	*Agrobacterium larrymoorei* strain DDBU3	PP412004		
3	PTL-3	*Solibacillus silvestris* strain DDBU6	PP412075	Leaf	
4	PTL-4	*Kocuria assamensis* strain DDBU9	PP412522		
5	PTR-5	*Alkalicoccobacillus gibsonii* strain PTR1DD	PQ780166	Root	
6	PTR-6	*Prescottella equi* strain PTR2DD	PQ780173		
7	HAL-1	*Pseudomonas oryzihabitans* strain HAL1DD	PP907921	Leaf	*Hygrophila auriculata*
8	HAL-2	*Proteus mirabilis* strain HAL2DD	PQ056532		
9	HAS-3	*Stenotrophomonas geniculata* strain HAS3DD	PP907950	Stem	

10	HAS-4	*Agrobacterium cavarae* strain HAS4DD	PP908465		
11	HAR-5	*Lysinibacillus macrolides* strain HARDD	PQ780158	Root	
12	GPL-1	*Alkalicoccobacillus gibsonii* strain GPDD1	PP907833	Leaf	*Glycosmis pentaphylla*
13	GPL-2	*Bacillus cereus* strain GP2DD	PP907840		
14	GPL-3	*Bacillus subtilis* strain GP3DD	PP907864		
15	GPS-4	*Bacillus cereus* strain GP4DD	PQ056567	Stem	
16	GPR-5	*Bacillus australimaris* strain GPRDD	PQ536475	Root	

4.4 Plant growth promotion activity of isolated endophytic bacteria

4.4.1 Phosphate solubilisation activity

In phosphate solubilisation test, seven isolates showed positive results out of sixteen isolates. Phosphate solubilization test was done by inoculating the isolates in Pikovskaya's agar medium and incubated for atleast 7 days and maximum for 10 days at 28 ℃. In this study, the ability of the isolates to solubilise calcium phosphate were determined. The clearing zone around the colony means positive result (Plate 12). Solubilisation index was also determined for the isolates which showed positive result. The clearing zone formed for isolates between 1 to 4 mm. Highest solubilisation index was observed in PTS-1 isolates followed by PTR-6, GPR 5 PTR-5, GPL-3, PTS-2, HAS-3 (Table 10).

4.4.2 Ammonia production activity

Ammonia production ability test for the isolates were done by inoculating the isolates in peptone water and incubated 5 days at 30 ℃. After the incubation Nessler's reagent is added to the tubes containing isolates. Production yellow color indicates positive results (Plate 11). In our study, almost all isolates showed positive for ammonia production test except PTS-1 and PTR-5 (Table 9).

4.4.3 IAA production activity

Isolates were cultured in LB broth supplemented with 400 µg/ml of L-tryptophan and incubated for 72 hours at 25°C to perform the IAA production test. The isolates were centrifuged at 3000 rpm for 30 minutes after the incubation period was over. Then, 2 ml of the supernatant were then mixed with orthophosphoric acid and Salkowski reagent, and the mixture was left to incubate for one hour at room temperature in a dark environment. The production of IAA is indicated by the appearance of the pink colour. At 530 nm, the optical density was measured. Out of sixteen, eleven isolates showed positive results for the IAA production ability. The amount of IAA determined using standard curve for authentic IAA (Fig 7). Highest production was observed in HA-5 followed by HA-1, PTL-3, GP-3, GP-4, GP-1, GP-2, PTS-2, PTL-4, GP-5, PTS-1 (Table 9).

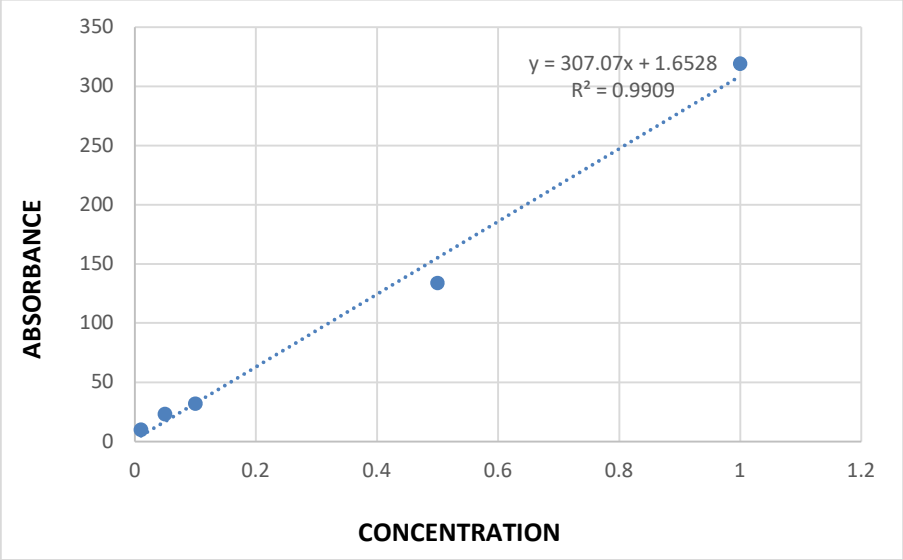


Figure 7: Standard curve of IAA production

PLATE 11

Ammonia production test results of the isolates of, (A) *P. thyrsiformis*; (B) *H. auriculata*; (C) *G. pentaphylla*

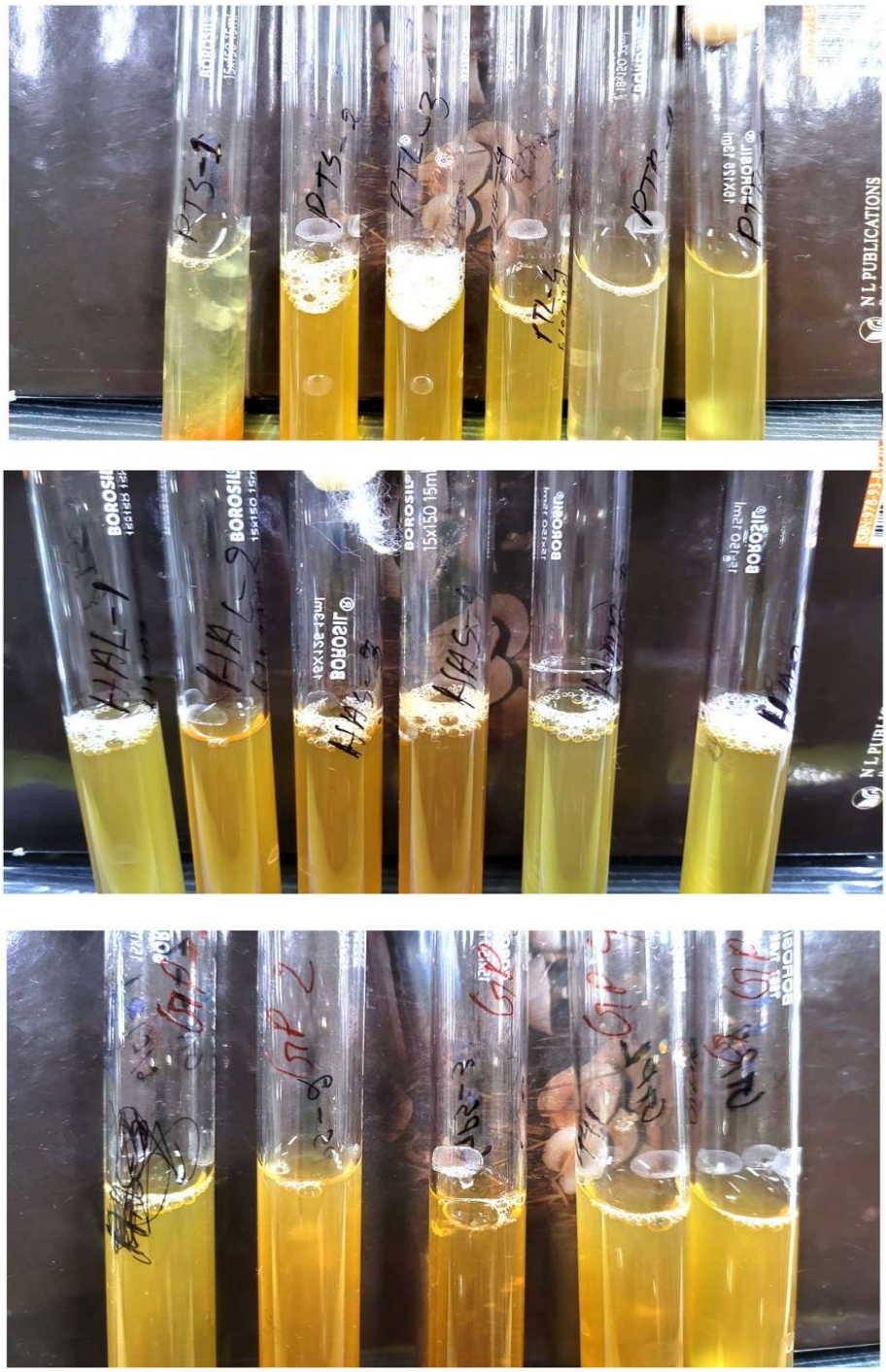


PLATE 12

Phosphate solubilisation ability of the isolates, (A) Plate used as control; (B) PTS-1; (C) HAS-3; (D) PTS-2; (E) GPR-5; (F) GPL-3

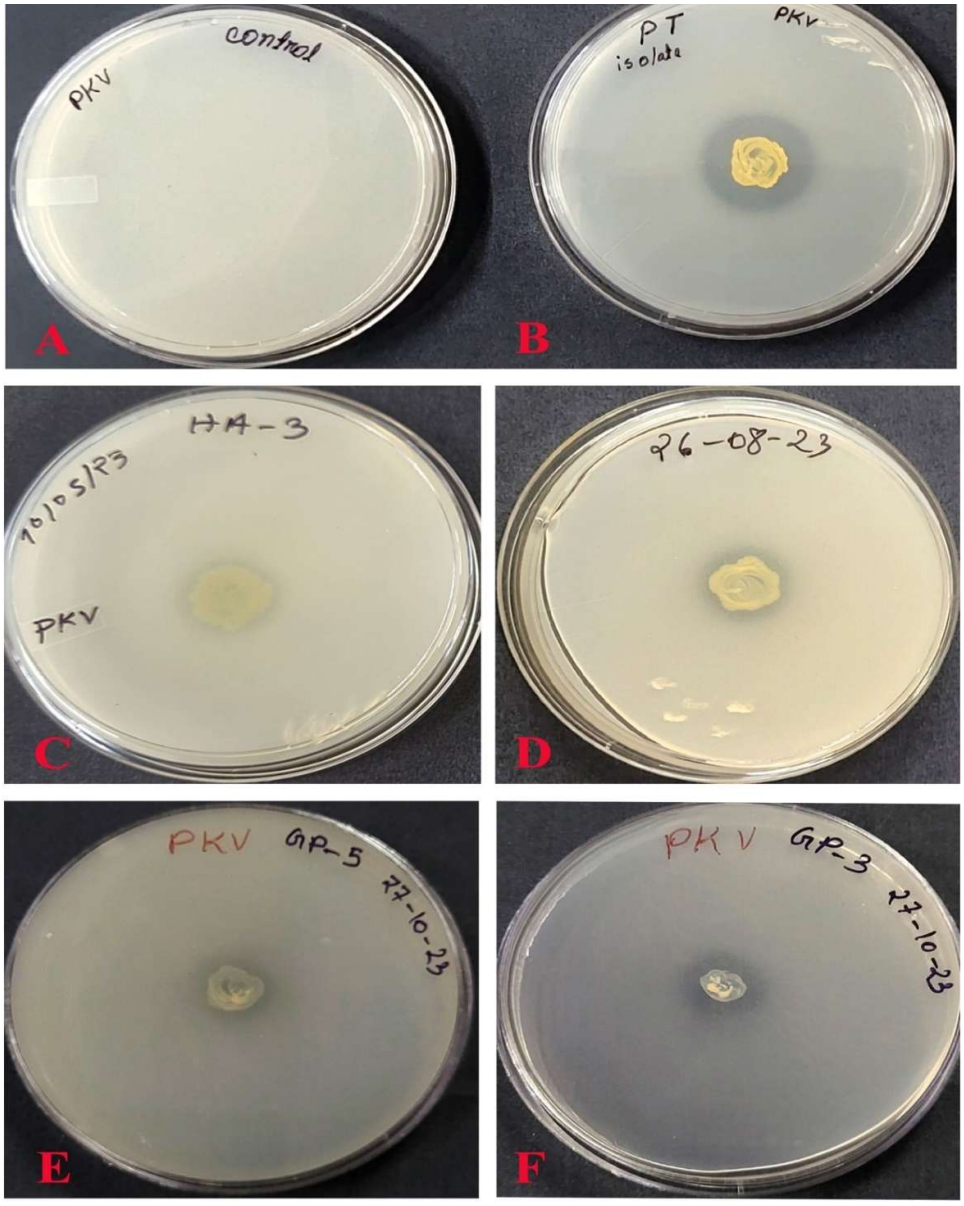


Table 9: Plant growth promotion activity of isolated endophytic bacteria

SL NO	Isolates	Ammonia production	IAA production (µg/ml)	Phosphate solubilization
1	PTS-1	**Negative**	15 ± 0.81	**Positive**
2	PTS-2	Positive	67 ± 1.24	**Positive**
3	PTL-3	Positive	81 ± 1.24	Negative
4	PTL-4	Positive	45 ± 0.47	Negative
5	PTR-5	**Negative**	**Negative**	**Positive**
6	PTR-6	Positive	**Negative**	**Positive**
7	HAL-1	Positive	121 ± 1.63	Negative
8	HAL-2	Positive	**Negative**	**Negative**
9	HAS-3	Positive	**Negative**	Positive
10	HAS-4	Positive	**Negative**	Negative
11	HAR-5	Positive	258.6 ± 2.05	Negative
12	GPL-1	Positive	76.33 ± 1.24	Negative
13	GPL-2	Positive	75.83 ± 1.31	Negative
14	GPL-3	Positive	80.16 ± 2.89	**Positive**
15	GPS-4	Positive	76.66 ± 2.49	Negative
16	GPR-5	Positive	31.66 ± 2.49	**Positive**

Table 10: Solubilisation index of the endophytic bacteria which showed positive results for phosphate solubilization activity

Isolates	Diameter of solubilisation zone (mm)	Colony diameter (mm)	SI
PTS-1	3.50 ± 0.11	1.14 ± 0.08	4.07
PTS-2	2 ± 0.05	1.5 ± 0.05	2.34
PTR-5	2.1 ± 0.12	1.1 ± 0.08	2.94
PTR-6	2.66 ± 0.28	1.2 ± 0.88	3.17
GPL-3	1.4 ± 0.08	0.88 ± 0.084	2.60
GPR-5	1.6 ± 0.12	0.8 ± 0.081	3.09
HAS-3	1.59 ± 0.06	1.2 ± 0.14	2.19

4.4.4 Salt tolerance ability

Isolated endophytic bacteria showed fantastic salt tolerance ability. The test was done by inoculating the isolates in the LB agar media containing NaCl from 1-10% and incubated for 7 to 10 days. The ability of the isolates to grow in those media showed their tolerance against saline environment. The highest tolerance was observed for PTS-2 isolates which showed tolerance up to 10 % NaCl. GP-4 showed tolerance up to 8% NaCl. GP-1, GP-2, GP-3 and HA-3 showed tolerance up to 7% NaCl. HA-1, PTR-6 showed tolerance up to 6% NaCl. GP-5, HA-2, PTR-5, PTL-4 and PTL-3 showed tolerance up to 5% NaCl. PTS-1 showed tolerance up to 4% NaCl. HA-4 showed tolerance upto 3%. The detailed results included in Table 11.

Table 11: Salt tolerance ability of the isolated endophytic bacteria

EB	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl	7% NaCl	8% NaCl	9% NaCl	10% NaCl
PTS-1	+	+	+	+	-	-	-	-	-	-
PTS-2	+	+	+	+	+	+	+	+	+	+
PTL-3	+	+	+	+	+	-	-	-	-	-
PTL-4	+	+	+	+	+	-	-	-	-	-
PTR-5	+	+	+	+	+	-	-	-	-	-
PTR-6	+	+	+	+	+	+	-	-	-	-
HAL-1	+	+	+	+	+	+	-	-	-	-
HAL-2	+	+	+	+	+	-	-	-	-	-
HAS-3	+	+	+	+	+	+	+	-	-	-
HAS-4	+	+	+	-	-	-	-	-	-	-
HAR-5	+	+	+	+	+	+	-	-	-	-
GPL-1	+	+	+	+	+	+	+	-	-	-
GPL-2	+	+	+	+	+	+	+	-	-	-
GPL-3	+	+	+	+	+	+	+	-	-	-

GPS-4	+	+	+	+	+	+	+	+	-	-
GPR-5	+	+	+	+	+	-	-	-	-	-

4.5 Hydrolytic enzyme production ability:

All isolated endophytic bacteria showed different hydrolytic enzyme production abilities. Details of the results were included in Table 12. The results for amylase activity were positive for PTL-4, PTR-5, GPL-3, HAL-1, HAL-2, and HAS-3. PTL-4 and HAS-3 revealed negative results for protease activity, while all other isolates showed positive results for protease production ability. For lipase activity, PTL-4, PTR-6, GPL-1, GPL-2, GPS-4, HAL-1, HAL-2, and HAR-5 showed positive results. PTS-1, PTR-6, GPL-2, GPL-3, GPR-5, HAL-1, and HAS-4 showed positive results for cellulase activity. Whereas for pectinase activity, PTS-1, PTS-2, PTR-6, GPL-1, GPL-2, HAL-1, HAS-3, and HAR-5 showed positive results, and for xylanase activity, PTS-1, PTS-2, PTL-4, PTR-6, GPL-1, GPS-4, GPR-5, HAS-4, and HAR-5 showed positive results.

Table 12: Hydrolytic enzyme producing ability of the isolated endophytic bacteria

Isolates	**Amylase activity**	**Protease activity**	**Lipase activity**	**Cellulase activity**	**Pectinase activity**	**Xylanase activity**
PTS-1	-	+	-	+	+	+
PTS-2	-	+	-	-	+	+
PTL-3	-	+	-	-	-	-
PTL-4	+	-	+	-	-	+
PTR-5	+	+	-	-	-	-
PTR-6	-	+	+	+	+	+
GPL-1	-	+	+	-	+	+

GPL-2	-	+	+	+	+	-
GPL-3	+	+	-	+	-	-
GPS-4	-	+	+	-	-	+
GPR-5	-	+	-	+	-	+
HAL-1	+	+	+	+	+	-
HAL-2	+	+	+	-	-	-
HAS-3	+	-	-	-	+	-
HAS-4	-	+	-	+	-	+
HAR-5	-	+	+	-	+	+

"+" means "Positive" and "-" means "Negative"

4.6 Antimicrobial activity test:

The disc diffusion method was used to evaluate the antibacterial potency of the isolated endophytic bacteria against the selected bacterial pathogens, and the findings are shown in Table 13 (Plate 13). All the isolates were tested for their antimicrobial activity against 6 pathogenic bacteria: *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). For the antibacterial activity test, a bacterial methanolic extract (400 mg/ml) was used. The best activity was shown by PTS-1, which showed antimicrobial activity against 5 pathogenic bacteria, with highest zone of inhibition against *E. aerogenes* which is 15.6 ± 0.43 mm. Out of sixteen, four endophytic bacterial isolates showed anti-bacterial activity against pathogenic microbe, *S. aureus* and PTS-1 showed highest activity by forming 14 ± 0.81 mm diameter zone of inhibition. Whereas PTS-1, PTS-2, GPL-1, GPL-2, GPS-4, and HAR-5 showed anti-microbial activity against *E. coli* and highest activity was shown PTS-2 with zone of inhibition 20.2 ± 0.58 mm. For *B. subtilis*, PTS-1, PTS-2, PTL-3, GPL-3, HAL-1, and HAL-2 showed inhibitory property where highest zone of inhibition was shown by PTL-3. Against *E. aerogenes*, PTS-1, GPL-1, and GPR-5 showed anti-bacterial activity. Here, the highest zone of

inhibition was shown by PTS-1 which formed 15.6 ± 0.43 mm diameter inhibition zone. PTS-1, PTS-2, and PTL-3 showed anti-bacterial activity against *P. aeruginosa* and PTL-4, PTR-6, GPR-5, and HAS-3 showed anti-bacterial activity against *K. pneumonia*.

Table 13: Anti-microbial activities of isolated endophytic bacteria against pathogenic microbes

Sl. no	**Isolates**	**Zone of inhibition against pathogenic microbes (mm)**					
		S. aureus	***E. coli***	***B. subtilis***	***E. aerogenes***	***P. aeruginosa***	***K. pneumonia***
1	PTS-1	**14 ± 0.81**	**12.6 ± 0.5**	**12.3 ± 0.4**	**15.6 ± 0.43**	**11.16 ± 0.86**	-
2	PTS-2	-	**20.2 ± 0.58**	**9.2 ± 0.61**	-	**10.76 ± 0.5**	-
3	PTL-3	**10.73± 0.5**	-	**16.13 ± 0.98**	-	**14.06 ± 0.29**	-
4	PTL-4	-	-	-	-	-	**16.1 ± 0.65**
5	PTR-5	-	-	-	-	-	-
6	PTR-6	-	-	-	-	-	**9.9 ± 0.73**
7	GPL-1	-	**15.8 ± 0.8**	-	**10.1 ± 0.29**	-	-
8	GPL-2	**10.36 ± 0.66**	**14.6 ± 0.99**	-	-	-	-

9	GPL-3	-	-	**16.1 ± 0.62**	-	-	-
10	GPS-4	**13.7 ± 0.52**	**7.9 ± 0.57**	**-**	-	-	-
11	GPR-5	-	-	-	**14.2 ± 0.52**	-	**10.5 ± 0.77**
12	HAL-1	-	-	**8.2 ± 0.71**	**-**	-	-
13	HAL-2	-	-	**14.16 ± 0.78**	-	-	-
14	HAS-3	-	-	-	-	-	**14.6 ± 0.48**
15	HAS-4	-	-	-	-	-	-
16	HAR-5	-	**11.16 ± 0.86**	-	-	-	-

PLATE 13: Antimicrobial activity of (A) Isolates of GP against *E. aerogenes*; (B) Isolates of GP against *S. aureus*; (C) Isolates of GP against *E. coli*; (D) Isolates of HA against *B. subtilis*; (E) Isolates of PT against *K. pneumonia*; (F) Isolates of GP against *P. aeruginosa*; (G) Isolates of GP against *E. coli*; (H) isolates of PT against *B. subtilis*; (I) Isolates of GP against *B. subtilis*; (J) Isolates of PT against *S. aureus*