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Plant Growth Promoting Potential of Bacterial Endophytes from Medicinal Plants

Krupali B. Ramanuj[1*] and Harsha N. Shelat[1]

[1]Department of Agricultural Microbiology and Biofertilizer Project, B. A. College of Agriculture, Anand Agricultural University, Anand-388110 (Gujarat), India.

Authors' contributions

This work was carried out in collaboration between both authors. Author KBR carried out all the research work, experimentation and writing protocol. Author HNS played role in guiding, planning the research and experimentation. Both authors read and approved the final manuscript.

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ABSTRACT

Endophytic bacteria were isolated from surface sterilized parts like leaf, stem, rhizome and fruit of medicinal plants *viz. Azadirachta indica, Zingiber officinale* Rosc*., Chlorophytum borivilianum, Curcuma longa, Tinospora cordifolia, Withania somnifera* (L) Dunal and *Ocimum sanctum* Lin. on various nutrient media. Out of thirty isolates, the best five cultures were selected on the basis of plant growth promoting abilities *viz.* phosphate solubilization, potash mobilization, production of IAA and siderophore, production of various enzymes like ACC-deaminase, cellulase, protease, lipase, chitinase; antifungal activity and biochemical tests. On the basis of morphological, physiological, biochemical and molecular ribotyping, endophytes were characterized as *Bacillus tequilensis* strain AAU K1, *Bacillus endophyticus* strain AAU K2, *Beijerinckia fluminensis* strain AAU K3, *Bacillus safensis* strain AAU K4 and *Pseudomonas aeruginosa* strain AAU K5. Moreover, later three endophytes showed presence of *nif*H gene indicating nitrogen fixing ability.

Keywords: IAA; PGP; medicinal plants.

**Corresponding author: E-mail: krupali09@yahoo.com;*

1. INTRODUCTION

Medicinal plants are gaining worldwide attention owing to the fact that the herbal drugs are cost effective, easily available and with negligible side effects. The beneficial effects of the medicinal plants in health care can be well judged from the WHO estimate that around 80% of the world population uses them in some form or the other [1]. It is established that plants harbor microorganisms, collectively known as endophytes. Microbial endophytes are plant associated microbes that colonize living internal tissues of plants without causing any visible symptoms or immediate negative effects. They can be isolated from surface disinfected plant tissues [2]. The diversity of endophytes ranges from Gram positive to Gram negative bacteria which include genera like *Achromobacter, Acinetobacter, Agrobacterium, Bacillus, Brevibacterium, Burkholderia, Chromobacterium, Curtobacterium, Enterobacter, Kocuria, Lysinibacillus, Methylobacterium, Microbacterium, Paenibacillus, Pantoea, Phyllobacterium, Pseudomonas, Rahnella, Rhodanobacter, Stenotrophomonas, Streptomyces, Xanthomonas etc*. However, in general, *Streptomyces* sp., *Bacillus* sp. and *Pseudomonas* sp. are the most predominant species [3].

Several endophytes have been shown to increase plant growth by an increase in nutrient uptake, production of phytohormones, enzymes, antimicrobial substances like antibiotics and siderophores. Other beneficial effects of endophytes to plants include - nitrogen fixation, increased drought resistance, thermal protection, survival under osmotic stress *etc*. [2]. Plant growth promoting bacteria utilizes a group of microbes for the plant growth enhancement and aids as biofertilizers with no side effects by direct or indirect mechanisms. Direct mechanisms involve fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores and synthesis of plant growth hormones. Indirect mechanisms involves the biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophore or through competition for nutrients and space which can further improve significantly plant health and promote growth, as evidenced by increase in seedling emergence, vigor and yield [4]. Endophytic bacteria are of agronomic interest because they can enhance plant growth and improve the nutrition of plants through nitrogen fixation and other mechanisms [5]. Several studies have shown the positive effects of endophytic bacteria inoculation in plants, e.g. sugarcane (*Saccharum* sp.), leading to increased contribution of biological nitrogen fixation, to promotion of root development, increased biomass and productivity; soybean (*Glycine max* (L.) Merr.), with bacteria capable to inhibit growth and sporulation of pathogenic fungi; tomato (*Lycopersicum esculentum* L.), with bacteria increasing plant height, leaf area, leaf number, together with fresh and dry plant weight [6].

The present investigation was undertaken with an aim of isolation and characterization of bacterial endophytes from medicinal plants.

2. MATERIALS AND METHODS

2.1 Isolation and Characterization of Endophytic Bacteria

For isolation of endophytic bacteria, medicinal plant samples (*viz*. *Azadirachta indica, Zingiber officinale* Rosc*., Chlorophytum borivilianum, Curcuma longa, Tinospora cordifolia, Withania somnifera, Ocimum sanctum* Lin.) were collected from Medicinal and aromatic plants research farm, Anand Agricultural University, Anand. Plant samples were washed under running tap water and cut into 2-3 cm pieces. Under aseptic conditions inside laminar air flow (LAF) these pieces were rinsed in sterile water and then surface-disinfected by soaking in 70% ethanol for 1 min. Then pieces were washed with sterile distilled water to remove traces of ethanol. Further, they were soaked in 0.1% $HgCl_2$ solution for 30 s. The disinfected plant parts were then rinsed extensively in sterile distilled water thrice and drained. After that, they were passed through bunsen burner flame for 2 s. To ensure complete surface sterilization of the sample, the last wash was streaked onto R2A agar plates using sterile nichrome wire loop. For isolation of endophytes, the surface disinfected plant sample was transferred into mortar in aseptic conditions in LAF and crushed using pestle by adding few drops of sterile distilled water. Once the plant sample was completely crushed into thick liquid paste, it was collected in sterile eppendorf vial. About 50 µl was spread using sterile glass spreader on different media such as Nutrient agar (NA), Starch casein agar (SCA), Glycerol asparagine agar (GAA), Kuster's agar (KUS) and Inorganic salt starch (ISP4) supplemented with cyclohexamide 50 ppm to avoid fungal

contamination. These plates were incubated at 28 ± 2°C for 24 to 60 h. Isolated colonies were subcultured and maintained at 4 ± 1°C for further study.

All bacterial isolates were preliminarily identified on the basis of cultural and morphological characteristics using 9th edition of Bergey's Manual of Determinative Bacteriology and The Prokaryotes.

2.2 PGP Traits of Endophytic Bacterial Isolates

Bacterial isolates were further screened for their plant growth promoting traits like phosphate solubilization on Sperber media [7], potash mobilization on Aleksandrov's media, enzyme production like cellulase on Carboxymethylcellulose (CMC) agar media [8], protease on Skimmed milk agar media [9], chitinase on 0.2% colloidal chitin media [10], lipase on tributyrin agar media [11] and ACC deaminase production on Dworkin and Foster (DF) salt minimal media [12], IAA production in Glucose phosphate broth (GPB) supplemented with L-tryptophan (1 µg/ml) [13], siderophore production on Chrome azurol S (CAS) agar media [14] and antifungal activity against plant pathogenic fungi *Alternaria alternata, Fusarium oxysporum, Aspergillus niger* and *Macrophomina phaseolina* by agar well diffusion assay.

2.3 Biochemical Characterization

On the basis of PGP traits, five prominent cultures were selected which were further characterized using biochemical tests. For biochemical tests, HiMedia kits (HiPure Bacterial Identification Kit) were used. Individual bacterial suspension (50 µl having 10^7 CFU/ml) was inoculated in each well of test strips and allowed to incubate for 24 h at 28 ± 2°C. After incubation, observations were recorded by applying appropriate reagents provided with the kit.

2.4 Molecular Identification of Selected Potential Isolates

To further confirm identity of prominent isolates, bacterial cultures were subjected to 16S *r*RNA sequencing which is considered as an effective tool for identification of microorganisms up to species level. Primers for 16S *r*RNA genes were selected from standard scientific literature, 27F - 5'AGAGTTTGATCCTGGCTCAG'3 and 1492R - 5'GGTTACCTTGTTACGACTT'3 [15,16]. The oligonucleotides were synthesized at MWG Biotech Pvt. Ltd., Germany.16S *r*DNA amplification was performed in a thermal cycler (Eppendorf Master cycler, Germany) with a 25 µl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTPs, 1 µM of each primer [17], 2.5 mM of MgCl2, and 1 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. The output sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify the cultures and to find out the nearest match of the cultures (http://www.ncbi.nlm.nih.gov). Additionally, *nif*H gene amplification was carried out using two primers Pol I 5' TGCGAYCCSAARGCBGACTC 3' and Pol II 5' ATSGCCATCATYTCRCCGGA 3' which was constructed by MWG Biotech Pvt. Ltd., Bangalore, India.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Endophytic Bacteria

Total thirty bacterial endophytes were isolated from various plants like *Azadirachta indica, Zingiber officinale* Rosc*., Chlorophytum borivilianum, Curcuma longa, Tinospora cordifolia, Withania somnifera* and *Ocimum sanctum* Lin. from various plant parts like leaf, stem, rhizome and fruit using different media *viz*. Nutrient agar (NA), Starch casein agar (SCA), Glycerol asparagine agar (GAA), Kuster's agar (KUS), King's B (KB) and Inorganic salt starch (ISP4) (Table 1).

Kumar et al. [18] isolated a total of 14 fungus and 7 bacteria from leaves and stem of three different medicinal plants *viz. A. indica*, *Terminalia arjuna*, and *Catharanthus roseus.* Aswathy et al. [19] confirmed two different strains of *Paenibacillus* sp. endophytically associated with the rhizome of *C. longa*. Mini screened endophytes from *W. somnifera* and found to be *Streptomyces* sp*., Nocardioides* sp*., Actinomadura* sp. and *Kitasatosporia* sp [20]. Panchal and Ingle isolated five bacterial species from *Chlorophytum borivilianum* [21]. Tiwari et al. [22] isolated endophytes from the leaves of the naturally soil grown *Ocimum* plants and an endophyte OS-11 was identified as *Bacillus subtilis* which showed significant enhancement in the yields.

All the isolates were subjected to Gram's staining for cellular characterization. Isolates A-1, A-2, A-6, Z-3, C-1, C-3, T-3, T-4, T-5, W-1, W-2, W-3,

W-4, O-5 and O-6 found to be Gram negative short rods occurring singly and/or in groups, whereas other isolates A-3, A-4, A-5, Z-1, Z-2, Z-4, CH-1, C-2, T-1, T-2, W-5, O-1, O-2, O-3 and O-4 were Gram positive big rods or filamentous (Table 2).

3.2 PGP Traits of Endophytic Bacterial Isolates

They were further screened for plant growth promoting traits (Table 3). Phosphate solubilizing activities were screened by measuring the clearing zone (due to the release of organic acids acting on inorganic phosphate source) surrounding the developed bacterial colony via calculation of phosphate solubilization index. All the isolates were able to solubilize phosphate on Sperber medium wherein isolate T-1 gave the highest solubilisation index (SI) 3.88. Potash mobilization SI index (3.0) was found higher in case of isolate W-1, followed by isolate Z-1 (2.67), CH-1(2.60), T-1 (2.56) and C-1 (2.45) Fig. 1. Enzyme production like cellulase, protease, chitinase and lipase by isolates were also detected. Twenty one isolates gave activity for cellulase production on CMC agar plates of which SI of cellulase was found the maximum for isolate C-1 (2.86) followed by isolate CH-1 (2.71), T-1 (2.63) and Z-1 (2.57). Eleven isolates were positive for protease activity, twelve for lipase and eighteen were found positive for chitinase (Fig. 2). Indole 3-acetic acid (IAA) production was carried out in the presence of tryptophan (1 mg ml^{-1}). IAA production was found to be the highest in isolate CH-1 (59.94 µg/ml) followed by C-1 (46.21 µg/ml), T-1 (38.92 µg/ml), Z-1 (26.32 µg/ml) and W-1(22.61 µg/ml). Sixteen isolates were found positive for siderophore production whereas ACC deaminase activity was found maximum in six isolates. Isolate W-1 gave antifungal activity against all the tested fungi namely *A. alternatae, F. oxysporum, A. niger* and *M. phaseolina.*

Table 1. Bacterial endophytes isolated from medicinal plants

Sr. no.	Isolate	Isolation source	Plant part	Media
1	A-1	*A. indica*	Leaf	ISP4
2	A-2	(Neem)		KB
3	A-3			SCA
4	A-4		Fruit	KUS
5	A-5			NA
6	A-6			GAA
7	Z-1	*Z. officinale* Rosc.	Rhizome	KUS
8	Z-2	(Ginger)		GAA
9	Z-3			NA
10	Z-4			NA
11	CH-1	*C. borivilianum* (Safed musli)	Rhizome	NA
12	C-1	*C. longa*	Rhizome	KUS
13	C-2	(Turmeric)		SCA
14	C-3			NA
15	T-1	*T. cordifolia*	Leaf	NA
16	T-2	(Guduchi)		KUS
17	T-3			GAA
18	T-4		Stem	NA
19	T-5			ISP4
20	W-1	*W. somnifera*	Leaf	NA
21	W-2	(Ashwagandha)		KUS
22	W-3		Stem	SCA
23	W-4			KB
24	W-5			NA
25	O-1	*O. sanctum*	Leaf	NA
26	O-2	(Tulsi)		KUS
27	O-3			KB
28	O-4		Stem	SCA
29	O-5			GAA
30	O-6			ISP4

Table 2. Colony characteristics of isolates

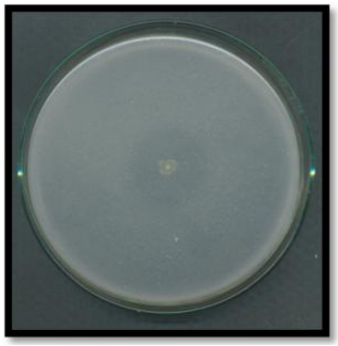
Isolate	Size	Shape	Margin	Elevation	Texture	Opacity	Pigment	Gram reaction	Arrangement
A-1	small	round	entire	flat	smooth	opaque	off white	negative	short rods singly, in groups
A-2	small	round	entire	slightly raised	smooth	translucent	none	negative	short rods in groups
A-3	medium	round	entire	slightly raised	rough	opaque	off white	positive	rods in chains
A-4	medium	round	entire	raised	smooth	opaque	yellow	positive	cocci in chain, groups
A-5	big	round	entire	raised	smooth	opaque	white	positive	rods in chains
A-6	small	round	entire	raised	smooth	translucent	none	negative	short rods in groups
Z-1	medium	round	entire	slightly raised	smooth	opaque	off white	positive	rods in chains
Z-2	medium	round	entire	raised	smooth	opaque	white	positive	rods in chains
Z-3	small	uneven	undulate	flat	rough	opaque	off white	negative	short rods in groups
Z-4	medium	round	entire	raised	rough	opaque	white	positive	filamentous
CH-1	medium	round	entire	slightly raised	smooth	opaque	peach	positive	rods in chains
C-1	small	round	entire	raised	smooth	translucent	none	negative	short rods groups, singly
C-2	medium	round	entire	raised	smooth	opaque	orange	positive	cocci in groups
C-3	small	round	entire	flat	smooth	opaque	off white	negative	short rods in groups, singly
T-1	medium	round	entire	slightly raised	smooth	opaque	white	positive	rods in chain
T-2	medium	round	curled	raised	rough	opaque	violet	positive	filamentous
T-3	small	round	entire	raised	smooth	opaque	off white	negative	short rods in groups, singly
T-4	small	round	entire	raised	smooth	opaque	brown	negative	short rods in groups, singly
T-5	small	round	entire	slightly raised	smooth	opaque	pink	negative	short rods in groups, singly
W-1	small	round	entire	slightly raised	smooth	translucent	green	negative	short rods groups
W-2	small	round	entire	slightly raised	smooth	opaque	yellow	negative	short rods in groups, singly
W-3	small	round	entire	slightly raised	smooth	opaque	red	negative	short rods in groups, singly
W-4	small	round	entire	raised	smooth	translucent	none	negative	cocci in groups
W-5	medium	round	undulated	flat	rough	opaque	white	positive	rods in chain
O-1	small	round	entire	raised	smooth	opaque	yellow	positive	big rods in chain
O-2	small	round	entire	slightly raised	smooth	opaque	peach	positive	big rods in chain
O-3	medium	round	entire	raised	rough	opaque	red	positive	filamentous
O-4	medium	round	entire	raised	smooth	opaque	white	positive	rods in chain
O-5	small	round	entire	Flat	smooth	opaque	yellow	negative	rods in groups
O-6	small	round	entire	Raised	smooth	translucent	none	negative	rods in groups

Table 3. PGP traits of bacterial isolates

Sr. no.	Isolates	Solubilization/Mobilization			Production of						Fungal growth inhibition %			
		P (SI)	K (SI)	Ce (SI)	Pr	Ch	Li	IAA (µg/ml)	Si	ACC deaminase	AA	FO	AN	MP
1	A-1	2.17	-	1.88	+	-	+	3.39	+	++	-	-	-	-
2	A-2	2.71	2.25	2.43	-	+	-	1.68	+	+	-	11	-	-
3	A-3	2.50	-	2.17	-	+	-	3.33	+	++	10	-	-	-
4	A-4	1.56	2.00	2.00	-	+	-	1.24	+	-	-	25	-	-
5	A-5	2.10	2.44	-	+	-	+	6.15	-	-	-	-	-	-
6	A-6	2.29	1.75	1.88	-	+	-	1.22	-	-	-	18	33	-
7	Z-1	3.63	2.67	2.57	+	+	+	26.32	+	+++	-	64.44	61.11	67.77
8	Z-2	1.89	2.57	2.17	-	-	-	4.64	-	-	-	-	-	-
9	Z-3	2.00	-	2.00	-	-	-	1.86	-	-	-	-	-	-
10	Z-4	2.17	2.14	1.88	-	-	+	3.54	+	+	-	-	-	-
11	CH-1	3.33	2.60	2.71	+	+	+	**59.94**	+	+++	12	-	**72.22**	-
12	C-1	3.00	2.45	**2.86**	-	+	-	46.21	+	+++	-	-	32	**73.33**
13	C-2	1.80	-	-	-	+	-	11.36	-	-	-	-	-	12
14	C-3	2.50	1.86	-	-	-	-	3.98	-	+	-	-	-	-
15	T-1	**3.88**	2.56	2.63	+	+	+	38.92	+	+++	-	42	17	-
16	T-2	2.29	1.71	-	+	+	+	4.51	-	+	-	-	38	-
17	T-3	1.56	2.20	-	-	+	-	10.08	+	++	-	21	-	-
18	T-4	1.80	2.14	2.14	-	-	-	3.19	+	-	-	-	-	-
19	T-5	1.86	2.57	2.17	+	+	-	5.96	-	-	-	-	12	-
20	W-1	3.67	**3.00**	2.71	+	+	+	22.61	+	+++	**66.66**	**68.75**	63.33	64.44
21	W-2	2.33	-	-	-	-	-	2.62	-	++	-	-	-	-
22	W-3	2.40	-	1.75	+	+	+	18.91	+	-	18	-	-	-
23	W-4	2.63	2.00	1.86	-	-	-	11.19	-	++	-	-	-	-
24	W-5	2.38	2.00	1.70	-	-	-	2.24	+	+	-	-	-	-
25	O-1	2.56	1.63	2.00	-	+	+	3.72	-	+++	-	-	23	18
26	O-2	1.70	-	-	-	-	-	10.40	-	-	-	-	-	-
27	O-3	3.14	2.00	-	+	+	+	4.22	+	-	-	-	10	-
28	O-4	3.00	2.00	-	+	-	+	12.54	+	+	-	-	-	-
29	O-5	2.33	-	1.71	-	+	-	8.02	-	++	-	21	-	-
30	O-6	1.60	1.73	1.64	-	+	-	6.33	-	-	-	-	-	11

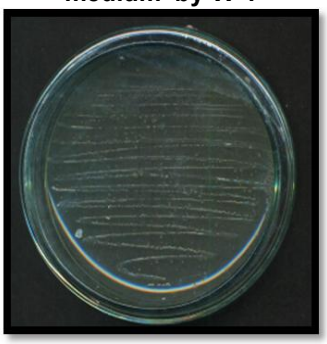
Note: P: phosphate, K: Potash, Ce: cellulase, Pr: protease, Ch: chitinase, Li: lipase, Si: siderophore, AA: Alternaria alternate, FO: Fusarium oxysporum, AN: Aspergillus niger and MP: Macrophomina phaseolina, '+' positive test, '-'negative test, '+++' highly positive, '++'moderately positive

a) Phosphate solubilisation on Sperber medium by T-1



b) Potash mobilization on Aleksandrov medium by W-1

c) Siderophore production on CAS agar plate by W-1



d) ACC deaminase production on DF medium by W-1

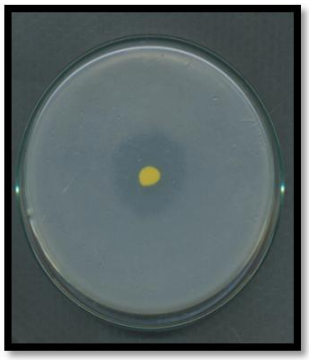
Fig. 1. Phosphorus solubilisation, Potash mobilization, Siderophore and ACC deaminase production by endophytic bacterial isolates

Phosphorus is the second most important nutrient for plants, after nitrogen. In soil it exists as mineral salts or incorporated into organic compounds of which majority of them occurs in an insoluble form. Phosphate solubilizing bacteria (PSB) secrete organic acids and phosphatases to convert the insoluble phosphates into soluble monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) ions, a process referred to as mineral phosphate solubilization. Arora *et al.* screened 20 endophytic bacterial isolates for plant growth promoting substances wherein, 2 isolates showed positive results for phosphate solubilisation [23]. Taurian et al. [7] screened a total of 433 bacterial isolates (304 endophytes and 129 epiphytes from root nodules, roots, stems and leaves) from *Arachis hypogaea* L. for phosphate solubilizing ability. Twenty five percent (110 isolates) showed this ability in Frioni´s and NBRIP media, being 73 of them endophytic and 37 epiphytic isolates. Diameter of solubilization halo in Frioni´s medium varied from 1 to 20.5 mm. Microbes especially endophytes produce cellulolytic enzymes which help them inorder to establish entry way within plants and also for the defense against harmful microbes. Jalgaonwala and Mahajan evaluated some endophytes for hydrolytic enzymatic activity from various parts of medicinal plants growing in Jalgaon, Maharashtra, India. A total of fourteen bacterial and twenty four fungal endophytes were isolated from the aerial and underground parts of seven medicinal plants [24]. Cellulolytic activity was observed in seven bacterial and three fungal isolates. Pandey et al. [25] studied the role of plant beneficial endophytic bacteria from *Mussaenda roxburghii* a very important ethnomedicinal plant, used for its various applications from the ancient period. Four isolates PAK1, PAK2, PAK3 and PAK8 were able to synthesize significant level of IAA in the presence of 5 μg mL^{-1} L-tryptophan. PAK1 was identified as the highest producer of IAA 16.86 ± 0.20 µg/ml and 15.26±0.20 in presence or absence of of L-tryptophan, respectively. Taurian et al. [7] reported peanut associated nine bacterial isolates (5 endophytes and 4 epiphytes) showing IAA production. Quantitative analysis of this phytohormone indicated values in a range from 6.2 to 37.4 µg/ml. Lin et al. [26] screened

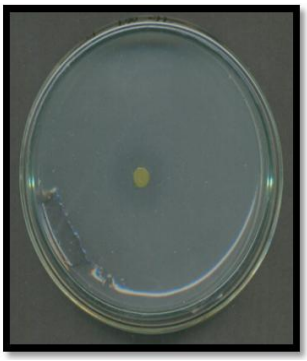
siderophore production of endophytic strains KDRE01 (*B. subtilis*) and KDRE25 (*B. megaterium*) wherein, positive reactions by the CAS method presented yellow halo surrounding the bacterial colonies under iron-limiting conditions. Jasim et al. [27] screened four isolates (ZoB1-ZoB4) from ginger rhizomes for the production of ACC deaminase on DF salts minimal medium amended with 0.2% ammonium sulphate. ZoB2 (*Pseudomonas* sp.) was found to be positive for ACC deaminase production as indicated by its growth in the media. The endophytic isolate *Klebsiella* sp. by Anisha et al. [28] was found to grow in DF minimal salt medium indicating the production of ACC deaminase enzyme. A key mechanism employed for plant growth promotion is by lowering the level of plant ethylene by production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme.

3.3 Biochemical Characterization

Five isolates namely isolate Z-1, CH-1, C-1, T-1 and W-1 selected on the basis of PGP traits were further studied for qualitative biochemical tests in HiMedia Lab identification kit. Reactions to tests for specific breakdown products and carbohydrate utilization are presented in Table 4. All the isolates showed variable response for specific breakdown products and utilization of various carbon sources showing that they are different from each other. Isolate Z-1 was found positive for Voges Proskauer's and urease test. Isolate CH-1 was found positive for ONPG, nitrate reductase and urease enzyme production. Isolate C-1 was found positive for ornithine utilization and urease enzyme production. Isolate T-1 was found positive for ornithine, citrate and malonate utilization as well as Voges Proskauer's test. Isolate W-1 was found positive for ornithine, citrate malonate utilization and urease enzyme production. The results of specific carbon substrate breakdown pattern of selected isolates are presented in Table 5. All chosen endophytic bacterial isolates were found positive for esculine hydrolysis. Isolate CH-1 was found positive for all the tested carbon sources utilization.



a) Lipase production on tributyrin agar plate by W-1



b) Protease production on skimmed milk agar by W-1

c) Chitinase production on colloidal chitin medium by W-1

d) Cellulase production on CMC agar by C-1

Fig. 2. Cell wall degrading enzyme production by endophytic bacterial isolates

Table 4. Biochemical qualitative tests of isolates

Sr. no.	Biochemical test	Isolate				
		Z-1	CH-1	C-1	T-1	W-1
1.	ONPG	-	+	-	-	-
2.	Lysine utilization	-	-	-	-	-
3.	Ornithine utilization	-	-	+	+	+
4.	Urease	-	-	+	-	-
5.	Phenyl alanine deamination	-	-	-	-	-
6.	Nitrate reductase	-	+	-	-	-
7.	H_2S production	-	-	-	-	-
8.	Citrate utilization	-	-	-	+	+
9.	Voges proskauer's	+	-	-	+	-
10.	Methyl red	-	-	-	-	-
11.	Indole	-	-	-	-	-
12.	Malonate utilization	-	-	-	+	+
13.	Oxidase	+	+	+	-	+

Table 5. Carbon substrate utilization by isolates

Sr. no.	Carbon source	Isolates				
		Z-1	CH-1	C-1	T-1	W-1
1.	Esculine	+	+	+	+	+
2.	Arabinose	-	+	+	+	-
3.	Xylose	+	+	+	-	+
4.	Adonitol	-	+	+	-	-
5.	Rhamnose	-	+	+	-	-
6.	Cellobiose	+	+	+	+	-
7.	Melibiose	+	+	+	-	-
8.	Sachharose	+	+	-	+	-
9.	Raffinose	+	+	-	-	-
10.	Trehalose	+	+	+	-	-
11.	Glucose	+	+	+	+	-
12.	Lactose	+	+	+	+	-

3.4 Molecular Identification of Selected Potential Isolates

To further confirm identity of isolates Z-1, CH-1, C-1, T-1 and W-1, bacterial cultures were subjected to 16S *r*RNA sequencing following DNA extraction as described by Nour et al. [29] and PCR amplification of 16S *r*RNA gene using universal primers (U27f and U1492r). These primers gave single band of ~ 1500 bp on 2% agarose gel. All the isolates successfully gave amplification of such band (Fig. 3). The primer pair selectively amplify partial universal region along with variable regions of the bacterial small ribosomal subunit. This region of ribosome is most frequently used to identify and to check diversity of the different group of bacteria.16S *r*RNA partial gene sequence of ~ 1500 bp was carried out (with technical support of Chromus Biotech, Mumbai) and the output data were stored in FASTA format as described in Table 6.

Selected five isolates were then identified on the basis of biochemical and molecular characterization. Isolate Z-1 was identified as *Bacillus tequilensis* strain AAU K1 Accn. MF034733, CH-1 as *Bacillus endophyticus* strain AAU K2 Accn. MF034734, C-1 as *Beijerinckia fluminensis* strain AAU K3 Accn. MF034735, T-1 as *Bacillus safensis* strain AAU K4 Accn. MF034736 and W-1 as *Pseudomonas aeruginosa* strain AAU K5 Accn. MF034737 (Figs. 4-8).

The presence of *nif*H gene was detected by PCR amplification of region coding for Fe-protein (component II) of nitrogenase enzyme complex using degenerate primers. This primer pair selectively amplified Fe-protein coding region depending upon the organism and its *nif* gene sequence [30]. Three out of five isolates gave single band of ~ 380 bp indicating these isolates have presence of *nif* gene providing ability to fix atmospheric nitrogen (Fig. 9).

Table 6. Identification of isolates by 16S *rRNA* sequencing

Sr. no.	Isolate	Length of 16S *rRNA* gene sequenced	GeneBank Accn.	Most closely related organisms*			
				Species	Accession description	% Gene identity	% Query coverage
1	Z-1	1384	MF034733	*Bacillus tequilensis* strain 10b	NR_104919.1	100	100
2	CH-1	1386	MF034734	*Bacillus endophyticus* strain 2DT	NR_025122.1	99	100
3	C-1	1264	MF034735	*Beijerinckia fluminensis* strain UQM 1685	NR_116306.1	100	100
4	T-1	1330	MF034736	*Bacillus safensis* strain NBRC 100820	NR_113945.1	99	95
5	W-1	1303	MF034737	*Pseudomonas aeruginosa* strain DSM 50071	NR_117678.1	99	100

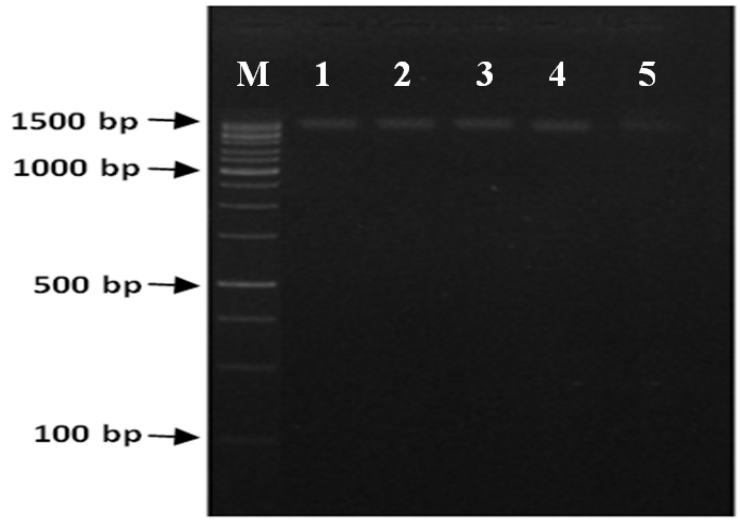


Fig. 3. 16S *r*RNA gene amplification from endophytic bacterial isolates. M-Marker, 1:Z-1, 2:CH-1, 3:C-1, 4:T-1 and 5:W-1

The *nif*H gene is widely used as marker gene for screening nitrogen fixing prokaryotes in soil [31]. Dhole et al. [32] isolated endophytic non rhizobial bacterial stain AM2 from root nodules of *Vigna radiate*. Phylogeny of 16S *r*RNA gene sequences revealed that the strain belonged to the genus *Chryseobacterium*, a member of family *Flavobacteriaceae*. This strain showed presence of *nif*H gene of ~380 bp. Muthukumarasamy et al. [33] isolated bacterial strains possessing nitrogen fixation, phosphate and potassium solubilization and their inoculation effects on sugarcane. Presence of *nif* genes in the representative isolates of both the groups namely *Roseateles terrae* and *Burkholderia gladioli* was confirmed by a PCR assay with *nif*H specific primers which yielded a product at about 360 bp. Andrade et al. [34] studied the analysis of the

abilities of endophytic bacteria associated with banana tree roots to promote plant growth. A total of 40 endophytic bacterial isolates obtained from banana tree roots were characterized for their biotechnological potential for promoting banana tree growth. Bacterial isolates were screened by PCR analysis for the presence of partial *nif*H gene sequences. Isolated DNA was amplified with the universal primers 19fF (5'-GGAATTCTGTGACCTAAAGCTGA-3') and

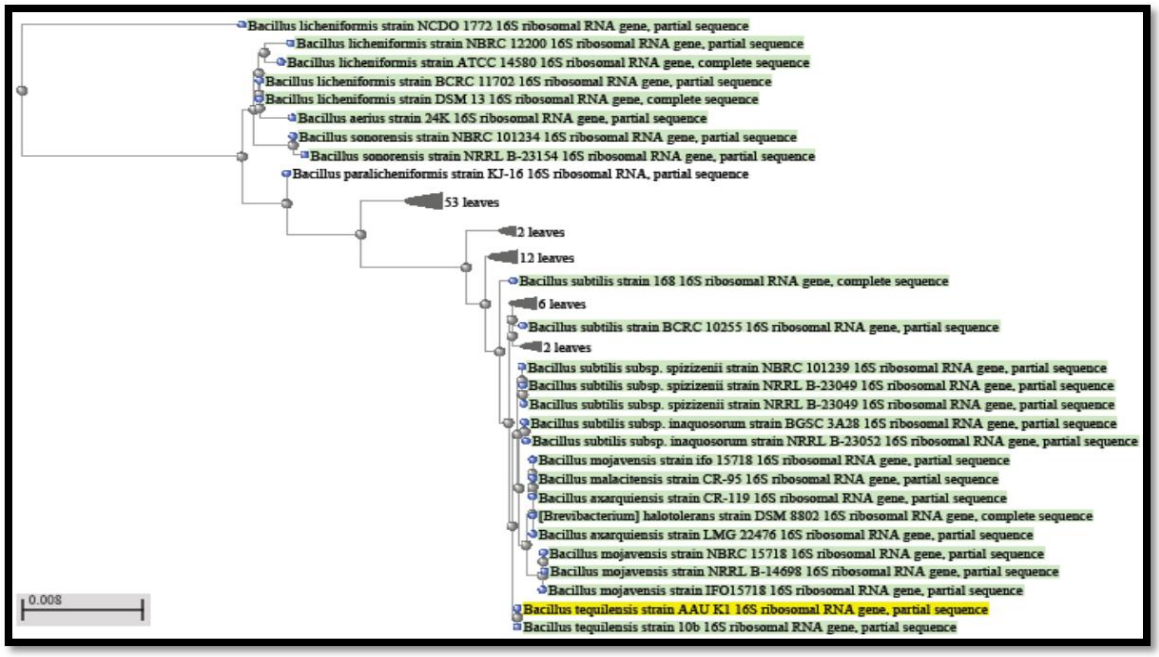


Fig. 4. Phylogenetic tree based on 16S *r*RNA sequence of isolate AAU K1

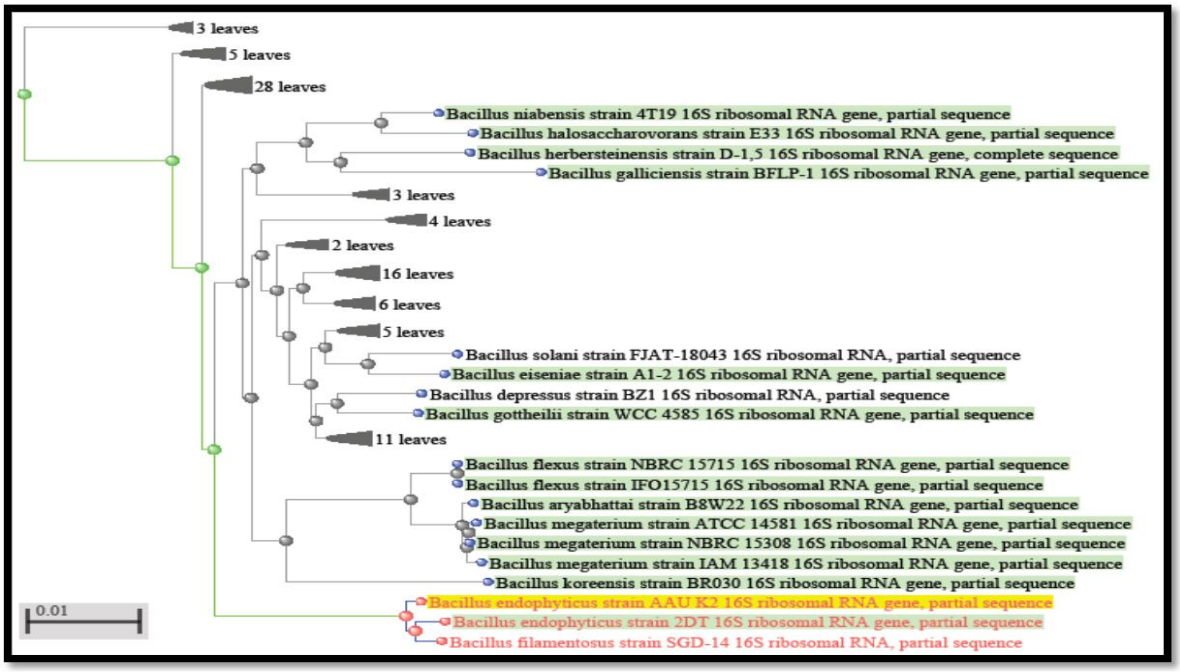


Fig. 5. Phylogenetic tree based on 16S rRNA sequence of isolate AAU K2

407R (5'-AGCATACATTGCCATCATTTCACC-3'), and an amplification product was detected in 24 of the 40 isolates evaluated. Ji et al. [35] isolated and characterized plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Through 16S *r*DNA sequence analysis, *nif*H genes were confirmed in the two species of *Paenibacillus*, three species of *Microbacterium,* three *Bacillus* species, and four species of *Klebsiella*.

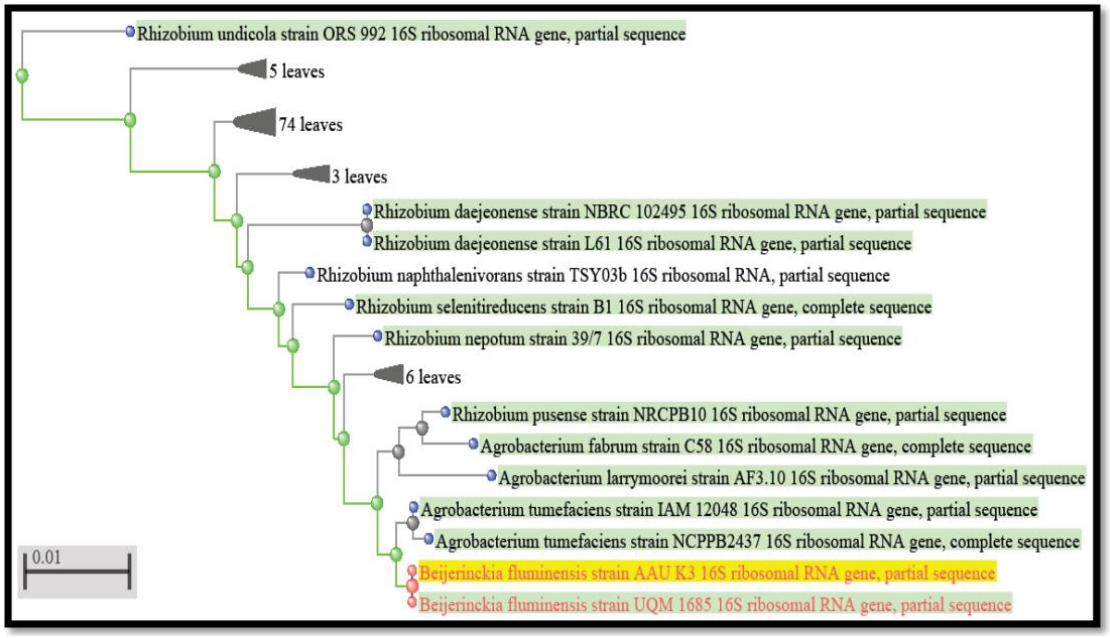


Fig. 6. Phylogenetic tree based on 16S *r*RNA sequence of isolate AAU K3

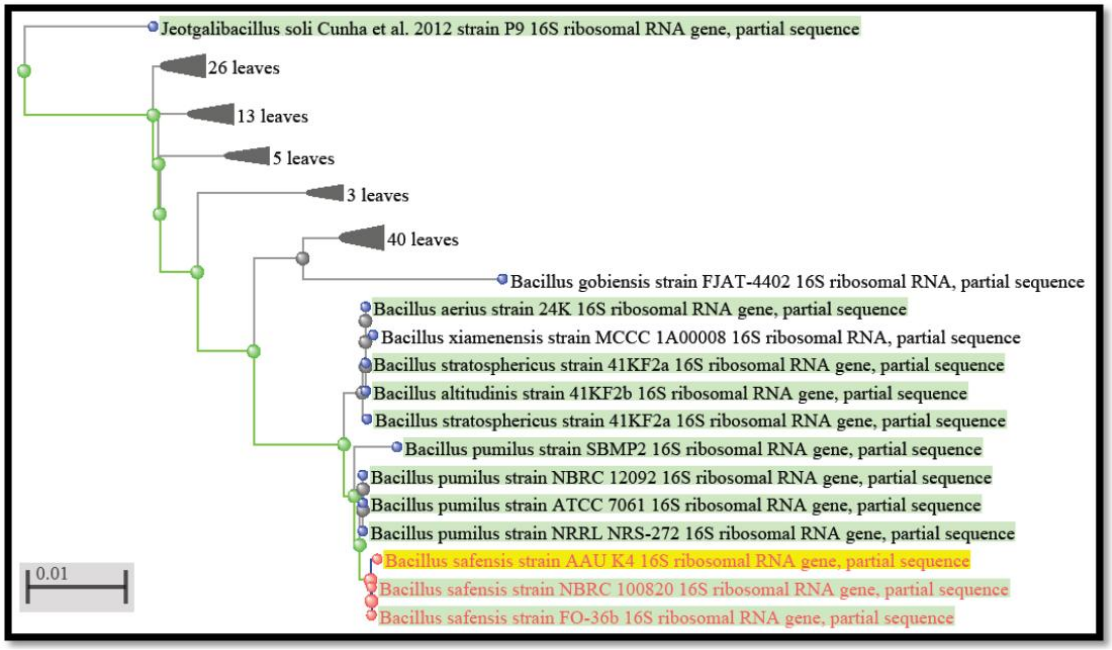


Fig. 7. Phylogenetic tree based on 16S *r*RNA sequence of isolate AAU K4

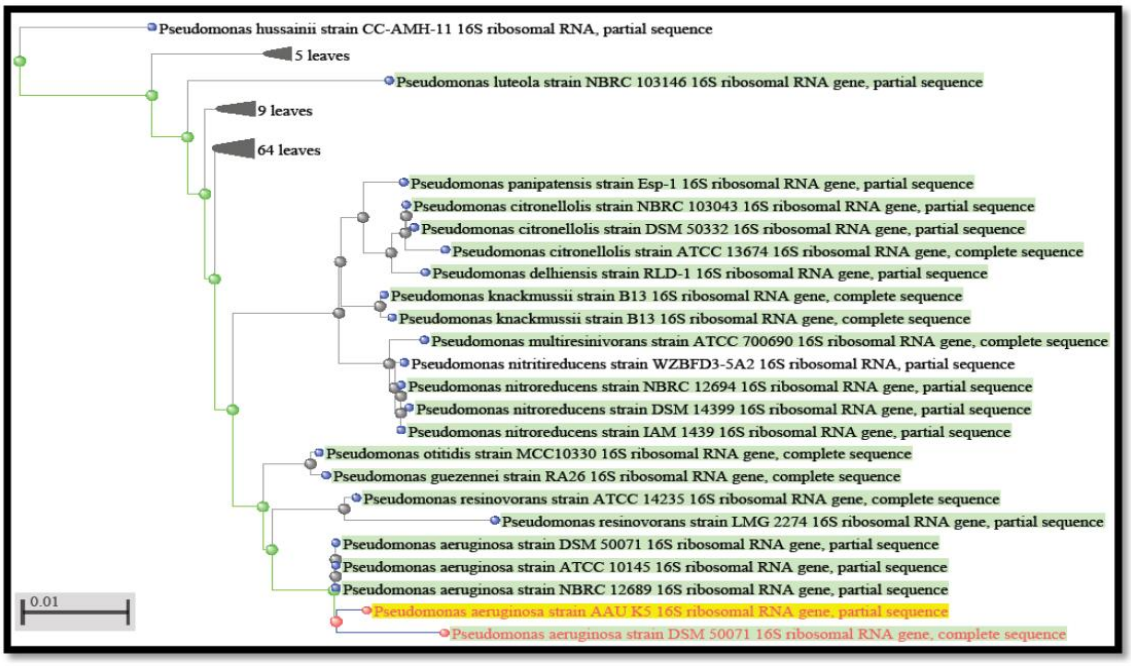


Fig. 8. Phylogenetic tree based on 16S *r*RNA sequence of isolate AAU K5

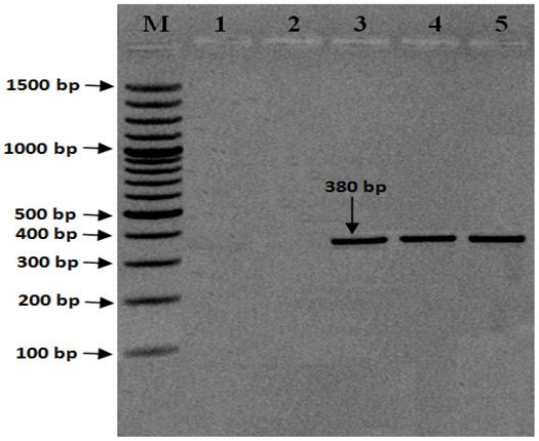


Fig. 9. Amplification of the nitrogen fixation (*nif*H) gene from endophytic bacterial isolates using degenerated universal *nif*H gene primer. Lane: M: Marker, 1:Z-1, 2:CH-1, 3:C-1, 4:T-1 and 5:W-1

4. CONCLUSION

The present study reports the isolation and characterization of endophytic bacteria with PGP properties *viz*. P solubilization, K mobilization, production of hydrolytic enzymes such as cellulase, protease, chitinase and lipase responsible for endophytic colonization, ACC deaminase activity for stress management, production of IAA, siderophore and possessing biocontrol activity. The five best isolates were characterized as *Bacillus tequilensis* strain AAU K1, *Bacillus endophyticus* strain AAU K2, *Beijerinckia fluminensis* strain AAU K3, *Bacillus safensis* strain AAU K4 and *Pseudomonas aeruginosa* strain AAU K5.

The use of plant growth promoting bacterial entophytic inoculants as biofertilizer provide an alternative to chemical fertilizers and could be ideal candidates for commercial applications. These isolates can be developed as new inoculants to utilize in agro industrial sector.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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