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Biochemical and molecular identification of *Solanum lycopersicum* L. temperature tolerant bacterial endophytes

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ABSTRACT

Endophytes are non-pathogenic microorganisms and potential natural bio-inoculant that reside within internal tissues of plant, without causing any apparent symptoms of infection and improve the plant health through acquisition of nutrients. The endophytic bacteria were isolated from the roots of *Solanum lycopersicum* L. surviving at 45–47 °C in the month of June 2013. On the basis of morphological, biochemical, carbon utilization pattern by Biolog and 16S rRNA gene sequence analysis, the isolates were identified as *Rhizobium pusense* (MS-1), *Bacillus flexus* (MS-2), *B. cereus* (MS-3), *Methylophilus flavus* (MS-4) and *Pseudomonas aeruginosa* (MS-5). Most of the endophytic bacterial strains showed plant growth promoting traits activities such as phosphate solubilization, production of IAA, siderophore, ammonia, and nitrate reductase. Carbon sources and other chemicals utilization pattern observed with GEN III microplate of Biolog exhibited strains specific responses. Ten antibiotics discs and 96 wells PM 11C plates were used for their antibiotic sensitivity assay. All the strains were sensitive to chloramphenical, erythromycin, rifampicin, piperacillin, imipenam and gentamycin, while *P. aeruginosa* (MS-5) was resistant to penicillin, linezolid, and clotrimazole discs. This is first report of *M. flavus*, a facultative methylotroph as an endophyte of tomato plant.

1. Introduction

Bacterial endophytes colonize the internal tissues of plants in every ecosystems plant crops' biotic and abiotic stressors by stimulating immune responses, excluding plant pathogens by niche competition, and participating in antioxidant activities and some of them are able to promote the plant growth (Santoyo et al., 2016; Ek-Ramos et al., 2019). They have been isolated from almost all the plant parts including fruits, leaves, stems, seeds and roots (Sturz et al., 2000) and enter into plant tissue primarily through the roots; however leaves may also be used for entry (Kobayashi and Palumbo, 2000). Endophytic bacteria create an enormous biodiversity and majority of them are a rich source of novel natural bioactive compounds (Tan and Zou, 2001). They have diverse effects on their hosts such as nutrient acquisition, growth promotion and bio-control (Brooks et al., 1994; Berg et al., 2005; Tan et al., 2006; Rijavec et al., 2007). The mechanisms of endophytes to counteract the changing environmental conditions may provide better survival strategies to the host plants (Jasim et al., 2016). Endophytic bacteria control plant diseases and also develop systemic resistance which includes the aggressive colonization of plant roots surfaces and adhering to soil interface (Ji et al., 2010; Goswami et al., 2016). Distribution of endophytic bacteria in a plant host is not restricted to a single species but spread to several genera and species (Ryan et al., 2008). The infection process is conditioned by biotic and abiotic factors and they may re-infect to disinfected seedlings. Plant-growth promoting bacteria (PGPB) inoculated to plant resulted in the production of plant growth regulators at the root interface and better absorption of water and nutrient from the soil which stimulated root development (Sharafzadeh, 2012).

Siderophore a low-molecular weight secondary metabolite is considered as indirect bio-control trait which limits the availability of iron trace metal to potential plant pathogens, when it is produced by endophytic bacteria facilitate the plant growth by providing iron to plants (Arora and Verma, 2017; Jin et al., 2014; Maheshwari et al., 2019). Phosphorus (P) is an essential macronutrient for the growth and development of the plant. Several bacterial endophytes solubilize tri-calcium phosphate, *Gluconacetobacter liquefaciens* qzr 14, an bacterial isolate from cucumber might stimulate rhizosphere bacterial community by solubilizing soil phosphorus (Wang et al., 2017; Ouattara et al., 2019). Indole acetic acid is one of the most important phytohormones

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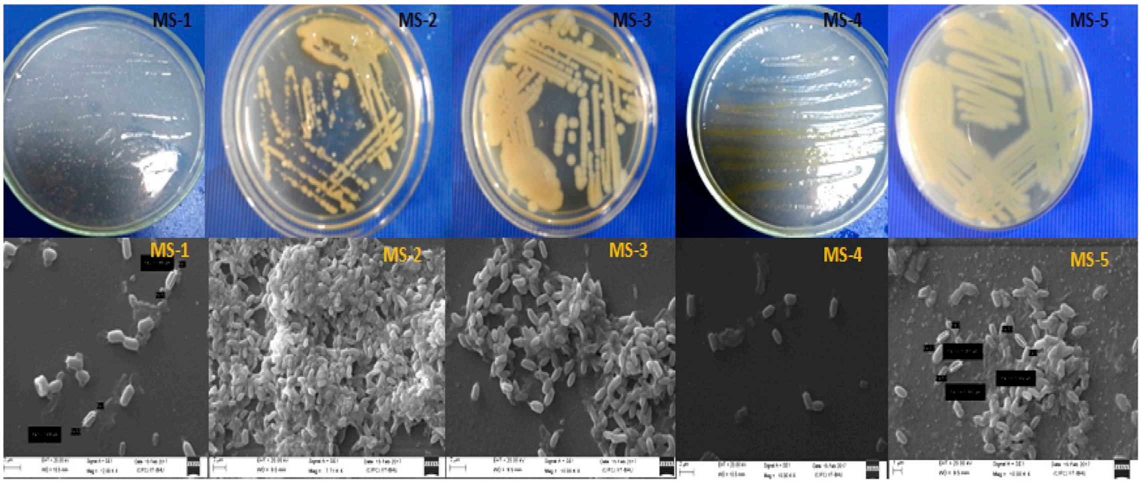

Fig. 1. Pure endophytic bacterial isolates on different agar media and their scanning electron microscopic images.

Table 1
Morphological characteristics of endophytic bacterial isolates from *Solanum lycopersicum* L. plant roots.

Isolates	Colony characteristics				
	Shape	Motility	Color	Consistency	Gram character
MS-1	Rod	Motile	Transparent	Viscid	Negative
MS-2	Rod	Motile	Off white	Mucoid	Positive
MS-3	Rod	Motile	Off white	Mucoid	Positive
MS-4	Rod	Non-Motile	Pale yellow	Mucoid	Negative
MS-5	Rod	Motile	Yellow with fluorescence	Mucoid	Negative

Table 2
Endophytic bacterial isolates viz. MS-1, MS-2, MS-3, MS-4 and MS-5 of *Solanum lycopersicum* L. with their accession no. and nearest phylogenic strain.

S. N.	Isolated strain	Accession no.	Nearest phylogenic strain	Identifaication by Biolog (GEN III plate)	Similarity %
1.	MS-1	MF977751	*Rhizobium pusense*	*Enterobacter aerogene*	99%
2.	MS-2	KX858538	*Bacillus flexus*	*Bacillus krulwichiae*	99%
3.	MS-3	KY018604	*Bacillus cereus*	*Bacillus patagoniensis*	99%
4.	MS-4	MF977752	*Methylophilus flavus*	*Serratia ficaria*	99%
5.	MS-5	KX817189	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	99%

enhances the root structure and growth of the host plant. Endophytic bacteria *Bacillus aryabhattai, B. megaterium* and *B. cereus* isolated from nodules of *Vigna radiata* were produced indole acetic acid (IAA) phytohormone (Bhutani et al., 2018). Bacterial endophytes *Pseudomonas* sp., *Microbacterium* sp. and *Xanthomonas* sp. isolated from rice exhibited promising plant growth promoting activities including production of IAA and siderophore, hormone modulation, nitrogen fixation, and phosphate solubilization (Walitang et al., 2017; Das et al., 2019).

Tomato is the second most widely consumed vegetable and it is an important source of bioactive compounds like lycopene, phenolics, vitamins and ascorbic acid, essential amino acids and dietary fibers (Singh et al., 2017a). It is widely grown vegetable worldwide and also one of the most common kitchen garden vegetable (Rana, 2008; Singh et al., 2014). Several endophytic bacteria isolated from tomato viz. *Stenotrophomonas maltophilia* CT12, *S. maltophilia* CT13, *S. maltophilia* CT16, *Pseudomonas geniculata* CT19, *B. amyloliquefaciens* CT32, *B. subtilis* subsp. *inaquosorum* CT43, *B. licheniformis* SV4, and *B. subtilis* SV5. All isolates were produced IAA and displayed pectinolytic activity. Phosphate solubilization ability was recorded in *S. maltophilia* CT13, *S. maltophilia* CT16, *B. subtilis* subsp. *inaqosorum* CT43 and *B. licheniformis* SV4. Healthy tomato plants naturally colonized by beneficial endophytic bacteria with growth-promoting potential useful

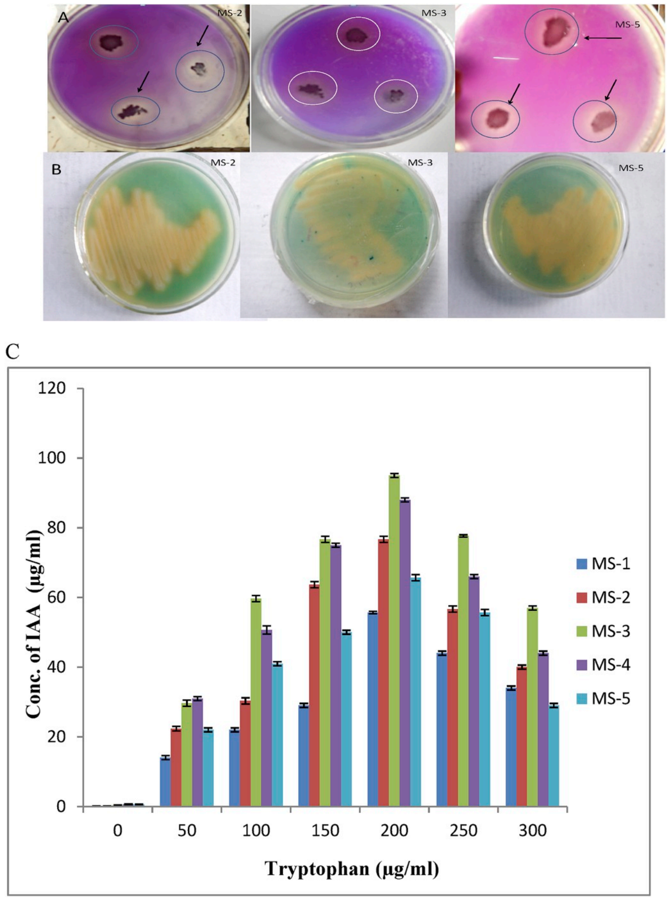

Fig. 2. Phosphate solubilization (A) siderophore production (B), Indole-acetic-acid production (C) of endophytic bacterial isolates at different concentration of L-tryptophan.

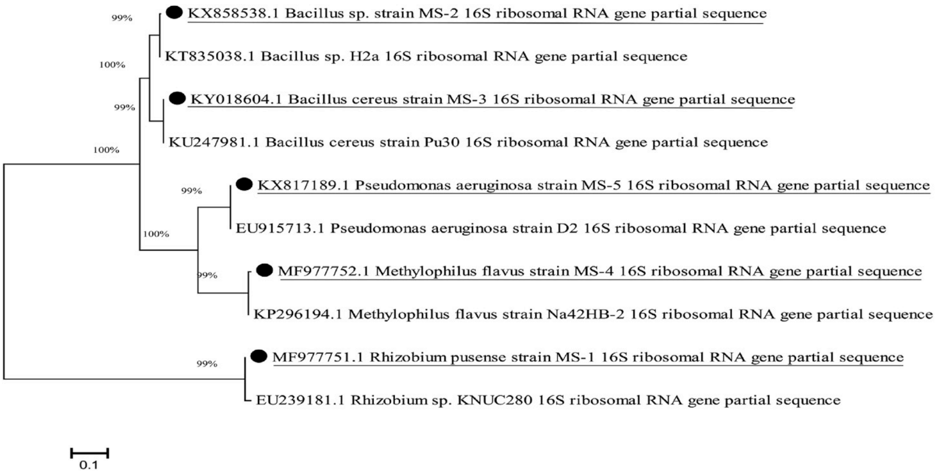

Fig. 3. Phylogenetic tree from analysis of 16S rRNA gene sequence of the endophytic bacterial strains of *Solanum lycopersicum* L. using neighbour joining approach. Each number on a branch indicates the bootstrap confidence values correspond to the scale bar of branch lengths. GenBank accession numbers of nucleotide sequences are shown along with the name of bacterial strain. Phylogenetic analysis were conducted in MEGA 7.0.

Table 3
Biochemical characteristics of endophytic bacterial strains isolated from *Solanum lycopersicum* L.

Biochemical tests	MS-1	MS-2	MS-3	MS-4	MS-5
Catalase	+	+	+	+	+
Oxidase	_	+	_	_	+
Phosphate solubilization	_	+	+	+	+
IAA production	+	+	+	+	+
Siderophore production	+	+	+	+	+
Ammonia production	+	+	+	+	+
HCN production	+	+	+	_	+
Starch hydrolysis	+	+	+	+	_
Nitrate reductase	+	+	+	+	+

Table 4
Antibiotic sensitivity (zone of inhibition in mm) of endophytic bacterial isolates of *Solanum lycopersicum* L.

Antibiotics	Endophytic bacterial isolates				
	MS-1	MS-2	MS-3	MS-4	MS-5
Penicillin (10 Units)	I (12)	S (22)	S (23)	I (17)	R (0)
Chloramphenical (30mcg/disc)	S (22)	S (26)	S (19)	S (20)	S (21)
Erythromycin (15mcg/disc)	S (17)	S (22)	S (21)	S (18)	S (21)
Rifampicin (5mcg/disc)	S (13)	S (16)	S (16)	S (11)	S (14)
Polymixin B, (300 unit/disc)	S (14)	S (12)	I (5)	1 (10)	S (12)
Piperacillin, (100 mcg)	S (19)	S (18)	S (20)	S (19)	S (21)
Linezolid (30 mcg)	S (25)	S (26)	S (24)	S (27)	R (0)
Clotrimazole (CC) 10mcg	S (19)	S (18)	R (9)	R (10)	R (0)
Imipenam (10μg/disc)	S (21)	I (17)	S (22)	S (21)	R (13)
Gentamycin(HLG 120 mcg)	S (18)	S (19)	S (19)	S (18)	S (21)

S: Sensitive.
I: Intermediate.
R: Resistant.

for the improvement of tomato growth (Abdallah et al., 2018). Eco-friendly and plant beneficial bacteria isolated from nature can be used as microbial inoculants to improve plant growth and systemic resistance. They have great potential to be applied to plant seeds or seedlings, or as foliar spray to promote plant growth and to produce bioactive compounds (Singh et al., 2017b). As a microbial endophytic bacteria may protect their host plants from pathogen infection (Akhdiya et al., 2014). Endophytic microbial abundance and diversity remain significantly higher in the organically grown vegetable crops indicating the superiority of in enhancing organic management practices over the modern agricultural practices (Gaiero et al., 2013). The re-inoculation of endophytic bacteria to tomato plants grown in a green house promoted plant growth indicating their potential application in agro-ecosystem (Xia et al., 2015). Although a large diversity pools of endophytic bacteria have been isolated, identified and characterized as potential microbial inoculant and source of bioactive compound from grain crops (Singh et al., 2017b), endophyte-plant interactions reports are limited in vegetable crops (Kumar et al., 2016; Singh et al., 2017).

The present study deals with the isolation of bacterial endophytes from hybrid and local varieties of tomato plants, grown in pots under natural environment surviving at 45–47 °C. Some selected endophytes were identified on the basis of phenotypic and biochemical characteristics and 16S rRNA sequence based phylogeny. Further the antibiotic sensitivity and carbon utilization pattern of bacterial strains were determined by Biolog Microstation system using GEN III microtitre and PM plates.

2. Materials and methods

Endophytic bacteria were isolated from the roots of *Solanum lycopersicum* L. in the month of June 2013 tolerating temperature 45–47 °C. The plants grown in pots (soil: sand: compost; 2:1:2) in the Botanical garden, Banaras Hindu University Varanasi, India (20^{0}180N and 80^{0}360E, elevation 80.71 m) seeds were selected for bacterial isolation.

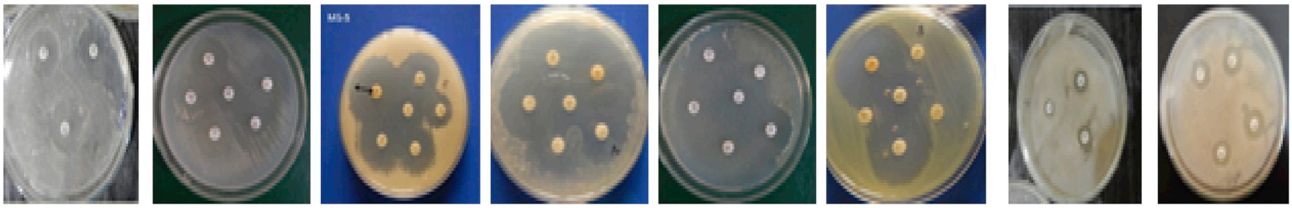

Fig. 4. Zone of inhibition of antibiotics on endophytic bacterial isolates lawn.

Table 5
Carbon source metabolic characteristics of endophytic bacterial strains in GEN III Microplate after 48 h of incubation.

S.N	Carbon source	MS-1	MS-2	MS-3	MS-4	MS-5	S.N	Carbon source	MS-1	MS-2	MS-3	MS-4	MS-5
A1	Negative control	-	-	-	-	-	**E1**	Gelatin	-	±	±	±	±
A2	Dextrin	±	+	+	+	+	**E2**	Glycl- L-proline	±	+	±	+	+
A3	Maltose	-	+	+	+	+	**E3**	L-alanine	+	+	+	+	+
A4	D-trehalose	±	+	+	+	+	**E4**	L-Arginine	+	+	+	+	+
A5	D-cellobiose	+	+	+	+	+	**E5**	L-Aspartic acid	+	+	+	+	+
A6	Gentobiose	±	+	+	+	+	**E6**	L-Glutamic acid	+	+	+	+	+
A7	Sucrose	+	±	+	+	+	**E7**	L-Histidine	+	+	+	+	+
A8	D-Turanose	+	±	+	+	+	**E8**	L-Pyroglumatic acid	+	+	+	+	+
A9	Stachyose	-	±	+	+	+	**E9**	L- Serine	+	+	+	±	+
A10	Positive control	+	+	+	+	+	**E10**	Lincomycin	+	+	-	+	+
A11	pH-6.0	+	+	±	+	+	**E11**	Guanidine hydroclanle	±	+	-	+	+
A12	pH-0	+	+	+	+	+	**E12**	Nicaproof 4	±	+	-	+	±
B1	D-Raffinose	-	±	±	+	+	**F1**	pectin	±	±	±	±	±
B2	D-Lactose	±	±	+	+	±	**F2**	D galacturonic acid	±	+	±	+	+
B3	D- Melibiose	-	+	+	+	+	**F3**	Lgalactonic acid galactone	+	+	±	+	+
B4	3- Methyl D-glucoside	-	+	+	+	+	**F4**	D gluconic acid	+	+	+	+	+
B5	D- Salicin	+	±	±	+	+	**F5**	D glucoronic acid	+	+	±	+	+
B6	*N*-Acetyl D-glucosamine	+	+	+	+	+	**F6**	glucuronamide	+	+	±	±	±
B7	*N*-Acetyl D-mannosamine	±	+	+	±	+	**F7**	Mucic acid	+	+	±	+	+
B8	*N*-Acetyl D-galactosamine	±	+	±	±	+	**F8**	Quinic acid	+	±	+	+	+
B9	*N*-Acetyl D-neuraminic acid	+	+	+	±	±	**F9**	D sacchric acid	+	+	+	+	+
B10	1% NaCl	+	±	±	+	+	**F10**	Vancomycin	+	+	-	+	+
B11	4% NaCl	+	±	±	+	±	**F11**	Tetrazoliumvoilet	-	+	-	+	+
B12	8% NaCl	-	±	±	+	±	**F12**	Tetrazolium Blue	-	+	-	+	+
C1	a D-Glucose	+	±	+	+	±	**G 1**	Hydroxyphenylacetic acid	+	±	±	+	+
C2	D-Mannose	+	±	+	+	+	**G2**	Pyruvic acid methyl ester	+	±	+	+	+
C3	D-Fructose	+	+	±	+	+	**G3**	D- Lactic Acid	-	±	+	±	+
C4	D-Galactose	+	±	+	+	+	**G4**	L-Lactic acid	±	+	+	+	+
C5	3-Methyl glucose	+	±	±	±	+	**G5**	Citric acid	-	+	+	+	+
C6	D-Fucose	+	±	±	±	±	**G6**	a Ketoglutaric acid	±	+	±	+	+
C7	L-Fucose	+	+	±	+	+	**G7**	D-Malic acid	+	+	+	+	+
C8	L-Rhamnose	+	+	±	+	+	**G8**	L- Malic acid	+	+	+	+	+
C9	Inosine	±	+	±	+	+	**G9**	Bromo succinic acid	+	+	+	±	+
C10	1% Sodium lactate	+	+	+	+	+	**G10**	Nalidixic acid	±	+	±	+	-
C11	Fusidic acid	+	+	-	+	+	**G11**	Lithium chloride	-	+	+	+	+
C12	D -serine	±	±	-	+	-	**G12**	Potassium tellurite	±	+	-	+	+
D1	D-Sorbitol	+	+	+	+	+	**H 1**	Tween 40	±	-	-	±	±
D2	D-Mannitol	±	+	+	+	+	**H2**	G- Amine N butyric acid	+	±	+	+	+
D3	L- Arabitol	+	+	+	+	+	**H3**	a Hydroxyl butyric acid	-	±	±	±	+
D4	Myo- Inositol	+	+	+	+	+	**H4**	β- Hydroxyl butyric acid	+	+	+	+	+
D5	Glycerol	+	+	+	+	+	**H5**	a ketobutyric acid	±	±	±	±	+
D6	D-Glucose 6 phosphate	±	+	+	+	+	**H6**	Acetoacetic acid	-	±	±	-	±
D7	D- fructose 6 phosphate	±	+	±	+	+	**H7**	Propionic acid	-	+	±	±	+
D8	D aspartic acid	±	+	±	±	+	**H8**	Acetic acid	±	+	±	±	+
D9	D serine	-	+	-	±	+	**H9**	Formic acid	±	±	±	-	+
D10	Troleandomycin	±	+	-	+	±	**H10**	aztreonam	±	±	±	+	+
D11	Rifamycin	+	±	+	+	+	**H11**	Sodium butyrate	-	+	+	+	±
D12	Minocycline	±	+	-	+	-	**H12**	Sodium bromate	-	±	-	+	-

'+' Positive, '-' Negative, '±' Partial.

Fresh root explants were washed thoroughly under running tap water and surface sterilized (70% C_2H_5OH, 3 min, 0.5% NaOCl, 3 min and 70% C_2H_5OH, 30 s) (Sun et al., 2008). Surface sterilization efficiency of the sterilizers was checked by inoculating surface sterilized unsliced plant roots on nutrient agar plate. The surface sterilized roots were sliced into thin sections and placed aseptically over different nutrient agar petri dishes. The plates were incubated at 45 ± 1 °C for 2–7 days in bacteriological incubator. The colonies surrounding root sections were picked up and streaked on the different nutrient media for the selection of clone.

2.1. *Morphological characterization of endophytic isolates*

The morphological characteristics of strains were determined by recording colony morphology, shape, margin, color and consistency and Gram-staining behavior according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Shape and size of isolated clones were confirmed from the scanning electron microscopy (Fig. 1). Isolates grown on their respective media Jenson's (MS-1), Luria- Bertani agar (MS-2 and MS-3), Nitrate minimal salt medium (MS-4) and King's B (MS-5) were examined for morphological and biochemical characteristics.

2.2. *Plant growth promoting activity*

The endophytic bacterial isolates were tested for their plant growth promoting potential including IAA, HCN, ammonia, siderophore production and phosphate solubilization.

2.2.1. *Phosphate solubilization*

All bacterial isolates were tested for tri-calcium phosphate solubilization by an agar assay using National Botanical Research Institute's phosphate (NBRIP) medium (Nautiyal, 1999). A loopful (10 μl) of each bacterial isolate was stabbed in triplicate using sterile micropipette on the centre of NBRIP growth medium. The halo colony diameters were measured after 7 days of incubation on agar plates at 45 ± 1 °C. Formation of clear zone indicated the phosphate solubilizing potential of bacteria.

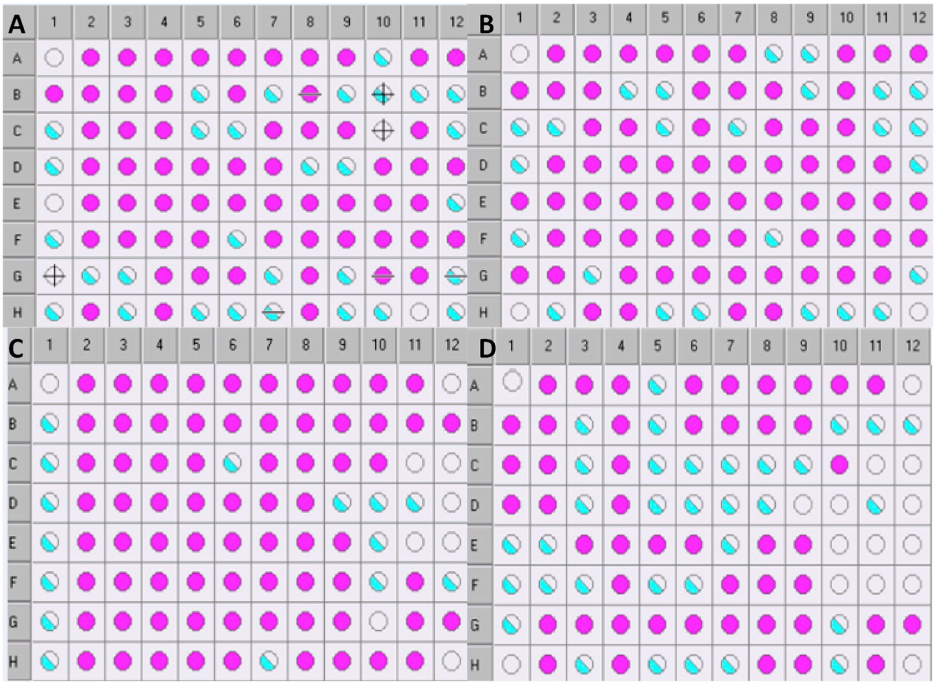

Fig. 5. Carbon utilization pattern in the GEN III microplate of Biolog microstation system after 72 h of incubation (A) MS-1, (B) MS-2 (C) MS-3 (D) MS-4.

2.2.2. Ammonia production

Ammonia production was determined in freshly grown cultures according to (Cappuccino and Sherman, 1992).

2.2.3. Indole-acetic-acid (IAA) production

IAA production was detected using the modified method described by Bric et al. (1991). Bacterial cell biomass grown for 48 h at 45 ± 0.1 °C in broth supplemented with 25–300 μg/ml of L-Tryptophan, was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant (2 ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent. After 1 h of incubation in dark at room temperature the absorbance of pink colour solution was measured at 530 nm and compared with standard graph of IAA (0–100 μg/ml).

2.2.4. Hydrogen cyanide production

Bacterial cultures were streaked on nutrient agar medium containing 4.4 mg per ml of glycine. A Whatman filter paper no.1 soaked in 0.5% picric acid solution (in 0.2% sodium carbonate) was placed inside the lid of a plate. The sealed plates were incubated at 45 ± 1 °C for 4 days. Development of light brown to dark brown colour indicated HCN production (Castric, 1975).

2.2.5. Catalase activity

Immediate gas bubbles formation was considered catalase positive when a single colony was mixed with 2–3 drops of 3% H_2O_2 on clean grease free glass slide.

2.2.6. Siderophore production

Formation of orange halos around the endophytic bacterial colonies grown on nutrient agar plates at 45 ± 1 °C containing the dye chromazurol S (CAS) were indicative for siderophore production (Schwyn and Neilands, 1987) which was considered as positive indication for siderophore production.

2.3. Identification of endophytic bacterial strains

2.3.1. 16SrRNA sequence analysis of endophytic bacterial isolates

The 16S rRNA gene partial sequences of the isolates were sequenced and compared with the RNA database. The resulting nucleotide sequences were assigned for bacterial taxonomic affiliation based on the closest match to the sequences available at National Centre for Biotechnology Information (NCBI) using Nucleotide Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST) program. The sequences showing 99% similarity were retrieved. The cluster analysis of the sequence was prepared using the multiple sequences alignment tool, Clustal X 2.1 version. The phylogenetic and molecular analyses were conducted using MEGA 7.0 software.

2.3.2. Antibiotic sensitivity test

Antibiotic sensitivity test was performed using antibiotic impregnated discs (6 mm diameter). The endophytic bacterial isolates were tested against penicillin, chloramphenicol, erythromycin, rifampicin, polymixin B, piperacillin, linezolid, clotrimazole and imipenam by Kirby Bauer disc-diffusion assay method (Kumar et al., 2016). The quantities of antibiotics used were 5–30μg/disc. On the basis of inhibition zone recorded, organisms were categorized as resistant or sensitive or intermediate according to Difco Laboratories (1984).

2.3.3. Carbon sources utilization pattern and identification of endophytic bacterial strain using the BIOLOG™ GEN III microplate

The carbon sources and some other chemicals utilization tolarance pattern of endophytic strains were determined and their identification was made by Biolog Microstation system using GEN III microplates. The test panel contains 71 carbon sources and 23 chemical sensitivity assays. GEN III dissects and analyzes the ability of the cell to metabolize all major classes of compounds, in addition to determining other important physiological properties such as pH, salt and lactic acid tolerance, reducing power, and chemical sensitivity. All the reagents applied were from Biolog, Inc. (Hayward, CA, USA). Freshly grown cultures of the isolates were prepared by removing bacterial colonies from the respective medium plate surface with a sterile cotton swab and agitating it in 5 ml of 0.85% saline solution. Bacterial suspension was adjusted in IF-A to achieve a 90–98% transmittance (T90) using a Biolog turbidity meter. Suspension (150 μL) was dispensed into each well of a Biolog GENIII microplate. The plates were incubated at 45 ± 1 °C in an Omnilog Reader/Incubater (Biolog). After incubation, the appearance of purple

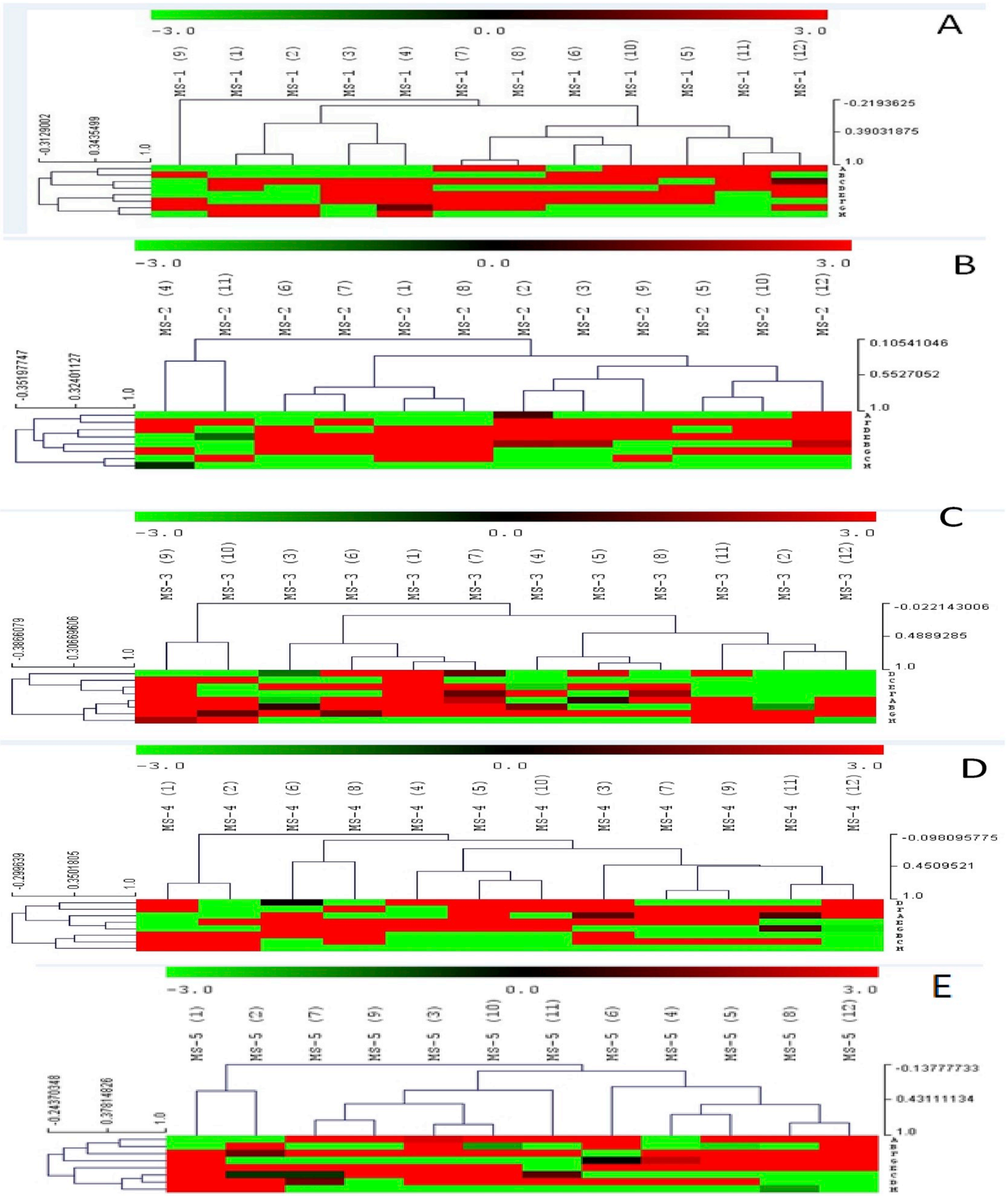

Fig. 6. Heatmap of five endophytic bacterial isolates on the basis of carbon utilization pattern of GEN III microplate of Biolog Microstation system, **A.** *Rhizobium pusense* (MS-1) **B.** *Bacillus flexus* (MS-2) **C.** *B. cereus* (MS-3) **D.** *Methylophilus flavus* (MS-4) **E.** *Pseudomonas aeruginosa* (MS-5).

wells was compared to the Biolog's extensive species library.

2.3.4. *Antimicrobial assay using phenotypic microarray plate*

Endophytic bacterial isolates grown overnight at 45 ± 1 °C on respective medium were suspended in 16 ml of Inoculating Fluid-0a (IF-10b), in a 20 ml sterile capped glass tube as above (the cell density equaled to 85% transmittance (T) on a Biolog turbidity meter). Later, the suspensions were transferred to a sterile reservoir and 100μl/well were added to PM11C plate (Dye included in plate) using Biolog multichannel pipette. The plates incubated at 45 ± 1 °C in the Biolog plate incubator and reader was monitored for any color change of the wells. Observations were recorded at 24, 48 and 72h for PM plate. Forty eight hours incubation of plate absorbance values was taken to access the data.

2.4. *Statistical analysis*

The data were analyzed with SPSS version 16.0 software. The heatmap analysis of carbon and 24 antibiotics utilization pattern by Biolog Microstation system were prepared with Meve software package (ver.16) and Microsoft office excel. Phylogenetic analysis was conducted in MEGA7.0.

3. Results

Twenty five endophytic bacterial clones were isolated from the roots of two tomato varieties (S-22 Hybrid, S-3619- Local) growing in the month of June (max. temp- 45–47 °C). The surface sterilized root did not show the any growth of bacteria which confirmed that the isolates were endophyte. Out of 25 bacterial isolates only five were selected and screened on the basis of their growth on Luria bertani agar medium, King's B nutrient agar medium, Jensen's N_2 free medium, and NMS agar medium. The clones growing on the respective agar medium were further checked for purity and finally transferred to the nutrient agar slants (Fig. 1).

Table 6
GEN III microplate with substrate: MS-1, MS-2, MS-3, MS-4 and MS-5 showed strong color intensity (absorbance) with substrates present in the wells. Data obtained using Heat map analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control	Dextrin	Maltose 🟣	D-trehalose	D-cellobiose 🔴🟡🟣	Gentobiose 🔵🟡🟣	Sucrose 🔴🟡🟣	D-Turanose 🔴🔵🟣	Stachyose 🔵🟡🟣	Positive control 🔴🟢🔵🟡🟣	pH-6.0 🔴🔵🟣	🔴🟢🔵🟡🟣 pH-5.0
B	D-Raffinose 🟢🔵🟡	D-Lactose 🟡🟣	D- Melibiose 🟡🟣	3- Methyl D-glucoside	D- Salicin 🔴	N-Acetyl D-glucosamine 🔴🟢🔵🟡🟣	N-Acetyl D-mannosamine 🟢🔵	N-Acetyl D-galactosamine 🟢	N-Acetyl D-neuraminic acid 🔵	1% NaCl 🔴🔵	4% NaCl 🔴🔵	8% NaCl 🔵🟣
C	a D-Glucose 🔴🟢🔵🟡🟣	D-Mannose 🟡	D-Fructose 🔴🟡🟣	D-Galactose 🔴	3-Methyl glucose 🔴	D-Fucose 🔴🔵	L-Fucose 🔴🟡	L-Rhamnose 🔴🟢	Inosine 🟢🔵🟡🟣	1% Sodium lactate 🔴🔵🟣	Fusidic acid 🔴🟢🟡	D –serine 🔴
D	D-Sorbitol 🔴🟢🔵🟡🟣	D-Mannitol 🟢🟣	L- Arabitol 🔴🟢🟡🟣	Myo- Inositol 🔴🟢🟡	Glycerol 🟢🔵🟣	D-Glucose 6 phosphat 🟢🔵🟣	D- fructose 6 phosphate	D aspartic acid 🟢	D serine 🟢	Troleandomycin 🟢🟡🟣	Rifamycin 🔴🔵🟣	Minocycline 🟢🟡
E	Gelatin 🔵🟡🟣	Glycl- L-proline 🟢🔵🟡	L-alanine 🔴🟢🔵🟡🟣	L-Arginine 🔴🟡🟣	L-Aspartic acid 🔴🟢🔵🟡🟣	L-Glutamic acid 🔴🟢🟡🟣	L-Histidine 🔴🟢🔵🟡🟣	L-Pyroglumatic acid 🔴🟢🔵🟣	L- Serine 🟢🔵🟡🟣	Lincomycin 🔴🟢🟡🟣	Guanidine hydroclanle	Nicaproof 4 🔴🟢🟣
F	Pectin 🔵🟡🟣	D galacturonic acid 🟢	L galactonic acid galactone 🔴🟢🟡🟣	D gluconic acid 🔴🟢🔵🟣	D glucoronic acid 🔴🟢🟡🟣	Glucuronamide 🔴🟢	Mucic acid 🟢🟡🟣	Quinic acid 🔴🟣	D sacchric acid 🟢🔵🟡🟣	Vancomycin 🔴🟢🟡🟣	Tetrazolium violet 🟢🟡🟣	Tetrazolium Blue 🟢🟡🟣
G	Hydroxyphenyl acetic acid 🔴🟢🔵🟣	Pyruvic acid methyl ester 🔴🔵	D- Lactic Acid Methyl ester 🔵	L-Lactic acid 🟢🔵🟡🟣	Citric acid 🟢🔵🟡🟣	a Ketoglutaric acid 🟢🟡	D-Malic acid 🔴🟢🔵	L- Malic acid 🔴🟢🔵🟣	Bromo succinic acid 🔵	Nalidixic acid 🟢🟡	Lithium chloride 🔵	Potassium tellurite 🔴🟢🔵🟣
H	Tween 40 🔴🟡🟣	G- Amine N butyric acid 🔴🔵🟡🟣	a Hydroxyl butyric acid 🟣	β- Hydroxyl butyric aci 🔴	a ketobutyric acid	Acetoacetic acid	Propionic acid	Acetic acid	Formic acid	Aztreonam 🔵	Sodium butyrate 🔵	Sodium broamate

🔴 *R. pusense* (MS-1), 🟢 *B. cereus* (MS-2), 🔵 *B. flexus* (MS-3), 🟡 *M. flavus* (MS-4), 🟣 *P. aeruginosa* (MS-5)

Table 7
Antibiotic sensitivity of endophytic bacterial isolates in PM 11C microplate after 48h

Well No. in PM11 C	Compounds and their targets		Results				
			MS-1	MS-2	MS-3	MS-4	MS-5
A1-4	Amikacin	Wall, lactam	±	±	+	±	_
A5-8	Chlorotetracycline	Protein synthesis, 30S ribosomal subunit, tetracycline	±	+	+	+	+
A9-12	Lincomycin	Protein synthesis, 50S ribosomal subunit, lincosamide	_	±	±	_	±
B1–B4	Amoxicillin	Wall, lactam	±	+	±	±	+
B5–B8	Cloxacillin	Wall, lactam	+	+	±	±	+
B9–B12	Lomefloxacin	DNA topoisomerase	+	+	+	±	+
C1–C4	Bleomycin	DNA damage, oxidation	±	+	+	_	±
C5–C8	Colistin	Membrane, cyclic peptide	+	+	+	+	±
C9–C12	Minocycline	Protein synthesis, 30S ribosomal subunit, tetracycline	_	±	±	+	±
D1-D4	Capreomycin	Protein synthesis	±	±	±	_	_
D5-D8	Demeclocyline	Protein synthesis, 30S ribosomal subunit, tetracycline	+	+	±	_	±
D9-D12	Nafcillin	Wall, lactam	+	+	+	+	+
E1-E4	Cefazolin	Wall, cephalosporin	±	+	+	±	±
E5-E8	Enoxacin	DNA topoisomerase	±	+	±	+	+
E9-E12	Nalidixic acid	DNA topoisomerase	+	+	+	+	+
F1–F4	Chloramphenicol	Protein synthesis, amphenicol	+	+	+	+	+
F5–F8	Erythromycin	Protein synthesis, 50S ribosomal subunit, macrolide	±	±	+	+	+
F9–F12	Neomycin	Protein synthesis, 30S ribosomal subunit, aminoglycoside	+	+	±	+	+
G1-G4	Ceftriaxone	Wall, cephalosporin	±	±	±	_	+
G5-G8	Gentamicin	Protein synthesis, 30S ribosomal subunit, aminoglycoside	±	+	+	+	+
G9-G12	Potassium tellurite	Toxic anion	_	_	±	+	±
H1–H4	Cephalothin	Wall, cephalosporin	±	±	+	±	±
H5–H8	Kanamycin	Protein synthesis, 30S ribosomal subunit, aminoglycoside	±	±	±	±	±
H9–H12	Ofloxacin	DNA topoisomerase	±	+	+	+	_

3.1. Morphological and biochemical characteristics

The morphological and biochemical characteristics of endophytic bacterial isolates have been presented in Table 1 and 2.

3.2. Phylogenetic analysis

On the basis of their morphological and biochemical characteristics isolates were identified as *Rhizobium* (MS-1), *Bacillus* (MS-2 and MS-3),

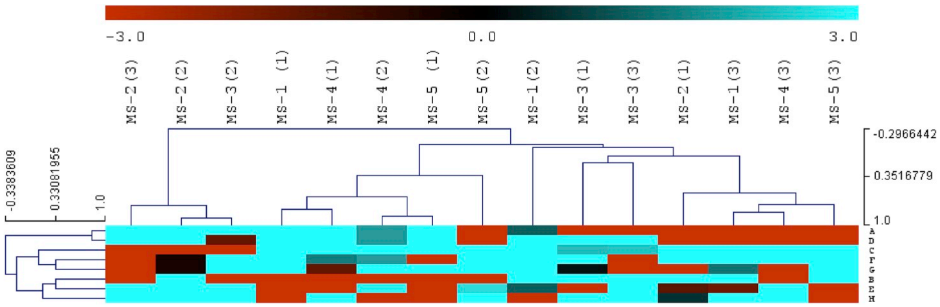

Fig. 7. Heat map of five endophytic bacterial strains on the basis of 24 antibiotics sensitivity pattern of PM 11 C microplate of Biolog Microstation system.

Table 8
Antibiotic assay of endophytic bacterial strains. The table showing strong color intensity of antibiotics by all five endophytic bacterial strains in Heat map.

PM11C plate	1(Well no.1-4)	2 (Well no.5-8)	3 (Well no. 9-12)
A	Amikacin 🟣🔴🟡	Chlortetracycline 🟢🔵	Lincomycin 🟢
B	Amoxicillin 🟢🔵	Cloxacillin 🟢	Lomefloxacin 🔴🔵🟣
C	Bleomycin 🔴🟣🟡🟢	Colistin 🟣🔴🟡	Minocycline 🟣🔴🟡
D	Capreomycin 🔴🟡🟣🔵	Demeclocyclin 🟢🔴	Nafcillin 🟢🔵
E	Cefazolin	Enoxacin 🟢🟡🔵	Nalidixic acid 🟢🟡🔵
F	Chloramphenicol 🔴🟢🔵	Erythromycin 🔴🔵🟣	Neomycin 🟣🔴🟡
G	Ceftriaxone 🟣🔴	Gentamicin 🔴🟡🟣🔵	Potassium tellurite 🟣
H	Cephalothin 🟡🔵	Kanamycin 🔵🟣🟢	Ofloxacin 🔵🔴🟣🟡🟢

🔴*R. pusense* (MS-1)
🟢*B. flexus* (MS-2),
🔵 *B. cereus* (MS-3),
🟡 *M. flavus* (MS-4),
🟣*P. aeruginosa* (MS-5)

Methylotroph (MS-4), and *Pseudomonas* (MS-5). Although the Biolog system yielded a characteristic pattern of substrate utilization for each isolates, GEN III database confirmed the identification of MS-1 as *Enterobacter aerogene*, MS-2 as *Bacillus krulwichiae,* MS-3 as *Bacillus patagoniensis*, MS-4 as *Serratia ficaria* and MS-5 as *Pseudomonas aeruginosa.* To confirm the identity of isolates, the molecular phylogeny using the 16S ribosomal gene sequencing of the isolates were performed. The results of their closely related strains from the NCBI gene data bank have been presented in Table 2. The phylogenetic tree of 16S ribosomal gene sequences as compared to the nearest closest strain and their percent of similarity have been presented in Fig. 3.

3.3. *Plant growth promoting traits*

All the endophytic bacterial strains (MS-1, MS-2, MS-3, MS-4 and MS-5) were catalase positive and showed IAA, siderophore and ammonia production. Endophytic strains *Bacillus flexus* (MS-2) and *Methylophilus flavus* (MS-4) were also oxidase positive, while all other strains showed HCN production except (MS-4). Endophytic bacterial isolates *Rhizobium pusense* (MS-1), *B. flexus* (MS-2), *Bacillus cereus* (MS-3) showed positive results for starch hydrolysis while *M. flavus* (MS-4) and *Pseudomonas aeruginosa* (MS-5) showed negative results. All endophytic bacterial strains showed positive results for nitrate reductase. (Table 3 and Fig. 2).

3.3.1. IAA production

All the five strains produced indole acetic acid. The rate of production measured in the presence of various concentrations (50–200 μg/ml) of tryptophan in the respective medium have been presented in Fig. 2. As the concentration of tryptophan increased, the production of IAA also increases. Two strains *B. cereus* (MS-3) and *M. flavus* (MS-4) were efficient producer of IAA followed by *R. pusense* (MS-1), *B. flexus* (MS-2) and *P. aeruginosa* (MS-5) isolates. IAA production measured after 48 h of incubation in different increasing tryptophan concentrations up to 200 μg/ml increased in all five bacterial strains in concentration dependent manner.

3.3.2. Antibiotic sensitivity

The endophytic strains were tested for their sensitivity to ten antibiotics (concentration mentioned in Table 4). The antibiotics discs were placed on the freshly inoculated lawns of isolates on their respective growth medium. The inhibition zone of antibiotics (mm) for each strain has been presented in Table 4. A differential sensitivity of isolates to different antibiotics revealed that the strains differed in their antibiotic sensitivity. *P. aeruginosa* (MS-5) was resistant to penicillin, linezolid, and clotrimazole. All the strains were sensitive to chloramphenical, erythromycin, rifampicin, piperacillin, imipenam and gentamycin (Fig. 4 and Table 4.).

3.4. Biolog GEN III and PM assays

Carbon utilization pattern of five endophytic bacterial strains were observed in Biolog Microstation System, using GEN III microplate and the results obtained after 48 h of incubation have been presented in Table 5 and Fig. 5. It is evident from the results that carbon sources utilization pattern of *P. aeruginosa* (MS-5) was more versatile as compared to the other isolates. Except *B. cereus* (MS-3) all strains showed growth at pH 5.0 and 6.0. *Rhizobium pusense* (MS-1) was less specific in the terms of carbon and nitrogen utilization pattern compared to the other strains. MS-1 and *M. flavus* (MS-4) were salt (NaCl) tolerant, while MS-4 was less tolerant as evident by partial growth in sodium chloride. The *R. pusense* (MS-1) was unable to utilize protein gelatin while rest others partially metabolized this protein. Also, all the strains partially metabolized the peptin. Although, the strains invariably utilized orbital, glycerol, mallic acid, β-hydroxyl butyric acid, sacchric acid, gluconic acid, histidine, asparagines, cellulobiose, arginine and alanine, but they differed in their carbon and nitrogen utilization pattern as well as in their metabolic activities, salt and pH tolerance.

Heat map clusters formed on the basis of carbon utilization pattern using GEN III microplate of Biolog Microstation system have been presented in (Fig. 6.) Heat map described the color intensity formed by log transformed value of absorbance of substrate present in the 96 wells of GEN III microplate. All the five endophytic bacterial strains formed two major clusters; one was small and second was large. The large cluster is further divided into two sub clusters. The clusters analysis based on the heat map of carbon utilization showed that *R. pusense* (MS-1) and *M. flavus* (MS-4) formed two major clusters which were different from *Bacillus flexus* (MS-2), *B. cereus* (MS-3) and *P. aeruginosa* (MS-5). *Bacillus flexus* (MS-2) and *B. cereus* (MS-3) showed similar cluster pattern, while *P. aeruginosa* (MS-5) showed different cluster pattern from these two.

The C- utilization by all endophytic bacterial isolates showed two major clusters in the heatmap dendrogram. The substrates utilized by individual strains strong colour intensity (green) on the basis of heatmap dendrogram have been presented in Table 6.

Antibiotic sensitivity of endophytic bacterial strains were also evaluated by PM 11C plate of Biolog Microstation system (Table 7). In the plate 24 antibiotics was taken for the preparation of heatmap (Fig. 7.). The data presented in Table 8 were prepared on the basis of colour intensity of antibiotics used by endophytic bacterial strains in heatmap. The heatmap dendrogram divided antibiotics into two major clusters. Cluster one formed with antibiotics used by *Bacillus flexus* (MS-2) and *B. cereus* (MS-3) bacterial strains. This cluster showed strong colour intensity with lincomycin, nafcillin, nalidixic acid, ofloxacin, chlortetracycline, cloxacillin, demeclocyclin, enoxacin, kanamycin, gentamycin and erythromycin while other sixteen antibiotics showed weak or no colour intensity. The cluster second was further divided into two sub clusters. These sub clusters constructed with all 24 antibiotics used by *R. pusense* (MS-1), *M. flavus* (MS-4) and *P. aeruginosa* (MS-5), while few antibiotics were utilized by *Bacillus flexus* (MS-2) and *B. cereus* (MS-3).

4. Discussion

The tomato root harbored rich taxonomic and functional diversity of bacterial endophytes (Marquez-Santacruz et al., 2010; Ottesen et al., 2013; Tian et al., 2015; Upreti and Thomas, 2015). Some endophytic bacterial strains of *Bacillus* spp., *Stenotrophomonas maltophilia*, and *Pseudomonas geniculata* were reported earlier from healthy tomato (*Solanum lycopersicum* L.) plants, they showed plant growth-promoting potential on tomato plants challenged with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) (Rania et al., 2018). In the present studyfive endophytic bacterial clones isolated from the roots of hybrid tomato variety Kashi Amrit belonged to four different genera and five different species viz. *Rhizobium pusense* (MS-1), *Bacillus flexus* (MS-2), *B. cereus* (MS-3), *Methylophilus flavus* (MS-4) and *Pseudomonas aeruginosa* (MS-5). *Pseudomonas, Bacillus* and *Rhizobium* sp., were already reported earlier as tomato endophyte (Tian et al., 2017). Endophytic bacteria isolated from *Typha angustifolia* L. and Tea (*Camellia sinensis* L.) roots and identified with 16S rRNA sequencing and BLAST analysis belongs to *Bacillus, Pseudomonas*, *Rhizobium*, *Brevibacterium, Paenibacillus* and *Lysinibacillus*sp. (Li et al., 2011; Borah et al., 2019). Some bacterial species were reported earlier, *Pseudomonas aeruginosa* and *Bacillus subtilis* also colonized internal root tissues of *Solanum lycopersicum* L. (tomato), *Abelmoschus esculentus* (okra), and *Amaranthus* sp. (African spinach) (Adesemoye et al., 2008). *Methyluphilus* is a species of restricted facultative methanol-utilizing bacteria, though *Methylophilus glucosoxydans* sp. has been isolated from rice rhizosphere (Doronina et al., 2012). This is a first report of *Methylophilus flavus* as an endophyte of tomato plant. Plant growth promoting endophytic bacteria are usually involved in mutualistic interaction with host plants. They promote plant growth directly, via production of phytohormones (Nivya, 2015). Indole acetic acid (IAA), one of the most physiologically active auxins, is a commonly produced by plant growth promoting bacteria (PGPB) (Lynch, 1985). The synthesis of IAA by some endophytic microorganisms may be one of the potential reasons for the increased growth of plants (Shi et al., 2009). IAA induced root growth and development may result in enhanced nutrient uptake (Patten and Glick, 2002). The endophytic bacterial strains *B. cereus* (MS-3) and *M. flavus* (MS-4) were the efficient producer of IAA. IAA production by endophytic *B. cereus*, *Pseudomonas putida* and *Clavibacter michiganensis* isolated from sweet potato and turmeric promoted plant growth (Khan et al., 2015; Rana et al., 2011; Kumar et al., 2016). The close interaction of plant growth-promoting endophytes with plant tissues facilitates nutrients acquisition and exchange and enzymes activity (Murphy et al., 2014; Khan et al., 2015). Endophytes possess vital ability to solubilize insoluble phosphate and provide nitrogen to their host plants (Shi et al., 2011; Matsuoka et al., 2013). Endophytic and rhizospheric bacteria have the ability to liberate orthophosphate (PO_4^{-3}) from organic phosphates or to solubilize insoluble inorganic phosphates (Oteino et al., 2015). All endophytic bacterial strains during the present study solubilized tri-calcium phosphate except *R. pusense* (MS-1). Salt tolerant and phosphate solubilizing endophytic bacteria *Acinetobacter* sp. ACMS25 and *Bacillus* sp. PVMX4 from *Phyllanthus amarus* also solubilized inorganic phosphate (Joe et al., 2016). Siderophores a small peptide molecules with side chains and functional groups bind the ferric ions (Goswami et al., 2016). All five endophytic bacterial strains *R. pusense* (MS-1), *B. flexus* (MS-2), *B. cereus* (MS-3), *M. flavus* (MS-4) and *P. aeruginosa* (MS-5) produced siderophores. *Azotobacter vinelandii*, a nitrogen fixing bacterium produced siderophores, used to acquire the nitrogenase co-factors molybdenum (Mo) and vanadium (V). Siderophore production in *A. vinelandii* increases under Fe-stress (McRose et al., 2017). Starch hydrolysis test depict the potential of micro organisms to utilized starch as a carbon source (De Oliveira, 2007). *Bacillus*, *Arthrobacter*, *Micrococcus* spp. isolated from *Piper nigrum* L. showed starch hydrolysis (Aravind et al., 2009). Endophytic bacterial isolates *R. pusense* (MS-1), *B. flexus* (MS-2), *B. cereus* (MS-3) showed positive results for starch hydrolysis. Drought affects nitrate reductase (NR) activity, due to lower uptake of nitrate from the soil (Caravaca et al., 2005). All endophytic bacterial isolates were positive for nitrate reductase activity. Antimicrobial activities were from the genera *Bacillus* and *Pseudomonas* were already reported (Tian et al., 2017). The all five strains were sensitive to antibiotic chloramphenicol followed by erythromycin rifampicin, piperacillin, imipenam and gentamycin while resistant to polymixin B. Biolog GEN III plates are increasingly used to characterize microorganisms and identification of bacterial species by determining the metabolic potential to oxidize various carbon sources. In the Biolog Microstation system, GEN III library database identified MS-1 as *Enterobacter aerogene*, MS-2 as *Bacillus krulwichiae,* MS-3 as *Bacillus patagoniensis*, MS-4 as *Serratia ficaria* and MS-5 as *Pseudomonas aeruginosa*, while in 16S rRNA phylogenetic analysis revealed that MS-1 as *Rhizobium pusense*, MS-2 as *Bacillus flexus*, MS-3 as *B. cereus*, MS-4 as *Methylophilus flavus* and MS-5 as *Pseudomonas aeruginosa*. The identification from GEN III plate of Biolog Microstation system was different from 16S rRNA sequence phylogeny because *Rhizobium* and *Methylophilus* sps. were not present in the library data base of Biolog Microstation system and Biolog data alone are not enough to identify bacterial species. The Heat map cluster was formed on the basis of carbon utilization pattern using GEN III microplate. The data-revealed the utilization of the substrates D-sorbitol, L-alanine, L-aspartic acid, L-histidine, *N*-acetyl D-glucosamine, α-D-glucose as evidenced by strong color intensity by all strains, D-galactose, 3-methyl glucose, D-salicin, hydroxyl butyric acid, D –serine were utilized only by *R. pusense* (MS-1),while*B. flaxus* (MS-2) utilized D–aspartic acid, D-serine, *N*-acetyl D-galactosamine, D galacturonic acid. *B. cereus* showed strong color intensity with D-lactic Acid, Bromo succinic acid, aztreonam, sodium butyrate and *N*-cetyl D-neuraminic acid. Only two substrates maltose and α-hydroxyl butyric acid were completely utilized by *P. aeruginosa* (MS-5). Dextrin, D-trehalose, D-fructose −6 −phosphate, β-methyl D-glucoside, guanidine HCl, α-ketobutyric acid, acetoacetic acid, propionic acid, acetic acid, formic acid, sodium bromate were poorly utilized by all endophytic bacterial strains. *Rhizobium* ACR 1 to 15 strains isolated from *Cicer arientinum* and their carbon utilization pattern was evaluated using Biolog (Sharma et al., 2017).

Phenotypic microarrays could be used as a new tool for identification of the multi-drug resistance of bacteria and for determination of the minimal inhibitory concentrations (MIC) (Chojniak et al., 2015). PM approach may be also used as a tool to determine variations in the chemical sensitivity of microorganisms that are used in industrial processes (Borglin et al., 2009). The PM 11C plates were used for the determination of endophytic bacterial strains sensitivity to 24 antibiotics. Heat map showed that all five endophytic bacterial strains were resistant to ofloxacin evidenced by strong color intensity while cloxacillin and lincomycin showed strong absorbance only with *B. flexus* (MS-2). Lincomycin is a narrow spectrum antibiotic with activity against Gram-positive bacteria. All the strains showed weak colour intensity with Cefazolin. The above results revealed the differential response of endophytic strains towards carbon utilization pattern and antibiotic sensitivity which could be used for identification of bacterial strains to some extent.

5. Conclusion

In the present study endophytic bacteria isolated from the root of *Solanum lycopersicum* L. plants belongs to genera *Bacillus*, *Pseudomonas*, *Rhizobium* and *Methylophilus.* This is a first report of *Methylophilus flavus* as an endophyte of tomato plant. Endophytic strains showed positive results for IAA, HCN, ammonia and siderophore production, nitrate reduction and hydrolysis of starch. Biolog's microarrays and 16S rRNA sequencing used for identification and characterization of endophytic bacterial strains isolated from tomato. Special attention was taken to present the GEN III and PM 11C microplate as a novel tool for identification of the different carbon sources and multi-antibiotic resistance of endophytic bacterial strains.

Research involving human participants and/or animals

This research article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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