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Isolation and Identification of a Siderophore Producing and Antagonistic Cold Tolerant (psychrotrophic) *Pseudomonas* sp. LHSP1

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Abstract: Present study was conducted for isolation and selection of cold tolerant bacteria with plant growth promoting attributes. Among sixty-one psychrotrophic bacteria isolated from cold environments soils of the Western Himalayas, India, seven were found positive for siderophore production at low temperature of 5°C. Of these seven isolates, LHSP1 was selected for further studies on the basis of its ability to produce highest amount of siderophore, showing largest orange halo around the growth in plate assay. Strain showed maximum production of siderophore, 82.5 and 87.1% siderophore unit (psu) after three days of incubation at temperature of 20°C and 28°C, respectively. It showed strong antagonistic activity against two fungal pathogens, *Fusarium equiseti* CZCU-2 and *F. solani* CZGN-9 showing up to 65.0 and 55.6% inhibition at 20°C and 28°C, respectively. In the medium, supplemented with 0.30% of tryptophan, it produced 47.25 µg mL^{-1} of indole acetic acid (IAA) after three days of incubation. This strain showed very good growth in the presence of 20% polyethylene glycol (PEG) 6000 and was able to grow and survive at 30% PEG 6000. The 16S rRNA gene-sequencing was carried out and the sequence was submitted to NCBI with accession number MW627227. Based on 16S rRNA gene-sequencing studies, isolate was identified as *Pseudomonas* sp. LHSP1.

Key words: Siderophore, antagonistic, psychrotrophs, cold environment.

Microorganisms being ubiquitous play an important role for maintaining the ecological balance in any ecosystem. The microorganisms which thrive in extreme environments (low/high temperature, pH, pressures, etc.) are known as extremophiles. Extremophilic microorganisms are of particular interest because of their adaptation to the prevailing harsh conditions. Extremophilic microorganisms, isolated from the niche areas, are potential candidates for exploration and exploitation for improving the growth and yield of crops grown in these areas as well as for obtaining the compounds of industrial importance from them (Kasana and Yadav, 2007; Yadav *et al.*, 2015). Among extreme environments, low temperature environments are important as approximately 80% of the biosphere and more than 90% of the marine environments have temperatures lower than 5°C (Brenchley,

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1996; Kasana and Gulati, 2011). These low temperature environments are inhabited by cold-adapted microorganisms, which include psychrophiles and psychrotrophs (Chauhan *et al.*, 2023).

Iron is an essential micronutrient which plays a critical role in metabolic processes such as DNA synthesis, respiration, and photosynthesis. Iron has been reported the third most limiting nutrient for plant growth and metabolism, primarily due to the low solubility of the oxidized ferric form in aerobic environments (Zuo and Zhang, 2011; Gulati *et al.*, 2008). Siderophores are high-affinity iron chelating compounds produced and secreted by some microorganisms for the uptake of environmental iron from inorganic phase by formation of soluble Fe^{3+}complex, which can be taken up by active transport mechanisms (Kraemer, 2004).

Globally the losses to various agricultural crops to the tune of 30% crop yield and amounting for U.S.$416 million occurs every year due to the plant diseases caused by various pathogens (Sayyed and Chincholkar, 2009). The microorganisms which synthesize siderophores can be used as biocontrol agents to inhibit plant pathogenic fungi and bacteria by iron competition (Sayyed and Patel, 2011). Siderophore producing bacteria have been reported to show good antagonistic activity against various plant pathogenic fungi (Solanki *et al.*, 2014; Kotasthane *et al.*, 2017; Srivastava *et al.*, 2022).

Low temperature is one of the factors among others that impede growth and development of crops under cold environment conditions prevailing in most of the areas worldwide. The cold desert of Ladakh is marked by a harsh and fragile mountainous terrain with poor availability of essential mineral nutrients in soil. Such soils are niche for isolation of cold adapted microorganisms (Acharya *et al.*, 2014; Yadav *et al.*, 2015). The soils in Leh and Kargil have been reported to be deficient in iron, along with zinc and manganese affecting the crop yield (Dwivedi *et al.*, 2005). The vast potential of microorganisms for improving productivity in the cold environments remains unexplored and untapped. The aim of the present study was to isolate a potential siderophore producing bacterium having antagonistic activity from the rhizospheric soil samples from crops growing in cold environments of Leh region of Ladakh and screening for other plant growth promoting traits and to identify the potential isolate and to evaluate its abilities to grow and produce siderophore and perform antagonistic activity under two different temperatures.

Materials and Methods

Sample collection and isolation

Soil samples were obtained from the wheat and pea growing fields in different locations situated at height of about 3300-3500 m in Leh region of Ladakh. For isolation of psychrotrophic bacteria, ten-fold serial dilutions of each soil sample were prepared in sterilized distilled water and 0.1 ml diluted sample was spread on the surface of nutrient agar medium (0.3% beef extract, 0.5% peptone, 0.5% NaCl, 1.7% agar, pH 7.0). Plates were incubated at 5°C for 7 to 10 days. Morphologically different colonies that appeared on the plates were selected and subjected to further streaking on the same medium for the isolation of pure cultures.

Screening for siderophores

Qualitative method: Siderophore production was detected using Chrome Azurol S (CAS) assay with slight modification (Schwyn and Neilands, 1987) as described in Arora and Verma (2017). Briefly, 121 mg CAS was dissolved in 100 ml distilled water and mixed with 20 ml of 1 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) solution prepared in 10 mM HCl. Then this solution was slowly added to 20 ml of hexadecyl trimethyl ammonium bromide (HDTMA) solution prepared by dissolved 72.9 mg of HDTMA in 40 mL of water. This blue-coloured solution was sterilized by autoclaving. After cooling to about 5°C, this solution (100 ml) was added drop wise to 900 mL of nutrient agar with stirring to mix the ingredients without formation of bubbles. Then CAS agar plates were prepared and spot inoculated with bacterial isolates. After inoculation, plates were incubated at 20°C for 5 to 7 days and observed for the formation of orange zone around the bacterial colonies.

Quantitative method: For quantitative estimation of siderophore production, the bacterial culture was grown in nutrient broth medium at two different temperatures *viz* 20°C and 28°C for 6 days. Apart from this, control

tube (uninoculated nutrient broth) was also maintained. After incubation of bacterial culture and control tubes, samples were taken at interval of 24 h and centrifuged at 7100 rpm for 15 min, cell pellets were discarded, and supernatant was used to estimate siderophore production. To 2.0 ml of supernatant of bacterial culture and uninoculated nutrient broth, 2.0 ml CAS reagent was added and after 20 min optical density was taken at 630 nm. Siderophore production was calculated by using the formula given by Payne (1993) as given below.

$$\textit{Percent siderophore unit (psu)} = \frac{(Ar - As)\ X\ 100}{Ar}$$

where Ar = absorbance of reference (CAS solution and uninoculated broth), and As = absorbance of sample (CAS solution and cell-free supernatant of culture).

Production of Indole-3-acetic acid

Indole-3-acetic acid production by bacterial isolates was determined following the methods of Gordon and Weber (1951). For indole-3-acetic acid production, bacterial isolates were grown on nutrient broth supplemented with different concentrations of tryptophan ranging from 0.05% to 0.30% and incubated at 28 ± 1°C for 3 days. Culture was then centrifuged at 5,000 rpm for 25 min and supernatant was collected. Two drops of o-phosphoric acid was added to 2 ml of supernatant and then 4 ml of Salkowski reagent was added. The sample was incubated for 25 min and appearance of pink colour confirmed the production of indole-3-acetic acid. For quantification of indole-3-acetic acid produced, the optical density was recorded at 530 nm.

Drought stress tolerance

The tolerance of the strain to desiccation was studied in nutrient broth amended with 0, 5, 10, 15, 20, 25 and 30 % PEG 6000 at pH 7 in 100-ml Erlenmeyer flasks containing 50 ml medium with 1×10^7 CFU ml^{-1} initial inoculum. The flasks were incubated at 20 ± 1 °C for 6 days in an incubator shaker. Optical density was measured at 600 nm of each sample after 1, 2, 3, 4, 5 and 6 days of incubation.

Confrontation assay of antifungal activity

One 5 mm disk of a pure culture of *Fusarium equiseti* CZCU-2 and *F. solani* CZGN-9 was placed at the center of petri plates containing potato dextrose agar. With the help of a funnel, a circle of 4 cm diameter of the bacterial suspension of LHSP1 was made surrounding the fungal cultures (Kotasthane *et al.,* 2017). Plates were incubated at 20°C and 28°C for 8 days. Growth diameter of the pathogenic fungi was measured and compared to control growth where the bacterial suspension was replaced with autoclaved water. Per cent inhibition of pathogen by LHSP1 was calculated using the formula given by Vincent (1947):

Per cent inhibition = [(Growth of pathogen in control - growth of pathogen with LHSP1 isolate)/growth of pathogen in control] x 100.

Identification of bacterial isolate

The identification of the selected isolate was carried out at the sequencing facility of National Centre for Microbial Resource, National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/ chloroform extraction method (Sambrook *et al.,* 1989), followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers so that each position was read at least twice. Assembly was carried out using Lasergene package followed by identification using the EzBioCloud database (Yoon *et al.,* 2017).

Phylogenetic and evolutionary relationship analysis

For phylogenetic analysis, the 16S rRNA gene sequences of the related type species of *Pseudomonas* were downloaded from NCBI data bases. Multiple sequence alignments of the sequence were performed using CLUSTAL W (Thompson *et al.,* 1994). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980), *Acinetobacter calcoaceticus* ATCC 23055

was used as out group. All ambiguous positions were removed for each sequence pair and evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

Results and Discussion

Isolation and identification of strain LHSP1

Sixty-one bacteria that could grow at 5°C were isolated from soil samples collected from cold environments of the Leh Ladakh region, India, and analysed for their siderophore production ability. Among the seven siderophore producing psychrotrophic bacterial isolates, strain LHSP1, which formed largest orange zone on Chrome Azurol S agar (Fig. 1), was selected as potent siderophore producer and used for further screening of other plant growth promoting attributes and antagonistic activity against fungal pathogens.

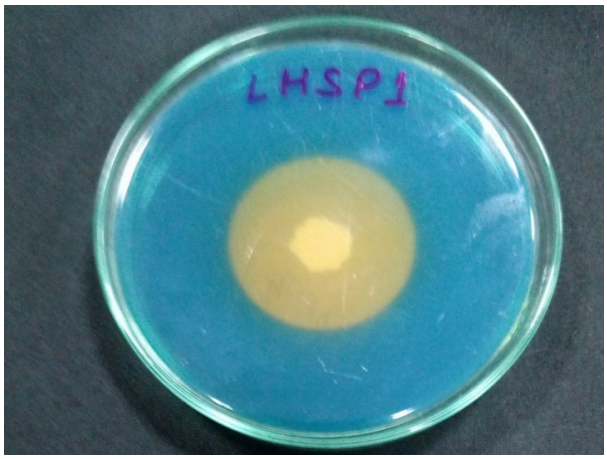

Fig. 1. Chrome Azurol S agar plate assay of Pseudomonas sp. LHSP1 siderophore.

Siderophore production by LHSP1

In the present investigation, strain LHPS1 was tested for siderophore production at two different temperatures of 20°C and 28°C. The strain showed good production of siderophore at both the temperatures. The maximum production of siderophore was shown after 3 days of incubation at both the temperatures, with 87.12 psu at 28°C and 82.48 psu at 20°C (Fig. 2). Concentration of siderophore produced by various bacterial strains varied from 7.97 to 69.81 psu (Arora and Verma, 2017). Studies on production of siderophore by fluorescent *Pseudomonas* isolates showed siderophore in the range of 22.36 to 80.15 psu (Kotasthane *et al.*, 2017). *Pseudomonas monteilii* strain MN759447 isolated from *Dalbergia sissoo* plantation forests showed siderophore production of 80.36 psu (Srivastava *et al.*, 2022).

Confrontation assay of antifungal activity

During the confrontation studies strain LHSP1 showed good antagonistic effect against two pathogens- *Fusarium equiseti* CZCU-2 and *F. solani* CZGN-9 at both the temperatures of 20°C and 28°C. The strain LHSP1 did not allow *F. equiseti* CZCU-2 to grow beyond it's growth even after 8 days of incubation, whereas in case of control plate whole plate was covered by the *F. equiseti* CZCU-2 (Fig. 3). Per cent growth inhibition against *F. equiseti* CZCU-2 at 20°C and 28°C ranged from 61.11 to 65.00 and 42.86 to 55.56, respectively. In case of *F. solani* CZGN-9 at 20°C and 28°C ranged from 60.00 to 63.16 and 41.18 to 55.56, respectively (Fig. 4). As the LHSP1 is a psychrotrophic (cold tolerant) the

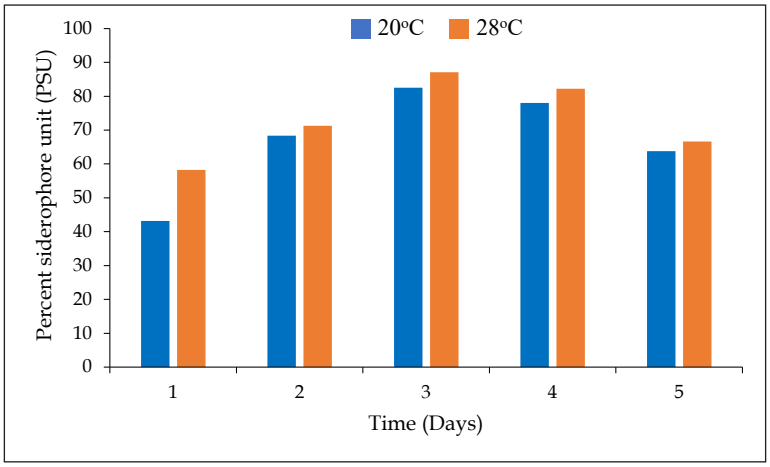

Fig. 2. Siderophore production by Pseudomonas sp. LHSP1 at two different temperatures.

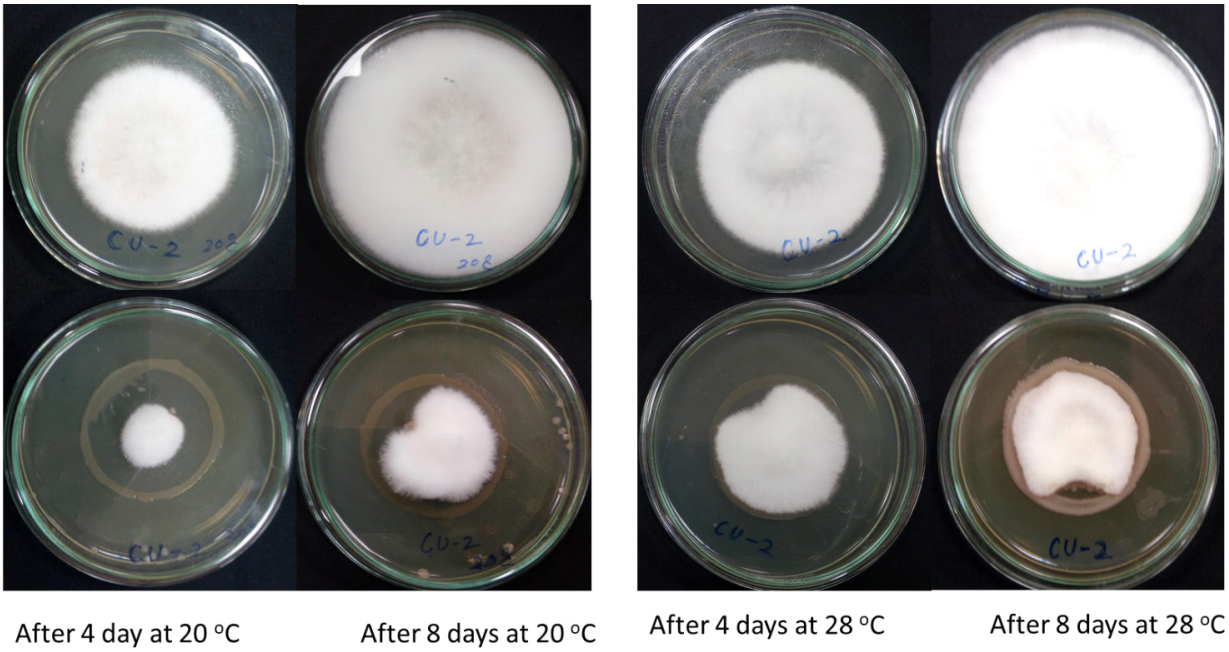

Fig. 3. Antagonistic activity of siderophore producing Pseudomonas sp. LHSP1 against Fusarium equiseti CZCU-2 tested at two different temperatures.

growth inhibition of fungi was more at low temperature of 20°C as compared to 28°C. Nine siderophore producing bacteria showed antagonistic activity against *Rhizoctonia solani* AG-4 in the range 36.71% and 71.0% inhibition (Solanki *et al.*, 2014). Siderophore producing *Pseudomonas monteilii* exhibited a broad range of antagonistic activity against *Aspergillus* (65%), *Fusarium* 41.66%), and *Talaromyces* (65.1%) (Srivastava *et al.*, 2022). *Bacillus subtilis* CAS15 which produce siderophore, showed strong antagonistic activity against 15 plant fungal pathogens with rates of inhibition ranging from 19.26 to 94.07% (Yu *et al.*, 2011).

Production of Indole-3-acetic acid

Indole acetic acid is one of the most important auxin which regulates plant growth and development through a variety of cellular mechanisms. Strain LHSP1 produced good amount of IAA, which varied from 11.42 to 47.25 µg mL^{-1} depending upon the concentration of tryptophan used in the medium (Table 1). Production of IAA increased when culture medium was supplemented with an IAA precursor; tryptophan confirming the results of earlier studies (Mutluru and Konada, 2007). Four siderophore producing bacteria, isolated

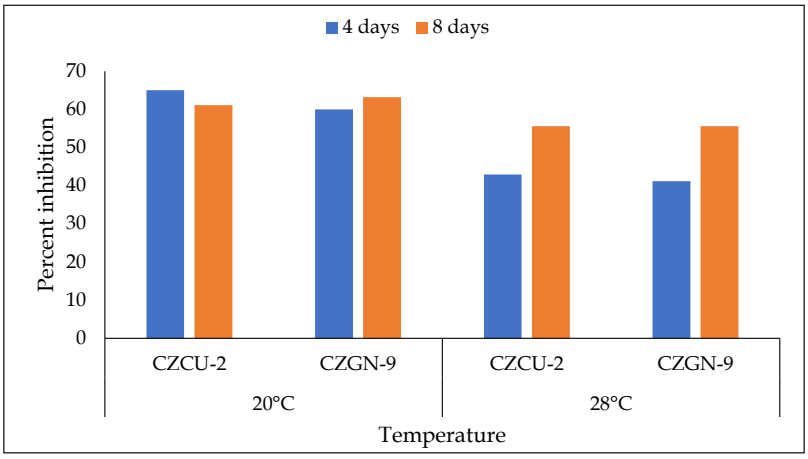

Fig. 4. Antagonistic activity of siderophore producing Pseudomonas sp. LHSP1 against Fusarium equiseti CZCU-2 and F. solani CZGN-9.

Table 1. Production of indole acetic acid by strain LHSP1

Concentration of Tryptophan	IAA production (µg mL^{-1})
0.00%	11.42
0.05%	20.58
0.10%	28.42
0.15%	32.58
0.20%	39.42
0.25%	47.25
0.30%	41.83

from the rhizosphere of *Vigna radiata* showed IAA production in range of 45.66 to 111.94 µg mL^{-1} (Kumari *et al.,* 2018). Optimization of indole acetic acid production by bacteria isolated from *Stevia rebaudiana* rhizosphere at different pH, temperature, carbon and nitrogen sources, showed maximum production with 104 µg mL^{-1} IAA production by isolate CA1001 (Chandra *et al.,* 2018).

Drought stress tolerance

Along with other stresses, drought stress affects the agriculture crop yield in arid and semiarid regions of the world (Niu *et al.,* 2018). Drought tolerance studies on strain LHSP1 showed that it was able to grow in the presence of 30% PEG 6000 during the six days of incubation (Fig 5). Maximum growth (OD 1.53) was observed after 48 h of incubation in the medium without PEG6000, whereas in medium supplemented with 5% PEG 6000 maximum growth (OD 1.46) obtained after 3 days of incubation. With increase in concentration of PEG 6000 there was decrease in growth and in 30% PEG 6000 after 6 days an OD of 0.43 was obtained. Among twenty seven bacteria from rhizosphere of various plants cultivated in the Kumaun Himalayas screened for drought tolerance nine showed growth in 15% PEG and four exhibited growth up to 35% PEG (Sati *et al.,* 2023).

Identification and Phylogenetic Analysis of Strain LHSP1

For identification and confirming its phylogenetic affiliation, genomic DNA was isolated from the bacterium LHSP1 and gene coding for 16S rRNA was amplified by polymerase chain reaction. The sequence was deposited in NCBI GenBank with the accession number MW627227. The sequencing of almost full-length 16S rRNA gene showed that it was closely related to genus *Pseudomonas* showing 99.2% homology to *Pseudomonas donghuensis*. Based on this comparison, the isolate was identified as *Pseudomonas* sp. LHSP1. Use of 16S rRNA gene sequences for bacterial identification has been by for the most common housekeeping genetic marker (Janda and Abbott 2007). The sequence of the *Pseudomonas* sp. LHSP1 was then aligned and compared with previously published sequences of the species from genus *Pseudomonas* with validly published names, and a neighbor-joining phylogenetic tree constructed (Fig. 6). In neighbour neighbor-joining tree *Pseudomonas* sp. LHSP1 make cluster with *Pseudomonas donghuensis*. Siderophore producing bacteria

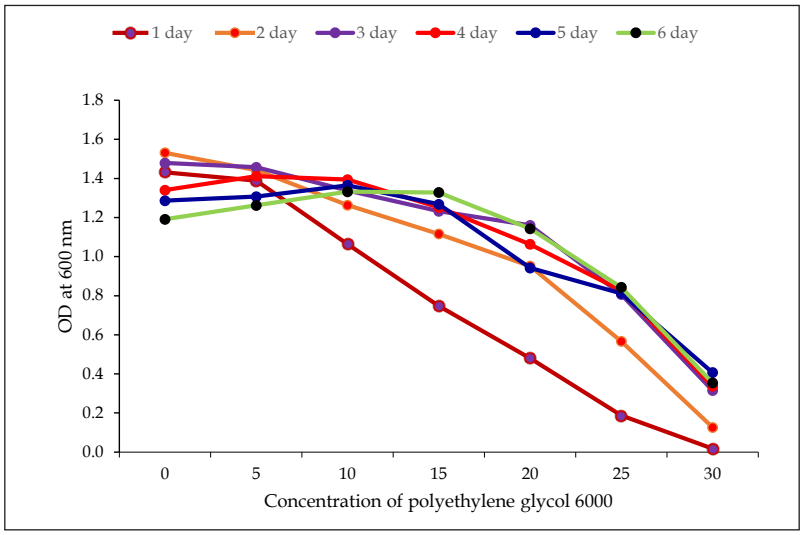

Fig. 5. Growth of siderophore producing Pseudomonas sp. LHSP1 in the presence of different concentrations of PEG 6000.

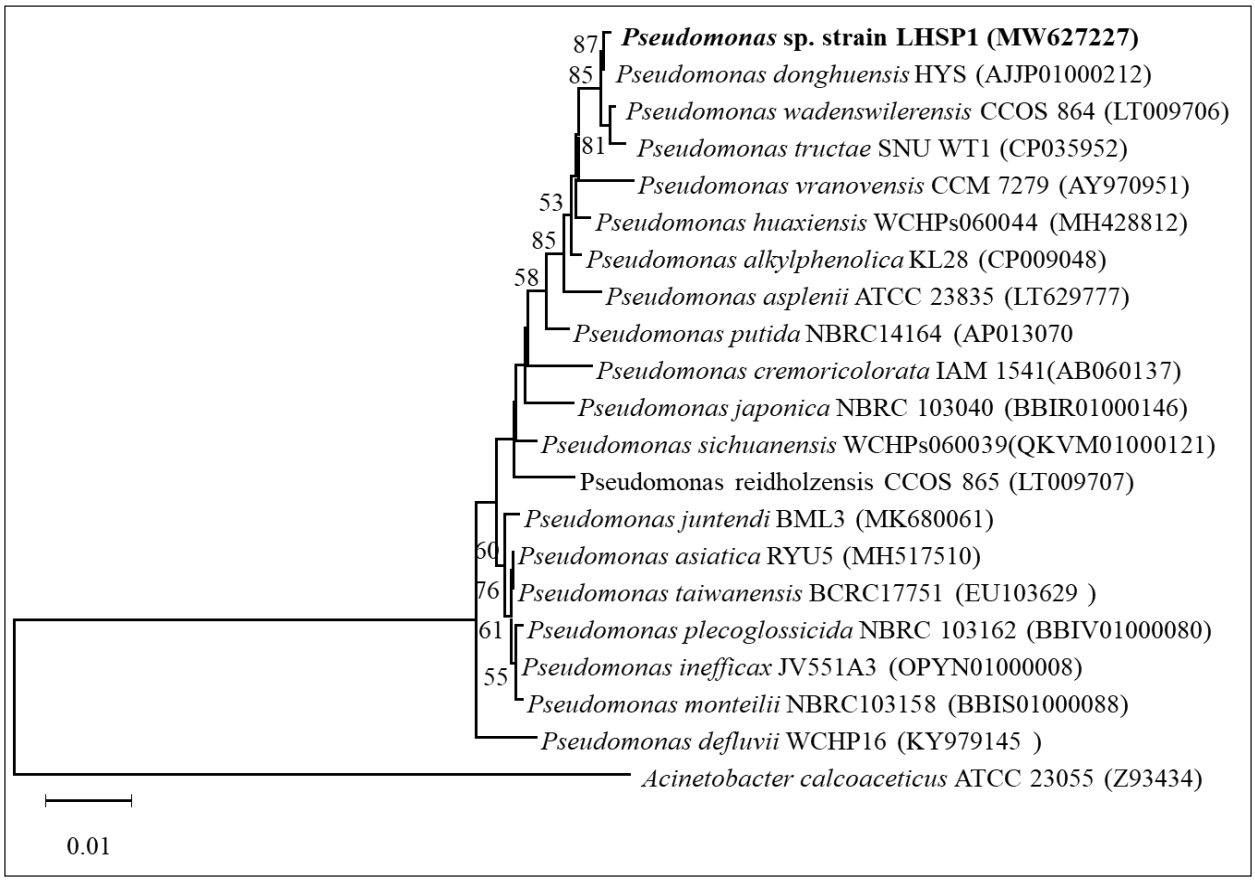

Fig. 6. Phylogenetic tree based on 16S rRNA gene sequences, drawn using the neighbor joining method and showing the relationship between Pseudomonas sp. LHSP1 and species from the genus Pseudomonas with validly published names. Acinetobacter calcoaceticus was used to root the tree. Bar, 0.01 substitutions per site.

belonging to various genera *Arthrobacter, Bacillus, Brachybacterium, Exiguobacterium, Lysinibacillus, Planococcus, Psychrobacter*, *Pseudomonas* and *Sanguibacter*, have been isolated from cold environment samples (Yadav *et al*., 2015, Gao *et al*., 2015). Strain LHSP1 is deposited at National Centre for Microbial Resource, NCCS, Pune as *Pseudomonas* sp. LHSP1 with accession number 4532.

Conclusions

In this study a psychrotrophic bacterium LHPS1 capable of producing siderophore at low temperature of 5°C was isolated and identified as *Pseudomonas* sp. LHSP1 by 16S rRNA gene sequencing. Strain also possesses plant growth promoting attributes like production of IAA, growth under drought stress and showed antagonistic activity against two plant pathogenic fungi. It can be explored for developing bio inoculant for increasing the plant growth and yield under the stressful conditions of cold arid environments.

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