International Journal of Microbial Resource Technology

Available at http://ijmrt.inpressco.com

IJMRT International Journal of Microbial Resource Technology

ISSN 2278 – 3822

Original Research Article

Potential plant growth promoting activity of *Bacillus licheniformis* UHI(II)7

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Accepted 4 Nov. 2014, Available online 1 Dec. 2014, **Vol.2, No. 3 (Dec. 2014)**

Abstract

The gram positive, endospore forming bacteria belonging to the genus Bacillus are found in most of the rhizospheric soils. One such isolate UHI(II)7 was found from the rhizospheric soil of Ocimum sp. from the vicinity of Haridwar, Uttarakhand (India) and was identified as Bacillus licheniformis. This isolate has shown to exhibit multiple plant growth promoting characteristics/traits. This isolate possess PGP traits such as ammonia production, indole acetic acid production, phosphate solubilization, catalase production, heavy metal tolerance and ACC deaminase activity. These plant growth promoting abilities can make this isolate a potential PGPR candidate for its application in sustainable agriculture.

Keywords: *PGPR, IAA, ACC deaminase activity, heavy metal tolerance, sustainable agriculture*

1. Introduction

Rhizosphere is a reservoir of microorganisms. To obtain potential PGPR which can be developed as efficient bio-inoculants, thereby enhancing the growth of plant and increasing the yields, this reservoir must be explored. PGPR are beneficial soil bacteria that colonize plant roots and helps in the enhancement of plant growth and yield. PGPR inoculants helps in growth promotion through different mechanisms such as suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers) or phytohormone production (termed Biostimulants) (Saharan and Nehra, 2011). Because of their effects on nutrient availability and soil conditions, they play a significant role in plant growth (Karakurt and Aslantas, 2010). Hence, there is a need to develop such PGPR strains as bio-inoculants which may prove successful in field conditions. Isolation and identification of *Pseudomonas* and *Bacillus* for their use as efficient PGPR has been well reported for crops such as maize, wheat, rye, *etc*. (Luey *et al.,* 2004).

Most studied bacteria which are increasingly marketed as the biological control agents belongs to genera such as *Bacillus*, *Pseudomonas*, *Streptomyces*, *Burkholderia* and *Agrobacteria* (Gutierrez Manero *et al.,* 2003). *Bacillus* sp. is one of the most potential genera due to their spore forming ability, thereby increasing the adaptation of *Bacillus* strain to commercial formulation and field application. Promotion of plant growth by *Bacillus* sp. (Ryu *et al.,* 2004) includes mechanisms such as phytohormone production and/or the solubilization of mineral phosphates (Calvo *et al.,* 2010; Viruel *et al.,* 2011) and their ubiquitous presence in rhizosphere (Gajbhiye *et al.,* 2010). *Bacillus subtilis* has been reported to be helpful in plant growth, vitality and protection from plant pathogens, resulting in higher yield. Another strain of *Bacilli*, *B. mucilaginous* has been documented for its capability in solubulizing potassium (Han *et al.,* 2006).

Application of PGPR for agricultural crops as well as medicinal plants is currently attracting many attentions of the international intellectual community. Therefore, the present study was undertaken to isolate and characterize rhizospheric *Bacillus* isolate for its multiple plant growth promoting activities under *in vitro* conditions such as production, of ammonia and indole acetic acid, phosphate solubilization, catalase test, ACC deaminase activity and heavy metal tolerance.

2. Materials and methods

2.1 Isolation of bacterial isolates

A total of 24 rhizospheric soil samples of *Ocimum* sp. were obtained from different regions of Delhi, Kurukshetra and Haridwar for the isolation of PGPR isolates. The soil samples were kept in polythene bags and stored in cool place to maintain their physiochemical properties.

Isolation was done by serial dilution technique. The soil samples were serially diluted up to 10^{-7} dilutions. The dilutions were plated on nutrient agar plates and incubated at 37 °C for 24h. Well isolated colonies were picked up, sub-cultured and preserved on Nutrient agar slants at 4 °C. All the isolates were characterized.

2.2 Screening of PGP traits of the potent isolate

2.2.1 Phosphate solubilization

Phosphate solubilizing activity was detected using Pikovaskaya's medium, consisted of 10 g glucose, 5 g tribasic phosphate ($Ca_5HO_{13}P_3$), 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, trace of $MnSO_4$ and $FeSO_4$, 0.5 g yeast extract, and 15 g agar, in 1000 ml distilled water.

Bacterial culture was spot-inoculated on the surface of plate containing Pikovskaya medium and incubated in an incubator at 28 °C for 7 days. P-solubilization was determined by the development of clearing zone around bacterial colony (Pikovaskya *et al*., 1948).

2.2.2 Production of Ammonia

Bacterial isolate was tested for the production of ammonia in peptone water. Freshly grown culture was inoculated in 10 ml peptone water in a test tube and incubated for 48-72 h at 36±2 °C.

Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour indicated positive test for ammonia production (Cappuccino and Sherman, 1992).

2.2.3 Production of Indole acetic acid

Bacterial culture was grown in LB medium amended with 100 mgL^{-1} tryptophan as the precursor of IAA by incubating in a shaker at 250 rpm at 28 ± 2 ^oC for seven days. Indole acetic acid (IAA) production was assayed colorimetrically by using Salkowski reagent (1ml of 0.5M $FeCl_3$ in 50ml of 35% $HClO_4$) and absorbance of the resultant pink color at 535nm in colorimeter.

Appearance of pink color in test tubes indicated IAA production. The concentration of IAA was determined by comparison with standard curve (Okon *et al.*, 1977).

2.2.4 Catalase test

Catalase test was performed by adding 3-4 drops of 3% (v/v) hydrogen peroxide (H_2O_2) to 48h old bacterial colony. The effervescence indicated catalase activity (Cappuccino and Sherman, 1992).

2.2.5 ACC-deaminase activity

The isolate was cultured in LG (N-free) medium at 28 ^oC for 2 days with shaking at 200 rpm until cell reached the early stationary phase. The cells collected by centrifugation at 5000 rpm for 5 min and washed twice with minimal medium. Cell pellets were suspended in 15 ml of minimal medium supplemented with 1 mM ACC (1-aminocyclopropane-1-carboxylate), and further incubated at 28 ^oC for 24 h with shaking at 200 rpm to induce ACC-deaminase enzyme production. ACC-deaminase activity was measured as described by Penrose *et al. (*2003).

2.2.6 Heavy metal tolerance

The selected isolate was tested for resistance to heavy metals by agar dilution method (Cervantes *et al.*, 1986). Heavy metal salts namely nickel, mercury, cobalt, cadmium, copper, lead, zinc and chromium at concentrations 25 µg/ml, 100 µg/ml and 400 µg/ml were used. Tolerance to these metals was determined by the appearance of bacterial growth after incubating the plates at room temperature for 24-48h.

2.3 16S rDNA sequencing

The 16S rDNA sequencing was performed. Selected bacterial 16S rDNA was amplified in full length by PCR using primers, 16SF Universal (AGA GTT TGA TCC TGG CTC AG) and

16SR Universal (ACG GCT ACC TTG TTA CGA CTT). To evaluate the phylogenetic analysis of 16S rDNA sequence, the resulting sequence was compared with the known sequences using the BLAST function of GeneBank in the National Centre Biotechnology information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and consensus sequences were computed using the program CLUSTAL W programmed at European Bioinformatics (EBI) site (http://www.ebi.eic.uk/clustalw) and the phylogenetic tree was constructed.

3. Results and discussion

3.1 Isolation of PGPR isolates

Total 266 PGPR isolates were obtained from 24 rhizospheric soil samples of *Ocimum* sp. Sites for soil sampling includes different regions of Kurukshetra, Delhi, and Haridwar (India). All these isolates were characterized and screened for various PGP traits. On the basis of maximum no. of PGP characteristics exhibited, isolate UHI(II)7 was chosen as potential PGPR.

3.2 PGP traits exhibited by UHI(II)7

Various plant growth promoting activities exhibited by the chosen isolate have been shown in Table 1.

Table 1: Plant growth promoting traits of the isolate UHI(II)7.

PGP traits	Response	Observation/justification
Ammonia production	+	Appearance of yellowish orange color
Phosphate solubilization	+	Zone size = 28 mm Solubilization index = 5.6
IAA production	+	28 µg/ml
ACC deaminase activity	+	3.90 µMol α-keto butyrate/ mg protein/ h
Catalase production	+	+
Heavy metal tolerance	+	Tolerance against metals such as nickel, cobalt, cadmium, copper, lead, chromium; No growth in presence of zinc and mercury

3.2.1 Phosphate solubilization

Some microorganisms have the ability to convert insoluble phosphorus (P) to an accessible form such as orthophosphate. This is an important trait in a PGPR for increasing the plant yields (Chen *et al.,* 2006; Rodriguez *et al.,* 2006). In this study, isolate UHI(II)7 showed clear visible halo around the colony on Pikovskaya agar media plates. The zone of phosphate solubilization was 28 mm and the solubilization index was found to be 5.6.

3.2.2 Ammonia production and catalase test

The isolate UHI(II)7 showed significant ammonia production by producing yellowish orange color in peptone water, on addition of Nesseler's reagent. The isolate have shown positive test for catalase also.

3.2.3 Production of Indole acetic acid (IAA)

IAA, a phytohormone, is a most important native auxin (Ashrafuzzaman *et al.,* 2009). Production of indole acetic acid (IAA) was observed by isolate UHI(II)7. Tryptophan is the main precursor for the biosynthesis of indole acetic acid in bacteria (Patten and Glick, 1996). So, the tryptophan acts as an important factor for *Bacillus* sp. isolates to produce IAA, when present in the media. Likewise, our isolate UHI(II)7 produced IAA in tryptophan supplemented medium. The isolate produced 28 µg/ml of IAA.

3.2.4 ACC-deaminase activity

One of the most considerable plant growth promoting trait is ACC deaminase activity. It has been reported that certain PGPR having ACC-deaminase enzyme activity breakdown the ACC in to α-ketobutyrate and ammonia (Glick *et al.,* 1998; Tahir *et al.,* 2006; Arshad *et al.,* 2007) thereby lowering the amount of ACC as well as ethylene outside the germinating seeds. These reduced levels of ACC further lessens the synthesis of endogenous ethylene, which in turn decreases the inhibitory effects of higher levels of ethylene (Glick *et al.,* 1998; Yuhashi *et al.,* 2000). This is how ACC deaminase containing PGPR helps

in growth promotion. The isolate showed activity of 3.90µMol α-keto butyrate/ mg protein/ h.

3.2.5 Heavy metal tolerance

Isolate UHI(II)7 was found to be tolerant to heavy metals such as nickel (Ni), chromium (Cr) cobalt (Co) and cadmium (Cd) at concentrations of 25, 100 and 400 µg/ml.
For metals such as lead (Pb) and copper (Cu), the isolate showed tolerance at 25 and 100 µg/ml but did not grow at 400 µg/ml. The isolate did not show any growth in presence of zinc (Zn) and mercury (Hg) (Table 2).

Table 2: Heavy metal tolerance pattern of isolate UHI(II)7.

Heavy metal	25 µg/ml	100 µg/ml	400 µg/ml
Nickel	+++	++	+
Zinc	-	-	-
Cobalt	+++	++	+
Cadmium	+++	+	+
Lead	+++	+	-
Copper	++	+	-
Chromium	+++	++	+
Mercury	-	-	-

+++ = Huge growth; ++ = good ; += poor ; - = No

The isolate has also been tested for production of siderophore, antifungal activity and HCN. But the isolate showed negative test for these traits.

On the basis of morphological and biochemical characterization, it was found that the isolate UHI(II)7 belongs to genera *Bacillus* (Table 3).

3.3 Molecular characterization and phylogeny

3.3.1 16S rDNA sequencing of PGPR isolate CHII(II)K7

On the basis of 16S rDNA sequencing, the isolate UHI(II)7 was identified as *Bacillus licheniformis*.

Sequence

GGCCCGGGAACGTATTCACCGCGGCATGCTG
ATCCGCGATTACTAGCGATTCCAGCTTCACG
CAGTCGAGTTGCAGACTGCGATCCGAACTGA
GAACAGATTTGTGGGATTGGCTTAGCCTCGC
GGCTTCGCTGCCCTTTGTTCTGCCCATTGTAG
CACGTGTGTAGCCCAGGTCATAAGGGGCATG
ATGATTTGACGTCATCCCCACCTTCCTCCGGT
TTGTCACCGGCAGTCACCTTAAAGTGCCCAA
CTGAATGCTGGCAACTAAGATCAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAACCATGCACCACCT
GTCACTCTGCCCCCGAAGGGGAAGCCCTATC
TCTAGGGTTGTCAAAGGATGTCAAGACCTGG
TAAGGTTCTTCGCGTTGCTTCGAATTAAACCA
CATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTCAGTCTTGCCACCGTACT
CCCCAGGCGGAGTGCTTAATGCGTTTGCTGC
AACACTAAAGGGCGGAAACGCTCTAACACTT
ATCACTCATCGTTTACGGCGTGGACTACCAA
GGTATCTAATCCTGTGCGCTCCCCACGCTTTC
ACGCCACACCGACACTTACAGACCAGAGAGT
CGCCCTCGACACTGGTGTTCGTCCACATCTCT
ACGCATTTCAGCGCTACACGTGGAAATACCG
CTCTCCTCTTCTGCACTCAAAGTCCACAGTTT
CAAATGACCCCTCCCCGGTAGAGCCGGGGGC
TATCACACACAACTAGAAAATACCGCGTGCG
CGCGCTTTACGCCCAATAATGTCGGTACAAC
GCATGCCACCTACGTAATACTTCGGCTGCCT
GGCACGTACCTAAGCAGTCGCTTACATGAGT
TAGGTTCACCCGTCTAGGTAGCGACGATATC
GGAGCGGTACGTGTTCTCCGTTAACAAGGAG
TGTAACGATCCGTAAATCTCACCACACAGCG
CCTGCTTCGCCGACTTCGTCATGGGAGATCCT
ACTGTGACTCCGAGGATCAGGACAGAGTT
TCAGTTCCAAGCTG

16S ribosomal DNA gene,
partial sequence Length: 1253 ; Score = 1554 bits (841) ; Strand=Plus/Plus

4. Conclusion

The present investigation suggests that the *Bacillus licheniformis* UHI(II)7 has the potential to be developed as a potent PGPR/bio-inoculant. This may, in near future replace chemical fertilizers thereby developing sustainable agricultural systems for crop production/ protection.

Table 3. Morphological, Cultural and Biochemical Characteristics of isolate UHI(II)7.

Characteristic(s)	**Observation(s)**
Colony	White, raised, irregular colony
Gram Reaction	+ve
Shape	rods
Indole	-
Methyl-red	+
VP	+
Citrate	-
Catalase	+
Oxidase	+
H_2S	-
Nitrate reduction	+
Growth at pH	
4	-
5	-
6	+
6.5	+
7	+
7.5	-
8	-
9	-
Utilization of sugars	
Glucose	+
Lactose	+
Sucrose	+
Mannitol	+
Starch	+
Fructose	+
Citrate	-
Growth at different temperatures	
10 °C	+
20 °C	+
28 °C	+
37 °C	+
45 °C	+

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Phylogenetic analysis

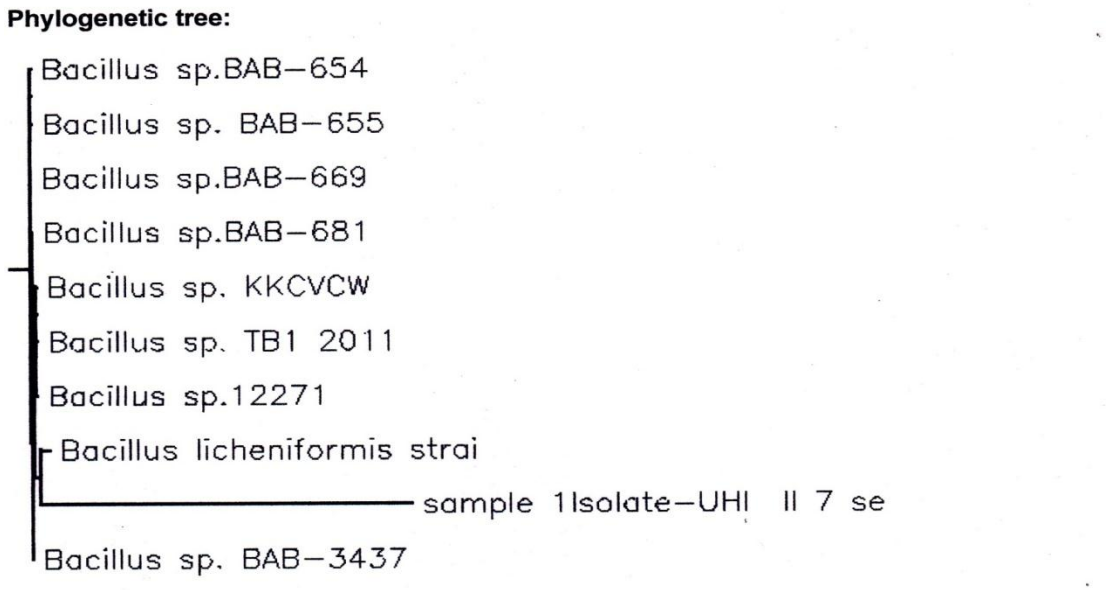

Fig. 1: Phylogenetic analysis based on 16S rDNA gene sequences of UHI(II)7