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# Exploring the potential of PGPR strain *Bacillus licheniformis* to be developed as multifunctional biofertilizer

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**ABSTRACT**

*Free-living soil bacteria beneficial to plant growth, usually referred to as plant growth promoting rhizobacteria (PGPR), are capable of promoting plant growth by colonizing the plant root. Strain ML3 was obtained from a weed rhizosphere soil sample collected from village Ladwa of Hisar on nitrogen-free Malate medium. The isolate had Gram positive rod shaped cells. Parameters assessed were indoleacetic acid (IAA) production, phosphate solubilization, ammonia excretion and siderophore (Fe-III chelating agent) production. The results showed that isolate ML3 produced IAA and ammonia in the range of 174.72 and 0.66 µg ml$^{-1}$, respectively. ML3 exhibited positive siderophore production and it was also able to efficiently solubilise tricalcium phosphate. The key functional nitrogenase gene nifH was detected in strain ML3. Partial 16S rRNA gene sequencing identified strain ML3 as Bacillus licheniformis which was confirmed by morphological and biochemical characterization. Possession of plant growth promoting traits make ML3 a promising strain to be developed as multifunctional biofertilizer.*

**Key words**: Rhizosphere, siderophore, nitrogenase gene, *Bacillus licheniformis*, biofertilizer

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## INTRODUCTION

Rhizobacteria that exert beneficial effects on growth and development of plants are termed as plant growth-promoting rhizobacteria (PGPR) [1]. The term rhizobacteria is used for bacteria that aggressively colonize the rhizosphere [2]. Although the mechanisms by which PGPR promote plant growth are not yet fully understood, many different traits of these bacteria are responsible for plant growth promoting activities [3]. Biofertilizers play a very significant role in improving soil fertility by fixing atmospheric nitrogen, both, in association with plant roots and without it, solubilise insoluble soil phosphates and produces plant growth substances in the soil. They are in fact being promoted to harvest the naturally available, biological system of nutrient mobilization. The role and importance of biofertilizers in sustainable crop production has been reviewed by several authors. Modern agriculture is heavily dependent on the application of chemical pesticides for disease control. Due to the concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought [4]. The interest in the use of biological approaches to replace hazardous pesticides in fertilizing soils or improve plant resistance against phytopathogens is steadily gaining worldwide acceptance. In this regard, the use of plant growth

promoting rhizobacteria (PGPR) has depicted potential in developing sustainable agricultural systems for crop production and protection [5,6]. The study of root-associated bacteria and their potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants but also for many biotechnological applications [7]. For example, strains from the genus *Bacillus* have been shown to enhance the growth of agricultural crops, wild plants, trees and model plants, through different mechanisms of plant growth-promotion [8,9,10,11,12]. Enhancement of plant growth by root-colonizing *Bacillus* sp. is well documented [13,14]. Studies reveal that *Bacillus* species are among the most prominent bacteria found to colonize plants root and soil populations [15]. *Bacillus* sp. may protect plants against pathogen by production of iron chelators such as Siderophores [16]. Promotion of plant growth by *Bacillus* sp. [17] includes production of bacterial phytohormones and/or the solubilization of mineral phosphates [18,19] and an ubiquitous presence in rhizosphere [20]. As such, keeping in view the aforementioned constrains, the present study was designed to isolate and characterize rhizospheric *Bacillus* isolate for its multiple plant growth promoting activities under in vitro conditions such as IAA production, Siderophore production, ammonia excretion, nitrogen fixation and P- solubilisation.

## MATERIALS AND METHODS

### Physiochemical analysis of rhizospheric soil sample and isolation of bacteria

Rhizosphere soil sample was collected from weed grown in saline fields of Ladwa. The soil sample was analysed for physiochemical parameters such as EC, pH, organic C, total N and available P. Bacterial cultures were isolated using serial dilution method with Malate's medium plates [21] which contained in g/L; Sodium malate (5.0), Yeast extract (0.2), $K_2HPO_4$ (0.2), $KH_2PO_4$ (0.8), $MgSO_4.7H_2O$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), $FeCl_3$ (0.02), $Na_2MoO_4$ (0.004), Agar (20.0).

### Measurement of IAA

IAA was quantified by the method of Patten and Glick [22]. The isolates were cultured in flasks containing 10 ml of nutrient broth supplemented with tryptophan (L-Trp) 0.2 mM and incubated at room temperature (25 to 28° C) for 48 h. The cultures were then centrifuged for 15 min at 10,000 rpm. Each 2 ml of the supernatant was mixed with 2 ml of Salkowski's reagent (150 ml $H_2SO_4$, 250 ml distilled water, 7.5 ml FeCl3.6H2O 0.5 M) and incubated at room temperature for 30 min. The presence of IAA was determined by the development of pink color and the IAA concentration was measured spectroscopically at 520 nm.

### Phosphate solubilization

Solubilization of tri-calcium phosphate was detected in Pikovskaya's Agar [23]. Each isolate was spotted on the surface of Pikovskaya agar medium and phosphate solubilizing activity was estimated after 1 to 5 days of incubation at room temperature. Phosphate solubilization activity was determined by the development of the clear zone around bacterial colony.

### Siderophore production

Siderophore production was tested qualitatively using chrome azurol S medium (CAS-medium) [24]. Each isolate was streaked on the surface of CAS agar medium and incubated at room temperature for 1 to 3 days. Siderophore production was indicated by orange halos around the colonies after the incubation.

### Ammonia excretion

For ammonia excretion, freshly grown culture was inoculated in 5 ml sterilized peptone water containing tubes and incubated at $28\pm2^oC$ for 4 days at stationary conditions. After 4 days, one ml of Nessler's reagent was added to peptone water and shaked thoroughly. Culture broth (1.5 ml) from this tube was centrifuged at 12000 rpm for 15 min. Supernatant was taken in a cuvette and absorbance was measured at 450 nm in a spectrophotometer [25].

### Genomic DNA extraction and *nif* H gene amplification

Whole cell genomic DNA was extracted by CTAB method [26]. The amplification of *nif* H was performed by the method of Sarita et al. [27] using primers *nifH* for (5'- TAY GGN AAR GGN GGHATY GGY ATC- 3') and *nifH* rev (5'-ATR TTR TTN GCN GCR TAV ABB GCC ATC AT-3'). The PCR reaction mixture (25 µl) contained 0.5 µl of dNTP mixture (10 mM), 0.5 µl of Taq DNA polymerase (3U $\mu l^{-1}$), 10X PCR assay buffer with 25 mM $MgCl_2$ (2.5 µl), 2 µl of each primer (10 pmol each), 2µl template DNA (0.1-.14 µg/µl) and 15.5 µl sterile water. PCR conditions were: initial denaturation at $95^oC$ for 4 min, 35 cycles of denaturation at $94^oC$ for 30 sec, annealing at

54°C for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR was then set on hold at 4°C. PCR product was separated by electrophoresis on 2% agarose gel stained with ethidium bromide and photographed under UV illumination using Gel Doc (DNR Bio-Imaging Systems).

**16SrDNA amplification:**

The 16S rDNA region was amplified using the universal bacterial 16 rDNA primers BAC 27 F (5'-AGA GTT TGA TCC TGG CTC AG – 3') [28] and BAC 1378 R (5'- CGG TGT GTA CAA GGC CCG GGA ACG - 3') [29]. The PCR amplification of the target sequence was carried out in a total volume of 50 µl of the following reaction mixture: 5 µl of PCR buffer (10X); 1.0 µl of dNTP mixture (10 mM); 2.0 µl of each of primers (10 µM); 1.0 µl of taq DNA polymerase (3U $\mu l^{-1}$) and 2.0 µl of template DNA (50 ng $\mu l^{-1}$ approx.) and 38 µl sterilized distilled water. Amplification conditions were as follows: 3 min of initial denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 sec, 50 °C for 30 s, and 72 °C for 1 min, with the last cycle followed by a 10 min extension step at 72 °C. PCR product was separated on 2% agarose gel, and photographed under UV illumination with Gel Doc (DNR Bio-Imaging Systems).

**16S rDNA gene sequencing and phylogenetic analysis**

The 16S rDNA product was got sequenced (Bioserve, Hyderabad). The sequencing reaction of 16S rDNA amplified product was performed by using both forward BAC 27 F and reverse BAC 1378 R primers. 16S rDNA sequence was compared with the GenBank database by using the algorithm BLASTN programme [30] to identify the most similar sequence of 16S rDNA. Phylogenetic tree was constructed using MEGA 4 software [31] through neighbour joining method [32]. The partial nucleotide sequence determined in this study was deposited in the GenBank database and got the accession number. .

**Morphological and Biochemical characterization.**

ML3 was further assayed for morphological characters such as Gram reaction and cell shape. Biochemical characterization was done by utilization of carbohydrates citrate, glucose, arabinose and hydrolysis of gelatin and starch.

## RESULTS AND DISCUSSION

Rhizospheric soil sample was physiochemically analysed as depicted in **Table I**. The selection of potential plant growth promoting isolate involved screening of over 20 bacterial isolates from the rhizosphere soil sample. The isolate was designated ML3. The plant growth promoting activity of the bacterium was accomplished by determining the IAA production, ammonia excretion, phosphate solubilisation and siderophore production (**Table II**). ML3 also has inherent nitrogen fixing ability which was detected by the presence of *nif* H gene as shown in **Fig I**. 16S rDNA amplification was confirmed with primers 27 F and 1378 R (**Fig II**). ML3 was identified on the basis of partial 16S rDNA gene sequencing. The sequence segment from the isolate was compared with the available nucleotide sequences in NCBI-nr database using the BLAST 2.2.9 system (www.ncbi.nlm.nih.gov/BLAST/). The sequence of the 16S rDNA gene of the isolate ML3 showed 100% identity with the 16S rDNA gene sequence of *Bacillus licheniformis*. This partial 16S rDNA gene sequence of strain ML3 (*B. licheniformis*) has been deposited in the GeneBank database under accession number JX495606. The phylogenetic analysis was performed by constructing a phylogenetic tree in which our strain ML3 fell within the cluster comprising *Bacillus* species (**Fig III**) with *Chlorella sorokiniana* was used as an outgroup. The identification was further confirmed by performing morphological and biochemical characterization of the isolate. ML3 exhibited gram positive rods and possession of biochemical traits such as utilization of citrate, glucose, arabinose, hydrolysis of gelatin and starch confirmed it as *Bacillus licheniformis* (**Table III**).

**Table I: Physiochemical analysis of rhizospheric soil sample**

| Soil Sample | Village (Source) | District | EC ($dS\ m^{-1}$) | pH | Organic C (%) | Total N (%) | Available P ($\mu g\ g^{-1}$ soil) |
|---|---|---|---|---|---|---|---|
| ML3 | Ladwa | Hisar | 20.00 | 7.63 | 0.67 | 0.15 | 0.15 |

**Table II: Plant growth promoting traits of ML3**

| Strain | *nif*H | IAA production (µg ml$^{-1}$) | Ammonia excretion (µg ml$^{-1}$) | Siderophore production | Phosphate solubilization |
|---|---|---|---|---|---|
| ML3 | + | 174.72 | 0.66 | + | + |

**Table III: Morphological and Biochemical characterization**

| Characteristics | ML3 |
|---|---|
| Gram stain | + |
| Cell shape | Rods |
| Utilization of: | |
| Citrate | + |
| Glucose | + |
| Arabinose | + |
| Hydrolysis of: | |
| Gelatin | + |
| Starch | + |

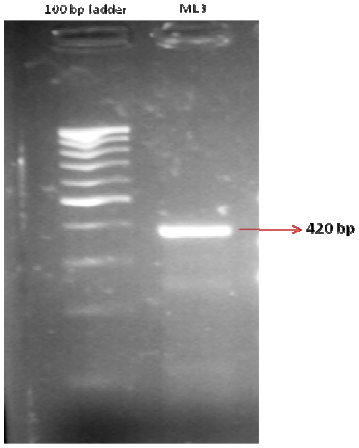


**Fig I: nifH gene amplification of ML3**

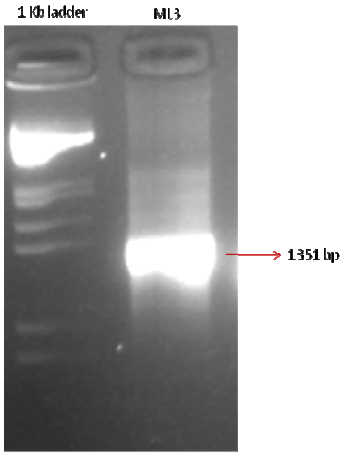


**Fig II: 16S rDNA gene amplification of ML3**

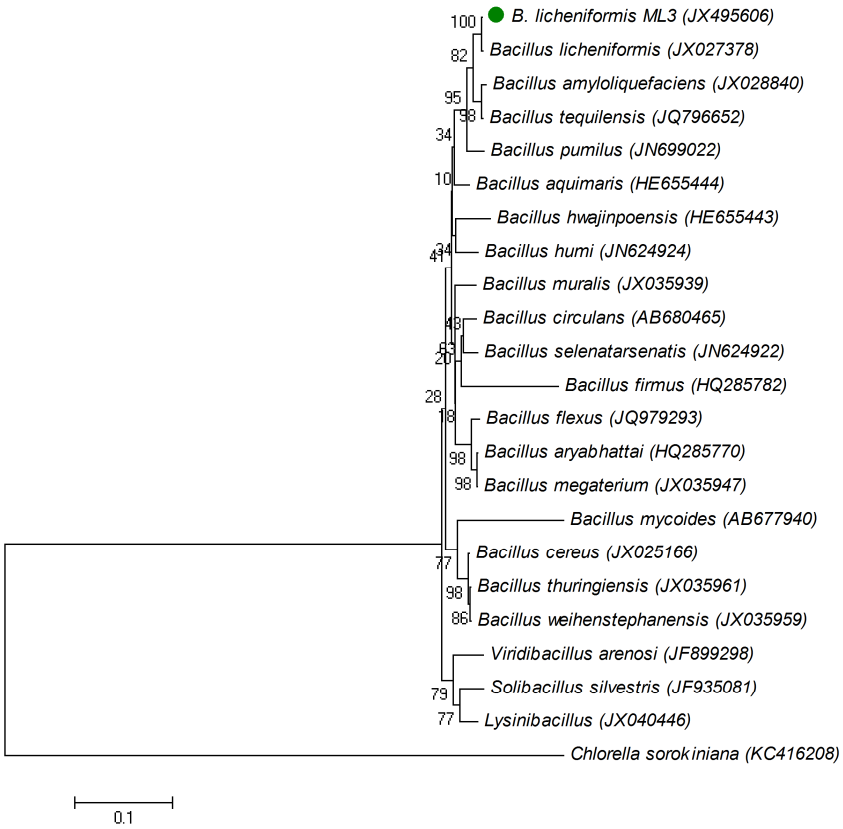


**Fig III: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of *Bacillus licheniformis* ML3 and other related taxa. *Chlorella sorokiniana* was used as an out-group. Bootstrap percentages (based on 1000 replicates) are shown at branch points. Bar, 0.1 changes per nucleotide position**

Isolation of *Bacillus* species from the rhizosphere area was widely studied previously. In this study, it was of interest to isolate and characterize rhizospheric *Bacillus* isolate for its multiple plant growth promoting activities under *in vitro* conditions so that it can be developed as a multifunctional biofertilizer. *Bacillus licheniformis* ML3 showed potential activity of producing IAA, solubilizing phosphate, producing siderophore and excretion of ammonia. The ability of some *Bacillus* sp. isolates to use tryptophan supplemented in the cultivation medium is one of the important points to determine IAA producing activity. Tryptophan is the main precursor of IAA biosynthesis in bacteria [22], therefore the presence of Trp in the medium is an important factor for *Bacillus* sp. isolates to produce IAA. In accordance with this report, our isolate *Bacillus licheniformis* ML3 produced IAA in tryptophan supplemented medium. ML3 was also efficient in solubilising tricalcium phosphate. The ability of several isolates to solubilize tricalcium phosphate *in vitro* shows the possible application of these isolates as biofertilizers in various crop fields. *Bacillus* and other phosphate solubilizing bacteria (PSB) were capable of increasing the availability of phosphorus in soil as demonstated by Rodriguez and Fraga in 1999 [33]. Ammonia excretion is also one of the important traits in plant growth promotion. *Bacillus licheniformis* ML3 was producing significant concentration of ammonia by developing a yellow color in peptone water. Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, including *Bacillus* sp. under iron deficient conditions. ML3 exhibited positive siderophore production which was indicated by yellow halo around the spotted culture. PGPR produces a range of siderophores which have a very high affinity for iron. Whipps in 2001 [34] reported that the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi.

## CONCLUSION

Overall, the present study suggested that the *Bacillus licheniformis* ML3 strain has the potential to be developed as a plant growth promoter or biofertilizer which in future can substitute the chemical fertilizers.

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