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# Identification and Characterisation of Potential Biofertilizer Bacterial Strains

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**Abstract.** In this study we aimed that isolation, identification and characterizations of PGPR strains from rhizosphere of legume plants. 188 bacterial strains isolated from different legume plants like clover, sainfoin and vetch in Erzurum province of Turkey. These three plants are cultivated commonly in the Erzurum province. It was screen that 50 out of 188 strains can fix nitrogen and solubilize phosphate. These strains were identified via MIS (Microbial identification system). According to MIS identification results, 40 out of 50 strains were identified as *Bacillus*, 5 as *Pseudomonas*, 3 as *Paenibacillus*, 1 as *Acinetobacter*, 1 as *Brevibacterium*. According to classical test results, while the catalase test result of all isolates are positive, oxidase, KOH and starch hydrolysis rest results are variable.

## INTRODUCTION

Bacteria, called PGPRs (Plant growth promoting rhizobacteria) are free-living bacteria and they countenance plant growth. These bacteria can be used as biological fertilizer, biocontrol agents and they can help soil remediation. PGPRs are generally divided into as microbial fertilizers, phytostimulators, rhizoremedators and biopesticides. Microbial fertilizers provide the nutrient uptake of plants, phytostimulators produce some group hormones that stimulate plant growth, rhizoremedators degrade many organic pollutants and biological control agents can help the plants to prevent from disease by producing some metabolites like antibiotics and inducing plant defense mechanisms [1].

Especially, N2-fixing and P-solubilizing bacteria are regarded as a possible alternative for inorganic fertilizers, and PGPR strains have previously been attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield [2, 3].

It is important that PGPRs produce some hormones like auxins, cytokinins and gibberellins that induce plant growth as well as they fix nitrogen [4]. Producing of plant hormones is not just a feature peculiar to plants; many bacteria related to plants can also produce one or more of these hormones. The most produced hormones by PGPRs quantitatively are auxins. Auxin production is considered as the main factor for providing plant growth and inducing root developing rather than nitrogen fixation [5]. It was known that soil microorganisms are capable of synthesizing of the cytokinins, auxins and gibberallins by relating to plants. PGPRs mostly are in the genus of *Acetobacter, Azospirillum, Azotobacter, Bacillus, Paenibacillus, Pseudomonas, Rhizobium, Rhodobacter, Rhodosprilum* and *Serratia* [6, 7]. Many bacteria from these genus, which grown in the soil rhizosphere, on or in the plants, are considered as plant growth promoting and they do that with different mechanisms. PGPRs have positive effect on the rising of vegetative and generative growth of plants (vegetables, fruits, grains, foliage or ornamental plants etc.) at different ratio [8, 9].

Considering all data summarized above; isolation, identification, characterization and researching of usage possibilities of the PGPRs are very important because of to ensure the sustainability of current agricultural systems. Beside, plant growth promoting traits of the bacteria should be defined well. Considering these, current research was designed to isolation and characterization of PGPRs from legume plants.

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## MATERIALS AND METHODS

### Isolation of PGPR Strains

We conducted a survey of PGPRs and took soil samples from rhizophere of legume plans in different location of Erzurum province. Breeding of legume plants are commonly done at all the areas. Rhizosphere soil samples were collected carefully by uprooting the root system and placed in a cool box for transport and stored at 4 °C. Ten grams of soil from each sample was aseptically weighed and transferred to an Erlenmeyer flask with 100ml sterile water, and was shaken for 30 min at 150 rpm. Immediately after shaking, a series of ten-fold dilutions of the suspension was made for each sample by pipetting 1 ml aliquots into 9 ml sterile water. The final dilution was 105- fold; 0.1 ml of each dilution of the series was placed onto a Petri dishes. Three replicate dishes were made for each dilution. Dishes were placed in an incubator at 28 °C for seven days (aerobically). NA was used all isolations, individual colonies were purified and then stored in LB- 30 % glycerol solution at -80 °C [10].

### Test of Nitrogen Fixation Ability

Nonsymbyotic nitrogen fixation ability of strains was tested on N-free solid malate sucrose medium (NFMM) modified from Döbereiner [11]. Modified NFMM medium contain (per liter): sucrose, 10.0 g; L-malic acid, 5.0 g; $MgSO_4 .7H_2O$, 0.2 g; $FeCl_3$, 0.01 g; NaCl, 0.1 g; $CaCl_2 . 2H_2O$, 0.02 g; $K_2HPO_4$, 0.1 g; $KH_2PO_4$, 0.4 g; $Na_2MoO_4 . H2O$, 0.002 g) with 18 g agar The medium adjusted to pH 7.2 with 1 N NaOH prior to agar addition and was then sterilized at 121 °C for 20 min in an autoclave. The bacteria to be tested was streaked a NFMM plate in the form of a line across the width of the plate. Then incubated 2-7 days at 27 °C. Bacterial growth on the plate was considered as positive result.

### Test of Phosphate Solubilization Ability

Phosphate solubilization activity of the bacterial isolates was detected on National Botanical Research Institute's phosphate growth medium (NBRIP-BPB). NBRIP-BPB contain (per liter): glucose, 20 g; $Ca_3(PO_4)_2$, 10 g; $MgCl_2 . 6H_2O$, 5 g; $MgSO_4 . 7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g, and bromophenol blue (BPB), 0.025 g. The pH of the media was adjusted to 7.0 before autoclaving, as described earlier. 5 ml of NBRIP-BPB medium was transferred to a sterile test tube and autoclaved. Autoclaved, uninoculated broth medium served as controls. The sterile liquid medium was inoculated with 500 ml suspension of the tested bacterial strains. The test tubes were incubated for 14 days at room temperature. At the end of the incubation period, change in pH (of the culture broth was recorded. All the pure isolates were also tested in triplicate [12].

### Biochemical Tests

The KOH string Test is done using a drop of 3% potassium hydroxide on a glass slide. A visible loop full of cells from a single, well-isolated colony is mixed into the drop. If the mixture becomes viscous within 60 seconds of mixing (KOH-positive) then the colony is considered gram-negative [13].

Catalase test conducted with $H_2O_2$ . Briefly, small amount of 24 h bacterial culture was placed onto a clean microscope slide. Then a few drops of $H_2O_2$ were added onto the smear and mix with a toothpick. Occurring bubbles rapidly were considered as positive result. No bulb or only e few scattered bubbles considered as negative result.

Oxidase tests were performed via discs containing 1% tetra methyl-p-phenylendiamine dihydrochloride. Firstly, the discs were moistened with sterile distil water, then one loop bacteria was smeared over the disc. Positive reaction is indicated by an intense deep-purple hue [10, 14].

Starch hydrolysis tests were conducted on NAS (Nutrient Starch Agar) medium. The bacteria to be tested was streaked a NAS plate in the form of a line across the width of the plate. Then plates were incubated 2-7 days at 27 °C. After the incubation period, a few drops of 10 % iodane solution were added directly onto the edge of colonies. 10-15 minutes later results were recorded. While the medium turn to dark, clear zone around of the bacteria was considered as positive result [10, 14].

### Extraction of Fatty Acid and FAME-Based Identification of Bacteria

Cells were streaked in a quadrant pattern and grown overnight on TSBA (Tyripticase soy broth agar). Approximately 50 mg of bacterial cells, harvested from the third and fourth quadrant streak of growth, were used for the extraction using standard extraction techniques (Sasser, 1990). FAME profiles were obtained by running samples on a Hewlett Packard Agilent GC 6890 GC fitted with a microprocessor containing the Sherlock Microbial Identification System (MIDI) Software (V.A. 06. 03). The FAME profiles were compared with the TSBA 50 aerobe library.

## RESULT AND DISCUSSION

Totally 188 bacterial strains were isolated from rhizosphere soil of legume plants. Then phosphate solubilization and nitrogen fixation abilities of strains were tested. According to test result 50 out of 188 strains have both of the abilities of nitrogen fixation and phosphate solubilization. These 50 strains were subjected to characterization studies and some basic traits of strains were defined. According to these; 43 of 50 strains are gram (+) and remains are gram (-), and all of the strains have catalase activity. Beside, positive and negative results are obtained from oxidase and starch hydrolysis tests. All results are given in Table 1.

It is well known, Biochemical characteristics or classical methods solely are not good enough for identification of bacteria. But these tests are so important, because they provide a pre-identification and help to define the molecular methods can be used for identification. For this reason, many researchers, working on identification and characterization of bacteria, take the advantage of classical methods [14, 15].

Molecular identification methods based on carbohydrates, lipids, proteins and nucleic acids profiles of microorganism. Many different methods are existing like PCR (polymerase chain reaction), ELISA (enzyme linked immunosorbent assay), BIOLOG, SDS-PAGE, MIS etc. [16, 17, 18, 19, 20, 21, 22]. The MIS system is one of the most common molecular methods. These methods identify the microorganism by using FAME profiles and routinely used to identify genera, species, and strains of bacteria. In current study, MIS were used to identification of bacterial strains. According to MIS identification results, most of the strains are within *Bacillus* and *Pseudomonas* genus. Various members of the two genuses are well known contributor for plant growth promoting and there are many research about on [23, 24, 25].

In consequence; Totally 188 bacterial strains were isolated from different legume plants' soil rhizosphere. It was screened that 50 out of 188 strains are capable of fixing the nitrogen and solubilizing the phosphate. MIS identification of these strains was done and some biochemical properties of them were defined. Of course many tests and experiments should do to define other properties of them. Here after; it is planned that finding quantitative nitrogen fixing and phosphate solubilizing ability of the strain. After that, the strains to be found most effective should be tested other desired traits like siderophor production, resistance to antibiotics and other chemicals, survival in the soil.

**TABLE 1.** MIS identification result and some traits of the strains

| Strain No | MIS identification result | SIM % | Location | G | K | O | NH | P | N |
|---|---|---|---|---|---|---|---|---|---|
| FDG-2 | *Bacillus-megaterium*-GC subgroup B | 71 | Erzurum | + | + | + | + | + | + |
| FDG-7 | *Pseudomonas fluorescens* biotype F | 11 | Erzurum | - | $K^+$ | + | - | + | + |
| FDG-20 | *Paenibacillus pabuli* | 34 | Erzurum | + | $K^+$ | - | - | + | + |
| FDG-31 | *Bacillus-megaterium*-GC subgroup B | 61 | Erzurum | + | + | - | + | + | + |
| FDG-37 | *Pseudomonas fluorescens* biotype F | 39 | Aşkale | - | + | + | - | + | + |
| FDG-38 | *Bacillus-megaterium*-GC subgroup B | 32 | Aşkale | - | $K^+$ | + | + | + | + |
| FDG-47 | *Bacillus-megaterium*-GC subgroup B | 48 | Aşkale | + | + | + | + | + | + |
| FDG-50 | *Bacillus-megaterium*-GC subgroup B | 15 | Aşkale | + | + | + | + | + | + |
| FDG-54 | *Bacillus-megaterium*-GC subgroup B | 46 | Aşkale | + | + | - | + | + | + |
| FDG-56 | *Bacillus-megaterium*-GC subgroup B | 58 | Aşkale | + | + | - | + | + | + |
| FDG-63 | *Bacillus-megaterium*-GC subgroup A | 53 | Aşkale | + | + | - | + | + | + |
| FDG-67 | *Bacillus-megaterium*-GC subgroup B | 54 | Aşkale | + | + | - | + | + | + |
| FDG-69 | *Bacillus-megaterium*-GC subgroup A | 39 | Aşkale | + | + | - | + | + | + |
| FDP-78 | *Bacillus-megaterium*-GC subgroup A | 64 | Aşkale | + | + | - | + | + | + |
| FDG-79 | *Acinetobacter johnsonii* | 35 | Pasinler | - | + | - | - | + | + |
| FDG-80 | *Bacillus-megaterium*-GC subgroup A | 31 | Pasinler | + | + | + | + | + | + |
| FDG-81 | *Bacillus-megaterium*-GC subgroup B | 66 | Pasinler | + | + | + | + | + | + |
| FDG-83 | *Bacillus-megaterium*-GC subgroup B | 44 | Pasinler | + | + | - | + | + | + |
| FDG-85 | *Bacillus-megaterium*-GC subgroup B | 43 | Pasinler | + | + | - | + | + | + |
| FDG-87 | *Pseudomonas chlororaphis* | 35 | Pasinler | - | + | - | - | + | + |
| FDG-89 | *Bacillus-megaterium*-GC subgroup B | 51 | Pasinler | + | + | - | + | + | + |
| FDG-90 | *Bacillus-megaterium*-GC subgroup B | 56 | Pasinler | + | + | - | + | + | + |
| FDG-99 | *Bacillus-megaterium*-GC subgroup A | 48 | Pasinler | + | + | + | + | + | + |
| FDG-100 | *Bacillus-megaterium*-GC subgroup A | 44 | Pasinler | + | + | + | + | + | + |
| FDG-101 | *Bacillus-megaterium*-GC subgroup A | 41 | Pasinler | + | + | + | + | + | + |
| FDG-102 | *Bacillus-megaterium*-GC subgroup A | 40 | Pasinler | + | + | + | + | + | + |
| FDG-108 | *Bacillus-megaterium*-GC subgroup A | 67 | Pasinler | + | + | - | + | + | + |
| FDG-117 | *Bacillus-megaterium*-GC subgroup B | 38 | Pasinler | + | + | - | + | + | + |
| FDG-119 | *Bacillus-megaterium*-GC subgroup A | 43 | Pasinler | + | + | - | - | + | + |
| FDG-121 | *Pseudomonas alcaligenes* | 30 | Pasinler | - | + | - | - | + | + |
| FDG-129 | *Paenibacillus pabuli* | 31 | Oltu | + | + | - | - | + | + |
| FDG-134 | *Bacillus-megaterium*-GC subgroup B | 34 | Oltu | + | + | - | + | + | + |
| FDG-136 | *Bacillus-megaterium*-GC subgroup A | 49 | Oltu | + | + | - | + | + | + |
| FDG-137 | *Bacillus-cereus*-GC subgroup B | 39 | Oltu | + | + | - | - | + | + |
| FDG-139 | *Bacillus-megaterium*-GC subgroup A | 38 | Oltu | + | $K^+$ | - | + | + | + |
| FDG-140 | *Bacillus-megaterium*-GC subgroup B | 51 | Oltu | + | + | + | + | + | + |
| FDG-141 | *Bacillus-megaterium*-GC subgroup B | 36 | Oltu | + | + | + | + | + | + |
| FDG-146 | *Bacillus-megaterium*-GC subgroup B | 40 | Oltu | + | + | - | + | + | + |
| FDG-150 | *Brevibacillus agri* | 47 | Oltu | + | + | - | - | + | + |
| FDG-153 | *Bacillus-megaterium*-GC subgroup A | 39 | Oltu | + | $K^+$ | - | + | + | + |
| FDG-157 | *Bacillus-megaterium*-GC subgroup B | 53 | Oltu | + | + | - | + | + | + |
| FDG-158 | *Bacillus-megaterium*-GC subgroup A | 40 | Oltu | + | $K^+$ | - | + | + | + |
| FDG-161 | *Bacillus-megaterium*-GC subgroup A | 60 | Oltu | + | + | - | + | + | + |
| FDG-164 | *Pseudomonas fluorescens* biotype F | 31 | Oltu | - | + | + | - | + | + |
| FDG-167 | *Bacillus-megaterium*-GC subgroup B | 44 | Oltu | + | + | - | + | + | + |
| FDG-174 | *Bacillus-megaterium*-GC subgroup A | 34 | Tortum | + | $K^+$ | - | + | + | + |
| FDG-180 | *Bacillus-megaterium*-GC subgroup A | 69 | Tortum | + | + | - | + | + | + |
| FDG-182 | *Bacillus-megaterium*-GC subgroup A | 36 | Tortum | + | $K^+$ | - | + | + | + |
| FDG-184 | *Paenibacillus* | 35 | Tortum | + | + | - | - | + | + |
| FDG-185 | *Bacillus psycrophilus* | 30 | Tortum | + | + | - | - | + | + |

**G:** Gram traits, **K:** Catalase activity: **O:** Oxidase activity, **NH:** Starch hydrolysis, **P:** Phospate solubulising, **N:** Nitrogen fixation, **$K^+$:** Strong posivive, **+:** Positive, **-:** Negative

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