Research Article

# Isolation and Identification of Bacterial Strains with Fatty Acid Methyl Ester (FAME) Analysis

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## ABSTRACT

Soil is an excellent source of micro-organisms, especially bacteria. In our present study, we isolated bacteria from a fertile agricultural soil IRCP (361) from Northern India and studied the morphological, cultural, and biochemical characteristics. We performed FAME (Fatty Acid Methyl Ester) analysis of the isolated strains. The isolated strains comprised of Gram- positive bacteria. Using FAME analysis, we confirmed majority of bacteria belong to Bacillus species and few belong to Pseudomonas species. Bacillus species enhance nitrogen fixation which increases soil fertility and protect plants from toxic effects of metals. Pseudomonas confers resistance against root-secreted antibiotics. Paenibacillus species possess potentiality for bioremediation of xenobiotics. This study shows that the soil is fertile due to the abundance of the Nitrogen fixing bacteria and the identification of the bacteria is fast and accurate using FAME analysis.

**Key words:** Soil fertility, FAME analysis, Gram-positive bacteria, Bacillus species, Pseudomonas species, Nitrogen fixation.

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## INTRODUCTION

A fertile soil has ability to sustain plant growth by providing essential nutrients. In the soil-root interface, soil micro-organisms associate with plant roots and soil constituents, resulting in the formation of a complex ecosystem known as the rhizosphere. Soil micro-organisms decompose organic matter and release vital nutrients into the soil, which are used by plants to grow and develop. Bacillus species are involved in nitrogen fixation in the atmosphere, mineral solubilization, stress response gene expression, nutrient uptake by plants, and as a biocontrol agent.[1,2] Pseudomonas species have ability to promote immune defense in plants by forming biofilm and confers resistance against several pathogens.[3] Bacterial identification is necessary to distinguish one organism from another and to group similar organisms under one criteria. The Gram staining technique is a preliminary step in initial characterization of bacteria. Biochemical tests are useful for identifying bacterial organisms based on their biochemical activities. FAME analysis is a relatively new technique for identifying bacterial species with greater speed and accuracy. Fatty Acid Methyl Esters are derived by transesterification of fats with methanol. Every micro-organism has its specific FAME profile (microbial fingerprinting) which is developed by using gas chromatography.[4] These profiles can be used as a tool for microbial source tracking (MST).[5] 1700 species of bacteria and yeast can currently be reliably classified using the Sherlock analysis programme. The Sherlock system's special configuration is tailored for gas chromatography study of fatty acids.

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## MATERIALS AND METHODS

**Collection of soil sample**: The soil sample IRCP (361) is collected from the agricultural field of Northern India by driving the auger to a plough depth of 15cm.

### Reagents

Gram staining**:** Crystal violet, Iodine solution/Gram's Iodine, ethanol, Safranin Water.

Indole test: Kovac's Reagent: p - dimethyl amino benzaldehyde - 10g, Iso amyl alcohol -150ml and Conc. Hydrochloric acid- 50ml. Media: Tryptone broth.

Methyl red test: Methyl red dye-0.1g, ethyl alcohol-300ml and water-200ml. Media: MR-VP broth.

Voges-Proskauer test: 40% Potassium hydroxide, alpha-naphthol. Media: Glucose phosphate broth.

Citrate utilization test: Bromothymol Blue: 0.4g and Sodium hydroxide: 0.05 N. Media: Citrate agar slant.

**FAME Analysis:** Reagent 1: 45g sodium hydroxide, 150ml methanol, and 150ml distilled water, Reagent 2: 325ml certified 6.0N hydrochloric acid and 275ml methyl alcohol, Reagent 3: 200ml hexane and 200ml methyl tert-butyl ether, Reagent 4: 10.8g sodium hydroxide dissolved in 900ml distilled water.[6]

The soil sample is serially diluted.[7] Pure cultures of bacteria are obtained by streak plate technique.[8]

Gram staining: Gram staining was performed by following the standard protocol.[9]

Indole test: 4 mL of tryptophan broth was drawn from sterilized test tubes. The growth from a 24-hr culture is used to aseptically inoculate the tubes. The tubes are incubated for 24 hr at 37°C. 0.5 ml of Kovac's reagent was added to the broth cultures.

Methyl red test: MRVP broth was prepared in test tubes. The broth was aseptically inoculated with 2 loopful of respective bacterial cultures. The test tubes are incubated for 48 hr at 37°C. In the incubated tubes, a few drops of methyl red indicator were added.

Vogues-Proskauer test: Cultures to be tested are inoculated into glucose phosphate broth and incubated for 48 hr. 0.6 ml of alpha-naphthol and 0.2 ml of 40% KOH was added to the test broths and shaken.

Citrate utilization test: The media was prepared and distributed into tubes about 5ml each. The tubes are filled to make slants and poured into a slanting position. The cultures are streaked on citrate agar slant and incubated at 37°C for 24 hr.

Fame analysis: A 4mm loop is used to collect about 40mg of bacterial cells from the streaked plate's third quadrant. Each tube is filled with 1ml of Reagent 1, then sealed with teflon lined caps, vortexed briefly, and heated in a boiling water bath. 2ml of Reagent 2 is applied to the cooled tubes after they have been uncapped. The tubes are vortexed momentarily before being heated at 80°C for 10 min. The aqueous step is pipetted out and discarded after 1.25ml of Reagent 3 is applied to the cooled tubes and gently tumbling on a clinical rotator for about 10 min. The organic process receives 3ml of Reagent 4 and the tubes are tumbled for 5 min. A GC vial is pipetted with around 2/3 of the organic phase.[10]

## RESULTS

The colony characteristics of the isolated strains are Dry, flat, irregular, white to cream color colonies, Irregular, rough, white, opaque colonies, Large, opaque, mucoid, flat colonies with irregular margins, Circular, smooth, opaque, dark brown colonies, Transparent, Round, white, smooth colonies for isolates 1-5 respectively (Figure 1).

The results of Gram staining technique showed that all the isolated strains are gram positive bacteria and have rod shape morphology when viewed under compound microscope (Figure 2).

The Biochemical tests shows negative for all the isolates for Indole and methyl red test and negative only for isolate 3 for VP test and positive for all the isolates in citrate utilization test (Table 1).

The biochemical tests shows that all the isolated strains are Indole test negative which indicates the absence of the enzyme tryptophanase enzyme. Tryptophanase enzyme is used to break the amino acid tryptophan to release indole which reacts with aldehyde producing blueish-green color. The absence of the enzyme results in no color production.[11] All the isolated strains are Methyl red test negative indicating the less production of acid by fermentation of glucose.[12] The isolates-1,2,4,5 are Voges-Proskauer test positive indicating the production of acetoin which reacts with Barritt's reagent producing red color whereas the isolate-3 showed no color change indicating the absence of acetoin production.[13] All the isolated strains showed positive result for citrate utilization test, suggesting that they use citrate as their sole source of carbon to meet their energy needs.[14]

In FAME analysis, the automated system identified isolate-1 as *Bacillus subtilis* with 0.713 SI, isolate-2 as *Bacillus licheniformis* with 0.609 SI, isolate-3 as *Pseudomonas aeruginosa* with 0.345 SI, isolate-4 as *Bacillus atrophaeus* with 0.516 SI and isolate-5 as *Paenibacillus macerans* with 0.138 SI. *Bacillus subtilis* has a FAME profile that is comparable to *Bacillus atrophaeus*, and vice versa. *Pseudomonas aeruginosa* subgroup B has a FAME profile

**Table 1: Biochemical Tests.**

| Isolate | Indole test | Methyl red test | Voges-Proskauer test | Citrate utilization test |
|---|---|---|---|---|
| 1 | - | - | + | + |
| 2 | - | - | + | + |
| 3 | - | - | - | + |
| 4 | - | - | + | + |
| 5 | - | - | + | + |


**Figure 1: Cultures of the isolated strains on nutrient agar media.**

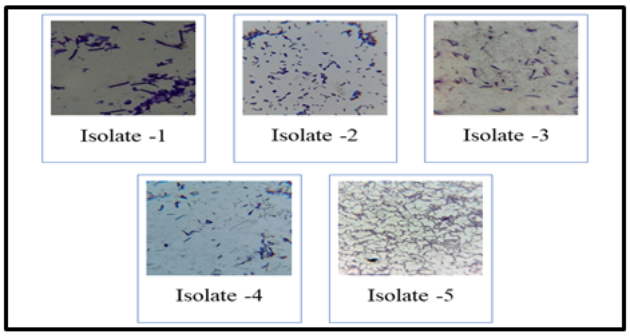


**Figure 2: Microscopic view of the isolated strains using compound microscope.**

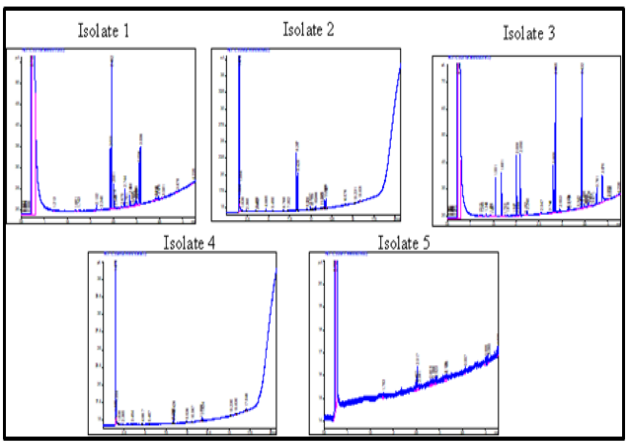


**Figure 3: Chromatogram of the isolated strains.**

that is similar to *Pseudomonas aeruginosa* subgroup A, and *Paenibacillus macerans* has a FAME profile that is similar to *Staphylococcus epidermidis*.

The FAME report generated by MIDI Sherlock system identified that Pentadecanoic Acid is present in highest concentration (38.02%) in *Bacillus subtilis*, 13-Methyltetradecanoic Acid is present in highest concentration (44.94%) in *Bacillus licheniformis*, (11Z)-11-Octadecanoic Acid and (12Z)-12-Octadecenoic Acid are present in highest concentrations (23.7%) in *Pseudomonas aeruginosa*, 12-Methyltetradecanoic Acid is present in highest concentration in *Bacillus atrophaeus* and *Paenibacillus macerans*. The percentage of 12-Methyltetradecanoic Acid in *Bacillus atrophaeus* is 43.05% and in *Paenibacillus macerans* is 31.72%.

The Chromatogram of the isolated strains indicated the solvent peak of *Bacillus subtilis* at $R_T$ (Retention time) value 0.729, *Bacillus licheniformis* at $R_T$ value 1.476, *Pseudomonas aeruginosa* at $R_T$ value 0.732, *Bacillus atrophaeus* at $R_T$ value 1.479 and *Paenibacillus macerans* at $R_T$ value 0.727 (Figure 3).

## DISCUSSION

Organic matter present in the soil is an important indicator of soil fertility and health. The nutrients present in the form of organic matter cannot be utilized by plants directly. Microbes present in the soil consume the organic matter and make the nitrogen available for plants. Organic acids in the soil come from a variety of locations, including plant roots, micro-organisms, and organic decomposition, and they are important for mineral solubilization and soil fertility. Soil organic acids play a significant part in soil nutrient availability and productivity.[15] Organic acids called fatty acids can be used to measure microbial biomass in soils. The greater the amount of fatty acids found in the soil, the greater will be the soil fertility. To determine the fertility of soil, one best way is by detecting the microbial biomass and fatty acid content by FAME analysis using Gas chromatography.[16] Fatty acid methyl ester (FAME) profiles extracted from soils are analysed rapidly to determine the composition of the soil microbial population.[17] The accurate identification of the bacterial species (Table 2) along with fatty acid content (Table 3) is possible by FAME analysis. The cultured bacterial species can be clearly demarcated on the basis of several factors like SI value, $R_T$ value, fatty acid concentration and chromatogram peak by means of FAME analysis. Such an identification is useful for assessing the fertility of soil for productivity. Bacillus and Pseudomonas species identified by FAME analysis

**Table 2 indicates the Sim index (SI), method of evaluation and closely related organism to the isolated strain.**

| Isolate | Method | Distance | Sim index | Cultured Organism | Closely relative organism |
|---|---|---|---|---|---|
| 1 | RTSBA6 | 2.920 | 0.713 | *Bacillus-subtilis* | *Bacillus atrophaeus* |
| 2 | TSBA6 | 3.537 | 0.609 | *Bacillus-licheniformis* | *Bacillus pumilus* |
| 3 | RTSBA6 | 5.182 | 0.345 | *Pseudomonas-aeruginosa*-GC subgroup B | *Pseudomonas aeruginosa*-GC subgroup A |
| 4 | TSBA6 | 4.089 | 0.516 | *Bacillus-atrophaeus* | *Bacillus subtilis* |
| 5 | RTSBA6 | 7.071 | 0.138 | *Paenibacillus-macerans* | Staphylococcus epidermidis |

**Table 3 shows the Fatty acid present in highest concentration based of the $R_T$ values of the solvent peak. Each species has a particular fatty acid in high concentration and their IUPAC name is indicated below.**

| Bacteria | Retention time (RT) value of solvent peak | Fatty acid in highest concentration | IUPAC name | Percentage |
|---|---|---|---|---|
| *Bacillus subtilis* | 0.729 | 15:0 | Pentadecanoic Acid | 38.02 |
| *Bacillus licheniformis* | 1.476 | 15:0 iso | 13-Methyltetradecanoic Acid | 44.94 |
| *Pseudomonas aeruginosa* | 0.732 | 18:1w7C and 18:1 w6C | (11Z)-11-Octadecenoic Acid and (12Z)-12-Octadecenoic Acid | 23.7 |
| *Bacillus atrophaeus* | 1.479 | 15:0 anteiso | 12-Methyltetradecanoic Acid | 43.05 |
| *Paenibacillus macerans* | 0.727 | 15:0 anteiso | 12-Methyltetradecanoic Acid | 31.72 |

are abundantly present in the soil sample, making it fertile. Strains of Bacillus and Pseudomonas are most efficient phosphate solubilizers due to their fatty acids. [18]

## CONCLUSION

Soil fertility is directly linked to the presence of microbial biomass. Microbes play a critical role in mineral solubilization and plant nutrient uptake. The presence of Bacillus and Pseudomonas species, which is rapidly and accurately identified by FAME analysis, makes the soil sample fertile enhancing its productivity.

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## CONFLICT OF INTEREST

The authors declare no Conflict of interest.

## ABBREVIATIONS

**FAME:** Fatty Acid Methyl Ester; **RT:** Retention Time; **SI:** Sim index.

## SUMMARY

A sample of bacteria is isolated from the agricultural soil (IRCP 361) through serial dilution method. The streak plate technique is used to create pure bacterial colonies. Gram staining, Biochemical analysis identified the bacterial strains which was further confirmed by FAME analysis as *Bacillus* and *Pseudomonas* species. Other studies shows that the soil fertility is improved by the presence of these micro-organisms.

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