Background: Fatty acids occur in nearly all living organisms as the important predominant constituents of lipids. While all fatty acids have essentially the same chemical nature, they are an extremely diverse group of compounds.

Materials and Methods: To test the hypothesis, fatty acids of alkaliphiles isolates, Bacillus subtilis SVNUM4, Bacillus licheniformis SVUNM8, Bacillus methylotrophicus SVUNM9, and Paenibacillus dendritiformis SVUNM11, were characterized compared using gas chromatography-mass spectrometry (GC-MS) analysis. Results: The content of investigated ten fatty acids, 1,2-benzenedicarboxylic acid butyl 2-methylpropyl ester, phthalic acid, isobutyl 2-pentyl ester, dibutyl phthalate, cyclotrisiloxane, hexamethyl, cyclotetrasiloxane, octamethyl, dodecylmethyl, heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-etracemethyl, 7,15-dihydroxydehydroabietic acid, methyl ester, di (trimethylsilyl) ether, hentriacontane, 2-thiopheneacetic acid, undec-2-enyl ester, obviously varied among four species, suggesting each species has its own fatty acid pattern.

Conclusions: These findings demonstrated that GC-MS-based fatty acid profiling analysis provides the reliable platform to classify these four species, which is helpful for ensuring their biotechnological interest and novel chemotaxonomic.

Keywords: Alkaliphiles, fatty acid methyl ester, gas chromatography analysis, muscovite mines

Introduction

The biological diversity of the Indian subcontinent is one of the richest in the world owing to its vast geographic area, varied topography and climate, and the juxtaposition of several biogeographical regions. Various types of diverse microenvironments and unique ecosystems such as boiling waters, deep sea vents, salts pans, acid mine drainages, cold environment, and deep subsurface environments are present in India that are home to diverse populations of microorganisms.

Muscovite ore granitic pegmatites are the source of muscovite sheet in sedimentary rocks. Formation of sedimentary rocks is one of the important parts of the rock cycle. These ultramafic rocks result in challenging environments for life in continental sites due to the combination of extremely high pH, low salinity, and lack of obvious electron acceptors and carbon sources. In sedimentary rocks, a variety of alkaliphilic microorganisms survives and grows. In these alkaline environments, pH increase is due to microbial ammonification and sulfate reduction and by water derived from leached silicates minerals. Recently, many bacteria capable of growing at a pH as high as 10.5 have been isolated for physiological interest as well as industrial applications.

Fatty acids are organic compounds commonly found in living organisms. They are abundant in the phospholipid bilayer of bacterial membranes. Their
diverse chemical and physical properties determine the variety of their biochemical functions. This diversity, which is found in unique combinations in various bacterial species, makes fatty acid profiling a useful identification tool.

The cellular fatty acid analysis for bacterial identification is based on the specific fatty acid composition of the cell wall. The fatty acids are extracted from cultured samples and are separated using gas chromatography (GC). A computer-generated unique profile pattern of the extracted fatty acids is compared through pattern recognition programs, to the existing microbial databases. These databases include fatty acid profiles coupled with an assigned statistical probability values indicating the confidence level of the match. This has become very common in biotechnology. Fatty acid methyl esters (FAMEs) have long been recognized as useful biochemical markers for bacterial classification and characterization. The types and relative abundances of fatty acids produced within a cell are largely determined by an organism’s genotype and can be used for identification of different species and strains and for discriminating between free spores and vegetative cells. Commercial systems that streamline fatty acid extraction and detection procedures have facilitated the extensive use of fatty acid profiling to identify bacteria in clinical, agricultural, and biodefense settings.

FAME analysis has been used to characterize microbial communities in aquifer environments and sediments in some studies. Consequently, phospholipid fatty acid analysis is generally preferred for microbiological studies of such environments. In contaminated soils that fatty acids are evaluated to estimate the structure of microbial community and metabolic activity, to identify the contamination of surface water polluted by soil which nearby agricultural fields and wooded riparian zones, to evaluate the application of microorganisms to a particular condition, to find the microbial community distribution in terms of structure to determine the relative changes in abundance of microorganisms like bacteria and fungi. The Bacillus sp. strain C6 is characterized by a high content of ramified (iso- and anteiso-C15:0 and C17:0) fatty acids that compose approximately 85% of the total fatty acid pool. The use of fatty acids as biomarkers to analyze the microbial community in air biofilters and polluted soils has also been proposed. This paper describes the fatty acid composition of alkaliphiles in the presence of various branched-chain organic acids.

**Materials and Methods**

**Micro organisms**

Four bacteria were chosen for this study: Bacillus subtilis SVUNM4, Bacillus licheniformis SVUNM8, Bacillus methylotrophicus SVUNM9, and Paenibacillus dendritiformis SVUNM11 were isolated from the subsurface environments and metamorphic igneous rocks, gudur division. Muscovite samples (1.0 g) were powdered and mixed with 10 ml of saline solution (0.8% NaCl) in a conical flask and were incubated on the shaker for 30 min at room temperature (30°C ± 2°C). The culture flasks were then allowed to stand for 15 min for the sediment to settle before serial dilution. The slightly turbid supernatant (1.0 ml) and water samples (1.0 ml) were serially diluted (10-fold serial dilution) with normal saline. A 0.1 ml of appropriate dilutions was spread onto the plates containing polypeptone-yeast extract-glucose (PPYG) agar (pH 10.5) for selective alkalphilic bacteria isolation.

**Categorization of alkaliphiles based on pH preference**

Purified predominant isolates from PPYG agar (pH 10.5) were inoculated into four sets of PPYG broth tubes of pH 7.0, 9.0, 10.0, 11.0, and 12.0 to obtain obligate alkaliphiles and incubated for 48 h at room temperature and the optical density values were recorded on a spectrophotometer at 630 nm. The isolates showing optimum growth at pH 10–12 were selected and considered as true alkaliphiles.

**Fatty acid methyl ester analysis**

Bacterial isolates were grown on trypticase soy agar at their optimum growth conditions. Whole cell fatty acids were extracted from cell material according to the Musical Instrument Digital Interface (MIDI) protocol. Overnight grown bacterial culture was taken (approximate 40 mg pellet) in a clean screw capped glass tube and 1 ml of Reagent I (45 g NaOH + 150 ml CH₃OH + 150 ml DW) was added to it. The tube was sealed with Teflon-lined screw caps, vortexed briefly, and heated in a boiling water bath for 5 min. The tube was vigorously vortexed for 5–10 s and returned to the water bath (100°C) to complete the 30-min heating (saponification step). The tube was cooled uncapped and 2 ml of Reagent II (325 ml of 6 N HCl + 275 ml CH₃OH) was added. The tube was capped again and briefly vortexed. After vortexing, the tube was heated for 10 min at 80°C (this methylation step is critical with time and temperature). Addition of 1.25 ml of Reagent III (200 ml hexane + 200 ml methyl tetra butyl ether) to the cooled tube was followed by recapping and gentle tumbling on a clinical rotator
for about 10 min. The tube was uncapped again and the aqueous (lower) phase was pipetted out and discarded (extraction step). About 3 ml of Reagent IV (10.8 g NaOH + 900 ml D/W) was added to the organic phase remaining in the tube; after that, the tube was recapped and tumbled for 5 min. Following uncapping, about 2/3 of the organic phase was pipetted into a GC vial which was capped and ready for analysis. Gas chromatographic analysis was performed on a GC Sherlock fatty acid identification system (New York, USA) fitted with cross-linked methyl silicon fused capillary column (25 m, 0.2 mm i.d.), flame ionization detector, and a sampler. Helium was used as carrier gas. The sample was injected at oven temperature of 50°C. After 1 min, the oven temperature was raised to 170°C at the rate of 30°C/min and then to 270°C at the rate of 2°C/min and finally to 300°C at 5°C/min.

**RESULTS**

Isolation of alkaliphiles from muscovite ore was carried out using PPYG medium with a pH of 10.5. Single, discrete colonies were picked up and purified by repeated streaking. A total of four predominant isolates were selected and named as SVUNM4, SVUNM8, SVUNM9, and SVUNM11 and purified by repeated streaking. Their cultural characteristics such as form, elevation, margin, color, and size were determined. The isolates’ size ranges from pinhead to large, without any extracellular or intracellular pigmentation. The colony form of the isolates ranges from circular to rhizoid; the colony margin ranges from entire, filamentous to lobate; the elevation of the isolates ranges from unrex, raised to flat. The color of the colonies ranges from white, yellow, light brown to purple.

The isolate SVUNM4 was in pin head, medium to large, purple, circular with entire margin and unrex elevation. The isolate SVUNM8 was medium to large, yellow, circular, with filamentous margin with raised elevation. The cultural characteristics of the isolate SVUNM9 include small in size, white, irregular, with entire margin and raised elevation. The isolate SVUNM11 exhibited the following structural characteristics. The size of the colonies was large, cream-colored, irregular, with filamentous margin and flat elevation.

**Categorization of alkaliphiles for pH preferences**

Alkaline-adapted microorganisms can be classified into two main groups; they are alkaliphilic and alkali tolerant according to Krulwich. Alkaline tolerant growth at the pH 7.0–9.5, but in capable of survival at pH above 9.5, whereas alkaliphiles show optimal growth at the pH range 10.0–12.0. In our study, the isolates were exposed to pH 7, 9, 10, and 12 to categorize them. Based on pH preference, the three isolates, namely, SVUNM4, SVUNM8, and SVUNM9 were alkaliphilic and one isolate, namely, SVUNM11 was alkali tolerant.

**Analysis of chemical signatures of alkaliphiles using fatty acid methyl esters analysis**

FAMEs were isolated from alkaliphiles *B. subtilis* SVUNM4 and were characterized by GC-mass spectrometry (MS) retention times and equivalent chain length (ECL) values. These isolates produced major fatty acids. The chromatogram analysis showed that they contained 1, 2-benzenedicarboxylic acid butyl 2-methylpropyl ester (42.743%), phthalic acid, isobutyl 2-pentyl ester (21.753%), dibutyl phthalate (35.684%) as major abundant fatty acid [Figure 1]. The abundance of each fatty acid is presented in Table 1. The fatty acid profiles agree with that of other members of species *B. subtilis*.

FAMEs were isolated from *B. licheniformis* SVUNM8 were characterized by GC-MS retention times and ECL values. These isolates produced major fatty acids. The chromatogram analysis showed that it contained cyclotrisiloxane, hexamethyl (50.089%), octamethyl (27.771%), dodecamethyl (15.794%), heptasiloxane 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl (2.607%), 7, 15-dihydroxydehydroabietic acid, methyl ester, di (trimethylsilyl) ether (3.74%) as major abundant fatty acid [Figure 2]. The abundance of each fatty acid is presented in Table 1B. The fatty acid profiles agree with that of other members of species *B. licheniformis*.

FAMEs were isolated from *B. methylotrophicus* SVUNM9 were characterized by GC-MS retention times and ECL values. These isolates produced major fatty acids. The chromatogram analysis showed that it contained hentriacontane (74.593%), 1, 2 benzenedi carboxylic

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**Table 1: Gas chromatography-mass spectrometry spectra of the fatty acids extracted from *Bacillus subtilis* sp.**

| Chemical name | Formula | RT | Abundance (%) | MW | CAS |
|---------------|---------|----|---------------|----|-----|
| 1, 2-benzenedicarboxylic acid, butyl 2-methylpropyl ester | C₁₆H₂₄O₄ | 17.579 | 42.743 | 278 | 17851-53-5 |
| Phthalic acid, isobutyl 2-pentyl ester | C₁₃H₁₆O₄ | 17.744 | 21.753 | 292 | 900315-48-6 |
| Dibutyl phthalate | C₁₈H₂₄O₄ | 18.075 | 35.684 | 278 | 84-74-2 |

RT: Retention time, MW: Molecular weight, CAS: Chemical abstract service
acid, mono (2-ethylhexyl) ester (25.388%) as major abundant fatty acid [Figure 3]. The abundance of each fatty acid is presented in Table 3. The fatty acid profiles agree with that of other members of *B. methylotrophicus*. 
FAMEs were isolated from *P. dendritiformis* SVUNM11 were characterized by GC-MS retention times and ECL values. These isolates produced major fatty acids. The chromatogram analysis showed that it contained 2-thiophenecacetic acid, undec-2-enyl ester (11.477%), hentriacontane, 1,2 benzene dicarboxylic acid, mono (2-ethylhexyl) ester (23.072%) as major abundant fatty acid [Figure 4]. The abundance of each fatty acid is presented in Table 4. The fatty acid profiles agree with that of other members of *P. dendritiformis*.

**DISCUSSION**

The microbial studies pertaining to deep surface often contain unique assets, and they are ideal for several biotechnological and environmental applications. Further, the microbial studies pertaining to muscovite ore are very scarce. The choice of indigenous microbes for this investigation is essentially due to possibility of their better acclimatization to the biobeneficiation environments. Alkaliphilic prokaryotes, in their rich phylogenetic diversity and metabolic versatility, are central participants.

**Table 2: Gas chromatography-mass spectrometry spectra of the fatty acids extracted from *Bacillus licheniformis* sp. SVUNM8**

| Chemical name | Formula | RT | Abundance (%) | MW | CAS |
|---------------|---------|----|---------------|----|-----|
| Cyclotrisiloxane, hexamethyl | C15H15O3Si3 | 3.644 | 50.089 | 222 | 541-05-9 |
| Cyclotetrasiloxane, octamethyl | C18H18O4Si4 | 5.650 | 27.771 | 296 | 556-67-2 |
| Cyclohexasiloxane, dodecamethyl | C21H21O5Si5 | 7.901 | 15.794 | 370 | 541-02-06 |
| Heptasiloxane 1,1,3,3,5,5,7,7,9,9,11,11,13,13-etradeamethyl | C16H16O6Si7 | 17.699 | 2.607 | 504 | 19095-23-9 |
| 7,15-dihydroxydehydroabietic acid, methyl ester, di (trimethylsilyl) ether | C27H46O4Si2 | 19.995 | 3.74 | 490 | 900292-80-2 |

RT: Retention time, MW: Molecular weight, CAS: Chemical abstract service

**Table 3: Gas chromatography-mass spectrometry spectra of the fatty acids extracted from *Bacillus methylotrophicus* sp. SVUNM9**

| Chemical name | Formula | RT | Abundance (%) | MW | CAS |
|---------------|---------|----|---------------|----|-----|
| Hentriacontane | C31H64 | 16.504 | 74.593 | 436 | 630-04-6 |
| 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester | C18H22O4 | 23.073 | 25.388 | 278 | 4376-20-9 |

RT: Retention time, MW: Molecular weight, CAS: Chemical abstract service
A range of pH values are used by different investigation to define extreme alkaliphiles, alkaliphiles, and alkaline tolerant bacteria. Extremely alkaliphilic bacteria are generally defined as those that grow at an external pH ≥10 (the more extreme strains growing at pH ≥12), moderate alkaliphiles as those that can grow in the pH 9–10 range, the alkaline tolerant bacteria as those that can survive and grow suboptimally at ~pH 9.\[28\] It has always been a very interesting and challenging area to explore microbes that reside in extreme environment. Alkaliphiles were isolated using culture-dependent analysis. Microbial communities in subsurface muscovite mine have escaped our attention so far. These mines harbor unique extreme environments for microorganisms, both natural and anthropogenic, including extreme temperature pressure, low oxygen concentration toxic heavy metals oligotrophic conditions, low water availability, and pH. The physical and chemical characterization of the sample also revealed its nature. The chemical signatures of the four alkaliphilic isolates were analyzed using FAME analysis. A total of ten fatty acids were identified in all the four isolates. The predominant fatty acid was 1,2-benzenedicarboxylic acid butyl 2-methylpropyl ester and hentricantane, phthalic acid, isobutyl 2-pentyl ester, dibutyl phthalate. The abundance of each fatty acid is presented in Table 5. Pujari\[31\] reported 12 fatty acids from marine bacteria. Cooney\[32\] reported 13 fatty acids from marine Fungi. Devi\[33\] reported ten fatty acids from marine Fungi. Thompson\[34\] due to the estimation of community diversity by FAME content of bacterial isolates exposed that the genetically modified *Pseudomonas fluorescens* had less impact on the bacterial community than the wild type. Haack\[35\] applied principal components

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**Figure 4:** Gas chromatography-mass spectrometry spectra of the fatty acids esters of *Paenibacillus dentriiformis* sp. SVUNM11

| Chemical name                                                                 | Formula      | RT     | Abundance (%) | MW  | CAS           |
|-------------------------------------------------------------------------------|--------------|--------|----------------|-----|---------------|
| 2-thiopheneacetic acid, undec-2-enyl ester                                    | C_{17}H_{26}O_{2S} | 15.318 | 11.477         | 296 | 900299-39-0   |
| Hentriacontane                                                                | C_{31}H_{64} | 6.469  | 79.762         | 436 | 630-04-6      |
| 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester                      | C_{16}H_{22}O_{4} | 8.660  | 23.072         | 278 | 4376-20-9     |

RT: Retention time, MW: Molecular weight, CAS: Chemical abstract service
Table 5: Fatty acid methyl esters profiles of the SVUNM4, SVUNM8, SVUNM9 and SVUNM11

| RT    | Chemical name                                      | SVUNM4 | SVUNM8 | SVUNM9 | SVUNM11 |
|-------|----------------------------------------------------|--------|--------|--------|---------|
| 17.579| 1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester | 42.743 | -      | 25.388 | 8.660   |
| 17.744| Phthalic acid, isobutyl 2-pentyl ester              | 21.753 | -      | -      | -       |
| 18.075| Dibutyl phthalate                                   | 35.684 | -      | -      | -       |
| 3.644 | Cyclotrisiloxane, hexamethyl                         | -      | 50.089 | -      | -       |
| 5.650 | Cyclotetrasiloxane, octamethyl                       | -      | 27.771 | -      | -       |
| 7.901 | Cyclohexasiloxane, dodecamethyl                      | -      | 15.794 | -      | -       |
| 17.699| Heptasiloxane 1,1,3,3,5,5,7,9,9,11,13,13‑etradecamethyl | -      | 2.607  | -      | -       |
| 19.995| 7,15-dihydroxydehydroabieticacid, methyl ester, di (trimethylsilyl) ether | -     | 3.74   | -      | -       |
| 16.504| Hentriantocane                                      | -      | -      | 74.593 | 79.762  |
| 15.318| 2-thiopheneacetic acid, undec-2-enyl ester           | -      | -      | 11.477 |         |

RT: Retention time

analysis of MIDI-FAMEs profiles in a clear separation of two different communities. They found comparative similarities and differences of microbial communities that differed in taxonomic status. Petersen and Klug[36] observed a major change in the fatty acid profiles in soil at a near-freezing temperature and 25°C, whereas Nazih[37] did not observe changes in FAMEs profiles at 22°C and 30°C. Kozdrój[38] used FAME analysis to assess microbial community structure in technogenic wastes such as coal mine spoil, nonferrous metallurgical slag, and coal fly ash. He noticed a high content of 18:2 ω6,9 in the metallurgical slag, indicating the domination of Fungi in this waste. In difference, representatives of the Cytophaga-Flavobacterium group, for which 16:1 ω5c fatty acid was used as a marker, dominated in the coal fly ash. Bossio and Scow[39] concluded that fatty acid profiles were sensitive indicators of changes occurring in the structure of soil microbial communities due to agricultural management. In previous cases, information obtained from lipid analysis provides insight into the community composition as well. It has been proposed that particular groups of microorganisms contain characteristic fatty acid profiles that can be used as biomarkers.[20] Mummey[40] applied FAME biomarkers to monitor the recovery of ecosystems following surface mine reclamation. In this study, it was found that the percentage of FAME bacterial to fungal biomarkers reflected changes in other indicators of soil health signifying that this ratio is a useful indicator of reclamation progress. Fatty acids from whole cells of Chlorobium are within the range of C12–C18, and the main ones are n-tetradeanoic (C14:0), hexadecanoic (C16:1), and n-hexadecanoic (C16:0).[41-43] Cha et al.[44] identified signature fatty acids of Nocardia amarae (19: 1o8, 16: 1o6c, i15: 0 2OH) and used their relative abundance to reveal their potential to quantitatively monitor the abundance of Nocardia in mixed liquor samples of activated sludge. The use of fatty acid patterns has also been applied to full-scale biological wastewater treatment plants to estimate activated sludge microbial communities, demonstrating that FAME profiles could be a valuable technique in evaluating the alters in bacterial communities when a wastewater treatment system is operated in a particular way.[45]

**Conclusions**

Although advances in the technologies for fatty acid analysis have been reported, the fatty acid analysis of alkaliphiles prokaryotes remains scarce, and it is very important to encourage the investigation of these microorganisms. The chemical signature of the four alkaliphilic isolates was analyzed using FAME analysis. A total of ten fatty acids were identified in all the four isolates. The predominant fatty acid was 1,2-benzenedicarboxylic acid butyl 2-methylpropyl ester and hentriantocane, phthalic acid, isobutyl 2-pentyl ester, dibutyl phthalate. With new sources of genetic materials from high pH bacteria and the application of new technologies – for example, lipidomics – which are more precise, it will be possible to discover new fatty acid structures and to evaluate the sources of these compounds of biotechnological interest and novel chemotaxonomic biomarkers.

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**Conflicts of interest**

There are no conflicts of interest.

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