Research Article

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European Journal of Experimental Biology
ISSN 2248-9215

2016
Vol.6 No.6:5

Fatty Acid Methyl Ester (FAME) Analysis of Moderately Thermophilic Bacteria Isolated from the Coramandal Coast, Chennai, Tamilnadu

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Received date: October 28, 2016; **Accepted date**: December 28, 2016; **Published date**: December 30, 2016

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Citation: Sharmili AS, Ramasamy P. Fatty Acid Methyl Ester (FAME) Analysis of Moderately Thermophilic Bacteria Isolated from the Coramandal Coast, Chennai, Tamilnadu.

Abstract

A total of 18 out of 44 moderately thermophlic bacteria isolated from water samples of the Coramandal Coast, Chennai, Tamilnadu, were analyzed for Fatty Acid Methyl Ester (FAME). The present study showed that the predominant fatty acid was 15:0i followed by 15:0a, which were characteristic of Bacillus. The results suggest that the order of decreasing abundance of terminally branched fatty acids is as follows: iso even-numbered acids namely 16:0i, 14:0i, iso odd-numbered acids, 15:0i, 17:0i, 13:0i, 19:0i and anteiso acids, 15:0a, 17:0a, 11:0a, 19:0a. Straight chain saturated fatty acids identified were 10:0, 12:0, 14:0, 16:0, 18:0. The remaining fatty acids components were present in negligible quantities. The isolates were also identified and classified using the comparison with the TSBA database as B. cereus-GC subgroup A (isolates ASR 29CIII, ASR 37CIII), *B. subtilis* (isolates ASR 6SII, ASR 21SI, ASR 25SV), *B. laevolacticus* (isolates ASR 4LIII, ASR 7SIII, ASR 11LVI, ASR 42SVII), *B. alcalophilus* (isolates ASR 14PI, ASR 18PIV), *B. pumilus* subgroup A (isolate- ASR 41PVIII), *Staphylococcus schleiferi* (isolates- ASR 2LII, ASR 5LIV), *S. gallinarum*-GC subgroup A (isolates ASR 13SIV, ASR 33SVI), *Kurthia sibirica* (isolate ASR 15PII), *Geobacillus stearothermophilus*-GC subgroup (isolate ASR 8LV).

Keywords: Moderately thermophilic bacteria; Bacillus; FAME; Fatty acids; 15:0i; 15:0a; 17:0a

Introduction

Microbial biomarkers are chemical components of microorganisms which can be analyzed in a given sample and interpreted (both quantitatively and qualitatively) in terms of in situ microbial biomass. The most useful biomarkers are membrane lipids and their related fatty acids as they are essential components of every living cell and have great structural diversity and high biological specificity [1]. Two common approaches are analysis of microbial lipids: (i) polar phospholipid fatty acid (PLFA) analysis and (ii) total fatty acid methyl ester (total FAME) [2]. PLFA assay provides information leading to identification and quantification of viable bacterial biomass [3]. Total FAME analysis, on the other hand, includes all saponifiable lipids present in the sample (including PLFAs). Polyunsaturated fatty acids and long chain fatty acids beyond 18 carbons are absent in Prokaryotes. Saturated or monounsaturated fatty acids of length 10 to 18 carbon are present in Eukaroytes [4,5].

Bacterial fatty acids are highly conserved due to their role in cell structure and function and are the major constituents of the lipid bilayer of bacterial membranes and lipopolysaccharides. They have been used extensively for taxonomic and identification purposes. Whole cellular FAME content is a bacterial profile and is a direct and stable expression of the cellular genome. The cellular fatty acid pattern is a phenotypic character that is not affected by mutations, acquisition or loss of plasmids. The use of fatty acid analysis by gas chromatography for the identification of bacteria is rapid, efficient, reproducible and used for the identification of both clinical and environmental isolates [6-10]. Fatty acids are mainly located in the cytoplasmic membrane as constituents of phospholipids and as lipopolysaccharides in the outer membrane of Gram-negative bacteria as well as lipoteichoic acids in Gram-positive bacteria. The importance of FAME analysis for the identification of bacteria is based on the large structural differences within these molecules viz., (i) variation in length (8 to 20 C-atoms), (ii) presence of saturated and monounsaturated fatty acids, (iii) occurrence of branched fatty acids (iso and anteiso fatty acids or methylated within the molecule), (iv) occurrence of cyclopropane fatty acids (17:0c, 19:0c), (v) occurrence of hydroxy-fatty acids with an OH-group at position two or three of the molecule. For classification or identification of bacteria the presence of distinct fatty acids and their relative amount is analyzed and compared with the fatty acid profiles of reference strains. The characteristic feature of a phylogenetic group of bacteria can be the dominating presence of a single fatty acid or a specific fatty acid pattern. As the fatty acid composition of bacteria is dependent on the growth phase, temperature and growth medium, standardization of these conditions is important. FAME assay is a powerful tool in the study of

bacterial phylogeny due to the quantity and specificity of the fatty acids [5].

FAME assay involved derivatization of cellular lipids into their corresponding fatty acid methyl esters, resolved and identified by gas chromatography [5]. The quantification of these lipid derivatives allows distinctions between and within prokaryotic genera to be elucidated. To obtain reproducible results with FAME analysis, growth conditions must be standardized and there must be a library of known bacterial FAME profiles with which to compare profiles from unknown microorganisms. Gas-liquid chromatography is used to identify fatty acids on the basis of their retention characteristics. The Sherlock Microbial Identification System employed by MIDI Labs Inc. (Newark, DE) uses a database containing over 100,000 such profiles [5]. Bacterial identifications are generated for each sample and ranked by similarity indices (SI). The similarity index (ranging from 0 to 1) is defined as the closeness of a match of the unknown bacterium to a library entry. Since identification is based on comparisons with a profile library; sample identification is not definitive. A similarity index of 0.6 is defined as an excellent match [11].

Fatty Acid Methyl Ester (FAME) Analysis

Fatty acid methyl ester (FAME) analysis of the bacterial isolates was done to identify the bacterial strain and analyse the fatty acid profile of the cell wall [12-14]. The microbial identification systems (MIS) rely on quantitative and qualitative analysis of the fatty acid of organisms. Fatty acids were identified based on their peak retention times and their relative peaks areas were determined using the MIS aerobe chromatographic program and peak naming table as supplied by MIDI. The FAME profile generated for each unknown bacteria analyzed was electronically compared to a computer-generated library containing the fatty acid profile of over 5,000 bacteria. Bacterial identification are generated for each sample and ranked by similarity indices. In the present study FAME analysis of 18 of the 44 moderately thermophilc bacteria isolated from the Coramandal Coast, Chennai, Tamilnadu was studied to identify the bacterial isolates and analyze total fatty acid methyl ester.

Materials and Method

Water samples were collected from the Adyar River, Adyar Estuary, Marina beach, the Buckingham Canal, Kovalam Estuary and Kovalam beach along the Coramandal Coastal regions, Chennai, Tamil Nadu, India. As no specific selective media are available for the isolation and cultivation of thermophiles, water samples were serially diluted and was plated on Nutrient agar medium (Hi-media Mumbai, India) and incubated at 37°C overnight. Following incubation, various bacterial isolates were picked based on morphological appearance. The selected cultures were inoculated in Nutrient broth and incubated at temperatures ranging from 20°C to 80°C for a maximum of 48 hours. Bacterial isolates, which had growth above 50°C to 55°C, were termed “moderately thermophilic bacteria”. FAME analysis was carried out as per method described by MIDI (Newark, De, USA) on Agilent 6980N network GC system for identification of the 18 moderately thermophilic bacterial isolates. Fatty acids were identified based on their peak retention times and their relative peaks areas were determined using the MIS aerobe chromatographic program and peak naming table as supplied by MIDI. The FAME profile generated for each unknown bacteria analyzed was electronically compared to a computer-generated library containing the fatty acid profile of over 5,000 bacteria. Bacterial identification are generated for each sample and ranked by similarity indices. The similarity index (SI) shows how closely the unknown bacterial sample compares to the FAME profiles of the known bacteria in the library collection.

Preparation of reagents for FAME

Saponification Reagent: Sodium hydroxide (45 g) (certified ACS) and Methanol (150 ml) (HPLC grade) were added to deionized distilled water (150 ml) and the solution was stirred well until the pellets were dissolved.

Methylation Reagent: 6.00 N Hydrochloric Acid (325 ml) and Methanol (275 ml) (HPLC grade) were mixed and the solution was stirred well.

Extraction Solvent: Hexane (200) (HPLC Grade) and Methyl tert-butyl ether (MTBE) (200 ml) (HPLC Grade) were mixed and the solution was stirred well.

Base Wash: Sodium hydroxide (10.8 g) (certified ACS) was dissolved in 900 ml of deionized distilled water.

Saturated NaCl: NaCl (40 g) was dissolved in 100 ml of deionized distilled water. Reagents were prepared freshly and stored at room temperature in bottles supplied with Teflon-lined caps. The purity of reagents was confirmed by preparing a reagent control blank (procedure without cells) with every batch of samples. All the bacterial cultures were grown in tryptic soy agar medium (Hi-Media, Mumbai) and their fatty acid was extracted as given below.

Harvesting: The quadrant streak method is designed to dilute the inoculum so that quadrant four will contain well-isolated colonies to serve as a check for purity. Bacterial colonies were harvested from the diluted quadrant exhibiting confluent growth (late log phase) along the streaking axis. This area of harvesting typically yields the most stable fatty acid compositions since the inoculum has been diluted enough to result in abundant growth of colonies without a limiting nutrient supply.

The cultured cells were removed from the plate by gently scraping the surface of the culture with a sterile 4 mm inoculating loop (approx. 40 mg of live wet cells). The loop with the cells was inserted into a clean, dry lower inner surface of the 13 mm × 100 mm screw cap culture tube.

Saponification: 1.0 ml of Reagent (a) the saponification reagent was pipetted into each of the culture tubes in the batch. Each tube was tightly sealed with a clean Teflon-lined screw cap. The tubes were vortexed for 5-10 sec. The racks of the batched sample tubes were placed into a boiling water bath at 95°C-100°C. After 5 min. the tubes were removed from the water bath and cooled slightly. Each tube was vortexed for 5-10 sec. The tubes were again placed in the water bath for an

additional 25 min. After a total of 30 min of saponification in the water bath, the rack of tubes were removed and set in a tray of cold tap water to cool.

Methylation: 2.0 ml of Reagent (b) the methylation reagent was added in each tube. The tubes were tightly capped and the solutions were vortexed for 5-10 sec. The tubes were heated at 80°C ± 1°C in a water bath for 10 ± 1 min. The tubes were removed and quickly cooled to room temperature by placing tubes in a tray of cold tap water.

Extraction: 1.25 ml of Reagent (c) the extraction solvent was added to each tube. The tubes were tightly sealed. The batch of tubes was placed in a laboratory rotator and gently mixed end-over-end for 10 min. The aqueous (lower) phase was removed and discarded.

Base Wash: 3.0 ml of Reagent (d) the base wash was added to each tube. The tubes were tightly capped and gently rotated end-over-end for 5 min. Brief centrifugation (3 min at 2000 rpm) was done to clarify the interface between the phases when an emulsion was present. About 2/3 of the organic (upper) phase from the tube was transferred to a clean GC sample vial for gas chromatographic (GC) analysis with MIDI Sherlock Version 4.5 (0209B).

RESULTS

Fatty Acid Composition of the Bacterial Isolates

The results of FAME analysis showed that from the 18 moderately thermophilic bacterial isolates, thirty-two different fatty acids with 10 to 18 carbon atoms were detected. Standard nomenclature is used to describe FAMEs detected by MIDI extraction procedure. Numbering the carbons begins at the aliphatic (w) end of the fatty acids molecule. The number of double bonds within the molecule is given after the colon. Cis and trans confirmations are designed with the suffixes “c” and “t”, respectively. Other notations are “Me” for a methyl group, “OH” for hydroxy, “cy” for cyclopropane groups, and the prefixes “i” and “a” for iso- and anteiso-branched FAMEs respectively.

All the 18 moderately thermophilic bacterial isolates produced 15:0i branched chain fatty acid as the major component of the total fatty acids (100%) and among the twelve terminally branched fatty acids, the anteiso fatty acids (15:0a, 17:0a) were found in most of the isolates **(Table 1)**. The present study showed that the predominant fatty acid was 15:0i and the second most abundant fatty acid were 15:0a, which is characteristic of Bacillus. The results suggest that the order of decreasing abundance of terminally branched fatty acids is as follows: iso even-numbered acids namely 16:0i, 14:0i, iso odd-numbered acids, 13:0i, 19:0i and anteiso acids, 19:0a. Straight chain saturated 10:0, 12:0, 14:0, 16:0, 18:0 were identified. The GC data of the bacterial isolates showed that the order of abundance of the identified components of the fatty acids vary with each isolates.

The moderately thermophilic bacterial isolates, ASR 13SIV alone was found to produce 10:0 and 18:0 saturated fatty acid. 12:0 was present in ASR 6SII, ASR 13SIV, ASR 14PI, ASR 15PII, ASR 29CIII, ASR 37CIII. Similarly, 14:0 was present only in ASR 29CIII, ASR 33SVI, and ASR 37CIII. The moderately thermophilic bacterial isolate ASR 29CIII, ASR 37CIII alone produced 13:0i whereas 14:0i was present only in 33SVI. The anteiso fatty acid component 11:0a was present in ASR 6SII, while 19:0a in ASR 21SI. The methyl monounsaturated fatty acid components, 11 methyl 18:1 w7c (ASR 18PIV and ASR 29CIII), 16:0 10 methyl (ASR 8LV), 17:0 10 methyl (ASR 13SIV, ASR 14PI, ASR 15PII, ASR 25SV, ASR 29CIII, ASR 33SVI, ASR 42SVII) were present in less abundance. The moderately thermophilic bacterial isolate ASR 33SVI was found to produce 16:1 w7c whereas ASR 11LVI produced 17:1 w7c. The component 17:1 w8c was produced only by ASR 6SII, ASR 13SIV, ASR 14PI, ASR 29CIII. 15:0 2 OH hydroxyl fatty acid was produced only in ASR 8LV while 15:0 3 OH was produced by ASR 4LIII, ASR7SIII, ASR 13SIV, ASR 25SV, ASR 33SVI. The fatty acid anteiso components, 17:1 w5c, 17:1 w9c were found only in ASR 33SVI and ASR 8LV respectively. ASR 8LV produced fatty acid component 18:3 w6c while ASR 18PIV was found to produce unique fatty acid component 17-cyclo. 19:0i fatty acid component was found unique in the moderately thermophilic bacterial isolate ASR 8LV and absent in all the other isolates. This is a characteristic fatty acid in Geobacillus sp. The present study has shown that the fatty acid pairs of the moderately thermophilic bacteria contained less of the lower melting point pair (15:0a, 17:0a) and more of the higher melting point pair (15:0i, 17:0i).

Thermophilic bacterial isolate ASR 29CIII had a similarity index (SI) of 0.057, ASR 37CIII had a SI of 0.023, were identified as *B. cereus*-GC subgroup A by comparing with the TSBA database. ASR 6SII (SI 0.133), ASR 21SI (SI 0.225), ASR 25SV (SI 0.142) were identified as *B. subtilis*. ASR 4LIII (SI 0.127), ASR 7SIII (SI 0.072), ASR 11LVI (SI 0.107), ASR 42SVII (SI 0.227) were identified as *B. laevolacticus*. Thermophilic bacterial isolate ASR 14PI (SI 0.012), ASR 18PIV (SI 0.010) was identified as *B. alcalophilus*. ASR 41PVIII with a SI of 0.533 was identified as *B. pumilus* subgroup A. Isolates ASR 2LII (SI 0.10), ASR 5LIV (SI 0.437) was identified as Staphylococcus schleiferi. ASR 13SIV (SI 0.079), ASR 33SVI (SI 0.062) were identified as *S. gallinarum*-GC subgroup A. ASR 15PII (SI 0.296) and ASR 8LV (SI 0.011) was identified as *Kurthia sibirica* and *Geobacillus stearothermophilus*-GC subgroup respectively.

Table 1 Percentage of fatty acid composition in the moderately thermophilic bacterial isolates.

	Bacterial isolates																	
Fatty acids (%)	ASR 2LII	ASR 4LIII	ASR 5LIV	ASR 6SII	ASR 7SIII	ASR 8LV	ASR 11	ASR 13	ASR 14PI	ASR 15PII	ASR 18	ASR	ASR 25SV	ASR 29	ASR 33	ASR 37CII	ASR 41PVIII	ASR 42SVII

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							LVI	SIV			PIV	21SI		CIII	SVI			
Straight chain saturated																		
10:0								2.29										
12:0				3.35				1.92	6.44	1.60				3.31		5.16		
14:0														1.97	1.34	7.66		
16:0	2.66	2.22	4.55		3.56	5.81	3.90	5.10	5.94			8.92		2.98	3.25	8.61	2.23	
18:0								2.46										
Terminally branched																		
13:0 iso														15.77		8.92		
14:0 iso															1.04			
15:0 iso	39.68	22.28	37.16	22.59	26.11	5.52	19.99	18.39	21.74	45.16	37.18	29.40	22.18	29.02	15.61	25.58	51.94	27.17
16:0 iso		3.22	2.83		3.73		9.36	4.23		3.32			5.39		3.63		1.87	5.35
17:0 iso	10.14	10.42	13.57	13.05		12.66	9.36	9.8		4.28		9.98	8.35	13.58	10.87	13.94		6.12
19:0 iso		3.94																
11:0 anteiso						9.22												
15:0 anteiso	21.50	28.68	29.13	35.02	24.49	20.1	27.59	25.34	27.54	22.55	22.84	33.65	33.38	4.11	24.19		30.00	37.81
17:0 anteiso	9.80	8.99	12.77	10.62	8.42	10.51	9.75	7.43	6.70	4.61		6.63	11.22		7.46		4.45	8.77
19:0 anteiso												6.59						
Monounsaturated																		
14:1 iso E		4.83			3.55						6.39		3.29	6.03	2.33			
16:1iso G							3.52							4.04	3.74			
18:1 iso H	2.54				2.64	3.74	4.35	2.88	9.96	2.39	3.84	4.85			1.67			
11:0 iso 3 OH															0.89			
15:0 2 OH						2.28												
15:0 3 OH		3.26			10.32	-		7.06					5.78		5.25			
16:0 N alcohol	9.01	3.46			7.70	3.57	2.11	2.53			13.16							
16:0 10 Methyl						1.89												

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17:0 10 Methyl								4.15	9.56	5.09			6.55	3.02	5.69			4.77
11 Methyl 18:1 w7c											10.27				1.64			
16:1w7c																6.40		
17:1 w7c							5.83											
17:1 w8c				6.17					3.30	2.44					2.49			
17:1 w5c Anteiso															2.24			
17:1 w9c Anteiso						9.00												
18:3 w6c (6,9,12)						7.22												
17:0 cyclo											3.26							

DISCUSSION

The importance of fatty acids in chemotaxonomy of the genus Bacillus was reviewed by few researchres [15,16]. Kaneda has documented the fatty acid composition of 19 Bacillus spp. and enabled classification of these microorganisms into six groups (Kaneda Groups A-F) [15].

Group A includes *B. licheniformis*, *B. alveli*, *B. brevis*, *B. circulans*, *B. macerans*, *B. megaterium*, *B. pumilus* and *B. subtilis*. The terminally branched saturates 15:0i, 15:0a, 16:0i, 17:0i and 17:0a are usually found in Gram-positive bacteria or anaerobic microbes [17]. In the current study it was found that terminally branched iso and anteiso saturated fatty acids were the predominant fatty acids among the moderately thermophilic bacterial isolates and these findings support the observations of Kaneda who reported that iso and anteiso saturated fatty acids are the major fatty acid components of lipids (60% to 90% of the total fatty acids) in the genus Bacillus [15]. The current study has shown that unsaturated fatty acids were present in small amounts (1.64%-13.33%) in only some of the isolates, and these observations also concur with the findings of [18]. In the current study, it was found that 15:0i occurred most abundantly (23.6%-68.83%) in the moderately thermophilic *B. cereus* isolates ASR 29CIII and ASR 37CIII when compared with the other isolates, while 15:0a was most abundant (11.59%-41.50%) among the bacterial isolates *B. subtilis* viz., ASR 6SII, ASR 21SI and *B. licheniformis* strains viz., ASR 2LII, ASR 5LIV, ASR 8LV, ASR 11LVI. The usefulness of 15:0a and 15:0i in the identification of the moderately thermophilic bacterial isolates of Bacillus spp. has thus been amply demonstrated in the current study. A similar observation was reported by Kaneda, who stated that 15:0a is the most abundant fatty acid present in the *B. subtilis* group whereas 15:0i is most abundant in the *B. cereus* group though these two groups are taxonomically closely related [19]. The predominant occurrence of terminally methyl branched iso and anteiso fatty acids having 12 to 17 carbons has been shown to be a characteristic feature of all species of Bacillus [20,21]. In contrast, in the current study it has been shown that terminally methyl branched iso and anteiso fatty acids with 12 to 17 carbons are present in negligible amounts in only three of the moderately thermophilic bacterial isolates viz., ASR 8LV, ASR 13SIV and ASR 18PIV while they were absent in the rest of the isolates. Fatty acids such as Myristic and Palmitic were also found only in minor amounts (>10%) in the moderately thermophilic bacterial isolates, and these observations concur with the finding of [15]. According to Shen et al. the fatty acids of chain lengths of 16 or 17 carbon atoms accounted for 80% to 90% of the total fatty acids in thermophilic Bacillus species [22]. By contrast, in the present study it was found that smaller amounts of 16:0 and 17:0 fatty acids occurred in all the moderately thermophilc bacterial isolates. Presence of the unique fatty acid 19:0 is characteristic of Geobacillus species along with the other saturated fatty acids in the study is in accordance with the reports of few authors [23-28]. Thus, although these bacterial isolates grow at elevated temperatures of 50-55°C they differed in their fatty acid profiles from the thermophiles described by [19]. In the present study the fatty acids were extracted from the moderately thermophilic bacterial isolates at 28°C according to the FAME extraction protocol and this may be a reason for the detection of smaller amounts of C16 and C17 fatty acids than expected. Alternatively, the fatty acids present could represent an adaptive feature for the survival of the bacteria at 50-55°C. The results of the current study corroborate the finding of many researchers that the pairs 15:0i, 17:0i and 15:0a, 17:0a (branched fatty acids) are the most abundant fatty acids in the genus Bacillus [5,15,22,23]. Considering the fatty acid pairs, the bacterial isolates from the present study contained less of the lower melting point pair (15:0a, 17:0a) and more of the higher melting point pair (15:0i, 17:0i) which is again consistent with the reports of Shen et al. and supports the theory that thermophiles contain lipids of higher melting point [22]. Similar to the present study, FAME analysis was used by Syed, for the identification of clinical isolated of Pseudomonas species and by Vijaya and Raashi, for the identification of *B. licheniformis* [29,30]. Higher percentage

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of branched fatty acids in Gram positive organisms has been reported by Morey [31].

Conclusion

The results of FAME analysis showed that of the 18 moderately thermophilic bacterial isolates, thirty-nine different fatty acids of length 10 to 20 carbon atoms were detected. Analysis of Fatty Acid Methyl Ester (FAME) of 18 bacteria showed that the predominant fatty acid was 15:0i followed by 15:0a, which are characteristic of Bacillus. The bacterial isolates contained less of the lower melting point pair (15:0a, 17:0a) and more of the higher melting point pair (15:0i, 17:0i). The terminally branched fatty acids were in abundance when compared to the straight chain saturated and monosaturated fatty acids. Based on the FAME profile, the isolates were identified by comparison with TSBA database as *B. cereus*-GC subgroup A, *B. subtilis*, *B. laevolacticus*, *B. alcalophilus*, *B. pumilus* subgroup A, *Staphylococcus schleiferi*, *S. gallinarum*-GC subgroup A, *Kurthia sibirica*, *Geobacillus stearothermophilus*-GC subgroup.

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