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Isolation, Characterization, Identification, Cataloguing and Fatty Acid Methyl Ester Analysis (FAME) of Some Bacteria from Selected Wetlands of North Bihar using the MIDI Sherlock Microbial Identification System

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A B S T R A C T

FAME analysis is used as a first line bacterial species identification method. Currently, this analysis is done by commercial systems such as Sherlock Microbial Identification (MIS, Microbial ID, Inc (MIDI), Newark, Delaware, U.S.A. However, the identification libraries of this system are not adaptable to the very rapidly changing and expanding bacterial taxonomy. This problem can be solved by the application of machine learning techniques, which are mathematical models that can learn from experience. Training these models with up to date information can very rapidly be achieved, and are, therefore very suitable to solve the present identification problem. Objective: The present paper deals with the detailed study on FAME analysis of four strains of bacterial genera Bacillus with MTCC no.9479, 9523, 9524 & 9526 and one actinomycetes of *Nocardiopsis* with MTCC no. 9525 from some selected wetlands of North Bihar. It also emphasizes on the detailed study of Similarity Index (SI) which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create the library entry listed as its match. Methods: The fatty acids were extracted by a procedure which consists of saponification in diliute sodium hydroxide/methanol solution followed by derivatization with dilute hydrochloric acid/methanol solution to give the respective methyl esters (FAMEs). The FAMEs were then extracted from the aqueous phase by the use of an organic solvent and the resulting extract was analyzed by GLC. Results: Strain SM8 showed a S.I. value of 0.021. Strain SM10 showed a S.I. value of 0.058 and 0.048. Strain SM2A showed a S.I. value of 0.413 and 0.253 & also 0.120 and 0.112 for different entry names in the library. Strain SM18A showed a S.I. value of 0.491 and 0.445. Strain BC7A showed a S.I. value of 0.376, 0.369 and 0.308. Conclusions: After the extensive study on the morphological, phyico-chemical and biochemical aspects of microbes isolated from selected wetlands of North Bihar, 5 test isolates were selected for FAME analysis. It was concluded that strain SM18A can be considered as good library comparisons. As compared to SM18A, rest three starins *viz* SM8, SM10 and SM2A could not be considered as good library comparisons. Strain BC7A could be considered as a good match, but an atypical strain.

Introduction

A total of 5 out of 30 moderately thermophlic bacteria isolated from selected wetlands of North Bihar and were analyzed for Fatty Acid Methyl Ester (FAME).The present investigation was carried out with the objective to isolate, characterize, identify, catalogue and perform fatty acid methyl ester analysis of some bacteria from selected wetlands of North Bihar using the MIDI Sherlock microbial identification system (MIDI, 2001; Kim *et al.,* 2000).

Lipid analysis has been used in Both for ecological as well as taxonomic studies lipid analysis is used (Minnikin and Goodfellow, 1981)**.** Lipids commonly examined include fatty acids and phospholipids. Differentially, lipids make up a large proportion of the cellular membrane and, therefore are compounds of fundamental importance to all cells. This allows direct comparisons between highly diverse taxa, in many cases, using a single method. While all fatty acids have essentially the same chemical nature, they are an extremely diverse group of compounds. These properties of occurrence and diversity have made fatty acid analysis an important factor in the chemotaxonomic examination of bacterial populations (Amann *et al.,* 1995). Quite a number of papers have been published retaled to the role of lipids in bacterial taxonomy. More recently lipid analysis has been applied to microbial ecological problems. With the use of high resolution gas-liquid chromatography, investigators have used fatty acid analysis to study diverse microbial community structure which potentially can be extended for the study of different habitats of the environment (Keller and Zengler, 2004; Ratledge and Wilkinson, 1998).

For the identification of anaerobic bacteria, since last so many years analysis of short fatty acid chains (volatile fatty acids, VFAs) has been routinely used (Erwin and Bloch, 1964; Haack *et al.,* 1994). In many of the scientific papers, the fatty acids between 9 and 20 carbons in length have also been used to characterize genera and species of bacteria, especially non-fermentative Gram-negative organisms. With the advent of fused silica capillary columns, it has become practical to use gas chromatography (Sasser, 2001) of whole cell fatty acid methyl esters to identify a wide range of organisms in DNA based technology for the identification of bacteria usually uses only the 16S r RNA gene as the basis for identification (Smibert and Krieg, 1994). Although the 16R r RNA technology, requires high level of technical proficiency, and costs per sample, as well as equipments costs are high (Chèneby *et al.,* 2000; Heyrman and Swings, 2001; Magnusson *et al.,* 1998). The technology is not well suited for routine microbial quality contro, but rather is suited for direct product failures. The 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 is due to the microbial biomarkers.

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. The most common approaches are analysis of microbial lipids: (i) polar phospholipid fatty acid (PLFA) analysis and (ii) total fatty acid methyl ester (total FAME). PLFA assay provides information regarding the identification and quantification of viable bacterial biomass. 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. A powerful tool in the study of bacterial phylogeny due to the quantity and specificity of the fatty acid is the FAME assay.

Fatty acid analysis has been performed on numerous types of organisms, most notably bacteria. In 1988, Myron Sasser developed a method that labeled whole-cell fatty acids of bacterial cells that could then be analyzed by an automated gas

chromatograph (GC) (Kunitsky *et al.,* 2005). This process hydrolyzes fatty acids from phospholipids, triacylglycerols, sterols, and various other lipid structures and then adds a methyl group to the carboxyl group of fatty acids which forms a methyl ester that acts as a label for the GC, which then reads and identifies those fatty acids. The system was termed FAME analysis since it utilizes fatty acid methyl esters.

Identification of fatty acids with this system is accomplished with measurements of retention time, which is the time for a specific fatty acid to pass through the GC column (Sherlock® Analysis Software, MIDI Systems, Inc.). The analysis software of the GC contains a library of retention times that it matches to the retention time of a fatty acid from an unknown sample.

The percentage of each fatty acid is calculated by the proportion of response (measured in the electrical response mV) produced when it passes though the detector at the end of the column over the total response of the sample. The basic structure of a fatty acid is a carbon skeleton usually containing at least 12 carbon atoms with a carboxyl group (-COOH) at one end and a methyl group (-CH3) at the other.

To identify the various fatty acids, a numbering scheme is used that starts at the carboxyl carbon – the number one or α (alpha) carbon – and ending at the terminal, or ω (omega), carbon; the carboxyl carbon is labeled C-1. The number of carbon atoms (x) are given, followed by a colon (:) and the number of double bonds in that molecule (y).

The double bond and any other modifications to the carbon backbone - methyl groups (-CH3), hydroxyl groups (-OH) – are indicated by the number of carbon atoms from the carboxyl end (z). For example, a fatty acid named 18:1 ω5c (x:y z) would contain 18 carbon atoms and a single double bond, the double bond located between the 13C-14C bond. Since the double bond begins at 13C in the 18:1 ω5c example, there are five carbon atoms from 13C to the ω carbon. Therefore, the ω5c notation indicates that the double bond is located five carbon atoms from the ω carbon.

In addition to the original purpose of this system to study bacterial populations, (Sasser, 1990) this system has been adapted for studying fungi also (Graham *et al.,* 1995).

The MIDI Sherlock System (Kunitsky *et al.,* 2005) identifies all the aerobic bacteria (Pendergrass, 1998) in its library using a standard sample preparation technique, so there is no need for upfront biochemical tests or a Gram stain to help decide which card or test strip to use. *Bacillus***,** (2) *Pseudomonas,* Gram-positive cocci and rods (such as coryneforms), Gram-negative non-fermenters (such as *Acinetobacter),* and unusual environmental organisms found in pharmaceutical facilities are some of the species that are discriminated well using FAME analysis. Fatty acids found in bacteria:

More than 350 fatty acids and related compounds have been found in bacteria analyzed in the MIDI Research and Development Laboratory. In this paper, all compounds will be referred to as fatty acids, even though the actual compounds may be aldehydes, hydrocarbons, or dimethyl acetals, and are typically analyzed as the methyl esters.

The presence of 10:0 3OH, 12:0 3OH, and/or 14:0 3OH fatty acids indicates that the organism is Gram-negative and conversely, the absence of the LPS and hydroxy fatty acids indicates that the organism is Gram-positive. Therefore, it is not necessary to perform the traditional Gram stain prior to FAME analysis.

The fatty acid composition is highly conserved genetically and that significant changes take place only over considerable periods of time. Thus, the same genus and species of bacteria anywhere in the world will have highly similar fatty acid profiles if the ecological niche is similar. The largest genus entry in the Sherlock libraries belongs to the genus *Bacillus* (42 unique species) (Kaneda, 1977).

Materials and Methods

Experimental Arrangement

Study area: In the present investigation, isolates of bacteria including a few actinomycetes were isolated by serial dilution method and plating on Nutrient Agar media which were then characterized morphologically, physico-chemically & biochemically from two selected wetland sites of North Bihar mainly. These were,

(1) *Sikandarpur Maun (Wetland), Dist-Muzaffarpur, North Bihar*

(2) *Jubba Sahni Bird Sanctuary (Barela Chaur), Dist-Vaishali, North Bihar*

Method of sample collection: Soil and water samples were collected in sterile polythene bags and dropping bottles during pre-monsoon and post monsoon from these two sampling sites. These were quickly brought to laboratory and kept in a refrigerator.

Isolation of microorganisms: Nutrient Agar (NA) media was used for isolation of microorganisms (bacteria and actinomycetes) as it is the most commonly used media for routine cultivation of bacteria and actinomycetes. Method used for isolation and enumeration of microorganisms (bacteria and actinomycetes) on these selective as well as non-selective medias was serial dilution method following incubation of plates at 37º±2ºC. (Joseph *et al.,* 2003; Kaeberlein *et al.,* 2002; Stevenson *et al.,* 2004; Tyson and Banfield, 2005).

Composition of media

For isolation of bacteria and actinomycetes 1. Nutrient Agar media; Peptone-5gm, Beef extract-3gm, NaCl-5gm, Agar-20gm, Distil water-1 litre, pH-7.0 was taken.

For biochemical characterization of isolates following medias were used for characterizing the isolates biochemically.

(1) Pikovaskay'smedia: Glucose-10gm, Tricalcium phosphate-5gm, Diammonium sulphate-0.5gm, Sodium chloride-0.2gm, Magnesium sulphate-0.1gm, Manganous sulphate-0.1gm, Potassium chloride-0.2gm, Yeast extract-0.5gm, Ferrous sulphate-0.002gm, Agar-15gm, Distil water-1-litre, pH-7.0. (2) Starch Agar Medium: Soluble starch-20gm, Peptone-5gm, Beef extract-3gm, Agar-15gm, Distil water-1 litre, pH-7.0 **(**3)Czapek Mineral Salt Agar Media (Cellulase Producing media): Sodium nitrate-2gm, Dipotassium hydrogen phosphate-1gm, Magnesium sulphate-0.5gm, Potassium chloride-0.5gm, Peptone-2gm, Carboxy methyl cellulose-5gm, Agar-20gm, Distil water-1litre, pH-6.5 (4) Skimmed milk agar media (Casein hydrolyzing media): Skimmed milk agar p owder-50gm, Distil water-1litre, pH-7.2 (5) Gelatin medium (Nutrient gelatin broth): Peptone-5gm, Beef extract-3gm, Gelatin-20gm, Distil water-1litre, pH-6.8 (6) Indole Broth: Beef extract-10gm, Peptone-10gm, Distil water-1 litre, pH-7.4,Kovac's reagent: p-dimethylaminobenzaldehyde-5gm, Iso amyl alcohol-75ml, Conc HCl- 25ml. (7) Methyl Red-Voges Proskauer Broth -Peptone-7gm, Dextrose-5gm, Di-potassium hydrogen phosphate-5gm, Distil water-1 litre, pH-6.9, MR Reagent-0.425gm, Distil water-1litre, VP Reagent 1-0.425gm, Distil water-1litre, VP Reagent 2-40%KOH.8)Simmon's citrate agar media: Ammonium dihydrogen phosphate-1gm, Dipotassium hydrogen phosphate-1gm, Sodium chloride-5gm, Sodium citrate-2gm, Magnesium sulphate-0.2gm, Bromothymol blue-0.08gm, Agar-15gm, Distil water-1 litre, pH-6.8 to 6.9 (9) Sulphide Indole Motility (SIM) Agar media: Peptone-30gm, Beef extract-3gm, Ferrous ammonium sulphate-0.2gm, Sodium thiosulphate-0.025gm, Agar-3gm, Distil water-1 litre, pH-7.3 (10) Nitrate broth: Peptone-5gm, Beef extract-3gm, Potassium nitrate-5gm, Agar-0.1%, Distil water-1 litre, pH-7.2 (11) Catalase test reagent-by using 3% H_2O_2. (12) Trypticase soy agar media (for oxidase test): Trypticase-15gm, phytone-5gm, NaCl-5gm, Agar-15gm, Distil water-1litre, pH-7.3 (13) Urea agar media: Peptone-1gm, NaCl-5gm, Potassium monohydrogen (or dihydrogen) phosphate-2gm, Glucose-1gm, Phenolred (0.02%)6gm, Urea

(20%aqueous solution)-100ml, Distil water-1litre, pH-6.814) Media for acid production: Di-potassium sulphate-2gm, Di-potassium hydrogen phosphate-0.25gm, Magnesium sulphate-0.25gm, Potassium chloride-0.1gm, Yeast extract-0.1gm, Bromocrescol purple-4 to 8 drops of 0.8%aqueous solution for 1 1litre, distil water-1litre, pH-6.8

Results and Discussion

The morphological, physico-chemical and biochemical characterization of the selected 5 test isolates were confirmed by IMTECH, Chandigarh and the results were as follows. These are mentioned in Table 3, Table 4 and Table 5.

Finally, the 5 test isolates viz SM8, SM10, SM2A, SM18A and BC7A were identified, and catalogued from IMTECH, Chandigarh and an MTCC accession no. (Microbial Type Culture Collection) was allocated to these isolates.

FAME Analysis results

FAME analysis of these isolates was done using C:\HPCHEM\1\METHODS\$MIDISA.M method at IMTECH, Chandigarh.

Following results were obtained after performing the FAME test of the five selected isolates and the matches were made from the library TSBA50 5.00 and CLIN50 5.00.

1. Strain SM8 (*Nocardiopsis flava*) viz MTCC no.9525, showed S.I.value of 0.021 for the entry name *Lechevalieria-flava (Saccharothrix, Nocardiopsis flava)* by matching with TSBA50 5.00 library.
2. Strain SM10 (*Bacillus species)* viz MTCC no. 9524, showed S.I. value of 0.058 for the entry name *Stenotrophomonas- maltophilia (Xanthomonas, Pseudomonas)* and 0.048 for the entry name *Staphylococcus-intermedius* by matching with TSBA50 5.00 library.
3. Strain SM2A (*Virgibacillus pantothentcus)* viz MTCC no.9479, showed S.I. value of 0.413 for the entry name *Virgibacillus-pantothenticus* (Bacillus) and 0.253 for the entry name *Bacillus-oleronius* by matching with TSBA50 5.00 library. Also, it was 0.120 for the entry name *Bacillus-coagulans*-GC subgroup C and 0.112 for the entry name *Dermabcter-hominis* by matching with CLIN50 5.00 library.
4. Strain SM18A (*Lysinibacillus sphaericus)* viz MTCC no. 9523, showed S.I. value of 0.491 for the entry name *Bacillus-spahericus*-GC subgroup E and 0.445 for the entry name *Bacillus-spahericus*-GC subgroup F by matching with TSBA50 5.00 library.
5. Strain BC7A (*Lysinibacillus sphaericus)* viz MTCC no. 9526, showed S.I. value of 0.376 for the entry name *Bacillus-spahericus*-GC subgroup E, S.I.value of 0.369 for the entry name *Bacillus-spahericus*-GC subgroup C and the S.I.value of 0.308 for the entry name *Bacillus-spahericus*-GC subgroup D by matching with TSBA50 5.00 library.

It can be concluded from the above findings and investigations that, after isolating a number of strains from these selected wetlands of North Bihar, only most five selected strains were morphologically, physico-chemically and biochemically characterized and were then identified & catalogued from IMTECH(Institute of Microbial Technology), Chandigarh. Among these five strains SM18A *(Lysinibacillus sphaericus*) viz MTCC no 9523 showed a Similarity Index (S.I.) of 0.491 and 0.445 by matching with TSBA50 5.00 library. It also showed a separation of 0.046 between first and second choice and therefore can be considered as good library comparisons. Among the other strains, SM8 (*Nocardiopsis flava)* viz MTCC no.9525, SM10 (*Bacillus species)* viz MTCC no. 9524, SM2A (*Virgibacillus pantothenticus)* viz 9479 and BC7A (*Lysinibacillus sphaericus)* viz MTCC no. 9526 showed a Similarity Index (S.I.) of 0.021(TSBA) for SM8, 0.058 & 0.048(TSBA) for SM10, 0.413 & 0.253(TSBA) and 0.120 & 0.112(CLIN) for SM2A and 0.376, 0.369, 0.308(TSBA) for BC7A.

Table.1

S.No.	Strain Designation	Isolate Identified (Genus and species	MTCC no.
1.	SM8	*Nocardiopsis flava*	MTCC 9525
2	SM10	*Bacillus species*	MTCC 9524
3	SM2A	*Virgibacillus pantothenticus*	MTCC 9479
4	SM18A	*Lysinibacillus sphaericus*	MTCC 9523
5	BC7A	*Lysinibacillus sphaericus*	MTCC 9526

Details of the FAME analysis for the 5 test isolates are as follows:

Isolate SM8

Table.2 FAME analysis result of SM8

Total Response Less Than 50000.0. Concentrate and Re-Run

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.444	2.704E+8	0.018	------	7.146	SOLVENT PEAK	------	<min rt	_____
1.541	16765	0.020	------	7.352		------	<min rt	_____
7.790	1217	0.034	0.969	14.623	15:0 ISO	35.03	ECL deviates 0.000	Referene-0.004
9.412	2294	0.039	0.953	15.627	16:0 ISO	64.97	ECL deviates 0.000	Refrence-0.003

ECL Deviation: 0.000 Reference ECL Shift: 0.003
Number Reference Peaks: 2
Total Response: 3511 Total Named: 3511
Percent Named: 100.00% Total Amount: 3365

Matches

Library	Sim Index	Entry Name
TSBA50 5.00	0.021	*Lechevalieria- flava* (Saccharothrix, *Nocardiopsis flava)*
CLIN50 5.00	----------	No match
ACTIN 1 3.80	----------	No match
MOORE5 5.00	----------	No match

Isolate SM10

Table.3 FAME analysis result of SM10

Profile: Total Response Less Than 50000.0. Concentrate And Re-Run

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.444	2.702E+8	0.018	------	7.147	SOLVENT PEAK	---------	<min rt	----------
1.541	11564	0.017	------	7.352	----------	--------	<min rt	----------
2.573	778	0.024	------	9.542	----------	---------	----------	----------
5.037	2373	0.027	1.018	12.614	13:0 ISO	6.72	ECL deviates0.000	Reference-0.004
6.837	1564	0.030	0.981	14.002	14:0	4.27	ECL deviates0.002	Reference-0.001
7.790	15635	0.033	0.969	14.622	15:0 ISO	42.13	ECL deviates-0.001	Reference-0.004
7.929	2681	0.034	0.967	14.712	15:0 ANTEISO	7.21	ECL deviates-0.001	Reference-0.003
9.797	2580	0.037	0.950	15.859	Sum in Feature 3	6.82	ECL deviates0.007	15:0 ISO 2OH/16:1w7c
10.034	4722	0.037	0.949	16.001	16:0	12.46	ECL deviates0.001	Reference-0.001
11.115	3256	0.035	0.943	16.629	17:0 ISO	8.54	ECL deviates-0.001	Reference-0.004
13.507	4558	0.042	0.935	18.000	18:0	11.86	ECL deviates0.000	Reference-0.002
--------	2580	-------	-------	-------	Summed Feature 3	6.82	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c

ECL Deviation: 0.003 Reference ECL shift: 0.003
Number Reference Peaks: 7
Total Response: 38155 Total Named: 37368
Percent Named: 97.94% Total Amount: 35950

Matches

Library	Sim Index	Entry Name
TSBA50 5.00	0.058	*Stenotrophomonas-maltophilia* (Xanthomonas, Pseudomonas)
	0,048	*Staphylococcus-intermedius*
CLIN50 5.00	_____	(No Match)
ACTIN1 3.80	_____	(No Match)
MOORE5 5.00	_____	(No Match)

Isolate SM2A

Table.4 FAME analysis result of SM2A

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	C\omment2
1.443	2.777E+8	0.017	_____	7.161	SOLVENT PEAK	_____	< min rt	_____
1.540	16972	0.015	_____	7.366	_____	_____	< min rt	_____
6.315	3430	0.030	0.990	13.617	14:0 ISO	2.68	ECL deviates -0.002	Reference - 0.004
6.837	1584	0.030	0.981	14.002	14:0	1.23	ECL deviates 0.002	Reference - 0.001
7.790	31012	0.034	0.969	14.623	15:0 ISO	23.73	ECL deviates 0.000	Reference - 0.003
7.930	41821	0.035	0.967	14.714	15:0 ANTEISO	31.94	ECL deviates 0.001	Reference - 0.002
9.006	2611	0.036	0.956	15.384	16:1 w7c alcohol	1.97	ECL deviates -0.003	_____
9.412	19564	0. 36	0.953	15.628	16:0 ISO	14.73	ECL deviates 0.001	Reference - 0.003
10.033	7002	0.36	0.949	16.002	16:0	5.25	ECL deviates 0.002	Reference - 0.002
11.115	4259	0.040	0.943	16.628	17:0 ISO	3.17	ECL deviates -0.002	Reference - 0.004
11.276	18450	0.037	0.942	16.722	17:0 ANTEISO	13.73	ECL deviates -0.001	Reference - 0.003
16.356	2136	0.040	0.936	19.635	20:0 ISO	1.58	ECL deviates 0.001	Reference - 0.012
18.012	4824	0.039	_____	20.594	_____	_____	> max rt	_____
18.168	4501	0.034	_____	20.684	_____	_____	> max rt	_____
18.301	1590	0.032	_____	20.761	_____	_____	> max rt	_____

ECL Deviation: 0.002 Reference ECL shift: 0
Number Reference Peaks: 9
Total Response: 131868 Total Named: 131868
Percent Named: 100% Total Amount: 126610

Matches

Library	Sim Index	Entry Name
TSBA50 5.00	0.413	*Virgibacillus- pantothenticus* (Bacillus)
	0.253	*Bacillus-oleronius*
CLIN50 5.00	0.120	*Bacillus-coagulans*-GC subgroup C
	0.112	*Dermabcter –hominis*
ACTIN1 3.80	_____	(No Match)
MOORE5 5.00	_____	(No Match)

Isolate SM18A

Table.5 FAME analysis result of SM18A

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.443	2.713E+8	0.018	-------	7.147	SOLVENT PEAK	--------	< min rt	_____
1.540	27025	0.028	-------	7.352	----------	--------	< min rt	_____
6.315	3209	0.030	0.990	13.618	14:0 ISO	1.73	ECL deviates -0.001	Reference - 0.004
7.791	81949	0.034	0.969	14.623	15:0 ISO	43.16	ECL deviates 0.000	Reference - 0.003
7.930	13432	0.034	0.967	14.713	15:0 ANTEISO	7.06	ECL deviates 0.000	Reference - 0.003
9.007	21129	0.036	0.956	15.384	16:1 w7c alcohol	10.99	ECL deviates -0.003	----------
9.411	23245	0.036	0.953	15.627	16:0 ISO	12.04	ECL deviates 0.000	Reference - 0.003
9.627	5798	0.036	0.951	15.757	16:1w11c	3.00	ECL deviates 0.000	----------
10.033	6218	0.037	0.949	16.001	16:0	3.21	ECL deviates 0.001	Reference - 0.002
10.692	7141	0.039	0.945	16.383	ISO 17:1 w10c	3.67	ECL deviates -0.005	_____
10.848	5117	0.037	0.944	16.474	Sum in Feature 4	2.63	ECL deviates -0.002	17:1 ISO I/ANTEI B
11.115	14642	0.037	0.943	16.629	17:0 ISO	7.50	ECL deviates -0.001	Reference - 0.004
11.2755	6396	0.039	0.942	16.722	17:0 ANTEISO	3.28	ECL deviates -0.001	Reference - 0.004
13.508	3430	0.039	0.935	18.001	18:0	1.74	ECL deviates 0.001	Reference - 0.002
--------	5117	------	------	-------	Summed Feature 4	2.63	17:1 ISO I/ANTEI B	17:1 ISO I/ANTEISO B/i I

ECL Deviation: 0.002 Reference ECL shift: 0.003
Number Reference Peaks: 8
Total Response: 191707 Total Named: 191707
Percent Named: 100.00% Total Amount: 183936

Matches

Library	Sim Index	Entry Name
TSBA50 5.00	0.491	*Bacillus-sphaericus*-GC subgroup E
	0.445	*Bacillus-sphaericus*-GC subgroup F
CLIN50 5.00		(No Match)
ACTIN1 3.80	_____	(No Match)
MOORE5 5.00	_____	(No Match)

Isolate BC7A.

Table.6 FAME analysis result of BC7A

Profile: Total Response Less Than 50000.0. Concentrate And Re-Run

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.442	2.711E+8	0.019	_____	7.1444	SOLVENT PEAK	_____	< min rt	_____
1.538	14582	0.019	_____	7.349	_____	_____	< min rt	_____
6.306	1565	0.031	0.992	13.619	14: 0 ISO	5.52	ECL deviates 0.000	Reference -0.008
7.781	18752	0.034	0.968	14.623	15: 0 ISO	64.47	ECL deviates 0.000	Reference -0.007
7.920	3308	0.033	0.966	14.714	15:0ANTEISO	11.35	ECL deviates 0.001	Reference -0.006
8.996	2255	0.035	0.954	15.383	16:1 w7c alcohol	7.64	ECL deviates -0.004	_____
9.400	3269	0.037	0.950	15.627	16:0 ISO	11.03	ECL deviates 0.000	Reference -0.007

ECL Deviation: 0.002 Reference ECL shift: 0.007
Number Reference Peaks: 4
Total Response: 29148 Totals Named: 29148
Percent Named: 100.00% Total Amount: 28163

Matches

Library	Sim Index	Entry Name
TSBA50 5.00	0.376	*Bacillus-sphaericus*-GC subgroup E
	0.369	*Bacillus-sphaericus*-GC subgroup C
	0.308	*Bacillus-sphaericus*-GC subgroup D
CLIN50 5.00	_____	(No Match)
ACTIN1 3.80	_____	(No Match)
MOORE5 5.00	_____	(No Match)

Table.7 Five Steps to Prepare Extracts.

Step	Purpose
Harvesting	Removal of cells from culture media
Saponification	Extraction of fatty acids
Methylation	Formation of fatty acid methyl esters (FAMEs).
Extraction	Transfer of the FAMEs from aqueous phase to organic phase.
Base Wash	Aqueous wash of the organic extract before chromatographic analysis.

Table.8 Sherlock Microbial Libraries.

Package	Library	Description
AEROBE	TSBA6	Aerobes,28°C, 24 hr on Trypticase Soy Broth Agar
	RTSBA6	
	CLIN6	Clinical Aerobes,35°C,24 hr on Blood Agar, Chocolate ,etc
	RCLIN6	
	BTR3	Bioterrorism Clinical Aerobes,35°C,24hr on Blood Agar Chocolate, etc.
	RBTR3	
	M17H10	Mycobacteria, 35°C,5-10% CO_2 on Middlebrook 7H10 Agar with OADC enrichment
ANAEROBE	BHIBLA	Anaerobes 35°C,48hr on BHIBLA plates in Gas Packs
	MOORE6	VPI Broth-grown Anaerobe Library, 35°C ,in PYG Broth
YEAST	YST28	Yeasts,28°C,24 hr, SAB Dextrose Agar
	YSTCLN	Yeasts,28°C,24 hr, SAB Dextrose Agar
	FUNGI	Fungi, 28°C,2-5 days, in SAB Dextrose Broth, 150 rpm shake culture
	ACTIN1	Actinomycetes, 28°C, 3-10 days, in Trypticase Soy Broth 150 rpm shake culture

Table.9 Morphological characterization of selected Bacterial isolates.

	SM8	SM10	SM2A	SM18A	BC7A
Colony morphology	Round	Round	Round	Round	Round, with scalloped margin
Configuration	Smooth, shiny(entire)	Smooth, shiny(entire)	Filamentous	Filamentous	Wavy
Margin	Raised	Raised	Ciliate	Irregular	Raised
Surface	Shiny	Smooth	Smooth	Smooth	Smooth
Density	Opaque	Opaque	Opaque	Opaque	Opaque
Gram's reaction	-ve	+ve	+ve	+ve	+ve
Cell shape	Mycelium	Rods	Rods	Rods	Rods
Size	Small	Small	Long	Long	Small
Spore staining	-ve	+ve	+ve	+ve	+ve
Motility	-ve	-ve	+ve	+ve	-ve

Table.10 Physico-chemical characterization of selected Bacterial isolates

	SM8	SM10	SM2A	SM18A	BC7A
Growth at temp.					
25ºC	+	+	+	+	+
37ºC	+	+	+	+	+
42ºC	+	+	+	+	+
55ºC	-	-	+	+	+
Growth on NaCl%					
0.87%	+	+	+	+	+
2%	+	+	+	+	+
5%	+	+	+	+	+
7%	-	+	-	-	-
10%	-	-	-	-	-
Growth on pH					
5.2	+	+	+	+	+
7.0	+	+	+	+	+
8.0	+	+	+	+	+
9.0	+	+	+	+	+
11.0	+	+	+	+	+

Table.11 Biochemical characterization of selected Bacterial isolates.

	SM8	SM10	SM2A	SM18A	BC7A
Starch hydrolysis	_	+	_	_	_
Casein hydrolysis	+	+	_	_	_
Gelatin hydrolysis	_	_	_	_	_
Indole test	_	_	_	_	+
Methyl-Red test	+	+	_	_	_
Voges-Proskauer test	_	_	_	_	_
Citrate test	_	_	+	+	_
H_2S production test	_	_	_	_	_
Nitrate test	+	+	+	+	+
Catalase test	+	+	+	+	+
Oxidase test	_	_	_	_	_
Urea hydrolysis	_	_	+	+	_
Phosphatase test	+	+	+	+	-
Cellulase test	+	+	+	+	+
Acid production from					
Arabinose	+	_	+	_	+
Fructose	_	+	+	_	_
Galactose	_	_	+	_	_
Glucose	+	+	+		
Mannitol					
Meso-inositol	_	_	_	_	_
Raffinose	_	_	_	_	_
Rhamnose	_	_	_	_	_
Sucrose	_	+	_	_	_
Salicin	_	_	_	_	_
Xylose	_	_	+	_	_

As compared to SM18A, rest of the four strains viz SM8, SM10, SM2A could not be considered as good library comparisons since the organism is not a species in the library, but the software will still indicate the most closely related species, which can be useful when a new species is encountered. Strain BC 7A could be considered as a good match, but an atypical strain. A minimum amount of lipids is to be present in the samples to create a library entry that can reliably identify an unknown sample as per the Sherlock® Analysis Software. Lipid concentrations are the limiting factor to creating library entries, but

not to identifying samples. Possiblities may arise that the sample may contain less than the amount of lipids to create a library entry; however, the software will attempt to match that sample to an existing library entry. Generating library entries based on samples containing concentrated lipids allows the system to detect minute fatty acids and produces a more robust fatty acid profile than using dilute samples.

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Conflicts of Interest

The authors have no conflicts of interest.

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