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FAME and 16srDNA sequence analysis of halophilic bacteria from solar salterns of Goa: A comparative study

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Abstract- Halophilic microorganisms present in the hypersaline environments of solar salterns present a potential source of industrial and biotechnological applications .The Rapid identification of these microorganisms which are accurate and cost effective is a necessity in applied microbiology. There have been several systems developed in the past few years for rapid microbial identification. In the present study, GC FAME(Gas chromatography- Fatty acid methyl esters) analysis and 16s rDNA sequencing were compared for identification of four halophilic bacteria. Four halophilic bacterial isolates obtained from solar saltern sediment were analyzed . The isolates were identified with 16srDNA sequencing as *Virgibacillus pantothenticus, Bacillus atrophaeus, Corynebacterium diphtheria* and ,*Idiomarina Zobellii* .However in FAME analysis , the RTBSA 6 library did not confirm any matches for *Idiomarina Zobellii.* Comparative analysis, indicated that the FAME analysis results correlates to genotypic sequencing results. However,a halophilic bacterial FAME library should be established to enhance the accurate , rapid and cost effective investigation of halophilic microorganisms.

Index Terms- Solar salterns, Halophilic bacteria, FAME, Sequencing, BLAST, Identification

I. INTRODUCTION

Determining the taxonomic composition, biomass, and physiological status of microbial assemblages is still one of the greatest challenges facing ,microbial ecologists. Classical approaches that utilize enrichment methods for the isolation of microorganisms from the environment continue to provide valuable information in biochemical, taxonomic, and autoecological studies. The primary limitations to such approaches are those of non culturability ,and the problem of characterizing and identifying statistically relevant numbers of isolates necessary to gain insight into the population ecology and community diversity of habitats(Thompson et al., 1999). Rapid and accurate identification of bacterial pathogens is a fundamental goal of clinical microbiology, but one that is difficult or impossible for many slow-growing and fastidious organisms(Tang, 1998).New and exciting molecular methods, using the 16S small sub-unit ribosomal nucleic acid molecules have added much to our knowledge of microbial diversity (Macrae A ,2000). For many years, sequencing of the 16S ribosomal RNA (rRNA) gene has shown that sequence identification is useful for slow-growing, unusual, and fastidious bacteria as well as for bacteria that are poorly differentiated by conventional methods. The technical resources necessary for sequence identification are significant. Despite the availability of resources, sequence-based identification is still relatively expensive(Patel 2001).Although it is generally regarded that routine identification of very common species using conventional methodologies are highly accurate, we now have a more convenient and precise mechanism for checking these identifications on a molecular basis. Such studies need to be performed and published(Janda , 2007).The 16S rDNA sequence analysis is the reliable method for halophilic bacterium identification.(Chookiwattana,2003).Recent advances in the biochemistry of microorganisms revealed that analysis of cell components, such as proteins and fatty acids, can be effectively applied to bacterial identification, providing the basis for chemotaxonomy(Goodfellow, 1985., Komagata, 1987).Fatty acids are one of the most important building blocks of cellular materials. In bacterial cells, fatty acids occur mainly in the cell membranes as the acyl constituents of phospholipids. These fatty acids are synthesized in certain bacteria from iso, anteiso, or cyclic primer and malonyl-CoA with or without a subsequent modification(Kaneda, 1977.,Lechevalier 1977. ,Wilkinson 1988).The occurrence of branched-chain fatty acids as major constituents in bacteria was first reported for *Bacillus subtilis*(Saito, K. 1960.)These days, fatty acids in bacterial lipids are routinely analyzed by gas-liquid chromatography. In addition, other methods such as nuclear magnetic resonance spectroscopy,infrared spectroscopy, mass spectrometry, and thin-layer chromatography have been used to aid the gas-liquid chromatographic identification of fatty acids (Toshi 1991).The unique pattern of fatty acids in bacteria is the basis of identification in FAME analysis.The MIDI (Microbial Identification Incorporation) Sherlock FAME analysis is a laboratory-based system that can be used on a routine basis to identify commonly isolated bacteria from clinical and environmental source(Leonard et al 1995) More than 300 fatty acids and related compounds have been found in bacteria are analyzed in the MIDI Research and Development Laboratory. Whole cell fatty acids are converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the unknown is compared to a library of known organisms in order to find the closest match. The peaks are automatically named and quantitated by the system .Branched chain acids predominate in some Gram positive bacteria, while short chain hydroxy acids often characterize the lipopolysaccharides of the Gram negative bacteria(Sasser, 2001) . The MIS, as the first automated CFA(Cellular fatty acid) identification system, is an

accurate, efficient, and relatively rapid method for the identification of microorganisms (Osterhout et al 1991).The identification of marine isolates, is a continuing challenge for marine microbiologists. The identification of marine bacteria is commonly based on a wide range of biochemical and physiological tests. The major difficulties found with this traditional approach are the need for an easily cultivable strain and the time required for the preparation of cultures (Akagawa-Matsushita, et al., 1992) Solar saltpans are found worldwide and are considered extreme environments with very restricted biology (Litchfield, 2002).It is surmised that marine environments like solar saltern may yield newer strains which may prove to be a rich resource of new metabolites. Microorganisms that thrive in these hypersaline environments are called halophilic microorganisms (that is, require salt for their viability .The domain bacteria typically contains many types of halophilic microorganisms that spread over a large number of phylogenetic subgroups and they are moderate rather than extreme halophiles (Oren, 2002). Sparked by the availability of microbial diversity data in these salterns an effort is made to study the moderately halophilic bacterial in these extreme environments. The bacteria are analyzed for their identity by two methods 16s rDNA sequencing and GC-FAME,.The similarity between these methods is comparatively studied.

II. MATERIALS AND METHOD

Four halophilic bacterial pure cultures were obtained from solar saltern sediment and designated as H1, H2, H3 and H 4 .The cultures were grown on halophile medium(10% salt) at 28°C for 48-72 hours. The fatty acids were extracted and methylated to form fatty acid methyl esters (FAME). These FAME's were analyzed using Gas Chromatography with the help of MIDI Sherlock software for FAME. Aerobic library (RTBSA 6.0) was referred for the analysis. The analysis was performed as per (Sasser, 2001). For 16s rDNA sequence analysis the genomic DNA was isolated. PCR amplifications of the 16S rRNA gene, from the purified genomic DNAs, were carried out using the primer sets 27F (5´AGAGTTTGATCCTGGCTCAG-3´) and1492R (5´-GGTTACCTTGTTACGACTT-3´). The amplified DNA was purified and sequencing of the target gene was done using Big Dye Chemistry, and performed as per the manufacturer's protocols (Applied Biosystems). The purified extension products were separated in the ABI 3730xl DNA Analyzer by capillary electrophoresis. Sequence data analysis was done using ChromasPro and Sequencing Analysis software. The bioinformatics analysis was performed using NCBI BLAST (http://ncbi.nlm.nih.gov/blast) identifying the microorganism using online databases. The analysis data from the Sequencing and FAME analysis obtained was comparatively studied.

III. RESULTS

The four halophilic microbial cultures obtained from saltern sediment were analyzed using FAME and sequencing approaches. On comparing FAME results with that of sequencing, it was found that both the methods correlate to each other in confirmation of identity of the isolates except for isolate H4. Figure 1 shows peaks corresponding to the fatty acids identified through FAME analysis of bacterial sample H1. The MIDI Sherlock microbial identification system using RTSBA6 method identified the organism to be *Corynebacterium diptheriae* with 0.596 Similarity index(SI). The sequence analysis also showed similar results (Table 1). After the completion of BLAST analysis the organism was identified to *Corynebacterium diphtheriae strain with* 73% identity match. FAME analysis identified H2 as *Virgibacillus Pantothenticus* with similarity index 0.698(Figure 2).Sequence analysis allied with this identification ,as the after BLAST analysis the isolate H2 was identified as *Virgibacillus Pantothenticus* strain OLO3.(Table 2).The methods also displayed parallel results for isolate H3. Wherein the H3 isolate was identified as *Bacillus atrophaeus* with FAME analysis (Similarity index 0.776. (Figure 3).The sequencing and BLAST identified the isolated as *Bacillus atrophaeus* with 100% identity match. However the isolate H4, identified as *Idiomarina Zobellii 100 %* identity match after BLAST analysis, did not find any matching chromatogram pattern in FAME RTBSA6 6.0 aerobic library.

IV. DISCUSSION

Identification of bacteria by conventional methods usually requires ≥48 h after a colony has been isolated. The identification of slow-growing or biochemically inert microbes to the species level is difficult and time-consuming by conventional methods. 16S rRNA gene sequences frequently provide phylogenetically useful information. Signature nucleotides allow classification even if a particular sequence has no match in the database, since otherwise-unrecognizable isolates can be assigned to a phylogenetic branch at the class, family, genus, or subgenus level. Cost is a critical issue in the evaluation of 16S rDNA sequence analysis as a diagnostic tool. However, sequencing costs will probably continue their rapid trend downward, bringing this technology within the reach of many microbiology laboratories (Tang, 1998). Roohi et al in 2012 studied the halotolerant and Halophilic bacteria from salt mines of karak, Pakistan with 16S rRNA gene sequence.As the phenotypic characteristics alone were not enough to differentiate the bacterial isolates and could lead to identification problems. The main reason for this is the standardization of a conventional method, when it was applied to halophilic bacteria because their growth characteristic highly dependent on many factors such as NaCl concentrations, temperature, pH and medium composition.All the four isolates were sucessfully sequenced and identified in the present study.The MIDI FAME system identified 3 out of 4 bacterial isolates to the correct species level. The results were in accordance to the findings of Chookiewattana K, 2003 .Wherein 14.1% of halophilic bacterial isolates were not identified by FAME analysis, differing the 100% identification by 16srDNA sequencing. Species level identification of microbes is possible through MIDI Sherlock microbial identification. Abel and colleagues first suggested that microorganisms could be classified by gas chromatographic analysis (Abel et al 1963). The Sherlock Microbial Identification System is an accurate and automated gas chromatographic system, which identifies over 1,500 bacterial species based on their unique fatty acid profiles. The system can analyze over 200

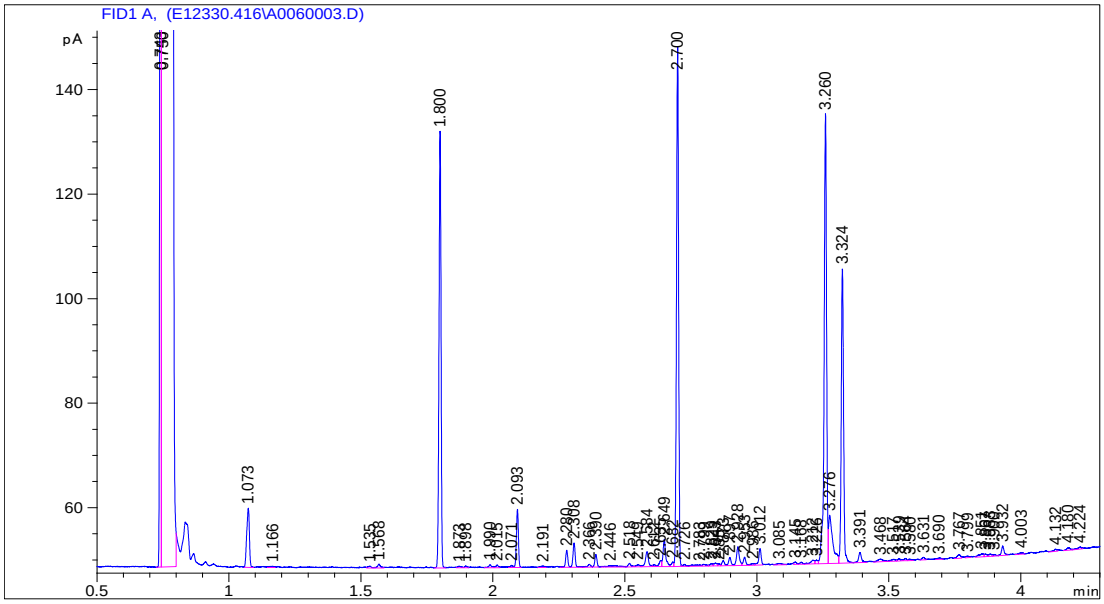
samples per day,uses a standardized sample procedure for all bacteria, and costs about $2.50 per sample in standard laboratory consumables (Sasser M 2001) .The MIDI's identification was listed with a confidence measurement (similarity index [SI]) on a scale of no match to 0.965.The Sherlock microbial identification system is solely based on computer comparison of the unknown organism's fatty acid methyl ester profile with the profiles of a predetermined library of known isolates with pattern recognition software (Tang et al 1998).The fatty acid analysis by the MIDI system can be a useful supplement and reference method, but cannot be recommended at this time for the routine identification of halophilic bacteria until a library of halophilic bacterium isolates is established in the MIDI database. Presently, the MIDI system does not contain a library for halophilic bacteria. The database RTBSA6 is the closet database for halophilic bacteria (Chookiewattana , 2003). The data and isolate obtained in this study can be used to develop the database in the MIDI system library for future identification of halophilic bacteria. The halophilic library can be constructed with ease as halophiles are studied extensively throughout the world . Recently marine red pigmented Bacteria isolated from nellore costal region of andhra Pradesh were studied for their fatty acid profile by FAME analysis(pabba et al .,2011). Similarly the fatty acid methyl esther profiles of 6 isolates belonging to bacteria domain representing different genera and species were analyzed by Guvenk et al in 2010.

V. CONCLUSION

Therefore, through the above study we can conclude that FAME analysis and 16s rDNA sequencing are rapid and accurate methods for bacterial identification .FAME analysis has an added advantage of being cost effective as compared to sequencing. Thus this technology should be implemented by microbiologists into routine practice. However, the MIDI FAME libraries should be upgraded with chromatogram profiles of more micro-organisms. As halophiles from extreme environments like solar salterns of Goa, are potential candidates for enzymatic and other industrial applications, a library of halophilic microbes should be established.The halophilic microbial library can assist faster and accurate identification of novel halophiles.

Accession	Description	Max score	Total score	Max ident
FJ409575.1	*Corynebacterium diphtheriae* strain CD450 16S ribosomal RNA gene, partial sequence	206	206	73%
FJ409574.1	*Corynebacterium diphtheriae* strain CD449 16S ribosomal RNA gene, partial sequence	206	206	73%
FJ409573.1	*Corynebacterium diphtheriae* strain CD448 16S ribosomal RNA gene, partial sequence	206	206	73%
FJ409572.1	*Corynebacterium diphtheriae* strain CD443 16S ribosomal RNA gene, partial sequence	206	206	73%
CP003217.1	*Corynebacterium diphtheriae* VA01, complete genome	195	975	73%
CP003215.1	*Corynebacterium diphtheriae* HC04, complete genome	195	975	73%
CP003214.1	Corynebacterium diphtheriae HC03, complete genome	195	975	73%

Table 1: Blast Similarity Search Results fo H1 :Similarity to *Corynebacterium diphtheriae* strain CD450 by 16S ribosomal rRNAgene, partial sequence.

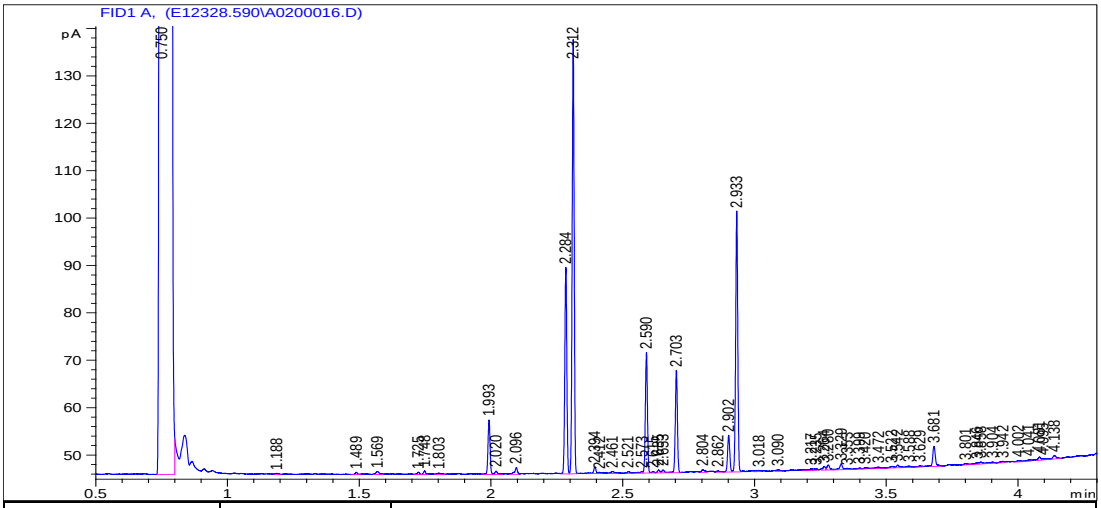


Library	Sim Index	Entry Name
RTSBA6 6.00	0.596	*Corynebacterium diphtheriae*

Figure 1: Chromatogram of bacterial sample H1 showing the fatty acid peaks through Agilent GC 6850.

Accession	Description	Max score	Total score	Max ident
JN791392.1	*Virgibacillus pantothenticus* strain OL03 16S ribosomal RNA gene, partial sequence	1483	1483	88%
AB617570.1	*Virgibacillus pantothenticus* gene for 16S rRNA, partial sequence, isolate: T8-4T	1483	1483	88%
AB305195.1	*Virgibacillus pantothenticus* gene for 16S rRNA, partial sequence	1483	1483	88%
AB196352.1	*Virgibacillus pantothenticus* gene for 16S rRNA, partial sequence, strain:PS11	1483	1483	88%
AB681789.1	*Virgibacillus pantothenticus* gene for 16S rRNA, partial sequence, strain: NBRC 102447	1456	1456	88%
AB489108.1	*Virgibacillus pantothenticus* gene for 16S rRNA, partial sequence	1456	1456	88%
AB039331.1	*Virgibacillus pantothenticus* gene for 16S rRNA, strain:M1-4	1447	1447	88%

Table2: Blast Similarity Search Results for H2:Similarity to *Virgibacillus pantothenticus* 16S ribosomal RNA gene, partial sequence



Library	Sim Index	Entry Name
RTSBA6 6.00	0.698	*Virgibacillus pantothenticus*

Figure 2: Chromatogram of bacterial sample H2 showing the fatty acid peaks through Agilent GC 6850.

Accession	Description	Max score	Total score	Max ident
JQ693811.1	*Bacillus atrophaeus* strain BF 3fF 16S ribosomal RNA gene, partial sequence	473	2196	100%
JN824998.1	*Bacillus atrophaeus* strain ZaK 16S ribosomal RNA gene, partial sequence	473	1604	100%
AB681057.1	*Bacillus atrophaeus* gene for 16S rRNA, partial sequence, strain: NBRC 16183	473	2196	100%
AB680855.1	*Bacillus atrophaeus* gene for 16S rRNA, partial sequence, strain: NBRC 15407	473	2202	100%
AB680488.1	*Bacillus atrophaeus* gene for 16S rRNA, partial sequence, strain: NBRC 13721 >dbj|AB680560.1| *Bacillus atrophaeus* gene for 16S rRNA, partial sequence, strain: NBRC 14117	473	2196	100%
HM744708.1	*Bacillus atrophaeus* strain LLS-M3-4 16S ribosomal RNA gene, partial sequence	473	2191	100%
JF411316.1	*Bacillus atrophaeus* strain KM40 16S ribosomal RNA gene, partial sequence	473	2196	100%

Table 3: Blast Similarity Search Results for H3:Similarity to *Bacillus atrophaeus strain BF 3fF* 16S ribosomal RNA gene, partial sequence

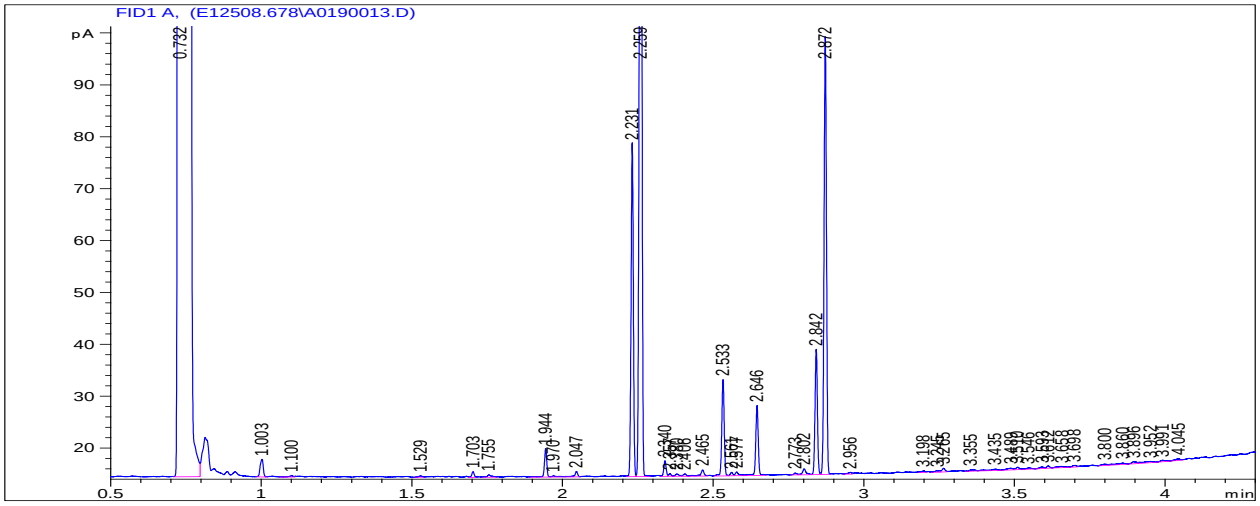


Figure 3: Chromatogram of bacterial sample H3 showing the fatty acid peaks through Agilent GC 6850.

Library	Sim Index	Entry Name
RTSBA6 6.00	0.776	*Bacillus atrophaeus*

Accession	Description	Max score	Total score	Max ident
GU397379.1	*Idiomarina zobellii* strain G5B 16S ribosomal RNA gene, partial sequence	592	2631	100%
AB619724.2	*Idiomarina sp*. TPS4-2 gene for 16S ribosomal RNA, partial sequence, strain: TPS4-2	588	3582	100%
EU440964.1	*Idiomarina seosinensis* strain PR58-8 16S ribosomal RNA gene, partial sequence	588	3617	100%
EU305725.1	*Idiomarina sp*. TBZ1 16S ribosomal RNA gene, partial sequence	588	3663	100%
DQ985042.1	*Idiomarina sp*. JL974 16S ribosomal RNA gene, partial sequence	588	3582	100%
DQ235548.1	Bacterium ABKPF4 16S ribosomal RNA gene, partial sequence	588	3542	99%
AY553079.1	*Idiomarina loihiensis* strain GSP37 16S ribosomal RNA gene, partial sequence	588	3511	100%

Table 4: Blast Similarity Search Results for H4:Similarity to *Idiomarina zobellii* strain G5B 16S ribosomal RNA gene, partial sequence

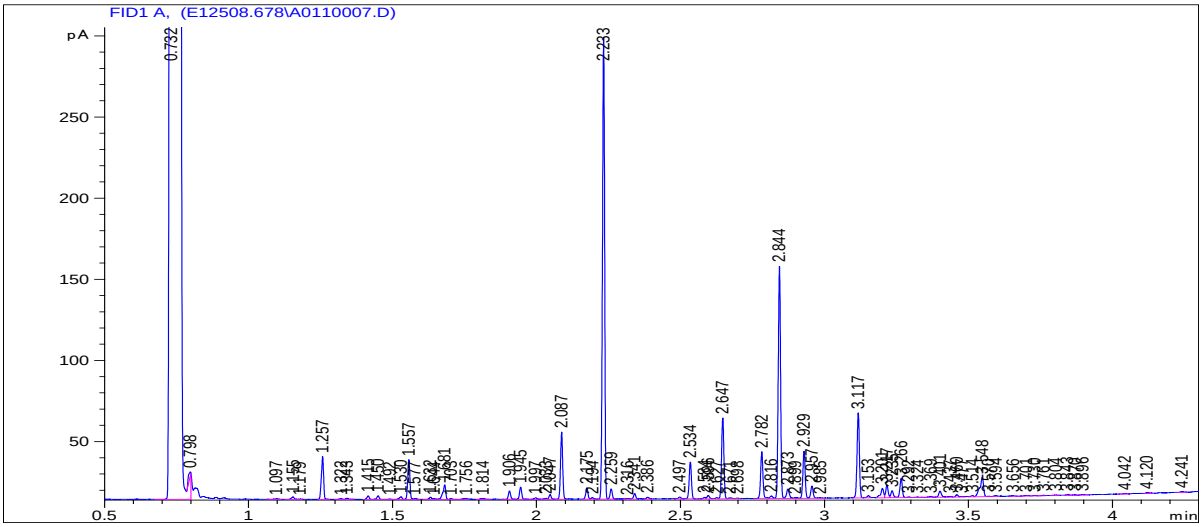


Figure 4 : Chromatogram of bacterial sample H4showing the fatty acid peaks through Agilent GC 6850.

No matches found in RTBSA6 6.0 Library

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