



## FAME FINGERPRINTS FOR IDENTIFICATION OF MARINE RED PIGMENTED BACTERIA ISOLATED FROM NELLORE COSTAL REGION OF ANDHRA PRADESH

SHIVA KRISHNA PABBA\*, BURRA SAMATHA, METUKU RAM PRASAD, SVSSSL HIMABINDU NIDADAVOLU AND M. A. SINGARA CHARYA

Department of Microbiology, Kakatiya University, Warangal – 506 009, Andhra Pradesh, India.

E mail: [shiva.krishnapabba@yahoo.com](mailto:shiva.krishnapabba@yahoo.com), \*Corresponding author

### ABSTRACT

The present study investigates on the marine bacteria; samples were collected from Nellore costal region Krishnapatnam beach in Andhra Pradesh, India, during the month of October, 2010. The selected bacterial isolates were identified by using FAME fingerprints i.e. fatty acid analysis using a commercially available device that directs a stream of compressed air across a section of the GC column, is used to focus peaks at the head of the column. When combined with a rapid sample processing method that uses smaller volumes of solvents, it becomes possibly the best method to identify bacteria. The bacterial strain collected was identified as *Vibrio gazogenes*.

**KEY WORDS:** FAME, GC column, Fatty acids.

### INTRODUCTION

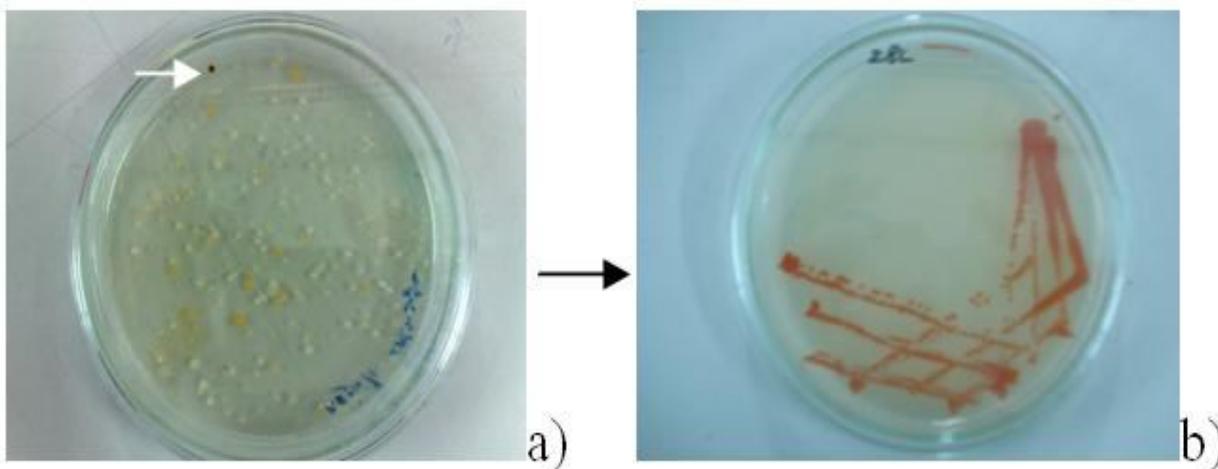
The Ocean, which is called the 'mother of origin of life', is also the source of structurally unique natural products that are mainly accumulated in living organisms. Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses. From the past few years, interest in marine bacteria has grown considerably. The importance of marine bacteria and as sources of valuable bioactive metabolites is very well established for more than 20 years. However, the identification of fresh marine isolates, a preliminary step in microbiological research, 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. The availability of miniaturized multi test systems that allow the simultaneous determination of numerous phenotypic characters has overcome the previous difficulties. However, the identification of environmental bacteria remains problematic because there is often a lack of agreement between the biochemical tests reported in the literature for the type strain and the results obtained in the laboratory with fresh isolates (1), (2), (3). The reason stems from the similar

physiological and biochemical characters shared by aerobic marine bacteria (1) and from the differences in the biochemical features of fresh and stored isolates. In fact, not only may plasmid DNA be lost during storage, but also fresh isolates may have greater enzymatic activity than their counterparts which have been stored on laboratory media (4) More sensitive and reliable techniques such as genomic fingerprinting hybridization analysis based on specific cleavage of DNA by restriction endonucleases and all PCR-based variants are generally difficult to perform, require many expensive reagents, and do not reduce the problem of long culture preparation times; therefore, they are not of practical use in routine analyses. 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 (5),(6). The microbial identification system produced by MIDI (Newark, DE, USA) is widely used for identification of microorganisms by fatty acid analysis (7),(8). The use of fatty acid analysis by gas chromatography for the identification of bacteria since its initial introduction of (9) has given results in agreement with DNA-DNA hybridization data(9)(10)(11)is currently widely used for the identification of both clinical and environmental isolates(12) ,(13),(14),(15),(16), (17). This technique has practical advantages, such as the simplicity of the analytical method,

the speed of analysis, and the low cost of materials. Moreover, the whole-cell fatty acid content is a direct and stable expression of the cellular genome. In fact, the cellular fatty acid pattern is a phenotypic character that is not affected by mutations or acquisition or loss of plasmids. A method for the rapid and reliable identification of marine bacteria has yet to be developed. In the present study, we describe a method for identification at the genus level of marine bacteria belonging to the genera *Vibrio* by using gas-chromatographic profiles of fatty acid methyl esters (FAME). To elaborate the FAME data the fatty acid pattern based on an

artificial neural network was chosen. Neural computing is the study of networks of adaptable nodes which, through a process of learning from task examples, store experimental knowledge and make it available for use (18) seek to reproduce the style of computing of the brain and can be regarded as computer models in which the nodes correspond to neurons and the connections between nodes correspond to synapses. Neural networks have the ability to identify unknown patterns after a training phase during which known patterns are shown to the net together with the expected identification.

**Figure1: a) The individual colonies isolated from sea water b) Plate shows the separated from the colony as streaking**



## **MATERIALS AND METHODS:**

### **Sample collection:**

The samples were collected from Nellore coastal region, Krishna Patanam beach in Andhra Pradesh, India, during the month of October 2010. 100µl of water sample was spreaded over the surface of the marine agar (Zobell Marine agar) with composition of Peptone 5.0 g, Yeast Extract. 1.0 g, Ferric Citrate 0.1 g, Sodium Chloride 19.45 g, Magnesium Chloride 8.8 g, Sodium Sulfate 3.24 g, Calcium Chloride 1.8g, Potassium Chloride 0.55 g, Sodium Bicarbonate 0.16g, Potassium Bromide 0.08 g, Strontium Chloride 34.0 mg, Boric Acid 2.0 mg, Sodium Silicate 4.0 mg, Sodium Fluoride 2.4 mg, Ammonium Nitrate 1.6 mg, Disodium Phosphate 8.0 mg, Agar 15.0. The individual colonies (Fig.1) were taken and maintained as stock cultures at 30°C in marine agar test tubes

slants. The selected strain was streaked on the petri plate and continued for the further process.

### **FATTY ACID ANALYSIS:**

#### **REAGENTS REPARATION:**

Four reagents are required to cleave the fatty acids from lipids: prepared according to the MIDI Sherlock protocol (19).

**Reagent 1:** Saponification—45g sodium hydroxide, 150ml methanol, and 150ml distilled water. Dispensing with an autopipette assures reproducibility and allows to perform large numbers of assays in a day.

**Reagent 2:** Methylation—325ml 6.0N hydrochloric acid and 275ml methyl alcohol to drop the pH of the solution below 1.5 and causes methylation (for the increased volatility in a partially polar column) of the fatty acid. The

fatty acid methyl ester is poorly soluble in the aqueous phase at this point.

**Reagent 3:** Extraction—200 ml hexane and 200 ml methyl tert-butyl ether. This will extract the fatty acid methyl esters into the organic phase for use with the gas chromatograph.

**Reagent 4:** Sample Cleanup—10.8 g sodium hydroxide dissolved in 900ml distilled water. This procedure reduces contamination of the injection port liner, the column, and the detector. More than 10,000 analyses can be performed on a column prior to needing any maintenance.

**SAMPLE PROCESSING:**

Steps to prepare GC ready extracts are illustrated in Figure 3.

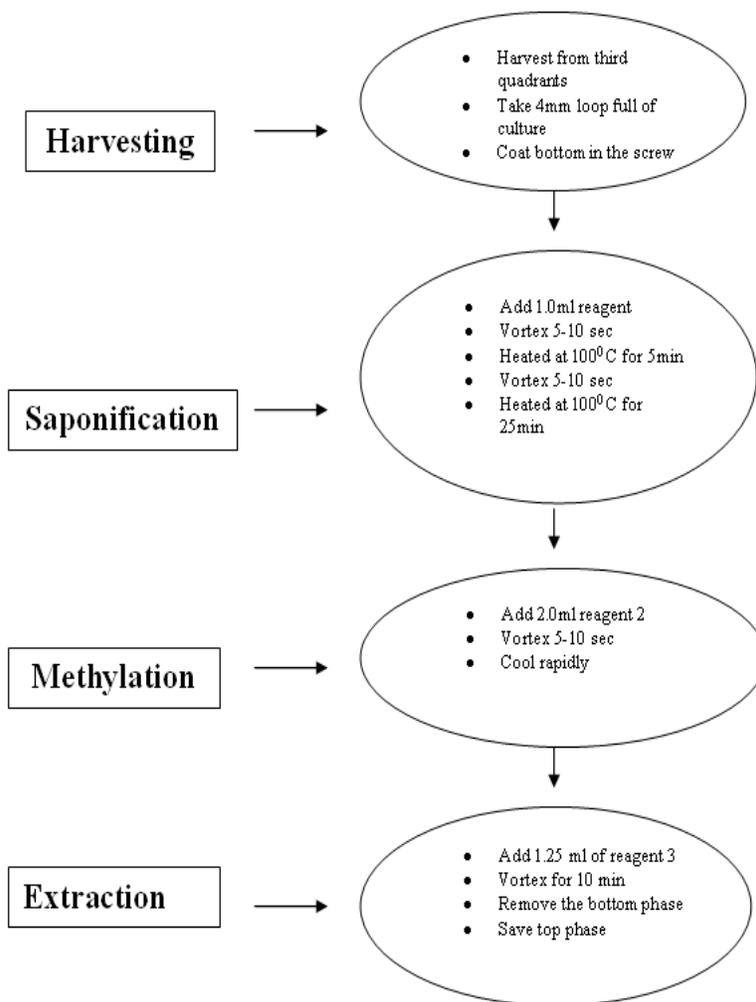


Fig3 : The flow chart explains the extraction process of fatty acids

**Harvesting**—a 4mm loop is used to harvest about 40mg of bacterial cells from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate. The cells are placed in a clean 13x100 culture tube. **Saponification**—1.0ml of Reagent 1 is added to each tube containing cells. The tubes

are securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for ca. 5 minutes, at which time the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating.

**Methylation**—the cooled tubes are uncapped, 2ml of Reagent 2 is added. The tubes are capped and briefly vortexed. After vortexing, the tubes are heated for  $10 \pm 1$  minute at  $80^\circ \pm 1^\circ\text{C}$ . (This step is critical in time and temperature.)

**Extraction**—Addition of 1.25ml of Reagent 3 to the cooled tubes is followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous (lower) phase is pipetted out and discarded.

**Base Wash**—About 3ml of Reagent 4 is added to the organic phase remaining in the tubes, the tubes are recapped, and tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase is pipetted into a GC vial which is capped and ready for analysis.

#### ESTIMATION OF FATTY ACID METHYLE ESTERS (FAME):

The FAME were analyzed by using Gas chromatography (GC)-mass spectrophotometry with an HP 6890 gas chromatograph equipped with an HP 5972A mass selective detector (Hewlett Packard Co., Porlo Alto California) and an HP Ultra 2 cross-linked 5% phenyl-methyl silicon capillary column (25m by 0.2mm 0.33m  $\mu$  m). The oven temperature was programmed with injection and 1-min hold at 80 C, followed by an increase to 160° C at 60 C  $\text{min}^{-1}$ , a hold at C for 28 min., and an increase at 5° C  $\text{min}^{-1}$  to 230 C. Individual FAME was identified by comparing their mass spectra with standard kits (Ana-labs North Heven, Conn.) the fatty acid content of the cells was calculated as the average of the three independent cultivations. The method presented here is RTSB50 (Table 1)

#### RESULT

The marine bacteria analyzed in the present study was to identify the bacteria based up on the fatty acid composition , the list of the fatty acids composition like 9:0 Pelargonic acid,10:0 capric acid,11:0 undecanoic acid,12:0 lauric acid,13:0 Tridecanoic acid,14:0 myristic acid,15:1 Pentadecenoic acid,16:1Palamitoic acid,17:1 iso mageraleic acid,18:1 Arachidonic acid, 20:4eicosenicacid,15:0 Pentadecanoic acid,16:0 Palmitic acid, 19:0Nanadoconic ,18:0steric acid was given clearly according to the GC report.

The chromatogram obtained in this experimental analysis fig 2 is more descriptive and

elaborative. It confirms and correlates the presence of saturated and unsaturated forms of fatty acids present in the bacterium with the predetermined fatty acids unknown fatty acids.

Our experimental data matches and establishes the similar results mentioned in the report of MIDI Sherlock software databases and given the similarity were matched with *Vibrio gazogenes* (RTSBA 66.00). The mostly concerned genus *Vibrio*. (20) Reported that 16:1, 16:0, and 18:1 were the major fatty acids in the *Vibrio* species and tested, with 16:1 being predominant.

Table 1

ITEM	RTSB50 Method
Column	Ultra 2,25m long x 0.2mm ID x 0.333 $\mu$ m film thickness
Carrier gas	Hydrogen,1.3ml/min, constant flow
Oven program(°C)	170to288at28/min 288to310at40/min
Split ratio	40:1
Injection volume	2 $\mu$ l
Valve 5(Air sharp)	None
Run time	5.83min

#### DISCUSSION:

Natural lipids consist of complex mixtures of molecular species, which are found in association with cell membranes, lipo proteins and other subcellular structures. The composition differs among different cell and tissue types. The GC of fatty acids by James and Martin 1956 provided the first success in dealing with the complexity of the hydrolysis products of fats and oils.

Fatty acid profiling is a popular method for characterizing microbial communities of natural systems. Direct extraction of microbial fatty acids *in situ* would be useful compared with methods that extracted lipids first and subsequently release fatty acids from lipids. The occurrence and abundance of microbial fatty acids have been used by many investigators for the identification of microorganisms in microbial communities (21). The Microbial Identification System (MIS, MIDI, Newark, DE) for fatty acid methyl ester (FAME) analysis is a standard method for identification of microorganisms. Whole cell fatty acids are converted to methyl

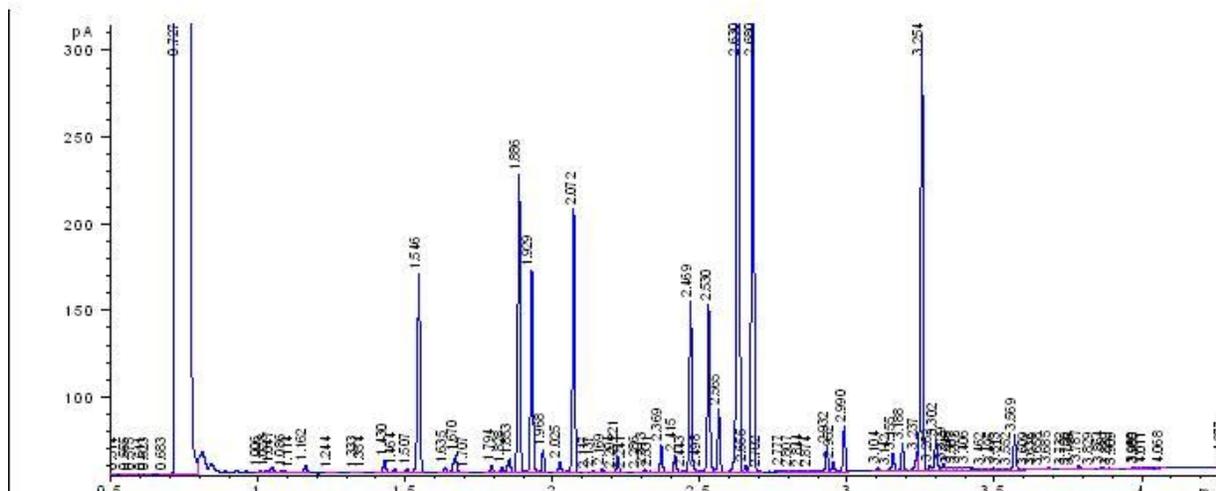


Fig: 3 Chromatogram of fatty acids

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.

## REFERENCES

1. Akagawa-Matsushita, M., T. Itoh, Y. Katayama, H. Kuraishi, and K. Yamasato. (1992). Isoprenoid quinone composition of some marine *Alteromonas*, *Marinomonas*, *Deleya*, *Pseudomonas* and *Shewanella* species. *J. Gen. Microbiol.* 1382275–2281.
2. Austin, B. (1982) Taxonomy of bacteria isolated from a coastal, marine fishrearing unit. *J. Appl. Bacteriol.* 53253–268.
3. Breschel, T. S., and F. L. Singleton. (1992) . Use of the API rapid NTF system for identifying nonfermentative and fermentative marine bacteria. *Appl. Environ. Microbiol.* 58 21–26.
4. Austin, B. (1985) Taxonomy of marine micro-organisms. In B Austin (ed.), *Marine microbiology*. Cambridge University Press, Cambridgepp. 45–78
5. Goodfellow, M., and D. E. Minnikin. (1985). Introduction to chemosystematics, *pln* M. Goodfellow and D. E. Minnikin (ed.), *Chemical methods in bacterial systematics*. Academic Press Ltd., London. 1–16.
6. Komagata, K., and K. Suzuki (1987) Lipid and cell-wall analysis in bacterial systematic. *Methods Microbiol.* 19 161–207.
7. Olson, W.P (1996). (Ed). *Automated microbial identification and quantitation*. Interpharm Press, Buffalo Grove, IL, USA,.
8. Abel, K., H. De Schmetzing, and J. I. Peterson. (1963) Classification of Microorganisms by analysis of chemical composition. *J. Bacteriol.* 85 1039–1044.
9. Huys, G., M. Vancanneyt, R. Coopman, P. Janssen, E. Falsen, M. Altwegg, and K. Kersters. (1996) Cellular fatty acid composition as a chemotaxonomic Continued. VOL. 62, identification of marine bacteria 2131 marker for the differentiation of phenospecies and hybridization groups in the genus *Aeromonas*. *Int. J. Syst. Bacteriol.* 44651–658.
10. Rainey, P. B., I. P. Thompson, and N. J. Palleroni. (1994) Genome and fatty acid analysis of *Pseudomonas stutzeri*. *Int. J. Syst. Bacteriol.* 44 54–61.
11. Yang, P., L. Vauterin, M. Vancanneyt, J. Swings, and K. Kersters. (1993) Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. *Syst. Appl. Microbiol.* 16 47–7
12. Bousfield, I. J., G. L. Smith, T. R. Dando, and G. Hobbs. (1983) Numerical analysis of total fatty acid profiles in the identification of coryneform, nocardioform and some other bacteria. *J. Gen. Microbiol.* 129375–394.

13. Guckert, J. B., D. B. Ringelberg, D. C. White, R. S. Hansonn, and B. J. Bratina. (1991) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J. Gen. Microbiol.* 137 2631–2641.
14. Henningson, P. J., and N. C. Gudmestad. (1991) Fatty acid analysis of phytopathogenic coryneform bacteria. *J. Gen. Microbiol.* 137 427–440
15. Osterhout, G. J., V. H. Shull, and J. D. Dick. (1991) Identification of clinical isolates of gram-negative non fermentative bacteria by an automated cellular fatty acid identification system. *J. Clin. Microbiol.* 29 1822–1830.
16. Stead, D. E. (1992) Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *Int. J. Syst. Bacteriol.* 42 281–295.
17. Stead, D. E., J. E. Sellwood, J. Wilson, and I. Vieny. (1992) Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *J. Appl. Microbiol.* 72 315–321.
18. Aleksander, I., and H. Morton. (1990) Introduction. In I. Aleksander and H. Morton (ed.), An introduction to neural computing. Chapman & Hall, Ltd., London. pp 120.
19. Myron Sasser. (2006). Bacterial Identification by Gas Chromatographic analysis of fatty acids methyl esters (GC-FAME). Technical note #101 .
20. Oliver, J. D., and R. R. Colwell. (1973) Extractable lipids of gram-negative marine bacteria: fatty acid composition. *Int. J. Syst. Bacteriol.* 23 442–458.
21. Schutter, M.E and Dick, R.P. (2000) Comparision of fatty acid methyl ester (FAME) methods for charecterising microbial communities. *Soil Sci .Soc of America J* 64 1659-1668.