Isolation and Molecular Characterization of acne causing *Propionibacterium acnes*

Roselin Polugari[1], Shailaja Raj Marla[1], Shailaja. D[2]

[1]St. Francis College for Women, Begumpet, Hyderabad, Telangana
[2]Gokaraju Rangaraju Institute of Technology, JNTUH, Telangana

Abstract- *Propionibacterium acnes* is the most common member of the Anaerobic or microaerophilic coryneform organisms found on the skin. The present study focused on the isolation of *Propionibacterium acnes,* the causative organism of acne and its characterization based on the cultural characteristics, biochemical tests, GC FAME (Gas Chromatography- Fatty acid methyl esters) analysis and 16 S r RNA sequencing. The results were compared with the standard organisms procured from IMTECH, Chandigarh, India. The FAME analysis report of the organism correlates with that of the genotyping of the isolate and the organism is identified as *Propionibacterium acnes.*

Index Terms- *Propionibacterium acnes*, Biochemical tests, FAME analysis, 16 S r RNA gene sequencing

I. INTRODUCTION

Acne is a chronic inflammatory disease of the pilosebaceous unit resulting from androgen induced increased sebum production, altered keratinization, inflammation and bacterial colonization of hair follicles on the face, neck, chest and back by *Propionibacterium acnes*. Facial scarring due to acne affects up to 20% of the teenagers. Acne can persist into adulthood, with detrimental effects on self-esteem.[1]

Propionibacterium acnes is relatively slow growing, aero tolerant anaerobic, Gram-positive skin microbe that colonizes sebaceous glands and pilosebaceous follicles.[2] This organism is considered to play a principal role in the development of acne vulgaris.[2] Acne has many different symptoms including comedones, papules, pustules, nodules, cysts and pilosebaceous inflammation. Among these, inflammatory lesions of acne are of the greatest concern to patients because they may lead to acne scarring, thereby inducing adverse psychological effects.[3] It can also cause chronic blepharitis and endophthalmitis, the latter particularly following intraocular surgery[4]. The bacterium is largely commensal and part of skin flora present on most healthy adult human skin[5]. It lives primarily on, among other things, fatty acids in sebum secreted by sebaceous glands in the follicles. It may also be found throughout the gastrointestinal tract in humans. [6] It accounts for approximately half of the total skin microbiota[7], with an estimated density of 10^2 to 10^5 or 10^6 organisms per cm^2 . [8, 9]

P. acnes bacteria live deep within follicles and pores, away from the surface of the skin. In these follicles, *P. acnes* bacteria use sebum, cellular debris and metabolic byproducts from the surrounding skin tissue as their primary sources of energy and nutrients. Elevated production of sebum by hyperactive sebaceous glands or blockage of the follicle can cause *P. acnes* bacteria to grow and multiply. [10]

P. acnes bacteria secrete many proteins, including several digestive enzymes. [11] These enzymes are involved in the digestion of sebum and the acquisition of other nutrients. They can also destabilize the layers of cells that form the walls of the follicle. The cellular damage, metabolic byproducts and bacterial debris produced by the rapid growth of *P. acnes* in follicles can trigger inflammation.[12] This inflammation can lead to the symptoms associated with some common skin disorders, such as folliculitis and acne vulgaris. [13, 14] The aim of this study is to isolate *P. acnes* and identify it based on its characteristics. There are many different ways of identifying bacteria. 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.[15] New and exciting molecular methods, using the 16S small sub-unit ribosomal nucleic acid molecules have added much to our knowledge of microbial diversity [16]. For many years, sequencing of the 16S ribosomal RNA (r RNA) 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. [17] 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[18].

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[19]. 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[20]. The occurrence of branched-chain fatty acids as major constituents in bacteria was first reported for *Bacillus subtilis*[21]. These days, fatty acids in bacterial lipids are routinely analyzed by gas-liquid chromatography. 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[22]. 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[23]. 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 [24]

The present study focuses on the isolation, characterization of *P. acnes* based on its cultural characteristics, biochemical tests, FAME and 16S r RNA typing.

II. Materials and Methods

2.1 Isolation of *Propionibacterium acnes*

P. acnes is isolated from acne. About 50 samples were collected from students aged between 18-21 years. The samples were collected using sterile swabs and then were inoculated into Brain Heart Infusion broth and Thioglycollate broth. A 1 cm^2 area of the facial skin of about 50 volunteers aged between 17-21 years was cleansed with a sterile swab, and the swab was placed in a test tube containing 10ml of Thioglycollate broth. The broth was incubated for 4days in Anaero Gas Pak. After incubation, the culture was then streaked onto BHI and Clostridia agar and incubated for 4 days at 37 ^{o} C. The colony morphology was observed and stained by gram staining. The isolated colonies were sub cultured anaerobically into BHI broth and were preserved on BHI agar slants and also inoculated by stab culture onto BHI agar tubes.

2.2 Characterization of the organisms:

2.2.3. Biochemical Characterization

The Biochemical characteristics of the strains of anaerobes isolated from the persons suffering with acnes were analyzed by looking for production of Indole, sugar fermentation tests, nitrate reduction and Gelatin hydrolysis [25]

Hemolysis:

The organisms were plated onto the Blood Agar plates containing 10% defibrinolysed sheep blood to test for Hemolysis. The plates were incubated anaerobically for 4 days.

2.2.4 .FAME Analysis:

FAME analysis was carried out using Agilent 6850 Series II. To perform this analysis, a bacterial culture was taken and the fatty acids were extracted and used to form methyl esters. The volatile derivatives are then introduced into a gas chromatograph and the patterns of the peaks helped identify the organism. Anaerobic library BHI was referred for the analysis. This is widely used in characterizing new species of bacteria and is useful for identifying the pathogenic strains.

2.2.5. 16S r RNA typing:

The 16S r RNA gene is used as the standard for classification and identification of microbes, because it is present in most microbes and shows proper changes. Type strains of 16S r RNA gene sequences for most bacteria and archaea are available on public databases. In the present study, the Ez Taxon database (a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences) was used. Ez Taxon is an open access database that is produced and maintained by Chun Lab, Inc. The Ez Taxon database contains sequences of type strains of prokaryotic species with validly published names, mainly used for the routine identification of prokaryotic isolates.

III. Results and Discussion

Isolation and Characterization of *P. acnes*

1.	Pa1	Round, slimy, opaque, small colonies	Gram positive cocci
2.	Pa2	Small, circular, raised, creamy mucoid colonies.	Gram positive bacilli
3.	Pa3	Circular, opaque, irregular and creamy colonies.	Gram positive bacilli
4.	Pa4	Small, Round, cream coloured pin head colonies	Gram positive bacilli
5.	Pa5	Slimy, round, cream colored colonies.	Gram positive bacilli
6.	Pa6	Small, round, opaque, mucoid colonies	Gram positive cocci
7.	Pa7	Big, round, elevated, creamy colonies	Gram positive bacilli
8.	1951	Round, opaque, raised, creamy pinhead colonies	Gram positive bacilli
9.	3297	Small, round, opaque, regular, creamy colonies.	Gram positive bacilli

Table: 1 Colony characterisctics and gram stain results

a **b**

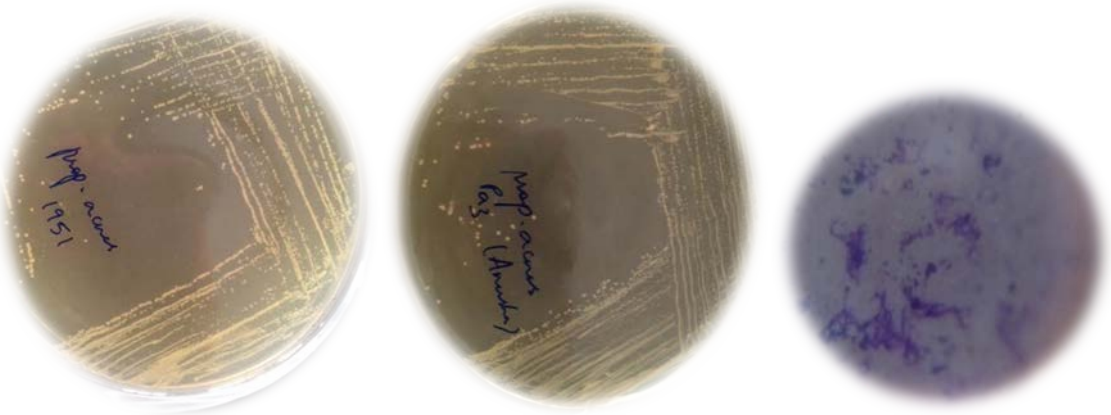


Figure 1: a) Colony morphology of 1951, Pa3 b) Microscopic observation of Pa 3

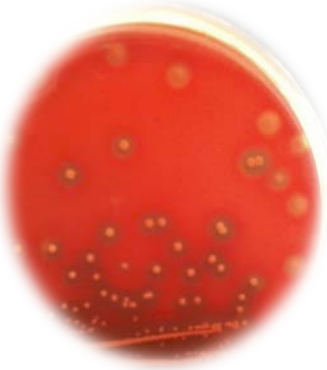


Figure 2: P.acnes showing Hemolysis on Blood Agar plate

Identification	Glucose	Maltose	Sucrose	Esculin*	Adonitole	Indole	NO_3^	Gelatin	Catalase
Pa2	-	-	-	-	-	+	+	+	+
Pa3	+	-	-	-	+	+	+	+	+
Pa4	+	-	-	-	+	+	+	-	+
Pa5	-	-	-	-	-	+	-	-	+
Pa7	+	+	+	-	-	-	+	+	-
MTCC1951	+	-	-	-	+	+	+	+	+
MTCC3297	+	-	-	-	+	+	+	+	+

Table 2: Typical reaction patterns of the isolates

*in the case of Esculin + indicates a positive ferric citrate test. The pH in these tubes did not change significantly
Fermentation of Carbohydrates in question is indicated by +
+ indicates production of Indole, reduction of nitrate to nitrite and liquefaction of Gelatin respectively in the last three columns

Out of the samples analyzed 5 samples showed colony morphology and Microscopic observation similar to that of the standard culture. These cultures were subjected to further analysis. The colonies were round, opaque, raised, creamy colonies and on gram staining, gram positive bacilli were observed (Table 1, Fig.1). Cultures Pa1, Pa6 were Gram positive cocci in clusters, hence could be *Staphylococcus aureus*. Pa3, Pa4 showed hemolysis on the blood agar plates as shown in Fig.2. From Table 2 we can infer that Pa3 and Pa4 showed sugar fermentation, Nitrate reductase, catalase test results similar to that of the standard cultures – *P. acnes* MTCC 1951, *P. acnes* MTCC 3297. Pa3 and Pa4 fermented glucose, adonitole. Were positive for indole, catalase and nitrate reductase. Gelatin was hydrolysed by Pa3 and not by Pa4. Similar results were reported by Cummins 1975[26]. The cultures Pa3 and Pa4 which showed most of the biochemical analysis similar to that of the standard culture were subjected to FAME Analysis and 16 S r RNA typing.

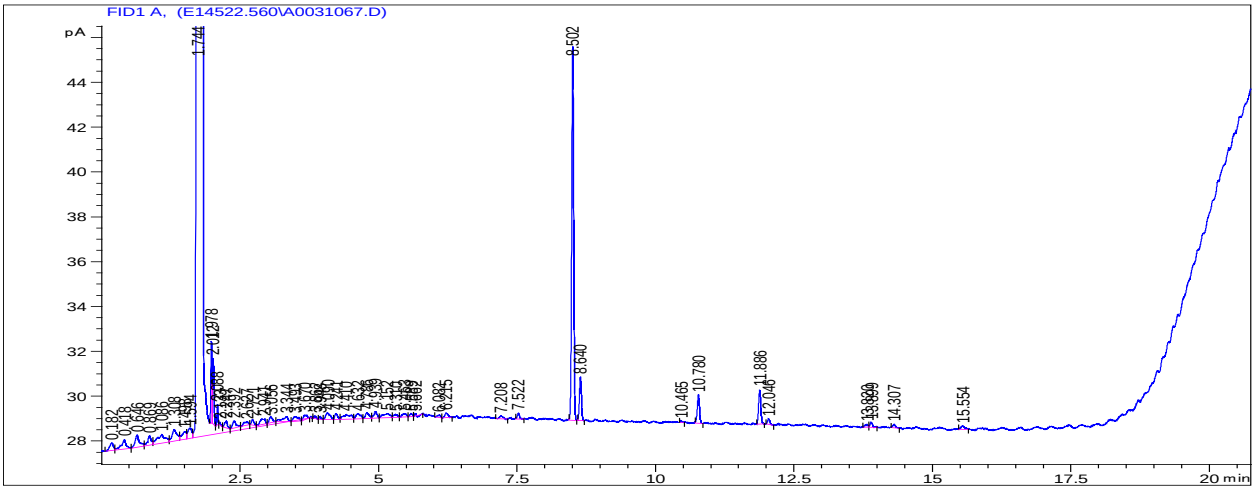
FAME Analysis and 16S r RNA:

The microbial culture Pa3 and Pa4 obtained from acne was analyzed using FAME and sequencing approaches. On comparing FAME results of Pa3 with that of sequencing, it was found that both the methods correlate to each other in confirmation of identity. Fig. 3. shows results corresponding to the fatty acids identified through FAME analysis of bacterial sample. The MIDI Sherlock microbial identification system using ANAER6 method identified the organism to be *Propionibacterium acnes* with 0.446 Similarity Index (SI). The SI is a numerical value, 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. The SI is not a "probability" or percentage, but an expression of the relative distance of the unknown sample from the population mean. Samples with an SI of 0.500 or higher and with a separation of 0.100 between the first and second choice are considered good library comparisons. If the SI is between 0.300 and 0.500 and well separated from the second choice (>0.100 separation), it may be a good match, but an atypical strain (it would fall very far away from the mean on the normal distribution curve).[27] The sequence analysis also showed similar results (Table 3). After the completion of BLAST analysis the organism was identified to be *Propionibacterium acnes with* 100% identity match with Propionibacterium acnes strain: DSM 1897(T) , Accession number AWZZ01000008 (EZ TAXON database). Pa4 did not find any matching chromatogram pattern in Anaerobic (BHIBLA) library.

The present study focused on the isolation, culturing and characterization of *Propionibacterium acnes* from acne. Identification was done based on the colony morphology, gram staining, biochemical tests, FAME analysis and sequencing. Identification of bacteria by conventional methods usually requires ≥48 h after a colony has been isolated. The identification of slow-growing fastidious microbes to the species level is difficult and time-consuming by conventional methods. 16S r RNA 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 [15, 28].

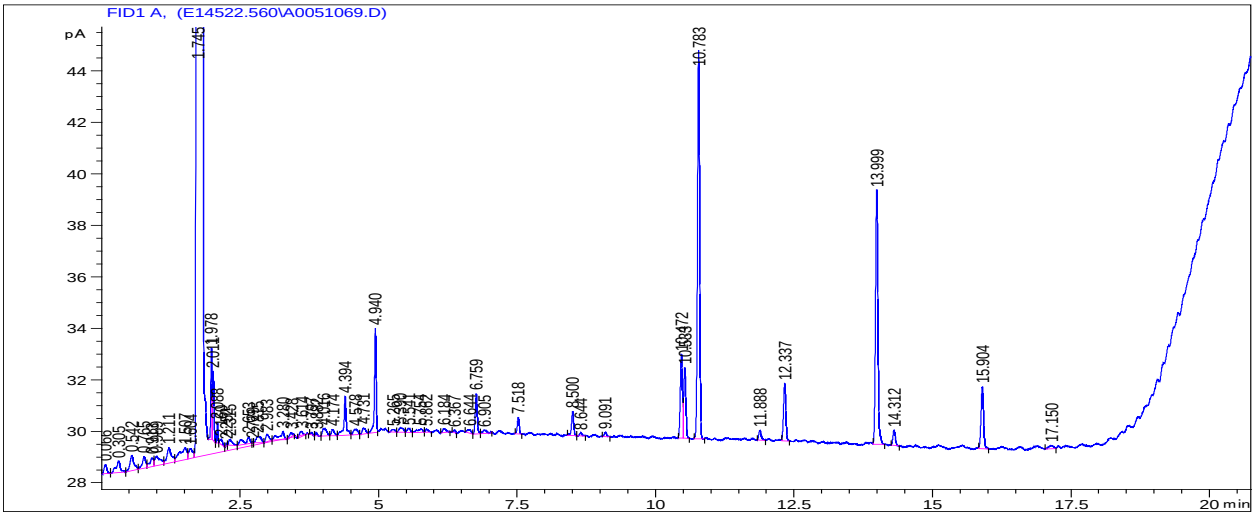
IV. Conclusion

Propionibacterium acnes was isolated from acne and the characterization and confirmation of the same was carried out by conventional methods (Cultural characteristics and biochemical tests), FAME analysis and 16 S r RNA gene sequencing. FAME analysis and sequencing were useful in characterizing the organism up to species level.



S.No	Sample Id	Analysis	Method Used	Result
1	Pa-3	FAME	ANAER6	*Propionibacterium acnes*

Figure 3: Chromatogram of bacterial sample Pa3 showing the fatty acid peaks through Agilent GC 6850



Sample Id	Analysis	Method Used	Result
Pa-4	FAME	ANAER6	*No Match*

Figure 4: Chromatogram of bacterial sample Pa4 showing the fatty acid peaks through Agilent GC 6850

Accession	Description	Pairwise similarity (%)	Different/ Total	Completeness (%)
AWZZ01000008	Propionibacterium acnes strain: DSM 1897(T)	100	0/1398	100
AFAM01000003	Propionibacterium humerusii strain: P08(T)	98.26	24/1397	100
AGBA01000019	Propionibacterium avidum strain: ATCC 25577(T)	96.44	49/1385	100
JQ283460	Propionibacterium olivae strain: IGBL1 (T)	94.4	76/1358	94.73
JQ283461	Propionibacterium damnosum strain: IGBL13(T)	94.36	77/1365	95.7
AOSS01000369	Propionibacterium granulosum strain: DSM 20700(T)	94.32	78/1374	100
AJ704569	Propionibacterium acidopropionici strain: NCFB 563 (T)	94.24	79/1372	98.85
AF234623	Propionibacterium microaerophilum strain: M5(T)	93.74	86/1373	100
AUDD01000055	Propionibacterium jensenii strain: DSM 20535(T)	93.74	86/1373	100
AJ704572	Propionibacterium thoenii strain : NCFB 568 (T)	92.79	99/1373	99.46

Table. 3: Blast Similarity Search Results for Pa3 by 16S r RNA sequencing

ACKNOWLEDGEMENTS

The authors thank UGC for financial assistance and Management of St. Francis College.

REFERENCES

[1] Hywel C Williams, Robert P Dellavalle, Sarah Garner. Acne Vulgaris. The Lancet . Science Direct. (2012) vol.379 (9813)361-372

[2] Bojar RA, Holland KT. Acne and Propionibacterium acnes. Clin Dermatol.2004;22:357–379

[3] Teruaki Nakatsuji, Mandy C. Kao, Jia-You Fang, Christos C. Zouboulis, Liangfang Zhang, Richard L. Gallo and Chun-Ming Huang Antimicrobial Property of Lauric Acid Against Propionibacterium acnes: Its Therapeutic

Potential for Inflammatory Acne Vulgaris. Journal of Investigative Dermatology (2009), Volume 129, 2480–2488

[4] Dali.P, Giugliano. E.R, Vellozi. E.M, Smith. M.A Susceptibilities of Propionibacterium acnes ophthalmic isolates to Moxifloxacin. Antimicrobial Agents and Chemotherapy (2001) 45(10): 2969-70

[5] Bruggeman .H, Henne. A, Hoster .F, Hujer. S, Durre. P, Gottschalk G. The Complete genome sequence of Propionibacterium acnes, a commensal of Human skin. Science (2004) 305 (5684): 671-73

[6] Perry, Alexandra, Lambert, Peter. Propionibacterium acnes: infection beyond the skin. Expert Review of Anti- infective therapy (2011) 9 (12):1149-56

[7] Tancrede, C.. Role of human microbiota in health and disease. Eur. J. Clin. Microbiol. Infect. Dis. 1992. 11:1012–1015

[8] Leyden, J. J., K. J. McGinley, and B. Vowels. Propionibacterium acnes colonization in acne and non-acne. Dermatology (1998)196:55–58.

[9] McGinley, K. J., G. F. Webster, and J. J. Leyden. Regional variations of cutaneous Propionibacterium. Appl. Environ. Microbiol. (1978) 35:62–66.

[10] Makrantonaki; et al. An update on the role of the sebaceous gland in the pathogenesis of acne. Dermatoendocrinology (2011) 22(5):360-6

[11] Holland, Carsten et al., Proteomic identification of secreted proteins of P. acnes. BMC Microbiology (2010) 10: 230.

[12] Kim, Christos C. Review of the innate immune response in acne vulgaris: activation of Toll-like receptor 2 in acne triggers inflammatory cytokine responses. Dermatology (2005) 211 (3): 191-2

[13] Zouboulis, Christos C. Acne and sebaceous gland function Clinical Dermatology (2004) 22 (5):360-6

[14] Understanding acne- News in Health, January 2010. Newsinhealth.nih.gov.

[15] TangY W , Ellis N M, Hopkins M K, Smith, DH, Dodge D E , Persing D H. Comparison of Phenotypic and Genotypic Techniques for Identification of Unusual Aerobic Pathogenic Gram- Negative Bacilli; Clin Microbiol. 1998. 36(12): 3674–3679

[16] Macrae A. The Use Of 16s rDNA Methods In Soil Microbial Ecology .Brazilian Journal Of Microbiology (2000) 31:77-82

[17] Patel J B. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol Diagn. (2001) 6(4):313-21

[18] Janda J M , Abbott S L. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J Clin Microbiol. 2007 September; 45(9): 2761–2764.

[19] Goodfellow, M., and D. E. Minnikin. Introduction to chemosystematics, pIn, Chemical methods in bacterial systematics. Academic Press Ltd., London. (1985). 1– 16.

[20] Kaneda, T. Fatty acids in the genus Bacillus: an example of branched-chain preference. Bacteriol. Rev. (1977). 41:391-418.

[21] Saito, K. Chromatographic Studies on Bacterial Fatty Acids. J. Biochem. (1960). 47:699-709.

[22] Leonard R. B., J. Mayer, M. Sasser, M.L. Woods, B. R. Mooney, B G Brinton, P. L. Newcomb-Gayman, And K C Carroll. Comparison of Midi Sherlock System And Pulsed-Field Gel electrophoresis In Characterizing Strains of Methicillin-Resistant Staphylococcus aureus From A Recent Hospital Outbreak. J Clin microbiol. (1995).33: 10 .

[23] Sasser M . Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids. (2001).MIDI Technical Note # 101

[24] Osterhout G J, Shull V H, Dick J D. Identification Of Clinical Isolates Of Gram-Negative Non fermentative Bacteria By An Automated Cellular Fatty Acid Identification System. J Clin Microbiol.(1991). ;29:1822– 1830

[25] Laboratory Exercises in Microbiology. Harley Prescott. Mc Graw Hill 2002. Pg 133-193.

[26] C.S Cummins. Identification of Propionibacterium acnes and related organisms by precipitin tests with Trichloroacetic acid extracts. Journal of Clinical Microbiology, (1975)p.104-110

[27] Craig Kunitsky, Gerard Osterhout, and Myron Sasser Newark, DE, USA Identification of Microorganisms Using Fatty Acid Methyl Ester (Fame) Analysis And The Midi Sherlock Microbial Identification System, MIDI, Inc. Newark, DE, USA, Encyclopedia of Rapid Microbiological Methods. www.pda.org/bookstore

[28] Surve V V, Patil M U, Dharmadhikari S M. FAME and 16 S r DNA sequence analysis of halophilic bacteria from solar salterns of Goa: A comparative study. International Journal of Scientific and Research Publications (2012) 2(8)

AUTHORS

First Author- Roselin Polugari, M.Sc., M.Phil.,(Ph.D), APSET, St.Francis College for Women, Begumpet, Hyderabad, Telangana, India
Email address: roselinpolugari@gmail.com
Second Author – Dr. M. Shailaja Raj, M.Sc., Ph.D., NET. St. Francis College for Women, Begumpet, Hyderabad, Telangana, India
Email address: shailarm@gmail.com
Third Author –Dr. D. Sailaja, M.Sc., Ph.D., Gokaraju Rangaraju Institute of Technology, Bachupally, Telangana, India
Email address: sailajadarbha1111@gmail.com

Correspondence Author – Roselin Polugari, M.Sc., M.Phil., (Ph.D), APSET ; St.Francis College for Women, Begumpet, Hyderabad, Telangana, India
Email address: roselinpolugari@gmail.com
Contact: 9985452043