Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BIOFILM PRODUCING BACTERIA

KOKKILIGADDA SUJANA, KARLAPUDI P. ABRAHAM, INDIRA MIKKILI AND KODALI V PRABHAKAR*

School of Biotechnology, Vignan University, Vadlamudi, Guntur-522213, Andhra Pradesh, India.

ABSTRACT

Biofilms have been proved to have useful effects on food chains, sewage treatment plants, to eliminate petroleum oil on oceans. They have been found to cause a wide variety of microbial infections in the body, such as urinary tract infections, catheter infection, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses. In the present study, the high yielding biofilm strains have been isolated from the soil. Microtitre plate assay was used to confirm the biofilm producing ability of these bacteria. Two high yielding isolates were identified and characterized. Both the isolates were named as sample 17 and sample 19. The isolates were characterized by biochemical and molecular techniques. The antibiofilm activities of various chemicals and plant extracts have been tested. Ethanol, formaldehyde, hydrogen peroxide showed considerable effect, iodine showed negligible effect on the biofilm formation. The extracts from *Prosopis juliflora, Nerium oleander, Eucalyptus globulus* and *Catharanthus roseus* showed significant reduction on biofilm formation. *Calotropis gigantea, Ocimum sanctum* and *Ricinus communis* did not show any effect on biofilm formation.

KEYWORDS: **Biofilm, Plant extracts, chemical agents, Exopolysaccharide, Microtitre plate assay, Tube method.**

DR. KODALI VIDYA PRABHAKAR
School of Biotechnology, Vignan University, Vadlamudi, Guntur-522213, Andhra Pradesh, India.

INTRODUCTION

Biofilms are composed primarily of microbial cells and exopolysaccharides (EPS)[1]. The role of the biofilm is to attach to abiotic surfaces, the epithelia of multicellular organisms, and interfaces such as that between air and water. Surface adhesion of bacteria is an essential step and it is required for the bacteria to arrange themselves favourably in their environment[2]. Some bacterial biofilms have been reported that they haduseful effects on food chains, sewage treatment plants, to eliminate petroleum oil/hydrocarbon spillage from the oceans[3]. Moreover, they have been found to cause a wide variety of microbial infections in the body, such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaques, gingivitis, coating contact lenses. Biofilms may form on a wide variety of surface, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems[4]. The use of chemical biocides (disinfectants, sanitizers and surfactants) is the common practice in the control of biofilms. Surfactants will interact with the EPS and cause detachment of the biofilm. In the medical industry, efforts to identify agents that prevent cell attachment or initiate detachment from a biofilm are currently being investigated in an effort to reduce infections caused by biofilms[5]. Anti-adhesion compounds have gained a lot of attention in controlling biofilm formation includes milk constituents, plant derived products, low molecular weight chitosan, polymers, dietary constituents, and furanone compounds[6] . The regular use and un-proper use of antibiotics may be lead to drug resistance and will make the drugs ineffective against common microbial infections[7]. Biofilm formation by the microbes is the major factor that is responsible for the microbes to develop resistance that leads to withstand extreme environmental conditions and antimicrobial agents. The EPS is a major feature of biofilms and plays a major role in their resistance. The EPS works as a diffusion barrier, molecular sieve and an adsorbent[8]. Due to the lack of penetration of antimicrobial agents the biofilm producing bacteria are resistant to antimicrobial agents[9]. Chemical antibiofilm agents show their activity through demonstrated nonspecific mechanisms[11]. Unfortunately, chemicals in higher concentrations cannot be used as drug molecules to treat the diseases associated with the biofilm[12]. The alternative to the chemical antibiofilm agents is natural source[13]. Plant derived compounds have been found to have potential applications in pharmaceutical industry. Recently, plant extracts and other biologically active compounds isolated from leaves, stems, and roots have gained interest in the antibiofilm activity [14]. In the present study, the high yielding biofilm strains have been isolated from the soil. Microtitre plate assay was used to confirm the biofilm producing ability of these bacteria. Two high biofilm yielding isolates have been identified and characterized. Both the isolates were named as sample 17 and sample 19. The isolates have been further characterized by biochemical and molecular techniques. The chemical and plant derived compounds that were able to inhibit the biofilm formation have been identified.

MATERIALS AND METHODS

(i) Sample collection and isolation of pure cultures

Sewage sample was collected from the municipal dump yard of Tenali, Andhra Pradesh. Pure cultures were isolated by serial dilutions using nutrient agar medium by streak plate method.

(ii) Microtitre plate assay

The overnight grown cultures were diluted to 1:100 in Nutrient broth medium with 1% glucose. From this 100 µl of medium was added to each of five wells of microtitre plate and incubated for 48 h at 30°C. Planktonic bacteria were removed and the wells were washed with sterile distilled water. 125 µl of crystal violet solution was added to each well and incubated for 10min at room temperature. Excess stain was removed by washing with sterile water. The plate was inverted over

paper towel and tapped vigorously to remove excess of liquid if any and allowed to dry. 200 µl of 95% alcohol was added to each well and allowed the dye to solubilize by incubating for 15 min at room temperature. The contents of each well were then briefly mixed by pipetting and then observed for optical density readings[16].

(iii) Tube method

Nutrient broth medium was prepared and 5 ml of the medium was transferred to each of the test tubes labelled. The tubes were autoclaved and they were inoculated with respective colonies. The inoculated tubes were incubated for 24 h on shaker at room temperature for growth of the organism. The tubes were decanted and washed with phosphate saline (PBS) buffer to remove the planktonic bacteria and were allowed to dry. The dried tubes were stained with 0.1% crystal violet solution, excess stain was removed using distilled water and the tubes were then dried and observed for biofilm production[16].

(iv) Strain identification

The high yielding strains (sample 17 and sample 19) were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Determinative Bacteriology. This is further characterized on the basis of 16S rRNA gene sequencing.

iv. a. Biochemical characterization

Catalase production and Voges– Proskauer reaction and methyl red tests were demonstrated using the standard methods[17]. The patterns of carbohydrate fermentations were determined (Table 1), and the identification of the major acid was done by a method as described earlier[18]. The isolates were tested for the ability to hydrolyze starch, citrate utilization, H_2S production, and indole production by the methods described by Smibert and Krieg[19].

v. 16S r RNA sequencing:

The high yielding strains (sample 17 and sample 19) were sent to Helini biomolecules, Chennai for 16s rRNA sequencing. Basic local alignment search tool (BLAST) was performed forthesequenceobtained(http://blast.ncbi.nlm.nih.gov/) and a phylogenetic tree was constructed to identify the isolated microorganisms(http://www.ncbi.nlm.nih.gov/About/primer/phylo.html).

vi. Extraction and estimation of the EPS

The overnight cultures of six high yielding strains were taken into vials and centrifuged at 10,000 rpm for 20 min at 4°C to remove bacterial cells. The obtained supernatant was collected into a fresh vial and precipitated with two volumes of absolute chilled ethanol by incubating the mixture at 4°C for overnight. The precipitated EPS was collected by centrifugation at 10,000 rpm for 20 min at 4°C and the supernatant was decanted. The pellet containing EPS was dried at room temperature[20]. The obtained EPS was estimated for the carbohydrate content using phenol sulphuric acid method[21].

vii. Preparation of plant extracts

Powdered plant material (1.0 g) was separately extracted with methanol (10 ml). Supernatant was filtered and concentrated to dryness at controlled temperature (60 ± 2°C). Total phenolic content of the extracts was measured by Folin-Ciocalteu method.

viii. Concentrations of the chemicals

The concentrations of the chemicals which are less than the minimum inhibitory concentration i.e., half of the MIC were calculated. The MIC intervals, which reduced bacterial populations over 6 log10, were: 39 to 246 mg/l of formaldehyde; 43750 to 87500 mg/l of ethanol; 1250 to 6250 mg/l of iodine in polyvinyl-pyrolidone complexes, and 469 to 2500 mg/l of hydrogen peroxide.

ix. Study on the effect of plant extracts and chemicals on biofilm formation

The effect of plant extracts and chemicals on the preformed biofilm was qualitatively estimated by growing cells. Plant extracts and chemicals of different concentrations (ethanol: 109 µl, formaldehyde: 0.6 µl, iodine: 0.6 µl, hydrogen peroxide: 0.6 µl) were added to the tubes and incubated for 24 h at 37°C. The concentrations of the plant extracts were ranged from 10 to 78 µg/ml. The medium

without extracts and chemicals were used as a control. After incubation, samples from each tube including control were collected on to their respectively labelled clean glass slides using inoculating loop and prepared a smear. Then the slides were air dried and stained with 0.1% (w/v) Crystal Violet [14] (Sigma-Aldrich, Germany).

RESULTS AND DISCUSSION

1. Isolation of microorganisms

Microorganisms were isolated from sewage sample and independent colonies were obtained by serial dilution. 24 Colonies with different morphological features were selected and the numbers were given to each colony.

2. Screening for biofilm producing ability of isolated bacteria

Based on the results obtained by microtitre plate assay method, the microbial colonies have been classified into three different biofilm producers' i.e., strong, moderate, and non producers. The colony that formed a visible film lined on the wall and bottom of the tube was considered as "Biofilm positive". The samples that showed strong biofilm production are 10, 11, 12, 13, 17 and 19. The moderately stained tubes indicating the moderate biofilm producers are 2, 3, 5, 6, 16, 20 and 24. The samples that showed no biofilm production are 1, 4, 7, 9, 14, 15, 18, 21, 22 and 23 (As shown in Fig. 1).

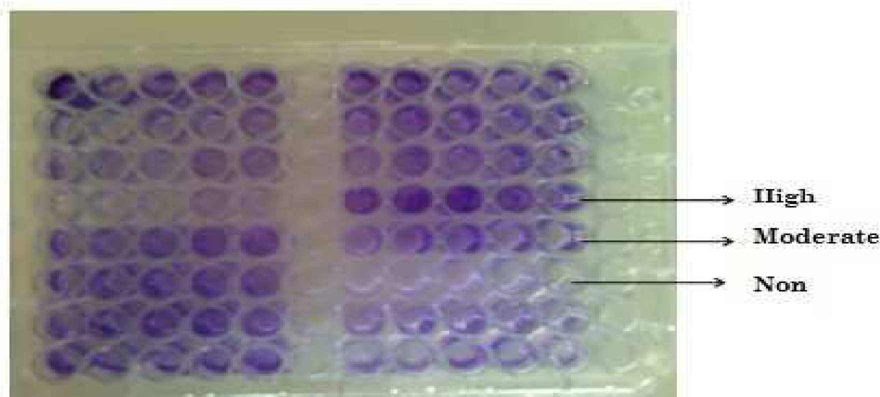

Figure1
Microtitre plate showing high, moderate and no growth of biofilm

The biofilm producing ability of the isolated colonies was tested. Colony numbers 17, 19 (Sample 17, 19) were found to be very high biofilm producing strains by microtitre plate (crystal violet) assay. The more concentrated the stain, the greater the biofilm (Fig. 2).

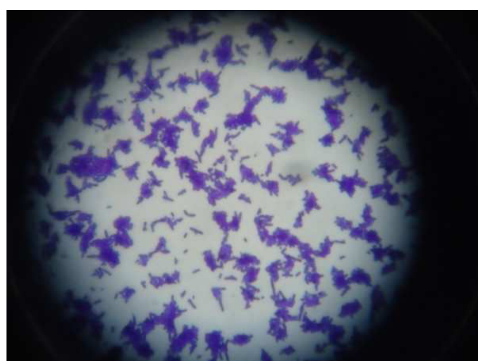

Figure 2
Microscopic picture of biofilm of high yielding strain

3. Confirmation of biofilm production by tube method

The samples that showed strong biofilm production are 10, 11, 12, 13, 17 and 19. The moderately stained tubes indicating the moderate biofilm producers are 2, 3, 5, 6, 16, 20 and 24). The samples that showed no biofilm production are 1, 4, 7, 9, 14, 15, 18, 21, 22 and 23.

Figure 3
Tubes with moderate Tubes with high Staining Tubes with no staining Staining

4. Biochemical characterization

Various biochemical tests have been performed for high biofilm yielding strains i.e sample 17 and sample 19. For carbohydrate metabolism sample 17 showed positive result for glucose, negative result for sucrose and lactose, sample 19 showed negative result for glucose, lactose and sucrose. Sample 17 and sample 19 showed negative results for Citrate utilization, Indole production, MRVP and H_2S production tests, both the samples 17 and 19 showed hydrolysis of starch. The results of biochemical tests were presented in Table No. 1.

Table 1
Biochemical characteristics of sample 17 and sample 19

S.No	Biochemical test	Sample 17	Sample 19
1.	Catalase	Positive	Positive
2.	Carbohydrate metabolism		
	a. Lactose fermentation	Negative	Negative
	b. Sucrose fermentation	Negative	Negative
	c. Glucose fermentation	Positive	Negative
3.	Citrate utilization	Negative	Negative
4.	H_2S production	Negative	Negative
5.	Starch hydrolysis	Positive	Positive
6.	Indole production	Negative	Negative
7.	MR	Negative	Negative
8.	VP	Negative	Negative

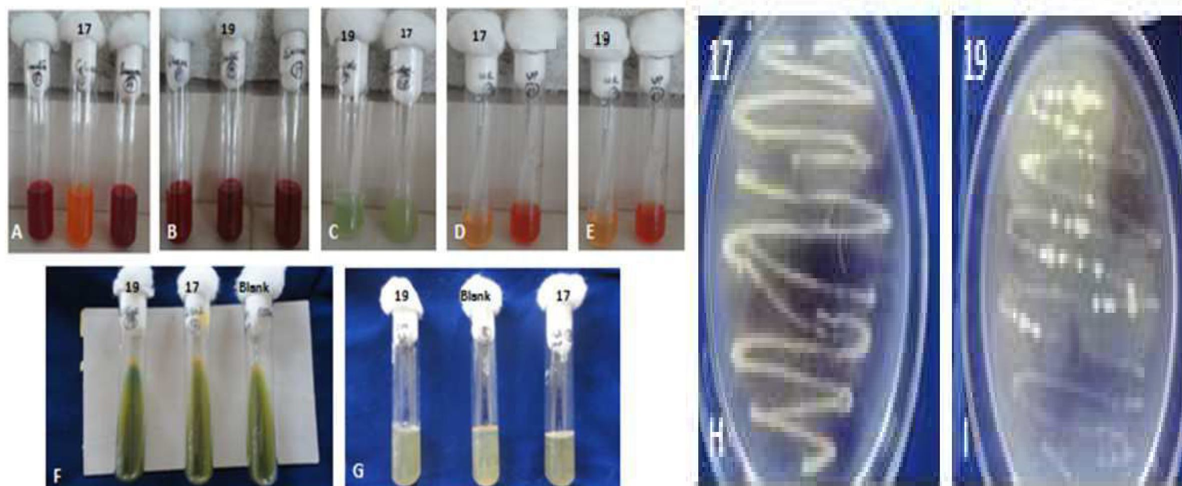

Figure 4

Images of results of various biochemical tests- carbohydrate metabolism: A-sample 17 showing positive result for glucose, negative result for sucrose and lactose; B:Sample 19: showing negative result for glucose, lactose and sucrose; C: Sample 17 and sample 19 showing negative result for Indole production; D&E: sample 17 and sample 19 showing negative result for MRVP respectively; F: sample 17 and sample 19 showing no utilization of citrate; G: sample 17 and sample 19 showing no H_2S production; H (sample 17), I (sample 19): showing hydrolysis of starch.

5. 16S r RNA sequencing

Since 16S rDNA sequences are highly conserved, multiple sequence alignment was done with DNA sequences of other Aceneto bactor sp. obtained from GenBank. The multiple alignments showed that among the high conserved region of 16S rDNA, a stretch of the DNA sequence at the starting of Sample 17 matches with *Acinetobacter sp. Sea-9 and sample* 19 matches with *Acinetobacter bouvetii* rather than other *Acinetobactor* strains (Fig. 1). Phylogenetic tree was also constructed with the same sequences of *Acinetobactor* sp. Using neighbor-joining method of Clustal W.

5. a. Fasta format of sample 17

The nucleotide sequence of sample 17 obtained after 16S r RNA analysis is

TGCCGTTACCGGCAAGCTAACACATGCAAGTCGAGCGGAGCGAGGGTGCTTGCACCTTAGCTTAGCG
GCGGACGGGTGAGTAAAGCTTAGGAATCTGCCTATTAATGGGGGACAACCTTCCGAAAGGGATGCTA
ATACCGCATACGTCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAAATGAGCCTAAGT
CGGATTACCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGA
TCCGCCACACTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCACCAGTGGGGAATATTGGACA
ATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAAAAGGCCTTTTGGTTGTAAAGCACTTTAA
GCGAGGAGGAGGCGCTCTAGGATAATACCCTAGATGCGTGGACGTTACTCGCAGAATAAGCACCGGC
TAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAA
GCGCGCGTAGGTGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATA
CTGGTTAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAACGGTGAAATGCGTATAGATCTG
GAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACACTGAGGTGCGAAAGCATGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGA
GGCTTTAGTGGCGCAGCTAACGCGATAAGTATACCGCCTGGGGGAGTACGGGTCGCAAGACTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGAGCATGTGGTTTAAATTCGATGCAACGCGAAGA
ATCCTATCCTGGCCTTGACATACAGAGAACTTTTCACGAGATGGAATTGTGCCTTCGGGAACTCTGATA
CAGTGCTGCATGGCTGTCGTCAACTCGTGTCCTGAGATGTTGGCTAAGTTCCCGCCACGAGCGCAAC
CTATTCTAACTGCAGCAATTCGATGCAACTACGATACTGCATGACACTGAGAGCGCACACGTCAGTCA
CATGCCTTACGACAGCTACAACGTGCC

This article can be downloaded from www.ijpbs.net

6. *Phylogenetic tree analysis of sample 17*

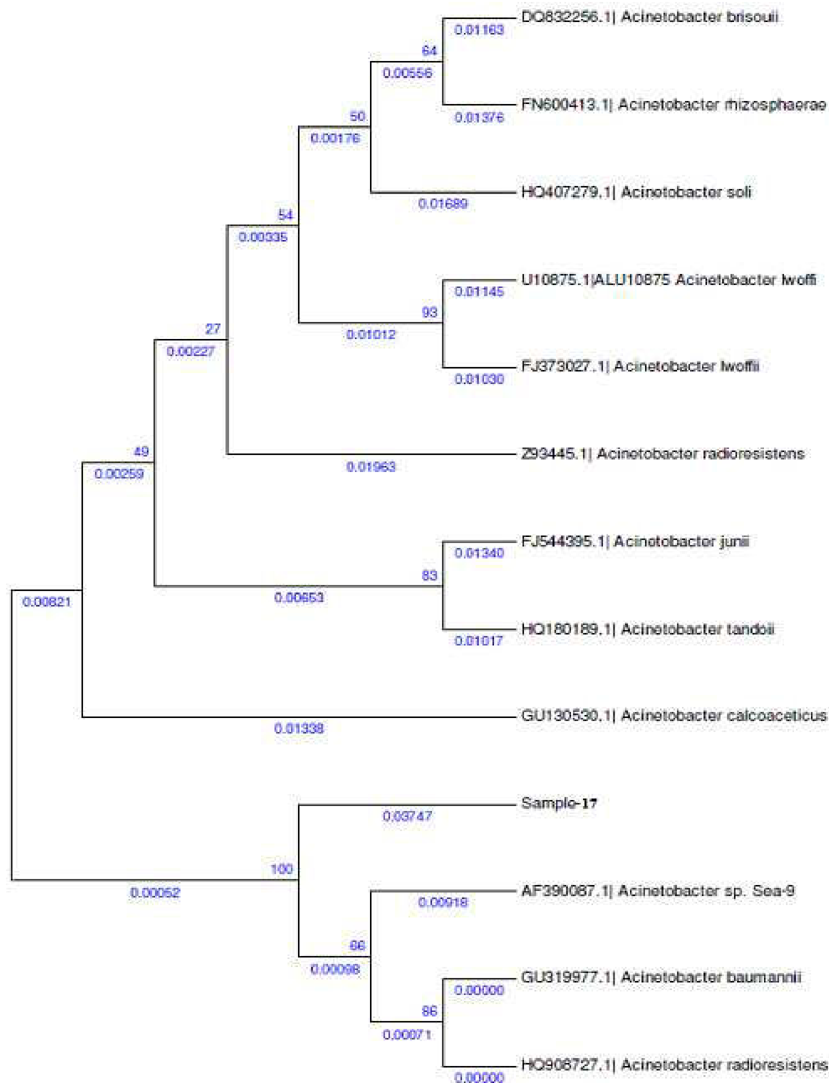

Figure 5

phylogenetic tree analysis of sample 17 Based on the phylogenetic tree analysis, sample 17 was closely related to Acinetobacter sp. Sea-9. The percentage similarity is 100% with the above species, so the isolate sample 17 could be Acinetobacter sp. Sea-9.

7. *FASTA format of sample 19*

The nucleotide sequence of sample 19 obtained after 16S r RNA analysis is

ACCATATCCCCACGGCCTTAAAAATGCAAGTCGAGCGGGGGGAAGGTAGCTTGCTATTTAACCTAGCG
GCGGACGGGTGAGAAGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGGATGCTAAT
ACCGCATACGCCCTAAGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTC
GGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGAT
CCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAA
GCGAGGAGGAGGCTACCGAGACTAATACTCTTGGATAGTGGACGTTACTCGCAGAATAAGCACCGGC
TAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAA
GCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATA
CTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCT
GGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTG

This article can be downloaded from www.ijpbs.net

AGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGATGCAACGCGAGAA
CCTTACCCTGGTCTTGACATAGTAAAGAACTTTCCAGAGATGGAATTGTGCCTTCGGGACTACATACAG
GTGCTGCATGCTGTCGTCAGCTCGTGTCGTGGAGATGTAGGGTTAAGTTCCCGCAACGGAGCGCACC
TTTCATACTTGCAGCACTTCGGATGGACCTTAGGATACTGCAGGTGACAACCTGAGATGGCT

8. Phylogenetic tree analysis of sample 19

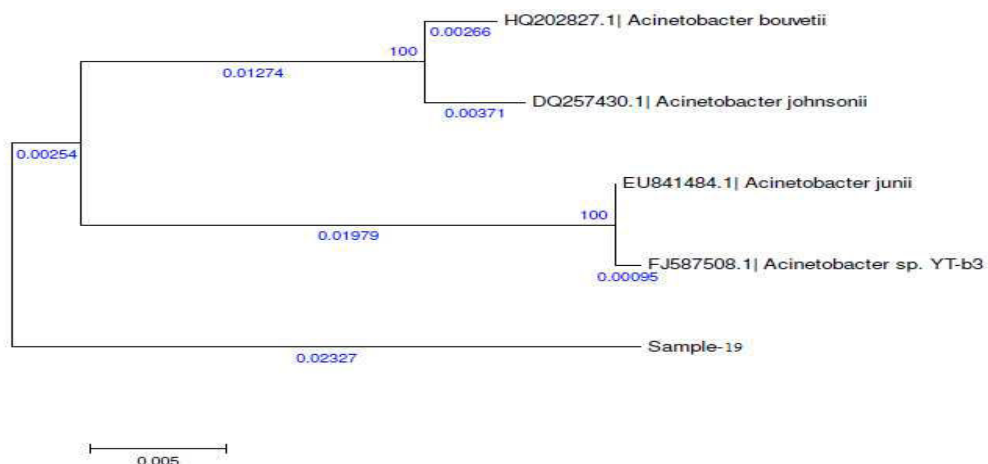

Figure 6
Phylogenetic tree analysis of sample 19

Based on the phylogenetic tree analysis, sample 19 was closely related to *Acinetobacter bouvetii*. The percentage similarity is 100% with the above species, so the isolate sample 19 could be *Acinetobacter bouvetii*. To establish the strain identity, 16S rDNA sequencing was performed, which is the most useful molecular chronometer to infer phylogenetic relationships, because they are present in all organisms. BLAST analysis revealed that the sample 17 has significant homology with other gene sequences of *Acinetobacter sp. Sea-9* strain and sample 19 has significant homology with other gene sequences of *Acinetobacter bouvetii*. Phylogenetic tree constructed using neighbor joining method further groups the *Acinetobacter* strains. Thus, the results of both multiple alignment and phylogenetic tree analysis provide a reasonable agreement in confirming the strains of *Acinetobacter species.*

9. EPS estimation

The total sugar concentration was determined by the phenol-sulfuric acid method using glucose as a standard. Sample 17 produced the EPS concentration of 78µg/ml and sample 19 produced a concentration of 34µg/ml and by this method rest of the samples showed undetectable amounts of EPS.

10. Effect of chemicals on pre-formed biofilm of samples 17 and 19

In the present study, out of the 4 chemicals used, ethanol, formaldehyde, hydrogen peroxide showed considerable effect, iodine showed negligible effect on the growth of the 24 hour culture of the sample 17. Formaldehyde, ethanol, hydrogen peroxide added tubes showed significant reduction in growth where as iodine showed negligible effect on the growth of the sample 19 (Fig. 7 A and B).

This article can be downloaded from www.ijpbs.net

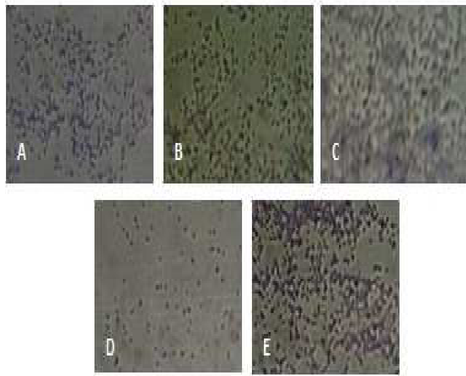

Figure 7A

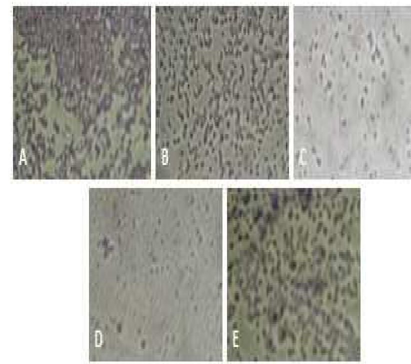

Figure 7B

Figure 7
Microscopic images of biofilms formed by sample 17 (fig: 7A) and sample 19 (fig: 7B) that are treated with different chemicals. A: control, B: Formaldehyde treated biofilm, C: Hydrogen peroxide treated biofilm, D: Ethanol treated biofilm, E: Iodine treated biofilm.

11. Effect of plant extracts on sample 17 and sample 19

Extracts from *Prosopis juliflora, Nerium oleander, Eucalyptus globulus* and *Catharanthus roseus* showed reduction in biofilm production significantly. *Calotropis gigantea, Ocimum sanctum* and *Ricinus communis* did not show any effect on biofilm formation by both sample 17 and sample 19 (Fig. 8). It was clearly indicated that there was tubes incubated with *Prosopis juliflora, Nerium oleander, Eucalyptus globulus* and *Catharanthus roseus* showed reduction in cell concentration/biofilm concentration indicated that there was reduction in biofilm production. The antimicrobial activity of these plant extracts may be due the presence of phenolics, alkaloids, flavonoids, terpenoids and polyacetylenes. Shan et al., 2007 reported that the antimicrobial activity of the plant extract is majorly attributed to the presence of phenolic compounds[22]. It would be very interesting to investigate the type of phenolic compounds responsible for the antibiofilm activity of plant extracts of *Prosopis juliflora, Nerium oleander, Eucalyptus globulus* and *Catharanthus roseus* and this would be future scope of our study[23, 24].

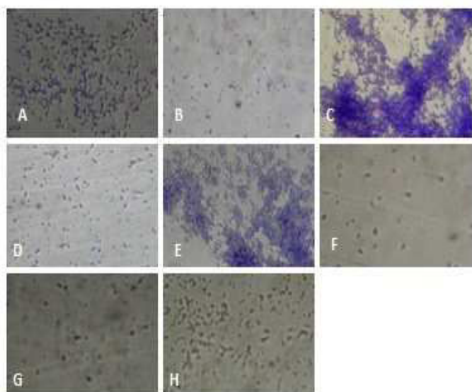

Figure 8A

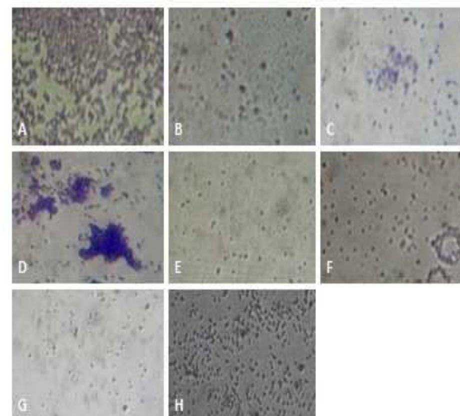

Figure 8B

Figure 8
Microscopic images of biofilms formed by sample 17 and sample 19 that are treated with different plant extracts. A: Control, B: Catharanthus roseus, C: Prosopis julifora, D: Ricinus communis, E: Ocimum tenui florum, F: Nerium oleander, G: Eucalyptus globulus, H: Calotropis gigantea

CONCLUSION

The main aim of this study was to isolate biofilm producing bacterial organisms and identification of the antibiofilm agents of plant origin. Many researchers are focused on the therapeutic and pharmacological effects of plant extracts. The antimicrobial compounds from plant source have increasing attention in recent years. Some of the selected plants, *Ricinus communis, Nerium Oleander, Prosopis juliflora, Ocimum tenuiflorum* and *Eucalyptus globulus* showed good antibiofilm activity. Hydrogen peroxide and ethanol in the case of chemicals exhibited significant antibiofilm activity. The EPS produced by the isolated strains can be further characterized by using various analytical techniques like HPLC, GC, GC-MS and NMR. The potential phenolic compounds of leaf extracts can further be characterized to develop these leaf extracts as drugs in curing the various infections caused by *Acinetobacter sps.*

ACKNOWLEDGEMENT

The authors would like to thank Management of Vignan University for providing laboratory facilities to carry out this study and other faculty members of the Biotechnology Department for their cooperation.

REFERENCES

1. Flemming HC, Wingender J, Griegbe and Mayer C, Physico-chemical properties of biofilms. In: Evans LV, editor Biofilms: recent advances in their study and control. Harwood Academic Publishers: 19–34, (2000).
2. Kodali VP and Sen R, Partial **s**tructural elucidation of an antioxidative exopolysaccharide from a probiotic bacterium, Journal of Natural Products, 8:1692–1697, (2011).
3. Djordjevic D, Wiedmann M. and Mclands borough LA, Microtitre plate assay for assessment of *Listeria monocytogenes* biofilm formation, Applied and Environmental Microbiology, 68:2950–2958, (1999).
4. Donlan RM, Microbial life on surfaces, Emerg Infect Dis, 8:881-890, (2002)
5. Carneiro , Helcio Silva dos Santos, Francisco Vassiliepe Sousa Arruda, Paulo Nogueira Bandeira , Maria Rose Jane Ribeiro Albuquerque , Maria Olívia Pereira ,Mariana Henriques , Benildo Sousa Cavada and Edson Holanda Teixeira, Casbane Diterpene as a Promising Natural Antimicrobial Agent against Biofilm-Associated Infection, Molecules, 16:190-201, (2011).
6. Essawi T and Srour M, Screening of some Palestinian medicinal plants for antibacterial activity, Journal of Ethnopharm, 70:343–349, (2000).
7. Dimopoulus G and Falagas ME, Gram-negative bacilli – resistance issues, Touch Briefing, 49–51, (2007).
8. Boyd A and Chakrabarty AM, Role of alginate lyase in cell detachment of pseudomonas aeruginosa, Appl Environ Microbiol, 60: 2355–2359, (1994).
9. Tichy J, Novak J, Extraction, assay, and analysis of antimicrobials from plants with activity against dental pathogens (*Streptococcus* sp.), J Altern Complement Med, 4:39-45, (1998)
10. Burton E, Yakandawala N, Lovetri L and Madhyastha, Microplate spectrofluorometric assay for bacterial biofilms, J Ind Microbiol Biotech, 34(1): 1-4, (2006).
11. Marshall M, Cancro L and Fischman S, Hydrogen Peroxide: A review of its uses in dentistry, J Periodontol, 66(9):786-96, (2006).

12. Costerton JW, Stewart PS and Greenberg EP, Bacterial biofilms: a common cause of persistent infections, Science, 284:1318-1322, (1999).
13. Absalon S, Kohl L, Branche C, Blisnick T, Toutirais G, Rusconi F,Cosson J, Bonhivers M and Robinson D, Bastin P Basal body positioning is controlled by flagellum formation in *Trypanosoma brucei*, PLoS ONE, 2:437, (2007).
14. Abraham PK, Srinivas J, Venkateswarulu TC, Indira M, John babu D., Diwakar T and Vidya PK, Investigation of the Potential Antibiofilm Activities of Plant Extracts, Int J Pharm and Pharm Sci, 4:282-285, (2012).
15. Xiao J, Zuo Y, Liu Y, Li J, Hao Y, Zhou X. Effects of *Nidus Vespae* extract and chemical fractions on adherence and biofilm formation of *Streptococcus mutans*, Arch Oral Biol, 52:869-875, (2007).
16. Christensen GD, Simpson WA, Bisno AL and Beachey EH, Adherence of slime–producing strains of *Staphylococcus epidermidis* to smooth surfaces, Infect Immun, 37:318–326, (1982).
17. Cowan ST, and Steel KJ Manual for identification of medical bacteria, 2nd edn. Cambridge University Press, Cambridge, 8–96(1974).
18. Smibert RM, Krieg NR Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA and Krieg NR, Methods for general and molecular bacteriology, ASM, Washington, DC, 607–654 (1994)
19. Baron JH, Gastric secretion, Mt Sinai J Med, 67:32–36 (2000).
20. Smitinont T, Tansakul C, Tanasupawat S, Keeratipibul S, Navarini L, Bosco M and Cescutti P, Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods, Isolation, identification and exopolysaccharide characterization, Int J of Food Microbiol, 51:105-111 (1999).
21. Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F, Colorimetric method for determination of sugars and related substances, Anal Chem, 28:350-356, (1956).
22. Shan B, Cai Y, Brooks JD and Corke H, The in vitro antibacterial activity of dietary spice and medicinal herb extracts. International Journal of Food Microbiol, 117:112–119 (2007).
23. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH and Koo H, Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms, FEMS Microbiol. Lett, 257:50-56 (2006).
24. Asish S and Sharma RA, Antibacterial and antifungal activity of various whole plant extracts of aerva tomentosa forsk. (amaranthaceae), Int J. Pharm. Biosci. 2: 184-192 (2011).