Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 4, 2012

Research Article

INVESTIGATION OF THE POTENTIAL ANTIBIOFILM ACTIVITIES OF PLANT EXTRACTS

KARLAPUDI P. ABRAHAM[1]., JAGARLAPUDI SREENIVAS[1], TIRUPATI C. VENKATESWARULU[1]., MIKKILI INDIRA[1]., DULLA JOHN BABU, TELLA DIWAKAR[2], KODALI V. PRABHAKAR[1,*]

School of Biotechnology, Vignan University, Vadlamudi, Guntur-522213, Andhra Pradesh, Department of Chemistry, Noble PG College, Machilipatnam 521001, Andhra Pradesh. Email: kodalividyaprabhakar@gmail.com.

Received: 19 July 2012, Revised and Accepted: 06 Sep 2012

ABSTRACT

Microbial biofilms cause a variety of microbial infections on human health, such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaques, gingivitis, coating contact lenses. In the present study, the high yielding biofilm strains have been isolated from the soil and characterized. Microtitre plate assay was used to confirm the biofilm producing ability of these bacteria. The antibiofilm compounds from different plant sources have been identified. The parameters that influence the biofilm formation were studied. The growth profiles of the isolated strains were monitored in different carbon and nitrogen sources and biofilm production at different pH and temperatures were screened. It was observed that the biofilm formation by these bacteria was growth dependent. The leaf extract of Pongamia pinnata showed significant antibiofilm activity.

Keywords: Biofilm, *Pongamia pinnata*, Plant extract, Exopolysaccharide, Microtitre plate assay.

INTRODUCTION

The biofilm is an extracellular matrix that surrounds microbial cells and is comprised of biological polymers such as exopolysaccharide (EPS), protein, and DNA [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 [2]. Surface adhesion of bacteria is an essential step and is required for the bacteria to arrange themselves favourably in their environment [3]. Some bacterial biofilms have been reported to have useful effects on food chains, sewage treatment plants, to eliminate petroleum oil/hydrocarbon spillage from the oceans [4]. 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 [5]. The formation of biofilms depends on physical factors, such as composition of the nutrient media, pH, and biological factors. Most of the bacterial biofilm formation is growth dependent. Hence, it is important to know the biofilm formation is growth dependent or growth independent [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]. The main factor contributing to microbial resistance is the biofilm formation by the microbes that allow them to withstand extreme environmental conditions and antimicrobial agents. The biofilm forming bacteria are resistant to antimicrobial agents due to the lack of penetration of antimicrobial agents [8].

In recent years, the much of the research have been focused to identify alternative medicines to treat the infections caused by the drug resistant organisms. Various chemicals have been tested for their antibiofilm activities. Unfortunately, those chemicals cannot be used as drug molecules to treat the diseases associated with the biofilm [9]. The alternative to the chemical antibiofilm agents is natural source [10]. Plant derived molecules have been found potential applications in pharmaceutical industry. Plant extracts and other biologically active compounds isolated from leaves, stems, and roots have gained interest in the antibiofilm activity[11]. In the present study, two soil bacterial isolates were isolated and identified as biofilm producing strains. Both the isolates were named as sample 17 and sample 19. The isolates are being characterized by biochemical and molecular techniques further. The biofilm producing ability and antibiofilm activity of plant extracts against sample 17 have been reported. The microbial strains have been characterized in terms of their biofilm formation ability, growth and pH profiles [12]. Different physical parameters that affect the biofilm formation were studied and various plant extracts have been investigated for their antibiofilm activity [13].

MATERIALS AND METHODS

Microorganisms and Biofilm assay

The isolate (Sample 17) was grown in nutrient broth (Hi media, Mumbai). The biofilm producing ability of the microbes was tested by staining the heat fixed cells with crystal violet. The slides were observed under the microscope (Olympus). The biofilm was further assessed by using the crystal violet (CV) assay done in microtitre plate [14]. The procedure involved washing the plates after incubation, three times with sterile distilled water to remove loosely associated cells. The plates were air-dried and then oven-dried at 60°C for 45 min. Following drying, the wells were stained with 100 µL of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed 5 times with sterile distilled water to remove unabsorbed stain.

EPS extraction

The biofilm was quantitatively estimated in terms of the quantity produced by the microbe. The EPS was extracted according to Smitinont et al [15]. The overnight culture of sample 17 was 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. The total carbohydrate content in the EPS was estimated by phenol-sulphuric acid method [16].

Study of the effect of nutrients on biofilm formation:

Different growth media (MRS broth, Czapek-dox broth, Saborouds broth, Nutrient broth, Mannitol broth) were used to understand the biofilm was nutrient dependent or not. A loopful of the culture broth of sample 17 was inoculated into 50 mL of each of the different media selected. The conical flasks were then incubated for 7 days at 37°C. For every 24 h sample was collected from each of the five different inoculated medium, O.D was measured at 540 nm and the EPS was estimated by phenol-sulphuric acid method [17].

Study of the effect of pH on biofilm formation

Nutrient broth (50 mL) with different pH (7, 8, 9 & 10) were inoculated with sample 17 and incubated for 7 days at 30°C. For every 24 h sample was collected from each of the medium, O.D was measured at 540 nm and the EPS was estimated by phenol-sulphuric acid method [17].

Optimization of temperature

Nutrient broth (50 mL) was prepared (4 flasks) and inoculated with sample 17. The conical flasks were incubated at different temperatures (25°C, 30°C, 37°C, 45°C), the sample from each flask was collected, O.D was measured for 7 days. For every 24 h sample was collected from each of the medium and observed O.D at 540nm and the EPS was estimated by phenol-sulphuric acid method [17].

Investigation of the antibiofilm activity of plant extracts

Plant material

Leaves of plants (*Coriandrum sativum, Mentha avensis, Pongamia pinnata, Azadiractha indica, Aloe vera, Eucalyptus globulus*) were obtained and were air-dried at room temperature and then ground with a grinder into fine powders which were stored into airtight containers at room temperature. The list of studied plants is given in Table 4.

Preparation of plant extract

Powdered plant material (1.0 g) was separately extracted with Methanol, 0.1 N HCl, and 0.1 N NaOH (10 mL). Supernatants were filtered through a funnel with glass wool and concentrated to dryness at controlled temperature (60 ± 2°C). Extracts of *Coriandrum sativum, Mentha avensis, Pongamia pinnata, Azadiractha indica, Aloe vera and Eucalyptus globulus* have been tested for their antibiofilm activities. Total phenolic content of the extracts was measured by Folin-Ciocalteu method [18,19].

Study of the effect of plant extracts on EPS formation

The effect of plant extracts on the formation of biofilm was qualitatively estimated by a method described by Xiao et al [20]. (40 µL of exponentially growing cells were dispensed in 96-well cell culture plates. Plant extracts of different concentrations were added to the wells and incubated for 24 h at 37°C. The concentrations of extracts were ranged from 27 to 73 µg/mL. The medium without extracts was used as the non-treated control. It was observed that only methanolic extracts showed significant antibiofilm activity. After incubation, media and unattached cells were decanted and washed with Phosphate Buffer Saline (PBS). Then the plate was air dried and stained with 0.1% (w/v) Crystal Violet (Sigma-Aldrich, Germany). In order to estimate the biofilm quantitatively, the overnight grown cultures of sample 17 was inoculated into test tubes containing 10 mL of MRS broth and incubated for 24 h for biofilm development. After 24 h of incubation the plant extract (0.1 mL) was added and the tubes were incubated for next 24 h. The biofilm formation was expressed in terms of EPS which was estimated by Phenol-Sulphuric acid method [17].

RESULTS AND DISCUSSION

Isolation of microorganisms and Biofilm Assay

Microorganisms were isolated from soil and independent colonies were obtained by serial dilution of soil sample. The numbers were given to each colony. The biofilm producing ability of the isolated colonies was tested. Colony number 17 (Sample 17) was found to be high biofilm producing strain. The microtitre plate (crystal violet) assay indicating the effect of extracts on biofilm formation. The more concentrated the stain, the greater the biofilm.

Isolation and Estimation of the EPS

The high biofilm yielding strain was grown in different nutrient media and the EPS was isolated and estimated in different time intervals. It was observed that the biofilm production by the microbe was increased with time till 72 h and the increment in the yield of EPS was not significant after 72 h. This indicated that the biofilm formation was growth dependent as the organism entered into the stationary phase in 60- 66 h. It was also observed that the EPS production was high in MRS medium when compared to the other media (Table 1).

Table 1: Biofilm formation by sample 17 in different nutrient media

Medium used	EPS concentration (µg/mL) in different time intervals [§]		
	24 h	48 h	72 h
MRS	6.2	18.4	26
Czapek-dox	1.9	3.6	4.0
Saborouds	4.3	13.3	17.6
Nutrient Broth	5.7	17.6	23
Mannitol Broth	2.0	3.7	4.2

[§]Average of three values

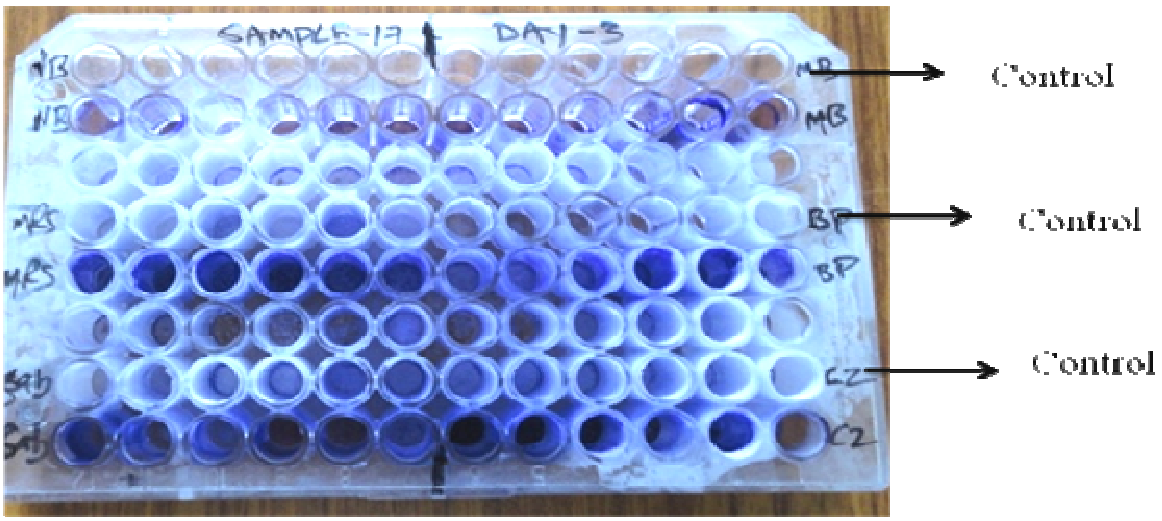

Fig. 1 Microtitre plate assay of biofilm formation for sample 17. (N.B- Nutrient broth; MRS-de Man Rogosa and Sharpe; SAB-Saborouds; CZ-Czapek-dox; MB-Mannitol; B.P-Beef peptone)

The EPS formation is maximum in MRS medium which was evident from microtitre plate assay (Fig. 1). The maximum growth of the isolate was observed in MRS medium which confirmed the biofilm formation was growth dependent. Hence, MRS medium was used for further studies of the bi ofilm formation.

Effect of pH on EPS production by sample 17

To understand whether the EPS formation was pH dependent or not, the organism was grown in different pH conditions. The results clearly showed (Table 2) that there was no significant difference on EPS production was observed at pH-7, pH-8 and pH -9 and pH-10 showed similar significant effect on EPS. There was no effect observed at pH-3, pH-4, pH -5 & pH-6. EPS production was maximum at 72 h and there was significant change observed between 24 h and 48 h of EPS production.

Effect of Temperature on EPS production

The biofilm production is temperature dependent. *Bacillus cereus* produces EPS at 37°C, *B. licheniformis* produces at 35°C [21]. This study was aimed to understand whether the biofilm production by the isolated microorganism was temperature dependent or not. The results showed that the EPS production was significant at 37°C and there was no significant EPS production was observed at 30°C. It was also noted that the isolated microorganism did not grow at 25°C & 45°C. EPS production is maximum during 72 h. There was a mild change observed between 24 h and 48 h. But there was significant change in EPS production was observed during 24 h and 72 h (Table 3). From these results, it was concluded that the isolated organism produced the EPS at stationary phase and the optimum temperature required for the EPS production was 37°C. The isolated organism cannot grow at 25°C and 45°C. The growth of the organism and EPS production were growth dependent.

Table 2: EPS production at different pH ranges incubated for 3 days at 37°c

S. No.	Day	EPS concentration (µg/mL) at different pH (MRS Medium)							
		3	4	5	6	7	8	9	10
1.	Day 1	No Growth				5.1	6.3	6.9	6.9
2.	Day 2					15	17.2	19	20
3.	Day 3					21	24	27	29

§ Average of three values

Table 3: EPS production at different temperatures incubated for 3 days

S. No.	Day	EPS concentration at different temperatures in MRS Medium (µg/mL)			
		25°C	30°C	37°C	45°C
1.	Day 1	No Growth	5.4	8.9	No Growth
2.	Day 2		14.7	16.6	
3.	Day 3		20.3	26	

§ Average of three values

Table 4: The effect of plant extracts (Methanolic) on biofilm (EPS) formation by sample 17 at 37°C

S. No.	Plant source	Concentrations used (µg/mL)	EPS concentrations for sample 17§ (µg/mL)
1.	*Coriandrum sativum*	27.0	7.7
2.	*Mentha avensis*	69.1	7.9
3.	*Pongamia pinnata*	61.5	5.1
4.	*Azadiractha indica*	73.0	7.2
5.	*Aloe vera*	51.0	7.7
6.	*Eucalyptus globulus*	66.7	7.6
7.	Control (without plant extract)	Nil	5.8

§ Average of three values

Effect of plant extracts on EPS formation of sample 17

Some of the polyphenolic compounds isolated from plants have been shown to have anticaries activity. This activity may be due to growth inhibition against oral bacteria [22]. In the present study, out of the 6 extracts used, extract from *Pongamia pinnata* significantly shown antibiofilm activity. *Coriandrum sativum, Mentha avensis, Azadiractha indica, Aloe vera and Eucalyptus globulus* did not show any effect on the EPS production (Table 4). It was clearly indicated that there was tubes incubated with *Pongamia pinnata* showed reduction in EPS production. There is no report on antibiofilm activity of *Pongamia pinnata*. The antimicrobial activity of *Pongamia pinnata* extract may be due the presence of phenolics, alkaloids, flavonoids, terpenoids and polyacetylenes. Shan et al [23] reported that the antimicrobial activity of the plant extract is majorly attributed to the presence of phenolic compounds. It would be very interesting to investigate the type of phenolic compounds responsible for the antibiofilm activity of *Pongamia pinnata* extract and this would be future scope of our study.

CONCLUSION

The natural products have been used as alternative medicines to conventional therapy and have gained interest in the researchers. This may be due to the perception that herbal products may be safe and have been used for many years as traditional medicines. Currently, researchers are focused on the therapeutic and pharmacological effects of natural products of plant origin. The antimicrobial compounds from plant source have increasing attention in recent years. Although there are many reports available on the antimicrobial properties of plants extracts, there are very few reports are available on the antibiofilm activities of plant extracts. Hence, the present study aimed to find the antibiofilm activities of different plant extracts.

ACKNOWLEDGEMENT

The authors would like to thank Dr. L. Rathaiah, Chancellor, Dr. V. Govardhana Rao, Vice Chancellor of Vignan University for providing laboratory facilities to carry out this study and other faculty members of Biotechnology Department for their cooperation.

REFERENCES

1. Flemming HC, Wingender J, Griegbe, Mayer C. Physico-chemical properties of biofilms. In: Evans LV, editor. Biofilms: recent advances in their study and control. Harwood Academic Publishers 2000:19–34
2. Khattar JI et al. Isolation and characterization of exopolysaccharides produced by the cyanobacterium *limnothrix redekei* PUPCCC 116. Appl Biochem Biotechnol 2010; 162:1327-38.

3. Absalon S et al. Basal body positioning is controlled by flagellum formation in *Trypanosoma brucei*. PLoS ONE 2007; 2:437.
4. Frederic coulon et al, Hydrocarbon biodegradation in coastal mudflats: the central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms. Appl Environ Microbiol 2012; 78:3638-3648.
5. Costerton JW. Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284:1318-1322
6. Donlan RM. Microbial life on surfaces. Emerg Infect Dis 2002; 8:881-890.
7. Dimopoulus G. Falagas ME. Gram-negative bacilli – resistance issues. Touch Briefing 2007; 1:49–51.
8. Frank JF, Koffi RA. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Protection 1990; 53: 550-554
9. Cowan MM. Plant products as antimicrobial agents. Clinical Microbiol Rev 1999; 12(4):564-582
10. Carneiro et al. Casbane Diterpene as a Promising Natural Antimicrobial Agent against Biofilm-Associated Infection. Molecules 2011; 16:190-201
11. Essawi T, Srour M. Screening of some Palestinian medicinal plants for antibacterial activity. Journal of Ethnopharm 2000; 70:343–349
12. Ahimou F, Semmens MJ, Haugstad G, Novak PJ. Effect of protein, polysaccharide, and oxygen concentration profiles on biofilm cohesiveness. Appl Environmental Microbiol 2007; 73:2905-2910
13. Tichy J, Novak J. Extraction, assay, and analysis of antimicrobials from plants with activity against dental pathogens (*Streptococcus* sp.). J Altern Complement Med 1998; 4:39-45
14. Djordjevic D, Wiedmann M. and Mclands borough LA. Microtitre plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl.Environ. Microbiol. 1999; 68:2950–2958
15. Smitinont T et al. Exopolysaccharide-producing lactic acid bacteria strains from traditional Thai fermented foods: isolation, identification and exopolysaccharide characterization. Int J. Food Microbiol 1999; 51:105-111
16. Kodali VP. and Sen R., Partial **s**tructural elucidation of an antioxidative exopolysaccharide from a probiotic bacterium, J Nat. Prod. 2011; 8:1692–1697
17. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal. Chem 1956; 28:350-356
18. Sathish M., Tharani C.B., Niraimathi V., Satheesh Kumar D., *In-vitro* antioxidative activity of phenolic and flavonoid compounds extracted from root of *clerodendrum phlomidis* (linn.) Int J Pharm Pharm Sci, 2012; 4: 288-291
19. Prasanth NV, Dilip C, Sanal Dev KT, Lis Augustine, Saraswathi R. Revaluation of in vitro cytotoxic and antioxidant activities of *Ipomoea batatas*. Int J Pharm Pharm Sci. 2010; 91-92
20. 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 2007; 52:869-875
21. Frengova G. I., Simova D. E., Beshkova D. M. and Simova Z. I., Exopolysaccharaides produced by lactic acid bacteria of keifer grains, *App. Microb.,* **45**, 805-810 **(2002)**
22. Duarte S et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. FEMS Microbiol Lett 2006; 257:50-56
23. Shan B, Cai Y, Brooks JD, Corke H. The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts. Int. J Food Microbiol. 2007; 117:112–119