

Research Article



Plant Extracts as Antibiofilm Agents

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ABSTRACT

Antimicrobial agents have been used for the treatment of different microbial infections on human health. Biofilm producing bacteria exist in biofilm and with stand harsh environmental conditions and antimicrobial agents. Most microbial infections caused by biofilm producing bacteria are resistance to conventional therapy. Researchers have been prompted to identify alternatives for the treatment of such infections. In the recent past, there has been an increased interest in the therapeutic properties of some medicinal plants and natural compounds which have demonstrated for their antibiofilm activities. Present study aimed to isolate and characterize the biofilm strain. Microtitre plate assay was used to confirm the biofilm producing ability of the bacteria. Different plant sources have been screened for their antibiofilm activity. Biofilm formation was studied using pH and temperatures. It was observed that the biofilm formation by these bacteria was growth dependent. The leaf extract of *Pongamia pinnata* showed significant antibiofilm activity.

Keywords: Anti-biofilm agents, Exopolysaccharides, Microtitre plate assay, *Pongamia Pinnata*.

INTRODUCTION

Biofilm is a community of cells attached to either a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS)¹. Biofilm producing bacteria has the ability to form biofilms, which is a most common feature. However, some forms of biofilms more readily than others. Biofilms allow micro-organisms to trap nutrients and withstand hostile environmental conditions which are a key feature for survival². Surface adhesion of the bacteria is an essential step and is required for the bacteria to arrange themselves favourably in their environment. Bacterial biofilms have been reported to have useful effects on food chains, sewage treatment plants to eliminate petroleum oil/hydrocarbon spillage from the oceans^{3,4}. 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. The resistance of cells in a biofilm system to antimicrobial agents including antibiotics, disinfectants and preservatives has been widely reported⁵. 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 independent⁶. The regular use and un-proper use of antibiotics may lead to drug resistance and will make the drugs ineffective against common microbial infections⁷. 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 agents⁸. Plant extracts have

been applied for the treatment of inflammations, wounds, certain forms of cancer, infections due to bacteria, virus or fungi and many more. Application of phytochemical methods has led to the isolation of a wide range of natural compounds from the various plant species. Researchers aiming on the isolation of tannins, hydrolysable tannins, triterpenoid structures, organic acids, numerous flavonoids, coumarins, polyprenols and other compounds of plant extracts. These compounds presumed to be safe and no toxic effects. In the recent past, there has been an increased interest in the therapeutic properties of some medicinal plants and natural compounds which have demonstrated for their antibiofilm activities. Synthetic substances and antibiotics have been used in the control of biofilms. Due to chemical nature these compounds cause many unexpected side-effects⁹⁻¹¹. 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 biofilm producing ability and antibiofilm activity of plant extracts against sample 19 have been reported. The microbial strains have been characterized in terms of their biofilm formation ability, growth and pH profiles. Different physical parameters that affect the biofilm formation were studied and various plant extracts have been investigated for their antibiofilm activity¹³.

MATERIALS AND METHODS

Biofilm assay

The isolate (Sample 19) 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



plate¹⁴. 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 Smitnont et al¹⁵. The overnight culture of sample 19 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 method¹⁶.

Study of the effect of nutrients, pH and Temperature on biofilm formation

Different growth media, pH and Temperatures were screened to understand the biofilm was nutrient dependent or not. A loopful of the culture broth of sample 19 was inoculated into 50 mL of each of the different media, pH and Temperatures selected. The conical flasks were then incubated for 7 days. For every 24 h sample was collected and the O.D was measured at 540 nm and the EPS was estimated by phenol-sulphuric acid method¹⁷.

ANTIBIOFILM ACTIVITY OF PLANT EXTRACTS

Plant material

Leaves of plants (*Coriandrum sativum*, *Mentha avensis*, *Pongamia pinnata*, *Azadirachta 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 extracts

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*, *Azadirachta indica*, *Aloe vera* and *Eucalyptus globulus* have been tested for their antibiofilm activities. Total phenolic content of the extracts was measured by Folin-Ciocalteu method¹⁸ as shown in figure 2.

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 al¹⁹. 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 19 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 method¹⁷.

RESULTS AND DISCUSSION

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 19 (Sample 19) 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 as shown in figure 1.

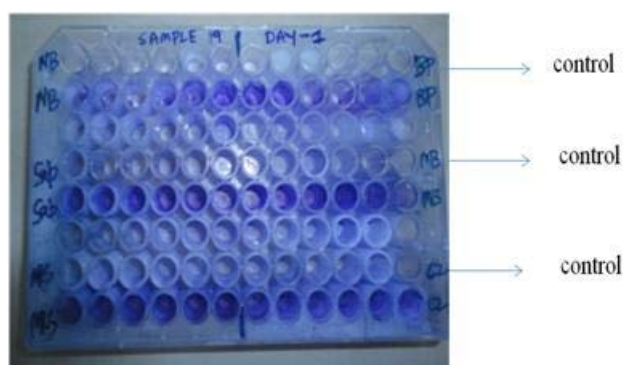


Figure 1: Microtitre plate assay of biofilm formation for sample 19 (NB- Nutrient broth; MRS-de Man Rogosa and Sharpe; SAB-Saborouds; CZ-Czapek-dox; MB-Mannitol; BP-Beef peptone)

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.

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 19 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 method¹⁷.

Effect of pH on EPS production by sample 19

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.

Table 1: Biofilm formation by sample 19 in different nutrient media

Medium used	EPS concentration (µg/mL) in different time intervals [§]		
	24 h	48 h	72 h
MRS	5.6	16.8	22
Czapakdox	0.9	1.2	1.3
Mannitol Broth	1.2	1.9	2.6
Nutrient Broth	3.8	13.2	16.9
Saborouds	2.9	9.8	11.8

[§] Average of three values

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

Day	EPS concentration (µg/mL) at different pH (MRS Medium)							
	3	4	5	6	7	8	9	10
Day 1	No Growth				5.5	6.7	7.6	8.9
Day 2					17.3	19.3	24.3	29.7
Day 3					25	27	38	43

[§] Average of three values

Effect of Temperature on EPS production by sample 19

The biofilm production is temperature dependent. *Bacillus cereus* produces EPS at 37°C, *B. licheniformis* produces at 35°C²⁰. 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 was 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.

Effect of plant extracts on EPS formation of sample 19

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 bacteria²¹. 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 al²² 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.

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

Day	EPS concentration at different temperatures in MRS Medium (µg/mL)			
	25°C	30°C	37°C	45°C
Day 1	No Growth	5.4	5.9	No Growth
Day 2		14.7	15.6	
Day 3		20.3	21	

[§] Average of three values

Table 4: Effect of plant extracts (methanolic) on biofilm (EPS) formation by sample 19 at 37°C

Plant source	Concentrations used (µg/mL)	EPS concentration for sample 19 [§] (µg/mL)
<i>Coriandrum sativum</i>	27.0	8.1
<i>Mentha avensis</i>	69.1	8.2
<i>Pongamia pinnata</i>	61.5	6.1
<i>Azadiractha indica</i>	73.0	8.2
<i>Aloe vera</i>	51.0	7.2
<i>Eucalyptus globulus</i>	66.7	7.9
Control (without plant extract)	Nil	6.9

[§] Average of three values



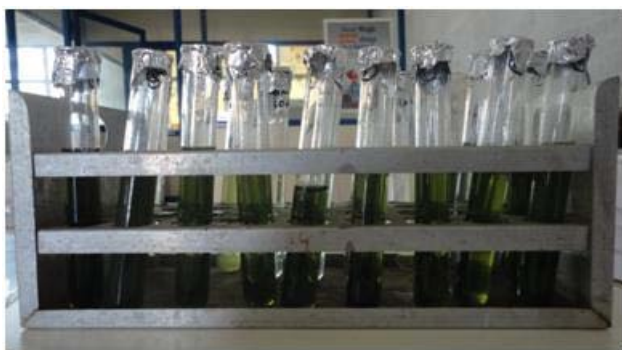


Figure 2: Phenolic content estimation of selected plant extracts

CONCLUSION

Plant products are the natural products which have been used as alternative medicines to conventional therapy and have gained interest in the researchers. This is 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.

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