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Screening of Biofilm-Forming Coral-Associated Bacteria using TC Plate By Crystal Violet Staining

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Abstract

Microbial communities are potentially very useful as indicators for water quality as they respond and be affected rapidly to environmental changes. Screening of biofilm-forming Coral-Associated bacteria can be used to study the natural and human factors affecting coasts and reefs. The objective of this experiment was to assess biofilm-forming in Coral-Associated bacterial isolates. Twelve strains of bacteria were grown in Tryptophan Soya Broth media and cultures were diluted into 100 folds, and then grown on a tissue culture (TC) plate. Crystal Violet staining was used to visualize biofilm growth. The procedure involved the addition of Crystal Violet solution, phosphate buffer saline (PBS) for washing and ethanol for fixation. The results show that 17% of the isolates displayed low biofilm growth patterns, while 25% of the isolates displayed medium biofilm growth patterns and 58% of the isolates displayed high biofilm growth patterns. When these results were compared with the appropriate controls, the *Pseudomonas aerogenosa* displayed high biofilm growth patterns while the negative control did not show any growth. In conclusion, Crystal Violet is a simple and easy method to evaluate the biofilm-forming differences among bacterial isolates, and in this experiment, it has been shown that formation of biofilm was very common in coral-associated bacteria.
Keywords: Biofilm, Coral-Associated Bacteria, Tissue culture Plate, Microbial Communities

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1. Introduction

Recent studies report potentially beneficial functions of the coral-associated bacteria. They cycle sulphur, fix nitrogen, produce antimicrobial compounds, inhibit cell-to-cell signaling and disrupt virulence in opportunistic pathogens (Krediet et al., 2013).
Natural and human factors influence the water quality along the coasts and reefs and may, in turn, affect coral reef communities. Associated bacterial biofilms respond rapidly to environmental conditions and are potential bioindicators for changes in water quality (Witt *et al.*, 2011).
The majority of microbes in biofilms are related to Alphaproteobacteria (40%), Gammaproteobacteria (14%), Bacteroidetes (13%), diatoms (8%) and Cyanobacteria (4%). Statistical analysis indicated that Cyanobacteria, Bacteroidetes and to some extent Alphaproteobacteria, are significantly more abundant in the offshore biofilm communities compared to inshore microbial communities (Kriwy *et al.*, 2011).
In a comparative DNA study on the diversity of microbes according to the state of coral health (i.e. healthy, diseased or bleached), the researchers found that healthy and bleached corals harbor similar dominant species, although bleached corals had higher proportions of Vibrio and Acidobacteria. Diseased corals generally had more Rhodobacter, Clostridia, and Cyanobacteria sequences, and fewer Oceanospirillum sequences (Mouchka *et al.*, 2010).
Formation of a biofilm is one of the important aspects of bacterial pathogenicity. Biofilm communities tend to exhibit significant tolerance to the antimicrobial challenge. Interestingly, some marine bacteria, especially coral-associated bacteria, are known for antibiofilm activity against pathogenic biofilm formation (Thenmozhi *et al.*, 2009; Gowrishankar *et al.*, 2012; Bakkiyaraj *et al.*, 2013).
Biofilm formation by *Pseudomonas aeruginosa* has been implicated in within the pathology of chronic wounds. Both the d and l isoforms of tryptophan inhibited *P. aeruginosa* biofilm formation on tissue culture plates, with an equal ratio of d and l isoforms producing the greatest inhibitory effect. Addition of d-/l-tryptophan to existing biofilms inhibited further biofilm growth and caused partial biofilm disassembly. Tryptophan significantly increased swimming motility, which may be responsible in part for diminished biofilm formation by *P. aeruginosa* (Brandenburg *et al.*, 2013).

2. Materials and Methods

Twelve strains of bacteria were inoculated in a 5 ml culture of Tryptophan Soya Broth (TSB) media and grown at 30°C to stationary phase. Two tubes were used as negative control (no inoculation) and two tubes were used as positive control (inoculated with *Pseudomonas aerogenosa*).
The culture was diluted 1:100 in the TSB media. One milliliter of each diluted culture was pipetted into each well of a fresh tissue culture (TC) plate. The plate was covered and incubated at the optimal growth temperature (30°C) for 24 hours.
Approximately 200 µl of 0.1% crystal violet solution (pre-filtered through a 0.44 µm membrane filter) was added to each well and left to stain for 10 min at room temperature. The non-attached bacteria were pipetted out by touching the top of tips at the corner of each well. The wells were washed successively with phosphate buffer saline (PBS) three times, and the plate was left to air dry for 15 min.
To fix the stain, an amount of 1 ml of 95% ethanol were added to each stained well. The dye was allowed to solubilize by covering the plates and incubating them for 15 min at room temperature. The content of each well was allowed to mix briefly using a pipet, then the TC plate was left to air dry. The results were compared

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visually with the appropriate controls and growth was estimated in the range of three levels: low, medium and high.

3. Results

Twelve strains of bacteria were grown in Tryptophan Soya Broth (TSB) media to stationary phase (**Figure 1**). Two tubes were used as negative control (no inoculation) and two tubes were used as positive control (inoculated with *Pseudomonas aerogenosa*). These cultures were diluted into 100 folds, and grown on a tissue culture (TC) plate (**Figure 2**).
Crystal Violet staining was used to visualize biofilm growth. The procedure involved the addition of Crystal Violet solution, phosphate buffer saline (PBS) for washing and ethanol for fixation as shown in (**Figure 3**).
The results show that 17% of the isolates displayed low biofilm growth patterns, while 25% of the isolates displayed medium biofilm growth patterns and 58% of the isolates displayed high biofilm growth patterns (**Figure 4**). When these results were compared with the appropriate controls, the *Pseudomonas aerogenosa* displayed high biofilm growth patterns while the negative control did not show any growth. The first two isolates

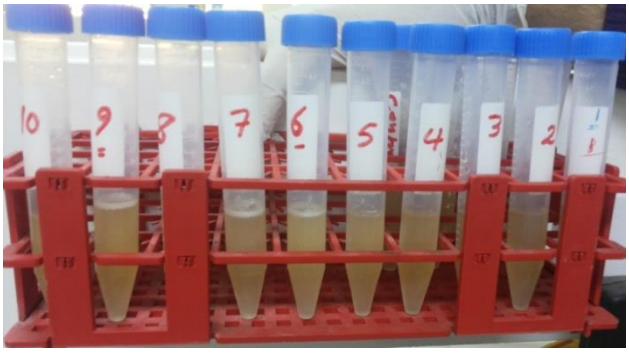

Figure 1: Bacterial growth after 24 hours of incubation in 5 ml of TSB media at 30°C.

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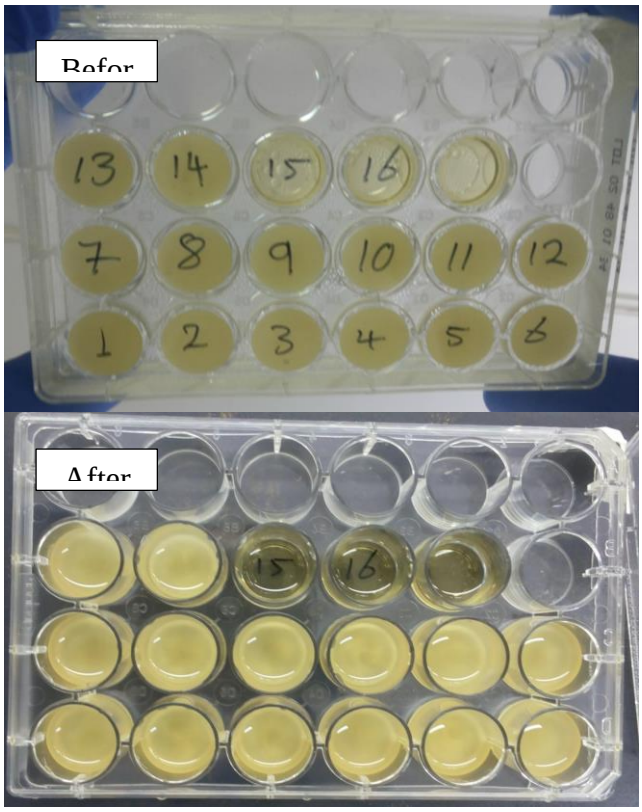

(1 and 2) displayed low biofilm growth, while three isolates (7, 9 and 12) displayed medium biofilm growth as shown in **Table 1**.

Figure 2: Bacterial growth (1:100 dilution inoculates) before and after 24 hours of incubation in 1 ml of TSB media at 30°C. The tubes 13 and 14 were used as positive control (inoculated with *Pseudomonas aerogenosa*) while the tubes 15 and 16 were used as negative controls.

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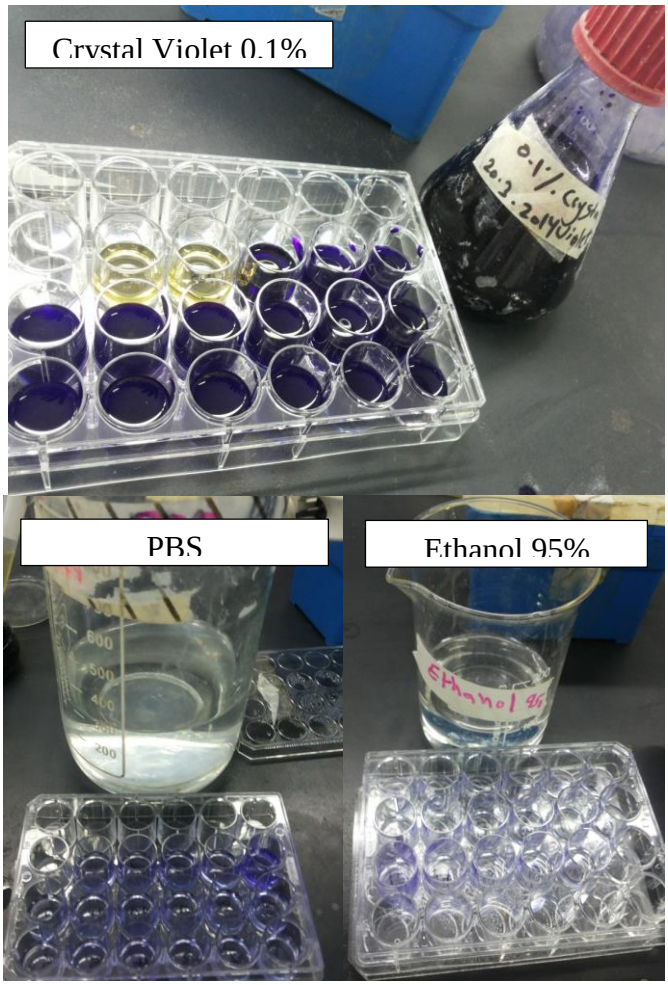

Figure 3: Crystal Violet staining procedure showing the additional steps of crystal violet, PBS, and ethanol.

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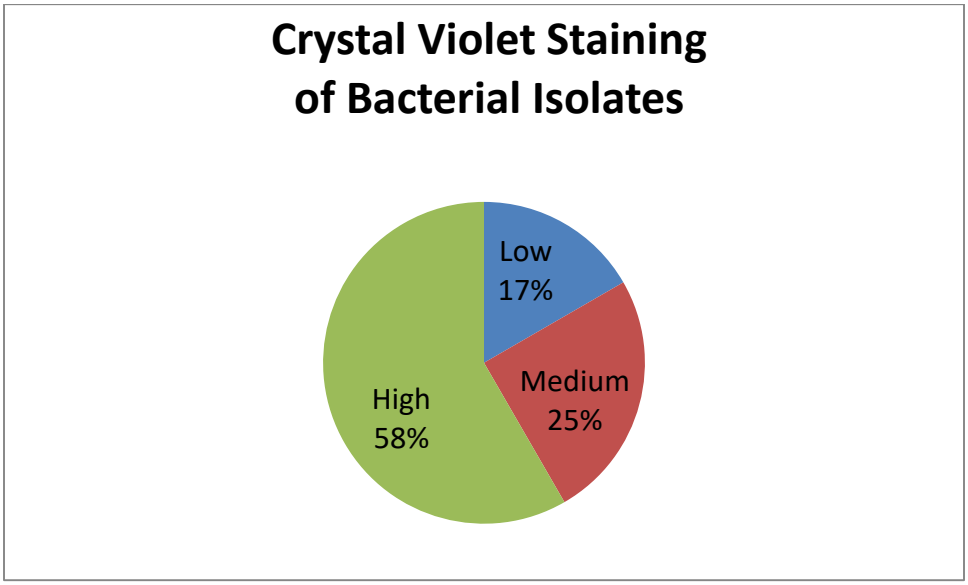

Figure 4: Crystal Violet staining of 12 bacterial isolates.

Table 1: Results of Crystal Violet Staining.

Inoculate	No Growth	Low	Medium	High
1		X		
2		X		
3				X
4				X
5				X
6				X
7			X	
8				X
9			X	
10				X
11				X
12			X	
+ve Control				X
+ve Control				X
-ve Control	X			
-ve Control	X			

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4. Discussion

Previous reports on Coral-Associated bacteria showed that bacterial biofilms can be quantified by crystal violet staining (Thenmozhi *et al.*, 2009). The results of this experiment showed that the formation of biofilm was very common in coral-associated bacteria. When these results were compared with *Pseudomonas aerogenosa*, a known biofilm-forming bacteria most isolates displayed similar patterns.

Coral-Associated bacteria are known for antibiofilm activity against pathogenic biofilm formation. A future experiment can utilize these bacteria for developing drugs against pathogenic bacteria. A suggested procedure will involve the use of coral-associated bacterial extract to screen for the formation of biofilms using TC plate and crystal violet in *Pseudomonas aerogenosa.*

It is important to note that crystal violet stains not only cells, but essentially any material adhering to the surface of the plate (e.g. matrix components), and therefore, crystal violet staining may overestimate the number of adherent bacteria. However, it is a good indicator of biofilm (matrix components) formation.

The study of biofilm formation has undergone a great deal of challenges due to structural heterogeneity which leads to temporal and spatial variation in cell density and gene expression (Gu *et al.*, 2013).

Further understanding of the mechanisms involved in biofilm formation will shed light on the process of eradicating biofilm-associated pathogens, and also further investigation is required in the field of coral-associated bacteria and their role in biofilm formation and anti-biofilm formation.

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