

# A Simple Technique to Assess Bacterial Attachment to Metal Surfaces

Sangeeta Sonak and Narayan Bhosle

Marine Corrosion and Materials Research Division, National Institute of Oceanography,  
Dona Paula 403004, Goa

## Abstract

There are several methods to assess bacterial adhesion to metal surfaces. Although these methods are sensitive, they are time consuming and need expensive chemicals and instruments. Hence, their use in assessing bacterial adhesion is limited. Therefore, a simple spectrophotometric method was developed to evaluate bacterial adhesion to metal surfaces. The method was calibrated using a bacterium *Vibrio* sp. and aluminium as a model organism and substratum, respectively. The method involves staining attached bacterial cells with crystal violet (0.5%). The crystal violet stain from the cells was eluted with an aqueous solution of sodium deoxycholate (2%). The absorbance of the crystal violet stain in deoxycholate was measured at 570 nm. The viable cell count of the attached bacterial cells was also measured using conventional plating technique. Relationship between the absorbance of crystal violet stained attached cells and the viable cell count of the attached bacteria was assessed using linear regression analysis. There was a highly significant relationship ( $r = 0.99$   $p < 0.001$ ) between these two parameters. This suggests that the method can be used to assess bacterial adhesion to metal surfaces.

## Introduction

Surfaces immersed in aquatic environment adsorb organic molecules thereby forming 'a conditioning film' (Costlow & Tipper, 1984). Such surfaces are then colonized by microorganisms including bacteria. Microbial films induce or enhance metamorphosis and settlement of invertebrate lar-

vae on surfaces (Maki and Mitchell, 1986), thus leading to macrofouling. Fouling increases the frictional resistance of ships, leading to rise in fuel consumption and loss of speed. A lot of money is wasted on drydocking to remove fouling. In heat transfer equipment as well, biofilms can lead to economic losses due to impairment of heat transfer efficiency (Aftring and Taylor, 1979). Biofilms even increase fluid frictional resistance in water distribution and waste water transport systems (Yoshikawa and Takiguchi, 1979). Bacteria are also believed to be associated with the corrosion of metals (Dexter, 1986). For these reasons, It is necessary to assess the bacterial adhesion to metal surfaces.

There are several techniques including adenosine triphosphate, lipid phosphate, muramic acids and viable and total cell count to evaluate bacterial biomass (Holm-Hansen, 1969; Hobbie *et al*, 1977; Paul *et al*, 1985; Balkwill *et al*, 1988). Some of these techniques have been employed to assess bacterial adhesion to surfaces (Davis and White, 1980; Jefferey and Paul, 1986; Balkwill *et al*, 1988). Although these methods are sensitive, they are time consuming and uneconomical. Moreover, they require specific instrumentation. Hence their use in routine monitoring of bacterial adhesion is limited.

Crystal violet microplate adhesion assay has been previously reported for the bacterial adhesion to non-metal surfaces (McEldowney and Fletcher, 1986; Shea and Williamson, 1990). However, this

method has not been used to evaluate bacterial adhesion to metal surfaces. Therefore, this paper describes a new application for the crystal violet adhesion assay and has been designed to evaluate bacterial adhesion to metal surfaces.

## Materials and Methods

Crystal violet, deoxycholate were purchased from Sigma Chemical Company, U.S.A.

0.5% crystal violet: 500 mg of crystal violet was dissolved in 10 ml ethanol and made to 100 ml using double distilled water. 2% sodium deoxycholate: 2g of sodium deoxycholate was dissolved in 100 ml of double distilled water.

Aluminium panels (size 3.5 × 1.5cm) were cleaned with 20% (v/v) hydrochloric acid, repeatedly washed with tap water, followed by double distilled water, dried in an oven and kept covered until used (Bhosle *et al*, 1990). Before using for the experiment, these panels were sterilized by autoclaving for 20 minutes at 121°C and 1b pressure.

Bacterial culture, *Vibrio* sp, isolated from aluminium panels immersed in estuarine water was used for the present study.

Nutrient broth prepared in basal salt solution (Bhosle, 1981) was inoculated with *Vibrio* culture and grown at room temperature (28±2°C) for 18 hrs. This culture was then used as a source of inoculum for another flask of nutrient broth. After fresh inoculation the flask was incubated at room temperature on rotary shaker at 150 r.p.m. for 18 hrs. As shown in the flow diagram (Fig. 1), the cells in the stationary phase were harvested, by centrifugation at 4000 r.p.m., washed thrice with 0.01 M P04 buffer and suspended in the same buffer. The optical density at 400 nm was adjusted to 0.2 using phosphate buffer. This corresponds to about  $2 \times 10^9$  CFU/ml.

Aliquot (100 ml) of the above bacterial suspension was dispensed in six 250 ml Erlenmeyer flasks. Replicate (4) aluminium panels were suspended in

each flask using nylon thread and the flasks were incubated over a period of 3 hours at room temperature. Panels (4) were retrieved at an interval of 30 minutes over a period of 3 hours. They were rinsed with water to remove any unattached cells. Two pannels were used for crystal violet staining and the remaining two were used for viable cell count of the bacterium using Zobell Marine Agar. In order to obtain reliable data the experiment was repeated at least 4 times.

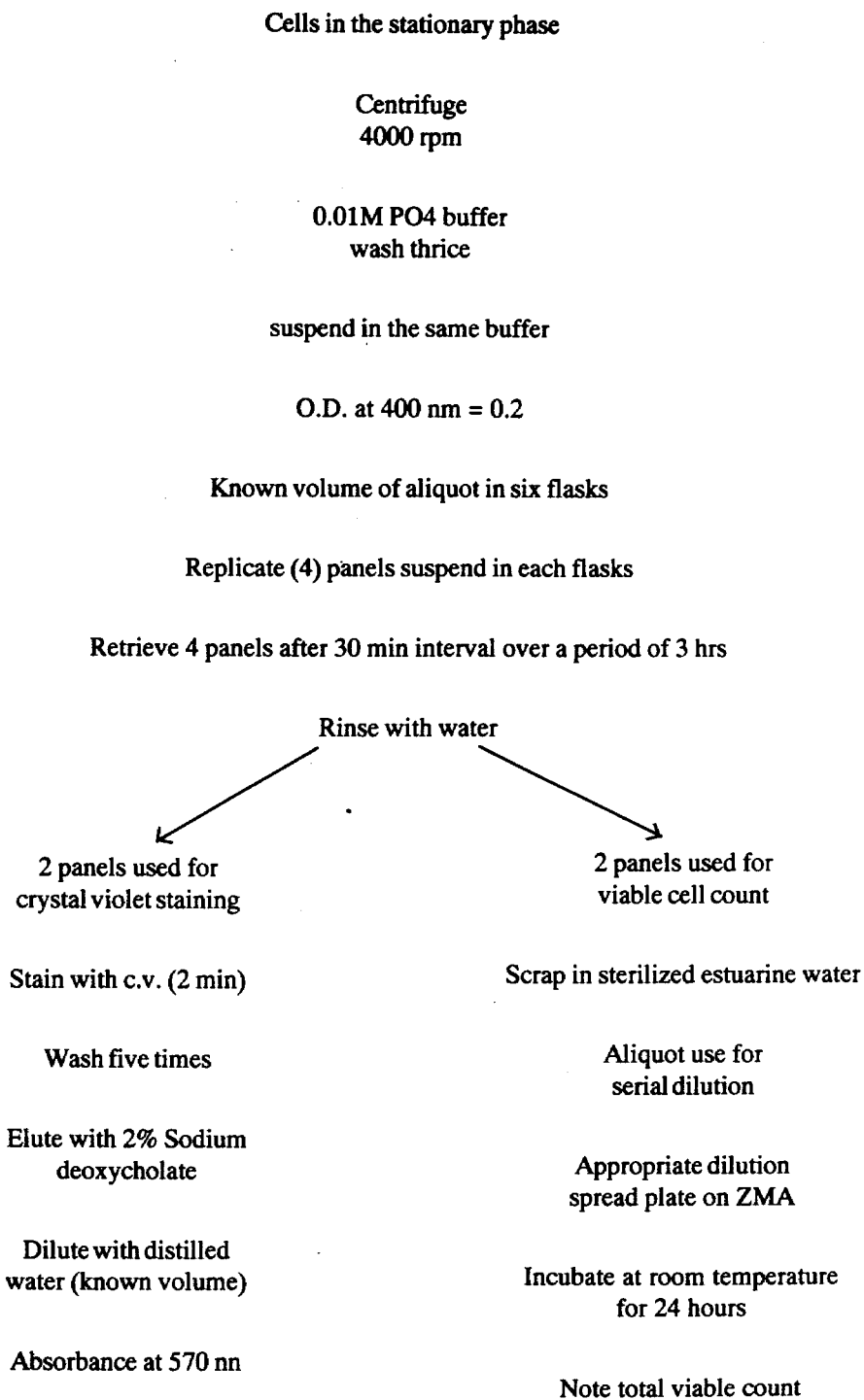
The panels with attached cells were stained with crystal violet (5gl-1) for two minutes, after which they were removed, washed five times in water (500 ml each time) to remove excess stain. The crystal violet stain from the attached cells was eluted with 2ml of 2% sodium deoxycholate. Subsequently, this was diluted with 3 ml of distilled water. Absorbance was measured at 570 nm using Beckmann DU 64 spectrophotometer. Flasks containing aluminium panels in phosphate buffer without bacteria were treated similarly and were used as controls.

Remaining two panels were used to assess viable cell count of the attached cells of *Vibrio*. Panels were scraped with a nylon brush using filter sterilized estuarine water (0.2 nm). A suitable aliquot (0.1ml) of this scraped material was used for serial dilution. Appropriate dilutions were spread plated on petri dishes prepared with Zobell Marine Agar. Petri dishes were incubated at room temperature for 24hrs after which total viable cell count was noted.

Relationship between the crystal violet stained attached cells and the viable count of the attached cells of *Vibrio*, was assessed by the linear regression analysis using the 123 LOTUS programme.

## Results and Discussion

Absorbance of crystal violet stained attached cells as well as the viable cell count of the cells attached to aluminium panels increased as a function of the incubation period (Fig. 2 &3). The observed increase in the viable cell count of bacteria



**Fig. 1.**  
**Flow diagram showing protocol for the method.**

over the period of incubation was due to the enhanced settlement of bacteria on the aluminium panels. The apparent relationship between the crystal violet stained cells and the viable counts of the attached cells was further evaluated using linear

regression analysis. Highly significant relationship ( $r = 0.99$ ,  $p < 0.001$ ,  $n = 6$ ) was found between crystal violet adhesion assay and the total viable count for the cells of *Vibrio* (Fig. 4).

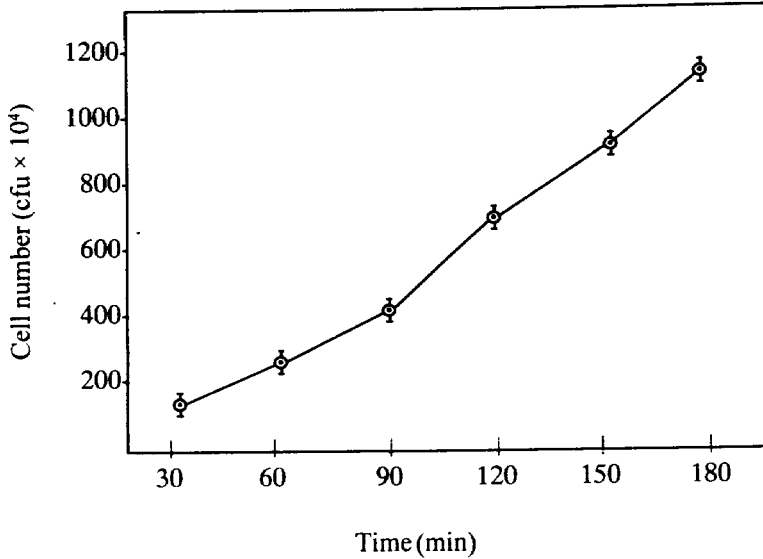


Fig. 2.

Attachment of *Vibrio* cells to the aluminium panels measured by crystal violet assay

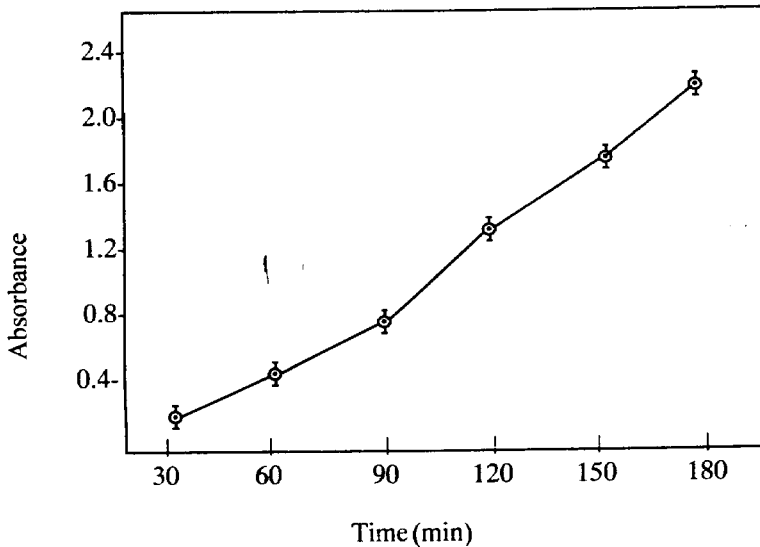


Fig. 3.

Attachment of *Vibrio* cells to the aluminium panels measured by viable cell count.

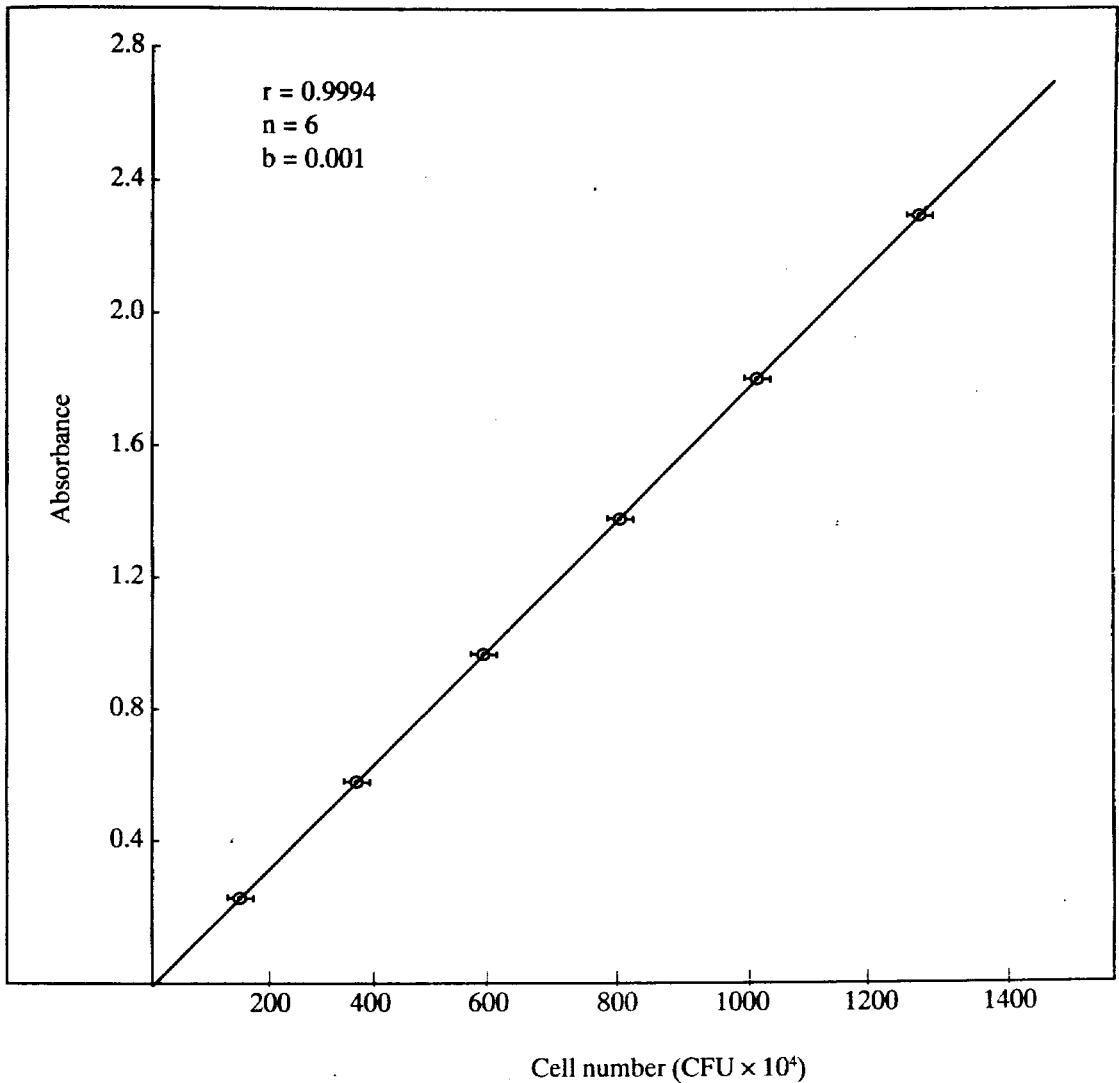


Fig. 4.

Relationship between the absorbance of the crystal violet assay and the total viable cell count for the attached cells of *Vibrio*.

This implies that the technique provides a reliable estimate of the bacterial attachment to surfaces and may be useful for assessing bacterial adhesion to metal surfaces. Crystal violet adhesion assay also has several attractive features. The stain is readily eluted from the cells with an aqueous

solution of sodium deoxyholate. The eluted dye is stable and is protected from evaporation.

Furthermore, crystal violet adhesion assay is relatively less time consuming, simpler and very economical. Many samples can be examined quickly.

Therefore, the technique is recommended for evaluating bacterial adhesion to metal surfaces. Applications for this assay include the effect of hydrophobicity on cell adhesion, quantification of bacteria, identification of adhesion mutants and evaluation of antifouling agents.

## References:

Aftring R P, Taylor B (1979): Assessment of Microbial Fouling in an Ocean Thermal Energy Conversion Experiment. *Appl Environ Microbiol* 38 : 734 - 739

Balkwill D L, Leach F R, Willson JT, Mc Nabb J F & White DC (1988): Equivalence of Microbial Biomass Measures Based on Membrane Lipid and Cell Wall Components, Adenosine Triphosphate and Direct Counts in Sub-surface Aquifer Sediments. *Microb Ecol* 16: 73 - 84

Bosle N.B. (1981): Microbial Degradation of Petroleum Hydrocarbons. PhD Thesis, University of Bombay.

Bhosle N B, Nandakumar K, Wagh A B (1990): Influence of Particulate Material on Microfouling Biomass in the Arabian Sea. *Biofouling* 2: 65 - 74.

Costlow J D, Tipper R C (1984) (eds): *Marine Biodeterioration: An Inter Disciplinary Study*. US Naval Institute, Annapolis, Maryland.

Davis W M, White D C (1980): Fluorometric Determination of Adenosine Nucleotide Derivatives as Measures of the Microfouling, Detrital and Sedimentary Microbial Biomass and Physiological Status. *Appl Environ Microbiol* 40 : 539-548

Dexter S C (ed) (1986) : *Biologically induced corro-*

*sion* (NACE reference book 8), Ntional Association of Corrosion Engineers, Houston

Hobbie J E, Daley R J & Jasper S (1977): Use of Nucleopore Filters for Counting Bacteria by Fluorescence Microscopy. *Appl Environ Microbiol* 33: 1225-1228.

Holm-Hansen O (1969): Determination of Microbial Biomass in Ocean Profiles. *Limnol. Oceanogr* 14 : 740-747

Jeffrey W H, Paul J H (1986): Activity of an Attached and Free Living *Vibrio* sp Measured by Thymidine Incorporation, pidonitrotreazolium Reduction and ATP/DNA Ratios. *Appl Environ Microbiol* 51 : 150-156

Maki J S, Mitchell R (1986): The Function of Lectins in Interactions among Bacteria, Invertebrates and Algae. pp 409 - 425 In : D Mirelman (ed) *Microbial lectins and agglutinins : properties and biological activity*, Wiley, New York

McEldowney S, Fletcher M (1986): Effect of Growth Conditions and Surface Characteristics of Aquatic Bacteria on their Attachment to Solid Surfaces. *J.Gen. Microbiol.* 132 : 513-523

Paul J H, Jeffrey W H, De Flaun M (1985): Particulate DNA in Subtropical Oceanic and Estuarine Planktonic Environments. *Mar Biol* 90: 95-101

Shea, C & J C Williamson (1990): Rapid Analysis of Bacterial Adhesion in a Microplate Assay. *Biotechniques* 8: 610-611.

Yoshikawa H, Takiguchi Y (1979): Attached growth of *Sphaerotilus natans* in Continuous-flow Apparatus and its Growth Inhibition by 9-β-D-arabino-furanosy ladenine. *Appl Environ Microbiol* 38: 200-204