FUNDAMENTAL STUDY OF THE INITIAL BACTERIAL ATTACHMENT OF

Pseudomonas aeruginosa, *Pseudomonas putida* AND *Escherichia coli*

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Thesis

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ABSTRACT

Microbial biofilm formation on surfaces leads to significant losses in terms of energy, equipment damage, product contamination, medical infections, etc. and also raises serious environmental concerns. To prevent formation of biofilms, a detailed investigation into the initial stage of bacterial attachment was conducted using three types of bacterial species; *Pseudomonas aeruginosa* PAO1, *Escherichia coli* and *Pseudomonas putida*.

Several approaches were evaluated and finally a reproducible procedure was adopted to study initial bacterial attachment. The procedure primarily involved monitoring and counting the number of attached cells on the glass walls of the flow chambers, through which a bacterial suspension was circulated and, subsequently, saline was passed for washing to remove loosely attached cells. Preliminary investigations in terms of fluid flow behaviors, consistency check, and the effect of various factors including the attachment locations within the chamber, on the attachment results were conducted prior to employing the flow chamber systems to study the initial bacterial attachment. Using the flow chambers systems, the effects of chamber wall shear, medium pH, chamber, and two potential antifoulants, Zosteric acid and Rhamnolipids, on attachments were examined.

The three bacterial species used showed similar behavior (peaking at different shear stress values) in response to varying shear conditions in their environment. The antifouling properties of zosteric acid were not convincing enough, however promising results were seen while studying rhamnolipids in the case of all three bacterial strains. The effect of other factors like pH, presence of zosteric acid impurities, surface conditioning were also explored. These conditions however proved to have no effect on the initial bacterial attachment but provided useful information for future studies.

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CHAPTER I

INTRODUCTION

Over a decade ago, biofilm was considered to simply be an amassing of bacterial cells. It was only in recent years, with the help of technological advances that it has been identified as the favored means of bacterial growth. Modern molecular-level techniques which adopt a non destructive mode of inspecting these active micro-organisms revealed a great deal about biofilms, such as the presence of water permeable channels that form the basic circulatory system of these structures (Wilson et al., 2003). It was also uncovered that one of the most important rationale behind this biofilm formation was defense from the external environment, be it other higher organisms or other potent substances like antibiotics (Prakash et al., 2003).

Solid surfaces that are most susceptible to biofilm formation are the ones submerged in water or in contact with water over a period of time. Biofouling is believed to occur due to the attachment of various organisms like bacteria, algae and invertebrates including barnacles and mussels. This is a common phenomenon which is seen on a variety of surfaces like that of heat exchangers, turbines, flow ducts, hulls of ships, prosthetic devices, teeth, and even in organ linings in higher organisms like humans.

In case of heat exchangers, the fouled surfaces cause corrosion and hence reduce the thermal efficiency. In addition to this energy loss and high operational/maintenance costs, the capital costs are also raised as various components of these equipments need to be constructed keeping in mind the possibility of fouling, as in it might prove to be more economical if the design includes larger sized parts to compensate for heat loss due to fouling. Added to these costs, fouling also causes degradation and limits the life time of a part or the equipment as a whole.

Pipes and flow channels which are also commonly effected by this phenomenon, may exhibit changes in the flow pressure and velocity due to blockage and this in turn causes power loss, increasing expenses and may also cause serious damage leading to cavitations. Other flow devices like orifice plates, venturi tubes affected by biofouling set off inaccurate flow rate measurements.

Marine surfaces like fishing equipment, underwater pipelines, offshore gas and oil equipment, and the most obvious, the hulls of ships are other surfaces vulnerable to this phenomenon. This growth on the ship's surface alters the roughness of the hull causing maneuverability problems and also increases the fuel consumption mainly due to an increase in the drag (Stanczak, 2004). Increase in the fuel consumption then leads to an increase in the release of greenhouse gases, thereby raising economic and environmental concerns. Biofouling also poses another major problem of spread of invasive species when biofouled surfaces pick up species and introduce them into non-native waters where they wreak havoc on native environments.

Microbial biofilms also play a vital role in human infections mainly those associated with the lungs and the gut. Prosthetic devices like catheters, implants and teeth

are common surfaces where biofilm formation is commonly observed (Wilson et al., 2003). Usually, biofilm that is formed on clinical devices has been found to have detrimental effects on the human body, since the bacteria growing in the biofilms are less susceptible to antibiotics. The extracellular polymeric substances secreted are believed to act as a physical/chemical barrier and prevent the antibiotics from infiltrating and affecting the embedded biofilm cells (Prakash et al., 2003).

Therefore it is simpler, cheaper and more convenient to prevent than to get rid of this highly resistant film once it has already formed and in order to prevent biofilm formation, we need to understand the phenomenon that is occurring at the solid surface. Biofilm formation begins with individual bacterial attachments, which then develops into colonization and finally growth and multiplication of the microorganisms through cell division and recruitment (Palmer et al., 2007).

The purpose of this thesis was to design a system to study this initial stage of bacterial attachment with consistent and reproducible results.

Static systems have been widely used to study the bacterial attachments and biofilm formations. However, in our case the use of static systems resulted in inconsistencies in final observations. In order to overcome the problems faced while using static systems, a flow chamber was designed to study the initial bacterial attachments. The flow system involved continuous circulation of the bacterial medium through a completely sealed chamber, dispelling issues regarding contamination, absence of shear and other problems faced while using stationary systems.

The main objectives of this study include:

- To determine the issues associated with using static systems for initial bacterial attachment.

- To design and construct a flow system to study the initial bacterial attachments.

- To study the attachment and biofilm formation of three types of bacterial strains.

- To examine the effects of shear on initial bacterial attachments.

- To study the effectiveness of two potential antifoulants; Zosteric acid and Rhamnolipids, on the initial bacterial attachments.

An outline of this thesis is as follows. The background information on biofouling, bacterial attachment and biofilm formation, the three species of bacteria: *Pseudomonas aeruginosa* strain PAO1, *E. coli*, *Pseudomonas putida*, and the two potential antifoulants: Zosteric Acid and Rhamnolipids will be presented in Chapter II. Chapter III will provide the details on the materials, equipments and methods used in this study. Experimental results obtained using the static system will be detailed in Chapter IV, while Chapter V will include the flow systems experimental results and discussions. Finally, the main findings of this study along with the suggestions for future work will be included in Chapter VI.

CHAPTER II

BACKGROUND

Prevention of biofilm formation has been under scrutiny for over two decades. In their quest to find a solution to this problem, scientists have come up with the use of signaling molecules which especially seem to be effective in inhibiting bacterial adhesion brought about by pili and flagella (Prakash et al., 2003). In some cases molecules interfering with the cell-to-cell communication have also been successfully used (Prakash et al., 2003). Examples found in nature, like certain sea weeds, namely *Delisea pulchra* and *Zostera Marina*, have been found to produce compounds like furanones (a class of signaling molecules) that are believed to interfere with the development of typical biofilm structures (Prakash et al., 2003). Surface finishing is another solution being touted for dealing with biofilms. Polishing, sandblasting and grinding are some of the surface treatments used typically to reduce biofilm buildup. Electropolishing involves passing electric current through an acid bath containing the substrate surface (usually steel) and has been found to furnish the best results among all the surface treatment techniques. Another fascinating prospect being researched is biofilm self-destruction, which has been achieved by depleting oxygen supply to the biofilm mass. A certain exopolysaccharide was found to be produced which digested the biofilm matrix and released the entrapped bacterial cells.

Although, biofilm prevention has received considerable attention as discussed, in certain cases such as bio-implants, bio-sensors and sewage treatment plants (Prakash et al., 2003) biofilm formation is advantageous. In order to come up with such constructive ways of using these biofilms or to prevent their formation and development, a deeper understanding of the phenomenon and species involved, devices commonly used for such investigations and finally the background of possible antifoulants is warranted. This chapter provides this basis for research carried out in this thesis.

2.1 Bio-fouling

Bio-fouling, as the name clearly indicates means biological fouling, and is different from other kinds of fouling, where the accumulation of various kinds of deposits, like products of corrosion, crystallization, chemical reactions, detritus, ice, etc. occur (Railkin et al., 2004). Colonization of a solid surface exposed to water for a period of time is mainly composed of 4 steps, with an overlapping time sequence, biochemical conditioning, bacterial colonization, unicellular and multi-cellular fouling. During the course of this fouling sequence, the processes are believed to progress from physical to biological (Wahl et al., 2003), as shown in Figure 2.1 below.
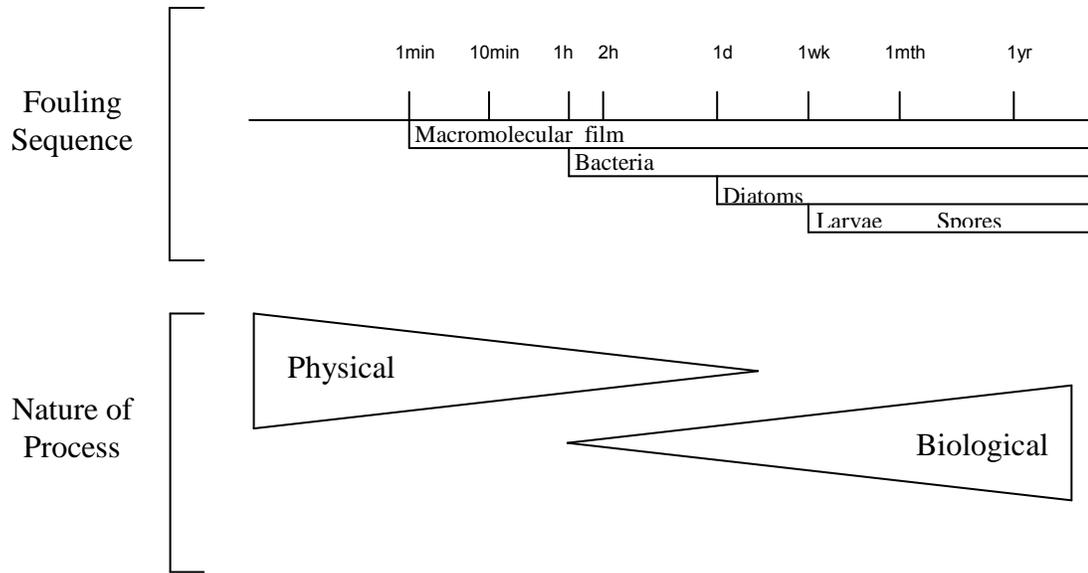
Figure 2.1. A fouling sequence leading to the formation of a fouling community (modified from Wahl, M. et al., 2003).

Bio-fouling has also been explained by dividing the process into 3 levels of organization, molecular fouling, micro fouling and macro fouling. Molecular fouling includes fouling of the submerged surface by all organic and inorganic materials in the solution. It is considered to be a rapid phenomenon which is easily reversible. Molecular fouling is also commonly known as the conditioning of the surface. Micro fouling consists of fouling by species like bacteria, diatoms, algal spores and protozoa and comes in the form of a layer of slime on the surface of vessels, plant and pipe work. A macro-fouling community consists of either "soft fouling" or "hard fouling" and may result from the development and growth of micro-fouling. Soft fouling comprises of algae and invertebrates such as soft corals, sponges, anemones, tunicates and hydroids, while hard fouling comprises of invertebrates such as barnacles and mussels (Callow et al., 2002).

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Macro fouling is considered to be a permanent irreversible process which involves the secretion of proteinaceous adhesives.

The complete step by step explanation of this biofouling process is elucidated in the next section, 2.2.

2.2 Bacterial Attachment and Biofilm Formation

During the first step, nutrients from the water absorb onto the solid surface and condition the surface. The nutrient molecules are carried to the solid surface from the bulk aquatic environment either by diffusion, turbulent flow, or sedimentation. This conditioning of the surface changes its physicochemical characteristics and is believed to help attract bacteria to the surface, mainly due to the high concentration of nutrients at the solid surface (Palmer et al., 2007). This initial step is a very fast phenomenon, occurring within a few seconds of the exposure of the surface to the medium. There have been conflicting reports about the importance of conditioning of the substrate surface. Jeong et al.(1994) suggested that conditioning of the surface with proteins would cause the bacteria to move to the surface for the essential nutrients, resulting in higher bacterial attachment. However, Barnes et al. (1999) have reported that the presence of the milk proteins on a steel surface has reduced the attachment of certain species including *Listeria monocytogenes* to steel surfaces. The reason for these conflicting opinions could be due to the different species being studied, different substrates being tested, different experimental conditions, different proteins and organic materials used for conditioning the surface (Plamer et al., 2007).

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The bacterial cells are then believed to travel or transported close to the surface by Brownian movement or convective mass transport. The initial attachment of the bacteria has been suggested to occur due to van der Waals forces, electrostatic forces and also due to various surface interactions such as hydrophobic interactions. This initial attachment is also believed to be affected by the availability of nutrients at the surface and the surrounding medium, and the growth stage of the bacterial cells. These attachments are believed to be weak and easily removed using shear forces, hence termed as reversible attachments. In the next step, various forces like dipole-dipole interactions, hydrogen, ionic and covalent bonding and hydrophobic interactions come into play, making the attachments irreversible (Kumar et al., 1998).

The individual irreversible attachments then give rise to micro-colonies, which grow in size with new cellular attachments. The attached cells at this point are believed to produce some adhesive compounds to keep them anchored to the substrate surface. *Pseudomonas* strains have been found to produce exopolysaccharide to help the colonies stay anchored to the surface. Multiple layers of these micro-colonies give rise to bacterial biofilm.

The biofilms thus formed may or may not be uniformly distributed throughout the surface (Kumar et al., 1998). Biofilms are not a continuous monolayers deposited on the surface. They are heterogeneous and are primarily composed of microbial cells and extracellular polymeric substance (EPS) which is the main matrix component (Lewandowski, 2000).

Therefore, numerous factors play a significant role in the final biofilm formation, right from the initial stages of attachment to the final biofilm matrix formation. Two such

important features such as the cell properties and characteristics of the growth medium are discussed below.

2.2.1 Cell Properties

The attachment of bacterial cells is effected by the hydrophobicity of the cell surface, and also by the presence of flagella and fimbriae (i.e. non-flagellar appendages that are involved in the transfer of bacterial nucleic acids) on its surface. The hydrophobicity of the cell surface is an important factor in adhesion. Hydrophobic interactions between the bacterial cell and the substrate surface tend to change proportionally with the non-polar nature of the cell and substrate surfaces. Though most bacteria are negatively charged, the presence of surface components such as fimbriae contributes to their surface hydrophobicity (Rosenberg et al., 1986). Such fimbriae were also observed on the surface of some bacteria found in the aquatic environment, assisting in the attachment of the bacterial cells to animal cells (Corpe, 1980) and substrate surfaces (Rosenberg et al., 1982).

Other properties of the cell such as motility also affect the attachment a great deal. Korber et al. (1989) studied the attachment of motile and non-motile strains of *P. fluorescens*. It was shown that motile cells attached a great deal more as compared to non-motile strains. This was proven even in the presence of flow or shear. It was also discovered that non-motile strains do not occupy the surface of substrate as evenly as motile strains, resulting in an uneven and slower biofilm formation. Bacterial cells which were motile were found to mainly posses some kind of surface structures like flagella, fimbriae, etc. There structures are believed to enable cells in the planktonic state to swim

towards nutrients exhibiting chemotaxis behavior and also helping the cells in physically adhering to the surface (Pratt et al., 1998)

Therefore, cell surface structures such as fimbriae, flagella and cell properties like motility all clearly play an important role, especially in the initial attachment process. The presence of these structures on the surface of microorganisms may also prove to be an advantage when a mixed community is involved.

The attachment of microorganisms to surfaces is a complex process, with many factors affecting the outcome. In general, it is believed that attachment occurs more readily on surfaces that are rougher, more hydrophobic, and conditioned by proteins and nutrients. An increase in flow velocity, water temperature, or nutrient concentration may also effect attachment, provided these factors do not exceed critical levels proving harmful to the microorganisms.

2.2.2 Characteristics of the Bacterial Suspension

The adhesion of bacteria to surfaces and the amount of EPS produced to form the biofilm matrix are affected by various characteristics of the bacterial suspension or the growth medium. These factors mainly include the temperature, pH, ionic strength, and nutrient levels. A seasonal effect on the bacterial attachment and biofilm formation has also been suggested by several studies (Fera et al., 1989). This effect may be attributed to the temperature of the bacterial suspension or other unmeasured, seasonally affected parameters. Fletcher studied the effect of the concentration of several cations (sodium, calcium, lanthanum, ferric iron) on the attachment of *Pseudomonas fluorescens* to glass substrate. It was suggested that with the increase in concentration of cations, the repulsive

forces between the negatively charged bacteria and the glass surface reduced, thereby allowing attachment (Fletcher et al., 1988). Cowan et al. (1991) proved experimentally that an increase in nutrient concentration resulted in an increase in the number of attachments.

The pH of the culture may also play a significant role on the ability of the cells to form micro colonies as it affects the ionization and therefore the binding and interaction of the various molecules present in the bacterial suspension. It also affects the solubility of a number of substances that the bacteria need for their growth and other activities. There is no specific pH level that would result in the maximum growth of bacteria in general, since the strains all differ in their evolution. Some bacteria like *H. pylori* may grow in highly acidic conditions, while most *Pseudomonas* strains prefer higher pH levels, closer to neutral pH (Palmer et al., 2007).

Therefore, the extent of the effect of the above mentioned conditions on bacterial attachment and biofilm formation depends a great deal on the type of bacterial species used, as different species or strains of bacteria respond in their own unique manner to the various changes in their environment.

2.3 Bacterial Species

Majority of the experiments carried out during this study have concentrated on the initial bacterial attachment of three main species, *Pseudomonas aeruginosa* (PAO1), *Escherichia coli* and *Pseudomonas putida*. The three bacterial species possessing clear similarities along with several marked differences were especially studied for their responses to several factors like shear, pH and potential antifoulants.

2.3.1 *Pseudomonas aeruginosa* – PAO1

Pseudomonas aeruginosa is a gram-negative, aerobic, rod shaped bacterium. Although classified as an aerobic organism, it is an environmentally versatile bacteria, which can thrive in conditions of partial or total oxygen depletion and has therefore adhered to a number of natural and artificial surfaces (Todar, 2008). Some strains have also shown anaerobic growth in the presence of nitrates. It is an important bacterium with a complex metabolism and is capable of degrading aromatic hydrocarbons, and producing biosurfactants called rhamnolipids. The *Pseudomonas aeruginosa* strains may also respond to changes in environmental conditions like pH, temperature, shear, presence of chemicals and gases, etc.

The *Pseudomonas* group bacteria are commonly found in the soil, in water and also on plant and animal surfaces. They are mainly believed to be plant pathogens, but certain strains are pathogenic to humans as well. They exist in nature either in the planktonic form or in a biofilm. In the planktonic form, the cells have been observed to have high motility and this has been attributed to their polar flagella. The initial stages of biofilm formation, which is individual attachments and formation of micro-colonies, have also been attributed to the interaction of these flagella with the substrate surface (O'Toole et al., 1998).

Pseudomonas aeruginosa forms biofilm on a number of surfaces like that of contact lenses and catheter lines. It is also found in the human body especially in those with compromised immune systems like patients with burn victims. AIDS, cancer, cystic fibrosis patients are also at increased risk of being infected by this bacterium. *Pseudomonas aeruginosa* is naturally resistant to a large range of antibiotics and

disinfectants, and may demonstrate additional resistance after unsuccessful treatment, making it a difficult pathogen to treat (Todar, 2008). The PAO1 strain used a great deal in our laboratory experiments is one of the most widely used *Pseudomonas aeruginosa* laboratory strain.

2.3.2 *Escherichia coli* (*E.coli*)

Escherichia coli are a large and diverse bacterial group which are a gram negative, rod shaped bacteria. These bacteria are versatile and are well-adapted to their characteristic habitats and are capable of growing in a media with glucose as the sole organic constituent. They can grow in aerobic as well as anaerobic conditions. Under anaerobic conditions it usually grows by means of fermentation or by utilizing nitrates (Todar 2008). This adaptable nature of *E. coli* allows it to grow in intestines (anaerobic) and in other environments (aerobic or anaerobic). *E. coli* too responds to environmental changes such as chemicals, pH, temperature, etc., in a number of significant ways (Todar, 2008).

Most *E. coli* strains are harmless and are found in the lower intestines of warm-blooded animals (including humans), but some can cause serious food poisoning in humans. Potent strains of *E. coli* may cause certain problems like gastroenteritis, urinary tract infections, and neonatal meningitis (Todar, 2008). But, they are not always confined to the body. When expelled, they are capable of surviving in water, sediment and soil. This ability to survive outside the body for brief periods makes their presence an ideal indicator of fecal contamination in environmental samples.

Many *E. coli* species are motile. Some use flagella which rotate allowing them to swim, however these flagella are believed to be different from those observed in the *Pseudomonas aeruginosa* strains. While *Pseudomonas aeruginosa* strains usually posses a single polar flagellum and swim in a straight mode, stopping to turn, the flagella seen in *E. coli* strains are about 5 to 10 in number originating at random points on their surface therefore causing a random awkward movement alternating between swimming smoothly and tumbling (Pratt et al., 1998). Others *E. coli* strains have been noticed to glide over surfaces by extending and retracting thin filament like structures called pili, that are believed to stick to the substrate surface (Berg, 2004). Therefore it has been hypothesized that these bacterial strains use different approaches leading to biofilm formation, and the development of a mature biofilm depends on the motility of the cells along the surface allowing the biofilm to spread (Pratt et al., 1998).

E. coli are easily grown in the laboratory and their genetics being simple, can be easily-manipulated or duplicated. Hence, they form an important species in biotechnology and microbiology.

2.3.3 *Pseudomonas putida*

Pseudomonas putida is a versatile environmental bacterium which is also rod shaped and gram negative. It is generally aerobic in its metabolism and is capable of growing in simple media at the cost of several organic compounds. This bacterium is capable of growing on aromatic hydrocarbons like benzene, toluene and ethyl benzene (Choi et al., 2003).

This species is very similar to *Pseudomonas aeruginosa*. It even has the pathogen's ability to resist antibiotics and can even withstand environmental toxins, either by breaking them down or forcing them out. However, unlike *Pseudomonas aeruginosa,* they are believed to be usually non-pathogenic as they lack the genes and enzymes to digest the membrane of cells. Therefore the microbe is considered to be benign (Nelson et al., 2002).

Pseudomonas putida strain is also believed to track chemicals in the soil environment, enabling it to respond to toxins when needed. It is also capable of the typical *Pseudomonas* bacterial group motility with the help of one or more polar flagella (Nelson at al., 2002).

One of the distinctive properties of this microorganism is its ability to cleanse the soil of organic pollutants as it can resist the adverse effects of these organic solvents. *P. putida* is also capable of breaking down hazardous chemicals like aromatic hydrocarbons generated by the burning of coal, tobacco, meat and other organic matter. The petroleum industry has therefore been looking into using *P. putida* as a cheap way of purifying fuel (Choi at al., 2003). Its resistance to antibiotics has enabled scientists to study its ability as a fertilizer by protecting plants from pests and helping them grow. The non-pathogenic nature of *P. putida* is the main reason behind its potential new applications in agriculture, biocatalysis, bioremediation and bioplastic production.

The conditions discussed so far would be perfectly applicable to stationary systems that have been studied in the initial stages of this research work. However, an improvement of the stationary system, i.e. the flow system was employed later in this study. Therefore, added to the several features listed in the previous sections, some more

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aspects need to be understood before the bacterial attachment and biofilm formation could be studied under flow conditions.

2.4 Bacterial Attachment and Biofilm Formation in Flow Systems

Flow systems have been increasingly used in recent times to study bacterial attachment, biofilm formation and development. Different from static systems, a number of additional factors like shear, flow development, etc. could play dominant roles in these systems. Hence it is important to be aware of the basic flow dynamic aspects and principles governing these flow systems.

2.4.1 Flow Dynamics

Theoretically, the velocity of the flow immediately next to the liquid-solid (substrate) interface is negligible and this zero velocity zone is called the hydrodynamic boundary layer. The thickness of this boundary layer depends on the linear velocity of the flow, i.e. as the velocity increases, the boundary layer thickness decreases to a certain extent. The region outside this hydrodynamic boundary layer within the flow is characterized by different degrees of turbulence.

In case of laminar flow regimes, the thickness of the boundary layer may considerably affect the cell-substrate interactions. The cells are brought close to the substrate surface due to the little turbulence caused by the flow and then must traverse the sizeable hydrodynamic boundary layer and associate with the surface. This interaction of the cells with the substrate surface also depends a great deal on the cell size and motility.

As the velocity increases, the boundary layer thickness decreases, and the cells experience greater turbulence. Higher linear velocities would imply greater association of the cells with the surface, until the velocity becomes high enough to exert significant shear forces on the attaching cells, preventing the attachments and sometimes also causing detachment of cells.

2.4.2 Some Common Flow Devices

Some common flow devices being used for bacterial attachment and biofilm formation are discussed in this section.

2.4.2.1 Robbins Device

Robbins device is a multi-port device commonly used to study early attachment and biofilm formation at the substrate surface. It allows multiple samples to be analyzed after different lengths of cultivation time, without disturbing the other surrounding samples.
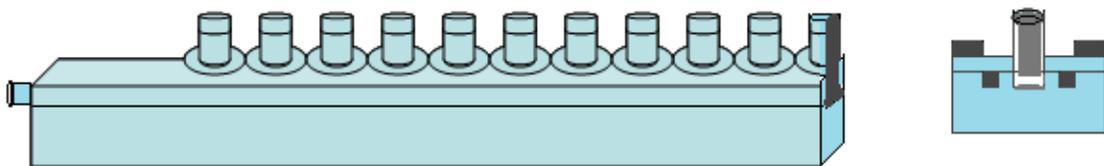
Figure 2.2. Cross sectional sketch of a Robbins Device (sketch modified from reference 37).

Several modified versions of this device have also been widely used in waste water treatment and industrial situations to study the biofilm formation on the surface of

various materials (Linton et al., 1999). One common variation to study the bacterial attachment to stents involved using catheters to flow and cultivate the bacterial suspension and finally cutting the catheters into sections to view under the microscope or other analyzing aids. Another such modification involves the use of rectangular flow chamber with a removable top to which the experiment surface is attached to study the bacterial adhesion on those specific substrata (An et al., 2000). These modifications of the basic design are based on the specific experimental requirements and the problems faced.

In addition to the above mentioned advantages of system balance and easy modification of the system, other advantages include simplicity and ease of operation. However, due to its fine construction the cost of this device is on the higher end (An et al., 2000).

2.4.2.2 McGlohorn's channel based laminar flow chamber

The McGlohorn's flow chamber is an eight channel flow chamber, with removable microscopic slides with attached test surfaces for biofilm formation analysis on specific surfaces. This flow chamber is equipped with identical mixing chambers at both ends to reverse the fluid flow and to control the turbulence of the flow at the entry. The channels are built such that all the channels experience an equal amount of fluid shear and flow rate. Another advantage of this design is the presence of air space between the lid and the fluid in the channels accommodating anaerobic conditions for the growth of thick biofilms (An et al., 2000).

2.4.2.3 Radial Flow Device

Another common flow device is the radial flow chamber, where the fluid flows radially from the center of a circular disk like fermenter towards the outer walls. The shear forces of the fluid have been measured to decrease in proportion to the square of the distance from the center of the disk. But it must be noted that with time as the biofilm growth increases, it affects the set up conditions especially due to increased frictional drag and dispersion of cells from the inner biofilm to the outer biofilm (An et al., 2000). Based on the studies and the problems targeted, some other flow chambers have also been devised. The laminar flow parallel plate flow chambers include those chambers where the biofilm is cultured between parallel plates (Habash at al., 1997; Poelstra et al., 2000). The laminar flow, radial "artificial mouth" systems, include flow systems which duplicate the dental plaque on teeth and other artificial surfaces (An et al., 2000).

The common flow chambers listed in this section mainly use glass slides or other metallic substrate surfaces inserted in the chamber which then need to be removed to image for biofilm formation. However in our case, one of the most important aims was to study initial bacterial attachment which warranted the use of a real time system. Also the underlying reason for designing the flow device was to avoid manual handling before imaging, since this tends to dislodge the initial bacterial attachment. Furthermore, most of the flow chambers talked about in literature employ high shear conditions (Boyle et al., 2007; Habash at al., 1997; Thomas et al., 2004), while we planned to study attachment under low shear. At the low shear conditions studied using the flow system, individual bacterial movement even against the flow could be observed. This suggests that the attachment was not purely dependent on the flow dynamics but was also prompted by the

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response of the bacterial cells to the environmental conditions. This was another reason for designing the flow chamber as explained in section 3.4.

Hence, the flow chambers were designed mainly to understand the reasons behind the increase or decrease in attachment in the presence of certain conditions. By better understanding the exact phenomenon occurring, its prevention would be that much easier and in order to prevent, two potential antifoulants have been investigated with the help of these flow systems, in the hope of finding a possible solution to this biofouling problem.

2.5 Potential Antifoulants

Currently, the search is on for antifoulants which have a repelling effect on the bio- organisms rather than a toxic one. The surface should be perceived to be unpleasant by the bio-organisms instead of causing cellular damage. A number of marine plants and animals have been found to possess a self cleaning ability, which keeps their surfaces clean in spite of spores and larvae found in the waters. Zosteric acid is a compound which has been isolated from the young shoots of one such sea grass called *Zostera Marina*. Another possible antifoulant being studied is Rhamnolipid, which is also a highly environment friendly compound.

2.5.1 Zosteric Acid

In order to survive, some plants produce compounds that deter or kill pathogens, or reduce the impact of environmental factors, such as harmful levels of UV radiation. Zosteric acid is one such natural compound which is produced by a sea grass namely

zostera marina. It contains a sulphate phenolic ester group at one end and at the other, a carboxylic acid group.
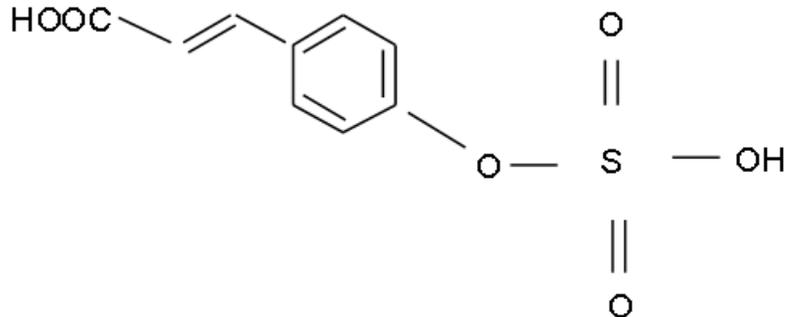
Figure 2.3. Structure of Zosteric Acid

The yield of zosteric acid obtained from natural extraction was only about 66mg from 1700g of dry biomass (Zimmerman et al., 1995). Hence, naturally obtaining zosteric acid was found to be economically too steep. The concept of extracting and purifying significant amounts of the compound from eel grass, apart from acquiring the consent of the government, drawing out a substantial quantity of the raw material from the aquatic environment proved to be too problematic and expensive.

Zosteric acid was then synthesized in the laboratory from chlorosulfonic acid and p-coumaric acid (Todd et al., 1993; Alexandratos, 1999). The properties of the two products, one obtained naturally and one from laboratory synthesis were tested and proved to be similar. Hence, the zosteric acid synthesized in the laboratory was used to study the antifouling activity of the compound (Hany et al., 2004).

Zosteric acid, based on its purity is in the form of an off white (relatively more pure) or light tan solid at ambient conditions. It is highly soluble in water (Todd et al., 1993) and shows a UV absorbance peak at 275nm (Sundberg et al., 1997).

The antifouling property of zosteric acid has been studied against a number of organisms like marine bacteria (Todd et al., 1993), fresh water bacteria (Barrios et al., 2004), algae (Calow and Calow, 1998; Shin et al., 2001;), fungi (Stanley et al., 2002), barnacle larvae (Haslbeck et al., 1996), and tubeworms (Shin et al., 2001; Sundberg et al., 1997).

2.5.2 Rhamnolipids

Rhamnolipids are environmentally friendly alternatives to synthetic and more crucially to the petroleum derived surfactants. Synthetic and petroleum derived surfactants necessitate a great deal of energy, in order to be produced and due to the depletion of world wide natural resources, now face huge price increases and limited supplies.

Rhamnolipids are naturally occurring glycolipids which are produced by the *Pseudomonas aeruginosa* species of bacteria. They can also be made from renewable resources. They contain carbon, hydrogen, and oxygen and are a combination of one or two rhamnose sugars (naturally-occurring sugars that have a hydroxyl group replaced by hydrogen) and fatty acids. In their dry pure form, they are in the form of a white powder, while in an aqueous solution they appear milky white or slightly tan in color. These surfactants reduce the surface tension of water by more than half, i.e. from 72mN/m to values below 30mN/m (Nitschke et al., 2005).

There are two major groups of rhamnolipids; mono-rhamnolipids and di-rhamnolipids. Mono-rhamnolipids have a single rhamnose sugar ring. The basic formula (which is most often produced by *P. aeruginosa*) is L-rhamnosyl-beta-hydroxydecanoyl-

beta-hydroxydecanoate (often referred to as Rha-C_{10}-C_{10}) with a formula of $C_{26}H_{48}O_9$. Di-rhamnolipids have two rhamnose sugar rings. The basic formula is L-rhamnosyl-L-rhamnosyl-beta-hydroxydecanoyl-beta-hydroxydecanoate (often referred to as Rha-Rha-C_{10}-C_{10}) with a formula of $C_{32}H_{58}O_{13}$. The critical micelle concentration (CMC) (or concentration above which the micelles form spontaneously) for mono-rhamnolipids was found to be close to 40mg/L, and for the di-rhamnolipids, 5mg/L. This has been explained by the presence of the larger fatty acid chains, as is the case in di-rhamnolipids. The larger fatty acid chains make the molecules more hydrophobic, which tend to aggregate in the form of micelles at much lower concentration than the mono-rhamnolipid molecules (Nitschke et al., 2005).

It has been recently discovered that *Pseudomonas aeruginosa* uses low concentration of rhamnolipids to maintain open channels and void spaces in biofilms. Once the open channels are formed, the *Pseudomonas aeruginosa* bacteria are believed to utilize these biosurfactants to actively maintain the void spaces allowing the distribution of nutrients and oxygen through the biofilm and the removal of metabolic end products (Davey et al., 2002). However, in the presence of higher concentrations of rhamnolipids, cellular attachments have been inhibited, preventing the formation of biofilm. It has also been suggested that rhamnolipids not only prevent attachment, but also facilitate the detachment of bacterial cells from biofilm into the planktonic form (Davey et al., 2002). Rhamnolipids have a wide range of applications as a natural surfactant, emulsifier, foaming agent, fungicide and even as an antibiotic. The use of rhamnolipids as fungicide and antibiotic, more specifically implies their use as a pesticide, food preservative, and in medical device and implant coating. As a pharmacological agent, rhamnolipids can be

used for treating age marks, bedsores, burn repair, and also in wound healing, sun damage, and wrinkle reduction. Rhamnolipid foaming can also be used to remove heavy metal contaminants from soils, wastewater, and other liquids. Hence, they are currently being used as the natural ingredient or "green" ingredients in soaps, shampoos, cosmetics skin care products, detergents, etc. It is also an FDA approved food additive and is used for flavors (Nitschke et al., 2005).

CHAPTER III

EXPERIMENTAL APPROACH

Essential details about the materials and equipment used, methods adopted while preparing and inoculating the cell culture for both static as well as the flow systems, design and construction of the flow chambers employed are provided in this chapter.

3.1 Materials and Equipment

Silicone coatings or devices were prepared using Sylgard® 184 Silicone Elastomer manufactured by Dow Corning. The Sylgard® 184 Silicone Elastomer was supplied as two separate components, the base and the curing agent, which needed to be mixed in a ratio of ten parts base to one part curing agent by weight.

The glass slides used in the preparation of hydrophobic surfaces or construction of flow chambers were purchased from VWR Scientific. The microscopic glass slides were plain and precleaned with dimensions 25mm x 75mm x 1mm. These glass slides were cut by drawing a line using a diamond pen and tapping the glass lightly to break it at that line.

The source of the two potential antifoulants studied is as follows, the rhamnolipid samples were obtained from Ecochem Canada Ltd, while zosteric acid was synthesized in

the lab from p-coumaric acid and chloro-sulfonic acid. After purification, the final product was in the form of an off-white powder after purification. The chemicals (p-coumaric acid, chlorosulfonic acid) and solvents (pyridine, diethyl ether, and methanol) used for the synthesis of zosteric acid were purchased from Sigma-Aldrich and used as received. The zosteric acid samples were subjected to UV-vis Spectroscopy, Mass Spectroscopy and Nuclear magnetic resonance (NMR) analysis before using.

The equipment used included a Vacuum Oven (National Appliance, Model No.5831), Hot Plate (Corning), Stirrers (Corning), Hot Plate / Stirrer (VWR Scientific), Plasma cleaner (Harrick ,Model No.PDC-32G), a shaker incubator (New Brunswick Scientific Co., Innova 4080), Electric Pressure Steam Sterilizer (All American, Model 25X), UV/Vis spectrophotometer (Shimadzu, Model 1601), pumps (Cole Parmer Instrument Company, Masterflex® L/S), Optical Microscope (Olympus IX70), and Dazzle Multimedia Hardware and Software.

3.2 Medium Preparation

For static systems, the medium prepared had a higher concentration of the bacterial cells as compared to the one prepared for the flow systems. This was mainly done to facilitate easy counting during imaging in case of the flow systems.

3.2.1 Static Systems

Pseudomonas aeruginosa PAO1 and other *Pseudomonas* strains (F04-63, H05-25, E03-36, E03-40), were maintained in frozen stocks of TSB medium (Sigma-Aldrich, St. Louis, MO) and 15% glycerol (Sigma-Aldrich). *E. coli* was maintained in Agar plates.

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When the microorganism used in the static system studies was *P. aeruginosa* PAO1, the bacterial cells were grown at 34°C for 24 h in a shaker incubator and were used as inoculum for the biofilm experiments. For the preliminary static experiments, 60-mL glass bottles containing 20 mL of medium and various concentrations of zosteric acid, including a zosteric acid-free control were used. The pH of all the systems was adjusted to 7.1 ± 0.1 before sterilization. Samples from each bottle were taken to study the cell concentration, degradation of both zosteric acid and p-coumaric acid, and extra-cellular proteins. The medium used for attachment experiments contained 3 g/L $NaNO_3$, 2 g/L KH_2PO4; 0.3 g/L $Mg_2SO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L NaCl; 0.01 g/L $MnCl_2 \cdot 4H_2O$ and 2 mL of a trace element solution that contained 0.08 g/L $FeCl_3 \cdot 6H_2O$, 0.75 g/L $ZnSO_4 \cdot 7H_2O$, 0.08 g/L $CoCl_2 \cdot 6H_2O$, 0.075 g/L $CuSO_4 \cdot 5H_2O$, 0.75 g/L $MnSO_4 \cdot H2O$, 0.15 g/L H_3BO_3, and 0.05 g/L Na_2MoO_4. Glucose (6g/L) was added to these samples as the carbon source.

When *E. coli* was the microorganism being studied, the bacterial cells were grown overnight at 37°C in a shaker incubator. The medium used was the same as that used for *Pseudomonas aeruginosa* PAO1. However, in case of *E. coli*, glucose was not added. Instead peptone 2.5g/L and yeast extract 2.5g/L were added to the samples for the cell growth.

In case of the other *Pseudomonas* strains, the conditions and procedure followed was exactly the same as that used in case of *Pseudomonas aeruginosa* PAO1.

3.2.2 Flow Systems

When the microorganism used in the flow system studies was *Pseudomonas aeruginosa* PAO1, the culture was maintained in frozen stocks of tryptic soy broth (TSB) medium containing 15% glycerol. The cultures were grown overnight in liquid medium at 34°C in a shaker incubator at 300 rpm before being used in the static or flow systems. The medium for flow system experiments contained 0.3 g/L $NaNO_3$, 0.07 g/L KH2PO4; 0.03 g/L $Mg_2SO_4 \cdot 7H_2O$, 0.001 g/L $CaCl_2 \cdot 2H_2O$, 0.002 g/L $FeSO_4 \cdot 7H_2O$, 0.05 g/L NaCl; 0.001 g/L $MnCl_2 \cdot 4H_2O$ and 0.2 mL of a trace element solution that contained 0.08 g/L $FeCl_3 \cdot 6H_2O$, 0.75 g/L $ZnSO_4 \cdot 7H_2O$, 0.08 g/L $CoCl_2 \cdot 6H_2O$, 0.075 g/L $CuSO_4 \cdot 5H_2O$, 0.75 g/L MnSO4·H2O, 0.15 g/L H_3BO_3, and 0.05 g/L Na_2MoO_4.

The medium for the flow-system experiments was essentially a 10-fold dilution of the medium described above for the static systems, with the following modifications: (1) no glucose was added and (2) the concentrations of KH_2PO_4 and $FeSO_4 \cdot 7H_2O$ were lowered to 0.07 g/L and 0.002 g/L, respectively, to minimize precipitate formation for clearer observation of cell attachment in the flow chambers. For these experiments, 75 ml of the medium was put in 125 ml flasks and autoclaved at 121ºC for 15 min. The pH was adjusted to 7.1 ± 0.1 before autoclaving (or to the desired value, with phosphate buffers, when studying the effect of pH on the attachment). To keep the cell concentrations similar in all experiments, the optical density of the culture grown overnight was measured at 600 nm using a UV/Vis spectrophotometer and the inoculation size adjusted accordingly. Very low inoculation levels, around 0.1%, were used to keep the cell concentrations low for easier counting of the attached cells.

When the microorganism used in the static system studies was *E. coli*, the bacterial cells were maintained in Agar plates. These bacterial cells were grown overnight at 37°C in a shaker incubator and were used as inoculum for the biofilm experiments. The medium for flow system experiments using *E. coli* was similar to that of *Pseudomonas aeruginosa* PAO1. The only difference being the addition of yeast extract 2.5g/L and peptone 2.5g/L for growth of the *E. coli* cells. *Pseudomonas putida* cells were grown using the exact same medium and procedure as that followed for *Pseudomonas aeruginosa* PAO1.

3.3 Coating Preparation

The regular sized microscope glass slides were cut in halves along the length, such that their width was reduced to 12.5mm and their length remained at 75mm. These cut slides were then cleaned by sonicating in ethanol, and rinsing with copious DI water, and finally drying them in a stream of nitrogen. The cleaned glass slides were then coated with a thin layer of silicone, the liquid mixture of the Sylgard$^{®}$ 184 Silicone elastomer base and its curing agent. During preparation, the liquid mixture of the elastomer base and its curing agent was thoroughly stirred to ensure that the curing agent was well-mixed with the base, to avoid partial curing. A drop of this mixture was placed on each of the cleaned glass slide and allowed to spread naturally. After coating the glass slides with a thin layer of silicone mixture, they were then allowed to cure for a period of 48 hours under the ambient condition to form a cross-linked film on the surface of glass. After curing, the silicone film covered about three-fourths of the surface of the glass.

3.4 Chamber Constructions

      The design and construction of the chambers used in this study are discussed in this section.

3.4.1 Silicone-Glass Cover slip Chambers

      A mixture of the Sylgard® 184 Silicone elastomer base and its curing agent was prepared as described earlier. This mixture was then poured into a plastic petri-dish and allowed to cure for 12 hours at a temperature of 40^oC. The thick sheet of silicone formed in the petridish after curing, was cut to give a square piece of dimensions ~ 10mm x 10mm x 0.75mm. A square cavity with a dimensions 1mm x 1mm x 0.75mm was then cut out of the center of this square silicone piece. This silicone piece with the cavity was then covered with a glass cover-slip by holding them together and applying some gentle pressure. The chamber was completely sealed using another piece of cover-slip after introducing the bacterial medium into the cavity.

3.4.2 Silicone-Glass Flow Chambers

      The chambers were prepared by curing the silicone over a mold (magnetic stirring bar) with glass tubes sticking out from either end forming the entrance and exit. Four chambers were prepared at once in a plastic container using silicone pieces to separate the four glass slides with molds placed in the center of each. Meanwhile, glass tubes were cut to ~ 1.5cm in length and sealed on one side by dipping the tube in the liquid silicone mixture and holding it above a hot plate at a temperature of ~ 150^oC. The other free end of the glass tubes was ensconced in plastic tubing. Metal wires were used to hold the

attached plastic tubing in an upright position forming an L shape. The glass tubes with the plastic tubing were positioned on the glass slides such that on removing the mold they would form the entrance and exit to the chamber. The glass tubes were held in place by taping the attached plastic tubing to the side wall of the container, with the free end of the plastic tubing sticking out of the plastic container to prevent liquid silicone from entering it during the curing process. The liquid silicone mixture was poured over this arrangement and allowed to cure at a room temperature of 20°C for 1 hour to let the air bubbles out (i.e. degas) and later placed in an oven at a temperature of 40°C for 12 hours. After 12 hours, the solidified silicone was removed from the plastic container along with the mold and the glass tubes as a whole, and cut to form four chambers. The magnetic stirring bars acting as molds were then removed from the silicone, and the chamber side walls were trimmed to form vertically straight walls, thus almost identical cavities of the four chambers. The silicone blocking the glass tubes was also removed to form a clear path for the flow of medium through the chamber. Each cut silicone piece containing a cavity was finally completely sealed by bonding a glass slide to the silicone surface. The glass slide was sealed onto the silicone piece after oxidizing the glass slides and the silicone surface using air plasma and then pressing the oxidized glass slide against the oxidized silicone surface. The silicone surface is said to become extremely hydrophilic immediately after air or oxygen plasma exposure by creating silanol bond (Si-OH), which promotes its adhesion to the oxidized glass surface. In the case of improper bonding of the glass and silicone piece at certain areas due to unevenness of the surface, a silicone gel was used to seal the chamber to avoid potential leakage.

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The procedure used can be better understood with the help of the sketch provided

below in Figure 3.1.

(a)

(b)
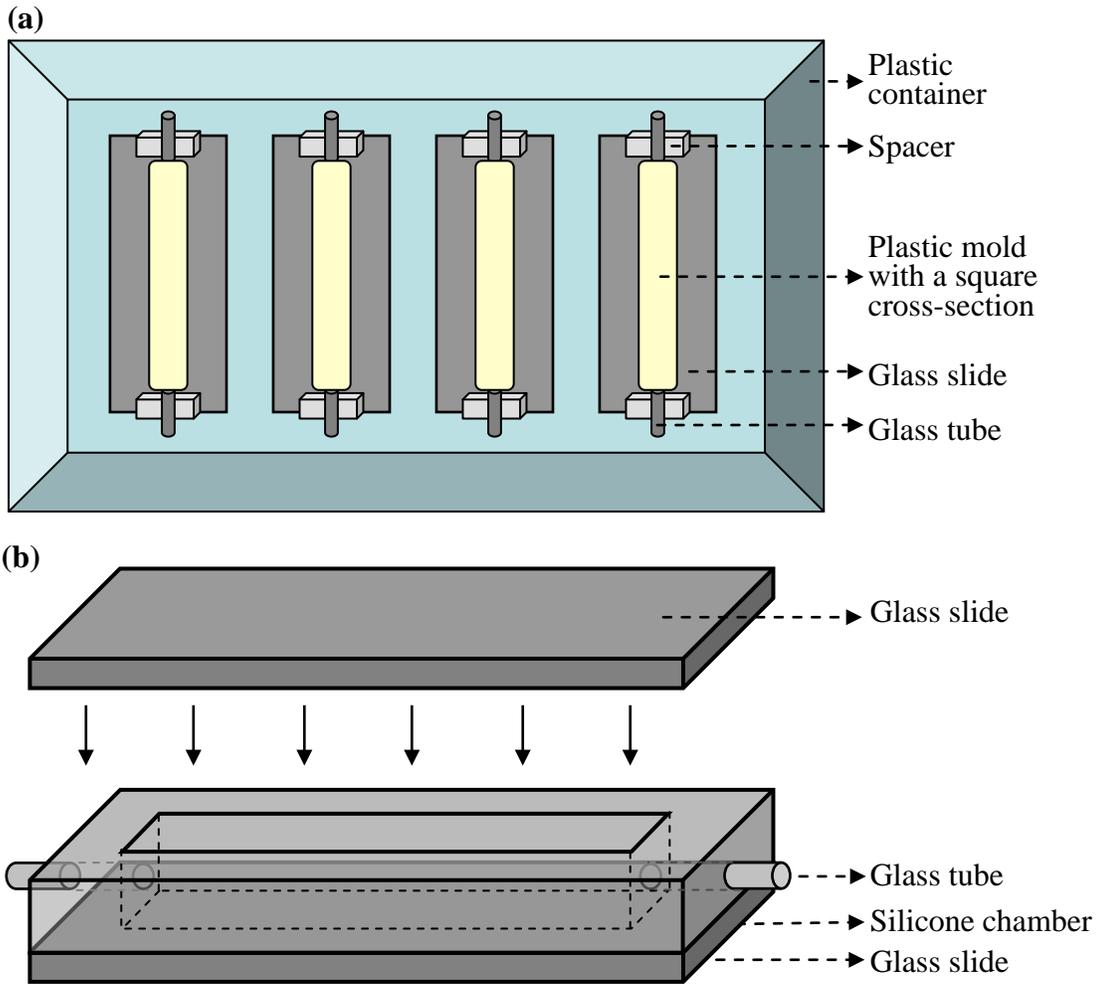
Figure 3.1. Schematic (a) shows the plastic container with the glass slides, molds (magnetic stir bars), spacers (to maintain the height of the glass tubes such that they would open into the chamber right in the center) and glass tubes (forming the inlet and outlet of the chamber). Liquid silicone mixture was poured over this set up and allowed to cure. (b) shows one silicone chamber, after being cut out from the cured silicone assembly removed from the plastic container. One side of the chamber has already been sealed with the glass slide during curing. The schematic shows the chamber being completely sealed to a second glass slide placed at the top.

3.4.3 Glass Flow Chambers

The silicone-glass chambers described in section 3.4.2 proved to be very useful when studying the bacterial attachments under a number of different conditions. However, the chamber construction was highly time consuming and elaborate. In order to reduce the time and effort, glass chambers were constructed using the commercially available square glass tubes. Square glass tubes (Friedrich & Dimmock, Inc.) with the internal cross section of 10mm x 10mm and a wall thickness of 1mm were cut to ~ 4 inches lengthwise. The cut glass tubes were then cleaned by soaking them in piranha solution (70% H_2SO_4 and 30% H_2O_2) for 45 minutes and rinsing them with copious amounts of DI water. Meanwhile, the circular glass tubes which formed the path to and from the chamber were sealed using silicone to prevent the liquid silicone from filling them up. Thick silicone sheets of thickness ~ 11mm were cured in a petridish with the sealed glass tubes sticking out perpendicularly. The curing process took ~ 3 hours. These cured flexible sheets were then cut to form stoppers for the square tube ends, with the circular glass tubes cleared off silicone to form a clear path for the flow of the bacterial medium. The silicone stoppers were inserted at the glass tube ends and their interface with glass was coated with a layer of liquid silicone to hold them together and prevent leakage. Silicone gel (silicone rubber compound from RS components) used in the silicone-glass chambers to prevent leakage was used for the same purpose around the ends of these chambers as well. The chamber construction is illustrated in Figure 3.2.
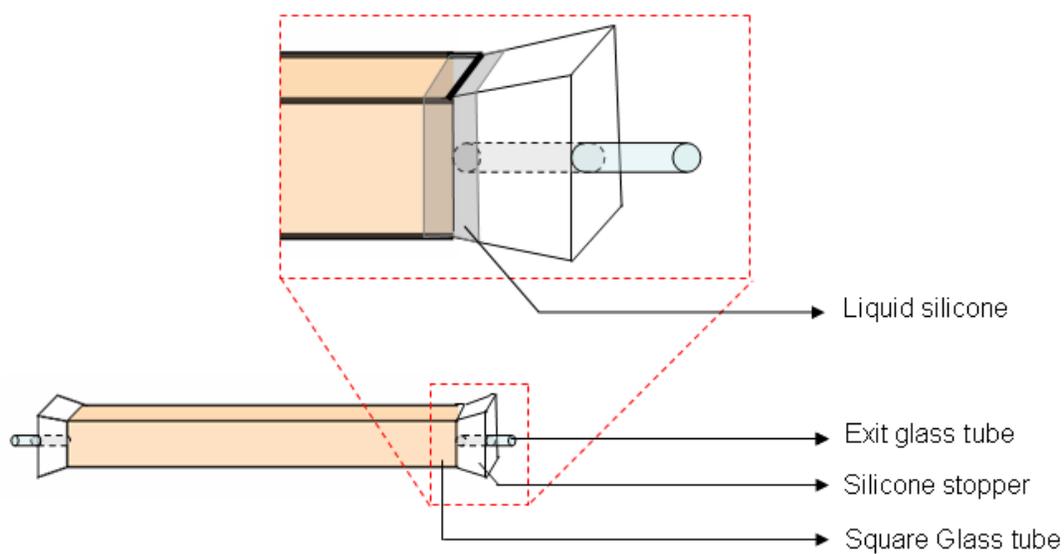
Figure 3.2. Schematic showing the construction of glass flow chamber. The magnified portion shows the liquid silicone used to seal the glass-silicone interface.

3.5 Bacterial Attachment and Biofilm Formation Study

Details of the basic procedure followed while setting up the experiments (both static and flow systems) are provided in this section.

3.5.1 Static Systems

For the first set of static systems, the amber glass bottles, glass slides with coatings and the medium without the cells were autoclaved in an electric pressure steam sterilizer at a temperature of 120°C. The bacterial cell culture was added to each of the bottles inside the laminar flow hood and the systems (bottles) were left undisturbed till the time of imaging.

The glass slides were imaged after a period of 2 hours, 24 hours, 48 hours and 7 days. During imaging, the glass slides were carefully removed from the bottles and rinsed

to remove the loose attachments and viewed under the optical microscope for bacterial attachments and biofilm formations. Two types of rinsing techniques were followed; dipping the glass slides in a DI water/saline solution three times, and then the surface which was facing down was imaged for cell attachments or biofilm formations. and holding the slides upright and allowing the DI water/saline solution to flow down the slide washing away the loose deposits for about 30 seconds.

The second set of static systems used polystyrene petridish (Fisherbrand, dimensions of diameter x height = 100mm x 15mm). The medium and glass cover slips (Fisherfinest, dimensions of 22mm x 40mm) were autoclaved, and set up by adding the bacterial cells in the laminar flow hood. The experiments were started by placing the autoclaved glass cover slips on the surface of the liquid in the petridish, allowing the cover slips to float. These cover slips were imaged after a period of 2 to 3 hours after rinsing them using the two techniques mentioned previously.

The third set of static systems comprised of the silicone – glass cover slip static chambers. The chambers after the autoclaving were filled with the bacterial medium inside the hood to prevent contamination. The silicone chambers were then completely sealed by placing the glass cover slip over the top and were continuously imaged using an optical microscope. The initial attachment followed by the formation of clusters leading to biofilm formation was recorded for a period of 5 hours. Systems with varying factors were compared to the control systems to determine the factors affecting the bacterial attachment and biofilm formation.

3.5.2 Flow Systems

For the flow systems, the flask containing the bacterial medium was connected to the flow chamber using plastic tubing (Masterflex® platinum-cured silicone tubing, L/S® 16), so that the medium could be continuously circulated. A flask containing the saline solution was also connected by a switch to the line leading to the entrance of the chamber, such that after running the experiment with the bacterial medium the saline solution could be used to wash away the loose attachments and drain the solution in one circulation. The flasks containing the medium without the cells, flasks containing the saline solution, the plastic tubing, and the flow chambers after being connected and sealed were all autoclaved at a temperature of $120^{o}C$ and a pressure of 20psi for 20 minutes, in order to sterilize the system. The bacterial cells were introduced into the system just before running the experiments inside the hood to prevent contamination.

The experiments were carried out immediately after adding the cells to the flasks containing the medium. The runs usually involved comparing four or six systems, such that each of the system had a duplicate to confirm the results obtained. Peristatic pumps (flowrate range 1.4ml/min to 87.7ml/min for the silicone tubing size L/S®16) were used to circulate the medium through the chamber. The medium was introduced to the flow chambers with a time gap of 10 to 15 minutes from one chamber system to another to allow ample time for imaging of the attachment of each chamber. Once the medium was introduced into each chamber, it was continuously circulated for the entire duration of the runs. The bacterial media were also continuously stirred (using Corning Stirrers) to avoid settlement of the bacterial cells in the flask before and during circulation. The top and bottom glass slides of each of the chambers were imaged using a transmission Optical

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Microscope for bacterial attachments. The attachments were counted manually and recorded for a few minutes every hour for 3 hours. At 3 hours of attachment, the individual attached cells started to form clusters and small biofilm patches, making it difficult to quantify the individual attachments. After 3 hours, the chambers were rinsed by circulating saline solution to remove loose attachments and the glass surfaces were once again imaged.

All experiments were conducted at room temperature (~ 24°C). For each set of experiments, the handling of the cell cultures, the pretreatment procedure and the time between the chamber assembly and bacterial attachment experiments were kept as consistent as possible.

The complete flow system set up is shown in Figure 3.3 below, for better understanding of the arrangement.
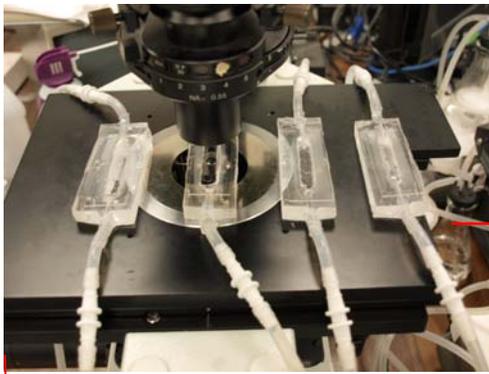
Flow chamber
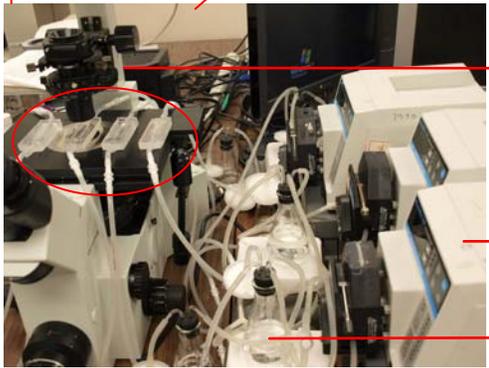
Optical Microscope

Pump

Flask carrying the
bacterial suspension

Figure 3.3. Flow system set up pictures showing the flow chambers under the optical microscope for real time imaging. Also shown in the pictures are the peristaltic pumps circulating the bacterial suspension and saline (for rinsing), and the stirring plates to keep the bacterial cells in the flasks from settling.

CHAPTER IV

STATIC SYSTEMS

Static systems were initially used to carry out some fundamental studies on bacterial attachment and biofilm formation. The effect of a potential antifoulant, zosteric acid (ZA) and other factors, mainly the bacterial medium and substrate surface hydrophobicity, on bacterial attachments was researched and the results of these studies are presented in this chapter.

4.1 Zosteric Acid Effect

In the process of investigating the antifouling effect of zosteric acid, several stationary system setups were designed, assembled and employed. The initial stages of this investigation involved long term surveys of the effect of zosteric acid. Towards the later part, the experiments were limited to a short time period since the focus was mainly on initial attachment.

4.1.1 Chemotaxis of ZA on PAO1

Before carrying out the bacterial attachment studies, a chemotaxis experiment was conducted to evaluate the toxicity of ZA on the three types of bacterial cells used in this

research, i.e. *Pseudomonas aeruginosa* PAO1, *Pseudomonas putida* and *E. coli*. Chemotaxis is the phenomenon due to which cells move based on the chemicals present in their environment. When the chemical (or compound), normally dissolved in water, injected into a capillary tube that contains certain bacteria, the bacteria will migrate toward the injection site when the compound is a food or poses no toxicity to the bacteria. If the compound is unfavorable or toxic to the bacteria, they will flee in the opposite direction. In order to substantiate the correctness of the experiment, glucose, a food source for bacteria, was injected from one end into a capillary tube that contained the bacterial suspension. Within a few minutes (< 5 minutes) of injection, it was observed that the bacterial density was higher near the injection site as compared to the other far end. The individual bacteria were also observed to be swimming towards the glucose concentrated region, under the optical microscope. Following the exact procedure, ZA solution in DI water (500ppm) was injected into a capillary tube already filled with the bacterial suspension, and observed continuously using an optical microscope during the injection procedure. In this case, no difference in the bacterial concentrations at the injection end and at the other end of the tube was noticed, and furthermore a random bacterial movement was observed. The experiment was repeated several times to confirm that ZA has no effect on the movement of the three bacterial strains, thus suggesting that ZA poses no or low toxicity to the bacteria used in our study.

4.1.2 Systems using amber bottles

Amber bottles containing the bacterial medium rich in nutrients and glucose were added with solutions of ZA to reach final concentrations of 10ppm, 50ppm, 100ppm,

500ppm, and 1000ppm. Medium free of ZA was also prepared and used as control. The pH of all the systems was adjusted to 7.1±0.1. Each system had at least one duplicate to verify the results.

After a certain period of time (2, 5, 7 and 14 days), the glass slides were removed and observed using the optical microscope. For the observation, the glass slides were dipped in a saline solution three times, and then the surface which was facing down was imaged for cell attachments or biofilm formations. The sketch in Figure 4.1 helps in better understanding the procedure used.
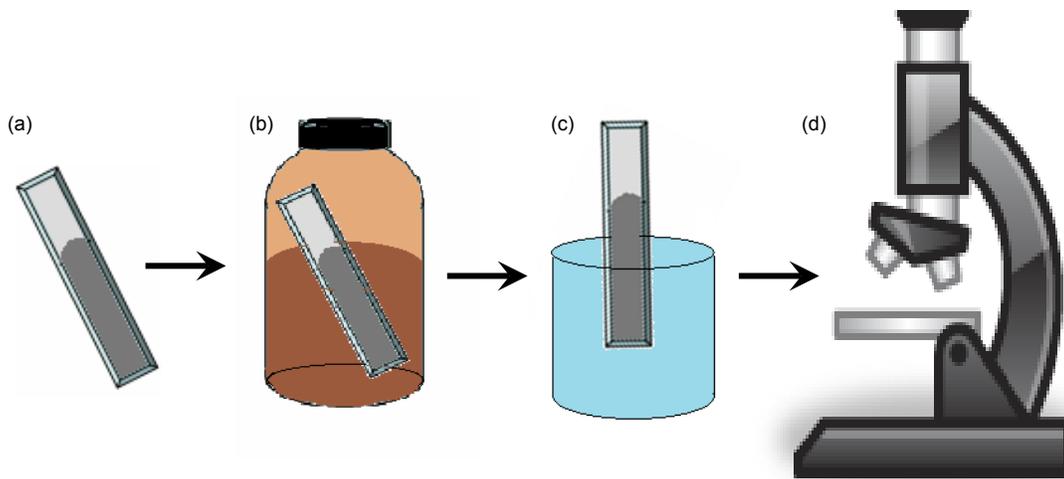
Figure 4.1. Illustration of the procedure followed in the static system experiments while using the amber bottle set up. The cartoons clearly explain the experimental sequence: (a) Glass slide coated with the hydrophobic silicone coating. (b) Experimental period of 2 hours during which the glass slide is placed in the amber bottle containing the bacterial suspension. (c) Dipping in DI water / saline. (d) Imaging of the glass substrate surface for bacterial attachment or biofilm formation.

Incase of the control systems, small thin biofilm patches were seen floating close to the surface with a very thin liquid layer between the biofilm and the substrate surface.

Free bacteria and other molecular particles were also seen swimming and floating around, respectively, in the liquid above the biofilm patches. Once the liquid on the surface dried up, all the particles adhered to the surface. It was also observed that the coated portion (i.e. hydrophobic silicone) had more attachments than the glass portion (i.e. hydrophilic silica) of the slide.

The biofilm layer observed initially in the ZA systems seemed to be more continuous and dense than the layer observed in the control systems. This was observed even in those systems containing a very low content of ZA (10ppm ZA) indicating that ZA may actually increase biofilm formation in the medium. Similar to the control, the solution taken from the ZA systems also contained free bacteria and other particles, indicating ZA did not have a toxic affect on the bacterial mobility and motility. However, later when the experiment was repeated several times, this difference in the density and thickness of the biofilm between the control and ZA systems could not be reproduced and the biofilm seen in the ZA systems was found to be similar to what was seen in the control systems. The reason for this lack of reproducibility was probably due to human error introduced during the dipping of the glass slides in saline solution before imaging. It was also noticed that when the samples were rinsed, often the entire biofilm layer came off.

The amber bottle set-up was also used to study the effect of ZA on other *Pseudomonas aeruginosa* bacterial strains, mainly F04-63, H05-25, E03-36, E03-40, and also on *E. coli*. Each of the different strains had two systems with ZA and two without ZA, acting as control. It was observed that *E.coli* and *Pseudomonas aeruginosa* strains F04-63 and E03-36 did not attach at all in the control systems and hence could not be

compared with their ZA counterparts, and the attachments seen in the ZA systems of other species, PAO1, H05-25 and E03-40 were not much different from those observed in the control, both before and after rinsing.

4.1.3 Systems with clear bottle set-up

An experimental procedure similar to the one using the amber bottles was repeated, except in this case, clear bottles were used instead. The clear bottles were used to favor observation of the system for differences in the turbidity of the solution and the number of bubbles formed due to de-nitrification. As explained earlier in the amber bottle set-up, systems with ZA (of different concentrations) and systems free of ZA were compared for differences.

No differences were seen between the ZA systems (of different concentrations) and the control systems. All the systems had similar features before and after dipping. The continuous layer, which was seen in the ZA systems during the preliminary studies using amber bottles, was not found during these observations. Therefore, differences have been observed in the ZA systems as compared to their control counterparts. However, these deviations could not be reproduced, pointing the blame either towards the system set ups or the ZA product used. Hence the impurities present in ZA were then scrutinized using a similar stationary set up.

4.1.4 Systems using the silicone-cover slip chamber set-up

A real time bacterial attachment and biofilm formation study was also carried out to follow the various stages of biofilm formation. Both glass cover slip surfaces of the

chamber were continuously imaged for attachments and/or biofilm formation. The bacterial medium introduced to the cavity was very dilute in terms of both the bacterial cell density and the nutrients, since a concentrated medium resulted in a rapid formation of biofilm in patches, making the chamber hazy and thus hindering imaging. Control and ZA systems were continuously imaged for about 4 hours, for differences in the biofilm formation, and again imaged after a period of 48 hours.

During the early stages, the individual bacteria cells could be observed spinning on the glass surface before attaching. The gradual formation of colonies before forming biofilm patches was also observed. Small scattered patches of biofilm were noticed in the solution as well as on the glass surfaces within 1 hour of the experiment. The time taken for the biofilm patches to appear in the solution as well as for the biofilm to attach to the bottom and top glass surfaces were similar for both control and ZA systems. However, the glass surface facing down (i.e. the top glass surface in contact with the bacterial solution) was comparatively clear of bacterial attachments for both the control and ZA systems. This was probably due to the extremely low concentration of bacterial cells in the system. Therefore, nothing very conclusive could be obtained from these experiments and these real time chambers were not very favored later while studying bacterial attachment and biofilm formation.

4.1.5 Systems using the petridish set-up

The effect of ZA on the bacterial attachment and biofilm formation was also studied using the petridish systems. The experiment was carried out by floating glass cover slips on the liquid surface in petri-dishes filled with medium. The ZA systems had

different concentrations of 10ppm and 500ppm. The cover slips were imaged for differences after 3 hours and 24 hours. Each of the ZA systems and the control systems had a duplicate to confirm the final results.

A significant difference between the ZA systems and the control systems was not observed. Furthermore, the control systems with the exact same conditions showed differences in attachments, indicating the presence of other factors affecting bacterial behaviors. The individual bacterial attachments also seemed to be very weak, as the attachments were easily swept away with the movement of drying liquid from the glass surface. When the surfaces were dipped in saline solution to rinse off the loosely attached bacteria, most of the attachments came off, rendering the surfaces clean. Attempts at rinsing were also made by holding the slides upright and gently dispensing DI water/saline solution at the top of the slides and allowing the liquid to flow down the slide washing away the loose attachments. However both the washing techniques implemented only seemed to add to the inconsistencies of the final attachment results. Therefore, the results obtained were inconclusive due to inconsistencies between similar systems, and the lack of reproducibility using the static systems hampers the further investigations on attachments of our bacterial strains and the effects of zosteric acid on bacterial attachment.

4.1.6 Systems using a modified petridish set-up

The petridish system used earlier was slightly modified since the attachments on the glass cover slips were extremely weak. It has been reported that hydrophobic surfaces lead to increased bacterial attachments (Zita et al., 1997). Hence, a small silicone sheet

(inclined at an angle) was placed on the inside lip of a petridish and the dish was filled with the bacterial medium. The surfaces of the silicone pieces attached to the petridish lip were imaged for biofilm formation. Little difference was observed in terms of the biofilm formed in the control and the ZA systems. But a noticeable difference in the amount of bubbles formed in the ZA contained petri-dish systems was detected (described in detail in the appendix).

4.2 Medium Effect

The PAO1 cell attachments were compared in systems with 6 different types of medium. The various media were; original medium (as described in the experimental section for static systems), original medium without sugar, original medium with low nitrate concentration (0.6 g/L), egg yolk medium with sugar, and egg yolk medium without sugar, tryptone soya broth (TSB) medium. The egg yolk medium consisted of peptone 3g/L, sucrose 2g/L, EDTA 0.01g/L, NaCl 5g/L, KCl 2.2g/L, and dry egg yolk 1.5g/L. This was done to find the optimum cell growth and attachment medium conditions, to be used as a control while studying various other factors and their effect on the cell attachments.

The amber bottle set up complete with the bacterial suspension and silicone coated hydrophobic glass slide was used for this study. Glass slides were removed from the amber bottles and placed on the microscope stage for imaging, with no prior rinsing. After 2 days, it was observed that the egg yolk (with and without sugar) and TSB systems had a very thick layer of biofilm, with a thin liquid layer between the substrate surface and dense biofilm layer. The other three systems, i.e. original medium with and without

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sugar and with low nitrate concentration were similar, without the presence of the aforementioned thick biofilm. The glass slides from these three systems, exhibited attachment and small patches of biofilm spread over the surface. Therefore, either no noticeable differences were found among these three systems, or the static systems used for these studies were not ideal while looking for such differences.

4.3 Surface Properties Effect

Surface properties have been known to have a significant effect on the bacterial attachments. From previous studies it is also known that different bacterial strains may behave distinctly towards different surfaces (Palmer et al., 2007). The interaction of a microorganism with a surface mainly involves three steps, adsorption onto the surface, bacterial attachment, and finally growth and colonization on the surface (Prakash et al., 2003). Therefore, the surface properties play an important role in bacterial attachments right from the earliest stage.

4.3.1 Effect of silane modified surfaces

In the present study, surfaces modified with octadecyltrichlorosilane(OTS, polyethyleneglycol(PEG) and aminopropyltriethoxysilane(APTES) were used. The OTS modified surfaces were tested since they have a -CH$_3$ terminated group making them hydrophobic, and it has been reported that hydrophobic surfaces favor bacterial attachment (Alavi et al., 2007; Muller et al., 2007). APTES has been known to have the ability to immobilize proteins and from studies on the conditioning of the surfaces, this surface was also expected to have a large number of attachments (Cunliffe et al., 1999;

Jeong et al., 1994). PEG surfaces, on the other hand, due to their amphiphilic nature have been reported to deposit onto the surface of various materials, and their hydrophilic ends are believed to repel proteins and cells from the surface (Harris et al., 2004; Kingshott et al., 2003; Sofia et al., 1998).

The substrate surfaces (microscopic glass slides) were therefore modified by OTS and APTES using the contact printing method, while the solution deposition method was used to modify the surface with PEG silane. Water contact angles of the surfaces were measured before starting the experiment in order to ensure the surfaces were modified as expected. The contact angle values were as expected; they were $95\pm5°$, $40\pm5°$, and $65\pm5°$ for the OTS, PEG, and APTES modified surfaces, respectively. After this preliminary study, mainly carried out to confirm the presence of silanes on the surface, the modified glass surfaces were placed in the bacterial medium in amber bottles containing the PAO1 bacterial suspension, and imaged after 2, 7, and 14 days while comparing them to control systems (containing unmodified glass slides) under similar time conditions.

The attachments on all the different modified surfaces appeared to be similar to each other as well as to the control, with mostly small patches of biofilm, a few larger patches and few individual bacterial attachments. The individual bacterial attachments, however seemed to be weak, as the attachments were swept along with the movement of drying water on the glass surface. Weak attachment was also suspected when the surfaces were dipped in saline solution to rinse off the loosely attached bacteria, and most of the attachments came off, rendering the surfaces clean.

4.3.2 Effect of hydrophobicity

Another attachment study was carried out to check if the hydrophobicity of the surfaces had an affect on the bacterial attachments. Surfaces were plasma treated to render them hydrophilic. These hydrophilic plasma treated surfaces were studied for attachments and compared with the untreated hydrophobic surfaces. The surface properties were verified using the contact angle method. The plasma treated hydrophilic surfaces had a water contact angle of approximately 0 ± 5°, while the untreated hydrophobic surfaces had a water contact angle of 75 ± 5°. These surfaces were also placed in amber bottles containing PAO1 bacterial suspension and compared for differences in attachment and biofilm. This study was also carried out in the petridish with cover slips floating on the liquid surface of the PAO1 suspension. Before imaging, both the glass slide surfaces and the cover slip samples were rinsed by dipping them in saline water (to remove the loose attachments).

There were no significant differences between the plasma treated (hydrophilic) and untreated (hydrophobic) surfaces, both in the case of the glass slides taken from the amber bottle set up as well as the cover slips from the petridish arrangement. Again the inconsistency in the wash procedure proved to be a major problem and the duplicate systems in some cases varied from their counterparts in terms of attachment observed.

4.4 Different bacterial species and strains

Different *Pseudomonas aeruginosa* strains (PAO1, F04-63, H05-25, E03-36, E03-40) and *E. coli,* were tested using the amber bottle set-up for differences in the attachment and biofilm formation. Systems containing the *Pseudomonas aeruginosa*

strains; PAO1, H05-25, E03-40 were not different from each other in terms of the attachment or biofilm formation on the surface and also from their ZA counterparts.

The *E.coli* and *Pseudomonas aeruginosa* strains F04-63 and E03-36 on the other hand did not attach at all in the control systems and hence could not be compared with their corresponding ZA systems. The bacterial cells in these systems were observed to be swimming around in the liquid medium, however their attachment to the surface was negligible. Therefore this indicates that the cells were still alive in the system, but their attachment was hindered due to the influence of some additional factor, necessitating the presence of a more controlled environment which is clearly absent in case of the static system set ups described so far.

Therefore several experiments were carried out using stationary systems however while using most of the previously mentioned static system set ups, a number of problems were encountered. They mainly were:

- A perfectly consistent system giving reproducible results could not be achieved.

- Inaccuracies resulted from inconsistent washing techniques, mainly used to remove the loosely attached particles.

- Contamination of the systems was a possible occurrence during observation, due to exposure.

- Effect of gravity on the bacterial attachments was a crucial factor due to the stagnant nature of the set up.

- The bacterial attachments and biofilm studies were being conducted in the absence of shear stress which is an important factor in the practical world, especially in case of bio fouling of pipelines, heat exchangers, ships, etc.

Hence in order to overcome the setbacks and difficulties mentioned above with regard to the static systems, flow systems were designed, constructed and utilized for the purpose of studying the initial microbial attachment and biofilm formation.

CHAPTER V

FLOW SYSTEMS

The design and construction of the flow chambers have been explained in section 3.4.2. This new set up was such that the possibility of error creeping in due to manual handling was minimal and furthermore the results obtained could be easily and consistently reproduced. The entire arrangement was sealed, preventing contamination and it allowed for easy real time imaging. Gravity was not as big an issue as in the case of stationary systems and shear could be easily manipulated in accordance to the factors being tested. However before these flow systems were implemented to study the bacterial attachment, some verification on the flexibility of the chambers were carried out.

5.1 Flow Chamber Flexibility Evaluation

The flow chambers were made to undergo some flow behavior analysis and a consistent check before they were utilized to study the bacterial attachment. This was chiefly done to gather information on the flow through the chamber, to ensure that the chamber design was dependable and useful for studying the initial bacterial attachment.

5.1.1 Flow Behavior Analysis

The flow system used most commonly in this study involved circulating the bacterial suspension at the rate of 22 ml/min through a roughly square chamber of 10 mm x 10 mm cross section. For such a system, the flow in the chamber was laminar with an average Reynolds number of about 41. The velocity profile near the chamber surface was measured by following the displacement of individual tracer particles at different distances away from the chamber surface, for both types of flow chambers used (Figure 5.1 (a) and (b)).
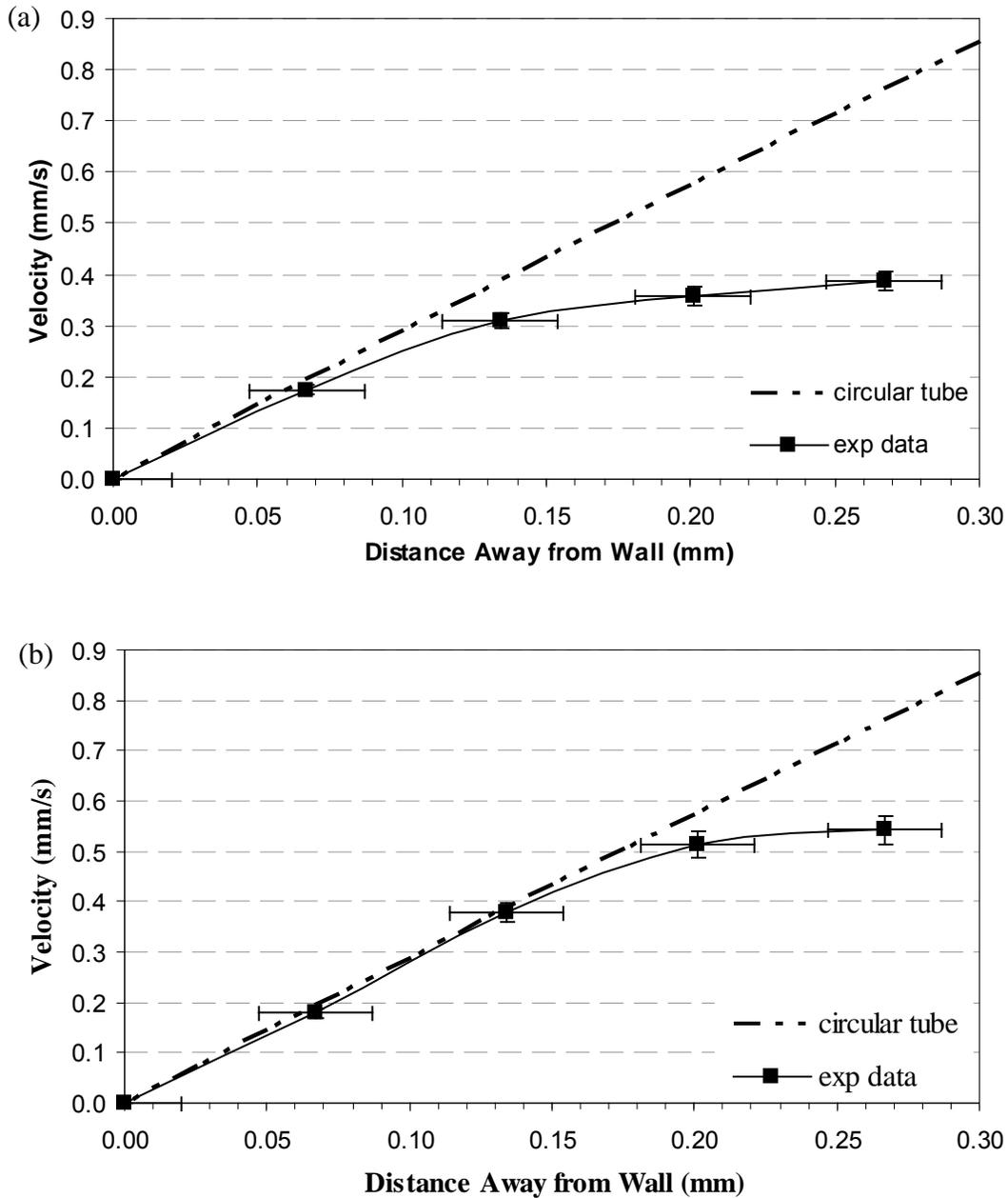
Figure 5.1. The velocities of fluorescent particles measured at different distances away from the chamber surface, (a) Using the silicone glass chamber. The circulation rate was 22 ml/min. The chambers had a rough square cross section of 10 mm x 10 mm. The best-fit velocity profile near the chamber surface was $v_x = -6.0z^2 + 3.0z$, with $r^2 = 0.998$, where z is the distance away from the chamber wall. (b) Using the square glass tube chamber. The circulation rate was 22ml/min. The chambers had a square cross section of 10mm x 10mm. The best-fit velocity profile near the chamber surface was $v_x = -5.8z^2 + 3.7z$, with $r^2 = 0.992$, where z is the distance away from the chamber wall.

In the immediate vicinity of chamber wall, the measured profiles coincide (in the case of both flow chambers) with the velocity profile predicted for a circular tube with the same cross sectional area. For the square glass tube chambers this similarity in the velocity profile with that of the circular tube is even more apparent (Figure 5.1(b)). The deviation of the measured profile from that predicted for the circular tube, at larger distances from the wall, could be due to the complex flow behavior in the chamber used. This complex flow behavior could be the result of (1) the flow expansion as it enters the chamber (from a small tube with a cross section area of 4.52 mm^2 to the chamber of 100 mm^2), (2) the periodic pulse action associated with the peristaltic pump, and (3) the square (not circular) cross section of the chamber.

The smallest distance observed was about 67 μm, which was the minimal separation required for two different focal planes (in this case, the flowing streamline levels) to be clearly distinguishable under the microscope employed. For the same reason, the different distances observed in this study were also separated by 67 μm. At these distances away from the chamber surface, particles were observed to move with practically constant speeds. On the contrary, the particles that settled on or moved very close to the bottom chamber surface (at distances of < 10 μm) exhibited erratic behaviors with occasional "stick-slip" and "forward-backward movements". The displacements of these particles were therefore not followed. Due to the working distance limitation of the microscope objective, the particles moving at more than 267 µm away from the surface were also not followed.

In Figure 5.1 the experimentally determined velocities (v_x) at different distances (z) were best fit with a second-order polynomial equation, of which the constant term was

fixed at zero to correspond to the assumed no-slip condition at the chamber surface. The resultant equation were

$$v_x = -6.0z^2 + 3.0z \qquad (r^2 = 0.998), \quad \text{for the silicone glass chambers}$$

$$v_x = -5.8z^2 + 3.7z \qquad (r2 = 0.992), \quad \text{for the square glass tube chambers}$$

Also shown in Figure 5.1 (a) and (b) was the velocity profile expected for a circular tube with the same cross sectional area, 100 mm^2 (Bird et al., 2002). The measured profile coincides very well with the predicted profile at the immediate vicinity of wall but differs increasingly with increasing distance from the wall. The flow in the actual system was too complex to model mathematically: the chamber was square (chosen for avoiding the curvature-caused interference to the microscopic observation of cell attachment) and complicated flow expansion occurred when the liquid entered the chamber (10 mm x 10 mm) from the much smaller entry tube (1.2 mm in diameter) (i.e. from a small tube with a cross section area of 4.52 mm^2 to the chamber of 100 mm^2). The periodic pulse action associated with the peristaltic pump also added to the complexity of the model.

Nevertheless, the good agreement of the two velocity profiles at the immediate vicinity of liquid-solid interface enabled us to reasonably estimate the shear stress at chamber wall (τ) at different circulation rates (Q) by using the equation derived for circular tubes (Bird et al., 2002), with the equivalent cross section area (πR^2) being replaced by that of the chamber (WH),

$$\tau = 4\sqrt{\pi} \, \frac{\mu Q}{(WH)^{3/2}} .$$

In this equation, μ is the viscosity and W and H are the width and height of the chamber, respectively. Once the flow dynamics and shear stress calculations were completed, the flow chambers were tested to ensure consistency.

5.1.2 Consistency Check

To check the consistency of the flow chamber results, three chambers were run simultaneously at the same flow rate of 22 ml/min. The obtained numbers of attached cells per frame (0.028 mm^2) are compared in Figure 5.2. Clearly the six surfaces, both the top and bottom surfaces of the 3 systems, had very similar attachments throughout the 3 hour period and even after rinsing. The results verified that the flow chamber systems gave much more reproducible and reliable results of initial bacterial attachments as compared to the static systems.
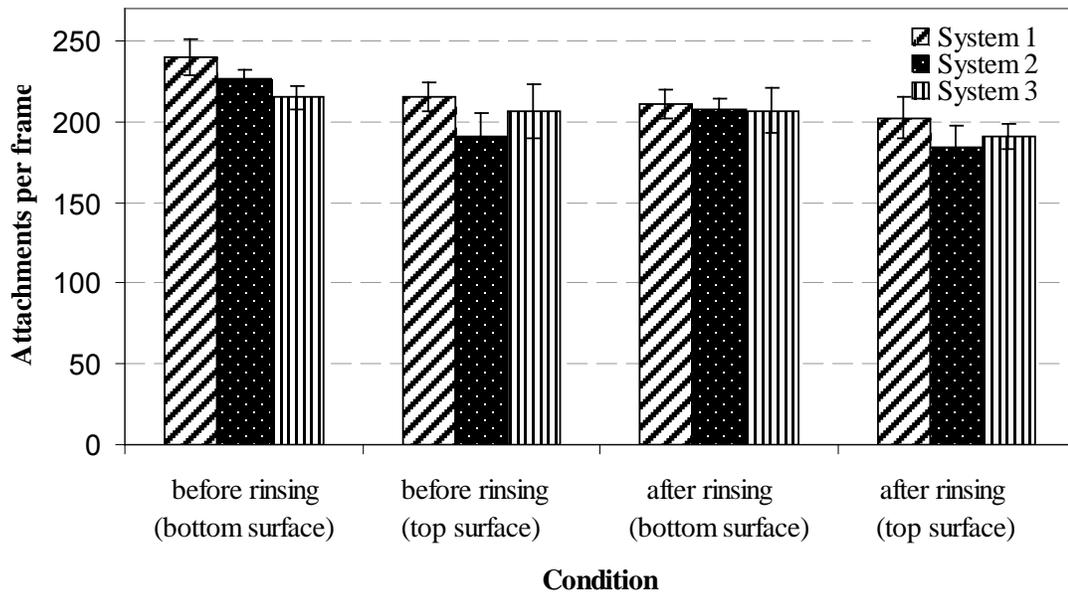
Figure 5.2. Comparison of the numbers of attached PAO1 cells per frame (i.e., 0.028mm^2 surface area) on the top and bottom surfaces, before and after rinsing, observed in three flow chambers (10mm x 10mm) operated at the same circulation rate of 22ml/min. The top surfaces had slightly less attachment than the bottom surfaces did, while all three systems had very similar attachments both before and after rinsing.

After this initial analysis, the flow chambers were utilized for investigating some fundamental aspects of bacterial attachment and also the ability of two probable antifoulants. During these experimental runs, a few general observations were made about the bacterial attachment. In the flow system experiments, the majority of cell attachments occurred within the first hour, irrespective of the bacterial species used. More cells attached in the second hour, while the increase in the third hour was usually small. Rinsing the chamber with the saline solution removed the loosely attached cells, reducing the attachments by ~ 10% - 20% (Figure 5.2). It was also noticed that in case of the PAO1 attachments the top glass surfaces had slightly (< 10%) fewer attached cells as compared to the bottom surfaces (Figure 5.3). However, in case of the *E. coli* and *P.*

putida systems the top surfaces had much (~ 65% - 75% in case of *E. coli* and 45% - 55% in case of *P. putida*) lower attachment (Figure 5.3).
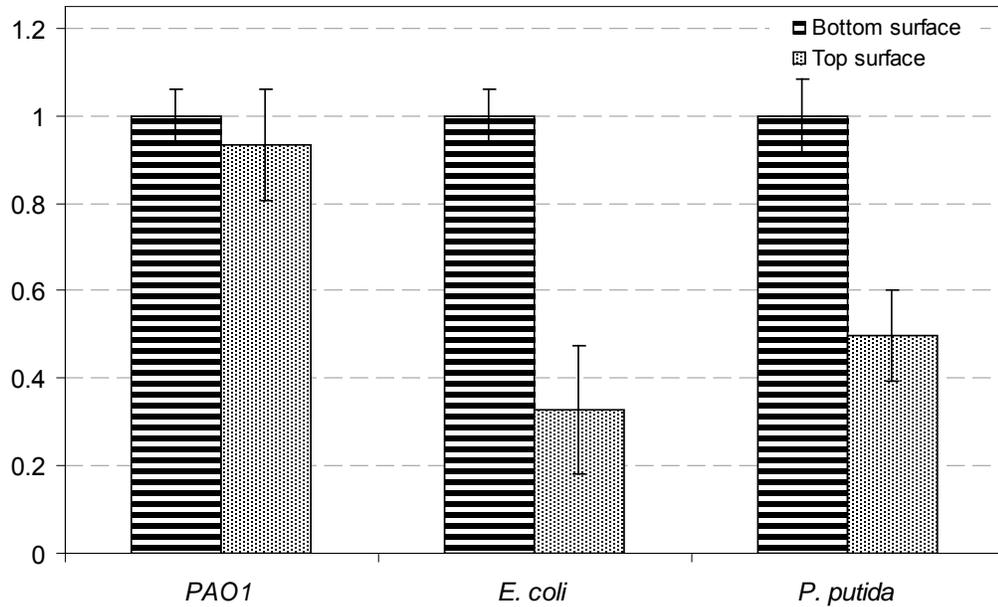
Figure 5.3. Comparison of Bottom and Top surface attachment for the three bacterial strains.

5.2 Shear Effect

It is generally expected that the bacterial attachment would be prevented or washed off the surface by high-shear fluid flow (Shive *et al.,* 1999, Wang *et al.;* 1995,). However, the opposite was observed in certain cases as reported by Thomas et al. (2004). They suggested that as the shear increased, the higher drag force induced the bacterial cells to form long lasting bonds with the substrate. This induced bond formation resulted in increased bacterial adhesion to the surface at higher shear. Similar increase in attachment with shear was observed up to a certain shear value in the case of the three bacterial species tested. After a certain threshold shear value, which was unique for each

of the bacterial strains, a decrease in attachment was observed in the case of all the three strains studied.

5.2.1 Shear effect on PAO1 attachment

The effect of shear on PAO1 attachment was studied using the flow chamber. Initially, the experiment was carried out at different circulation rates of 15, 22 and 30ml/min. The corresponding shear stresses at the chamber walls were calculated using the shear stress formula (explained in the Flow Behavior Analysis section; 5.1.1) and found to be 1.8, 2.6 and 3.5mN/m^2. The numbers of attached PAO1 cells increased with increasing shear between this shear range.

The effect of shear on PAO1 was further investigated by increasing the shear stress experienced at the chamber walls by varying the circulation rates. Shear stress of 2.6, 13 and 26mN/m^2 (circulation rates of 22ml/min, 110ml/min, and 220ml/min respectively) were applied to learn if the attachment of PAO1 bacteria at the chamber walls would still increase with shear. When this more wide ranged shear study was conducted, the bacterial attachments decreased with increasing shear, with the system at 220ml/min (shear of 26mN/m^2) free of bacterial attachments.

A narrower range of circulation was then carried out in order to obtain a more accurate trend of this shear effect on PAO1 bacterial attachment. Three systems experiencing shear values 2.6, 5.2 and 10.4mN/m^2 (circulation rates: 22ml/min, 44ml/min and 88ml/min respectively) were then utilized.

The data were collected from the three sets of experiments and normalized against its corresponding control (flow system at 22ml/min circulation rate) to get a more

accurate idea of the shear effect on the initial PAO1 attachment. Normalization of the data was needed to compare the results obtained from three runs of the experiment over three different days, as the absolute attachment numbers changed with different batches of experiments, presumably because of the different cell properties despite our efforts to follow the same procedures. The mechanism(s) responsible for the increased attachment, with increasing flow rate or shear, remains to be clearly delineated.

It was found that the PAO1 bacterial cell attachment increased with shear upto a shear of $5.1mN/m^2$ corresponding to the circulation rate of 44ml/min and then decreased as the shear was further increased resulting in no attachment at a high shear of $25.3mN/m^2$ corresponding to the circulation rate of 220ml/min. Thus the shear effect on PAO1 attachments is explained by the curve shown in Figure 5.4. The attachment seen before and after rinsing showed this similar trend of the attachment initially increasing with shear and then falling after a certain shear stress value was attained.
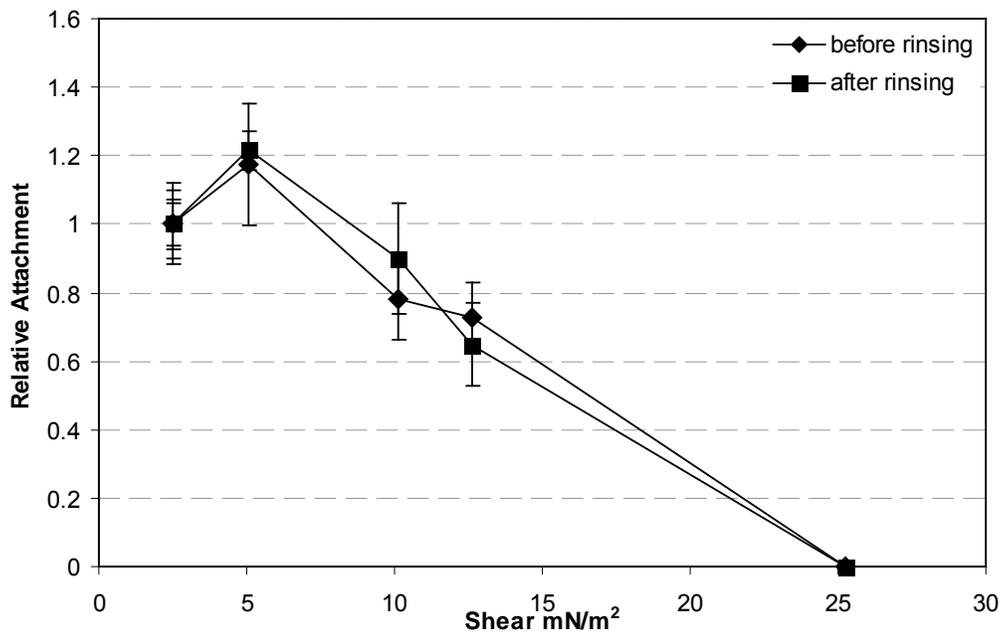
Figure 5.4. Effect of shear (at the chamber wall) on the initial attachment of *Pseudomonas aeruginosa* PAO1. Initially the attachment increased with increasing shear, in the range of wall shear stress 2.6 to 5.1mN/m^2, after which the attachment decreased with the rise in shear.

5.2.2 Shear effect on *P. putida* attachment

The effect of shear on *Pseudomonas putida* was tested with the shear at the chamber walls ranging from 2.6 to 26mN/m^2 (i.e. circulation varying from 22 to 220ml/min). As the shear increased from 2.6 to 26mN/m^2, the attachments observed on the glass substrate surfaces decreased. Hence a lower shear of 1.3mN/m^2 (circulation rate of 11ml/min) was included in the study and it was then noticed that attachment increased upto a shear of 2.6mN/m^2, however as the shear increased beyond 2.6mN/m^2 through 26mN/m^2, the attachments began to decrease. The effect of shear on attachments of *P. putida* is shown in Figure 5.5.

Figure 5.5. Effect of shear on the initial attachment of *Pseudomonas putida.* The attachment increased with increasing shear, in the range of wall shear stress 1.3 to 2.6mN/m^2, after which the attachment decreased with the rise in shear.

5.2.3 Shear effect on *E.Coli* attachment

     E. coli, when placed in an environment of high shear, have been reported to have reacted unexpectedly by increasingly attaching to the substrate as compared to a situation when the surrounding fluid shear was low. Thomas et al. (2004) have reported that as the shear increased from 10mN/m^2 to 400mN/m^2, the bacteria accumulated on the surface increased but dropped when the shear was increased further. However these experiments were different from those undertaken in this study in terms of the substrate surface used. Here, an unmodified glass surface was used while Thomas et al. used monomannose-coated surface, and this difference is important and cannot be ignored as the surface

characteristics have been suggested to play an important role in bacterial attachment (Palmer et al., 2007; Prakash et al., 2003).

The shear effect on *E. Coli* attachment was initially tested between a range similar to that used for investigating the effect on PAO1, i.e. a shear range of 2.6 to 26mN/m^2 (corresponding to flow rates 22ml/min to 220ml/min). For this range, as the shear increased, the attachments increased throughout the 3 hour experimental run. However, one major problem faced while studying the shear effect, was the high flow rate used. Due to the large dimensions of the chamber, a flow rate of 220ml/min had to be used to increase the shear ten fold. This caused an increased vibration of the chambers, making imaging difficult. Hence, chambers of smaller dimension, i.e. 10mm (width) X 1mm (height) were used. This eliminated the excessive vibration, while allowing a shear 50 times higher even. Hence, a much wider shear range of 23.6 to 118.2mN/m^2 could be tested. It was then noticed that attachment behavior similar to the one seen in case of PAO1 was observed (Figure 5.6), but the curve peaked at a higher shear (26mN/m^2), as compared to PAO1(5.1mN/m^2).
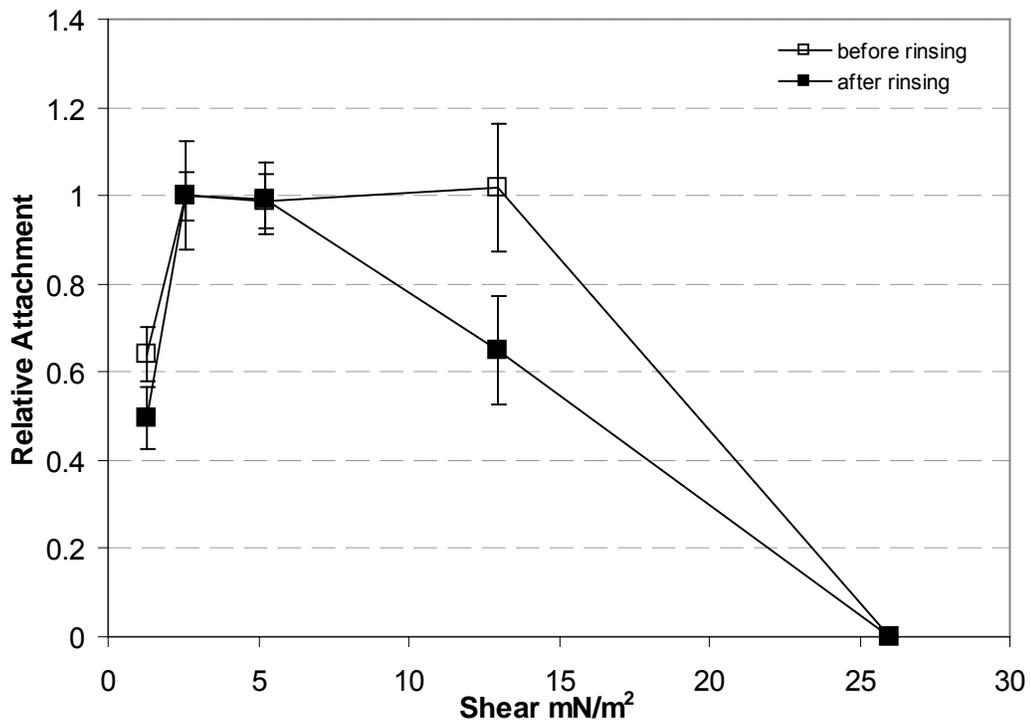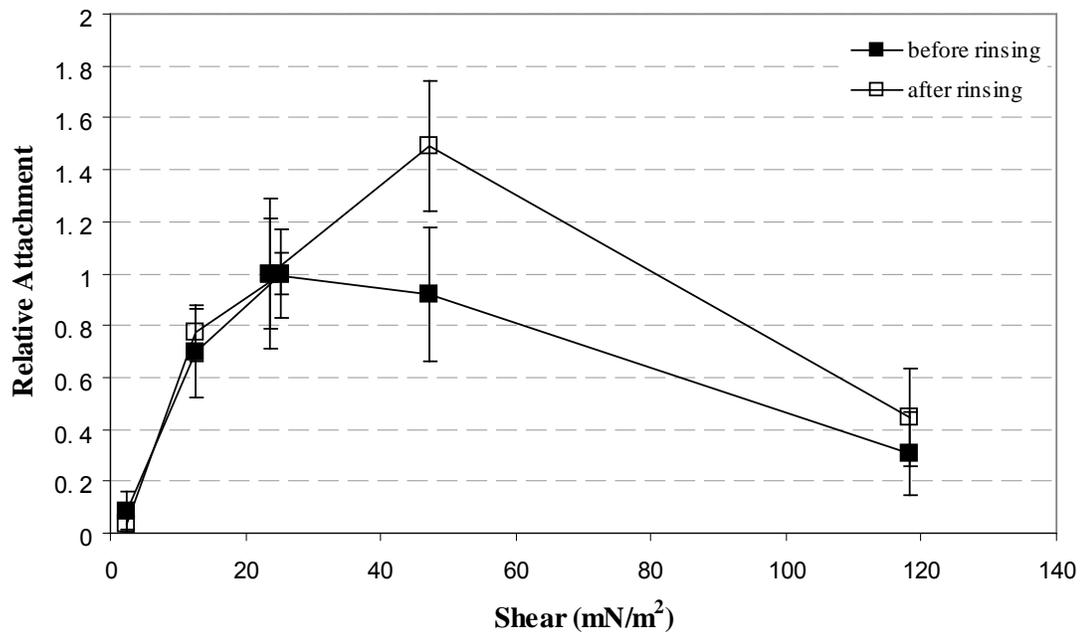
Figure 5.6. Effect of shear on initial attachment of *E. coli*. The attachment increased with increasing shear, in the range of wall shear stress 2.6 to 26mN/m^2, after which the attachment decreased with the rise in shear. Attachment in *E. coli* peaked at a much higher shear stress value (26mN/m^2).

Further investigations were conducted to understand this large shift in the attachment peak. A set of experiments was carried out to check if the high shear conditions in the environment were in some way altering the cell surface properties. The *E.coli* bacterial cell suspension was made to circulate through plastic tubing with the help of a pump at a high rate of 220ml/min for a period of 4 hours and then this highly activated cell suspension was passed through the flow chamber setup at a rate of 22ml/min for the usual 3 hour period. This system (along with its duplicate) was compared to the control system (and its duplicate), which involved just circulating the bacterial suspension through the flow chamber setup at 22ml/min for 3 hours. The result obtained for this experiment is shown in Figure 5.7. It was observed that the two systems

were very similar in terms of the attachment seen through the 3 hour period. Thus the hypothesis that modifications in the bacterial cell surface characteristics in the presence of high shear may not be behind this bacterial attachment behavior.
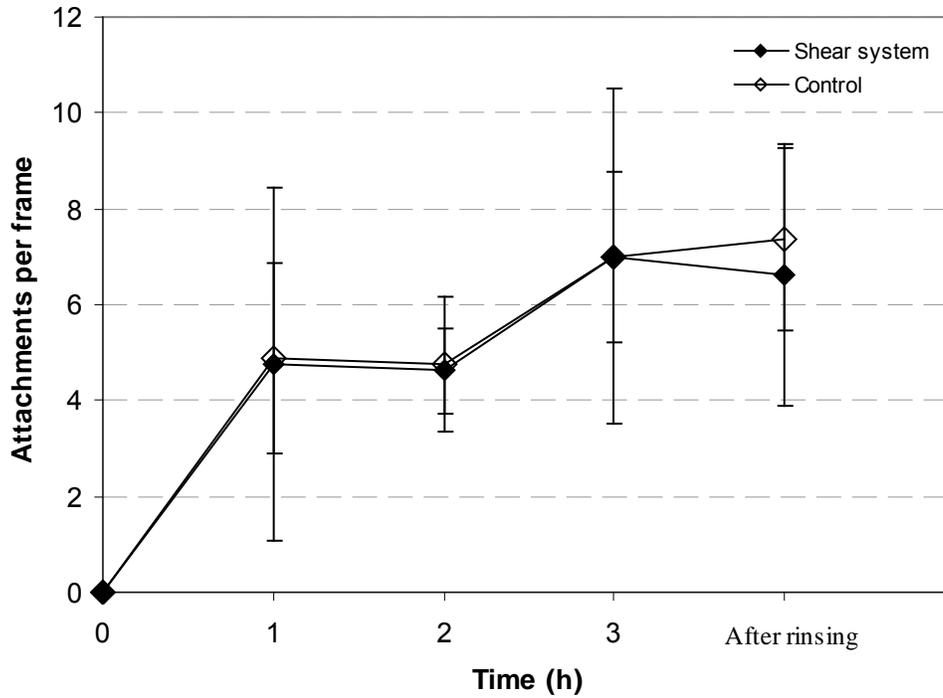
Figure 5.7. Study to determine if the high shear has an effect on the surface properties of *E. coli* cells. In the shear system, bacterial cell suspension was made to circulate at a high rate of 220ml/min for a period of 4 hours before carrying out the standard 3 hour run at 22ml/min through the chamber. In case of the control system, *E. coli* bacterial suspension was just circulated at 22ml/min through the chamber for the usual 3 hour period.

Based on the results obtained from the experiments conducted using the above mentioned three bacterial strains, it can be inferred that bacterial attachments may increase with shear up to a certain threshold, exclusive to the bacterial strain being used, and then fall to a minimum. The varying shear threshold values for different strains may

be attributed to the differences in the cell surface characteristics, i.e. the presence/absence of fimbriae, flagella (Korber et al., 1989), surface charge (Fletcher et al., 1988), hydrophobicity (Rosenberg et al., 1986). The shape of the bacterial cells have also been reported to play an important role on the shear at which the curve would peak (Feldner et al., 1983). Hence, the differences in size and shape of the three bacterial species used in the flow study were investigated. The results obtained from the AFM images of the three bacterial cells are shown in the table below.

Table 5.1. Comparison of the three bacterial cell dimensions in µm.

Bacteria	Length	Width	Height
PAO1	2.27±0.02	0.99±0.03	0.38±0.03
P. putida	2.19±0.02	0.93±0.03	0.59±0.06
E. coli	1.93±0.06	1.24±0.05	0.97±0.07

From the dimensions listed above it is clear that the *P. aeruginosa* PAO1 and *P. putida* bacteria are rod shaped while *E. coli* are more ellipsoidal in shape. This difference in the shape and size could also lead to the differences in their responses towards shear in the environment, since the geometry of the cells may have an effect on the manner in which the cells are transported to the substrate surface before adhesion. In the initial stages such physical characteristics have been suggested to play a governing role in transferring the cells to the substrate surface (An et al., 1998).

Detachment of bacterial cells from the substrate is another factor that needs to be addressed while analyzing the effect of shear on bacterial attachment. Generally, the probability of cells detaching is said to decrease as the contact time between the bacteria and a substrate surface increases, indicating an increase in bond strength with time (Rodriguez et al., 2004). The bond strength is said to differ for different bacterial strains (Boks et al., 2009). Hence, these differences in bond strengths may also cause the bacterial cells to respond differently to varying shear conditions in their environment.

Therefore, further investigation needs to be carried out into the cause behind these attachment differences among the three bacterial strains under the influence of shear. The exact reasons behind this initially increasing and then decreasing behavior also need to be studied further.

5.3 Study of Potential Antifoulants

The ability of two potential antifoulants on preventing/reducing the attachment of three different bacterial species was evaluated in this study. The two potential antifoulants are: zosteric acid and rhamnolipids, and the results are provided below.

5.3.1 Zosteric acid Effect

The flow chambers were used to study the effect of ZA on *Pseudomonas aeruginosa* PAO1, and also on *E. coli* attachments. The experiments were repeated several times for conclusive results which are explained in this section.

5.3.1.1 Effect of zosteric acid on PAO1 attachment

The effect of ZA on PAO1 was tested several times using the silicone-glass flow chambers and glass flow chambers. The glass surfaces which were observed for attachments were also plasma treated and compared to the untreated ones, in the hope of observing clearer attachment differences between the ZA and ZA free (control) systems. However, no clear differences were observed between the ZA and control systems even after an extended period of 4 hours. The presence of ZA was verified by Mass spectroscopy, UV visible spectroscopy and NMR. Samples from different batches of synthesis were also tested for their effect on attachments, but the results still remained the same. Therefore, for PAO1, ZA has no visible effect on its attachment as shown in Figure 5.8.
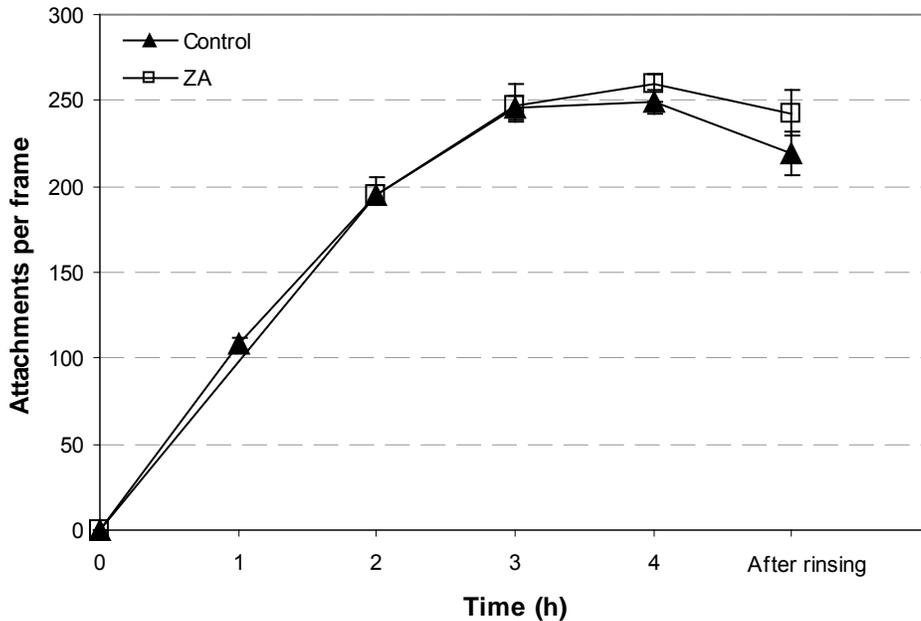
Figure 5.8. Effect of ZA [500mg/L] on *Pseudomonas aeruginosa* PAO1 attachment.

5.3.1.2 Effect of zosteric acid on *E. coli* attachment

The antifouling property of ZA was also tested on *E.coli* attachments using the flow system set ups. Separate experiments were carried out utilizing the silicone-glass based chambers and the glass chambers. While using both chambers, the ZA systems were found to be no different than the ZA free control systems (Figure 5.9). One of the major problems faced during these set of experimental studies was the low attachment number of *E. coli* as compared to those seen in the PAO1 systems. Low attachment numbers are undesirable as the differences caused by the factors being studied such as ZA in this case may not be very distinctive.
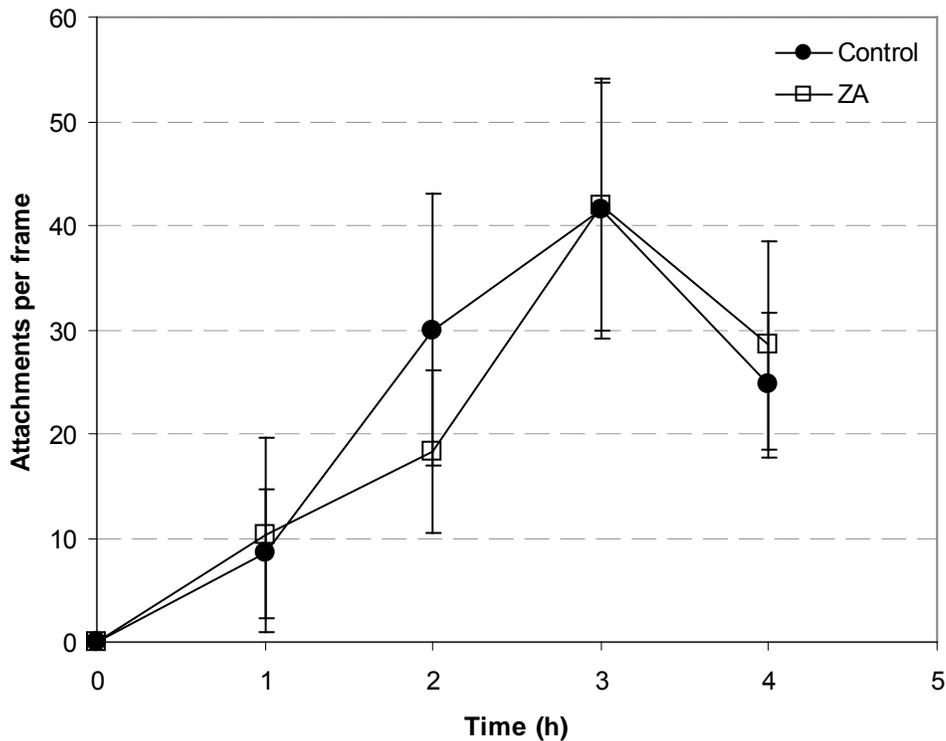
Figure 5.9. Effect of ZA [500mg/L] on *E. coli* attachment.

5.3.1.3 p-Coumaric acid

When ZA was tested on PAO1 bacteria using static systems, on certain occasions it was noticed that the ZA in the system seemed to enhance the initial attachment as well as biofilm formation. However, this result could not be verified using flow systems, and in case of static systems on repeating the experiments, this effect of ZA was not always observed. Hence p-Coumaric acid was tested, since it is an important component in the synthesis of ZA and 5 to 10% of it still remains in the ZA final product even after repeated purification. On the first experimental run, the systems containing coumaric acid had relatively much lower attachment as compared to the coumaric acid free control systems as shown in Figure 5.10. On further investigation it was realized that the coumaric acid systems had a low pH of 4.4±0.1, as compared to the control systems which revealed a value of 6.8±0.1.

Figure 5.10. Effect of Coumaric acid on PAO1 attachment. pH of Coumaric acid system = 4.4±0.1. pH of Control system = 6.8±0.1.

The effect of pH on the attachment of PAO1 was next scrutinized. Several literature reports have suggested that factors such as the composition of the media (Farber & Sharpe 1984, Fletcher 1976), temperature (Fletcher 1977; Herald & Zottola 1988) and pH (Kroll 1985; Litopoulou-Tzanetaki *et al*. 1989) may have significant effects on the adhesion of bacteria to a surface. The effect of medium pH on PAO1 attachment was investigated in the pH range of 4.6 to 7.4, in two sets of experiments. Bacterial systems containing medium at different pH values were compared for attachment. The relative attachments, normalized against the cross-reference system of pH 7.0 ($\pm$ 0.05), are shown in Figure 5.11. While the average values appeared to show lower attachments at pH away from neutrality, the pH effect, in the range examined, was not very statistically significant with a *p* value of 0.055 from the analysis of variance (ANOVA).
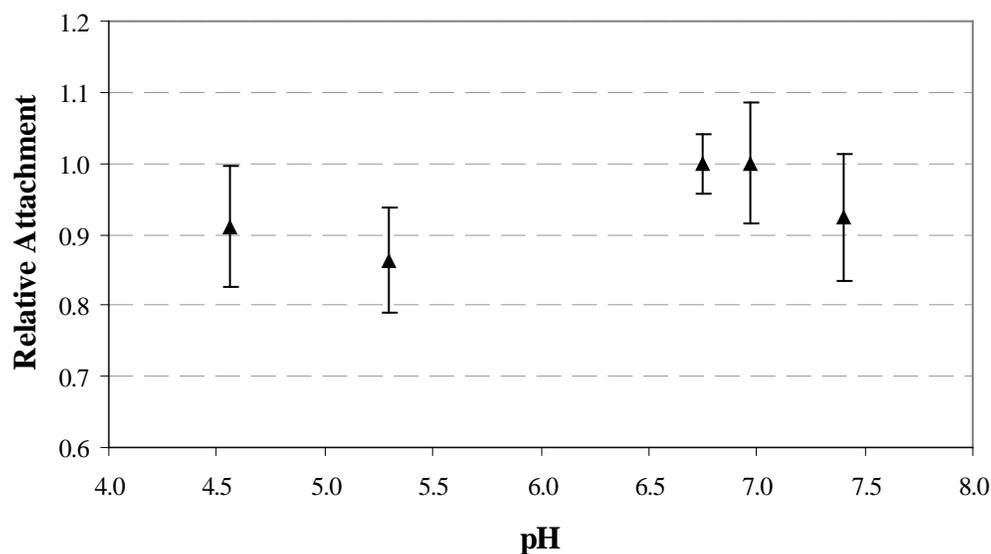
Figure 5.11. Effect of medium pH on initial PAO1 attachment. The effect of pH was found to be statistically insignificant with a *p* value of 0.055 in the analysis of variance (ANOVA).

Another set of experiments were carried out where the coumaric acid systems were raised to a pH similar to that of the control systems, i.e. to a value of 6.8±0.1. This was done to uncover the real reason behind the earlier observed potent effect of coumaric acid on attachment, i.e. to verify if the low attachment seen was really due to coumaric acid. Hence a study was carried out where the pH of the coumaric acid system was raised to a value similar to that of the control system, i.e. a pH of 6.8±0.1, using phosphate buffers. The result of this study is shown in Figure 5.12, and from this plot the lack of effect of coumaric acid, at neutral pH, on attachment is quiet apparent.
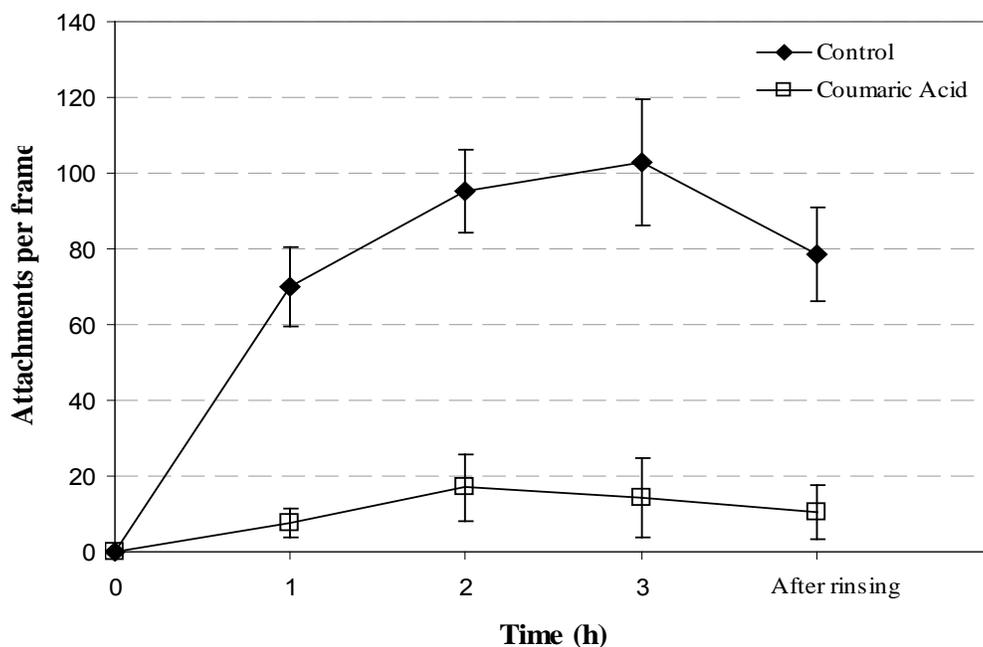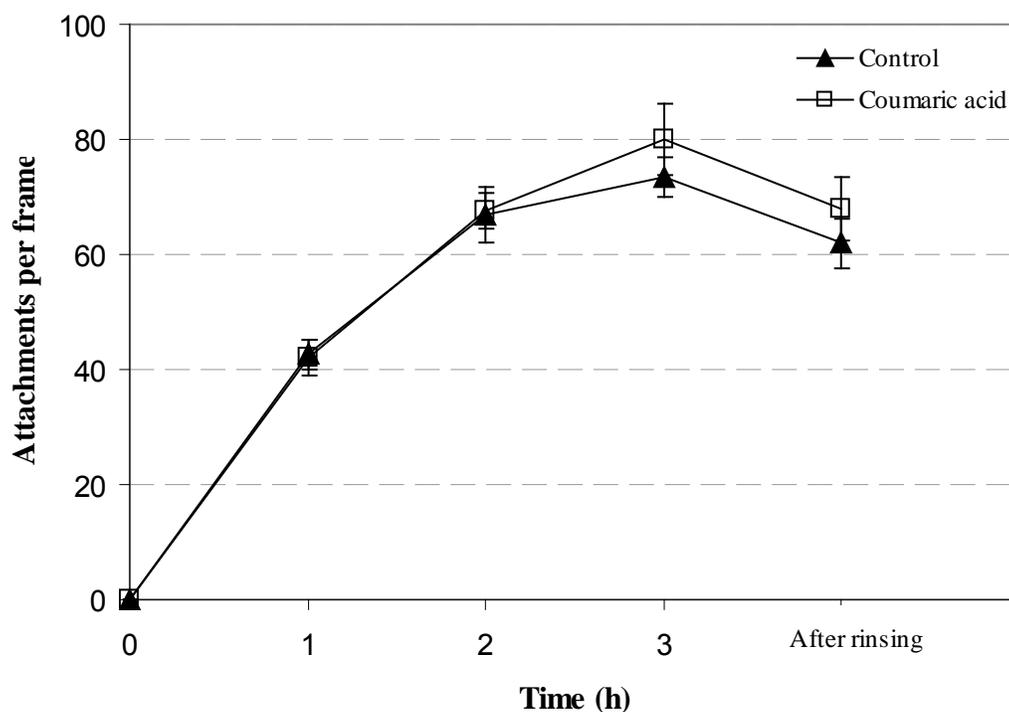
Figure 5.12. Effect of Coumaric acid on PAO1 attachment. pH of Coumaric acid system = 6.8±0.1. pH of Control system = 6.8±0.1.

Another set of experiments were carried out with the pH of the coumaric acid as well as control systems lowered to 4.23±0.1 and 4.5±0.1 respectively, using a phosphate buffer solution. This was done to ascertain that the low attachment seen in case of the coumaric acid system (pH = 4.4±0.1) was not due to some enhanced behavior of coumaric acid at low pH levels. Figure 5.13 shows the result for this particular experimental run. In spite of the acidic condition of the medium, coumaric acid showed no effect on the attachment of PAO1. These results rule out the possibility of the effect of coumaric acid under low pH conditions.
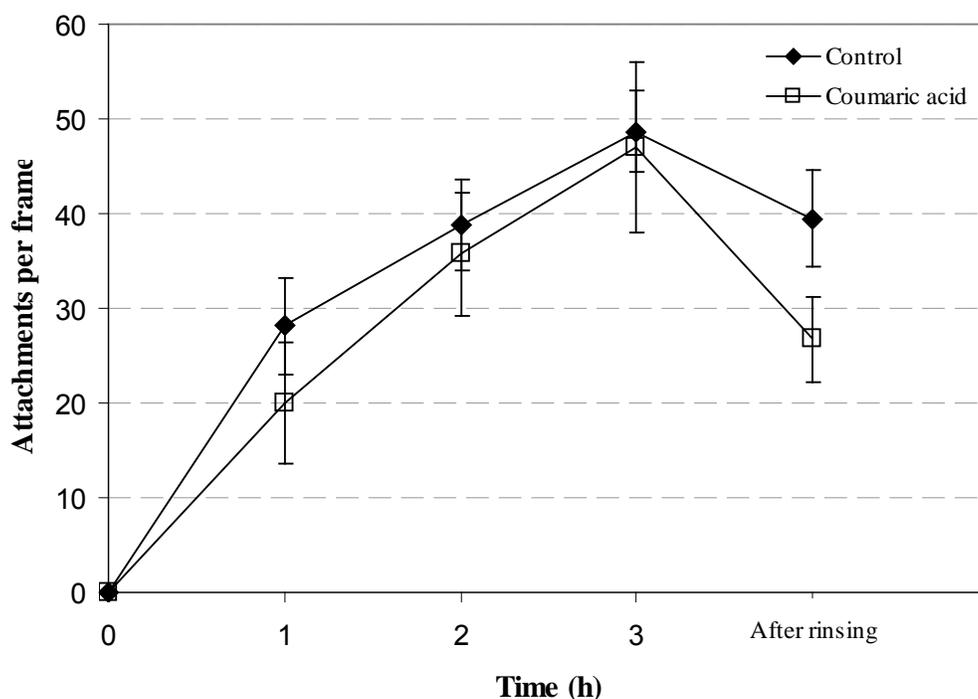
Figure 5.13. Effect of Coumaric acid on PAO1 attachment. pH of Coumaric acid system = 4.23±0.1. pH of Control system = 4.5±0.1.

Therefore, the reason behind the low attachment number seen in case of the coumaric acid system could not be uncovered. This indicates the presence of still some unknown factors affecting the attachment in the flow system. Hence, in future a deeper investigation is required to reveal the other causes that may affect the attachment in this system.

Hence, zosteric acid (or even p-coumaric) acid could not be found to have convincing antifouling properties and therefore the search for another antifoulant to reduce the attachment of PAO1, as well as other species used in this study, was pursued. Rhamnolipid was the next alternative antifoulant to be tested.

5.3.2 Rhamnolipid Effect

The effect of rhamnolipids on the attachment of *P. aeruginosa* PAO1, *E. coli* and *P. putida* was studied and results are discussed in this section.

5.3.2.1 Effect of Rhamnolipids on PAO1 attachment

The flow chambers were also used to study the effect of rhamnolipids on the PAO1 bacterial attachment at a flow rate of 22ml/min (i.e. a shear force of 2.6mN/m^2). In our study, the initial attachment of PAO1 cells was compared with the cell suspensions added, respectively, with 0 (control), 13 and 270 mg/L of rhamnolipids. The systems containing rhamnolipids, even at only 13 mg/L, showed very few attachments per frame (i.e. per 0.028 mm^2 surface area) as compared to the control systems (Figure 5.14 (a) and (b)). Rhamnolipids were shown to be a very potent antifoulant for PAO1. It was worth to note that few aggregates, which were clearly larger in size as compared to the bacteria, were seen in case of the 270 mg/L system. The systems containing a rhamnolipid concentration of 10 mg/L were also carefully monitored for the presence of these aggregates, but none were observed even after an extended period of 4 hours.
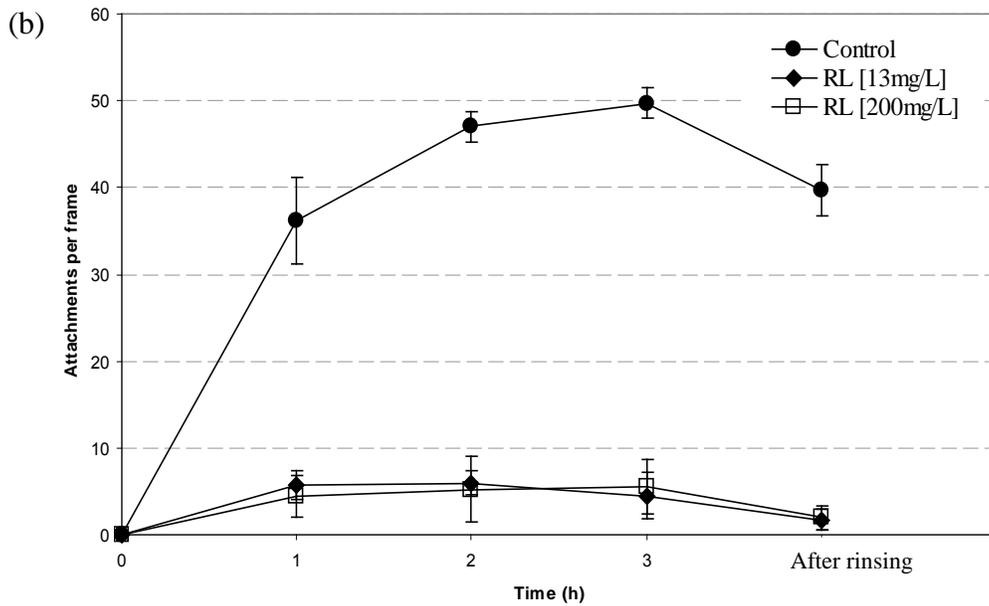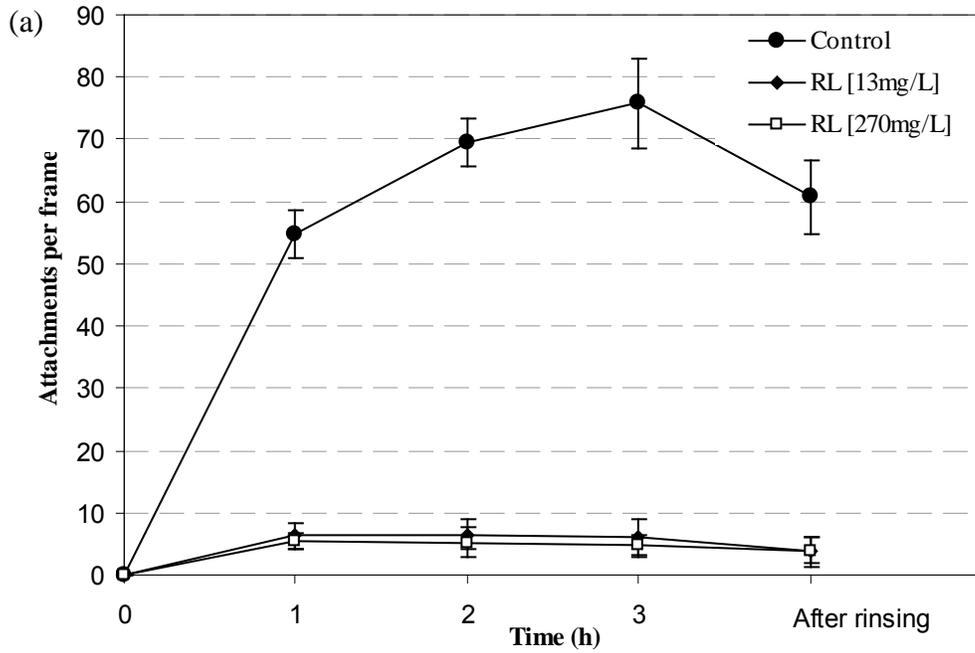
Figure 5.14. Effect of Rhamnolipids on initial bacterial attachment. The presence of rhamnolipids significantly reduced the initial attachment of PAO1, when tested using both the chamber setups. Same attachment prevention effect was found with the two rhamnolipid concentrations, 13 and 270mg/L. (a) Effect of Rhamnolipids on *Pseudomonas aeruginosa* PAO1 attachment as a function of time, using the silicone glass flow chamber setup. (b) Effect of Rhamnolipids on *Pseudomonas aeruginosa* PAO1 attachment, as a function of time, using the square glass tube chamber setup.

5.3.2.2 Effect of Rhamnolipids on *E. coli* attachment

The effect of rhamnolipids on *E. coli* was also studied using the flow chamber set up at a flow rate of 20ml/min (i.e. a shear force of $2.4mN/m^2$). Systems containing rhamnolipids at concentrations of 10mg/L and 200mg/L were compared to the rhamnolipid free control for differences. The attachments observed in case of the 10mg/L and 200mg/L rhamnolipid systems were 25 ± 9.6 % and 58 ± 9.7 % lower, respectively, than those observed in the control, as shown in Figure 5.15.
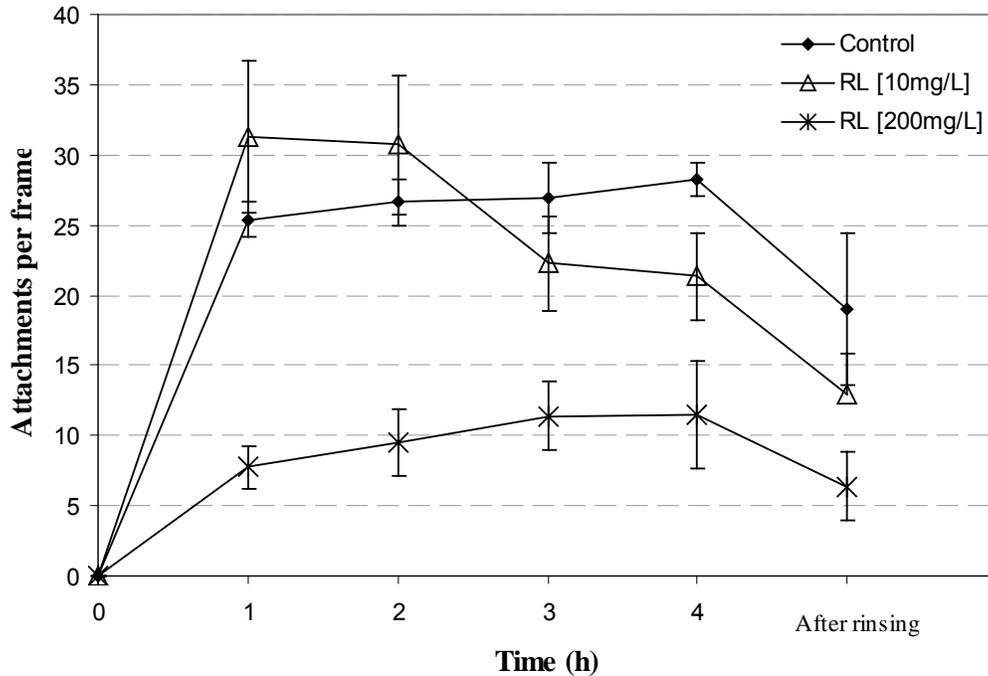
Figure 5.15. Effect of Rhamnolipids on *E. coli* attachment as a function of time. The presence of rhamnolipids reduced the initial attachment of *E. coli*. The system with 200mg/L of Rhamnolipid concentration had a stronger effect on attachment in comparison to the system at 10mg/L rhamnolipid concentration. An interesting observation made in case of the 10mg/L system is the decrease in attachment seen only after the 2nd hour. This may be due to a delayed rhamnolipid effect due to its low concentration.

This clearly indicated that the presence of higher concentration of rhamnolipids (200mg/L) in the bacterial suspension inhibited bacterial attachment twice as strongly as the much lower (10mg/L) rhamnolipid concentrated system. In case of *E. coli*, the attachment was followed for a period of 4 hours before rinsing the system with saline. The study was extended by an hour to get clear profiles, especially of the 10mg/L rhamnolipid. The attachment in this system was observed to decrease after the first two hours, suggesting the delayed effect of rhamnolipids in this lower concentration system.

Another important observation made during these experiments was the presence of large concentrations of the micelle like aggregates in the 200 mg/L rhamnolipid systems as shown in Figure 5.16. These aggregates were observed in large numbers in the 200mg/L rhamnolipid systems right at time 0, while in case of the systems containing 10mg/L rhamnolipids the aggregates began to form only after a period of 2 hours. This may be due to the presence of increased number of fatty acid chains in case of the systems containing a higher concentration of rhamnolipids, which makes them hydrophobic and causes these aggregates to form. The aggregates in case of the 200 mg/L rhamnolipid system increased in size with time. They started with an ellipsoidal shape and increased in length gradually, forming aggregates with long cylindrical sausage like limbs. These aggregates were mostly observed in the solution, and not attached on the surface. Very few of the aggregates were seen stuck to the surface by one end, with the other larger portion of their body fluttering with the flowing fluid. Therefore, rhamnolipids had a different effect on *E. coli* attachments as compared to the effect on the attachments of PAO1. Furthermore, the aggregates which were so conspicuous in the *E. coli* system containing 200 mg/L rhamnolipids were rare in the PAO1 system

containing the same concentration of rhamnolipids. A low concentration of these aggregates were also observed in the *E. coli* system containing 10 mg/L rhamnolipids after 2 hours of circulation through the flow set up, while they were completely absent in case of the PAO1 system with the same concentration of rhamnolipids.
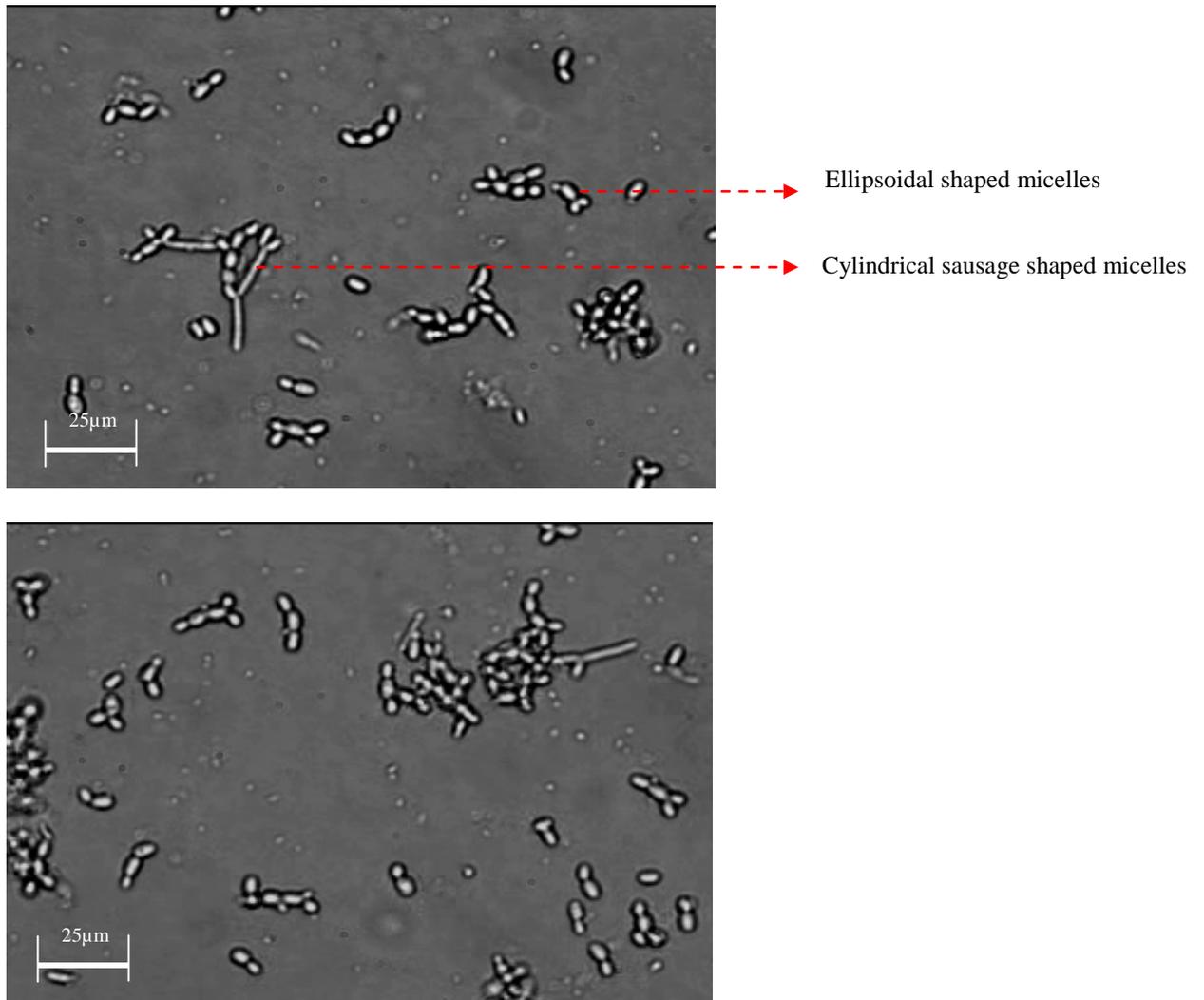
Figure 5.16. Images of micelles observed in the 200mg/L system.

5.3.2.3 Effect of Rhamnolipids on *P. putida* attachment

The antifouling property of rhamnolipids which was observed in case of PAO1 and *E. coli* systems was tested for Pseudomonas putida bacteria using a similar flow set up at 20ml/min (i.e. a shear force of 2.4mN/m^2). In case of the *P. putida* bacteria, the attachment observed in the 10 mg/L and 200 mg/L was lower than that seen in the control by 41.2 ± 15 % and 64.6 ± 6.8 %, respectively, as observed in the Figure 5.17.
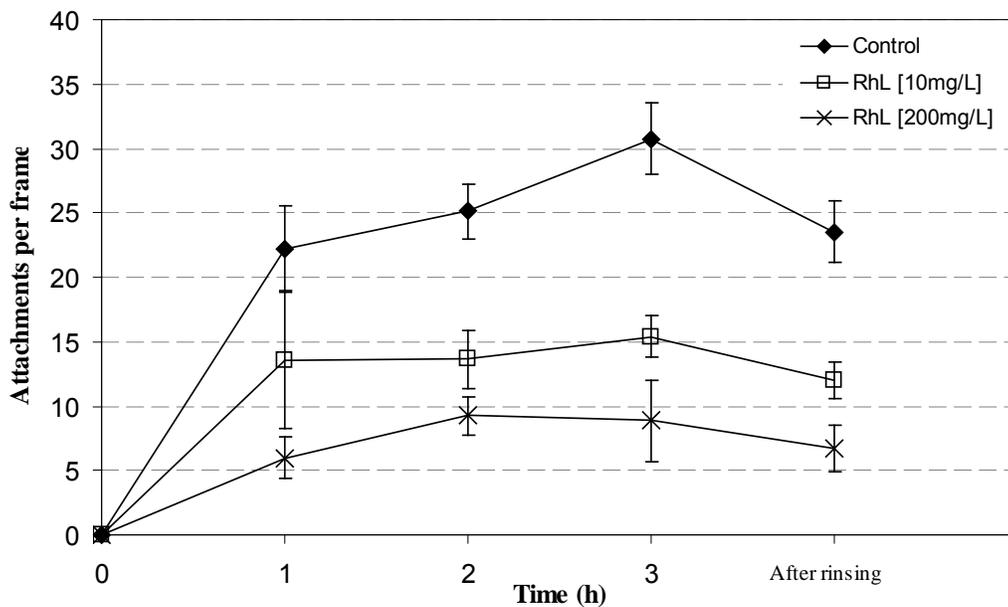
Figure 5.17. Effect of Rhamnolipids on *P. putida* attachment as a function of time. The presence of rhamnolipids reduced the initial attachment of *P. putida*. The system with 200mg/L of Rhamnolipid concentration had a stronger effect on attachment in comparison to the system at 10mg/L rhamnolipid concentration.

In case of *P. putida* as well the attachment seen in the 200mg/L rhamnolipid concentrated system was lower than that seen in the 10mg/L rhamnolipid system, indicating higher concentration of rhamnolipids prevent attachment better. The aggregates seen in case of the *E. coli* system containing 200mg/L of rhamnolipids were also seen in the *P. putida* equivalent system. The growth and behavior, i.e. their presence

in the solution rather than attached to the substrate surface was also very similar. A low concentration of these aggregates were also observed in the 10mg/L rhamnolipid system at 2 hours, identical to the phenomenon observed in the *E. coli* 10mg/L rhamnolipid system.

Therefore, rhamnolipids exhibit antifouling properties in case of all the three species tested. However, differences have been observed between different species and strains of bacteria. One glaring difference observed was the presence of aggregates in the *E. coli* and *P. putida* systems which were almost absent in case of the equivalent *P. aeruginosa* PAO1 strain. This could be attributed to the different metabolic activities of the bacterial strains and their varied behavior to chemical and biomolecules in the surrounding environment. However, the exact phenomenon behind this antifouling behavior and differences between the species is still not very clear. Future studies on the antifoulant effect of this bio-surfactant are hence warranted.

CHAPTER VI

SUMMARY AND CONCLUSIONS

6.1 Summary

The main purpose of our study was to investigate the phenomenon leading to the formation of the highly undesirable biofilm layer, or in other words to examine the initial attachment of microbial species.

Initially, static systems were employed to study the bacterial attachments and biofilm formation. Various set ups including amber bottles and glass slides coated with a hydrophobic silicone layer, polystyrene petridish and glass cover slips, real time silicone – glass cover slip chambers, were utilized to investigate this attachment process. The effect of a natural antifoulant namely, zosteric acid was tested repeatedly using the arrangements mentioned above. Other aspects such as the substrate surface properties including the effect of the presence of silanes with characteristic functional end groups and surface hydrophobicity, different bacterial species, various medium compositions were also explored. Every one of the static system experiments were repeatedly carried out, but due to the lack of consistency in the results, nothing conclusive could be drawn from the findings.

In the process of finding a consistent system, the various flaws or disadvantages associated with static systems were recognized and carefully documented. Consequently, a flow chamber system was designed and improved upon gradually, eliminating the majority of the common problems faced while using static systems. The entire flow chamber system is completely sealed to minimize manual handling and reduce the various uncertainties from creeping into the final results. Furthermore, the flow chamber systems allowed the real time study of the attachments, enabling us to better understand the mechanism behind the attachments.

Once the chambers were designed and built, the fluid flow behavior was studied thoroughly by analyzing the type of flow (i.e. laminar or turbulent), development of the flow, velocity profile and the characteristic flow parameters like Reynolds number, shear force exerted by the flowing liquid on the walls of the chamber, etc. The velocity profile, determined experimentally was compared to the theoretical analysis of tubing with conventional cross sections, and the equation for shear stress at the chamber walls was derived.

The effect of the estimated shear on the initial attachments when tested for three bacterial strains: *P. aeruginosa* PAO1, *E. coli* and *P. putida* and plotted, showed an initial increase in attachment with increase in shear and then a decrease in attachment with further increase in the shear. The only difference observed between the three strains was the shear at which these curves peaked. *Pseudomonas aeruginosa* PAO1 attachment peaked at a shear of $5mN/m^2$, *E. coli* at $45mN/m^2$, and *Pseudomonas putida* at $2.5mN/m^2$. The lack of effect of pH, in the range of 4.6 to 7.4, was also uncovered using the flow system set up.

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The antifouling property of zosteric acid was reanalyzed using this more consistent set up. However, a reduction in the attachment was not observed due to the presence of zosteric acid. Coumaric acid, which forms the major impurity in the zosteric acid tested, was also reexamined for its ability to reduce/prevent bacterial attachment to the surface. When initially tested, coumaric acid had a reducing effect on the attachment. However on repeatedly testing each of the individual factors which could have caused this reduced attachment, conclusive results could not be obtained. Rhamnolipids on the other hand, showed a strong effect on initial attachment of all three bacterial species, especially on *Pseudomonas aeruginosa* PAO1.

The flow system set up hence, was found to have a number of advantages over the static systems. However, the flow dynamics could be affected due to variation in the entrance length as a function of flow rate. Bacterial attachment in the fully developed flow region was found to differ from that in the non-developed region (Appendix E).

6.2 Future Work

Though the effect of shear and rhamnolipids has been investigated extensively using the three bacterial strains, a deeper investigation into the phenomenon behind these effects is warranted. Though the cell surface characteristics, surface charge, cell and surface hydrophobicity, size and shape dependence of the bacterial cells have been discussed as a possible reason for the attachment difference under shear conditions, a more detailed study is needed to confirm or reject this hypothesis. The effect on coumaric acid also needs to be studied further to understand the reduced attachment seen initially.

The effect of silane modified surfaces and presence of glucose and nitrates in the medium on bacterial attachment can be further looked into using the more reliable flow chamber set up. Patterned surfaces, both surface chemistry and topography, can also be used to study bacterial attachment.

The peristaltic pumps used in the flow systems resulted in a pulsating flow of the fluid through the chambers. A deeper investigation as to whether this pulsation actually does affect the attachment needs to be carried out. If the attachment is found to be effected by this flow behavior, pulsation can be avoided by using two parallel pumps each correcting the lag of the other. Also a gravity based reservoir system or syringe pump can be used for passing the fluid through the chamber setup.

Surfaces covered using the transparent hydrogel (a colloidal gel in which water is the dispersive medium) coating can also be tested, as this hydrogel covering has been reported to prevent the microorganisms from recognizing the surface (Cowling et al., 2000).

Several studies can be carried out using AFM and SEM to obtain more detailed information on the bacterial cell homogeneity and topography and also the adhesion forces between these cells and the substrate surface.

Several other studies like the effect of washing the cells prior to attachment, age of the bacterial cells, bacterial surface charge and surface hydrophobicity also need to be investigated for a more comprehensive idea of the events leading to biofilm formation.

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APPENDICES

APPENDIX A

FUNGI (*Trichoderma reseei*) AND ALGAE (*Ochromonas danica*) ATTACHMENT

Static system set ups were utilized to study the attachment behavior of various other bio-organisms like algae and fungi under different conditions. The effect of ZA was also tested on these species.

Experiments investigating the attachment behavior of fungi and algae were carried out using petri-dish systems with cover slips floating on top of the solution after inoculation. The effect of ZA on the species behavior and attachment was also studied. The attachments on the cover slips of the ZA and control systems were observed under the microscope after 3 hours.

In the case of fungi, no obvious differences were seen with respect to the attachment or growth characteristics (the length of their filaments, their side-branches, and their nodules) between the control and the ZA systems. Very few attachments were seen, in both types of systems.

In the algae systems, initially it was suspected that the algae observed in the ZA system were more circular and round as compared to the ones in the control, which were more oval in shape. But on repeating the experiment, it was noticed that this difference was due to the rinsing with water prior to imaging. When the experiment was repeated

using saline to rinse the coatings, there were no significant differences among the systems with and without ZA.

The fungi and algae were also used for an attachment study, in different media, that is in the regular media (control used in most of the previous studies) and in the rich media (TSB). These experiments were carried out using the petridish set-up. The cover slips were imaged for differences after a 3 hours and 24 hours. Again, no significant differences were encountered between the corresponding control and ZA systems. The only difference noted was between the rich and the regular media systems. In case of fungi, the attachment was till very low in both kinds of systems. While in case of algae, the rich medium systems, showed more attachments as compared to the regular medium systems, indicating better growth and development in the presence of abundant nutrients.

APPENDIX B

DIFFERENCE IN THE BUBBLES OBSERVED DURING A PETRIDISH

ATTACHMENT STUDY

An experiment was carried out using the modified petridish system to study attachment and biofilm formation and the surfaces of the silicone pieces attached to the petridish lip were imaged for biofilm formation. The bubbles at the top of the liquid level formed in the control and ZA systems were observed to differ and were hence followed as well.

In terms of the biofilm formed, there was little difference between the control and the ZA systems, but there was a slight difference in the bubbles formed in the ZA contained petri-dish systems. The ZA systems (both 10 and 500ppm) had more bubbles seen at the top of the liquid layer and near the silicone pieces than those of the control systems. The experiment was repeated and the difference in the bubbles formed was noted for 4 days. A small difference was observed in the systems (Figure B1), in terms of the bubbles formed mainly on the 2nd and 3rd days. The nitrate in the systems was measured to check for differences in the de-nitrification rate but no marked differences were found between the ZA and control systems. However, on repeating this study to ascertain that the ZA and control systems did differ in terms of the bubbles formed, the above described variation was not observed.
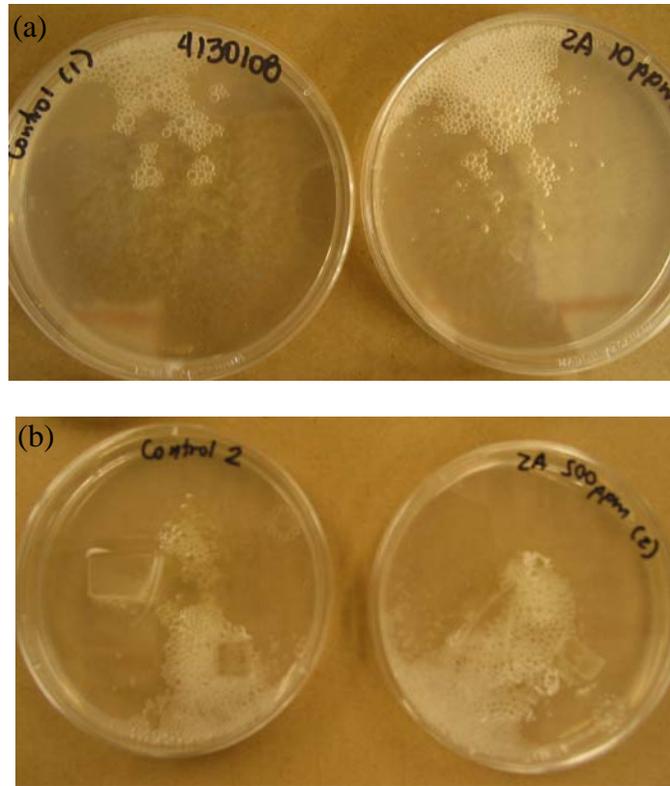
Figure B.1. Comparison of bubbles in the control and zosteric acid. Higher number of bubbles observed in the ZA system (irrespective of the concentration) as compared to the control. (ZA) systems. (a) Control and ZA (10ppm) comparison. (b) Control and ZA (500ppm) comparison.

APPENDIX C

COMPARISON OF FLOW CHAMBERS USED FOR THE FLOW SYSTEM STUDIES

During the various flow system studies, mainly two kinds of chambers were utilized. The first one was constructed primarily with silicone and glass as explained in section 3.4.2, while the second kind of chamber was mainly just glass as explained in section 3.4.3. A comparison was made between the two chambers to ensure no major differences in the flow behavior were observed while using the second kind of chamber (square glass tube chamber). The exact same bacterial suspension was passed through both kinds of chambers and an attachment study was carried out to assess the comparison of the two types of chambers to one another. The results for the attachment study are shown in Figure C1.
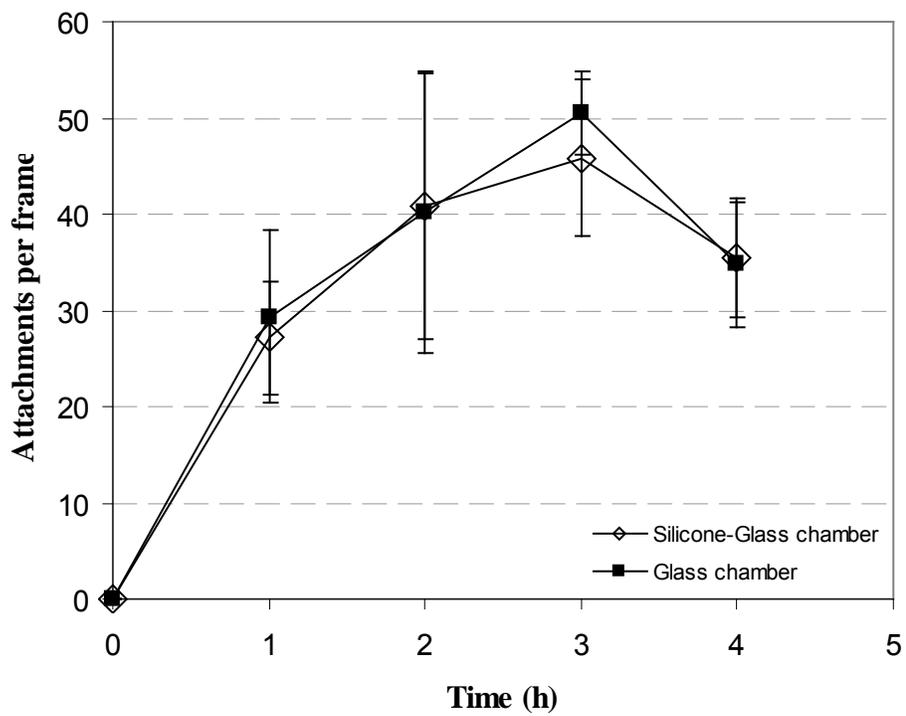
Figure C.1. Comparison of the two flow chambers used for the flow system studies.

APPENDIX D

EFFECT OF SURFACE CONDITIONING ON *E. coli* ATTACHMENT

The flow chamber surface was conditioned with nutrients prior to exposing it to *E. coli* cells. The first kind of system was conditioned with a combination of yeast extract (2.5g/L) and peptones (2.5g/L). Peptone is rich in amino acids and peptides, while yeast extract is rich in nucleic acid, lipids and other nutrients essential for the growth of *E. coli* cells. A medium rich in these peptones and yeast extract was circulated through the chamber for 1 hour and then the bacterial cells were introduced into the system. The second system was conditioned with a glucose rich medium for an hour before introducing the cells. The initial attachment numbers observed in case of the above two systems were compared to that seen in the control system which did not involve any surface conditioning prior to the introduction of cells.

The control and glucose conditioned system was found to be very similar in terms of the initial attachment number seen during the three hours of the experimental run. The peptone-yeast extract system however differed a great deal from the other two systems, as shown in Figure D1. The attachment in the peptone-yeast extract system was less than 20% of those seen in the control and glucose system.

However, when this experiment was repeated no such difference in the peptone-yeast extract system was noticed. All three systems were found to be similar.
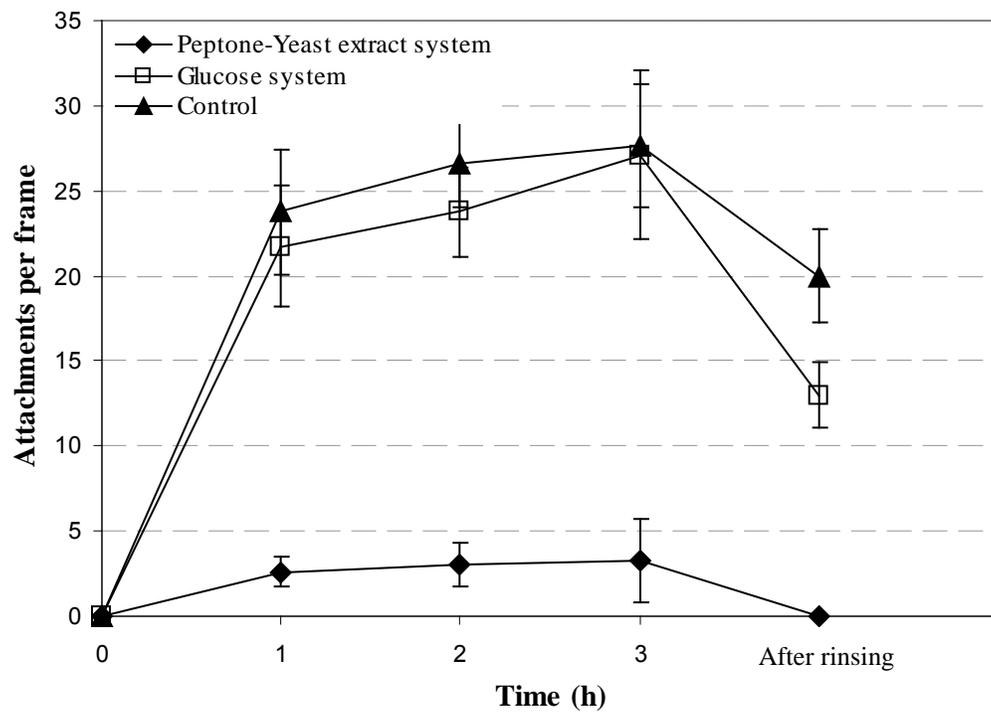
Figure D.1. Effect of conditioning the surface prior to the *E. coli* attachment study.

APPENDIX E

ATTACHMENT OBSERVATION WITHIN THE CHAMBER

A study was carried out to determine the best possible location within the chamber where the attachment could be studied. This was done to ensure development of flow in the chamber at the point where the attachment was being counted. Two identical systems at a flow rate of 20ml/min were examined for attachment at three locations within the chamber. The three observation points chosen, 15, 40, 80mm from the entrance were examined for attachment for 3 hours and also after rinsing. *Pseudomonas aeruginosa* PAO1 microorganism was used for this study.

It was noticed that at 15mm from the entrance the attachment was higher than at 40mm and 80mm (as shown in Figure E1). This region (15mm) in the flow chamber where the laminar flow is not fully developed, may experience formation of eddies near the substrate surface exhibiting reverse flow properties (Blevins et al., 1984, Schlichting et al., 2000). The increased attachment at the 15mm position from the chamber entrance may be due to this erratic flow before the flow develops. The bacterial cells may experience differences in the force applied when they come into contact with the substrate surface, therefore affecting their adhesion (Rodriguez et al., 2004). This agreed well with the calculation for development of flow within a chamber of 10mm X 10mm dimension at a flow rate of 20ml/min.
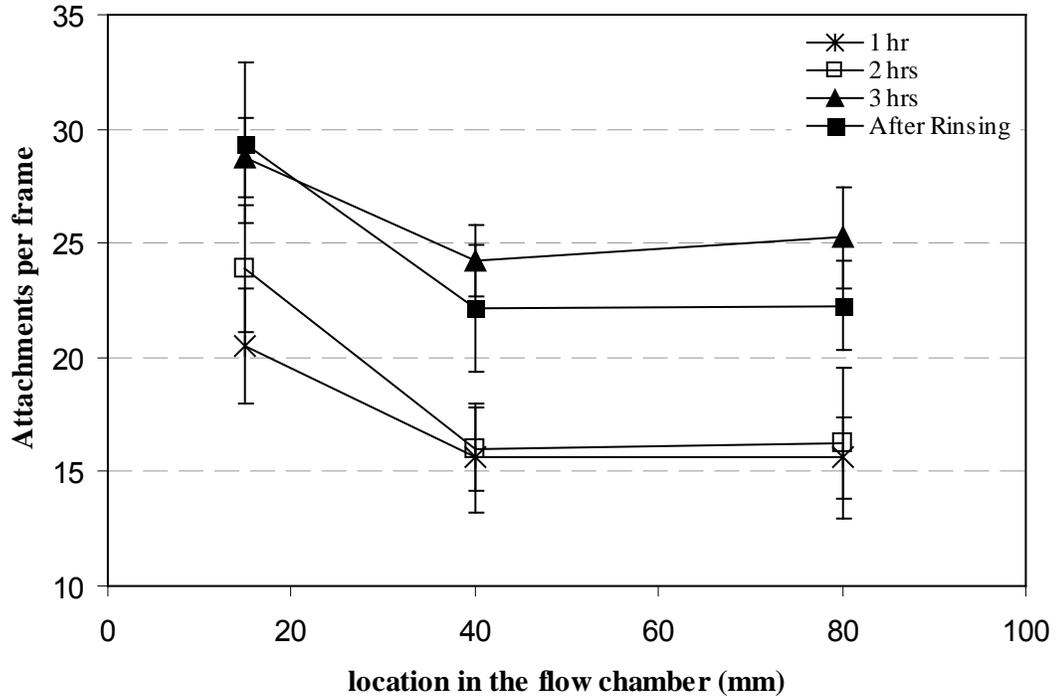
Figure E.1. Attachment of PAO1 in the flow chamber as a function of time and location along the length of the chamber.

The calculated value for the location where the flow is fully developed was at 25mm from the entrance (based on entrance length of circular tubes equation; $El_{laminar} = 0.06$ Re (Johnson, 1998) where Re is the Reynolds number calculated with the equivalent cross section area (πR^2) of the circular tube replaced by that of the chamber (WH). Hence the final equation for entrance length for fully developed laminar flow is;

$$le = \frac{0.24}{\Pi} \cdot \frac{\rho}{\mu} \cdot Q$$

In above equation, *le* is the entrance length and Q is the volumetric flowrate. From this equation it is clear that the entrance length is dependent solely on the flowrate, irrespective of the dimensions of the chamber used. Therefore, the usual point of

attachment observation within the chambers at 50mm from the entrance was within the fully developed region.