University of Alberta

Initial Adhesion of EPS Producing Bacteria *Burkholderia cepacia* – the Impact of Cranberry Juice

by

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ABSTRACT: The impact of cranberry juice was investigated with respect to the initial adhesion of three isogenic Burkholderia cepacia bacteria with different extracellular polymeric substance (EPS) producing capacities: a wild-type cepacian EPS producer PC184, mutant bacteria PC184rml with reduced EPS production, and PC184bceK with a deficiency in EPS production. Adhesion experiments demonstrated that in the presence of cranberry juice, the adhesive capacity of PC184 was largely reduced, while cranberry juice had little impact on the adhesion of either mutant. Thermodynamic modeling supported results from adhesion experiments. For PC184, the surface free energy change ΔGadh switched from negative in the absence of cranberry juice to positive when cranberry juice was added. Surface force apparatus (SFA) and scanning electron microscopy (SEM) studies demonstrated strong adsorption of cranberry juice components to bacterial EPS. It was concluded that cranberry juice components could impact bacterial initial adhesion by adhering to EPS and impairing bacterial adhesive capacity.

KEYWORDS: bacterial adhesion; cranberry juice; EPS; surface free energy; interaction forces.

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List of Symbols, Nomenclature, or Abbreviations

EPS: extracellular polymeric substances

SFA: surface force apparatus

SEM: scanning electron microscope

CBJ: cranberry juices

DLVO: Derjaguin, Landau, Verwey and Overbeek theory

PBS: phosphate buffered saline

Chapter I Introduction

1.1 Overview

Microorganisms have a natural tendency to attach to beneficial surfaces as a survival mechanism. This can occur in both natural and engineering environments, including the living host, natural water systems, and industrial systems. The general result of bacterial colonization on substrata is the establishment of biofilms, which consist of immobilized microorganism embedded in extracellular polymeric substances (EPS).

It is known that biofilms provide significant protection to bacterial cells against unfavorable environmental factors. The stratified structure of environmental biofilms harboring mixed microbial communities protects microbial processes occurring underneath the surface layer of biofilms and make biofilm reactors up to 500 times much more resistant to toxicity than suspension cultures (Characklis, 1990). This is not only due to the physiological changes of biofilm bacteria enhancing their resistance to biocides, but also to a barrier function of the EPS. Other effects include nutrient reserve and digestion, resistance to toxic and antibiotic substances, and hydrate conservation.

In the engineered systems, formation of microbial biofilms is unfavorable in many occasions and causes physical, chemical and biological problems. For example, in water conduits, biofilms may result in unusual high fluid frictional resistance losses. Biofilms formed inside of heat transfer tubes significantly

reduce the heat transfer rate (Characklis, 1981). Undesired biofilm accumulation increases the operation costs, introduces health risks and reduces the instrument performance. As a result, those biofilms must be removed or inhibited. However, the practice of biofilm control and removal still stays in its developing step.

Traditional practice for removing microbial biofilms in engineered systems relies mainly on the disinfectant treatment, including chlorine, ozone and ultraviolet light. However, because of the wide distribution and high resistance of microbial biofilms to disinfectants, novel strategies are needed. Bacterial adhesion to a solid surface is an essential step for biofilm formation, and this step is mainly dependent on van der Waals, electrostatic, and acid-base interactions. These interactions are influenced by physicochemical properties of the substratum and the bacterial surface, such as hydrophobicity, surface charge, and electron donor-electron acceptor properties. More research is now focused on reducing biofilm formation through inhibiting bacterial initial adhesion. These attempts include the exploration of desirable inhibitors to bacteria adhesion or colonization, and surface modification to produce incompatible surface for bacteria settlement. These types of approach are desirable as mechanical methods are not needed.

Cranberry juice is known to inhibit bacterial initial adhesion (Eydelnant and Tufenkji, 2008). However, the mechanisms controlling its impact on bacterial initial adhesion are still unknown. For instance, EPS producing bacteria represent one of the most common types of bacteria widely distributed in natural and engineered environments, which infect plant and animal tissues, establish

biofilms, and coaggregate into flocs. The EPS play a key role in mediating adhesion and colonization. However, though cranberry was recognized to impair bacteria adhesion capacity, a complete understanding at the nano-scale of how cranberry components interact with bacteria and reduce their adhesive capability is lacking.

The research reported in this thesis was undertaken to elucidate the mechanisms involved in EPS producing bacterial initial adhesion in the presence of cranberry juice, to determine the ability of cranberry juice to reduce the extent of bacterial adhesion, and to gain insight into the impact of bacterial surface EPS on their response to cranberry juice treatment.

1.2 Objectives

The objectives of this study were to gain a fundamental understanding of the mechanisms controlling the impact of cranberry juice on EPS producing bacterial initial adhesion in aquatic systems. Specific objectives are as follows:

1. To examine the impact of bacterial EPS on their adhesion in the presence of cranberry juice.

2. To explore the fundamental mechanisms involved in the initial stages of EPS producing bacteria adhesion in the presence of cranberry juice, and to gain

insight into the extent to which bacterial surface polymers influence bacteria adhesion.

3. To model bacterial initial adhesion mechanisms using DLVO and thermodynamic models.

1.3 Organization of the dissertation.

Following the introduction, Chapter 2 will review recent publications and background theories in the research area, including background information on biofilm establishment, bacteria adhesion process and the inhibitory properties of cranberry juice.

Chapter 3 will introduce the experimental approaches to investigate the adhesion process of bacteria and anti-adhesive mechanisms of cranberry juice. Chapter 4 will describe the results of each experiment. Chapter 5 will discuss the results of the adhesion research and offer several conclusions.

Findings from this master's research are summarized in Chapter 6, some thoughts for future research are also provided.

Chapter II Literature Review

2.1 Principles of biofilm formation and bacteria adhesion

2.1.1 The distribution, formation and function of biofilms

Microorganisms are able to attach tightly onto almost any surface submerged in an aqueous environment. The microbial cells are immobilized on surfaces, reproduce and produce EPS, developing a tangled matrix of fibers that provides the supporting structure of a biofilm (S.P.Denyer et al., 1993). Biofilms can consist of either a monolayer of cells or can be as thick as 3-4 mm. A thick biofilm can contain both aerobic and anaerobic environments due to oxygen diffusion limitations within the biofilm (Characklis and Marshall, 1990). Biofilm processes are manifest in many forms and are studied by researchers from a wide variety of disciplines.

Biofilms can serve beneficial purposes in the natural environment as well as in modulated or engineered systems. For example, biofilms are responsible for the removal of dissolved and particulate contaminants from natural streams in wastewater treatment plants, such as trickling filters, rotating biological contactors (RBC) and fluidized beds (Tchobanoglous et al., 2003). Figure 2-1 shows an SEM image of microbial biofilms obtained from the Devon wastewater treatment plant in Alberta.

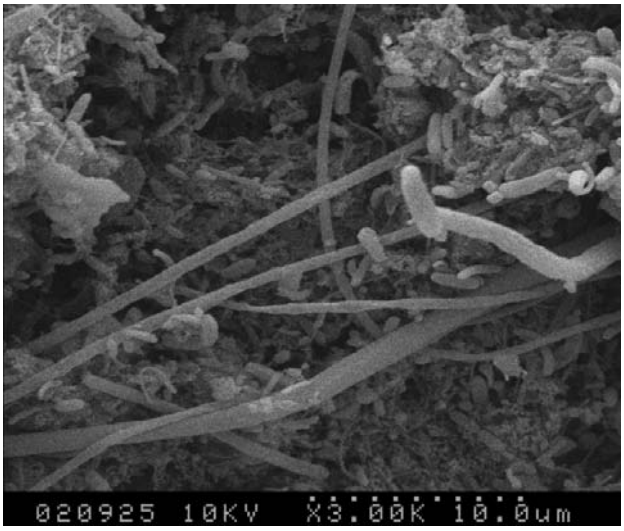


Figure 2- 1 Scanning electron micrographs of biofilms from Devon wastewater treatment plant, Alberta, Canada

However, not all biofilms are favorable or beneficial. Biofilm accumulation in plants and animals causes diseases, and they frequently cause operational problems in engineered systems. Biofilms accumulate in water and wastewater conduits, porous media, and ship hulls, causing higher fluid frictional resistance, increased energy losses, and reduced performance. Biofilms on sensors, separation membranes, filter medium, submarine periscopes and sight glasses reduce the performance of these devices. Public health risks may be increased by the formation and detachment of biofilms from drinking water distribution systems and cooling towers (Flemming and Schaule, 1996).

Biofouling is a problem in water pipes and industrial cooling water systems, as well as submerged engineering surfaces, even when anti-fouling protection is employed. In industrial cooling systems, fouling is primarily due to microbial biofilms and particulate matter, which reduces the heat transfer efficiency and

increases the pressure drop in the system, leading to the increased operation costs. Biofouling of submerged metal structures, such as oil platforms, leads to corrosion and metal fatigue. Fouling of moving structures such as ships by biofilms composed chiefly of diatoms, leads to losses of operating efficiency due to increased frictional resistance and drag. Table 2-1 summarizes the effects and environmental relevance of undesired biofilms in engineering systems.

Table 2- 1 Effects and environmental relevance of undesired biofilms

Process	Effects
Biofilm accumulation inside of heat exchangers, cooling towers (Lee and West, 1991; Liu et al., 2009), and wastewater pipelines (Costerton and Lashen, 1984)	Increasing heat transfer resistance, energy losses, causing corrosion on materials, and reducing system performance and lifetime
Biofilm formation on sensors, separation membranes (Ivnitsky et al., 2007; Chae et al., 2008), and filter media (Williams and Edyvean, 1998)	Causing biofouling, porous block,; decreasing equipment lifetime and water quality
Biofilm accumulation and detachment in drinking water distribution systems	Decreasing water quality and raising public health risks
Biofilm accumulation on ship hulls, cooling towers, pipelines (Costerton and Lashen, 1984), and oil platforms (Ferreira et al., 2006)	Increasing frictions and drag; leading to head losses, metal fatigue and higher costs
Biofilm accumulation on teeth(Sen et al., 1997), urinal and intestinal tract, medical devices and implants (Nickel et al., 1985; Jacques and Costerton, 1987)	Health risks and diseases

The formation of a biofilm in natural or engineered environments follows the same strategy, starting with the attachment of free suspended microorganisms to a surface. Figure 2-2 provides a scheme of initial bacterial adhesion and colonization, followed by which biofilms can develop. Usually, these colonies

adhere to the surface initially through weak, reversible interactions including van der Waals forces or electrostatic forces. If the colonies do not immediately detach from the surface, they can anchor themselves more tightly using cell adhesion structures such as pili or fimbriae (S.P.Denyer et al., 1993).

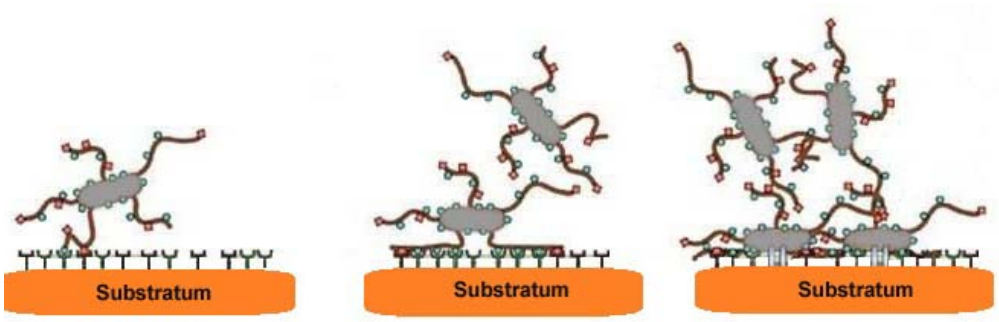


Figure 2- 2 Three stages in biofilm establishment

These first colonies facilitate the arrival of following cells by providing various adhesion sites and by initiating the buildup of the EPS matrix that holds the microorganisms together. Once colonization has begun, the biofilm develops by cell reproduction and attachment. The final stage of biofilm formation is known as maturity, in which the biofilm is established and may only change in shape and size. The development of a biofilm may allow for the aggregated cell colonies to be increasingly antibiotic resistant (Jost Wingender, 1999).

2.1.2 The principles and processes of bacterial initial adhesion

Bacterial initial attachment is the first step in the colonization of surfaces and precedes the process of consolidation, during which the initially weak adhesive

forces are strengthened by EPS formation and, finally, by reproduction to form an established biofilm. This adhesion of microorganisms onto various substrata is a complicated physico-chemical and biological process, which serve an important role in the survival of microorganisms in niches, tracts, and pipelines. The attachment process involves an interaction between complementary molecules on the approaching surfaces of the cell and the substratum. The rate of attachment and the affinity of the interaction reflect an interplay between microbial and substratum surface characteristics, and fluid shear stress. Macromolecules or polymers expressed by organisms participate in the adhesion process, and are controlled by several regulatory mechanisms. Different bacteria have different cell surface properties and secrete different biopolymers (i.e. fimbriae, lipopolysaccharide (LPS)), which function as adhesins or onto which adhesions can assemble (Cuatrecasas and Greaves, 1980; Tuomanen, 1986).

Bacteria adhere to substrata only if the repulsive forces are overcome by adhesive forces. They adhere by ionic interactions (Coulombic interactions that result from interactions between molecular groups that contain net permanent opposite charges) , hydrogen bond (the attractive force between the hydrogen attached to an electronegative atom of one molecule and an electronegative atom of another molecule) (Pimente and McClellan, 1990), hydrophobic effects (Doyle and Rosenberg, 1990), or coordination complexes involving multivalent metal ions. Figure 2-3 shows several typical attractive forces facilitating bacterial adhesion and their operation distances. The interaction free energy

profile described the change of contribution to the total energy that is caused by the approaching of the bacterial and substratum surfaces.

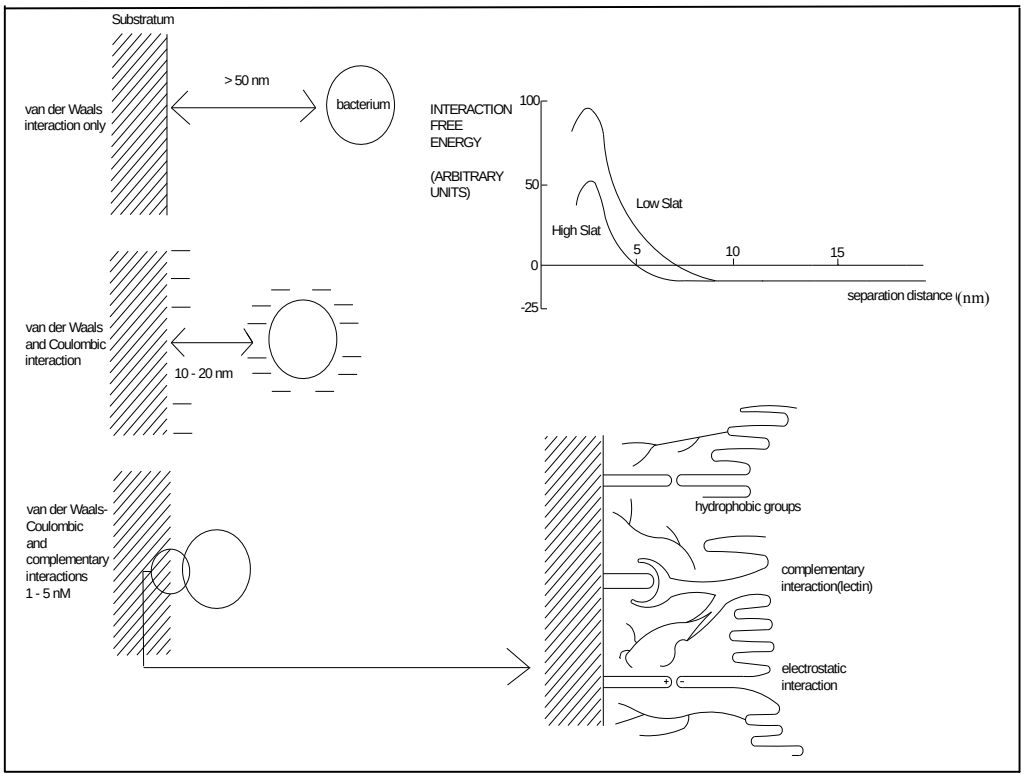


Figure 2- 3 Descriptions of the bacteria-substratum adhesion (modified from Busscher and Weerkamp, 1987)

The bacterial adhesion process is a complex composite driven by forces from various resources. According to the interaction types, bacterial adhesion can be divided into two types, specific and non-specific. Specific interactions only exist between adhesin-receptor pairs. A typical example for the specific interaction in bacterial adhesion is that between a lectin and a carbohydrate (Ofek et al., 1977; Mirelman, 1986). In contrast, a much less specific adhesion is the case of a hydrophobic bacterium that binds to any hydrophobic surface.

Non-specific interactions exist widely between bacteria and various surfaces, which include Lifshitz-van der Waals (LW) forces, electron-donor /electron-acceptor, and electrostatic interactions (as shown in Figure 2-3). Although non-specific interactions are usually much weaker than specific interactions, they determine whether bacteria will be able to approach close enough to a surface.

2.1.3 Bacterial EPS: characterization, structure and function

Most microorganisms live and reproduce in aggregated forms such as biofilms and flocs. The common feature of these phenomena is that the microorganisms are embedded in a matrix of EPS. EPS form a three-dimensional, gel-like, highly dehydrated and often charged biofilm matrix in which the microorganisms are embedded and more or less immobilized. EPS are mainly responsible for the structural and functional integrity of biofilms and are considered as the key components that determine the physico-chemical and biological properties of biofilms.

Definition of EPS

EPS were defined as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates" (Geesey, 1982). Biofilm forming microorganisms tend to produce EPS when they attach to a surface, or when they are surrounded by high numbers of bacteria (J. Wingender, 1999). The EPS are implicated in the initial bacterial attachment to surfaces and subsequent biofilm formation. EPS are comprised of polysaccharides (Costerton

et al., 1981), proteins (Frolund et al., 1996), phospholipids, humic substances, and nucleic acids (Frolund et al., 1996; Nielsen et al., 1997). These substances form a layer of slime on the surface of many gram-negative bacteria (Stephen M. Hammond, 1984).

Composition and function of EPS

From various analyses of activated sludge flocs, biofilms, or exopolymers from fresh water bacteria, a very heterogeneous composition of the EPS was obtained (Brown and Lester, 1980; Urbain et al., 1993; Jahn and Nielsen, 1995). Main organic fractions are proteins and carbohydrates (Horan and Eccles, 1986), and humic substances (Liu and Fang, 2002). Considerable amounts of extracellular deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were discovered in microbial biofilm EPS as well (Steinberger and Holden, 2005).

According to the physical state of the EPS, determined by environmental factors such as pH and ions, EPS are divided into “bound EPS” and “soluble EPS” (Nielsen et al., 1997). Bound EPS include sheaths, capsular polymers, condensed gel, loosely bound polymers, and attached organic material. Soluble EPS are soluble macromolecules, colloids and slimes (Hsieh et al., 1994). Figure 2-4 shows the configuration of bound EPS and soluble EPS.

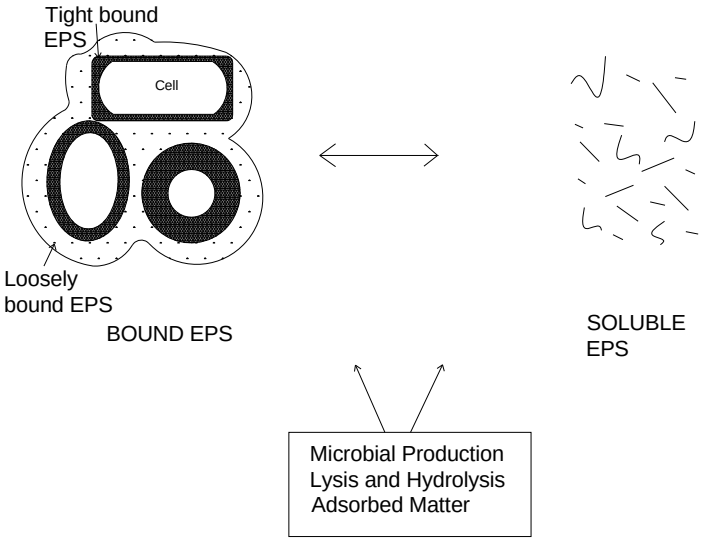


Figure 2- 4 Definition of bacterial EPS (modified from (Jost Wingender, 1999)

EPS in biofilms

In general, one of the most important roles of EPS is to provide a fundamental adhesive matrix responsible for the mechanical stability of biofilm, which is mediated by non-covalent interactions (Flemming and Schaule, 1996; Mayer et al., 1999). The EPS matrix promotes bacterial adhesion to inert substrata and also enables the biofilm cells to attach to the surface. Another function frequently attributed to EPS is their protective effects on biofilm organisms against unfavorable abiotic and biotic substances in the environment. The ability of certain EPS to absorb toxic metals such as Cd, Zn, Pb, Cu and Sr suggests that the presence of these polymers would protect other microbial cells in a mixed culture biofilm (Norberg and Persson, 1984). Also, EPS are able to increase the

biofilm resistance to biocides, by delaying or preventing biocides from reaching target cells within the biofilm. For example, previous research showed that the penetration of chlorine into biofilm matrix can be significantly reduced by the presence of EPS (Debeer et al., 1994).

In addition, EPS in biofilms can indirectly store and digest nutrients. The main function of the proteins in EPS was suggested to be producing certain types of enzymes, which are often used to digest exogenous macromolecules and particulate material in the microenvironment of the immobilized cells. Macromolecules enmeshed in the biofilm matrix could be degraded by extracellular enzymes and converted to usable oligomers or monomers. Further, the EPS matrix is highly hydrated, and may thus assist in the cell survival under desiccation conditions (Hughes et al., 1998).

EPS in bacterial adhesion

EPS compose bridges for cell-cell or cell-substratum interactions, and can mediate bacterial initial adhesion or colonization on surfaces, facilitate the aggregation of bacterial cells and the formation of flocs and biofilms, and result in adhesion of pathogens to plants or animals. It was proven that bacteria with EPS exhibit a higher adhesive capacity than those with a low EPS producing capacity. EPS serve as adhesins and increase bacterial adhesive forces (Quintero and Weiner, 1995; Fang et al., 2000).

EPS may mediate the adhesion of bacteria to tissue surfaces and protect bacteria from the host defense mechanisms, allowing for bacterial growth or biofilm formation (Jost Wingender, 1999). In addition, EPS are believed to promote a spatial organization of cells that promotes the interactions between cells and other surfaces (Jost Wingender, 1999). Previous studies showed that ionic strength, pH, and the presence of multivalent ions could impact bacterial EPS configuration and charge, thus interfering with bacterial adhesion to solid surfaces (Liu et al., 2007; Liu et al., 2008).

2.2 Biofilm control and bacterial adhesion inhibition

As discussed above, biofilms introduce various problems in engineering and public health, which can lead to significant increases in maintenance and operational costs. This highlights the need for effective strategies to control or minimize biofilm formation. However, biofilms are highly resistant to disinfectant or antibiotics due to the EPS protection, and are difficult to remove once initial adhesion occurs. Current disinfection technologies used to deal with biofilms are often ineffective, costly and often based on toxic biocides.

2.2.1 Biofilm control and removal in environmental engineering

Traditional disinfection methods for biofilm removal include chlorine, ozone and ultraviolet light (UV). However, problems such as high dosage rates and extended contact time make these processes less feasible for biofilm disinfection.

Biocides are currently the primary mitigation method to control biofilm formation. However, biofilms serve as protective niches for bacteria inside and are highly resistant to the bactericidal action of antibiotics. In addition, the use of antibiotics may give rise to the variation of antibiotic resistant strains (S.P.Denyer et al., 1993). For instance, in drinking water systems, injecting monochloramine or free chlorine into water systems has been the most popular choice for water disinfection by many water undertakings for a long time. The secondary disinfecting residual concentrations of monochloramines routinely range between 0.1 and 0.5 mg/L, which is regarded as sufficient to control coliform problems (Mara and Horan, 2003). There is, however, increasing evidence showing that even at relatively high concentrations, these disinfectants are still ineffective in controlling either heterotrophic bacteria or coliforms. It was reported that *Legionella pneumophila*, *E. coli* and *Aeromonas. hydrophila* survive in an established biofilm at a monochloramine concentration of 0.2 - 4 mg/L for weeks (Williams and Braun-Howland, 2003). Moreover, the feasibility of injecting sufficient disinfectant into water distribution systems or pipelines is still questionable due to the health risks and ecology concerns.

In view of the wide-ranging implications of microbial fouling for industries, coupled with increased environmental awareness resulting in the decreased use of biocides to control fouling, there have been interests in developing non-biocidal methods to prevent biofilm establishment.

Mechanical method is one of the most commonly used alternative in industrial and engineering biofilm removal. For example, high pressure water jets (Granhag et al., 2004) can be calibrated to remove spores from the various topographic structures. Vortex mixed with air and water is adopted by the Gold Bar wastewater treatment plant in Edmonton, Alberta to remove the biofilms from pipelines. However, this operation requires pauses of relevant processes and disrupts regular operations. This method is not suitable for more delicate sensor components.

2.2.2 The inhibition of bacterial adhesion

Since initial bacterial adhesion is an essential step in biofilm formation, the interruption of bacterial initial adhesion to solid surfaces may become an effective alternative for biofilm control.

Surface modification with the addition of anti-adhesive compounds makes substrate surfaces less attractive for microorganisms and therefore prevents bacterial adhesion and biofilm formation. Non-sticky coatings with low surface energy polymers, and biocide coatings impregnated with biocides were developed to protect surfaces from biofouling (Whelan and Regan, 2006). Silicone, polydimethylsiloxane, fluoropolymers, phosphorylcholine and polyethylene oxide have low surface energies and were investigated to prevent and ease biofouling (Whelan and Regan, 2006). Nanocomposite coatings were produced by using anisotropic nanoparticles (Wong and Ho, 2009) to inhibit bacterial adhesion and showed promising results in fouling-release properties.

However, due to the limitations of coating materials, coating techniques, durability, scales and costs, the surface modification is still in its infant stage, and their application is only limited in sensor membrane protection (Wisniewski and Reichert, 2000). Moreover, the life time of the modification coatings and the influences of the shedding coating materials are still unknown. Consequently, effective, safe and economical strategies are still needed to decrease the adhesion of bacteria on surfaces.

2.2.3 Cranberry – the natural inhibitor of bacterial adhesion

The American red cranberry (*Vaccinium macrocarpon*) has been recognized for inhibiting the bacterial adhesion to solid surfaces (Howell and Foxman, 2002; Eydelnant and Tufenkji, 2008; Liu et al., 2008). However, the wide application of cranberry for preventing initial bacterial adhesion in engineered systems is hindered due to a lack of understanding of cranberry anti-adhesive mechanisms. A complete understanding of how cranberry components interact with bacteria and reduce their adhesive capability is lacking.

Several recent studies showed that cranberry influences bacterial initial adhesion through altering bacterial surface characteristics or gene expression (Ahuja et al., 1998; Liu et al., 2006; Johnson et al., 2008; Liu et al., 2008). Incubation in media supplemented with cranberry juice or high molecular weight proanthocyanidin (PAC) from cranberries resulted in an interfacial tension increase between bacterial cell surfaces and polyvinyl chloride (PVC) and polytetrafluoroethylene (PTFE), making attachment thermodynamically

unfavorable (Ahuja et al., 1998). Cranberry juice has also been shown to change bacterial morphology and inhibit *Escherichia coli* P-fimbriae expression leading to reduced fimbrial adhesion (Ahuja et al., 1998; Liu et al., 2006). Additional gene expression studies demonstrated that the morphology change in *E. coli* is due to the down regulation of flagellar basal body rod and motor protein (Johnson et al., 2008). The mechanisms of the phenomenon were not clear, and several studies revealed that cranberry juice may change bacterial surface morphology, inhibit the expression or regulation of fimbriae (Ahuja et al., 1998; Liu et al., 2006; Wu et al., 2009).

2.2.4 *Burkholderia cepacia*

The *Burkholderia cepacia* complex (BCC) is a closely related family of gram-negative betaproteobacteria that are metabolically diverse and highly antibiotic resistant (Seed and Dennis, 2005), and are found in various environments (Mahenthiralingam et al., 2005). BCC species are ecologically beneficial in the natural environment (Parke and Gurian-Sherman, 2001), but are an important group of bacterial pathogens to animals and humans (Seed and Dennis, 2005; Lynch and Dennis, 2008). Because BCC bacteria are able to utilize various compounds as carbon sources, including groundwater pollutants and chlorinated aromatic substrates that are found in pesticides and herbicides, they have been widely used as bioremediation agents (Lessie et al., 1996; Parke and Gurian-Sherman, 2001). *B. cepacia* was also isolated from drinking water. Characterization and biofilm cultivation showed that *B. cepacia* had higher

growth rate and motility than most of other microorganisms, indicating their competitive advantage in co-cultured biofilms.

Most *B. cepacia* isolates produce considerable amounts of EPS, which are believed to facilitate the bacterial infection in niches such as the human body (Bartholdson et al., 2008). In recent publications (Cerantola et al., 2000; Richau et al., 2000), *B. cepacia* EPS were characterized and found to produce mainly one kind of EPS, which has the primary structure shown in Figure 2-5. This type of EPS is composed of a branched acetylated heptasaccharide repeating unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, in the ratio 1:1:1:3:1 (Cescutti et al., 2000).

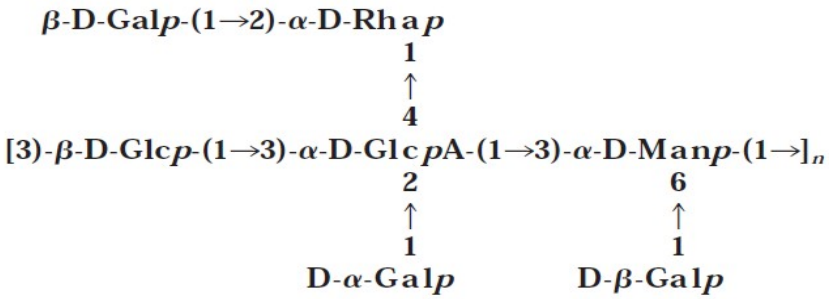


Figure 2- 5 The repeating unit of EPS of *B. cepacia* (Cescutti et al., 2000)

2.3 Adhesion models

A number of models have been developed to characterize the adhesion of bacteria to surfaces or substrata, such as the Derjaguin, Landau, Verwey and Overbeek (DLVO) model and its extensions (Simoni et al., 1998; Ong et al.,

1999), the thermodynamic (surface tensions) model (van Oss, 1994), and the steric model. By resorting to these models, the essential process and mechanisms of bacterial adhesion can be investigated. In this study, both DLVO and thermodynamic models were adopted because of their wide application and relative accuracy.

The DLVO model is based on the DLVO theory named after Derjaguin, Landau, Verwey and Overbeek, describing the forces between charged surfaces interacting through a liquid medium (Hermansson, 1999). It takes the effects of the van der Waals attraction and the electrostatic repulsion into account. The electrostatic part of the DLVO interaction is computed in the mean field approximation in the limit of low surface potentials and the radius of the approaching spheres.

The thermodynamic model calculates the surface energy change for the adhesion of small particles or microbial cells from a suspension onto a solid substratum. This model follows that the extent of adhesion is determined by the surface properties of all three phases involved, i.e., the surface tensions of the adhering particles, the substrate, and the suspending liquid medium (van Oss, 1994).

Chapter III Methodology

3.1 Materials

3.1.1 Cranberry juice cocktail.

Cranberry juice cocktail (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA), containing 27 wt. % cranberry juice (Liu et al., 2006), was obtained commercially, and dilutions of the cocktail to 9 and 18 wt. % cranberry juice with 0.01 M phosphate buffered saline (PBS) were prepared. The cranberry juice cocktail (27 wt.% cranberry juice) has an ionic strength of 0.955×10^{-2} M (Liu et al., 2006). Sodium chloride was added into the cranberry juice to equalize the ionic strength of cranberry juice with 0.01 M PBS through electronic conductivity measurements. The pH of cranberry juice solutions were measured using a pH meter, and are 4.00, 2.98 and 2.56 for 9, 18 and 27 wt.% cranberry juice, respectively.

3.1.2 Bacterial culture.

Deposition on polycarbonate coupons was examined in *B. cepacia* complex strains PC184, PC184*rml,* and PC184*bceK* obtained from Dr. Jonathan Dennis, Department of Biological Science, University of Alberta. PC184 is a wild-type cepacian EPS producer. PC184*rml* is an isogenic mutant strain of PC184 with a rhamnose producing deficiency (Vinion-Dubiel and Goldberg, 2003) that causes the mutant to produce a reduced amount of EPS. PC184*bceK* is an isogenic

mutant of PC184 with no EPS producing capability (Moreira et al., 2003). Of the three strains tested, PC184*bceK* produces the least amount of EPS.

Bacteria were cultured in Luria-Bertani (LB) broth at 37ºC and harvested at mid-log-growth phase (16 hours). Cells were harvested by centrifugation (Avanti J-20I, Beckman Coulter, CA) at 4000 rpm at 4°C for 10 min. The growth medium was decanted and pellets were resuspended in cranberry juice solutions (9, 18, and 27 wt. %). The centrifugation-resuspension process was repeated three times to remove traces of growth media. A final cell density of approximately 10^8 CFU/mL was determined by optical density (OD) of 0.1 using a UV/visible spectrophotometer (Varian, Inc., CA) at a wavelength of 600 nm.

3.1.3 Polycarbonate surfaces

In this study, polycarbonate surfaces were adopted to test the adhesive capacity of wild type *B. cepacia* (normal EPS production) and isogenic mutants (reduced EPS production) in the absence or presence of cranberry juice. Polycarbonate is a type of plastic widely applied in medical devices, food processing, electronics industry , construction and other engineering areas, with high-performance, great thermo-oxidative stability, optical transmittance, solvent resistance, and toughness (Stokes, 1995). Being easily shaped, moulded, and thermoformed, polycarbonate is frequently molded into tubes, membranes, packing materials and resins. In this study, polycarbonate was obtained from the Biosurface Technologies (MT, USA) and cut into round disks, with a thickness of 1 mm and a diameter of 10 mm.

3.2 Experimental Methods

3.2.1 Bacterial adhesion.

Bacterial initial adhesion to polycarbonate coupons was examined using a dual channel parallel plate flow chamber (dimensions, L × W × H: 39.5 × 13 × 0.32 mm, Model FC271, Biosurface Technologies, MT). Polycarbonate coupons (10 mm diameter × 2 mm thickness) soaked in 70% ethanol were sonicated in sterilized deionized (DI) water for 15 min to remove contaminants, and then mounted in the flow cell channel. The system was equilibrated with bacteria free 0.01M PBS or cranberry suspension (9, 18, or 27 wt. %) at 3 mL/min for 20 min. Bacteria suspensions (1×10^8 CFU/mL) were injected into the flow cell at a constant flow rate of 3 mL/min for 10 min. Disks were carefully removed from the flow cell and rinsed 3 times with bacteria free 0.01M PBS or cranberry suspension to remove unbound cells. To remove surface-attached bacteria, the sampling coupons were placed in a 2 mL centrifuge tube containing 1 mL of 0.01 M PBS, ultrasonicated for 10 minutes (FS 30H, Fisher Scientific Inc., IL), and then vortexed (Genie 2, Fisher Scientific Inc., IL) at maximum speed for 30 seconds. This ultrasonication-vortexing process has been shown to efficiently dissociate bacteria from coupons (Liu et al., 2007; Liu and Li, 2008; Liu et al., 2008). Viable bacterial cell counts were obtained using the drop plate method (Liu and Li, 2008). A series of 10-fold dilutions was performed and 10 μL of each dilution was plated onto an LB agar plate; dilutions were plated in triplicate. Bacteria counts were obtained after incubating the plates at 37°C for 16 h.

Bacteria adhesion experiments were conducted at room temperature (23°C) and were repeated at least 4 times.

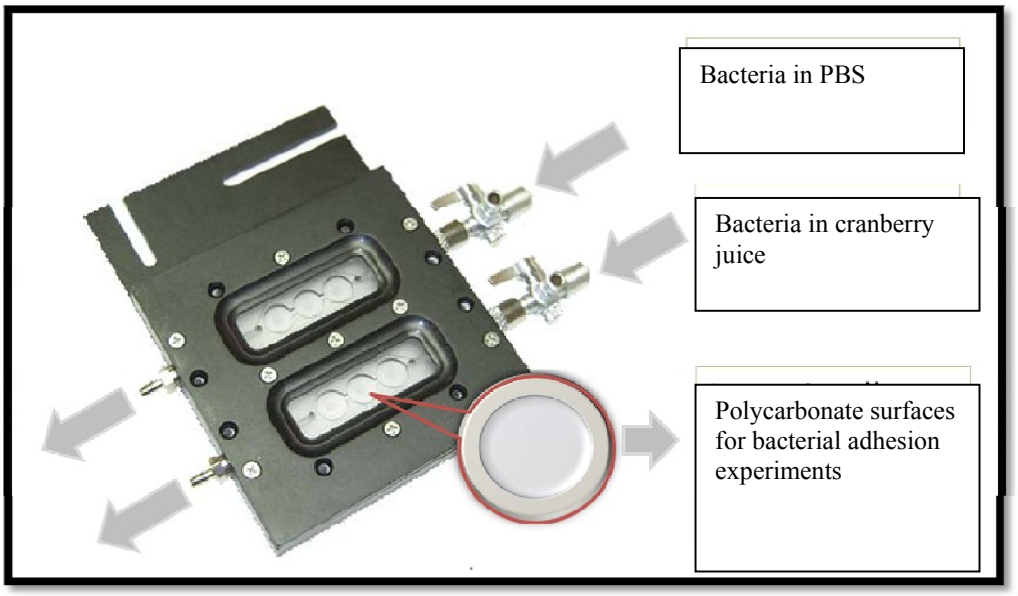


Figure 3- 1 Electrokinetic characterization of bacterial cells (figure of flow cell obtained from Biosurface Technologies)

The chemical and physical properties of cells in 0.01M PBS or cranberry juice (9, 18, and 27 wt. %) were characterized by measuring the zeta potential and electrophoretic mobility of the solutions at room temperature with a ZetaPALS, Zeta Potential Analyzer (Brookhaven Instruments Corp, NY). At least three samples were tested for each condition, and the measurements were replicated 10 times with 30 cycles for each assay.

3.2.3 Contact angle measurements.

The interfacial tensions of individual substrata were derived from the contact angles of three probe liquids, one nonpolar (diiodomethane) and two polar

(glycerol and ultrapure water), using the sessile drop method (Miller et al., 1993). Bacterial cells were incubated with 0.01 M PBS or cranberry juice (9, 18, or 27 wt. %) for 2 h. Lawns of bacteria were prepared by filtering 5 mL of cell suspension (10^8 cells ml^{-1} in early stationary phase) onto a cellulose acetate filter (0.45 μm pore size, 25 mm diameter, Millipore, USA) via vacuum. When a vacuum was applied, bacterial cells were compacted into a homogeneous lawn, reducing deviation in contact angles (θ) caused by the roughness of the lawn surface, and preventing permeation of sessile droplets of liquid. The lawns were further exposed to air at room temperature for 1 h to evaporate excess residual liquid adhering to the bacterial lawn (Vanloosdrecht et al., 1987). Previous studies have shown that contact angle measurements on bacterial lawns are stable for 3 h (Vanloosdrecht et al., 1987). 2 μL droplets of the probe solutions were placed on the bacterial lawns; images were captured and analyzed with a goniometer at room temperature and ambient humidity within 2 s. The contact angle was measured and given by the goniometer based on the shape of the sessile drop. At least three replicated measurements were conducted per probe liquid per filter, and four filters were analyzed for each combination of bacteria and probe liquid.

3.2.4 EPS extraction.

EPS components were isolated from the bacterial surface by ethanol extraction (Gong et al., 2009). A detailed description of an EPS extraction procedure was reported by Gong et al.(2009), and is briefly summarized here. Freshly harvested

bacterial cells were resuspended in 10 mL of 8.5% sodium chloride containing 0.22% formaldehyde and incubated at 4°C for 2 h. The suspension was then centrifuged at 3,700 × g at 4°C for 15 min, and the cell pellets were resuspended in 10 mL DI water. The centrifugation process was repeated to remove remaining cellular material (non-EPS). Pellets were collected and weighed, and resuspended in 50 mL DI water per gram of pellet. The resuspensions were sonicated and centrifuged to collect purified EPS in the pellet. Pellets were treated overnight at 4°C in 5 mL 10^{-2} M KCl and 10 mL pure ethanol, and centrifuged again to collect the pellet containing pure EPS.

3.2.5 Scanning Electron Microscope (SEM) imaging.

Fresh harvested bacteria were suspended in PBS or 18% cranberry juice. The bacterial suspension was dropped on a microscope glass cover, and fixed with 2.5% glutaraldehyde in PBS buffer for 30 min. Bacterial suspension was then washed 3 times with PBS and were further fixed with 1% OsO4 in PBS buffer for 30 min. Bacteria were dehydrated in a serial of 50%, 70%, 90% and 100% ethanol, and followed by critical point drying with CO2 at 31 °C for 5 min. The samples were sputter coated with gold (Edwards, Model S150B, U.K.) and then examined with the Hitachi Scanning Electron Microscope S-2500. Each sample was examined at least 10 times under several different magnitudes (×20,000; ×15,000; ×12,000), and the images were compared.

3.2.6 Surface force measurement.

Interaction forces and normal force-distance profiles between EPS and cranberry juice were determined using a surface force apparatus (SFA) (Israelachvili, 1992; Leckband, 1995; Israelachvili et al., 2010) that measured the force F as a function of absolute surface separation D and the local geometry of two interacting surfaces with a force sensitivity of ~1 nN and an absolute distance resolution of 0.1 nm measured in situ (Israelachvili, 1992; Leckband, 1995; Leckband and Israelachvili, 2001; Zeng et al., 2006; Zeng et al., 2007; Zeng et al., 2008; Israelachvili et al., 2010). An SFA 2000 (Surforce LLC, Santa Barbara, CA) was used to investigate the fine details of interactions between EPS and cranberry juice. A detailed setup for SFA experiments has been previously reported (Israelachvili, 1992; Zeng et al., 2007; Israelachvili et al., 2010). Basically, a thin mica sheet of 1–5 μm was glued onto a cylindrical silica disk (radius R = 2 cm). The two curved and coated mica surfaces were then mounted in the SFA chamber in a crossed-cylinder geometry, which roughly corresponds to a sphere of radius R approaching a flat surface based on the Derjaguin approximation: $F(D) = 2\pi RW(D)$, where $F(D)$ is the force between the two curved surfaces and $W(D)$ is the interaction energy per unit area between two flat surfaces (Israelachvili, 1992). The measured adhesion or "pull-off" force F_{ad} is related to the adhesion energy per unit area W_{ad} by $F_{ad} = 2\pi RW_{ad}$ for rigid (undeformable) surfaces with weakly adhesive interactions, and by $F_{ad} = 1.5\pi RW_{ad}$ (used in this study) for soft deformable surfaces with strong adhesive contact (Israelachvili, 1992). In the experiments, 30–50 mL of EPS in PBS

solution with or without cranberry juice was injected between the two mica surfaces (as illustrated in Figure 3-2). The absolute surface separation *D* was monitored in real-time during the force measurement using multiple beam interferometry employing fringes of equal chromatic order (FECO) (Israelachvili, 1992; Israelachvili et al., 2010). Experiments were performed at room temperature (23°C).

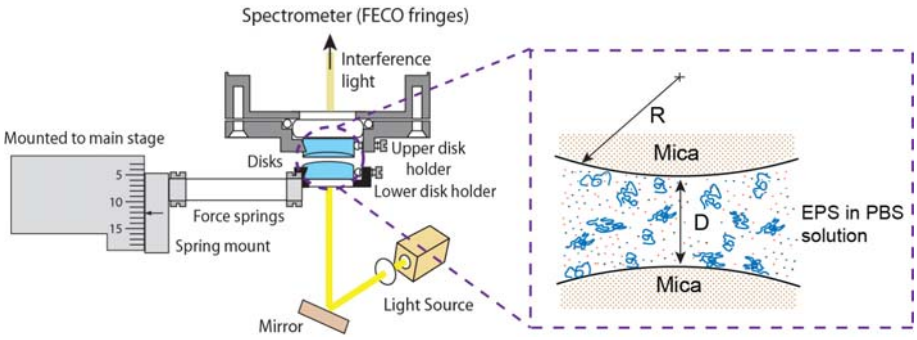


Figure 3- 2 Schematic diagram of the SFA setup

3.2.7 Model development.

The van Oss-Chaudhury-Good thermodynamic approach. Surface free energy change, ΔG_{adh}, representing the Gibbs free energy change for interactions between two different surfaces, can be determined by the surface tension γ, and the relative contributions to γ from Lifshitz–van der Waals (γ^{LW}) and Lewis acid base (γ^{AB}) components can be calculated by a method developed by van Oss et al (van Oss, 1994). Thus, interfacial tension can be expressed as:

$$\gamma = \gamma^{LW} + \gamma^{AB} \qquad (1)$$

The AB component of the surface tension (γ^{AB}) is defined to comprise all electron-acceptor–electron-donor, or Lewis acid-base interactions, given by

$$\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}} \tag{2}$$

The LW, electron donor and electron acceptor components, γ^{LW}, γ^{-}, and γ^{+}, can be calculated from the contact angle measurement. Contact angles θ for 2 μL droplets of water, diodomethane, and glycerol on different bacterial lawn surfaces are shown in Table 1. When a drop of a liquid (L) is deposited on a solid surface (S), the contact angle between the drop and the surface (θ_L) is a function of the components and parameters of the surface tensions of the liquid (γ_L) and the solid (γ_S). The Young–Dupre equation (van Oss, 1994) describes the relations among these elements:

$$\gamma_L(\cos\theta_L + 1) = 2\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + 2\sqrt{\gamma_S^{+}\gamma_L^{-}} + 2\sqrt{\gamma_S^{-}\gamma_L^{+}} \tag{3}$$

To solve the surface tension components of the solid, γ_S^{LW}, γ_S^{-} , and γ_S^{+} , three probe solutions must be used to yield three equations. Surface tension components for liquid, γ_L^{LW}, γ_L^{-} , and γ_L^{+}, are characteristic of the three probe liquids used; surface tensions of water, diiodomethane, and glycerol are designated $\gamma_W, \gamma_D, \gamma_G$, respectively. Substituting the values of γ_L^{LW}, γ_L^{-} , γ_L^{+} and θ_L intoEquation (3) and combining three equations together, we obtain a matrix to solve for the solid surface tensions γ_S^{LW}, γ_S^{-} , and γ_S^{+}:

$$\begin{bmatrix} \gamma_S^{LW} \\ \gamma_S^{+} \\ \gamma_S^{-} \end{bmatrix} = \left\{ \left[2 \begin{pmatrix} \sqrt{\gamma_W^{LW}} & \sqrt{\gamma_W^{-}} & \sqrt{\gamma_W^{+}} \\ \gamma_D^{LW} & \gamma_D^{-} & \gamma_D^{+} \\ \gamma_G^{LW} & \gamma_G^{-} & \gamma_G^{+} \end{pmatrix} \right]^{-1} \begin{pmatrix} \gamma_W[\cos\theta_W + 1] \\ \gamma_D[\cos\theta_D + 1] \\ \gamma_G[\cos\theta_G + 1] \end{pmatrix} \right\}^2 \quad (4)$$

The total energy change ΔG_{adh} can be expressed as a sum of LW and AB components. LW terms and AB terms can be collected to provide an alternate way to represent the different contributions to the Gibbs free energy change upon adhesion in aqueous media (van Oss et al., 1987):

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (5)$$

where

$$\Delta G_{adh}^{LW} = \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_{PC}^{LW}}\right)^2 - \left(\sqrt{\gamma_B^{LW}} - \sqrt{\gamma_W^{LW}}\right)^2 - \left(\sqrt{\gamma_{PC}^{LW}} - \sqrt{\gamma_W^{LW}}\right)^2 \quad (6)$$

where γ_B and γ_{PC} represent the surface tension of bacterial cells and polycarbonate coupons, respectively, and

$$\Delta G_{adh}^{AB} = 2[\sqrt{\gamma_W^{+}}\left(\sqrt{\gamma_B^{-}} + \sqrt{\gamma_{PC}^{-}} - \sqrt{\gamma_W^{-}}\right) + \sqrt{\gamma_W^{-}}\left(\sqrt{\gamma_B^{+}} + \sqrt{\gamma_{PC}^{+}} - \sqrt{\gamma_W^{+}}\right) - \sqrt{\gamma_B^{+}\gamma_{PC}^{-}} - \sqrt{\gamma_B^{-}\gamma_{PC}^{+}}] \quad (7)$$

Chapter IV Results

4.1 The effect of cranberry juice on bacterial survival.

To examine the effect of cranberry juice on bacterial survivability, bacterial cells were incubated in 0.01 M PBS, or cranberry juice (9, 18, or 27 wt. %) for 3 h at room temperature. As demonstrated in Figure 4-1, we verified that bacterial cells remained viable after exposure to cranberry juice for 3 h by comparing the remaining culturable bacteria number to the culturable bacteria count obtained from dilutions without cranberry. We did not detect any loss in bacterial viability indicating that the experimental concentrations of cranberry are not lethal to bacterial cells in 3h.

Table 4- 1 Viable bacteria cell counting in 0.01 M PBS and 27 wt. % cranberry juice

	Initial bacteria concentration ($\times 10^8$ CFU/ml)	Bacteria concentration after incubation of 2h ($\times 10^8$ CFU/ml)
PC184		
0.01M PBS	3.7±1.5	2.3±0.6
27 wt.% CBJ	3.3±0.6	1.7±0.6
PC184*rml*		
0.01M PBS	3.3±1.5	2.7±1.2
27 wt.% CBJ	4.3±2.5	2.6±1.5
PC184*bceK*		
0.01M PBS	1.3±0.5	0.7±0.6
27 wt.% CBJ	1.7±1.5	0.7±0.6

4.2 The effect of cranberry juice on bacterial electrokinetic potential.

The variation in zeta potentials of bacterial cells as a function of cranberry juice concentration is shown in Figure 4-1. Despite the difference in magnitude, all strains exhibited negative zeta potentials indicating negative surface charge under the conditions tested. In the absence of cranberry juice, the absolute magnitude of zeta potential increased with reduced EPS coverage on bacterial surfaces. The reduced spatial negative charge density of the EPS producing bacterial cells can be attributed to the neutral cepacian EPS of *B. cepacia*, which is composed of a branched acetylated heptasaccharide repeating unit of D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, in a 1:1:1:3:1 ratio (Cescutti et al., 2000; Lagatolla et al., 2002).

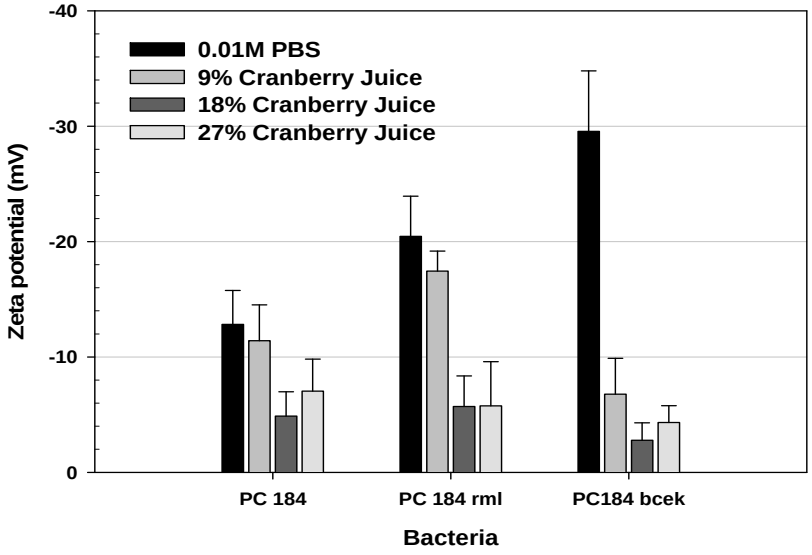


Figure 4- 1 Surface zeta potential of three *B. cepacia* strains: PC184 (wild-type EPS producng strain), PC184*rml* (mutant with deficiency in rhamnose EPS production), and PC184*bceK* (mutant with deficiency in EPS production) as a function of cranberry concentrations. Error bars represent standard deviations of 10 replicate measurements.

Upon exposure to cranberry juice, the zeta potential of all bacterial cells shifted in a positive direction, indicating that the bacterial surface charge was reduced in the presence of cranberry juice; this could be due to a reduced solution pH (the pH of the 27 wt. % cranberry juice used in our experiments was 2.56) or to the adsorption of cranberry components to bacterial surfaces. To evaluate the impact of pH on bacterial surface charge, experiments were performed to compare bacterial electronic kinetic charge in 0.01 M PBS at pH 7.4 and pH 2.6 (the pH of 0.01 M PBS was adjusted to 2.6 with 0. 1 N HCl). A pH drop to 2.6 had only a small impact on the surface charge of EPS producing bacteria, PC184 and PC184*rml* (-7.4±2.2, -12.8±2.9 for PC184 in pH 2.6 PBS and pH 7.0 PBS, -7.6±1.5, -20.5±2.7 for PC184*rml* in pH 2.6 PBS and pH 7.0 PBS, respectively), but significantly affected the surface charge of non-EPS producing bacteria PC184*bceK* (-5.6±1.5, -29.55±3.1 PC184*bceK* in pH 2.6 PBS and pH 7.0 PBS, respectively). Slight reduction in surface charge with decreasing pH has been reported for EPS producing bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Flavobacterium* sp. (Liu et al., 2009). An alternative explanation for neutralization of bacterial charge with addition of cranberry juice would be adsorption of cranberry juice components onto the bacterial cell membrane and EPS. Cranberry juice contains abundant fructose, mannose, and condensed tannins, which are more positively charged than cepacian EPS (Habash et al., 2000), and are able to bridge negatively charged groups of EPS components and shift the zeta potential from negative to neutral (Habash et al., 2000). In the

present study, pH change and cranberry component adsorption may both contribute to the bacterial cell electrokinetic property change observed.

4.3 Contact angles and Gibbs free energy changes

4.3.1 Contact angles and surface tension changes in PBS and cranberry juice.

Water contact angles increased in the wild type strain *B. cepacia* PC184 after exposure to cranberry juice (from 31.11° in 0.01 M PBS to 41–46° in all cranberry juice solutions as shown in Table 4-2). The diiodomethane and glycerol contact angles increased more significantly than the water contact angles after cranberry juice treatment. Using the contact angle values of the three probe liquids, interfacial tension components were calculated as a function of cranberry juice concentration for the three bacterial strains. As shown in Table 4-3, for PC184 bacteria, γ^{LW} decreased from 37.68 to 29.00 mJ m^{-2} and γ^{AB} increased by an order of magnitude, from 1.29 to 14.70 mJ m^{-2}, when PBS buffer was switched to 9 wt. % cranberry juice. Notably, the γ^{AB} of PC184 in 18 wt. % cranberry juice increased to 23.56 mJ m^{-2}, indicating the acid-base interaction between bacteria and polycarbonate coupons was greatly increased in cranberry juice.

Table 4- 2 Contact angles of bacteria in 0.01 M PBS and 9, 18 or 27 wt. % cranberry juice with three probe liquids

	Contact angles (degree)*		
	θ_W	θ_D	θ_G
PC184			
0.01M PBS	31.11±2.21	43.77±3.21	52.41±2.10
9 wt.% CBJ	44.33±2.49	58.13±2.32	69.18±4.53
18 wt.% CBJ	45.72±3.23	56.97±3.41	78.64±3.71
27 wt.% CBJ	41.63±1.89	59.27±3.02	69.28±6.57
PC184*rml*			
0.01M PBS	34.92±1.07	41.31±4.02	72.62±1.53
9 wt.% CBJ	39.29±4.81	36.79±2.87	82.76±6.90
18 wt.% CBJ	47.43±4.78	40.8±5.57	85.77±5.05
27 wt.% CBJ	41.45±3.96	43.29±5.27	78.99±3.55
PC184*bceK*			
0.01M PBS	44.11±4.95	46.44±2.64	82.44±1.79
9 wt.% CBJ	49.88±4.72	44.23±3.11	84.80±5.28
18 wt.% CBJ	49.58±4.46	40.89±4.92	88.52±5.23
27 wt.% CBJ	51.47±3.31	43.00±5.31	85.13±4.36

* θ_W, θ_D, θ_G: contact angles of water, diiodomethane, and glycerol.

Table 4- 3 Surface tension components of three bacteria strains in 0.01 M PBS and 9, 18, or 27 wt. % cranberry juice

	Surface tension parameters (mJ/m^{-2})**				
	γ^{LW}	γ^{+}	γ^{-}	γ^{AB}	γ^{total}
PC184					
0.01M PBS	37.68 ±1.72	0.0072 ±0.0023	58.05 ±0.04	1.29±0.72	29.59±0.24
9 wt.% CBJ	29.64 ±1.33	0.79 ±0.19	67.63 ±3.70	14.70±2.28	44.35±3.61
18 wt.% CBJ	30.32 ±1,97	2.02 ±0.36	73.47 ±0.01	24.37±2.16	54.69±0.20
27 wt.% CBJ	29.00 ±1.75	0.57 ±0.46	70.05 ±4.31	12.59±4.02	41.59±3.18
PC184*rml*					
0.01M PBS	38.49 ±1.66	2.72 ±0.07	81.58 ±1.25	29.80±0.63	58.10±0.63
9 wt.% CBJ	41.18 ±1.40	7.14 ±1.13	90.74 ±8.67	50.92±5.78	92.13±8.19
18 wt.% CBJ	39.21 ±2.94	6.82 ±0.91	79.32 ±1.21	46.51±2.66	85.72±1.80
27 wt.% CBJ	37.92 ±2.89	4.22 ±1.11	80.47 ±6.82	36.84±6.67	74.75±8.25
PC184*bceK*					
0.01MPBS	36.23 ±1.39	4.90 ±0.79	81.57 ±4.17	39.99±2.98	68.29±5.68
9 wt.% CBJ	37.42 ±4.72	5.43 ±1.52	72.71 ±6.98	39.73±6.03	77.15±7.30
18 wt.% CBJ	39.16 ±2.59	7.90 ±2.76	79.34 ±5.53	50.08±7.23	89.23±6.79
27 wt.% CBJ	38.07 ±2.84	5.50 ±0.71	69.54 ±5.28	39.12±3.98	77.19±4.02

**γ^{LW},Lifshitz-van der Waals component of interfacial tension; γ^{+}, γ^{-}, electron acceptor and electron donor components of interfacial tension; γ^{AB}, Lewis acid-base component of interfacial tension $\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}}$; γ^{total}, total surface tension $\gamma^{total} = \gamma^{LW} + \gamma^{AB}$

There was no significant difference in contact angles between 0.01 M PBS treated and cranberry juice treated PC184*bceK*. Hence, the surface free energy components remained fairly constant before and after cranberry juice treatment (Table 4-3). Moreover, water, diiodomethane, and glycerol contact angles of PC184*rml* changed more significantly than those of PC184*bceK* with cranberry juice treatment. When PC184*rml* was exposed to 9% cranberry juice, γ^{LW} varied from 37.92 to 41.18 mJ m^{-2} and γ^{AB} increased from 29.80 to 50.92 mJ m^{-2}.

4.3.2 Gibbs free energy changes.

In 0.01 M PBS, ΔG_{adh} was negative for PC184 and positive for PC184*rml* and PC184*bceK* (Table 4-4). This difference showed that EPS could facilitate a bacterial cell approach to polycarbonate surfaces, and consequently play an important role in bacteria adhesion. The similar ΔG_{adh} values of PC184*rml* and PC184*bceK* in 0.01 M PBS suhahaggest that, in the absence of cranberry juice, the thermodynamic model cannot discriminate between these two mutants in terms of adhesion energy changes. ΔG_{adh} values of PC184 were the most sensitive to cranberry juice concentration. The ΔG_{adh} of PC184 peaked at 18 wt.% cranberry juice, jumping above zero, indicating the attachment behavior of PC184 to polycarbonate varied from favorable to unfavorable as the concentration of cranberry juice increased from 0 to 18 wt. %. In contrast, the positive ΔG_{adh} observed for PC184*bceK* and PC184*rml* changed only slightly in the presence of cranberry juice, indicating unfavorable adhesion.

Table 4- 4 Gibbs free energy change of three bacteria strains in 0.01 M PBS and 9, 18 or 27 wt. % cranberry juice

	Gibbs free energy change		
	ΔG_{adh}^{LW}	ΔG_{adh}^{AB}	ΔG_{adh}^{total}
PC184			
0.01M PBS	0.74±0.07	-12.07±0.08	-11.33±0.15
9 wt.% CBJ	0.39±0.06	-4.86±1.75	-4.47±1.81
18 wt.% CBJ	0.42±0.09	-0.40±0.44	0.02±0.53
27 wt.% CBJ	0.36±0.08	-4.83±2.49	-4.48±2.57
PC184*rml*			
0.01M PBS	0.78±0.07	3.11±1.85	3.89±1.92
9 wt.% CBJ	0.83±0.11	10.54±3.37	11.18±3.48
18 wt.% CBJ	0.81±0.11	7.36±4.08	8.16±4.19
27 wt.% CBJ	0.75±0.12	4.85±2.59	5.60±2.71
PC184*bceK*			
0.01MPBS	0.68±0.06	5.97±2.46	6.66±2.52
9 wt.% CBJ	0.73±0.19	3.98±2.67	4.72±2.86
18 wt.% CBJ	0.80±0.11	8.37±3.37	9.18±3.48
27 wt.% CBJ	0.76±0.12	3.10±1.80	3.87±1.92

4.3.3 Lifshitz–van der Waals and acid–base components of ΔGadh.

In order to compare the relative strengths of LW and AB interactions, ΔG_{adh}^{AB} and ΔG_{adh}^{LW} were estimated. As shown in Table 4-3, ΔG_{adh}^{AB} values were much greater than ΔG_{adh}^{LW} values, suggesting that Lewis acid-base interactions are stronger than Lifshitz–van der Waals interactions in controlling bacterial adhesion to polycarbonate surfaces. For the interactions between *B. cepacia* PC184 and polycarbonate, ΔG_{adh}^{AB} became more positive between 0 and 18 wt. % cranberry juices, suggesting that bacterial adhesion became more unfavorable as the percentage of cranberry juice increased in this concentration range. Although negative ΔG_{adh} was observed when cranberry juice concentration increased to 27 wt.%, the ΔG_{adh} of 27 wt.% cranberry juice was still higher than the control. The general tendency was clear that the presence of cranberry juice increased the ΔG_{adh} of PC184.

4.4 Adhesion experiments in flow cells.

Parallel plate flow cell experiments were performed to determine the effect of cranberry juice in preventing bacterial initial adhesion to biomaterial surfaces. As shown in Figure 4-2, the presence of EPS on wild type strain PC184 increased bacterial adhesion over the mutant strains PC184*rml* with little EPS and PC184*bceK* with no EPS. The presence of cranberry juice had little impact on the deposition behavior of EPS deficient strains PC184*bceK* and PC184*rml*. However, the adhesion behavior of EPS producer PC184 was significantly

impacted by the addition of cranberry juice despite the slightly reduced surface charge indicated by zeta potential measurements (Figure 4-1). Our results showed that 9 wt. % cranberry juice reduced the adhesion of EPS producer PC184 by 2.5 log units 10 min after the experiment started. This study revealed that the antiadhesion impact of cranberry juice was more effective on EPS producing strain PC184 than on its EPS deficient counterparts. Specific components in cranberry juice may interact with and alter bacterial surface EPS causing EPS producing strain PC184 to be less adhesive.

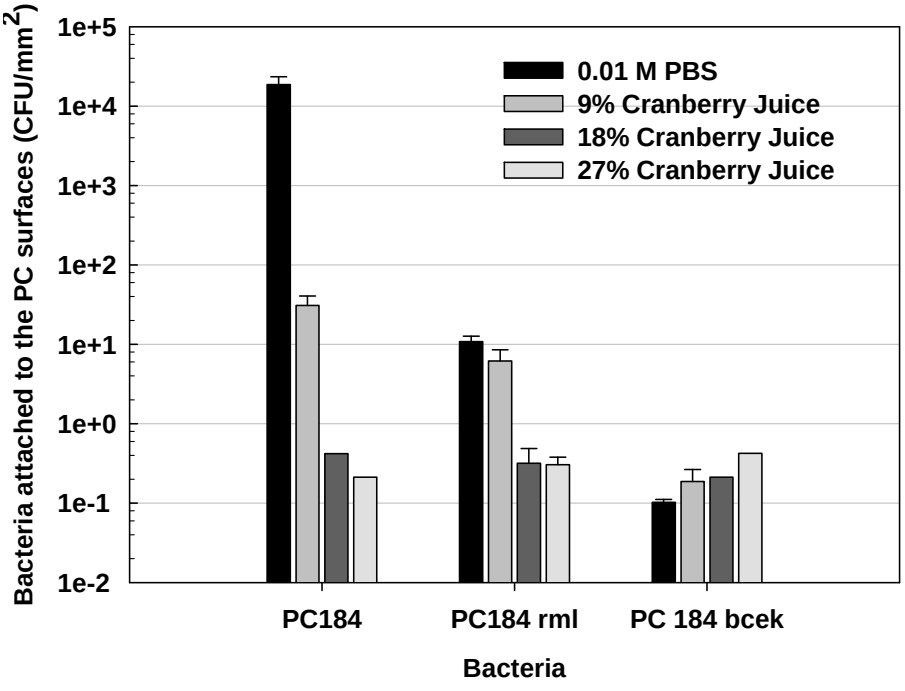


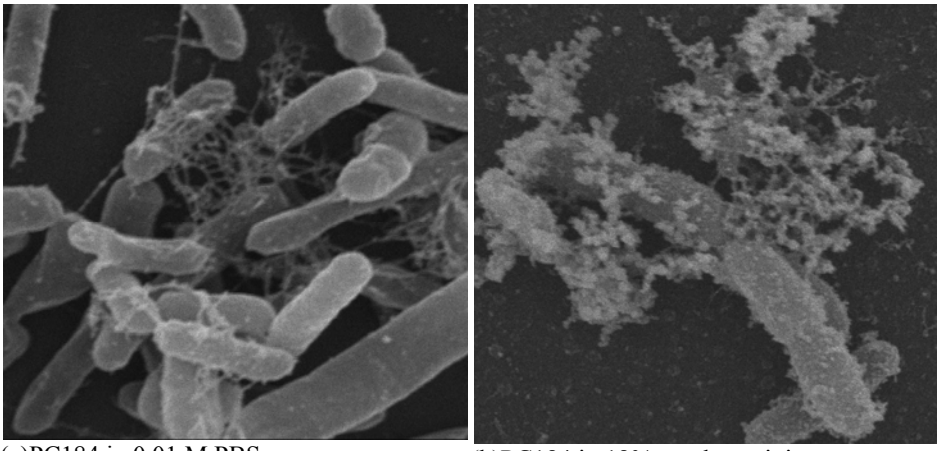
Figure 4- 2 Adhesion behaviors of three *B. cepacia* bacteria on polycarbonate disks in flow cells. Error bars represent standard deviations of four replicated experiments.

In general, a free energy calculation of the bacteria adhesion process provided results similar to the actual bacteria adhesion behavior under the study

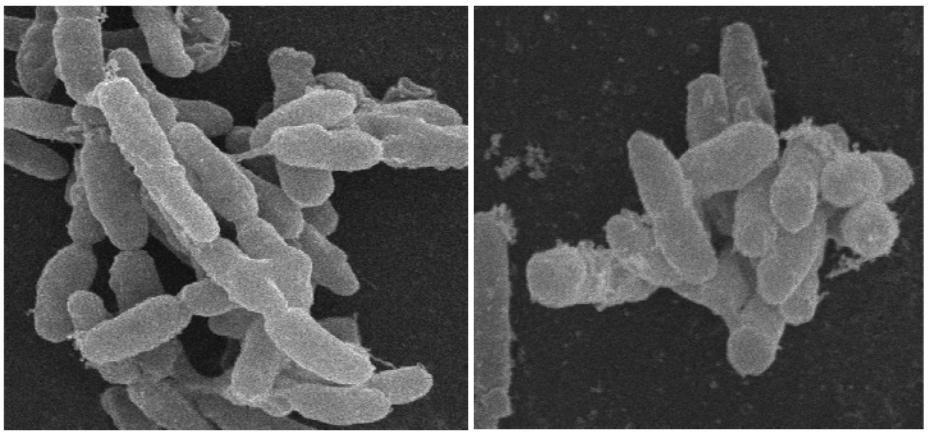
conditions. As shown in Table 4-3, in the absence of cranberry juice, the ΔG_{adh} was negative for EPS producing strain PC184, indicating favorable adhesion. In the presence of 18 wt. % cranberry juice, ΔG_{adh} became more positive indicating a reduction in affinity between PC184 and polycarbonate surfaces. For PC184 mutants, ΔG_{adh} was positive in the absence of cranberry juice and at all concentrations of cranberry juice tested, and the value of ΔG_{adh} was fairly insensitive to cranberry juice concentration. This result indicates that a thermodynamic model may be used to quantitatively explain cranberry anti-adhesive effects on bacterial cells.

4.5 SEM images of bacteria cells.

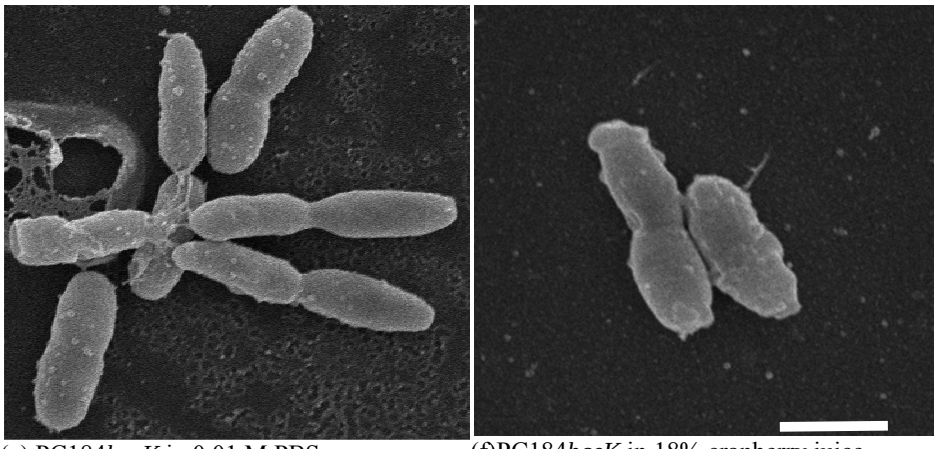
Figure 4-3 shows SEM images of three *B. cepacia* bacterial cells in 0.01 M PBS and 18 wt. % cranberry juice. As shown in Figure 4-3 (a, c, e), in 0.01 M PBS, PC184 bacteria contained the highest amount extracellular slimes on their cell surface. In the presence of cranberry juice (Figure 4-3b), PC184 bacterial cells and EPS became much rougher, indicating a considerable amount of cranberry components has bonded to the surface of bacterial cells and EPS. On the contrary, cranberry component sorption was not observed for the mutant bacteria PC184*rml* and PC184*bceK*.



(a)PC184 in 0.01 M PBS (b)PC184 in 18% cranberry juice



(c) PC184*rml* in 0.01 M PBS (d)PC184*rml* in 18% cranberry juice



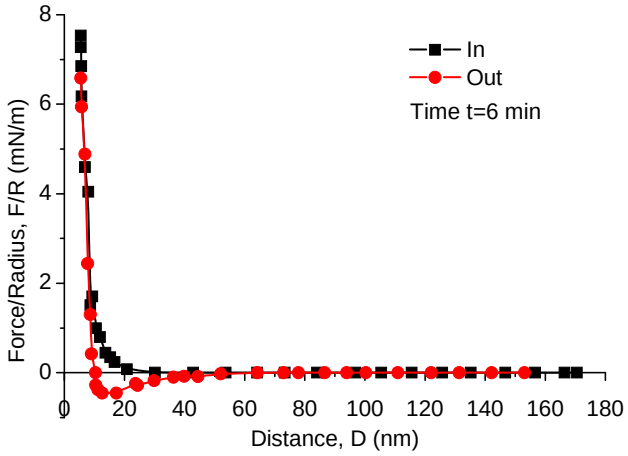
(e) PC184*bceK* in 0.01 M PBS (f)PC184*bceK* in 18% cranberry juice

Figure 4- 3 Scanning electron micrographs of three *B. cepacia* bacterial cells in 0.01M PBS (a, c, e) and in 18 wt. % cranberry juices (b, d, f). Scale bar = 1µm.

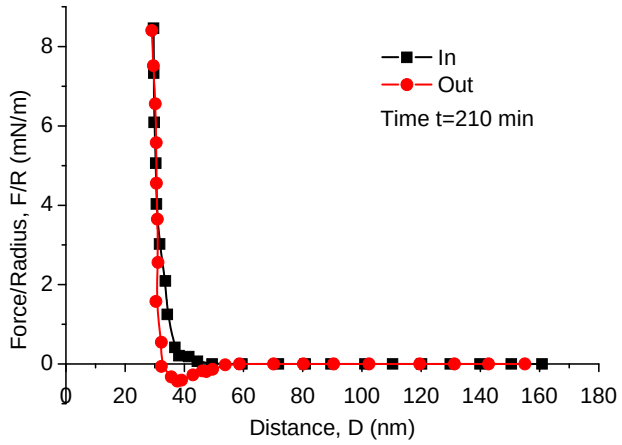
4.6 Surface force measurements using an SFA.

A surface force apparatus (SFA) was employed to investigate the interaction between EPS and cranberry juice. 30–50 mL of 20 μg/ml EPS in 0.01 M PBS was injected between two mica surfaces in the geometry shown in Figure 4-4 (a). During a typical normal force measurement, the two mica surfaces were brought close to reach a "hard wall," which is defined as the mica-mica separation at which the thickness of the confined polymers becomes asymptotic with increasing normal load or pressure; then the mica surfaces are separated. The normal force-distance profiles determined during the approach (■) and separation (●) are shown in Figure 4-4. The initial force measurement was taken ~5 min after injecting the solution, and an adhesion force of -0.5 mN/m was measured during the separation. Successive measurements showed the same adhesion force. The hard wall distance shifted from ~5 nm for the initial measurement to ~30 nm after about t = 3.5 hours adsorption, while the adhesion force remained almost unchanged.

The normal force-distance profiles for two mica surfaces across 0.01 M PBS buffer with 4.5% cranberry juice (no EPS presence) are shown in Figure 4-5. The hard wall distance remained unchanged (~6 nm) even after injecting the solution for 2 hours, and only repulsion forces were measured.

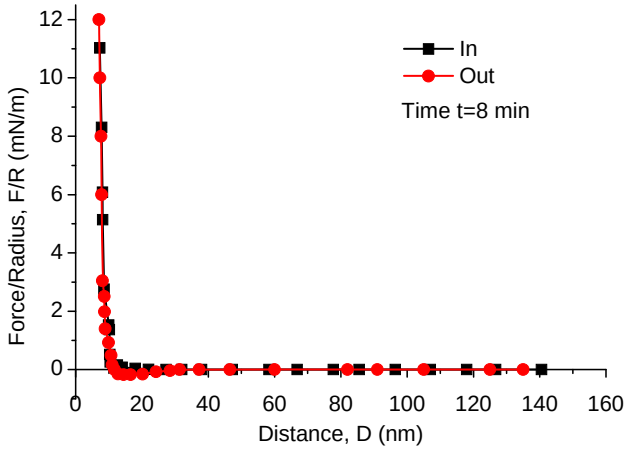


(a)

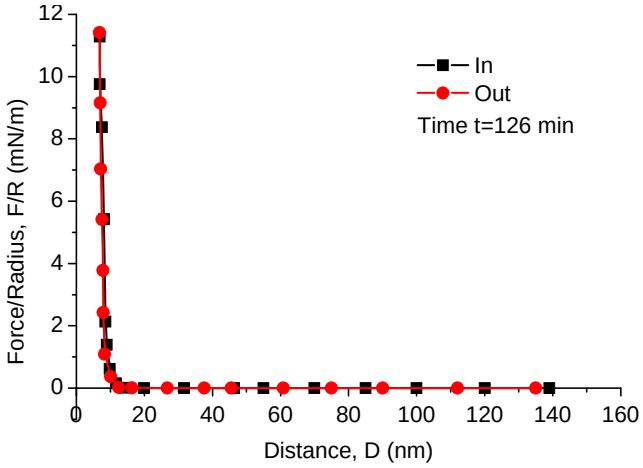


(b)

Figure 4- 4 Normal forces F (normalized by the radius of the surfaces R) measured for two approaching and separating mica surfaces as a function of surface separation D, in a 0.01 M PBS solution of EPS (20 µg/mL): (a) t = 6 min after injecting the EPS solution, and (b) t = 210 min after injecting the EPS solution.



(a)



(b)

Figure 4- 5 Normalized normal forces F/R measured on two approaching and separating mica surfaces as a function of surface separation D, in 0.01 M PBS buffer with 4.5 wt. % cranberry juice for (a) t = 8 min and (b) t = 120 min after injecting the solution.

Figure 4-6 shows the normal force-distance profile for a mixture of EPS and 4.5% cranberry juice in 0.01 M PBS. No adhesion was measured, while the hard wall distance was ~50 nm at t = 15 min, larger than the values measured for PBS solution with only EPS or cranberry juice. Evolution of the hard wall distances with time for the three different cases are summarized in Figure 4-7, which shows that the hard wall distances: (1) barely increase for PBS solution with 4.5% cranberry juice, (2) increase from ~5 to ~30 nm in 4 hours for EPS in PBS solution, and (3) increase from 50 to ~150 nm in 2 hours for EPS in PBS solution with 4.5% cranberry juice.

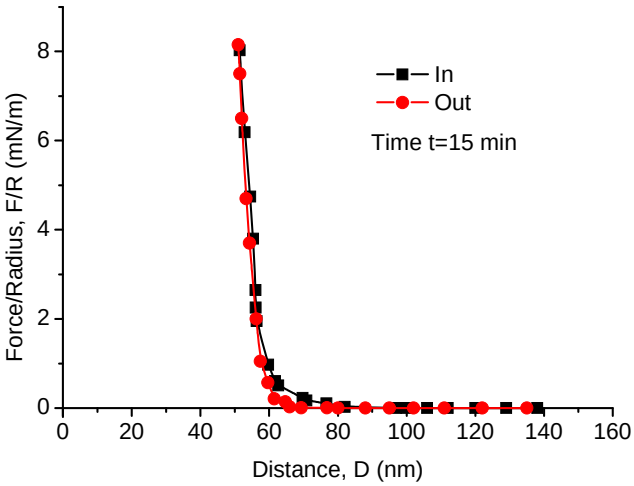


Figure 4- 6 Normalized normal forces F/R as a function of surface separation D, in a 0.01 M PBS solution of EPS with 4.5% cranberry juice 15 min after injecting the solution.

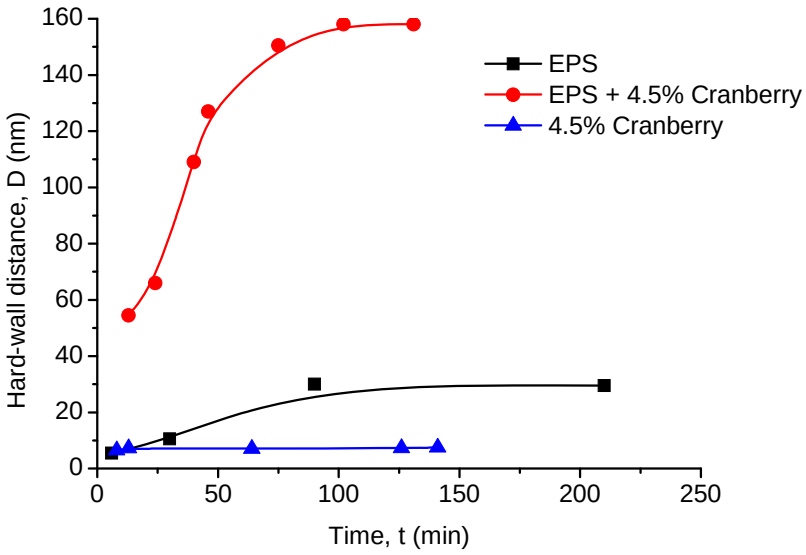


Figure 4- 7 "Hard wall" distance vs. time after solution injection between two mica surfaces (n.b. the "hard wall" is defined as the mica-mica separation at which the thickness of the confined polymers becomes asymptotic with increasing the normal load or pressure) for three different cases: EPS in 0.01 M PBS (■), EPS in 0.01 M PBS with 4.5% cranberry juice (●), and 0.01 M PBS with 4.5% cranberry juice (▲).

Chapter V Discussion

5.1 Impact of surface EPS on bacterial surface characteristics.

We tested bacteria strains with three different EPS producing capabilities and observed significant variations in surface characteristics and adhesive behaviors in the absence and presence of cranberry juice. Our results showed that in 0.01M PBS, the presence of cepacian EPS reduced the absolute values of bacterial surface charge (zeta potential) and water contact angles indicating an increase in bacterial surface hydrophicility. Variations in bacterial surface zeta potential and contact angles among different bacterial strains may be caused by the surface characteristics of cepacian EPS, which is mainly composed of neutrally charged, hydrophilic sugars (Stack, 1988; Cescutti et al., 2000). *B. cepacia* are gram-negative bacteria whose outer surface membrane is mainly composed of lipid and protein (Hancock and Nikaido, 1978). With little or no EPS coverage on cell membranes, we expected that surfaces in the mutant strains PC184*rml* and PC184*bceK* would be more negatively charged and more hydrophobic than the wild type EPS producing strain PC184.

When cranberry was introduced into the cell suspension, the wild type PC184 strain manifested the least change in cell zeta potential, and all three bacteria yielded similar zeta potentials around -5 mV. In contrast, the greatest change in water contact angle was observed in the PC184 strain, and water contact angle values of all three bacteria strains were similar in the presence of cranberry juice. The equalized zeta potentials and surface tensions of the three bacteria strains

may be explained by the adsorption of cranberry components onto bacterial membranes and EPS. The changes in bacterial surface characteristics can further be explained by interactions between EPS and cranberry components as discussed in the following sections.

5.2 Impact of cranberry components on bacterial surface EPS.

The adsorption of cranberry components on bacterial surface EPS may have caused the bacterial surface characteristics and adhesive capacity changes observed in our experiments. Previous studies showed that proanthocyanidins (PACs) from cranberry had a binding affinity to bacterial lipopolysaccharide (LPS) (Delehanty et al., 2007; Johnson et al., 2008), however, little information is available on cranberry adsorption on bacterial EPS. Our SFA force measurements showed that the bridging force of bacterial EPS was attenuated in the presence of cranberry juice. We propose that these effects were attributed to the binding of components in cranberry juice to bacterial EPS. The hydrophobicity of the EPS is expected to change on binding with cranberry components and the polarity of the ‘‘modified’’ EPS would change as well, resulting in a decrease in adhesive forces between the solid surface and bacterial cells. The PC184 bacteria surface was then observed with SEM after treatments with 0.01 M PBS and cranberry juice. As shown in Figure 4-3, these SEM images offered direct evidence that cranberry components were able to absorb on the surface of PC184 EPS. To evaluate the impact of cranberry adsorption on

bacterial adhesion, bacterial cells were treated with cranberry juice for 2 hours, washed with 0.01M PBS, and resuspended in PBS for the flow cell adhesion study (results showed in Table 5-1). A 2 log unit decrease in bacteria adherence to polycarbonate coupons was observed for bacteria treated with cranberry juice compared to untreated bacteria, indicating that cranberry components were retained on the treated bacteria and thus were positioned to inhibit bacterial adhesion to the polycarbonate surface.

Table 5- 1 Adhesion results of PC184 treated with and without 18 wt. % cranberry juice

	Number of bacteria attached to the polycarbonate surfaces in flow cell (CFU/ml)
PC184 in 0.01M PBS	$6.7\pm1.5\times10^{5}$
PC184 treated by 18 wt. % cranberry juice and resuspended in 0.01M PBS	$1.3\pm0.6\times10^{3}$

Cranberry juice may reduce bacterial adhesive capacity by altering EPS conformation. EPS are composed of highly hydrophilic monosaccharides. Adsorption of cranberry components to these molecules might disrupt hydrophilic interactions, changing EPS length or folds, and thus reducing the affinity between cell and polycarbonate surfaces. Previous work on *E. coli* fimbriae revealed that exposure to cranberry juice resulted in a more compressed bacterial fimbriae (Liu et al., 2006).

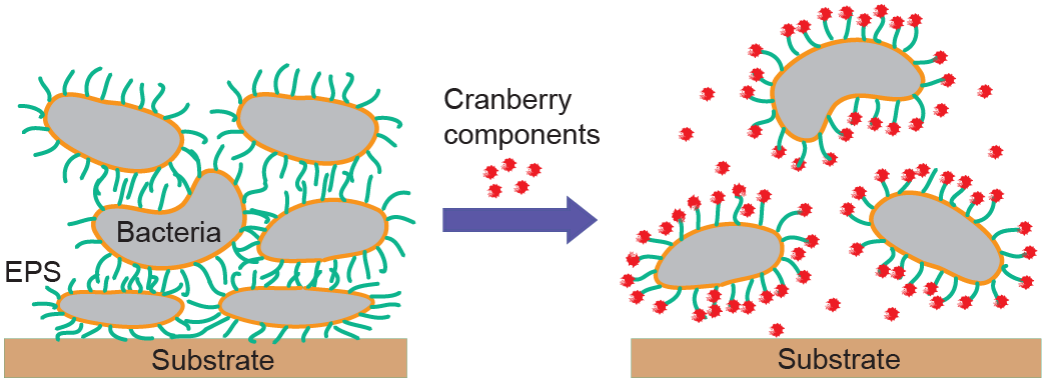


Figure 5- 1 Illustration of how cranberry components affect and reduce bacteria adhesion by attaching to the bacterial EPS.

Cranberry components may also change bacterial adhesive capacity by removing EPS from the bacteria cell surface or by inhibiting EPS production. Although no literature is available on bacterial EPS removal, cranberry components have been found to inhibit the production and catalytic activity of matrix metalloproteinases (Ahuja et al., 1998) and to genetically reduce fimbria production (La et al., 2009). To test the effect of cranberry juice on EPS density, we extracted PC184 EPS before and after 2 hour incubation in 27 wt. % cranberry juice and measured the mass (protein contents displayed in Figure 5-2); there was no significant difference in mass values before and after cranberry juice treatment. As EPS was not removed from the bacterial surface during a 2 hour exposure to 27 wt. % cranberry juice, it was concluded that inhibition of bacteria/polycarbonate adhesion observed in the presence of cranberry juice was due to other interactions between cranberry components and bacterial EPS.

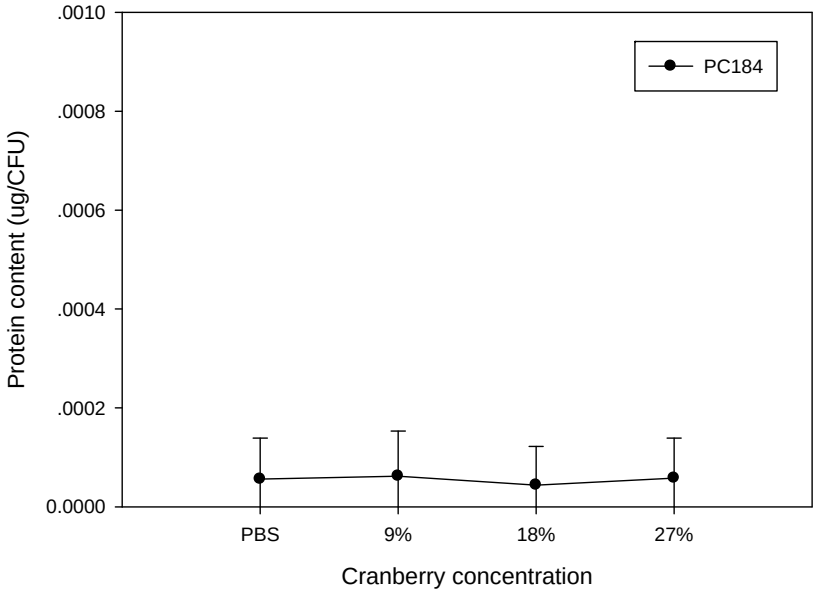


Figure 5- 2 Protein contents of EPS extracted from PC184 treated with and without 18 wt. % cranberry juice.

5.3 DLVO theory and bacterial adhesion.

DLVO (Derjaguin, Landau, Verwey, Overbeek) theory describes the force between charged surfaces interacting through a liquid medium. It combines the effects of van der Waals attractive forces and electrostatic repulsion (Bhattacharjee et al., 1998; Hermansson, 1999; de Kerchove and Elimelech, 2005). According to DLVO theory, less negatively charged bacterial cells are more prone than more negatively charged cells to attach to a negatively charged polycarbonate surface (Kim et al., 1997) due to the long-range repulsion between bacterial cells and the polycarbonate surface. In our study, the surface charges of

all bacteria cells became less negatively charged in the presence of cranberry juice. However, the adhesive capacity of these bacterial cells was impaired in the presence of cranberry juice, indicating DLVO theory cannot be used alone to explain the observed bacterial adhesion.

Extended DLVO theory, which was developed to account for surface hydrophobicity when predicting the forces between two surfaces (Hermansson, 1999; de Kerchove and Elimelech, 2005), also failed to explain our results. Cranberry juice increased all three bacteria water contact angles indicating an increased bacterial surface hydrophobicity. The increased bacteria hydrophobicity was expected to facilitate cell adhesion to polycarbonate (water contact angle 62.61º); however, weaker adhesion was observed in experiments with PC184 and PC184*rml* bacteria.

Thus, it can be concluded that the adhesion of PC184 and its mutants was governed at least partially by one kind of non-DLVO mechanism (de Kerchove and Elimelech, 2005; Liu et al., 2007). Non-DLVO interactions that may govern bacterial adhesion including electron-donor and electron-acceptor forces between polar molecules (van Oss, 1994); steric repulsion and bridging interactions between polymers (Netz and Andelman, 2003; Huang and Ruckenstein, 2004) may be stronger than DLVO forces (Lee and Belfort, 1989) and hence may affect bacteria adhesion.

5.4 Thermodynamic model and bacterial adhesion.

We applied the van Oss-Chaudhury-Good thermodynamic approach to model bacteria and solid surface interactions. A thermodynamic model of interactions in aqueous systems offers a straightforward examination of the cell surface adhesion energy change, and allows total energy to be decoupled into several component parts. The thermodynamic model has been used successfully by other researchers to predict nonspecific adhesion between bacterial cells and biotic and abiotic surfaces (Burgers et al., 2009). In the present study, cranberry treatment significantly reduced the adhesive capacity of EPS producing bacteria, while the adhesive capacity of mutants whose EPS were removed or reduced was only moderately reduced or not affected by cranberry juice. Our results showed that Gibbs free energy ΔG_{adh} increased to values close to zero and became positive at the cranberry concentration of 18 wt.% for PC184. For both PC184*rml* and PC184*bceK*, ΔG_{adh} values remained positive with little variation at all cranberry juice concentrations tested.

Agreement between flow cell studies and thermodynamic model predictions were generally good. However, there were quantitative discrepancies between Gibbs free energy values and observed adhesion phenomena. For instance, we predicted positive ΔG_{adh} values for mutant bacterial adhesion but observed a low extent of mutant adhesion. Similar results have been reported in other studies (Vadillo-Rodriguez et al., 2005). In part, this observation can be attributed to inherent physical and chemical/charge heterogeneity of bacterial and solid

surfaces, and the complex nature of bacteria-surface interactions which are not fully accounted for in the thermodynamic model (Chen and Strevett, 2003). Bacterial deposition may occur preferentially on energetically favorable sites (Gregory and Wishart, 1980), resulting in bacterial deposition even though the total energy change indicates unfavorable adhesion. Also, bacteria may attach reversibly to a surface at the secondary energy minimum, as discussed in previous studies (Tufenkji and Elimelech, 2005; Tufenkji, 2007). The thermodynamic prediction of adhesion potential based on physicochemical properties gives useful information about the possible real-life microbial behavior. However, mechanisms other than cellular physicochemical surface properties, such as the influence of fimbrae and production of EPS (Flint et al., 1997; Donlan, 2002), may determine bacterial adherence. Thermodynamic modeling only focuses on the initial and final status of cellular physicochemical surface tensions, offering no information about the actual process. In addition, bacteria may encounter an insurmountable energy barrier when approaching a solid surface (Ong et al., 1999), and may not be able to bind although the total energy change indicates preferable adhesion.

5.5 Implications of SFA adhesion measurement.

Adhesion forces were measured during the separation of two mica surfaces in EPS solution (Figure 4-4). The adhesion arose from bridging interactions between the two mica surfaces due to the adsorption of EPS; this is supported by

the sustained adhesion associated with increased hard wall distance with longer adsorption time. The SFA measurement shows quantitatively that the normalized adhesion between the EPS of PC184 in 0.01 M PBS buffer is F_{ad}/R ~-0.5 mN/m, or the adhesion energy $W_{ad} = F_{ad}/1.5\pi R$ ~ -0.11 mJ/m^2. This value is close to the adhesion energy measured using atomic force microscopy (AFM) between EPS from *P. atlantica* cells and polymer membrane surfaces such as polyvinylidene fluoride, regenerated cellulose, and polyethersulfone (Fang et al., 2000; Frank and Belfort, 2003; Li and Logan, 2004).

Cranberry juice components do not have the same bridging or adhesive capabilities as EPS polymer, as shown in Figure 4-5. The small hard wall distance (~6 nm) remained unchanged with interaction time due to the confinement of EPS polymer between the two mica surfaces as previously observed for many other polymers (Israelachvili, 1992; Leckband and Israelachvili, 2001; Zeng et al., 2007; Zeng et al., 2007). Figure 4-5 shows that the bridging adhesion forces of EPS can be attenuated by addition of cranberry juice to the PBS solution. This demonstrates that cranberry juice reduces or prevents the initial adhesion of EPS and blocks biofilm formation. The larger hard wall distance for the PBS solution with EPS and cranberry juice (Figures 4-6 and 4-7) implies that EPS and cranberry juice components form aggregates. The aggregates were confined between the two mica surfaces during the approaching process but showed no adhesive capability, as previously observed for nanoparticles using an SFA (Akbulut et al., 2007; Min et al., 2008). The SEM images in Figure 4-3 show the changes in bacterial surface morphology

before and after interacting with cranberry juice. The bacterial surfaces became much rougher after interacting with the cranberry solution; this observation agreed with SFA measurements.

As illustrated in Figure 5-1, EPS mediate initial bacterial attachments to substrates or tissue surfaces and subsequent biofilm formation since they present at the outermost layer of the cell. Contact angle measurements, SEM, and SFA results were consistent with each other, and suggested that the binding of cranberry juice components to bacterial EPS blocked bacterial biofilm formation. Cranberry juice components bound to the outermost EPS layer of the bacterial cell forming a thin coating that showed no bridging adhesive capability according to SFA measurements. This coating significantly reduced the adhesion of bacteria cells to polycarbonate surfaces and prevented the formation of biofilm. Our study probed the adhesion and surface interactions of bacteria and EPS and tested the impact of cranberry juice on the initial nanoscale adhesion of *B. cepacia*. The results obtained in this study provide an insight into the development of novel treatment strategies to block the biofilm formation associated with bacterial infection.

Chapter VI Summary and Recommendations for Future Research

In this thesis, studies were carried out to investigate the impact of cranberry juice on the adhesion of EPS producing bacteria *B. cepacia*, with a quantitative explanation for the role of cranberry juice in disrupting the binding between *B. cepacia* EPS and polycarbonate surfaces through a thermodynamic model and SFA measurements.

The adhesion experiment conducted in a flow cell revealed the effects of cranberry juice on *B. cepacia* adhesion capacity directly, validating our hypothesis on the anti-adhesive effect of cranberry on bacteria EPS. Further investigations on bacterial surface properties yielded significant changes in zeta potential and contact angle values of *B. cepacia* PC184, while those properties remained relatively constant for the PC184 mutants with a deficiency in EPS biosynthesis. Resorting to the thermodynamic model, the changes in bacteria surface properties were correlated with the bacterial adhesion process, and the effect of cranberry juice on preventing bacterial adhesion was estimated quantitatively. At last, in order to explore the actual impact between cranberry juice and bacteria EPS, SEM imaging and SFA measurement were introduced to make a thorough probe into the interactions between them. All experimental results were compiled together and analyzed to conclude the adsorption of cranberry on EPS and adhesion capacity debase.

Another achievement in this thesis was attributed to the set up of the thermodynamic model – SFA measurement system by which we could gain fundamental knowledge towards understanding on how certain factors prevents bacterial adhesion to surfaces. Our work provides evidence of the biophysical effects of cranberry juice on bacteria EPS–polycarbonate interactions, and this system can be adopted to investigate the effect of various factors (active compounds, ionic strength, pH, flow rates) on the bacterial adhesion in aqueous solution.

The work in this thesis points to several directions that deserve further investigation. The observations from this study are based on the model bacteria *B. cepacia*, which represents common Gram-negative bacteria, but may not present all bacteria. Future work can be done to investigate other EPS producing microorganisms, such as *P. aeruginosa*.

Another challenge facing the application of cranberry juice in various environmental systems is the lack of understanding on the active anti-adhesive compounds in the cranberry juices. It would be important to identify these active compounds in the cranberry juices, and identify the critical dosage necessary to impart anti-adhesive effects.

For further work, it would be interesting to explore the nature of the interaction or absorption between bacteria EPS and cranberry components. According to our results, the interactions between cranberry juice and EPS seemed strong and rapid, indicating the anti-adhesive effect acted immediately in the presence of cranberry juices. However, the detailed chemical bonds for the cranberry-EPS

interactions are still unclear, hindering the further exploration and application of the active components in cranberry juices or its analogs. A thorough understanding of the chemical nature of the interaction will assist to explore the use of cranberry juice in preventing formation or removing biofilm on various materials.

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