COMMITTEE MEMBERSHIP

TITLE: Analysis of Biofilm Remediation Capacity for Octenyl Succinic Anhydride (OSA), a Bioactive Food Starch Modifier Compound

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DATE SUBMITTED: June 2020

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ABSTRACT

Analysis of Biofilm Remediation Capacity for Octenyl Succinic Anhydride (OSA), a Bioactive Food Starch Modifier Compound

Matthew R. Borglin

This thesis demonstrates efficacy of Octenyl Succinic Anhydride (OSA), as a biofilm sanitizer. Biofilms allow bacteria to adhere to solid surfaces with the use of excreted polymeric compounds. For example, surfaces found in food production or processing facilities such as the interior of a raw milk holding tank, are some of the most susceptible to biofilm contamination. When present, biofilms can cause a variety of negative effects, which include; reduction of product shelf life, corrosion, and outbreaks of foodborne illnesses. The close association of biofilms with the majority of foodborne illness cases led the US Environmental Protection Agency (EPA) to create a new category of sanitizer specifically designed for treatment of mature biofilms. The efficacy of sanitizers in this new regulatory category is determined by the EPA protocols MB19 and MB-20. The EPA’s protocols outline methods for cultivating, treating, and measuring effects on Pseudomonas aeruginosa biofilms in a continuous flow stir bar bioreactor. Biofilm modification by OSA was verified by the presence of octenyl esters on OSA treated biofilms with single point Raman spectrophotometry. OSA modified biofilm's antimicrobial properties were first investigated with crystal violet staining in 96-well microtiter plates with inconclusive results. However, effective antimicrobial properties where apparent when using the CDC Biofilm Reactor. OSA treatments consistently returned a 6-log CFU/coupon reduction in biomass compared to controls. Inhibition of planktonic and/or biofilm regrowth was demonstrated using the 96-well plate methodology. This thesis demonstrated the effectiveness of OSA chemical esterification reaction as a biofilm treatment. In doing so, this work suggests a new approach for biofilm remediation by chemically modifying the structural components of biofilm.

Keywords: Biofilm, Sanitization, Octenyl Succinic Anhydride (OSA), Extracellular Polymeric Substances (EPS), Food Starch, Environmental Protection Agency (EPA), Chemical Modification
ACKNOWLEDGMENTS

I would like to acknowledge Baker Koob Endowment, Sigma Xi, and Cal Poly Bio Department for their financial support for this project. Also, to Pancho Ibarra for help in developing this idea, and Jason Lorenz for the hours of help in the laboratory.
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1.1 Biofilm History and Definition

Microorganisms were noticed in the aggregated biofilm state near the origin of the microscopic study of life by Antonie Von Leeuwenhoek in 1684. Von Leeuwenhoek wrote to the Royal Society of London describing the "microbial aggregates" he observed from scraping samples off his teeth. However, it was not until far more recently that Bill Costerton coined the term "Biofilm" in 1978 (Chandki, Banthia, & Banthia, 2011). Scientists and society are now facing a paradigm shift in our understanding of how microorganisms interact with the physical world. For instance, it was ignorantly agreed upon that the bacterial world consisted of isolated cellular units in products, environment, and in laboratories. Currently, it is well understood that microorganisms, especially bacteria, tend to live and grow in a general aggregate phase of biofilms, also known as flocs (free floating "planktonic" biofilms), or sludge. All of these denotations are generally referred to as "biofilm" (Fridjonsson et al., 2011; Heydorn et al., 2000). In 2012, the word biofilm was officially defined by International Union of Pure and Applied Chemistry (IUPAC) as ‘aggregates of microorganisms in which cells are generally embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface’ (Teh, Flint, Brooks, & Knight, 2015).

1.2 Biofilm Lifecycle

Biofilm formation is a complex process initiated when a free-floating microorganism contacts a surface. Contact occurs in any liquid-solid interface in which nutrients are available, but adhesion occurs more frequently if the solid interface is rough, scratched, cracked, or corroded (Mortensen, 2014). Additionally, various physical conditions influence the initial adhesion of bacteria such as the substrate’s pH,
electrolyte concentration, and surface ionic charge. Regardless of the physical properties of the substrate and the environment’s properties, attachment happens in a series of defined steps.

As the bacterial cell approaches the substrate, it is hit with a variety of nonspecific physiochemical forces such as Lewis acid-base, Van der Waals, and electrostatic forces, which in combination, create a net force promoting adhesion to the surface (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014a). During the process of adhesion, the bacterial cell initiates an enormous suite of genetic alterations converting the cell from the planktonic to the biofilm associate state. These genetic alterations begin once the first point of contact is made by surface proteins or in some cases proteinoid appendages such as flagella. These contact points serve as operative signals, for a series of signaling cascades resulting in changes to regulation for roughly 40% of the bacterial genome (Cunningham, Lennox, & Ross, 2010; Fridjonsson et al., 2011; Karatan & Watnick, 2009). Though no genes are altered, they undergo a dramatic shift in regulation; so much so that the two bacterial life states, planktonic and biofilm, can be looked at as entirely different organisms. This transformation occurs during the initial attachment process and includes morphological changes through down regulation of genes for structures that are no longer needed such as flagella, and upregulation of membrane proteins that secrete supporting exopolymers (Cunningham et al., 2010; Mortensen, 2014).

After the initial bacterial attachment, maturation into a multilayered biofilm is a fast process involving the replication of cells and secretion of polymeric material or EPS which binds the cellular colony more securely to the solid surface. This also allows for capturing of organic and inorganic material in a process called conditioning (Mortensen, 2014). The polymeric material that forms the EPS encases the overall structure, as well
as providing a complex architecture complete with channels, in which the bacterial community can share resources and information (Donlan & William Costerton, 2002). EPS plays many very important roles, but a significant role in the early stages is its alteration of the physio-electric properties of the substrate and biofilm surface. These alterations create higher net attraction forces (electrostatic and van der Waals) and in turn allow for greater planktonic microbial adhesion (H. C. Flemming et al., 2016). Newly added microorganisms then contribute to the existing EPS to make a three-dimensional matrix of organic and inorganic components (Mortensen, 2014).

The last step in the biofilm life cycle is dispersion, in which cells leave the community and repeat the process of adhesion on another surface. This process of dispersion is mainly influenced by environmental events such as a decrease in resource availability or another type of stress condition (Abdallah et al., 2014a).

Over the past decade more of the biofilm life cycle has been defined and it is now understood as a dynamic process greatly influenced by environmental conditions such as pH, nutrients, temperature, and shear force among other factors (Karatan & Watnick, 2009).

1.3 Biofilm Composition

Biofilm morphology is dynamic and depends on the life cycle stage, bacterial composition, and environmental properties. Biofilms have been described as smooth, flat, fluffy, rough, filamentous, mushroom-like, and varying in degree of porosity (H. C. Flemming & Wingender, 2010). The vast array of environments that biofilms are found in create morphologically unique structures, but all are composed of a community of microorganisms (unicellular, eukaryotic and prokaryotic), EPS, multivalent cations, biogenic and inorganic particles (Donlan, 2002; H.-C. Flemming, Wingender, Griebe, & Mayer, 2000). On average the microcolonies of bacterial cells within a biofilm can take
upwards of 20% of its dry mass. The rest of the biofilm’s weight is made up of a nonliving matrix that the embedded organisms produce or capture from the environment (Socransky & Haffajee, 2002). Most mature biofilms are visible to the naked eye and can be described as a bacterial paste or sludge. However, the morphology of the biofilm is complex and variable depending on the environmental conditions present during its formation. Conditions such as shear force can influence the overall structure. Low shear force will cause a towering mushroom-like structure, while high shear-force will result in elongated structures with microcolonies appearing to resemble rolling mounds on the surface (H. C. Flemming & Wingender, 2010). Additionally, the biofilm’s inhabitants play a role in influencing the degree of porosity, charge, and concentration of various polymeric substances. Despite the morphological differences, all biofilms serve to hold fast a diverse microbiota and promote their interactions in a small-scale community (Socransky & Haffajee, 2002).

1.4 Quorum Sensing

For a biofilm community of either monoculture or diverse bacteria to work, a means to coordinate cellular activity is required. Over the past forty years scientists have published a great deal of research on cell-to-cell signaling pathways utilizing low molecular weight compounds that can easily diffuse through cell membranes and are collectively known as autoinducers (Galié, García-Gutiérrez, Miguélez, Villar, & Lombó, 2018; Kim, Lee, Byun, & Park, 2015). Autoinducers are constantly produced by bacteria, but in the planktonic state these signaling molecules cannot accumulate to a high enough concentration to be effective. However, in an aggregated state such as a biofilm, the concentration of autoinducers can become high enough for a regulatory effect, which is essential for biofilm success. This type of signaling, called quorum sensing (QS), is widespread in bacteria (Cunningham et al., 2010; Galié et al., 2018).
QS molecules are categorized as either specific or generic: specific QS molecules are used only by the same species, and generic QS are capable of being used by other species. Both categories of QS molecules are utilized to induce cellular activities within biofilm communities allowing the coordination required to perform vital functions. These functions include maintaining water and nutrient channels, growing in a certain direction, or producing protective and toxic compounds in response to environmental factors (Cunningham et al., 2010). The importance of QS molecules is a constant focus for the research community, investigating the control of biofilm formation. Current QS related strategies for controlling biofilm formation include outcompeting signaling molecules by flooding the biofilm with competitive binding inhibitors, enzymatically degrading QS molecules, stopping the translation of the QS cascade using ribosomal binding sRNA, and/or preventing QS synthesis (Galié et al., 2018; Pérez-Martínez & Haas, 2011; Rasmussen et al., 2000).

1.5 Model Biofilm Species *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an opportunistic human pathogen posing a significant problem in industry and receiving a great deal of scientific attention. Clinically, *P. aeruginosa* is associated with the genetic lung deteriorating disease known as cystic fibrosis (Donlan & William Costerton, 2002). Additionally, *P. aeruginosa* is commonly found contaminating medical device equipment (Mann & Wozniak, 2012). As a common model organism for biofilms, *P. aeruginosa* has helped our understanding of biofilm attachment, life cycle, and the important role EPS plays in biofilm functions (Borlee et al., 2010; Colvin et al., 2012; Klausen et al., 2003; Nivens, Ohman, Williams, & Franklin, 2001; Passos da Silva et al., 2019; Reichhardt, Wong, da Silva, Wozniak, & Parsek, 2018; Yang et al., 2007).
1.6 Biofilm Resistance to Environmental Stressors

Defensive strategies used among microorganisms in a planktonic state are overshadowed by the benefits of the biofilm’s protective matrix. For example, studies comparing antibiotic susceptibility between planktonic and biofilm *Pseudomonas aeruginosa* phenotypes found a large increase in antibiotic resistance when in a biofilm state. For example, *P. aeruginosa* treated with an antibiotic called Imipenem, has an Minimum Inhibitory Concentration (MIC) of 1 ug/ml in the planktonic form, but increased to greater than 1,024 ug/ml MIC once in a biofilm (Ceri et al., 1999). The mechanisms behind a biofilm’s enhanced resistance include delaying antimicrobial penetration, and removal of antimicrobial targets through the altered life cycle and physiological properties of the encased bacteria (Donlan & William Costerton, 2002). The delay of penetration results from the EPS acting as a diffusion layer, protecting the cells by reducing the reactive properties of the antimicrobial agents or encouraging reactions with non-cellular components of the biofilm (Socransky & Haffajee, 2002). With both time and a high concentration, antimicrobial agents are still able to reach bacterial cells. However, uptake of antimicrobial agents is greatly reduced in biofilm-associated cells due to their significantly slower growth rate compared to a planktonic phenotype. Slower bacterial cell growth is one of the physiological stress responses invoked by the lack of oxygen and nutrients in the biofilm depths (Donlan & William Costerton, 2002). Through these mechanisms and others not mentioned, biofilm-associated bacteria are more resistant to environmental toxic substances than planktonic microorganisms (Chandki et al., 2011).
1.7 EPS Components

The biofilm EPS is a mix of excreted biopolymers primarily comprised of a suite of proteins, lipids, DNA, and polysaccharides (Seviour et al., 2019). Exopolymers make up the majority of the mass in biofilms, and are responsible for the morphology, integrity, and physiochemical properties of the bacterial communities (H.-C. Flemming et al., 2000). The wide array of environmental biofilm communities display diversity in the specificity and nature of the individual components of the EPS to meet the needs of their life-cycle stage within their given environment. Despite differences in exopolymers, the particular roles of EPS constituents are conserved across biofilms. For example, all biofilms utilize exoenzymes in the EPS for specific functions, such as digestion of exogenous macromolecules for nutrients and degradation of EPS for cell dispersal (H. C. Flemming & Wingender, 2010). Additionally, all biofilms utilize exopolysaccharides for key functions including but not limited to: surface adhesion, cell-to-cell adhesion, mechanical stability, water retention, protection, and absorption (H. C. Flemming & Wingender, 2010).

Biofilm exopolysaccharides are an essential component of the EPS due to their use for substrate adhesion, structural stability, functionality, formation, and antimicrobial resistance (H. C. Flemming & Wingender, 2010). The utilization of polysaccharides for these key functions is ubiquitously confirmed via electron microscopy and biochemical analyses across a wide range of environments (J. P. Kamerling, 2007). Exopolysaccharides are long and can be either the same repeating sugar-derived monomer leading to a linear structure, or branching polymers made from different repeating monomers. The majority of EPS-exopolysaccharides are comprised of branched heteropolysaccharides containing neutral and charged sugars (H. C. Flemming & Wingender, 2010). Heteropolysaccharides contain organic and inorganic components giving the diverse biological and physical properties needed for their EPS functions.
(Lembre, Lorentz, & Di, 2012). For example, polycationic heteropolysaccharides such as β-1,6-linked N-acetylglucosamine (Figure 1), are essential for cell-to-cell adhesion and early life-cycle stages of some biofilms (Göttz, 2002).

**Figure 1: Structure of PIA**, a positively charged linear homoglycan composed of N-acetylglucosamine/glucosamine residues (Lembre et al., 2012).

A model organism for biofilm research, *P. aeruginosa*, when placed in different environments will produce diverse three distinct exopolysaccharides groups: Alginates, mannose-rich polymers named Psl, and glucose-rich polymers named Pel (Mann & Wozniak, 2012). Alginates (Figure 2) are comprised of sugar monomers known as Uronic acid residues, which are of high-molecular weight, with functional properties dependent on the ratio of two non-repetitive monomers: Beta-1,4 linked L-guluronic(G) and D-mannuronic acids(M) (Ryder, Byrd, & Wozniak, 2007). For example, high monomer alginates found in *P. aeruginosa* are used for adhesion and structure (Boyd & Chakrabarty, 1995) An overexpression of alginate drastically alters the architecture and morphology of a biofilm, making it more resistant to antimicrobials. Alternatively, inhibiting Alginate polymers does not prevent formation of a mature biofilm (Nivens et al., 2001). When removed, the Alginate functions are taken over by mannose-rich polymers named Psl, short for polysaccharide synthesis locus (Ryder et al., 2007). The mannose rich Psl polymer is a neutral penta-saccharide subunit containing mannose, rhamnose and glucose units in a 3:1:1 ratio (Passos da Silva et al., 2019). When present, Psl
appears throughout the biofilm, and is important for maintenance of the biofilm’s post attachment structure through contributing to cell-to-surface, and cell-to-cell interactions (Ma, Jackson, Landry, Parsek, & Wozniak, 2006; Matsukawa & Greenberg, 2004). When biofilms form in liquid-air interfaces on structures called pellicles, they will contain very little Psl and a large portion of glucose rich polymer group appropriately name Pel (short for pellicle) (H. C. Flemming & Wingender, 2010). Generally, the Pel polymers are only found in association with pellicle biofilm growth, Pel and Psl exopolysaccharide groups both contribute in similar functions; therefore, they are generally not present together in the same biofilm (Lembre et al., 2012). All of these polymer groups rely on polycationic heteropolysaccharides know as polysaccharide intercellular adhesion (PIA); named after their function in cell-to-cell interactions. PIA (Figure 1), is observed in all biofilm types and is composed of β-1,6-linked N-acetylglucosamines with partly deacetylated residues. Bacteria in the biofilm are embedded in PIA which helps protect the individual cell and allows for interactions with other cells and other exopolymers of the EPS such as proteins (Götz, 2002).

**Figure 2: Structure of Alginate** exopolysaccharide found within a biofilm. The two nonrepetitive monomers are shown with Beta-1,4 linked L-guluronic (labeled G) and Dmannuronic acids (Labeled M) (Lembre et al., 2012)

Biofilm-associated proteins (BAPs) in the EPS provide a variety of key functions exopolysaccharides cannot perform. BAPs function in planktonic cell recruitment, biofilm
structural maintenance, and exopolysaccharide bridging (Cucarella et al., 2001). For example, a well-studied matrix protein called CdrA, present on bacteria in the planktonic cell, will bind to Psl exopolysaccharide, aiding in cell recruitment into the biofilm (Borlee et al., 2010). Additionally, the CdrA matrix protein aids cell-to-cell adhesion, which promotes biofilm formation and can lead to a proteinoid biofilm matrix devoid of polysaccharides (Reichhardt et al., 2018). Other important BAPs are extracellular carbohydrate-binding proteins called lectins, which stabilize the polysaccharide matrix and provide links between bacterial surfaces and extracellular EPS (Passos da Silva et al., 2019). Alongside Lectins and matrix proteins, large proteinaceous appendages such as pili, fimbriae, and flagella act as structural elements within the matrix by cross-linking with exopolysaccharides, creating a rigid hydrophobic EPS (Zogaj, Nimtz, Rohde, Bokranz, & Römling, 2001).

Along with structural BAPs, there are a wide range of extracellular enzymes found in the EPS. Exoenzymes are responsible for nutrient acquisition through degradation of environmental macromolecules, and the breakdown of EPS biopolymers (Xiaoqi Zhang & Bishop, 2003). EPS degradation by matrix bound enzymes allows for both the dispersion step in the biofilm life cycle, and a biofilm to serve as a form of extracellular nutrient storage (H. C. Flemming & Wingender, 2010).
Chapter 2
SIGNIFICANCE OF BIOFILMS

2.1 Humans and Biofilms

The vast majority of biofilms are not directly associated with humans, but rather live in all walks of life. Biofilms can be found in pristine alpine lakes, and as the first colonizers of deep hydrothermal vents. They serve an important role in plant-microbe relationships in the rhizosphere, and the base of food chains in the harsh arctic. It has been suggested that greater than 98% of bacteria live in biofilms, roughly the equivalent of more than 50% of the earth’s entire biomass (Reid, Dupraz, Visscher, & Sumner, 2003).

Once in biofilm, bacteria are phenotypically more resistant to environmental stressors, such as our cleaning efforts, than in their free-floating state (Donlan & William Costerton, 2002). This raises critical issues when biofilms grow on industry surfaces, which provide agreeable environments for attachment and growth (Simões, Simões, & Vieira, 2010). For example, biofilms reduce flow and corrode industrial pipelines, which is believed to have cost the US half a trillion dollars each year fighting biofilm-related contamination (de Carvalho, 2018; O’Toole, 2002). The U.S. Centers for Disease Control and Prevention (CDC) approximated the annual cost that the United States pays for biofilm related foodborne illness to be 21.6 billion dollars (Han et al., 2017; Minor et al., 2015).

Within the past century high-volume food production factories have introduced a large array of surfaces for bacterial contamination and biofilm formation to occur. Food production facilities are designed for an increase in food production to support the continually increasing world population (Everts, 2014). To accommodate this demand, a shift from local suppliers to specialty factories was necessary. However, these facilities
provided a substrate with a constant supply of nutrients ideal for biofilms and their potentially pathogenic inhabitants. Food safety concerns from US citizens and government officials started to rise as pathogenic bacteria such as Listeria started to be recorded in our food products. These concerns were the beginning of our now extensive sanitization regulations on food preparation and distribution by governmental agencies, like the FDA (Holbrook, 2009). Despite regulatory efforts, bacterial contamination still occurs. The most persistent contamination is from biofilm-associated bacteria as a biofilm increases physical, mechanical, and chemical resistance, ultimately allowing bacteria to survive sanitization processes (Galié et al., 2018).

2.2 Importance of Surface Materials in Biofilm Formation

The type of material and the state of surface roughness can be the most influential factor contributing to attachment and formation of biofilm in food processing plants. Industrial surfaces include stainless steel, glass, rubber, polyurethane, Teflon, nitrile butyl rubber, and/or wood. Materials of choice depend on the type of food produced and the level of money invested (Srey, Jahid, & Ha, 2013). Of these surfaces, wood tends to accumulate the most biofilm due to its highly porous and absorbent properties. In contrast glass is a preferred contact surface for its corrosion resistant properties (Chia, Goulter, McMeekin, Dykes, & Fegan, 2009). Stainless steel is commonly used and contains desirable impact resistant qualities as well as hygienic welding, joints, and cornering. Despite these positive traits, stainless steel equipment is vulnerable to corrosion, which increases surface roughness, and thus increases bacterial adhesion (Howell & Behrends, 2006).

For food producing facilities, biofilm contamination raises many concerns. Of great concern are the human pathogenic biofilm-forming species. Examples include: *Bacillus cereus* a diarrhea and vomit inducer, *Listeria monocytogenes* which causes health
complications such as abortion, and the deadly *Salmonella enterica* (Galié et al., 2018). These species attach and grow readily on industrial steel, polyethylene, wood, glass, polypropylene, and rubber surfaces. Another concern for food facilities is non-pathogenic biofilms growing on surfaces due to the fact that they harbor pathogenic bacteria and lead to corrosion (Abdallah, Benoliel, Drider, Dholster, & Chihib, 2014b). Damage of surfaces and products from biofilms is of particular interest in facilities with tight profit margins. Facilities such as dairies have to balance the retention of microorganisms that make their products, and removal of problematic biofilms from milk tanks, pipelines, pasteurizers and packaging tools (Galié et al., 2018).

### 2.3 Food Safety Modernization Act (FSMA)

The FDA’s Food Safety Modernization Act (FSMA) was signed into law in response to the Centers for Disease Control and Prevention 2011 report showing the impact food borne illness had on the American population. According to the report, 48 million Americans became sick, 128,000 were hospitalized, and 3,000 died each year from food borne illnesses alone (Del Portal & Karras, 2013; FDA, 2011). FSMA is the current regulatory act that governs the food production industry and encompasses the FDA’s four priorities; prevention, inspection and compliance, response, and imports (FDA, 2011). This allows the FDA through FSMA to focus on food born disease prevention when selling local, national, and/or international foods, by providing authority to enforce these new laws (Reader et al., 2002). Within the new preventative approach, the FDA is requiring all qualified food manufactures to create food safety plan which include formalizing safety plans for prevention, processing, sanitation, allergens, and supply chains as well as a full hazard analysis. (Food Safety Plan, 2015).
2.4 Biofilms in Dairy Processing Plants

Much like other industries, biofilms in the dairy industry are of rising concern. Globally, the dairy industry produces a range of perishable foods, such as milk and cream, nonperishables such as cheese and butter, and food ingredients, such as milk powders and whey protein concentrates (Teh et al., 2015). The concern about biofilm contamination in these products is apparent from the dramatic increase in funding and biofilm related talks at national and international conventions such as the International Association for Food Protection. Microorganisms associated with raw milk plants can be categorized into three major categories: spoilage, pathogenic, and beneficial (Teh et al., 2015). Biofilms are the greatest source of both spoilage and pathogenic microorganisms.

In the US, dairy plants contain wide variety of surfaces like filter membranes, internal piping systems, or heat-exchangers, in which biofilms rapidly establish and grow due to a constant supply of nutrients available from the milk (D. Z. Liu, Jindal, Amamcharla, Anand, & Metzger, 2017). Overall, the presence of biofilms on these surfaces decreases product safety, quality, stability, and value (Galié et al., 2018).

The dairy industry is focusing on reducing the health risk associated with pathogenic bacteria containing biofilms. Reducing the presence of these bacteria is problematic because of the industry’s dependency on bacterial cultures for production and flavoring. In order to retain product quality, dairy plants are required to pasteurize milk in a continuous flow system at 72°C for 15 seconds. While this will kill most pathogenic bacteria, a higher temperature of around 85°C is needed to kill remaining cells (Bansal & Chen, 2006). Suboptimal pasteurization steps are ideal for a great tasting product but are inefficient at killing heat-resistant spore forming bacteria. These spore formers include the pathogenic organism, *Bacillus cereus*, found at levels of $10^3$ to $10^{10}$ colony forming units (CFU) per gram of food in recorded outbreaks (Gopal et al., 2015). High
temperature pasteurization milks in the market reduce the spoilage and pathogenic microorganisms allowing for a longer shelf life and storage at room temperature. However, a random survey of these ultra-high temperature pasteurization milks in the United States, showed *B. cereus* at $10^1$ to $10^3$ CFU/g of food of in 33% of the products (Chaves, De Paiva, Rabinovitch, & Vivoni, 2017). Therefore, biofouling by biofilm formation remains a serious quality concern in even highly pasteurized products. Both microorganisms entering in the milk production system, and those being dispersed from already present biofilms represent a source of contamination that reduces product quality. This expensive and constant battle to prevent, control, or remove, biofouling encompasses up to 80% of all operating cost in dairy plants (Bansal & Chen, 2006). However, the costs associated with prevention are minimal compared to the cost of contaminated product leaving the facilities. In an economic analysis, it was calculated that the average recall event reduced the shareholder’s wealth by 1.15 percent within five days after the recall had been issued, equivalent to a drop of 109 million dollars (Pozo & Schroeder, 2016).

2.5 Current Sanitization Methods in the Dairy Industry

Hazards Analysis and Critical Control Point is a voluntary protocol the FDA has laid out to help reduce foodborne illnesses in the United States (*FDA, 2006*). The guidelines include hazard analysis, identification of critical control points, CFU limits, validations, monitoring, corrective actions, record keeping systems, and verification procedures. Most of the regulations are built from CDC studies of biofilm forming species throughout commercial (>5000 gallons/day), and experimental dairy plants (<5000 gallons/day). The studies are continuous, and the last published data still found a detectible amount of spoilage and pathogenic bacteria from the genera *Bacillus*, *Lactobacillus*, *Lactococcus*, and *Staphylococcus* (M. Sharma & Anand, 2002). The most alarming data was the
presence of bacteria in post-pasteurization lines, including pathogenic *Staphylococcus aureus* (M. Sharma & Anand, 2002). Present day commercial dairy plant quality control managers utilizing HACCP protocols find them essential, but incomplete, since they do not incorporate protocols to manage the current prevalence of biofilms in the plant system (Record, 2019; D. Sharma & Malik, 2012).

2.6 Current Strategies for Controlling Biofilm Formation

2.6.1 Clean-in-Place

The two main FSMA strategies for maintaining sterile food processing surfaces are Clean-in-Place (CIP), and Clean-Out-of-Place (COP). Neither strategy is perfect, and the facility manager will need to determine whether a CIP or COP is better for their plant’s operation. COP procedures include a plant shut down, followed by the disassembly and individual cleaning of equipment interiors. This is expensive, time consuming, and keeps production shut down for long periods of time.

In contrast, the CIP method can be run between production cycles in a fully assembled plant allowing a much faster process. Due to the efficiency of the CIP method, it is the generally preferred method of sanitization in for-profit companies. CIP includes a series of cleaners followed by a rinse to make sure cleaning steps solutions do not end up in the product. For dairy, the most common CIP sequence begins with a caustic cleaner like sodium hydroxide to remove any proteins and carbohydrates (Flint, Van Den Elzen, Brooks, & Bremer, 1999). This is followed by an acid wash to remove any alkaline agents and mineral scale leftovers from the caustic step. The acid wash results in drying and growth delay for any left-over microbes (Bremer, Fillery, & McQuillan, 2006). The following step in the process utilizes a sanitizer, which is traditionally chlorine, but other sanitizers are now being used such as quaternary
ammonium compounds, anionic acids, and iodophors. The CIP is then completed with a water rinse to remove any leftover sanitizer compounds (Bremer et al., 2006).

The CIP system for cleaning various milk collection tanks, pipes, and pasteurizers was created for removal of contaminating agents such as leftover protein, fat and minerals. However, CIP was not designed for biofilm removal and control (Thomas & Sathian, 2014). With increased awareness of biofilm contamination, dairies are trying to adapt already approved CIP methods by lengthening the three steps. Lengthening of wash steps causes a drastic increase in on water usage. For instance, a typical daily cleaning cycle can cost a large facility 40,000 gallons of water (Record, 2019). Additionally, the lengthening of CIP protocols inherently increases the amount of time food processing equipment is exposed to corrosive chemicals.

### 2.6.2 Reagents used in Dairy Sanitization

With the aforementioned shortcomings of CIP biofilm management, dairies find themselves incorporating additional EPA registered disinfectants like peracetic acid (PAA). Registered disinfectants are approved after careful antimicrobial efficiency tests, where model bacterial organisms are utilized in a planktonic form. PAA is an antimicrobial registered disinfectant, a class of disinfectants successfully used to remove pathogens in a wide variety of industries. PAA is commonly used due to its strong oxidizing capabilities and environmentally favorable break down residues, making it an appealing cleaner for many industries. Applications of PAA to biofilms will kill cells and destroy matrix materials. Although PAA shows promise as a biofilm controlling agent in both destruction of matrix materials and cells, it does not fall under the new antibiofilm disinfectant category (Chino, Nukui, Morishita, & Moriya, 2017).
2.6.3 Testing Efficiency of Biofilm Sanitizers

The increase in biofilm related food poisoning cases in the US led the EPA to recognize the bacterial biofilm structure as a human health hazard, prompting a new EPA classification for antibiofilm sanitizers (Chino et al., 2017). This classification created a need for standardized testing protocols to assess biofilm controlling agents, and a way to accurately measure biofilm biomass. Many biofilm biomass analysis methods exist, including viable cell counts, growth with turbidity measurements, quantification of cells using qPCR, or EPS quantification using specific dyes (Stiefel et al., 2016). Stains, including crystal violet (CV), are also used in biofilm research as a cheap and fast colorimetric assay for indirect biofilm biomass quantification (Burton, Yakandawala, LoVetri, & Madhyastha, 2007). This indirect method uses bacterial cells and negatively charged components of the EPS as a proxy for biofilm biomass. Although this method does not take into account the diverse EPS to cell ratio across biofilms, researchers still use indirect methods like CV to investigate biofilms using 96-well flat bottom microtiter plates (Stiefel et al., 2016). The 96-well format’s large quantity of sampling surfaces allow for affordable investigation of a broad array of antibiofilm solutions at one time. Being able to test a large number of potential cleaners makes the 96-well plate a great presumptive test for quickly determining potential for biofilm removal. However, 96-well assays are not used for efficiency testing of biofilm sanitization due to high levels of variation within and between experiments. The variability arises from the use of small sampling surfaces, which make consistent biofilm growth, application of treatments, and quantification of the remaining biofilm very difficult.

To reliably investigate a novel cleaner, a method must produce consistent biofilm development across sampling surfaces and a reliable way to measure the biofilm is required. However, the development of a biofilm is a dynamic and hard to predict process, which makes the cultivation of identical biofilms very difficult. Additionally, due
to heterogenous biofilm structures, a quick and effective way to measure the amount of biofilm on a sampling surface is also very difficult. With this in mind, researchers attempted countless methods for controlling the many conditions influencing biofilm development. These conditions include temperature, inoculation culture, development time, and sheer force. All of these conditions play a large role in the development of the biofilm; especially the sheer force, which is a major determinant of shape, size, and orientation of the biofilm matrix (Williams & Bloebaum, 2010).

The EPA selected a continuously stirred tank reactor developed by the University of Montana, known as the Centers for Disease Control and Prevention (CDC) Biofilm Reactor (Epa Ocspp, 2017). The CDC bioreactor’s success comes from its ability to cultivate biofilm under controlled environment with constant sheer force along with other important growth factors. This control leads to the high repeatability and low deviation of biofilm growth across sampling surfaces (Buckingham-Meyer, Goeres, & Hamilton, 2007).
Figure 3: BioSurfaces Technologies® CDC biofilm reactor.

2.7 Brief Summary of CDC Biofilm Reactor

The CDC biofilm reactor is a one-liter vessel designed to produce consistent biofilm growth by controlling key environmental conditions. The vessel (Figure 3), contains 24 sampling surfaces, called coupons, constructed from a variety of industrial materials, such as polycarbonates, stainless steels, and glasses. The coupons are secured in the reactor by eight independent rods each holding three coupons. The rods allow coupons to be positioned and oriented in the medium so that each sampling surface is influenced by the same shear forces invoked by the rotation of the stir bar. Along with the stir bar, the reactor is designed to have liquid growth medium
continuously pumped into and out of the vessel. By having both the continuous nutrient flow and a stir bar the reactor is categorized as a continuous stirred tank reactor (CFSTR).

Use of this CFSTR reactor includes four distinct steps from inoculation to data collection as outlined in the EPA’s protocol for determining the efficiency of a product for biofilm removal of *Pseudomonas aeruginosa*. These steps begin with the batch phase, where the assembled reactor containing sterile growth media is inoculated by a 1ml of a bacterial culture at $10^8$ CFU/ml. *P. aeruginosa* is grown in batch phase for 24 hours while the baffle rotates at 125 rpm. The second phase of the operation is a continuous flow phase in which media is pumped through the reactor to maintain a 30-minute residence time: the average amount of time any given media is left within the reactor. This is less than half of the bacterial doubling time to allow wash out of bacteria growing suspended in media. To achieve this residence time over the entire 24-hour continuous flow phase in the one-liter vessel, 16.8 liters must to be pumped through the reactor at 11.67 mL/min. After the continuous flow phase, coupons are collected and aseptically deposited in individual 50 mL conical tubes for the treatment phase. Coupons in the conical tubes are submerged by 4 mL of the assigned treatment for a ten-minute contact time, then the treatment is neutralized with 36 mL of the appropriate neutralizing solution, resulting in a final volume of 40 mL. Following neutralization, the contents of the conical tubes underdo three rounds of vortexing and bath sonication to disassociate the adherent biofilm and cells from the coupons. The disaggregated biofilm is serially diluted from control coupons in dilution buffer and then 0.1 mL aliquots are platted on TSA agar. Samples might also need to be serially diluted if necessary, to reach a countable filter in the target range of 20-200 CFU/filter. The treated coupon wash is filtered through 0.45 µm PES membrane filter, and then the filters are gently rolled onto TSA agar. Both
control and test plates are incubated for 24 hours to encourage cell growth for viable cell count. After incubation, the colony forming units are recorded and used to calculate the log CFU/coupon of the control and treatment coupons in order to determine a log CFU reduction for a treatment’s antibiofilm efficiency.

2.7.1 EPA Listed Biofilm Sanitizer, Sterilex® Disinfectant

The first chemical agent approved with the EPA’s standard operating procedure for determining the efficacy of disinfectants against bacterial biofilms (SOP: MB-20-03) comes from a chemical cleaning company known as Sterilex®. Their patented cleaner is a two-part biofilm controlling agent utilizing a foaming quaternary ammonium compound which, when applied with force, effectively removes adherent biomass. This mechanical and foaming application of the Sterilex® cleaning agent restricts its application to COP procedures, though it is mainly used clean accessible areas such as drains and large holding tanks. If applied within a piped system, the pressure can lead to cavitation (intense vibrations from high pressure cavities) and cause massive equipment damage (Arndt, 1981).

2.7.2 Investigation of Novel Biofilm Controlling Agent

This thesis investigates a new approach for removal of biofilms from surfaces like those used in the dairy industry. The approach is based on common food starch chemical modifications used for dispersal effects due to chemical similarities between the polysaccharides found in food products and the exopolysaccharides in biofilms.
3.1 Comparison of Food Starch and Biofilm Polysaccharides

Despite complex structures in a diverse array of biofilms, most are primarily comprised of polysaccharides that greatly influence the biofilm’s adhesion and formation (H. C. Flemming et al., 2016). The influence exopolysaccharides have on the viability of biofilms and their chemical structure make them a good target for novel biofilm remediation research (H. C. Flemming & Wingender, 2010). The inspiration for the direction of this thesis work came from the similarities between the chemical structure of exopolysaccharides found in EPS (Figure 1) and the food starch structure (Figure 4). In particular, the polymers share hydroxyl functional groups targeted in the food industry for chemical modification (esterification) to create desirable physical and chemical characteristics (Sweedman, Tizzotti, Schäfer, & Gilbert, 2013). Due to the similar structures on EPS exopolysaccharides, chemical modifications used to emulsify starch polymers could be used to alter biofilm EPS, leading to biofilm dispersal. In particular, this thesis investigates the capacity of esterification reactions to produce polymers with amphiphilic properties from the exopolysaccharides found in biofilms.

![Native starch polymer backbone](image1)

![Octenyl succinate substituent esterified to the starch polymer backbone](image2)

Figure 4. Octenyl succinic anhydride (OSA) modification of a food starch (Nilsson & Bergenstahl, 2007).
3.2 Food Starch Modification

Food-starch modifications enhance properties desired for industry such as creating stable emulsions. Food products commonly bring together ingredients that are normally unstable with each other and will separate after mixing. This in turn has created a need for an emulsifier that keeps components from falling out of solution. Stable emulsions can be produced using inexpensive, naturally occurring, and biodegradable chemical modifiers of starch that are also safe to use (Dona, Pages, Gilbert, & Kuchel, 2010). These chemical modifications add a functional group to starch’s exposed hydroxyl groups to create useful physical property changes (Ashogbon & Akintayo, 2014). This process is known as esterification and can change the naturally hydrophilic native starch into an amphiphilic compound (Figure 4). The amphiphilic compounds stabilize emulsions by increasing steric hindrance, or the inhibition of emulsion aggregation using nonionic molecule interactions. Additionally, the amphiphilic compounds act in the between hydrophilic and hydrophobic interfacial region to lower surface tension as a surfactant. (BeMiller, 2019; Prochaska, Kedziora, Le Thanh, & Lewandowicz, 2007).

3.2.1 Succinic Anhydride Modification (OSA and Derivatives)

Alkyl- succinic anhydrides including hydrophobic alkyl chain lengths of one to ten carbon units are traditionally used to increase starch affinity for hydrophobic substances and thus decrease its hydrophilic affinity. Different alkyl- carbon chain lengths alter the starch modification efficiency and resultant chemical properties of the starch (Ačkar et al., 2015). The eight-carbon octyl- succinic anhydride (OSA), has the greatest desired effect in industrial settings because the modified starch exhibits a peak in esterifying abilities at the ideal 20-40ºC temperature range (Sweedman et al., 2013).

Historically the effectiveness of OSA was not recognized until 1953, when it was patented for its ability to create amphiphilic starches (Caldwell, Carlyle G.; Hills, Forest;
Since then, the application of OSA for starch modification is considered safe and the FDA guidelines dictate that up to 3% OSA can be added to react with starch for food applications (Sweedman et al., 2013). OSA modification of starch is a common practice and is proving useful in a variety of other situations outside the food industry. Creative uses of OSA are reflected by an increase in patents for the modifier over the past decade (Sweedman et al., 2013).

### 3.2.2 Antimicrobial OSA Modified Polysaccharides

A recent study described antibacterial properties of an OSA modified fructan called inulin (Stevens, Meriggi, & Booten, 2001). Inulin is a term covering a range of renewable and environmentally friendly $\beta(2 \rightarrow 1)$ linear fructans, a category of undigestible dietary fibers (Roberfroid, 2005). Their abundance and environmental sustainability caused fructans to be a common target of bioactivate enhancement modifications (Stevens et al., 2001). In particular, one study investigated the antimicrobial properties of OSA modified inulin through Scanning Electron Microscopy (SEM). This study visualized a membrane disruption mechanism for the antimicrobial action of the modified fructan in both gram negative and positive bacteria (Stevens et al., 2001). The same research team also treated bacteria with silver ions and viewed condensed substances within the cells using Tunneling Electron Microscopy (TEM) (Figure 5). This internal aggregation indicates that OSA-modified compounds can enter the cell, leading to an adverse effect on cellular protein and nucleic acids (Xiaoyun Zhang et al., 2015).
Figure 5: Negative staining electron microscopy photomicrographs of *E. coli* cultured without (CK₁, CK₂) and with (T₁, T₂) 0.5% In-OSA. The *E. coli* that was not treated showed full and smooth surface in an aggregate and diploid state. However, after treatment notable pores are formed with release of intracellular components apparent. Additionally, the treated cells showed a dispersal of the cells from one and another (Xiaoyun Zhang et al., 2015)
3.2.3 Detection of OSA Esterification Through Raman Spectrophotometry

The homogeneity of OSA-modified starches is assessed for commercial production using Raman spectrophotometry, which allows for investigation of OSA modification at the molecular and granular level (Besheer, Hause, Kressler, & Mäder, 2007; W. Liu, Li, Goff, Nsor-Atindana, & Zhong, 2018). In particular, the characteristic peaks of octenyl succinic esters at wavenumber 1670 and 1726 cm⁻¹ can differentiate levels of modification when comparing single-point Raman spectra of OSA-modified and unmodified starch granules (Bai, Shi, & Wetzel, 2009; W. Liu et al., 2018).

3.3 OSA-Modification of EPS

Chemical similarities of the exopolysaccharides in the EPS and food starch polymer, suggest the potential similar physical and chemical property outcome from chemical modifications such as OSA. OSA modification of biofilm EPS could induce dispersing and antimicrobial properties that would be desirable in biofilm remediation.

3.3.1 Detection of OSA Modifications Using Raman Spectrophotometry

OSA-esterification reactions occur at hydroxyl functional groups, found in all biofilm exopolysaccharides (Passos da Silva et al., 2019). Although OSA modification will not discriminate between exopolysaccharides, the ideal modification target is the adhesion and structural support polymers such as Psl and Pel polysaccharide groups in P. aeruginosa (Colvin et al., 2012). Biofilm dispersal should be encouraged by applying food starch OSA modifier to structural polysaccharide components. Hydrophilic polysaccharides will take up the same amphiphilic characteristics when OSA replaces free hydroxyl functional groups with hydrophobic octenyl succinic group (Figure 4). The added amphiphilic properties should disrupt the EPS by creating surfactant properties which lower the interfacial energies (Prochaska et al., 2007). Once disrupted, the OSA
exopolysaccharides will act as emulsion stabilizers, by inhibiting particle aggregation through steric hindrance (Tesch, Gerhards, & Schubert, 2002).

3.3.2 Bactericidal

OSA-modified exopolysaccharides are also desirable for biofilm remediation due to the antibacterial properties shown in OSA-inulin compounds (Xiaoyun Zhang et al., 2015). However, instead of the OSA hydrophobic alkyl chain attaching to the hydroxyls on inulin, it would be attaching to the exopolysaccharides in the EPS. In doing so, biofilm structural polysaccharide components should become antibacterial, killing the inhabitants and preventing regrowth.
Chapter 4

INVESTIGATION OF OSA IN BIOFILM REMEDIATION

4.1 Hypothesis
The Food-Starch esterifying compound OSA, can chemically modify biofilm exopolysaccharides in a way that creates antimicrobial properties and disrupts adhesion.

4.2 Predictions
1. Applying OSA to mature biofilm will result in esterification reactions between OSA and the exopolysaccharides in the EPS.
2. OSA modified biofilms will be more easily dislodged and washed away than biofilms that have not been modified reducing the speed of bacterial recolonization of the treated surface.
3. OSA modification of biofilms on the CDC biofilm reactor will meet the EPA required 6-log reduction from the control coupons by creating antimicrobial EPS properties.

4.3 Objectives
1. Verify the presence of the OSA ester band at 1720 cm\(^{-1}\) and 1670 cm\(^{-1}\) in a single point Raman spectrum of a biofilm treated with OSA.
2. Test the biofilm sanitizing capabilities of OSA on *Pseudomonas aeruginosa* biofilms by using 96-well plate assays and then confirm with the BioSurfaces Technologies® CDC biofilm reactor, as outlined in the EPA guidelines.
3. Test biofilm sloughing capabilities of the commonly used food starch esterification modifier, Octenyl Succinic Anhydride (OSA), on *Pseudomonas aeruginosa* biofilms by using 96-well plate assays and then confirm with the BioSurfaces Technologies® CDC biofilm reactor.
4.4 Methods

4.4.1 Raman Spectrophotometry

Raman Spectrophotometry was used to confirm esterification of OSA onto the biofilm EPS. *P. aeruginosa* biofilms were grown in 50 ml conical tubes on stainless steel coupons. The biofilms were either treated with or without OSA solution for a 5-minute contact time, and then rinsed before drying. Dried coupons were then analyzed with a Thermo DXR Smart Raman Spectrometer with the aperture set to 25 µm and low laser power. Spectra of both OSA modified and native food starch were saved to identify the characteristic Raman bands for OSA esters at 1670 cm$^{-1}$ and 1727 cm$^{-1}$ (Wetzel, Shi, & Schmidt, 2010).

4.4.2 Bactericidal Investigation

4.4.2.1 96-Well Microtiter Plate Assay

Prior to assessing the effectiveness of the food-starch modifier (OSA) on the CDC Bioreactor, a 96-well flat bottom microtiter protocol used to determine biofilm-killing capabilities (Stiefel et al., 2016). This was done by comparing the amount of the simple stain, crystal violet (CV), bound in the OSA treated wells to the same in no-treatment control wells. The biofilm bound CV within the wells was decolorized with ethanol, and the absorbance of the resultant solution was then measured with a Multi-Detection Microplate Reader at 595 nm (Stiefel et al., 2016). Before staining with CV, the absorbance was also taken at 600 nm as a proxy for bacterial and biofilm presence.

Biofilm cultivation: *P. aeruginosa* was grown overnight in TSB and diluted to OD600 of 1.0. Each of the sampling wells were then inoculated with 500 µl of the diluted sample. After inoculation, the culture’s cell density recorded at 600 nm with a MultiDetection Microplate Reader. Following the reading, the plate was then covered and incubated at 37°C. After 24 hours of incubation, planktonic cells on the plate were
removed with a water rinse, and then dried by leaving them upside down. Once dried a multichannel pipette was used to apply 500 µl of treatment for a 15-minute contact time. After treatment, the excess treatment solutions were removed with a water rinse and dried upside down for 5 minutes. Once dry, the wells were stained for 10 minutes by 500 µl of 0.5% CV solution. Excess CV not bound to biomass was then removed from the wells with 350 µl of 1% NaCl solution. Once dried the biomass bound CV was removed with 500 µl of 95% ethanol, and absorbance for the resultant solution was read at 595 nm by a Multi-Detection Microplate Reader.

### 4.4.2.2 Bioreactor Protocol

The CDC Biofilm Reactor was run following the EPA’s published standard operating procedures MB-19-05 and MB-20-03 with a few alterations. The cultivation of *P. aeruginosa* biofilm occurred during the 24-hour batch phase under room temperature on borosilicate glass, 304 stainless steel, and polycarbonate disc coupons. The continuous flow phase followed, in which a peristaltic pump (Fisher Scientific Cat No. 12-876-2) was used to pump 14.96±0.6 l of 100 mg/L TSB at a rate of 11.33±0.5 mL/min for 22 hrs from a 20 L carboy through the reactor and into a 20 L waste carboy. At the end of the continuous flow phase, the rods containing biofilm covered coupons were rinsed in a 50 mL conical tube containing 30 mL of sterile buffered water. Once rinsed they were then oriented over a sterile 50 mL conical tube containing a splashguard, to allow coupons to fall into the tubes once unscrewed. The splash guards were then removed, and the conical tubes containing coupons where randomly assigned to either control or treatment groups. Four mL of previously prepared disinfectants (treatments) were slowly pipetted into the conical tubes, submerging the coupons. After a 10 min contact time, 36 mL of neutralizer was added and vortexed after the conical tube was capped. Control coupons received 40 mL of dilution buffer followed by a brief vortex.
After treatment and neutralization, the coupon’s biofilm was removed and disaggregated by three rounds of alternating treatments of vortexing at the highest setting for 30±5 s, and sonication at 45±5 kHz for 30±5 s using a bath sonicator. The resulting 10^0 dilution was then assayed for remaining live cells. Aliquots of 10^0 dilutions were filtered through a 0.45 µm PES membrane and incubated overnight at 37ºC for CFU enumeration. Serial dilutions were plated, and then incubated overnight at 37ºC for CFU enumeration. For this experiment, a drop plating method was selected instead of the MB-20-03 recommended spread plating method to reduce the number of agar plates used for each experimental run. Up to eight successive dilutions were quantified by pipetting five 10 µl drops into subdivided agar plates. After incubation, the resulting colonies were inspected for purity and then counted to determine the coupon’s log CFU density. The log CFU reduction was calculated by subtracting the control coupon log density from the treatments.

4.4.3 Investigation of Regrowth After OSA Treatment

Investigation of dispersal properties was assessed by altering the protocol for the 96-well assay described above by the addition of a second inoculation step after treatments. Biofilms were grown as described in 4.5.2.1. At random time intervals during the 24 hours of growth, OD 600 reading were taken. After growth, the plates were randomly assigned to either receive 200 µL of water or OSA. After a 10-minute contact time the plates were rinsed three times with sterile TSB media, and then reinoculated with 200 µL of P. aeruginosa diluted to an OD 600 of 0.4. After inoculation, the 96-well plates where incubated in 37ºC incubator along with incremental OD 600 absorbance measurements for an additional 24 hours.
4.5 Raman Spectrophotometry Results

The 1670 cm\(^{-1}\) peak representing an octenyl ester was detected in the OSA treated biofilm sample, as well as peaks that represent CH\(_2\) presence (1450 cm\(^{-1}\)), and the increased presence of C-C or glyosidic links at 868 cm\(^{-1}\) wavelengths (Figure 6) (Wagner, Ivleva, Haisch, Niessner, & Horn, 2009)

![Figure 6: Comparison of Single Point Raman Spectra of OSA treated P. aeruginosa biofilm (red line) against no treatment (blue line). Dashed lines mark the octenyl ester presence at 1670 cm\(^{-1}\).](image-url)
4.6 Bactericidal Results

4.6.1 96-Well Assay

Absorbance readings taken at OD 595 of OSA, PAA, and water treated wells showed no significant difference (Figure 7). OSA treated wells displayed the greatest range of OD measurement, contrasting the similar range of PAA and water treatments.

Figure 7: Comparison of OSA treatment effect on OD 595 absorbance readings from wells treated with either OSA (n=28), PAA (n=32), and water (n=38). Bars represent the data range excluding the outliers which are expressed as black points.
4.6.2 CDC Biofilm Reactor Results

The CDC reactor protocol was run ten times over the course of three months, during which OSA and PAA were tested and optimized to remove biofilm from polycarbonate, 304 stainless steel, and borosilicate glass discs. The different material coupons were divided into different experimental categories including controls and treatment groups. The reactor produced consistent growth on the control coupons, and eight out of the ten experimental runs were within the EPA requirement of 8.0 to 9.5 log CFU/coupon (Figure 8). Glass control coupons consistently produced log CFU densities within the EPA range with a mean (and standard deviation) of 9.126 ± 0.357 log CFU/coupon. In contrast, plastic and metal control coupons had means of 9.886 ± 1.169 log/coupon and 9.515 ±1.04 log CFU/coupon respectfully.

![Box plots for control coupons](image)

**Figure 8:** Average log CFU density for the CDC bioreactor control coupons. Three box plots representing the mean, quartiles, maximums and minimums for the Glass (blue), Plastic (green), and the Stainless Steel (red) coupons.
With the EPA requirement for biofilm growth met, OSA and PAA’s efficiency for biofilm sanitization was determined by calculating the log CFU reduction; the value used by the EPA to determine the efficacy of biofilm sanitization. The log CFU reduction values calculated by subtracting the average log CFU/coupon for treated coupons from the average log CFU/coupon for control coupons. OSA treatments (Fig. 9) had an average log CFU reduction of 6.44 ± 0.35 (glass coupon), 6.169 ± 0.12 (stainless steel coupon), and 6.67 ± 0.508 (plastic coupon). PAA treatments had an average log CFU reduction of 6.4 ± 0.9 (glass coupon) and 6.67 ± 0.69 (stainless steel coupon).

![Box plot of log CFU reduction for OSA and PAA treatments](image)

**Figure 9: Mean log CFU reduction of OSA and PAA treatments.** The box plots representing the mean, quartiles, maximums and minimums for the Glass (blue) and the Stainless Steel (red) coupons. Plastic coupons were only tested with OSA treatment. Data is compiled from five separate biofilm reactor runs.
4.7 Regrowth After OSA Treatment Results

OD 600 turbidity readings allowed for a visualization of the biofilm growth curve before and after treatment (Figure 10). Data shows effect on OD600 after inoculation of treated wells. There were no significant differences between the OSA-treated wells until after 9.5 hours from the treatment application, at which the OSA treated wells showed a drastic reduction in turbidity.

**Figure 10: The long-term effect of OSA treatment.** OSA shown in blue, and Water treatment shown in red on OD 600 over the 24-hours period of regrowth after re-inoculation. represented by the grey bar starting at 22 and finishing at 24 hours after the initial inoculation. Data from 5 separate experiments is shown. Colored bars shown represent data range for the given treatment excluding outlier which are shown as the colored dots.
4.8 Discussion

4.8.1 Confirmation of Esterification Reaction

The undefined Raman spectra demonstrated the complex chemical heterogeneity of the both biofilms by its lack of defined peaks (Figure 6). However, an unmistakable intensity shift occurred at 1670 cm\(^{-1}\), which is the characteristic band used to distinguish the octenyl succinic ester, the resulting compound of the OSA esterification reaction (Bai et al., 2009). Presence of this peak in OSA treated biofilm suggests that a component of \(P. \ aeruginosa\) biofilm was chemically modified by OSA.

4.8.2 OSA Antimicrobial Activity

The 96-well microtiter plate investigation of OSA’s antimicrobial properties was inconclusive due to an inability to accurately measure the change in total biomass using CV staining (Figure 7). Despite inconclusive results in 96-well experiments, OSA and PAA treatments were investigated using the CDC Biofilm Reactor by following the EPA’s guidelines outlined in MB-19 and MB-20. The consistently >6 log CFU reduction recorded in the CDC biofilm reactor confirmed an antimicrobial action on OSA treatment coupons that exceeds EPA requirements, indicating OSA modification has potential for a biofilm sanitizing claim (Figure 8). However, the mechanism of OSA’s antibiofilm action is still unclear. Raman data suggests that a component of the EPS is being esterified by OSA in the same way in which food-starch is esterified, producing the described octenyl ester. The detection of the OSA ester, and the chemical similarities between the two polymers, suggests that OSA-Biofilm antimicrobial mechanisms could be similar to those of OSA-inulin (Figure 5).
4.8.3 Effect of OSA Treated Biofilm on Regrowth

A mature biofilm structure not only provides a protective and nourishing habitat but even after most biofilm is removed, remnants on surfaces can also behave as site for future contamination. Therefore, when the 96-well plate received a second bacterial inoculation, both planktonic growth and biofilm regrowth in the wells after treatment could influence OD 600 measurements. The early increase in OD 600, up to 9 hr. after re-inoculation, could be attributed to planktonic bacterial growth, particularly because it indistinguishable in both treated and untreated wells, and began to level off after 9 hrs. (Figure 10). However, the effect of OSA treatment on regrowth can only be seen in OD 600 measurements after 20 hr of regrowth. Thus, delay in treatment effect may result from the inability to form new biofilm in the OSA treated wells. This would be true if the increase in OD 600 seen in untreated wells was due to increased biofilm biomass, while this was not possible in the treated wells, and overall bacterial concentration declined due to either the antimicrobial effect of OSA-biofilm, or the buildup of other toxic growth byproducts.

4.8.4 Next Steps

The antimicrobial mechanisms of OSA-modified biofilm could be investigated using Scanning Electron Microscopy and Tunneling Electron Microscopy as described in Xiaoyun Zhang et al. (2015) to confirm similar antimicrobial action.

Further investigation of OSA dispersing properties using the CDC biofilm reactor were planned, but access to laboratory space was restricted during the SARS-COVID pandemic. If allowed, the EPA protocol will be altered to allow for a second batch and continuous flow phase with OSA treated coupons. A second batch and continuous flow phase allows for OSA-treated coupons, coupons with untreated biofilms, as well as clean
coupons to be compared for biofilm regrowth. This comparison will give a greater insight on whether or not OSA treated biofilms have a long-term effect on the biofilm’s success.

4.9 Conclusions

This thesis demonstrated the effectiveness of the chemical esterification reaction done with OSA as a biofilm treatment. In doing so, suggests a new approach of biofilm remediation by chemically modifying the structural components of biofilm.


