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Maintaining and Controlling an Extrinsic Biofilm for Pathogen Removal in Dental Unit Water Lines

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering
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Abstract

The control of biofilm formation is a major concern for industrial, environmental, and public health. Undesirable biofilms can harbor different disease-causing pathogens and shorten the operational life of different equipment. On the other hand, beneficial biofilms are also used in various applications and managing its growth and activity can be desirable. Killing the biofilm does not usually incorporate the removal of the dead biofilm structure that is adhered to the surface. Therefore, the aim of this thesis is to control biofilm formation; to be able to remove, inhibit, and enhance biofilm formation. This thesis investigated the use of norspermidine, D-amino acids, and selected enzymes for the control of biofilm formation. Biofilm was pre-grown in 96-well microtiter plates and the different treatments were applied for 24 h. Biofilm formation was quantified before and after treatments using crystal violet stain. The results obtained in this thesis showed that norspermidine had a dual effect on biofilms formation. A concentration of 1 mM norspermidine removed 39% of nonactive biofilm, while for active biofilm norspermidine enhanced the biofilm growth by 73%. D-amino acids can inhibit biofilm growth at a low concentration of 5 µM. The two D-amino acids used in this study, D-tryptophan (15 mM) and D-tyrosine (20 µM), removed 28% and 31% of biofilm, respectively. No clear synergetic effects were noticed from mixed D-amino acids treatment. The enzymes Savinase and Pectinex showed the highest biofilm removal among the different tested industrial enzymes. Savinase removed 68% and 84% while Pectinex removed 74% and 55% of biofilm formed by *Bacillus species* and *Pseudomonas fluorescens,* respectively. The optimized

enzymatic treatment containing both Savinase (19.6 and 23.7 U/mL) and Pectinase (63.8 and 48.8 U/mL) showed the highest biofilm removal for *Bacillus sp.* biofilm at pH 6 and *P. fluorescens* at pH 8.

Keywords

Biofilm, norspermidine, D-amino acid, D-tyrosine, D-tryptophan, D-leucin, D-methionine, enzymes, Savinase, Protamex, Subtilisin A, Trypsin, Pectinex, Cellic-CTec2, Cellic-HTec2, Carezyme, optimization, central composite design, response surface methodology, model.

Co-Authorship Statement

This work was accomplished under the supervision of Dr Lars Rehmann. This thesis includes four coauthored research articles. The contribution of the coauthors can be found below.

Section 3.2 was coauthored by three collaborators, Bilal Al-Bataina, Dr Erin Johnson, and Dr Lars Rehmann. In specific, Bilal Al-Bataina designed and performed all experimental work and drafted the manuscript. Both of Dr Erin Johnson and Dr Lars Rehmann provided technical advice and revised the manuscript.

Sections 3.3-3.5 were coauthored by two collaborators, Bilal Al-Bataina, and Dr Lars Rehmann. Specifically, Bilal Al-Bataina designed and executed all experimental work and drafted the manuscript. Dr Lars Rehmann provided technical advice and revised the manuscript.

Dedication

To my late grandfather, Atwan Mofadi Al-Bataina, a man who believed in science as the only way for humanity to advance. To my father, Prof. Barakat Atwan Al-Bataina, and my mother, Nimah Radwan Arefaie, for your ongoing love and endless support, you are all my reasons.

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Chapter 1 - Introduction

1.1 Research Background

Nosocomial infections obtained at dental clinics are a major rising concern (Shatokhin, 2010). There is a growing interest in the contamination of dental unit waterlines (DUWLs) in the dental community due to rising awareness of infection control. Large quantities of water are used daily in dental procedures for rinsing the mouth, cooling the instruments, and other procedures. This water is being supplied to the dental units from the municipal water network or in some other cases from a private water tank owned by the dental clinic (Pederson, 2002; Rowland and Voorheesville, 2003). One old practice was warming the water to reach body temperature for the comfort of the patient; this is not encouraged anymore because it provides a favorable environment that will increase the microbial contamination. Biofilms are formed by bacterial communities that adhere to surfaces where moisture and nutrients can be found. Dental unit's water lines are one good example of an optimal environment for bacterial microorganisms to dwell (Rowland and Voorheesville, 2003). Noncoliform bacteria in recreational and drinking water cannot exceed 500 CFU/mL according to Safe Drinking Water Act standards. However, untreated DUWLs can contain anywhere between 10^4 and 10^5 CFU/mL, in fact, a study on DUWLs in a dental school revealed a value of 2×10^5 CFU/mL after 5 days only from the first use of newly installed DUWLs (Barbeau *et al.*, 1996). The fact that DUWLs are dramatically higher in bacterial load when compared with the municipal water network comes from four main factors that act together to provide an optimal environment for biofilms, these are nutrition, surface area to volume ratio, surface chemistry, and laminar flow

(Rowland and Voorheesville, 2003). Drinking water supplied by municipal water network usually contains minerals that deposit inside the DUWLs such as calcium carbonate (Mills, 2000). Free bacterial cells in the water will then adhere to the minerals and create biofilms, this is more promoted during the stagnation periods when dental units are turned off after working hours (Pederson, 2002).

One of the main characteristics of biofilms is their higher resistance to antibiotic and other chemical treatments due to the protective shield provided by the biofilm matrix to the cells living underneath in what is called “the protective mode”, one study reported that biofilms can be up to 1000-fold more resistant to chemical treatments (Gilbert and McBain, 2001). Another characteristic of biofilms is the fact that they are multicellular communities that can contain different types of microorganisms (Coleman *et al.*, 2010). One interesting characteristic of biofilm is quorum sensing, which is the cell-to-cell communication system used among bacteria living within the biofilm through signaling molecules, this communication system is used to coordinate the collective behavior of the biofilm community (Atkinson and Williams, 2009). Biofilms can vary in their thicknesses depending on the shear forces exerted from the surrounding flow, biofilm forming under higher shear conditions are denser and have less permeability and are much harder to remove (Stoodley, Boyle and Lappin-scott, 1999).

Most of the bacterial microorganisms that dwell in DUWLs are harmless water bacteria, the remaining harmful pathogenic microorganisms are obtained from patients during dental procedures as a result of backflow in the suction devices. These pathogenic microorganisms can cause serious health problems to dental staff and patients, especially those who are

immunocompromised (Rowland and Voorheesville, 2003). A list of microorganisms that have been detected in DUWLs is presented in *Table 1–1*.

Table 1–1. Microorganisms detected in DUWL (Rowland and Voorheesville, 2003).

Acanthamoeba	*Klebsiella aerogenes*	*Pseudomonas fluorescens*
Lactobacillus	*Klebsiella pneumoniae*	*Pseudomonas maltophilia*
Legionella dumoffi	*Achmmobacter xyloxidans*	*Pseudomonas pickettii*
Actinomyces	*Acinetobacter calcoaceticus*	*Pseudomonas posimobilis*
Aeromonas	*Legionella pneumophila*	*Pseudomonas putida*
Moraxella urethalis	*Brevindimonas vesicularis*	*Pseudomonas testosteroni*
Alcaligenes faecalis	*Burkholderia cepacia*	*Pseudomonas stutzeri*
Bacillus subtilis	*Burkholderia pickettii*	*Pseudomonas vesicularis*
Bacteroides	*Alcaligenes dentrificans*	*Psychrobacter phenylpyruvica*
Micrococcus luteus	*Myroides odoramm*	*Seromonas salmonicida*
Morabella osloensis	*Streptococcus mitis*	*Moraxella phenylpyruvica*
Serratia marcescens	*Flavobacterium odoratum*	*Sphingomonas paucimobilis*
Candida	*Flavobacterium indologenes*	*Staphylococcus capitus*
Cephalosporium	*Pasteurella haemolytica*	*Staphylococcus cohnii*
Cladosporium	*Pasteurella multocida*	*Staphylococcus saprophyticus*
Cloaca	*Pasteurella paucimobilis*	*Staphylococcus wameri*
Enterococcus	*Pasteurella pneumotropica*	*Streptococcus faecalis*
Nocardia	*Pseudomonas acidovorans*	*Ochromobacterium anthropi*
Fusobacterium	*Pseudomonas aeruginosa*	*Streptococcus salivarius*
Weillonella	*Pseudomonas cepacia*	*Xanthomonas maltophilia*

Some of the alarming pathogens that have been detected in DUWLs are *Legionella sp.*, aquatic *nontuberculosis mycobacterium*, and *Pseudomonas aeruginosa*. *Legionella sp.* can cause several diseases including the legionnaire's disease, pontiac fever, and pneumonia (Bartram *et al.*, 2007). Each year between 10,000 and 15,000 cases caused by *Legionella* are reported in the United States with mortality rates of 5 to 15% (Rowland and Voorheesville, 2003). Aquatic *nontuberculosis mycobacterium* can cause respiratory diseases and are associated with wound

infections (Griffith *et al.*, 2007). Different types of infections can be caused by *Pseudomonas aeruginosa*, in addition to pneumonia especially in individuals that suffer from cystic fibrosis (Young and Armstrong, 1972). Gram-negative bacteria are the most common microorganisms found in DUWLs and they are associated with endotoxins, which can lead to serious immune reactions. Endotoxins have been reported in DUWLs with concentrations up to 500 endotoxin units/mL and up to this day, there is no legislation on the standard levels of endotoxins in water (Rowland and Voorheesville, 2003). Documented cases of infections that were related to contaminated DUWLs are not abundant, in two cases patients with immunocompromised conditions acquired wound infections caused by *Pseudomonas aeruginosa*, and both patients completely recovered (Mills, 2000). In one other case, a dentist lost his life to Legionnaire's pneumonia caused by aerosols from DUWLs contaminated with *Legionella dumoffii* (Atlas and Williams, 1995).

Although several organizations and governmental agencies provided guidelines for the water used in dental clinics, there are no regulations or laws that set water quality standards for water used in dental clinics. In 1993, the Center for Disease Control and Prevention published their guidelines with three main relevant recommendations, the use of backflow prevention valves (later proven not to be effective due to negative pressure), if the treatment is associated with cutting bone sterile solutions should be used, and flushing the DUWLs for 20 to 30 sec between different patients and for several minutes at the end of each day (Kohn *et al.*, 2003). In 1996, following the results of a study on hemodialysis that found rapid colonization occurs in fluids containing more than 200 CFU/mL heterotrophic bacteria; the American Dental Association recommended that water used in dental treatments should not contain more than

200 CFU/mL heterotrophic bacteria (Depaola *et al.*, 2002). The American Public Health Association (APHA) are alert to DUWLs associated problems, they encouraged the CDC to perform studies on the DUWLs associated health effects. It is worth mentioning that none of the aforementioned recommendations was directed towards prevention or control of biofilms in DUWLs.

Common cleaning procedures used to clean DUWLs can be summarised in four main methods: drying or flushing DUWLs, chemical agents, filtration devices, and the use of autoclavable or disposable water delivery systems (Rowland and Voorheesville, 2003). Drying the DUWLs will not reduce the microorganism load in the DUWLs and had no effect on biofilms. While flushing the DUWLs can reduce the microorganisms count in the DUWLs temporarily until the microorganisms repopulate again, the process has no effect on biofilms in DUWLs (Kettering *et al.*, 2002). Flushing the DUWLs between consecutive treatments can discharge microorganisms acquired during treatments from patients and help in preventing cross-contamination between patients (Cobb *et al.*, 2002; Fiehn and Larsen, 2002). The most applied method is the use of chemical agents to kill the microorganisms in DUWLs. Treatments are applied either by the shock-treatment in intermittent periods at high concentrations or as a continuous low concentration treatment. Dental units that use the shock treatment method must have their own water reservoir, the germicidal treatment is applied for a certain period of time and that is followed by a complete system flush with sterile water, in this way the germicidal treatment is completely removed from the DUWLs before treating the patients (Rowland and Voorheesville, 2003). Although the microorganisms that survive the germicidal treatment will repopulate again between the treatments. These germicidal treatments have

negative effects on the DUWLs components and can impose toxicity risks on the staff due to the harsh chemicals that are used in their manufacture (Rowland and Voorheesville, 2003). In the continuous treatment, the germicidal treatment is applied at low concentrations, since these germicidal treatments are continuously present in DUWLs, the chemicals used must be chosen carefully as they must not impose any health effects on patients (Rowland and Voorheesville, 2003). Moreover, these germicidal agents can also damage the DUWLs components and they can interfere with the bonding agents used in dental treatments. One common used germicidal agent is sodium hypochlorite (bleach). Other effective germicidal agents used are sodium fluoride, hydrogen peroxide, and commercial mouth rinses (Wirthlin and Marshall, 2001; Kettering *et al.*, 2002; Tuttlebee *et al.*, 2002; Rowland and Voorheesville, 2003).

The only method that can lower the microorganisms count to 200 CFU/mL or even lower is filtration. Filters used are usually 0.22 µM pore size. The locations of the filters are usually chosen to be as close as possible to the endpoint of the DUWLs (i.e. just before the handpiece or the sonicator). The limitations on using filters can be summarised by DUWLs clogging, biofouling, and no control on the release of the harmful endotoxins (Mills, 2000).

New research is now focused on developing novel methods for reducing the microorganisms count and biofilm control in DUWLs. Germiphene corporation, the industrial partner in this project, is a privately owned Canadian company located in Brantford, Ontario. Germiphene is licensed by FDA and Health Canada. They are specialized in developing and manufacturing medical devices, infection control products, and dental pharmaceuticals with a special interest in the dental market. Germiphene started to implement a new development

plan to improve a number of its current products to become more environmentally friendly. One of their many products is a biofilm control product called Gobble, which has been in the dental market for many years. In their product, Germiphene developed a new "out of the box" method for the reduction of pathogenic microorganisms count and biofilm control in DUWLs. It is a fact that biofilms are going to grow inside the DUWLs for the many reasons mentioned previously, and since that is inevitable, a beneficial biofilm will be grown, using the heterotrophic, cannibalistic, and yet non-pathogenic microorganism, that microorganism is a gram-positive *Bacillus species*. Biofilms by *Bacillus sp.* will form inside the DUWLs and they will cannibalize the pathogenic microorganisms they will get in contact with using self-secreted toxins or antibiotics. Researchers discovered this phenomenon during a study on natural matrix inducers. In their study, they co-cultured a *Bacillus* strain that contained a reporter for matrix gene expression with other similar soil microorganisms. Although there were a variety of bacterial microorganisms tested in the soil samples they used, the majority of matrix inducing microorganisms were found to be *Bacillus species* (Liu *et al.*, 2010; Shank *et al.*, 2011; Vlamakis *et al.*, 2013).

1.2 Problem Description and Motivation

Dental clinics sanitation relies on chemical disinfectants to lower the microorganisms count in their DUWLs, the presence of pathogenic biofilms in the DUWLs will make that extremely hard to achieve (Barbeau, Bokum and Gauthier, 1998; Rowland and Voorheesville, 2003). Pathogenic biofilms formation in dental units has been reported by several researchers. Biofilms can form in tanks, pipes, tubing, sinks, suction hoses, hand pieces, air and water syringes, and ultrasonic scalers (Barbeau, Bokum and Gauthier, 1998; Coleman *et al.*, 2009,

2010). Pathogenic biofilms can incorporate different mixtures of microorganisms such as bacteria, viruses, fungi, algae, amoebae and protozoa (Coleman *et al.*, 2010; Vasickova *et al.*, 2010). The biofilm extracellular matrix and the outer layers of cells form a shield that protects the microorganisms living underneath, consequently resulting in a biofilm community that is more resistant to disinfectants, antibiotics, and detergents. It has been reported that bacteria are one thousand-fold more resistant when they live within a biofilm community (Barbeau, Bokum and Gauthier, 1998; Coleman *et al.*, 2010). In dental clinics, pathogenic biofilms are responsible for high contamination levels in water used for invasive procedures (Barbeau *et al.*, 1996; Barbeau, Bokum and Gauthier, 1998). Pathogens released from the biofilms can be passed around with aerosols from dental tools such as the hand piece (Barbeau, Bokum and Gauthier, 1998; Pederson, 2002). Moreover, wet dental devices such as the evacuation system (suctioning apparatus and saliva ejectors) are bases of cross-contamination among the patients due to tubing backflow (Barbeau, Bokum and Gauthier, 1998). These dental instruments are used to drain blood, saliva, tissue, and restoration debris that result from dental procedures (Barbeau, Bokum and Gauthier, 1998). The evacuation system consists of a filter, plastic tubing, and a mouth piece. Although the mouth piece is disposable, the filter and plastic tubing are not and can be easily contaminated with pathogens. Since the evacuation system is not regularly cleaned or disinfected, clearly not between consecutive patients, they can become a source of cross-contamination among patients (Barbeau, Bokum and Gauthier, 1998). One other concern from biofilm growth in dental settings is the interference with the mechanical functions of the dental devices and the clogging of tubes and pipes. Based on the aforementioned biofilm

growth control is a major concern for dental clinics hygiene and dental devices industry (Barbeau *et al.*, 1996).

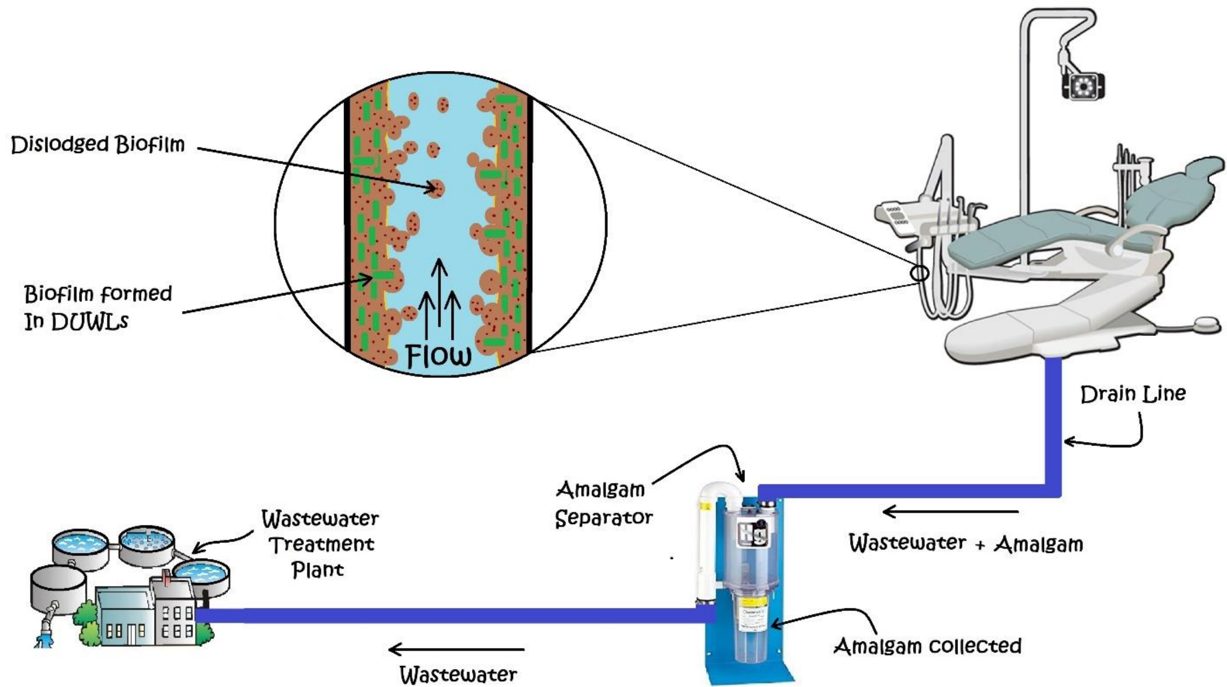

Figure 1-1. Schematic for biofilm formation in dental unit water lines (DUWLs) and the use of amalgam separators for the remove of amalgam from the wastewater.

Amalgam separators are a specialized type of filters used to recover amalgam waste from the liquids removed from patient’s mouth during procedures involving amalgam containing

fillings. Without these separators amalgam will travel through the DUWLs to the vacuum lines and end of in the wastewater discharged to the municipal sewer system. The main problem occurs from mercury that is being released from the amalgam. The amount of mercury in the sludge of a wastewater treatment plant as well as the amount being discharge in wastewater are regulated by Canadian Environmental Protection Act, 1999.

Germiphene developed a product for cleaning DUWLs known commercially as Gobble. Gobble utilizes a beneficial biofilm formed from the non-pathogenic *Bacillus Species* to cover the inner walls of the DUWLs. This beneficial biofilm is safe for human consumption and will prevent the formation of pathogenic biofilm. It is useful to enhance the formation of such beneficial biofilm especially with new commissioned DUWLs. However, some dental clinics have been experiencing excessive shedding of biofilm. These biofilm aggregates that peel from the inner walls of the DUWLs have been travelling through the DUWLs and accumulating in the amalgam separator. This accumulation of biofilm aggregates in the amalgam separator requires a more frequent replacement of the filter. These filters are expensive to replace. Also, the proper disposal of the amalgam filter is expensive and must be contracted to a specialized company that carries a certificate of approval to handle biohazard and hazardous wastes. The ability to better control biofilm growth could therefore lead to developing an overall better product.

It must be emphasised here that the aim of this research is not to kill the biofilm nor completely remove it, but control its formation in the DUWLs. In this thesis, the term “control”, as in control biofilm formation, implies the ability to either enhance, inhibit, or remove biofilm

formation. There is abundant of previous knowledge that can be employed in this research to find effective methods to control biofilm formation.

1.3 Research Objectives

1.3.1 General Research Objective

The general objective of this thesis is to thoroughly investigate the capability of different suggested treatments for controlling biofilm formations. To be able to inhibit, enhance, and most importantly eradicate biofilm formation.

The main problem faced from the presence of biofilm in DUWLs is the physical barrier provided by the biofilm extracellular structure. This structure provides a diffusion barrier that harbors different pathogens from chemical treatments, and it interferes with the physical properties of the dental devices. The intention of this work is not to kill biofilms or the microorganisms living within the biofilm community, as by doing so, the biofilm structure "skeleton" remains intact and can facilitate the attachment of new microorganisms, and the biofilm cycle continues building on the extracellular structure causing more problems.

1.3.2 Specific Research Objectives

1.3.2.1 Investigate Biofilm Control Using Norspermidine

There is current apparent controversy in the literature on the interaction of norspermidine with biofilms. It has been reported that norspermidine is self-produced in mature biofilm formed by *Bacillus subtilis* to trigger biofilm disassembly (Kolodkin-Gal *et al.*, 2012). While other researchers reported that norspermidine is not self-produced by *Bacillus subtilis* and when added to biofilms it enhanced growth (Hobley *et al.*, 2014). Moreover, to the best knowledge of

the authors, present literature is lacking for quantitative studies that investigate the interaction of norspermidine with biofilms. Therefore, it was deemed necessary to further investigate the interaction between norspermidine and biofilms, and whether norspermidine can be used to dismember biofilm formation or not.

1.3.2.2 Investigate Biofilm Control Using Amino Acids

Previous researchers have reported that four D-amino acids (D-tyrosine, D-tryptophan, D-methionine, and D-leucine) are self-produced in late stages of biofilms formed by *Bacillus subtilis* to trigger biofilm disassembly (Kolodkin-Gal *et al.*, 2010). To the best knowledge of the authors, no quantitative studies that investigated biofilm removal using D-amino acids exist today. Hence, it was considered important to further investigate the removal of biofilms using D-amino acids, and if any synergetic effects can be noticed for combined treatments that can lead to optimization experiments.

1.3.2.3 Investigate Biofilm Control Using Enzymes

Enzymatic treatments are being suggested as relatively new method for biofilm removal due to their environmentally friendly nature, high efficiency, and low cost (Cortés, Bonilla and Sinisterra, 2011; Taraszkiewicz *et al.*, 2013; Meireles *et al.*, 2016). There exists today abundant information in the literature on different types of enzymes that are being used in different applications. To the best of the author's knowledge, current published work related to the use of enzymes for biofilm removal lacks a comprehensive approach to the problem. Most of the published studies deal with a specific type of microorganism, enzyme(s), and substratum (Johansen and Falholt, 1997; Loiselle and Anderson, 2003; Molobela, Cloete and Beukes, 2010; Marcato-Romain *et al.*, 2012). The diversity in the biofilm-forming microorganisms is coupled

with diversity in biofilms constituents (Branda *et al.*, 2001; Morikawa *et al.*, 2006; Molobela, Cloete and Beukes, 2010), which should be accounted for in the selection of proper enzymatic treatments (Molobela, Cloete and Beukes, 2010). Although biofilms are complex in their composition, the two main constituents of biofilms reportedly are polysaccharides and proteins (Branda *et al.*, 2001; Morikawa *et al.*, 2006; Molobela, Cloete and Beukes, 2010). Therefore, it was considered important to further investigate the available enzymes that can target those two constituents and degrade them. The published literature will be revisited for the selection of specific enzymes that have been reported to degrade biofilms by targeting the polysaccharides and proteins constituents (polysaccharide-degrading enzymes and proteases). The selected enzymes will be further investigated to select the most effective in biofilm removal.

1.3.2.4 Optimization of Biofilm Removal Using Enzymatic Treatments

The use of enzymatic treatments as anti-biofilm agents have proven to be successful (Taraszkiewicz *et al.*, 2013; Thallinger *et al.*, 2013; Meireles *et al.*, 2016). Enzymes present a more green, efficient, and affordable alternative to chemical treatments used to eradicate biofilms (Cordeiro, Hippius and Werner, 2011; Cortés, Bonilla and Sinisterra, 2011; Srey, Jahid and Ha, 2013). To obtain a higher biofilm removal efficiency, previous researchers have recommended the use of anti-biofilm enzymatic treatments consisting from mixtures of different enzymes targeting different substrate types (Augustin, Ali-Vehmas and Atroshi, 2004; Cordeiro, Hippius and Werner, 2011; Torres *et al.*, 2011). To the best knowledge of the authors, no such studies exist in the literature. Consequently, it was considered vital to conduct a “first of its kind” optimization study for the use of proteases and polysaccharide-degrading enzymes

for biofilm removal. The top two of each, proteases and polysaccharide-degrading enzymes, that will show the highest biofilm removal efficiency in the preceding objective (Section 1.3.2.3) will be further utilized in the optimization study.

1.4 Thesis Overview

This thesis contains four chapters. The first chapter is the introduction which presents the research background, the problem description and motivation, research objectives (general and specific), and the thesis overview. The second chapter is the literature review which will provide the reader with the related background information on bacteria and biofilms, biofilm life cycle, bacterial strains used (*Bacillus species* and *Pseudomonas fluorescens*), factors governing biofilm formation, polyamines, amino acids, and enzymes. The third chapter is the experimental results where the methodology used and results obtained are presented and discussed. The fourth chapter is the summary and conclusions of the thesis.

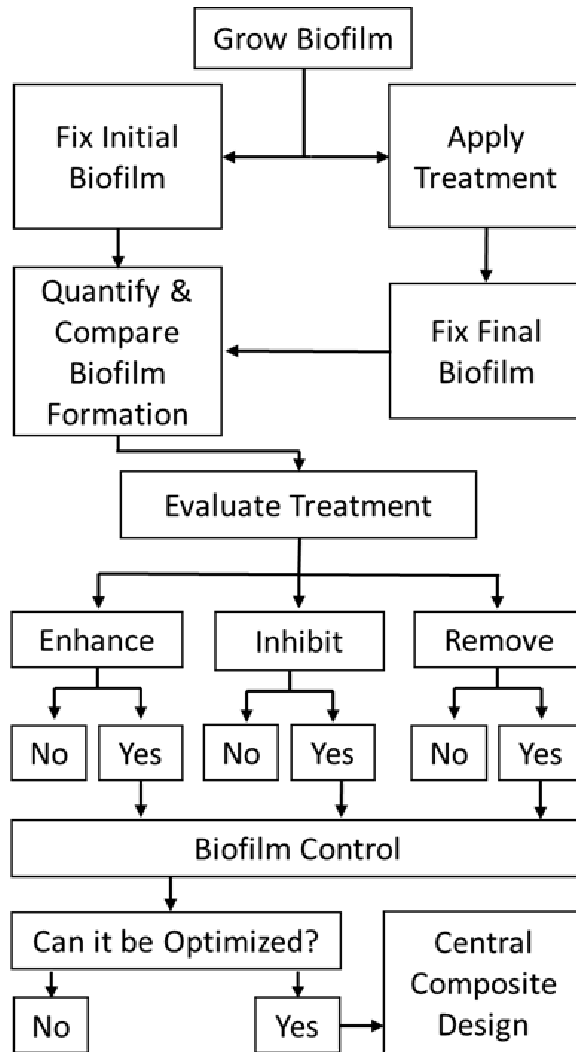

Figure 1-2. Overview of the procedure used for evaluating the efficiency of the different biofilm control agents tested in this thesis. Fixed (killed) biofilm is treated with methanol to preserve biofilm formation at a certain stage to be quantified at a later stage.

This thesis investigated different biofilm control agents. Each biofilm control agent investigated in the subsections of chapter three had a specific preparation procedure and application method and unique results. Nevertheless, the assessment of the effectiveness of each different biofilm control agent tested was done following the flow chart presented in *Figure 1-2*.

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Chapter 2 - Literature Review

2.1 Preface

This thesis aims to investigate different potential biofilm control agents and their ability to control biofilm formation. Therefore, a literature review was prepared to provide the reader with the relevant background information on bacteria, biofilm, and biofilm control agents. The literature review presented in this chapter is divided into seven subsections (subsections 2.2-2.7) reviewing the major related topics covered in this thesis. Section 2.2 will provide information on bacteria and biofilms, Section 2.3 will provide information on biofilms life cycle, Section 2.4 will provide information on the bacterial strains used in this research, Section 2.5 will provide information on the factors governing biofilm formation, Section 2.6 will provide information on polyamines and the previous work that used them for biofilm control, Section 2.6 will provide information on amino acids and their related previous research as biofilm control agents, and finally Section 2.8 will provide information on enzymes and their use as biofilm control agents.

2.2 Bacteria and Biofilms

Bacterial microorganisms are single-celled living beings, also known as prokaryotes, which have been considered to be "simple" when compared to other multicellular organisms. Relatively recent studies have shown that these simple microorganisms can perform complex processes and behaviors. Few examples of that can be the spore formation of *Bacillus subtilis*, and bacterial biofilms which are both used by bacteria as survival mechanisms (Karatan and Watnick, 2009). In the spore formation, vegetative cells will generate multiple internal and

external signals to produce a new morphological shape that can adapt to harsh environments. When free planktonic bacterial cells find them self in a favorable environment (i.e. abundant nutrients, surface area) they tend to form biofilms, and once the surrounding environment becomes less favorable they will disassemble their biofilms and return to their free planktonic form and start a new quest to find another favorable location to start a new biofilm, and so on the cycle continues (Karatan and Watnick, 2009; Romero and Kolter, 2011). While in the biofilm formation bacterial cells aggregate and get attached together to form a protective layer that shelters the cells underneath it, by doing that bacteria have evolved to tolerate low concentrations of antibiotics and antimicrobials that they could not have survived in their free individual cell form (O'Toole, Kaplan and Kolter, 2000; O'Toole and Stewart, 2005). Biofilms are defined as complex multicellular communities that form by bacterial aggregation on abiotic and biotic surfaces. Bacterial cells in a biofilm are connected together by an extracellular matrix, which consists of exopolysaccharides and amyloid protein fibers, and sometimes DNA, with the exopolysaccharides and proteins being the main constituent (Hall-Stoodley, Costerton and Stoodley, 2004; Bryers, 2008; Coleman *et al.*, 2010; Molobela, Cloete and Beukes, 2010). Biofilm formation is part of the life cycle of bacteria and is vital for its survival, biofilms provide the bacterial cells located underneath is with a protective shield from surfactants and antibiotics, in addition to the optimum positioning of the bacteria to access nutrients (Costerton *et al.*, 1987; Cava *et al.*, 2011). Biofilms have a complex architectural shape that contains channels for the flow of food and nutrient within and throughout their community. Moreover, there is an interdependent relationship among the biofilm members including communications between different species through different signalling compound (Rowland and Voorheesville, 2003).

Biofilms are complex and hard to characterise, in example, it was found that in dental units water lines only 4% of bacterial cells within a biofilm community are cultivable with the rest being non-cultivable or dead (Depaola *et al.*, 2002).

In aquatic systems, bacteria tend to form biofilms at interfaces with abiotic and biotic surfaces. Biofilms can adhere to solid surfaces in the liquid-solid interface, they can also form floating biofilms called pellicles in the liquid-air interface of static cultures (Hall-Stoodley, Costerton and Stoodley, 2004; Bryers, 2008; Cava *et al.*, 2011; Kolodkin-Gal *et al.*, 2012). Biofilm formation starts after the initial layer of colonies are attached to the surface through the reversible and weak van der Waals forces. More attachment sites will become available for the free microorganisms on the initial biofilm layer and the attachment becomes more permanent due to other cell attachment methods. Consequently, biofilm growth is promoted and the protective extracellular matrix secretion is initiated (Coleman *et al.*, 2010).

2.3 Biofilms Life Cycle

Biofilms go through different stages in their life cycle which can be summarised in: free planktonic cells, Initial attachment, early biofilm, mature biofilm, biofilm disassembly, and return into its original planktonic form (Palmer and White, 1997; O'Toole, Kaplan and Kolter, 2000). Environmental stress is the main reason for biofilms formation with the availability of nutrients being the main driving force; some other environmental factors can trigger biofilm initiation such as temperature, pH, iron, and oxygen with all these factors being microorganism specific (O'Toole, Kaplan and Kolter, 2000). Planktonic cells are transferred from the liquid media to the surface boundary by physical forces or by bacterial movement through their

flagella. If the environmental condition promotes biofilm formation, planktonic cells will be induced to attach to the surface and they will change from motile cells into non-motile. This attachment is governed by different environmental factors such as surface structure, temperature and pressure. When bacterial cells are attached to each other the term cohesion is used, while the term adhesion is used when bacterial cells are attached to a surface (Garrett, Bhakoo and Zhang, 2008). External physical forces affect the bacterial adhesion to surfaces. These forces are: Electrostatic interactions, Van der Waals forces, steric interactions, all together known as the DVLO (Derjaguin, Verwey, Landau and Overbek) (Coleman *et al.*, 2010). After initial adhesion of the bacteria to the surface some bacterial cells will become permanently adsorbed to the surface and form the initial attached biofilm layer. The initially attached cells will divide and facilitate the cohesion of additional free planktonic cells that will get attached to the initial layer (Coleman *et al.*, 2010). Consequently, new bacterial cells will grow sideward and outward to form a mushroom like shape. Bacterial cells are connected together and to the surface by the exopolysaccharides and protein fibers (O'Toole, Kaplan and Kolter, 2000), forming the early biofilm which will grow and adapt to the surrounding environment that will develop into a mature biofilm. Bacterial cells at this point are characterised with and increased synthesis of exopolysaccharides, higher antimicrobial resistance, UV light tolerance, and formation of bacterial spore cells (Habash and Reid, 1999; O'Toole, Kaplan and Kolter, 2000; Rowland and Voorheesville, 2003; Garrett, Bhakoo and Zhang, 2008; Coleman *et al.*, 2010; Vlamakis *et al.*, 2013). At late stages of the mature biofilm, bacterial cells will start the secretion of small molecules such as polyamines and D-amino acids. These small molecules will target the protein fibers and the exopolysaccharides that connect

the bacterial cells in the biofilm, initiating the biofilm disassembly and the release of planktonic cells and bacterial spores as a survival mechanism. Released planktonic cells and spores will migrate to find another favorable location and initiate a new biofilm community (Kolodkin-Gal *et al.*, 2010, 2012).

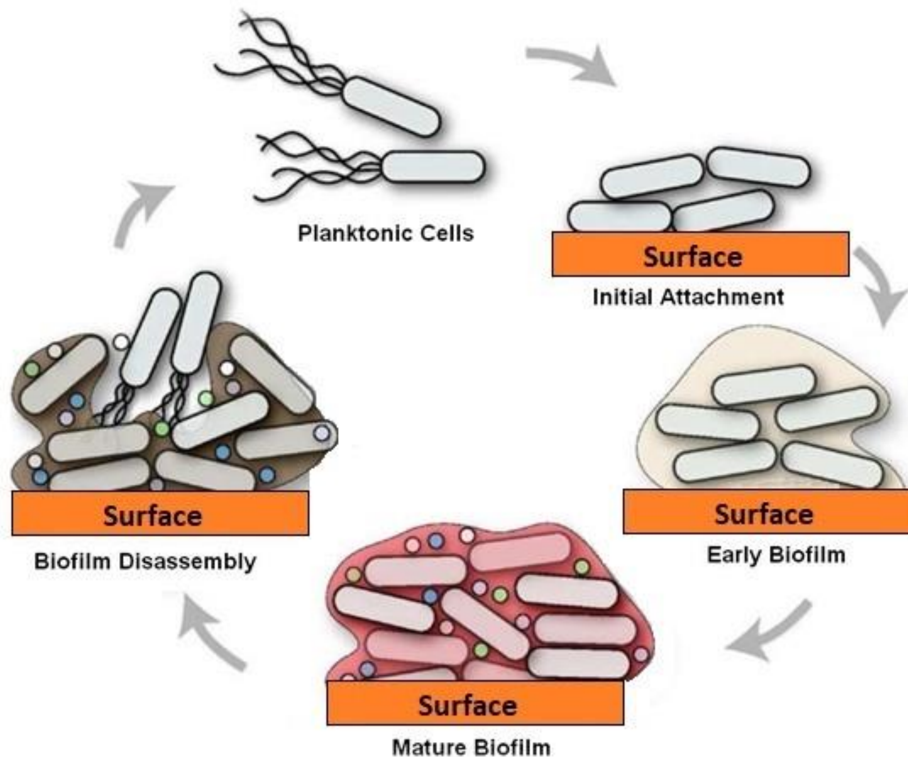

Figure 2-1. Biofilm life cycle for *Bacillus subtilis*, modified from (Cava *et al.*, 2011).

2.4 Bacterial Strains

2.4.1 Bacillus Species

The *Bacillus species* (*sp.*) strain used is known commercially as Unizyme 20X CSB 20 NF SPORE, and it was obtained from the industrial partner (Germiphene Inc.). Many researchers have employed *Bacillus* bacteria in different research studies, which made it a model

microorganism for understanding biofilm development (K P Lemon *et al.*, 2008). *Bacillus sp.* is a rod-shaped, motile, gram-positive, spore-forming, biofilm forming, and non-pathogenic bacteria that are well known for their ability to highly compete with other types of microorganisms as a response to nutrients depletion and increased population densities. *Bacillus sp.* are usually found in terrestrial (soil and vegetation) and aquatic environments, this ability to adapt to a wide range of environments makes it clear why this bacterial species is abundant in the environment. They grow in the mesophilic temperature range with an optimal temperature of growth ranging between 25 to 37 ^{o}C (Grossman, 1995; Maglott *et al.*, 2007; Earl, Losick and Kolter, 2008).

The cell wall of *Bacillus sp.* bacteria has a rigid structure, made of a polymer composed of sugars and amino acids known as peptidoglycan also known as murein in bacteria. *Bacillus sp.* uses its flagella to swarm around. (Schaechter, Ingraham and Neidhardt, 2006). *Bacillus* cells can produce toxins (enzymes) that kill different species they encounter in a mixed culture (Liu *et al.*, 2010).

It had been conceived previously that *Bacillus sp.* are strictly aerobic bacteria, which means that oxygen is required for their growth. However, later studies have shown that they can grow in anaerobic conditions. Nitrite and nitrates are used as electron acceptors in the absence of oxygen during the cell respiration (Nakano and Zuber, 1994; Marino *et al.*, 2001).

Bacillus sp. forms biofilms on the surface of plants roots (Earl, Losick and Kolter, 2008), evidence exist that this is a favorable association for the plants and it promotes plant growth due to the following reasons: (a) it activates the plant immune system making the plants ready

to overcome other harmful pathogens; (b) their biofilms will overgrow and kill other potential pathogens that can affect the plant health; (c) they make key nutrients to the plants (phosphorus and nitrogen) more readily available to the plant (Cazorla *et al.*, 2007; Nagórska, Bikowski and Obuchowski, 2007; Earl, Losick and Kolter, 2008). In animals, *Bacillus subtilis* is considered to have beneficial effects when ingested by maintaining a healthy bacterial community in their gastrointestinal tract (Hong, Duc and Cutting, 2005). Several popular fermented food products in Japan such as fermented soybeans contain *Bacillus subtilis*, these types of food have been thought to carry many health benefits (Inatsu *et al.*, 2006). Other industrial uses of *Bacillus subtilis* in the manufacturing of many types of enzymes such as xylanase, lipase, protease, amylase (Westers, Westers and Quax, 2004).

2.4.2 *Pseudomonas Fluorescens*

The *Pseudomonas fluorescens* strain used in this research was purchased from DSMZ in Germany, it is known commercially as *Pseudomonas fluorescens* (Migula 1895) DSM no. 50090, Type strain. *Pseudomonas fluorescens* is considered a model microorganism for studying biofilms (Rossignol *et al.*, 2008). *Pseudomonas fluorescens* is a gram-negative, rod-shaped, non-spore forming, motile (via flagella), biofilm forming, non-pathogenic bacteria (Rossignol *et al.*, 2008; Scales *et al.*, 2014). They are considered obligate aerobes, but they can survive in anaerobic conditions by using nitrate as an electron acceptor during cellular respiration. It grows in mesophilic temperatures (Optimal temp. 25 to 30 ^{o}C) (Donnarumma *et al.*, 2010), due to its ability to resist a wide range of antibiotics and disinfectants and its high adaptation, this bacterium is largely reported in many locations in the environment and in hospitals (Spiers, Buckling and Rainey, 2000; Rossignol *et al.*, 2008; Scales *et al.*, 2014). Previous research has

reported that *Pseudomonas fluorescens* biofilms are mainly composed of proteins (Molobela, Cloete and Beukes, 2010). Previous researchers showed that when compared with biofilms formed by other pathogenic gram-negative microorganisms (*Staphylococcus aureus, Pseudomonas aeruginosa, and Staphylococcus epidermidis*), *Pseudomonas fluorescens* biofilms has shown the highest resistance to treatments, which makes their biofilm the worst case scenario for biofilm removal (Johansen and Falholt, 1997).

2.5 Factors Governing Biofilm Formation

2.5.1 The Effect of Nutrients

Bacteria require a source of energy and raw materials to grow. Bacterial cells that can obtain energy from sunlight are called Autotrophs (self-feeding); while bacterial cells that obtain energy from oxidizing inorganic and organic materials are called heterotrophs (other-feeding). The heterotrophs are the most common types in humans and medical applications (Todar, 2012). For a bacterial cell to generate energy and carry on biosynthesis the surrounding environment must provide all the required substances. These substances that are utilized for bacterial growth are usually referred to as nutrients. Bacterial cells grown in laboratories are always grown in a culture media that provides them with all the essential nutrients for growth and development (Todar, 2012). Major elements required for bacterial growth are the same elements used in the composition of the bacterial cell itself and those are: C, H, N, O, P, S, Ca, Mn, K, Mg, Fe and some trace concentrations of Mo, Co, Zn, Cu. These molecules have structural or functional roles in the bacterial cells and are usually found in inorganic compounds, water, and other molecules. Trace concentration of metals are required by certain bacterial cells and their presence is not necessary in the culture medium, they work as co-

factors for enzymatic reactions in the cells (Todar, 2012). It must be stated that nutrients are bacteria-specific and what one bacterial cell can utilize for energy another bacterial cell may not be able to utilize, since some metabolizing enzymes may not be present or the fact that these molecules cannot be transported through the cell wall membrane of that specific bacteria (Todar, 2012).

Bacterial cells in a biofilm cannot utilize the available energy source in its surrounding environment without being able to transport it into the biofilm cells. Usually, nutrients are dissolved and must be able first to diffuse through the mass transfer boundary layer this is known as the external mass transfer, following that the nutrients need to diffuse through the biofilm matrix and this is known as the internal mass transfer to finally reach the bacterial cells. The decrease in the thickness of the boundary layer is reversely correlated with the nutrients diffusion rate (Characklis and Marshall, 1990; Ghannoum and O'Toole, 2004).

2.5.2 The Effect of Temperature

The ideal temperature for bacterial growth and development is related to the rate of nutrients intake, a higher rate will result in a rapid biofilm formation (Stepanovic *et al.*, 2003). The metabolism of nutrients is dependent on the usage of enzymes, based on that the rate of biofilm formation and development of biochemical and physiological systems depends on the availability and reaction rates of enzymes (Garrett, Bhakoo and Zhang, 2008). Optimal growth of bacterial cells and their biofilms requires ideal temperatures, and consequently when temperatures deviate from the ideal temperature that reduces their growth efficiency. Due to reduction in the reaction rates of the bacterial enzymes, and the change in the physical properties in the surrounding environment to the cells (Garrett, Bhakoo and Zhang, 2008).

A study by Fletcher et al. (Fletcher, 1977) investigated how the attachment of cells in the stationary phase is affected by temperature in the marine Pseudomonad, results showed that temperature and cell adhesive properties are proportional to each other. That was related to the reduction in the bacterial surface polymers and the reduction in surface area at lower temperatures. On the other hand, a study by Herald et al. (Herald and Zottola, 1988) observed that the bacterial cell flagella counts is temperature dependent, several flagellas were noticed at 10 ^{o}C, whilst two or three flagellas were noticed at 21 ^{o}C, and only one flagella was observed at 35 ^{o}C. Based on that, initial interactions between the bacterial cells and the attachment surface will increase at lower temperature, which likely will increase the chances of initial attachment of bacterial cells. Moreover, at lower temperatures there are more uniform properties of the exopolysaccharides which in turn enhances the bacterial cells attachment. It was also found that the use of high temperatures (80 to 90 ^{o}C) have negative effects on biofilm removal due to the "baking effects". Results showed that high temperatures are not effective for biofilm removal; in fact, high temperatures increased the adherent nature of the biofilms to the attachment surface (Marion-Ferey *et al.*, 2003).

2.5.3 The Effect of pH

The manipulation of the pH is common for detergents and disinfectants used to kill bacteria due to its direct effect on bacterial growth (Garrett, Bhakoo and Zhang, 2008). Bacterial cells possess proton pumps in their membrane that are used to expel protons from the cytoplasm to generate the proton motor force, which is used to regulate the cytoplasm pH (Rowland and Voorheesville, 2003; Garrett, Bhakoo and Zhang, 2008). A sudden change in external pH can cause influx of protons into the cytoplasm and that is problematic to the bacterial cells (Booth,

1985). Large variations in the pH of the surrounding environment of a bacterial cell can have fatal effects on it. Bacterial cells overcome the changes in pH by adjusting the synthesis and activity of proteins in cellular processes (Olson, 1993). Researchers have shown that a gradual decrease in pH will increase the bacterial cell survival when compared to a sudden drop in pH (Li *et al.*, 2001).

Although bacterial cells can adapt with gradual changes in pH, some cellular activities cannot adapt with pH changes. An example on that will be the excretion of the exopolysaccharides. An optimum pH value for exopolysaccharides secretion is species dependent; usually for most bacteria it is in the neutral range (Oliveira *et al.*, 1994).

2.5.4 The Effect of Flow Dynamics

The common testing methods used for biofilm control usually employ the use of microtiter plates (6, 12, and 96 well) in static conditions (Kolodkin-Gal *et al.*, 2010; Cava *et al.*, 2011; Brandenburg *et al.*, 2013; Leiman *et al.*, 2013), the use of microtiter plates has been evaluated previously and found to be appropriate for testing biofilm control agents (Stiefel *et al.*, 2016). Biofilm forming in static conditions are different from biofilms forming in a dynamic fluid system. One example on biofilm formation inside tubes and pipes is the biofilm formation inside DUWLs. Flow inside DUWLs is described to be laminar, flow velocity near the inner DUWLs wall approaches zero due to friction, which dramatically reduce the shear forces exerted on the biofilm developing on the inner walls of the DUWLs. This in fact reduces the chances of biofilm dislodging from the inner wall of DUWLs (Rowland and Voorheesville, 2003). A higher flow velocity is usually associated with higher shear forces exerted on the biofilm inside the DUWLs. Fluid dynamics has direct effects on biofilm formation; a biofilm will form in

different shapes with specific characteristics depending on the fluid dynamics under which it formed (Stoodley, Boyle and Lappin-scott, 1999; Buckingham-Meyer, Goeres and Hamilton, 2007). It had been reported that when flow was turned off biofilm thickness increased 38% in comparison to biofilm grown at 1.5 m s^{-1} flow velocity (Stoodley, Boyle and Lappin-scott, 1999). Biofilms forming under higher shear forces are denser, more strongly attached to the surface, have lower profile thickness, and have lower internal diffusivity when compared with biofilms formed in static conditions or under lower shear conditions (Vieira, Melo and Pinheiro, 1993; Pereira *et al.*, 2002).

When compared to municipal water network, DUWLs represent an ideal environment for biofilm growth for many reasons other than the abundance of nutrients. Flow rates inside DUWLs can be as low as 2 mL min^{-1} or as high as 100 mL min^{-1} and it turns into static conditions over night and when the dental units are not in use (Rowland and Voorheesville, 2003). This flow is considered to be very low when compared to flow rates of 5000 mL min^{-1} in usual municipal water networks (Swift, 1999; Pederson, 2002). Another factor is the surface area to volume ratio. Normally DUWLs have an inner diameter ranging between 1/16 and 1/8 inch. The inner diameter of DUWLs has an inverse relationship with the area available for bacterial attachment and growth, as the inner diameter of the DUWLs decrease the surface area to volume ratio will increase dramatically (Pederson, 2002).

2.5.5 The Effect of Surface Type

Previous literature has presented the effect of surface physical and chemical characteristics on bacterial adhesion to surfaces. Surface roughness is being used over a large

scale of measurements. The surface patterns that have been reported to reduce biofilm formation was in the scale of the bacterial cells (i.e. between 1 and 2 μm) (Graham *et al.*, 2013).

In a previous study by Graham et al. 2013, the attachment of *Escherichia coli* (*E. coli*) was evaluated on surfaces related to medical applications made from titanium, silicon and, glass. The researchers reported that the attachment of *E. coli* on the different surfaces was proportional to wettability of the surfaces quantified by measuring the surface contact angle using deionized water droplets. Interestingly the researchers reported that the inherent surface roughness did not correlate with the attachment of *E. coli* cells. The same researchers evaluated the effect of engineered surface roughness on the attachment of *E. coli*. Different topographical features were formed from silicon surfaces and tested at static and microfluidic flow conditions for *E. coli* attachment, the highest attachment observed was on un-patterned flat surfaces, while linear patterned surfaces showed less bacterial attachment. They also reported that surfaces containing holes further reduced attachment of *E. coli* when compared to surfaces with linear patterns. The authors concluded that the shape of the surface features, spacing, size have high effect on the cell-surface interactions which should be considered in the design of anti-biofilm surfaces (Graham *et al.*, 2013).

Previous studies have shown that surfaces that are formed from repetitive microscale patterns can reduce bacterial attachment to surfaces (Whitehead and Verran, 2006). A study by Schumacher et al. 2007 suggested that the use of microscale biometric patterns that are similar to ones on shark skin reduced bacterial attachment to surfaces significantly lower than similarly sized different patterns. The same researchers reported that engineered surfaces with spacings

that are equal to the size of the individual bacterial cells significantly enhanced the attachment of bacterial cells to surfaces (Schumacher *et al.*, 2007).

Other research focused on different coatings that can be used to reduce bacterial attachment to surfaces such as immobilized enzymes (Cordeiro, Hippius and Werner, 2011), this method was not seen effective for the use in dental treatments as enzymes can be denatured due to different chemical treatment that can pass through DUWLs. Other research focused on the use of active coatings that have bactericidal effects (such as copper) to prevent biofilm formation on surfaces (Zeiger *et al.*, 2014), these treatments have shown to be ineffective in DUWLs applications due to physical degradation by debris released from dental treatments passing through the DUWLs. More information can be found in the review by Tuson et al. 2013 on surface modifications and the interactions between bacteria and surfaces (Tuson and Weibel, 2013).

The research that focuses on modifying surfaces to reduce bacterial attachment can produce very useful results that can be used by the different manufacturers of dental and medical equipment. On the other hand, this method is not applicable for the removal of pre-existing biofilm from surfaces.

2.6 Polyamines

Polyamines are organic compounds that have two or more amino groups. Polyamines are found naturally in all types of bacteria and in most animal cells, they are considered to be important growth factors in microorganisms. In microorganisms, they are made from putrescene, while in mammals they are made from arginine (Lawrence, 2004). Polyamines are important for the growth and development of cells; it is no surprise they occur in high

concentrations in milk and other dairy products, they can also be found in other food products such as: fish, meat, fruits, and nuts. In addition to the polyamines that exist in nature, there are many other synthetic industrial polyamines (Lawrence, 2004). There is an increased interest in polyamines due to their high distribution among most living cells and their high concentrations especially in rapidly growing tissue (Tabor and Tabor, 1984).

The life span of biofilms formed by *Bacillus subtilis* is limited. After three days of incubation in a biofilm growth medium at 22 ^{o}C the biofilm matures, and after eight days the biofilm disassembles and start releasing the free planktonic cells (Kolodkin-Gal *et al.*, 2010; Romero *et al.*, 2011). It was previously found by Kolodkin-Gal et al. (2010) that an eight day old bacillus subtilis culture contained factors that inhibited the formation of pellicles when mixed with fresh cultures, these factors were mixtures of D-amino acids (Kolodkin-Gal *et al.*, 2010). Following the same procedure, the same researchers announced the discovery of a new biofilm disassembly factor that was found in the eight-day *Bacillus subtilis* biofilm growth medium, this factor is the polyamine norspermidine, which was found at concentrations ranging between 50 and 80 μM in the eight-day biofilm growth media culture which makes that the minimum required concentration for pellicle formation inhibition. Furthermore, norspermidine was present in the three day biofilm growth media culture only at a lower concentration of less than 1 μM and that concentration had no inhibition effects on pellicle formation (Kolodkin-Gal *et al.*, 2012).

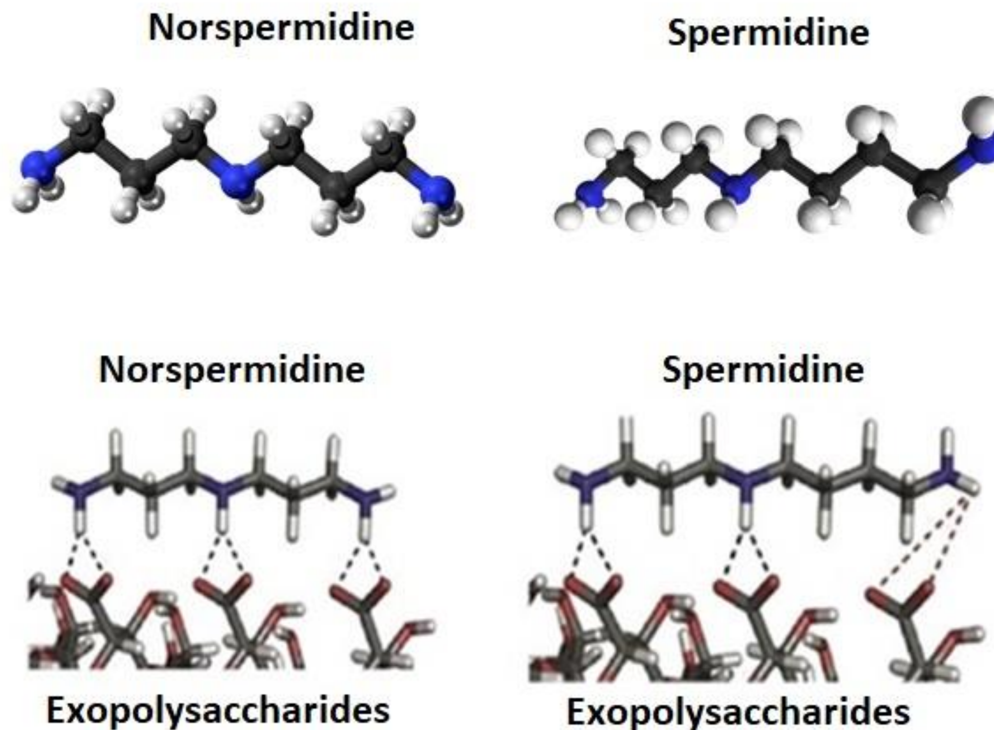

Figure 2-2. Specificity in structural activity of polyamines with exopolysaccharides, modified from (Kolodkin-Gal et al., 2012).

There is evidence of direct interaction between norspermidine and the exopolysaccharides, this interaction was specific to norspermidine that other similar polyamines such as spermidine that only have one extra methylene group did not show any biofilm inhibiting activity at concentration as high as 2 mM. Moreover, *Bacillus subtilis* mutant cells that could not produce norspermidine and D-amino acids formed long living pellicles. In their research paper Kolodkin-Gal et al. 2012 presented data showing extreme reduction in the average diameter of the exopolysaccharides aggregates before and after the addition of norspermidine (570 nm and 85 nm respectively) similar results were obtained from norspermine. Using molecular modeling (Figure 2-2) the researchers suggested that the amines in norspermidine and other polyamines that are similar in structure and charge ("three methylene groups flanked by two positively

charged amino groups") interacts with the negatively charged residues (i.e. uronic acid) or the neutral sugars with polar groups (i.e. poly-N-acetyglucosamine) that exist in the secondary structure of the exopolysaccharides. They hypothesised that norspermidine interacts directly with the exopolysaccharides within the biofilm community. This interaction disrupts the biofilm formation and diminishes the pre-existing biofilm and causes it to collapse in biofilms communities from *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* (Kolodkin-Gal *et al.*, 2012).

A later study by Hobley et al. 2014, revisited the findings of Kolodkin-Gal et al. 2012. In contrast, they found that norspermidine can not naturally exist in biofilms formed by *Bacillus subtilis*, therefore norspermidine is not self-produced *by Bacillus subtilis*. They also found that the gene responsible to produce norspermidine in *V. cholera* was missing in *Bacillus subtilis* genome. The researchers suggested that norspermidine does not have a native role in the physiology of *Bacillus subtilis* biofilms. Moreover, the researchers stated that spermidine has an essential role in robust biofilm formation from *Bacillus subtilis* and norspermidine can substitute spermidine efficiently for that role. Moreover, results showed that biofilm formation from *Bacillus subtilis* wild type was inhibited by norspermidine at a minimum concentration in the range of 250 to 300 µM. Pellicle formation was completely inhibited at a minimum concentration of 500 µM. When norspermidine concentrations were lower than 250 µM, biofilm formation was enhanced (Hobley *et al.*, 2014).

2.7 Amino Acids

The word chirality is used to describe objects when their mirror reflected image cannot be superimposed on their original image. One good example on that is the human hand, which in fact where the word was derived from in Greek. The term enantiomers have been used to refer to the two mirror images of a chiral molecule. Although enantiomers share almost the same physical properties (i.e. molecular weight, solubility…), they have different interactions when they are subjected to plane-polarized light. The common basis of chirality in molecules is a carbon atom that is bonded to four groups; this carbon atom is called the chiral center or stereocenter (Cava *et al.*, 2011). The arrangement of the atoms bonded to the chiral center in three-dimension is used to describe the chirality of a molecule. For amino acids and sugars the D or L are the nomenclature used to describe their chirality (Meierhenrich, 2008). The structures of D- and L- enantiomers of Tyrosine are presented in *Figure 2-3*.

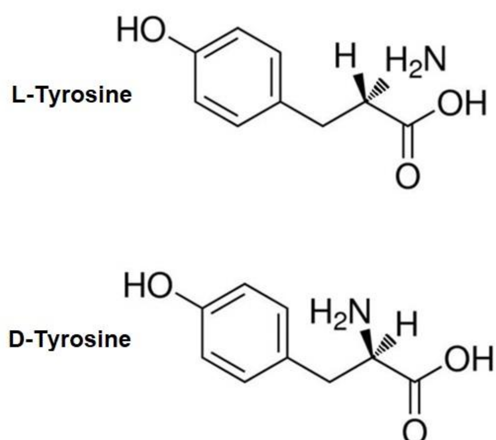

Figure 2-3. Chirality of Tyrosine.

Unlike chemical synthesis that can usually produce both enantiomers, enzymes show clear substrate selectivity, and based on that biochemical processes usually use and produce particular enantiomers. One good example on that is the fact that L-amino acids are the building blocks of proteins in the ribosomal synthesis, at the same time D-amino acids cannot be utilized into proteins though ribosomal synthesis. Another example on that are the enantiomers of alanine. While L-alanine is considered to be a germinant, and in the presence of L-alanine and other nutrients bacterial spores can reactivate their metabolism and grow again, it was discovered that D-alanine is a strong inhibitor of *Bacillus* spore germination (Hills, 1949). Following that discovery researchers have shown that *Bacillus* bacteria uses D-alanine as an auto inhibitor for spore germination at spore densities that are considered high when compared to available nutrients. This is considered a survival mechanism in *Bacillus* bacteria to prevent premature germination of bacterial spores in low nutrient environments and high spore density which will lead to cell death due to rapid consumption of available nutrients (Halvorson and Spiegelman, 1952; McKevitt *et al.*, 2007).

Among the different enantiomers of amino acids, the L-amino acids are the most common and naturally existing in nature and in many living organisms. L-amino acids are utilized for the manufacture of D-amino acids. The process of changing the L- amino acid to its D-amino acid enantiomer is called racemases; within racemases the stereochemistry of the chiral α-carbon atom is changed (Tanner, 2002).

Bacteria show great resistance to chemical, biological, and physical assaults. Mostly, due to the existence of the peptidoglycan cell wall, this is also known as the murein, which is located outside of the cytoplasmic membrane of most bacteria (Park, 1996; Holtje, 1998; Nanninga,

1998). The peptidoglycan cell wall is a flexible strong polymer that has a net-like shape, it is composed of linear glycan strands formed from disaccharide chains and cross linked by short polypeptides (Cava *et al.*, 2011). The peptidoglycan cell wall provides the bacterial cell with many benefits such as: protecting the contents of the bacterial cell, strength to hold its shape, resists the osmotic pressure, provides a framework structure to anchor cell envelop components (Holtje, 1998; Young, 2006; Dramsi *et al.*, 2008; Vollmer, Blanot and de Pedro, 2008). One interesting feature of the peptidoglycan cell wall composition is the D-amino acids that are present in the polypeptides (Nagata *et al.*, 1998). These D-amino acids help in forming the structure of the peptidoglycan cell wall and provide resilience to the known proteases (Cava *et al.*, 2011). The most common D-amino acids found in peptidoglycan cell wall are D-alanine and D-glutamate. However, the peptidoglycan cell wall of other bacteria incorporate other D-amino acids such as D-serine in *Staphylococcus aureus* (vancomysin-resistant bacteria), and D-aspartate in *Enterococcus* and *Lactococcus* (Sieradzki and Tomasz, 1996; de Jonge, Gage, and Xu, 2002; Reynolds and Courvalin, 2005; Bellais *et al.*, 2006; Veiga *et al.*, 2006). These incorporations are thought to increase the bacterial resistance to antibacterial agents (de Lencastre, Oliveira and Tomasz, 2007).

Previous researchers have concluded that D-amino acids are self-produced by *Bacillus subtilis* to disassemble their biofilms in the stationary phase of their life cycle (Kolodkin-Gal *et al.*, 2010). This is triggered when nutrients are limited and the products of metabolic waste have accumulated, which makes it more favorable to escape into the free planktonic cells form. In specific, D-tyrosine, D-tryptophan, D-leucine, and D-methionine have shown capabilities in

biofilm growth inhibition in both solid and liquid mediums (Lam *et al.*, 2009; Kolodkin-Gal *et al.*, 2010; Leiman *et al.*, 2013).

A study by Kolodkin-Gal et al. 2010 reported the existence of self-produced D-amino acids in mature biofilm growth medium (6 to 8 days old). In their experiments, they showed that a minimum concentration of 3µM, 5 mM, 8.5 mM, and 2 mM for D-tyrosine, D-tryptophan, D-leucine, and D-methionine respectively must be used to inhibit biofilm formation by *Bacillus subtilis*. Moreover, a mixture of the aforementioned D-amino acids with a concentration of 10 nM for each D-amino acid was potent and had synergetic results that dismembered the pre-existing biofilms. They also suggested that amyloid protein *TasA* fibers that connects the bacterial cells within the biofilm are anchored to the cell wall, when D-amino acids are introduced to the biofilm they incorporate them self into the cell wall by replacing the pre-existing D-alanine, and consequently the protein fibers are disengaged from their anchors which causes the biofilm to disassemble (Kolodkin-Gal *et al.*, 2010). They also stated that the corresponding L-amino acids had no effects on *Bacillus subtilis* biofilm disassembly, and that the presence of D-alanine will mitigate the inhibitory effects of the aforementioned D-amino acids. They also found similar results when same treatments were tested on biofilms from *Pseudomonas aeruginosa* (Kolodkin-Gal *et al.*, 2010). However, the direct interaction between the D-amino acids and the protein amyloid fibers is not the only mean by which biofilm disassembly is promoted by D-amino acids. The same researchers stated that D-amino acids dispersed biofilms from bacterial species that are not known to have amyloid protein fibers in their biofilm matrix such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Another study by Leiman et al. 2013 stated that D-tyrosine, D-tryptophan, D-leucine, and D-methionine can inhibit *Bacillus subtilis* biofilms. They suggested that the major inhibitory effect of the mentioned D-amino acids on biofilm growth arises from their interference with protein synthesis by their misincorporation into the protein synthesis. They also stated that D-tyrosine in specific is a metabolic inhibitor for *Bacillus subtilis*. It was also proposed that the presence of L-amino acids will mitigate the inhibitory effects of the D-amino acids on *Bacillus subtilis* biofilm. They also said that a mixture of the four amino acids at a concentration of 10 nM each was not sufficient for *Bacillus subtilis* biofilm inhibition, and a higher concentration (500 nM) is required for such inhibitory effects. They also suggested that the presence of D-alanine did not counter the effects of the four D-amino acids in contrast to the presence of L-tyrosine which countered the effects of the D-amino acids (Leiman *et al.*, 2013). This contradicts what was presented by Kolodkin-Gal et al. 2010.

Brandenburg et al. 2013 conducted a study that aimed to study the effects of amino acids on biofilm formation and motility of *Pseudomonas aeruginosa*. Results obtained showed that only D-tryptophan and D-tyrosine possessed inhibitory effects with D-tryptophan being most effective, while D-methionine and D-leucine interestingly increased biofilm growth. Moreover, at a concentration of 10 mM both tryptophan isoforms (D and L) were effective in biofilm inhibition with the L-tryptophan being more effective than D-tryptophan (86% and 71% at 24 hr respectively). A mixture with an equimolar ratio of both tryptophan isoforms (total concentration of 10 mM tryptophan) resulted in higher inhibitory effects (93% at 24 hr). They hypothesised that tryptophan enhances bacterial cell swimming motility (~40%) by increasing the flagellar activity of bacterial cells, which results in biofilm growth inhibition and

disassembly. Since bacterial cells that were under flagellar arrest before introducing tryptophan will prefer to detach from the biofilm (Brandenburg *et al.*, 2013). These results contradict what was presented in the previously mentioned two studies.

In summary, D-amino acids can inhibit biofilm formation and disperse pre-existing biofilms through different mechanisms which can be characterised by the following: (i) misincorporation of D-amino acids into the cell wall by replacing the D-alanine in the polypeptide chains, and consequently the amyloid protein fibers are disengaged from their anchors, (ii) D-amino acids can interfere with protein synthesis by their misincorporation into the protein synthesis, (iii) D-amino acids increase the flagellar activity of bacterial cells and promote free planktonic cells form. The fact that D-amino acids can be used to control biofilm formation is significantly promising due to the lack of toxicity and the favorable pharmacokinetic properties of D-amino acids (Jayaraman and Wood, 2008).

2.8 Enzymes

Enzymes are biological catalyst made from amino acids strings, they accelerate chemical reactions by lowering their activation energy, similar to other catalyst, enzymes do not get consumed within the reactions they catalyze (Berg, Tymoczko and Stryer, 2002). Enzymes have a limitless number of possible applications such as but not limited to cellular metabolism, detergents, food industry, animal feed, textiles, drugs, pulp, personal care, baking, and paper (Kirk, Borchert and Fuglsang, 2002). There is a current interest in enzymes to be used as anti-biofilm agents, this was after their reported promising results in the removal of biofilm from industrial surfaces (Taraszkiewicz *et al.*, 2013; Thallinger *et al.*, 2013; Meireles *et al.*, 2016).

Previous research have described enzymes as affordable, efficient, and echo-friendly alternative to the chemicals that have been used in eradicating biofilms (Cordeiro, Hippius and Werner, 2011; Cortés, Bonilla and Sinisterra, 2011; Srey, Jahid and Ha, 2013). Enzymes have been approved by regulatory agencies, and there has been no side effects reported from using enzymes for cleaning surfaces and in industry (Schmidt, 2012; Meireles *et al.*, 2016).

Enzymes mitigate biofilm formation by breaking the extracellular matrix components and weakening its structural integrity (Molobela, Cloete and Beukes, 2010). There are different factors that affect enzymes efficiency such as the substrate type and concentration, enzyme concentration, pH, temperature, cofactors or inhibitors, and presence or absence of activators (Madhumathi, 2007; Meireles *et al.*, 2016). The enzymes used to mitigate biofilm related problems has been categorised into anti-quorum sensing, oxidative enzymes, polysaccharide degrading enzymes, and proteolytic enzymes (Thallinger *et al.*, 2013).

Quorum sensing is the intercellular communication system used by bacterial cells in communities with high cell density (i.e. a Biofilm), it is used among bacterial cells to regulate many of their physiological activities including biofilm formation(Pearson, Van Delden and Iglewski, 1999; Meireles *et al.*, 2016). This communication system operates through the release, and detection of small self-produced molecules (autoinducers) used as signalling molecules among different bacterial cells within the biofilm community (LaSarre and Federle, 2013). Anti-quorum sensing enzymes such as lactonases and AHL acylase can prevent the signaling molecules from binding to their target (transcriptional regulators), this is done by the hydrolyses of the transcriptional regulators and/or the hydrolyses of the signalling molecule

respectively, eventually preventing them from bonding (Thallinger *et al.*, 2013). More information about quorum sensing can be found in previous work (Kalia, 2015).

Oxidative enzymes such as xanthine oxidase, deoxyribonuclease, and lipoxygenases can produce superoxide anions that are used to destroy invading pathogens, it can also disperse biofilms by targeting the DNA in the extracellular matrix of the biofilm (Valko *et al.*, 2007; Thallinger *et al.*, 2013; Okshevsky and Meyer, 2015a). This is similar to the defence system used in the human body defence system against pathogens (Thallinger *et al.*, 2013). Further information can be found on oxidative enzymes in many previous valuable studies (Hall-Stoodley *et al.*, 2008; Thomas *et al.*, 2008; Okshevsky and Meyer, 2015b).

Proteases (proteolytic enzymes) are enzymes that hydrolyse peptide bonds in proteins, they contain a range of enzymes that vary in their mechanisms and targeted substrates (Hedstrom, 2002). There are two major groups of proteases, exopeptidases are proteases that hydrolyse the peptide bond at the C-terminal and the N-terminal (i.e. Aminopeptidase and Carboxypeptidase), and endopeptidases are proteases that hydrolyse the peptide bond at the inner regions within the molecule (i.e. Savinase, Protamex, Subtilisin A, and Trypsin) (Obayashi and Suzuki, 2005). Previous researchers have reported promising results on the use of Savinase as an anti-biofilm agent (C Leroy *et al.*, 2008; Molobela, Cloete and Beukes, 2010).

A study by Marcato-Romain et al. 2012 investigated the effectiveness of different enzymes in reducing biofilm formation in paper industry. The extracellular matrix material (found to be mainly composed from proteins) was extracted from six industrial biofilms and treated with different enzymes (glycosidases, lipases, and proteases) at varying concentrations and contact

time. Only proteases were found to be effective in biofilm reduction, in specific, Savinase was found to be the most effective in biofilm reduction which exceeded 80% (Marcato-Romain *et al.*, 2012).

Protamex is an enzyme introduced first in fisheries for its ability to hydrolyse proteins (Dumay *et al.*, 2009; Nguyen *et al.*, 2011; Minh, 2015). To this date, no previous studied could be found on the use of Protamex as an anti-biofilm agent. Due to its wide use for protein hydrolysis in the fish industry, It was considered in this work as a potential anti-biofilm removal agent.

A study by Cordeiro et al. 2011 has investigated the effect of immobilized enzymes on the initial attachment of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on poly(ethylene-alt-maleic) anhydride copolymer surfaces. The enzymes Subtilisin A and Cellulase were immobilized by covalent bonding on the surfaces, and test control were prepared by using heat-inactivated enzymes on the surfaces. The tested surfaces were submerged in a suspension containing bacterial culture (10^6 CFU/mL) and incubated at 37°C for 24 hrs. The surfaces are then washed and the remaining attached cells were plated in serial dilutions for viable cell count quantification. The immobilized Subtilisin A reduced the attachment of *P. aeruginosa* by 44%, no effect on the attachment of *S. epidermidis* was noticed. The authors concluded that different biomolecules are involved in the initial attachment step among different bacterial strains which requires a wide spectrum of enzymes coatings to control biofilm attachment to surfaces (Cordeiro, Hippius and Werner, 2011).

Chaignon et al. 2007 investigated the use of the protease Trypsin for removing biofilms from different bacterial species (*S. epidermidis RP62A, S. epidermidis 444, S. epidermidis 5, S. lugdunensis 47, S. aureus 383, S. lugdunensis 18a*). The biofilms were grown in 96-well microtiter plates and then treated with Trypsin (100 µL) at 1 mg mL^{-1} in 20 mM Tris buffer (pH 7.5) for 2 h. Biofilms were measured indirectly using safranin stain (5%) and quantified via absorbance measurements at optical density of 492 nm. Their results showed that Trypsin was able to remove more than 70% of biofilms formed by *S. lugdunensis 47, S. epidermidis 444, S. aureus 383, S. lugdunensis 18a*. In contrary, Trypsin hand no effect on biofilm formed by *S. epidermidis 5* and *S. epidermidis RP62A.*, the authors hypothesised that this was due to their biofilm composition being rich in polysaccharides, which is not a suitable substrate for Trypsin. The authors stated that the removal of the polysaccharide-rich biofilm formed by *S. epidermidis 444* using Trypsin was a result of the interdependent roll of proteins and polysaccharides in the structural integrity of biofilms formed by this strain of bacteria (Chaignon *et al.*, 2007). In a similar study by Rohde et al. 2007, 98% of pre-grown biofilms from *Staphylococcus epidermidis* and *Staphylococcus aureus* were removed using Trypsin (100 µg/mL applied for 16 hrs at 37^oC) (Rohde *et al.*, 2007).

Polysaccharides and polysaccharide-degrading enzymes are naturally abundant, with bacteria being the main source for polysaccharide-degrading enzymes, these enzymes are used by bacteria to takedown host defences in nature (Jedrzejas, 2000). There are two main types of polysaccharide-degrading enzymes lyases and hydrolases. Lyases enzymes degrade polysaccharides using the β-elimination process that is based on the mechanism of proton donation and acceptance, more information can be found in previous research (Jedrzejas and

Chantalat, 2000). Hydrolases enzymes degrade polysaccharides by the hydrolysis of the glycosidic bonds between the building blocks of the sugar, some of the hydrolases enzymes use the mechanism of direct-displacement where a water molecule replaces the leaving group from the substrate (Jedrzejas, 2000). The polysaccharide-degrading enzyme Cellulase is vastly used as an anti-biofilm enzyme for the removal and prevention o biofilms (Loiselle and Anderson, 2003; Cordeiro, Hippius and Werner, 2011).

Cordeiro et al. 2011 reported that Cellulase was effective in reducing the attachment of *S. epidermidis* by 67%, while it had no effect on biofilms formed by *P. aeruginosa*, the researchers followed the same procedure explained previously for subtilisin A (Cordeiro, Hippius and Werner, 2011).

A study by Loiselle et al. 2003 investigated the use of Cellulase for inhibiting biofilm formed by *P. aeruginosa*. The biofilm was gown on a glass slide for four days inside a flow chamber. Biofilm formation was quantified using viable cell counts (CFU) and by biomass areal density (mg cm^{-2}). The biofilm tested was grown in the presence of Cellulase at different concentrations and pH values. Controls were prepared using deactivated Cellulase. The results presented showed that Cellulase could partially inhibit biofilm. The highest biofilm areal density removal achieved using Cellulase was 88% at concentration of 75.2 U mL^{-1} at pH 5. While viable cell counts was reduced 60% at concentration of 9.4 U mL^{-1} and pH 5, no effect of concentration increase was noticed for viable cell counts (Loiselle and Anderson, 2003).

Pectinases are heterogenous mixtures of enzymes that hydrolyse pectic substances, they are abundant in plants and microorganisms (Jayani, Saxena and Gupta, 2005).

Pectic compounds are polysaccharides that are negatively charged, acidic, macromolecules that have high molecular weight, they are abundantly found in the plant kingdom (Jayani, Saxena and Gupta, 2005).Pectinases have an important role in the plant kingdom in the degradation of the plant cell wall (Ward and Moo-Young, 1989). They also have an important role at plant maturation in softening the plant tissue (Sakai, 1992), they also have an ecological role in the degradation and recycling of plant waste material (Lang and Dörnenburg, 2000).

A study by Johansen et al. 1997 investigated the use of Pectinex as an anti-biofilm agent on biofilms formed by *Staphylococcus aureus ATCC 25923, Pseudomonas fluorescens AH2, and Pseudomonas aeruginosa ATCC 10148*. Discs made from steel and polypropylene were vertically immerged in growth medium and the biofilm was allowed to form on both sides of the discs while stirring continuously at 200 rounds per minute for four days at 26°C. The loosely attached cells were then removed by washing the discs using phosphate buffer (pH 7), following to that, the discs were incubated in different concentrations of Pectinex prepared in phosphate buffer for 15 min, static, at 20 ^{o}C. Control samples were prepared using phosphate buffer solution containing no Pectinex. The biofilm removal was assessed using the total number of cells and the number of respiring cells, which were quantified using fluorescence microscopy. The presented results showed that Pectinex was successful in removing biofilms formed by all tested bacterial strains. The cell number for *P. aeruginosa* and *S. aureus* were reduced by two log reductions, while the cell number count for *P. fluorescens* was reduced one log reduction, at a Pectinex concentration of 180 PSU mL^{-1} (Johansen and Falholt, 1997).

The first obstacle to overcome in biofilm removal is the extracellular matrix which shields and covers the bacterial cells living within the biofilm community. Due to the abundance

of available enzymes, the different biofilm composition among different bacterial strains, and the substrate specificity of enzymes (Simões, Simões and Vieira, 2010), it is important to investigate and compare the ability of different available enzymes to remove biofilms. Although biofilms have a complex composition, they are composed mainly from polysaccharides and proteins, which makes polysaccharide-degrading enzymes and proteases the most suitable enzymes for their removal, this due to their substrate compatibility (Meyer, 2003). Hence, polysaccharide-degrading enzymes and proteases will be investigated in this work.

2.9 References

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Chapter 3 - Experimental Work

3.1 Preface

The results of the experimental work conducted in this thesis are presented in Chapter Chapter 3. The experimental work is presented in four subsections (subsections 3.2-3.5), each subsection represents a separate contribution. Each subsection was directed toward one of the four specific research objectives presented previously in Sections (1.3.2.1-1.3.2.4).

3.2 Biofilm Control Using Norspermidine

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3.2.1. Preface

Section 3.2 presents the experimental work conducted to fulfill the specific objective presented in Section 1.3.2.1. The work presented in this section provides a possible explanation for the apparent contradiction in previous work presented by different researchers on the role of norspermidine in removing biofilm. Based on the results presented in Section 3.2 norspermidine can remove nonactive biofilms, and enhance the growth of active biofilms. The apparent controversy in previous published research was attributed to the different viable state of the treated biofilm. Section 3.2 provides quantitative information on biofilm removal and biofilm growth enhancement using norspermidine.

3.2.2. Introduction

When free planktonic bacterial cells find a surface in a favorable environment they tend to colonize it by forming biofilms (Henrici, 1933; Torres *et al.*, 2012; Daniel *et al.*, 2016). While in biofilm formation, bacterial cells aggregate to form a protective layer that shelters the cells underneath. In doing so, bacteria have evolved to tolerate low concentrations of antibiotics and antimicrobials (O'Toole, Kaplan and Kolter, 2000; O'Toole and Stewart, 2005). Bacterial cells in a biofilm are connected together by an extracellular matrix, which consists of polysaccharides, protein fibers, and in some cases DNA, with the exopolysaccharides and proteins being the main constituent (Donlan, 2002; Hall-Stoodley, Costerton and Stoodley, 2004; Molobela, Cloete and Beukes, 2010; Daniel *et al.*, 2016). Biofilm formation, a vital part of the bacteria life cycle, in harsh environments, also positions the bacteria to access nutrients (Costerton *et al.*, 1987; DeBeer, Stoodley and Lewandowski, 1994). Biofilms have a complex architectural shape that contains channels for the flow of nutrients within and throughout their community (DeBeer, Stoodley and Lewandowski, 1994). Naturally occurring biofilms are difficult to fully characterize, for example only 4% of the bacterial cells living in biofilm communities in dental units water lines (DUWL) are cultivable, with the rest being non-cultivable or dead (Barbeau *et al.*, 1996; Barbeau, Bokum and Gauthier, 1998). Biofilms can adhere to solid surfaces at the liquid-solid interface and can also form floating biofilms called pellicles in the liquid-air interface of static cultures (Bryers, 2008; Cava *et al.*, 2011; Kolodkin-Gal *et al.*, 2012). Biofilm formation in medical and industrial settings have been problematic, this is due to the adverse effects of the pathogens they can potentially harbor on public health (Rowland and Voorheesville, 2003), and

due to their negative interference with the mechanical functions of industrial settings (Torres *et al.*, 2012).

The typical ‘life cycle’ of a biofilm can be summarized in five main cyclical stages: free planktonic cells, initial attachment, early biofilm, mature biofilm, biofilm disassembly. After biofilm disassembly, many bacteria return to their original free planktonic form (Palmer and White, 1997; O’Toole, Kaplan and Kolter, 2000). Environmental stress promotes biofilms formation with the availability of nutrients being the main driving force; some other environmental factors can affect biofilm formation such as temperature, pH, iron, and oxygen with all these factors being microorganism specific (O’Toole, Kaplan and Kolter, 2000). At late stages of the mature biofilm, bacterial cells have been reported to secrete small molecules such as polyamines and D-amino acids. These molecules have been reported to target the exopolysaccharides and protein fibers in the extracellular matrix that connect the bacterial cells in the biofilm, initiating the biofilm disassembly and the release of planktonic cells and bacterial spores as a survival mechanism. Released planktonic cells and spores will migrate to find another favorable location and initiate a new biofilm community (Kolodkin-Gal *et al.*, 2010, 2012).

Controlled studies are typically undertaken with single species biofilms. The timeline of such lab experiment can differ substantially from natural biofilms (Jahid *et al.*, 2015). The life span of biofilms formed by *Bacillus subtilis* in lab studies is in the order of days (Kolodkin-Gal *et al.*, 2010, 2012; Hobley *et al.*, 2014), while biofilms in natural environments can form over longer durations (Jahid *et al.*, 2015). In lab studies biofilms of *B. subtilis* can matures in three days, and after eight days it disassembles and start releasing the free planktonic cells (Kolodkin-

Gal *et al.*, 2010; Romero *et al.*, 2011). Biofilms formed over longer durations exhibited some variations from biofilms formed over short durations (i.e. higher tolerance to harsh environments) (Jiang *et al.*, 2017) .

Polyamines are believed to function as growth factors in microorganisms, thus they are found naturally in all types of bacteria and in most animal cells (Tabor and Tabor, 1984). There has been an increased interest in the recent years in polyamines due to their debated role in biofilm formation (Hobley *et al.*, 2014; Nesse, Berg and Vestby, 2015; Cardile *et al.*, 2017).

There exists today some high-profile controversy among researchers on the interactions between the polyamine norspermidine and biofilms formed by *B. subtilis*. It was initially identified as an agent involved in biofilm disassembly in a highly-cited study, which was later retracted (Kolodkin-Gal *et al.*, 2012). While other researchers proposed norspermidine to play an essential role in the formation of a robust biofilm of the same organism (Hobley *et al.*, 2014). There is a lack of quantitative studies on the interaction between norspermidine and biofilm formation. It was therefore the objective of this study to clarify the effects of norspermidine on *Bacillus sp.* biofilm formation by investigating its effect on biofilms of different viable states.

3.2.3. Experimental Procedures

3.2.3.1. Materials

3.2.3.1.1 Macronutrients Concentrate

To prepare 100 mL macronutrients, 100 mL DI-Water, 1.9 g $MgCl_2$, 1.03 g $CaCl_2.2H_2O$, 0.1 g $MnCl_2.4H_2O$, 0.1 g $FeCl_2.4H_2O$, 0.0014 g $ZnCl_2$, sterile filter.

3.2.3.1.2 Amino Acids and Thiamine Concentrate

To prepare 100 mL, 100 mL DI-Water, 0.5 g L-tryptophan, 0.5 g L-phenylalanine, 0.5 g L-threonine, 0.006 g Thiamine-HCL, sterile filter.

3.2.3.1.3 10X Tbase

To prepare 250 mL, 4.95 g $(NH_4)_2SO_4$ (0.15M ammonium sulphate), 34.84 g K_2HPO_4 (0.8M potassium phosphate dibasic), 14.97 g KH_2PO_4 (0.44M potassium phosphate monobasic), 2.5 g Trisodium citrate (34 mM sodium citrate), add DI-Water to bring the total volume to 250 mL, autoclaved for 20 min.

3.2.3.1.4 Biofilm Growth Media 1 (BGM1)

To prepare 1 L, 900 mL of DI-Water, 10 g tryptone, 10 g NaCl, 5 g yeast extract, 0.04 g D-glucose), 0.012 g $MgSO_4$, and finally add 10 mL of 10X Tbase, autoclave for 20 min.

3.2.3.1.5 Biofilm Growth Media 2 (BGM2)

To prepare 1L, 0.871 g K_2HPO_4, 20.9 g MOPS free acid, 2 mL of 10M KOH, 5 g glycerol, 5 g glutamic acid, bring volume to 1L using DI-Water, sterile filter the mixture, remove 1 mL from the mixture and add 1 mL micronutrients concentrate, remove 1 mL from the mixture and add 1 mL amino acids and thiamine concentrate.

3.2.3.1.6 Wash Buffer (Sterile)

To prepare 1 L, 900 mL DI-Water, 1 ml of 1M $MgSO_4$, 100 mL of the 10X Tbase solution, autoclave for 20 min.

3.2.3.1.7 Crystal Violet Stain

To prepare 1 L of 0.1 % (w/v) crystal violet stain, 1 gm of crystal violet powder, 1 L of wash buffer. Stir the mix until the crystal violet dissolve completely.

3.2.3.1.8 Sodium Phosphate Buffer (SPB)

To prepare sodium phosphate buffer at pH 7.6, first prepare 60 mM of $Na_2HPO_4.7H_2O$ solution by dissolving 16.1 g in 1 L DI-Water, then prepare 60 mM of $NaH_2PO_4.H_2O$ solution by dissolving 8.28 g in 1L DI-Water. Mix the two solutions until the desired pH is obtained (the desired pH was obtained by mixing 12.5 mL of 60 mM Na_2HPO_4.7H2O to 1 mL of 60 mM $NaH_2PO_4.H_2O$).

3.2.3.2. Methods

3.2.3.1.9 Bacterial Strain

The bacterium used in this research is a *Bacillus sp.* known commercially as Unizyme 20X CSB 20 NF SPORE, obtained from Germiphene Inc. Brantford, Ontario, Canada.

3.2.3.1.10 Seed Culture Preparation (sterile procedure)

Four milliliters of *Bacillus sp.* spore suspension are poured into a small vial. Using a pipette 1 mL of the poured spore suspension is transferred into a sterile microcentrifuge vial, centrifuge at 10,000 rounds per minute for 60 seconds. At this point, bacterial spores should be visible to the naked eye as a white precipitated powder in the bottom of the vial. The supernatant is removed and 1 mL wash buffer is added. The vial is then mixed using a vortex until the bacterial spores are completely dissolved, following that the sample is centrifuged again, pour off the supernatant, another 1 mL of wash buffer is added, vortex again, centrifuge, pour off supernatant. Now the spore suspension is washed two times using the wash buffer. One

milliliter of biofilm growth media is added to the vial containing the washed *Bacillus sp.* spores and the sample is mixed using a vortex until completely dissolved. Half a milliliter of the *Bacillus sp.* washed spores and re-suspended in biofilm growth media is added into a sterile 125 mL flask with a cover that contains 20 mL of biofilm growth media and a stir bar. The mixture is then placed on the magnetic stirrer at 300 rounds per minute inside a 37 ^{o}C humidified incubator oven for 270 min until optical density at 600 nm is between 1 and 1.6 when Milli-Q water is used as blank.

3.2.3.1.11 Inoculating the Microtiter Plates with Seed Culture (sterile procedure)

Biofilm was grown in microtiter plates, a common method used in biofilm related studies (Kolodkin-Gal *et al.*, 2012; Hobley *et al.*, 2014; Liu *et al.*, 2015). A 200-mL glass petri dish with a flat bottom is filled with biofilm growth media (each plate requires 10 mL) and a stir bar. The prepared *Bacillus sp.* seed culture is then diluted in a 1:100 ratio in biofilm growth media. The petri dish is then placed on a magnetic stirrer at 300 rpm. It should be assured that no bubbles are formed, if that happens the rpm should be lowered until no more bubbles are formed. A multichannel pipette is then used to transfer 100 µL from the petri dish while stirring into wells in the microplates. Also, 100 µL of sterile biofilm growth media are transferred into control wells. Each plate is then covered with a sterile thin film (25 µm) to seal it and reduce evaporation, one hole is poked in the center of each well using a 22-gauge sterile needle. Separate needles should be used for each of the inoculated wells and control wells to ensure no cross contamination will occur. The microplates are then incubated at 37 ^{o}C humidified oven in static conditions for 32 h. Following that, plates are washed with wash buffer.

3.2.3.1.12 Washing Microtiter Plates with Buffer Solution

The thin film placed on the microplate is removed and then the microplate is placed in the Tecan Hydroflex Plate Washer. Each well in the plate is filled with 100 µL of wash buffer solution using the drip mode and then wash buffer is aspirated. This will remove the remaining media and free cells (Stepanović *et al.*, 2000). The device must be calibrated not to touch the sides or the bottom of the wells. Following that 150 µL of wash buffer solution is dispensed and aspirated same as the previous step, the process is repeated for a total of three times. Finally, the microtiter plate is gently flicked and the face of the microtiter plate is turned facing down on a paper towel to release any excess liquid.

3.2.3.1.13 Fixing Biofilm Growth

This step in performed to fix initial biofilm growth that will be compared with the biofilm remaining in other wells after applying treatment to them. Fixed biofilms are assumed to be nonactive or dead. This process is repeated at the end of the treatment duration to treated wells and prior to biofilm quantification. Using a multichannel micropipette, the volume of 200 µL of methanol (99%) are dispensed into desired wells. The microtiter plate is then covered with aluminum foil to prevent evaporation. After 20 min, the methanol is removed using the multichannel micropipette. Plates are then left to air dry at room temperature (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010).

3.2.3.1.14 Applying Treatments

After the thin film is removed and the wells are washed with the buffer solution. Treatment is applied by dispensing 125 µL of treatment solution into desired wells using the multichannel micropipette. The microtiter plates are then covered with thin film and holes are poked in the

center of each well using the 22-gauge sterile needle as described previously. The microtiter plates are then incubated in a humidified oven with the temperature set at 37 ^{o}C for the decided treatment duration.

3.2.3.1.15 Total Biofilm Quantification Using Crystal Violet Stain

Crystal violet binding assay has been used in abundant by researchers to quantify biofilms formation from gram-positive bacteria such as *Bacillus Subtilis* (O'Toole, Kaplan and Kolter, 2000; Kolodkin-Gal *et al.*, 2010, 2012; Brandenburg *et al.*, 2013). This method was initially described by Christensen et al. 1985, which was followed with many improvements to increase its accuracy and to allow biofilm quantification in the microtiter plate wells (Stepanović *et al.*, 2000).Crystal violet is a basic dye that binds to the exopolysaccharides and negatively charged surface molecules and stains them with a violet color, following that crystal violet can be easily resolubilized using an acetic acid solution(Li, Yan and Xu, 2003; Negri *et al.*, 2010). Due to the fact that crystal violet binds to both dead and alive cells in addition to the extracellular matrix, this method remains not suitable for evaluating the ability to kill biofilm cells or to measure disinfectants efficiencies on biofilms (Li, Yan and Xu, 2003).

To quantify biofilm formation in the microtiter plates, 150 µL of 0.1 % (W/V) crystal violet is dispensed into each well using the multichannel micropipette. The microtiter plate is covered to prevent evaporation and incubated at room temperature for 60 min (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010). Microtiter plates are washed 4 times by dispensing 200 µL DI-water in drip mode and aspirating using the Tecan Hydroflex plate washer (washing the plates 4 times have proven to produce a washing waste liquid that is free of stain). Microtiter plates are then left to air dry at room temperature. The crystal violet is then resolubilized by dispensing

200 µl 30% acetic acid into each well using the multichannel micropipette (Stepanović *et al.*, 2000). The microtiter plates are then covered and incubated without shaking at room temperature for 20 min. Using the multichannel pipette, transfer 100 µL from each well to new hard polystyrene plate with the clear flat bottom for quantification. Absorbance at OD 570 nm is measured using a TECAN infinite M200 Pro plate reader (Stepanović *et al.*, 2000). The mean OD 570 nm value for the media only controls is subtracted from the OD 570 nm of the treated wells, this represents the environmental noise in the measurements.

3.2.3.1.16 Biofilm Viability Using Fluorescein Diacetate

Biofilm viability following treatment will be measured using the fluorescein diacetate (FDA) method. The FDA method has been used by several researchers for viability measurements (Clarke *et al.*, 2001; Wanandy *et al.*, 2005; Armour, Powell and Boyce, 2008). A stock solution of FDA is prepared with a concentration of 10 mg mL^{-1} FDA in acetone. FDA Stock solution samples are then stored in the freezer at -80 ^{o}C.

The prepared FDA stock solution samples are taken out from the -80 ^{o}C freezer. The FDA stock solution is then diluted (1:50) using sodium phosphate buffer (60 mM, pH 7.6), this will result in a 0.2 mg mL^{-1} FDA concentration. Using a multichannel pipette, 100 µL of the sodium phosphate buffer (60 mM, pH 7.6) is dispensed into each tested well. The diluted FDA stock solution is vortexed and then 100 µL is dispensed into each tested well (FDA concentration in each well is now 0.1 mg mL^{-1}). Microplates are covered with thin film and a single hole is poked at the center of each tested well. The microplates are then covered with foil for darkness and incubate at 37 ^{o}C for 1 h with shaking at 150 rpm. Transfer 100 µL from each well to a rigid flat bottom black fluorescent plate for reading. All fluorescence measurements were quantified

using M1000 Plate Reader ($\lambda_{excitation}$ = 405 nm, $\lambda_{emission}$ = 520 nm, top mode, optimal gain, and no lid). The response of the assay is enhanced by norspermidine addition; hence a washing step was introduced prior to the addition of FDA. The effectiveness of the washing was verified experimentally (data not shown).

3.2.3.1.17 HPLC analysis

The supernatant in the microtiter plates was collected following the formation of biofilms amended with different concentrations of norspermidine. Samples collected and prepared standards were benzoylated using the procedure explained in Morgan (1998) (Morgan, 1998). In the benzoylation process, a benzene ring is attached to the polyamine to be able to detect it using the HPLC system. The HPLC analysis utilized an Agilent 1260 Infinity instrument equipped with an autosampler and an Agilent 1260 Infinity Diode Array Detector (DAD) for UV-detection set at 229 nm. The column used was a ZORBAX 300SB-C18 (4.6 x 250mm, 5µm). The sample run duration was 10 min, isocratic with 45% acetonitrile in water, sample injection volume of 40 µL, flow rate of 0.8 mL/min, and the column temperature was 25 ^{o}C.

3.2.4. Results

3.2.4.1. Biofilm Formations in Different Microtiter Plates are Comparable

All biofilm studies were conducted by cultivating *Bacillus sp.* in microtiter plates based on a modified method (Stepanović *et al.*, 2000; Branda *et al.*, 2001; Kwasny and Opperman, 2010). Reproducibility of biofilm growth and removal within the envisioned parameter space is crucial. It was therefore evaluated first, in order to validate comparisons between different wells within the same microtiter plate and between different microplates prepared from the same batch. Biofilm growth was quantified in 80 wells per plate in three separate plates

following a 32 h incubation time. A one-way ANOVA test was performed for inter-plate comparison of biofilm formation after 32 h incubation time. The results showed no significant difference (p-value = 0.34) among the three plates. The average biofilm formations in the three plates (based on 80 wells/plate) with a 95-per-cent confidence interval were 1.66 ±0.041, 1.69 ±0.037, and 1.71 ±0.043 Absorbance Units (based on crystal violet staining assay), respectively. The standard deviation between the three different plates' averages was found to be 0.022.

3.2.4.2. Biofilm Formation Increased for Active Biofilms in the Presence of Norspermidine, Crystal Violet Staining.

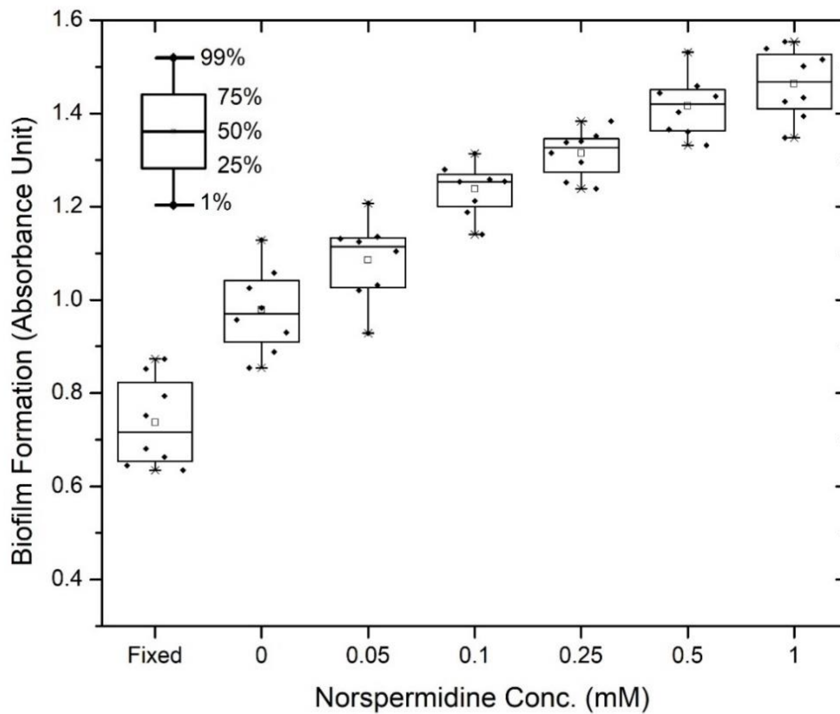

Figure 3-1. Biofilm Formation Increased Following the Addition of Norspermidine to Active Biofilm.

To investigate the effects of norspermidine on active biofilm, microtiter plates were inoculated with *Bacillus sp.* and the biofilm was grown for 32 h at 37°C. Following that, the plates were washed to remove existing media and one column (8 wells) was inactivated and fixed using methanol (the fixed column represent initial biofilm growth) (Stepanović *et al.*, 2000). The remaining wells in the microtiter plates were then filled with 150 µL fresh media (BGM2, only promoting slow biofilm formation compared to BGM1) and different concentrations of norspermidine and incubated at 37°C for an additional 24 hrs. At the end of the treatment time, the amount of biofilm was quantified.

The results of the quantification (crystal violet staining analysis, *Figure 3-1*) show that additional biofilm formation occurred (denoted with 0 mM norspermidine in *Figure 3-1*) when compared to the biofilm that was fixated after 32 h. The amount of biofilm increased with increasing the amount of norspermidine in the medium, up to 73% at 1 mM norspermidine.

3.2.4.3. Biofilm Formation Decreased for Nonactive Biofilms in the Presence of Norspermidine, Crystal Violet Staining.

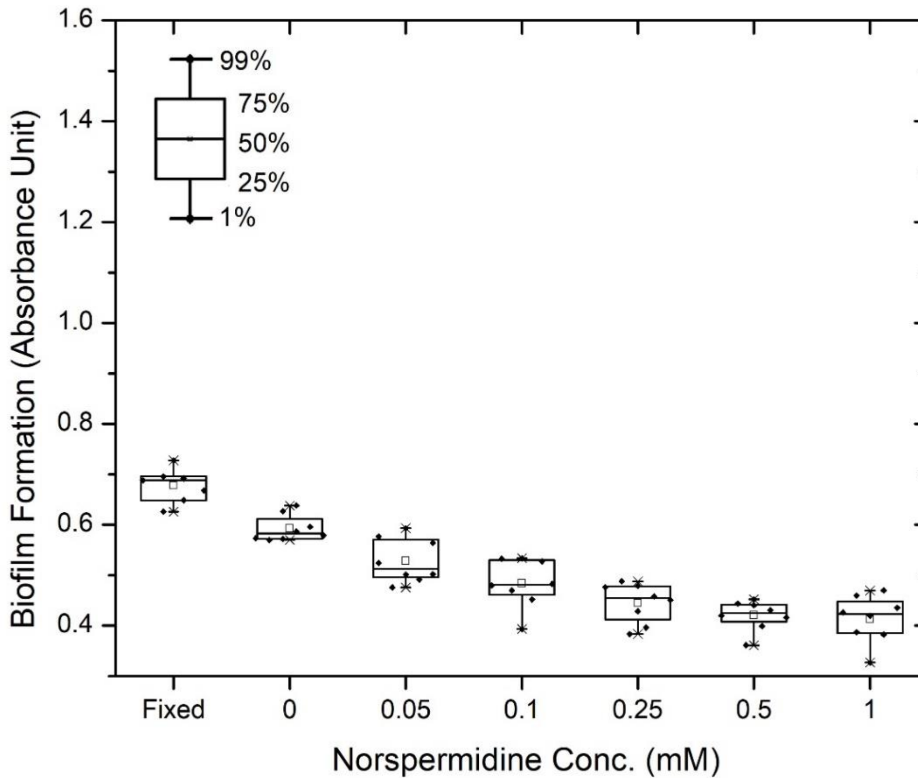

Figure 3-2. Biofilm Formation Decreased Following the Addition of Norspermidine to Fixed Biofilm.

The effects of norspermidine on non-active biofilm was investigated in a similar way as described above, only that all wells were inactivated with methanol. Quantification of the biofilm after incubation in the presence of norspermidine (crystal violet staining) is shown in *Figure 3-2*. Under the employed conditions, a slight decrease was observed in the amount of quantifiable biofilm for the control wells without norspermidine, while this effect was substantially increased with increasing concentrations of norspermidine. Up to 39% removal

was achieved for the norspermidine concentration of 1 mM on fixed, non-active biofilm (*Figure 3-2*).

3.2.4.4. Norspermidine in Growth Media was Consumed When Added to Active Biofilms

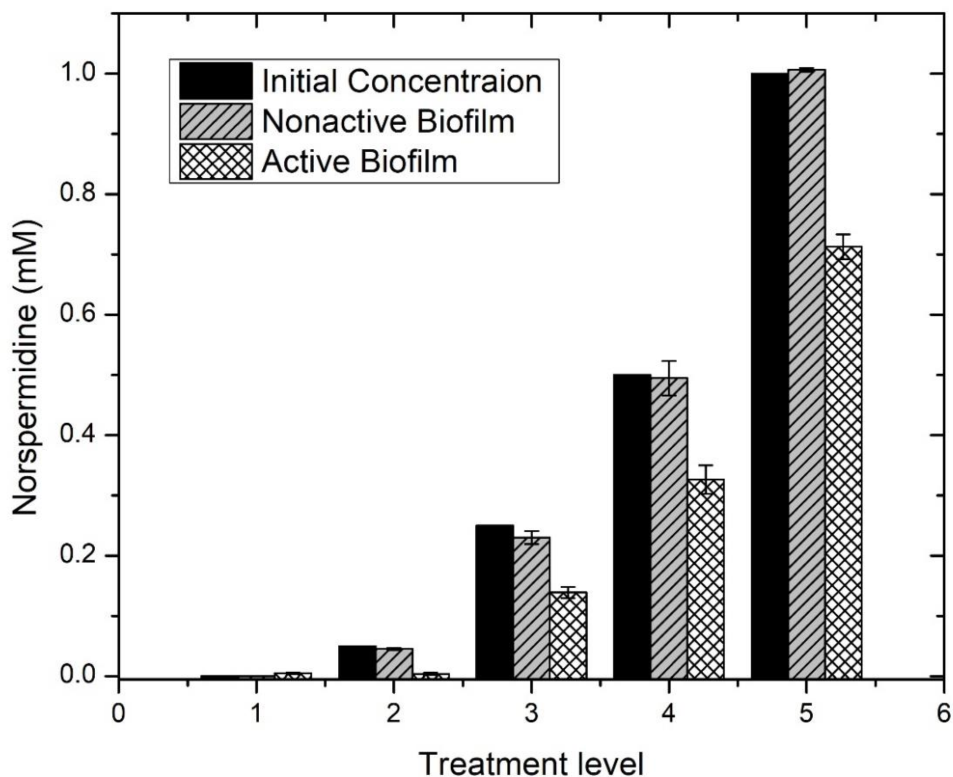

Figure 3-3. Norspermidine Concentration following 24 h Norspermidine Treatment Decreased with Active Biofilm, But Not for Nonactive Biofilm. Error bars represent 95% confidence interval (n=12).

In order to understand the nature of the interaction between norspermidine and the biofilm, the supernatant remaining in the microtiter plates following the 24 h treatment was collected and analyzed for remaining norspermidine via HPLC (Morgan, 1998). It can be seen from *Figure 3-3* that the amount of supplemented norspermidine was reduced in the active

biofilm samples. Residual norspermidine could be detected, however, the more biofilm was formed (*Figure 3-1*), the more norspermidine was reduced (*Figure 3-3*). This effect was not observed in the presence of inactive biofilm (fixed), where the norspermidine concentration after incubation is similar to the initially available amount.

3.2.4.5. Verification of Previous Results with Secondary Activity Assay (FDA testing)

The biofilm quantification via crystal violet staining, does not discriminate between living or dead cells. The observed increase in biofilm formation in the presence of norspermidine was therefore further investigated through the fluorescein diacetate (FDA) viability assay.

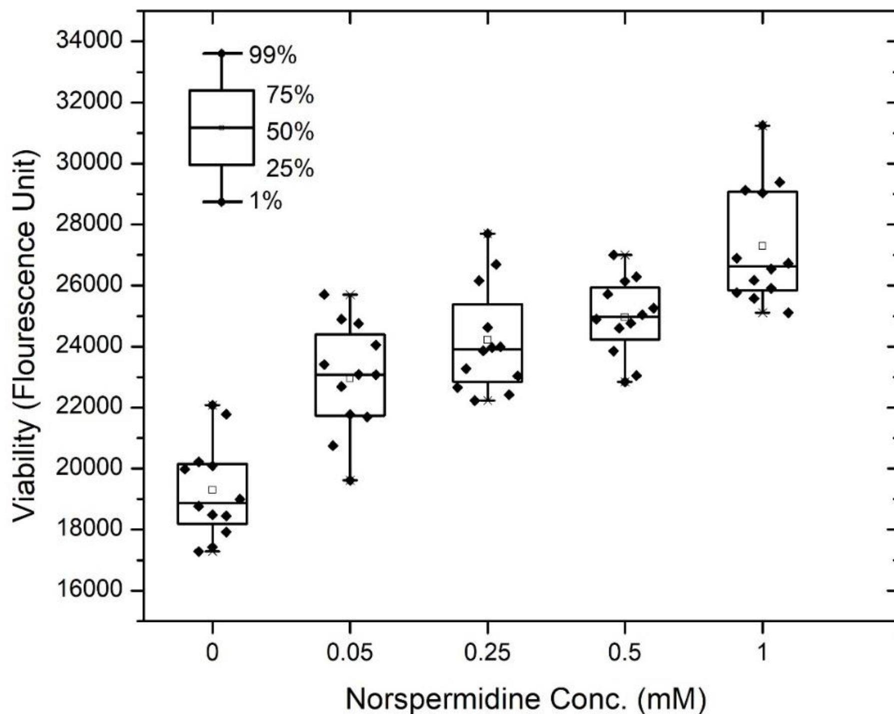

Figure 3-4. Norspermidine Addition Enhanced Biofilm Viability.

The intra-plate reproducibility of the FDA testing was evaluated to validate the comparison between the different treatments within the same microtiter plate. Biofilm grown within the same microtiter plate under the same conditions was tested using the FDA method. A one-way ANOVA test was performed for intra-plate comparison of biofilm viability. The results showed no significant difference (p-value = 0.19) among the ten-tested columns (each column contain 8 wells) within the same microtiter plate (data not shown).

Following the growth of biofilms for 32 h, norspermidine treatment was applied with different concentrations for 24 h. The biofilm grown in the microtiter plates was washed five times using sodium phosphate buffer saline, this was to assure that norspermidine was not left behind, interfering with the FDA test results. Following that, the enzymatic activity of the biofilm was measured using the FDA viability test and the results are presented in *Figure 3-4*. Biofilm treated with higher concentration of norspermidine showed higher enzymatic activity with a significant difference between concentrations of 0 and 1 mM norspermidine. The data follows the same trend as the data generated through total biomass quantification via crystal violet staining (*Figure 3-1*), hence verifying via a different detection mechanism.

3.2.5. Discussion

The effect of increased norspermidine concentrations on active biofilm was associated with an increase in biofilm formation (*Figure 3-1*). No inhibitory effects were detected for norspermidine concentrations on biofilm formation, up to 1 mM. These results appear to be in contradiction with findings reported by Kolodkin-Gal et al. (2012), showing that an inhibitory effect of norspermidine on biofilm formation at concentrations as low as 0.05-0.08 mM. While the results of this study partially agreed with Hobley et al. (2014) whom found that the addition

of norspermidine at concentrations 0.01- 0.025 mM resulted in more robust biofilm formation, they also reported inhibitory effects on biofilm formation at concentrations higher than 0.25-0.30 mM, which was not observed here. The results obtained in *Figure 3-1* suggests that norspermidine addition can be beneficial in situations where rapid biofilm growth is favored. Norspermidine can also be utilized in other applications were biofilm formation is beneficial (i.e. plant biocontrol agents, inhibitor of mild steel corrosion, bioreactor, and bioremediation) (Morikawa, 2006), for more information please refer to Morikawa (2006).

Naturally occurring polyamines, such as, spermidine and spermine, are reported in higher concentrations in fast growing tissues and are involved in the process that controls cellular growth in prokaryotic cells such as bacteria (Tabor and Tabor, 1984; Rodriguez-Garay, Phillips and Kuehn, 1989). Polyamines modulate membrane fusion and protect the functionality and structural integrity of the bacterial organisms (Meers *et al.*, 1986; Marton and Morris, 1987). Since the shorter polyamine, norspermidine can replace the essential role of spermidine in the formation of robust biofilms (Burrell *et al.*, 2010; Hobley *et al.*, 2014), this provides a possible explanation for the increase in biofilm formation associated with the increase of norspermidine concentration (*Figure 3-1*). The biological utilization of norspermidine in different cellular processes offers a possible explanation for the increase in biofilm formation, which was associated with the depletion of norspermidine concertation in the supernatant extracted from the microtiter plate wells containing actively growing biofilms (*Figure 3-3*), and an increase in viability of the biofilm (*Figure 3-4*).

Previous researchers have referred to norspermidine as a biofilm disassembly agent that prevents biofilm formation and disassembles pre-existing biofilm (Kolodkin-Gal *et al.*, 2012;

Böttcher *et al.*, 2013; Oppenheimer-Shaanan, Steinberg and Kolodkin-Gal, 2013). This apparent contradiction can likely be contributed to the different vital stages of the biofilms in question. This study clearly shows that norspermidine accelerates biofilm dissolution when its biological activities were artificially stopped by fixing the biofilm using methanol. The results of the interactions between norspermidine and the pre-existing nonactive biofilm are the direct opposite of the results found with the active biofilm. Since the biofilm was no longer actively growing, the interaction between the added norspermidine concentrations and the nonactive (fixed) biofilm was of a pure chemical nature and it is assumed that no biological process take place. When the concentration of added norspermidine increased, the pre-existing nonactive (fixed) biofilm removal increased (*Figure 3-2*). It is reported that norspermidine targets the exopolysaccharides component in the biofilm. Norspermidine binds to the negatively charged groups (i.e. uronic acid) or to the neutral sugars with polar groups (i.e. poly-*N*-acetylglucosamine) in the secondary structure of the exopolysaccharides through Coulombic attraction and hydrogen bonding (Kolodkin-Gal *et al.*, 2012; Oppenheimer-Shaanan, Steinberg and Kolodkin-Gal, 2013). These interactions between polyamines and biofilms are structure and charge specific; only polyamines with three methylene groups and flanked by two amino groups that are positively charged were successful in dismembering the biofilms (i.e. norspermidine, norspermine, …etc) (Kolodkin-Gal *et al.*, 2012). The aforementioned mechanism provides a possible explanation for the removal of the pre-existing nonactive (fixed) biofilm noticed in *Figure 3-2* following the addition of norspermidine.

3.2.6. Conclusions

Based on these results, with regards to biofilm formation and disassembly, it could be concluded that norspermidine can serve two functions. When norspermidine was added to an active biofilm, the biofilm formation increased due to the biological utilization of norspermidine, with no inhibition of biofilm formation at concentrations as high as 1 mM. On the other hand, when norspermidine was added to a pre-existing nonactive (fixed or dead) biofilm, biofilm formation was disassembled due to chemical interactions between norspermidine and the exopolysaccharides part of the biofilm. The results obtained in this research emphasizes that when it comes to investigating and testing of biofilm removal agents, the biological activity of the biofilm must be carefully considered. The interaction between the biofilm and norspermidine was dependent on its biological activity. When added to an active biofilm, biological process dominated the chemical interaction between norspermidine and the exopolysaccharides, which resulted in the increase of biofilm formation (*Figure 3-1*). The chemical interaction between the exopolysaccharides and norspermidine were likely not significant until the biological processes had ceased (after fixing the biofilm with methanol) and this resulted in the disassembly of the pre-existing nonactive biofilm (*Figure 3-2*) due to the interactions between norspermidine and the exopolysaccharides in the pre-existing biofilm. The fluorescein diacetate testing results also supported these findings.

3.2.7. Acknowledgments

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3.3 Biofilm Control Using Amino Acids

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3.3.1. Preface

Section 3.3 presents the experimental work performed to fulfill the specific objective presented in Section 1.3.2.2. The presented results in Section 3.3 provided a quantitative evaluation of biofilm removal using D-amino acids. The results obtained in Section 3.3 suggests that D-amino acids have limited biofilm removal capabilities, while they were found to be more suitable for inhibiting biofilm growth. No clear synergetic effects were noticed from the combined treatment.

3.3.2. Introduction

Biofilms are multicellular communities formed by bacterial aggregation on surfaces. The bacterial cells within the biofilm are connected together by an extracellular matrix consisting of exopolysaccharides, protein fibers, and DNA (Costerton, Stewart and Greenberg, 1999; Donlan, 2002; Hall-Stoodley, Costerton and Stoodley, 2004). The formation of biofilms is vital for bacterial survival and a natural part of their life cycle, they protect the bacterial cells living within from harsh environments, they also provide optimal positioning for the bacterial cells living within to access nutrients (DeBeer, Stoodley and Lewandowski, 1994; Costerton, Stewart and Greenberg, 1999). Bacterial cells in aquatic systems can form biofilms on both biotic and abiotic surfaces. They can adhere to solid surfaces at the liquid-solid interface, and they can form floating biofilms known as pellicles at the liquid-air interface (Hall-Stoodley, Costerton and Stoodley, 2004; Bryers, 2008; Cava *et al.*, 2011). The main driving force for biofilm formation is the availability of nutrients. Environmental stress promotes biofilm formation, factors such as temperature, pH, iron, and oxygen affects biofilm formation, although they are microorganism specific (O'Toole, Kaplan and Kolter, 2000). The architectural shape of biofilms is complex, it contains channels for the flow of nutrients within and through their community (DeBeer, Stoodley and Lewandowski, 1994). In addition to connecting the bacterial cells together, the extracellular matrix connects the bacterial cells to the surface, this will form the initial biofilm which will grow to a mature biofilm and adapt to its surrounding environment. The bacterial cells within the biofilm at this point are characterised by an increase synthesis of exopolysaccharides, UV light tolerance, higher antimicrobial resistance, and formation of bacterial spores (Habash and Reid, 1999; O'Toole, Kaplan and Kolter, 2000; Hall-Stoodley and

Stoodley, 2002; Rowland and Voorheesville, 2003; Garrett, Bhakoo and Zhang, 2008; Coleman *et al.*, 2010; Vlamakis *et al.*, 2013).

One example of problematic biofilms is the potential pathogenic biofilm that form in dental settings. Several researchers have reported the formation of pathogenic biofilms in dental settings. Biofilms can form in sinks, tanks, suction hoses, tubing, pipes, handpieces, air and water syringes, and ultrasonic scalers (Barbeau, Bokum and Gauthier, 1998; Coleman *et al.*, 2009, 2010). Different pathogenic microorganisms (viruses, bacteria, algae, and fugai) can be found inside biofilms (Coleman *et al.*, 2010; Vasickova *et al.*, 2010). Pathogenic biofilms are responsible for high contamination levels in water used for invasive procedures in dental clincs (Barbeau *et al.*, 1996; Barbeau, Bokum and Gauthier, 1998). In dental clinics, cross-contamination among different patients with pathogens released from biofilms can occur through aerosols from dental tools, and from tubing backflow in wet dental devices such as the evacuation system. These dental tools are used to drain blood, tissue, saliva, and other debris from dental procedures (Barbeau, Bokum and Gauthier, 1998). Biofilm growth inside dental settings can have adverse effects on dental equipment by interfering with their mechanical functions, and clogging tubes and pipes. Based on the aforementioned, it is a major concern to control biofilm formation in dental clinics to protect both the patients and the dental settings (Barbeau *et al.*, 1996).

When the biofilm is mature, bacterial cells will secrete small molecules such as amino acids and polyamines. These small molecules will target the exopolysaccharides and protein fibers in the extracellular matrix, which initiates the biofilm disassembly and the release of bacterial spores and free planktonic cells (Kolodkin-Gal *et al.*, 2010, 2012). Biofilms formed from *Bacillus*

subtilis mature after three days incubation in biofilm growth medium at 22 °C, and after eight days the biofilm disassembles and release free planktonic cells (Kolodkin-Gal *et al.*, 2010; Romero *et al.*, 2011).

Bacteria has high resistance to physical, biological, and chemical assaults, this is due to their peptidoglycan call wall (also known as the murein), in most bacteria it is located outside the cytoplasmic membrane (Park, 1996; Holtje, 1998; Nanninga, 1998). The peptidoglycan cell wall is a strong and flexible polymer with a net-like shape, composed from short polypeptides (proteins) that cross link linear glycan strands (formed from disaccharide chains) (Cava *et al.*, 2011). The peptidoglycan cell wall has many benefits such as protecting the contents of the bacterial cell, resists osmotic pressure, holds the bacterial cell shape, and provides a framework to anchor the components of the cell envelop (Holtje, 1998; Young, 2006; Dramsi *et al.*, 2008; Vollmer, Blanot and de Pedro, 2008). One interesting fact about the polypeptides in the peptidoglycan cell wall is that they contain D-amino acids (Nagata *et al.*, 1998). These D-amino acids help forming the structure of peptidoglycan cell wall and provide it with resilience to different proteases (Cava *et al.*, 2011). The two most common D-amino acids found in the peptidoglycan cell wall are D-alanine and D-glutamate, other D-amino acids have been reported also such as D-serine and D-asp (Sieradzki and Tomasz, 1996; Reynolds and Courvalin, 2005; Bellais *et al.*, 2006; Veiga *et al.*, 2006). Their incorporation into the peptidoglycan cell wall is believed to increase its resistance to antibacterial agents (de Lencastre, Oliveira and Tomasz, 2007).

Previous researchers presented evidence that D-amino acids are produced by *Bacillus subtilis* to dismember their own biofilms at a late stage of the biofilm life cycle (Kolodkin-Gal *et*

al., 2010). The release of D-amino acids is triggered by the accumulation of metabolic waste and the low concentration of nutrients, at this point it is more favourable for the bacteria to dismember its biofilm, and release free planktonic cells that will migrate to a new favorable location to establish a new biofilm (Karatan and Watnick, 2009). The D-amino acids D-tyrosine, D-leucine, D-tryptophan, and D-methionine have shown promising results in biofilm growth inhibition (Lam *et al.*, 2009; Kolodkin-Gal *et al.*, 2010; Leiman *et al.*, 2013). The probability that D-amino acids can be used to control biofilm formation is significantly promising due to the lack of toxicity and the favorable pharmacokinetic properties of D-amino acids (Jayaraman and Wood, 2008).

Kolodkin-Gal et al. 2010, have reported self produced D-amino acids in the growth medium of mature biofilms. The results of their study showed that to inhibit biofilm formation by *Bacillus subtilis* a minimum concentration of 2 mM, 8.5 mM, 5 mM, and 3 μM is required for D-methionine, D-leucine, D-tryptophan, and D-tyrosine respectively. Their results also showed that a mixture of the four mentioned D-amino acids was potent to biofilm growth and can dismember pre-existing biofilms. The researchers suggested that the D-amino acids will be miss-incorporated into the cell wall and disengage the biofilm. They reported that the corresponding L-amino acids did not have any effect on *Bacillus subtilis* biofilm disassembly. It was also reported that D-alanine did mitigate the inhibitory effects of the four mentioned D-amino acids. Similar results were obtained, by the same researchers, when the treatments were tested on biofilms of Pseudomonas aeruginosa. The researchers also suggested that the interaction between the D-amino acids and the protein amyloid fibers is not the only way by which D-amino acids disassemble biofilms. As they found that D-amino acids were successful in

dispersing biofilms from bacterial species that are not known to have amyloid protein fibers such as Pseudomonas *aeruginosa* (Kolodkin-Gal *et al.*, 2010).

Another study by Leiman et al. 2013 stated that D-leucine, D-tryptophan, D-tyrosine, and D-methionine can inhibit biofilms formed by *Bacillus subtilis*. The minimum reported concentrations required to inhibit biofilm formation for D-leucine, D-tryptophan, and D-tyrosine were 8.5 mM, 5 mM, and 6 µM respectively, while no information was provided for D-methionine. The researchers suggested that the four mentioned D-amino acids interfere with protein synthesis by their misincorporation into the process, which results in the inhibition of the biofilm growth. The researchers stated that D-tyrosine is a metabolic inhibitor for *Bacillus subtilis.* It was also suggested that the inhibitory effects of the D-amino acids on *Bacillus subtilis* biofilm can be mitigated in the presence of L-amino acids. They also reported that a mixture of the four D-amino acids at a concentration higher than that obtained by Kolodkin-Gal et al. 2010 was required to inhibit biofilm formation. The results they obtained also showed that the inhibitory effects of the four D-amino acids on biofilm formation were not countered in the presence of D-alanine. Moreover, they found that the presence of L-tyrosine did counter the inhibitory effects of the four D-amino acids (Leiman *et al.*, 2013). These results contradict the findings of Kolodkin-Gal et al. 2010.

Brandenburg et al. 2013, investigated the effects of amino acids on the formation and motility of biofilms produced by Pseudomonas *aeruginosa*. The results obtained in their study showed that only D-tyrosine and D-tryptophan had inhibitory effects at concentrations of 2.5 mM for each, with the latter being most effective. The biofilm growth was enhanced with the addition of D-methionine with concentrations between 2.5 and 10 mM. The addition of D-leucine with

concentrations between 2.5 and 10 mM had no significant effect on biofilm formation. Moreover, at a concentration of 10 mM, both D-tryptophan and L-tryptophan were found to be effective in inhibiting biofilm growth, with L-tryptophan being more effective than D-tryptophan in inhibiting biofilm formation (86% and 71% respectively). A mixture containing an equimolar ratio of both tryptophan isoforms with a total concentration of 10 mM tryptophan had higher inhibitory effects (93% at 24 hr), which contradicts what was reported by the two previously mentioned studies. The researchers hypothesised that tryptophan enhances the swimming motility (~40%) of bacterial cells by enhancing the flagellar activity of the bacterial cells which will prefer to detach from the biofilm and swim freely, consequently resulting in the inhibition of biofilm growth and promoting biofilm disassembly (Brandenburg *et al.*, 2013).

Even though previous research has provided abundant information on the interactions between D-amino acids and biofilms, there is a lack of quantitative studies on the ability of D-amino acids to remove pre-exiting biofilms. In this research, the effects of D-amino acids on biofilm formation and the dispersion of pre-existing biofilm will be further investigated.

3.3.3. Experimental Procedures

3.3.3.1. Materials

3.3.3.1.1. Amino Acids and Thiamine Concentrate

200 mL DI-Water, 0.012 g Thiamine-HCL, 1 g L-tryptophan, 1 g L-threonine, 1 g L-phenylalanine, the mixture was sterile filtered.

3.3.3.1.2. Macronutrients Concentrate

200 mL DI-Water, 0.2 g $MnCl_2.4H_2O$, 0.2 g $FeCl_2.4H_2O$, 0.0028 g $ZnCl_2$, 3.8 g $MgCl_2$, 2.06 g $CaCl_2.2H_2O$, the mixture was sterile filtered.

3.3.3.1.3. 10X Tbase

116 mL of DI-Water, 1.25 g Trisodium citrate (34 mM sodium citrate), 2.48 g $(NH_4)_2SO_4$ (0.15M ammonium sulphate), 7.49 g KH_2PO_4 (0.44M potassium phosphate monobasic), 17.42 g K_2HPO_4 (0.8M potassium phosphate dibasic), the mixture was autoclaved for 20 min.

3.3.3.1.4. Biofilm Growth Media 1 (BGM1)

225 mL of DI-Water, 2.5 mL of 10X Tbase, 2.5 g tryptone, 0.01 g D-glucose, 2.5 g NaCl, 1.25 g yeast extract, 0.003 g $MgSO_4$, the mixture was autoclaved for 20 min.

3.3.3.1.5. Biofilm Growth Media 2 (BGM2)

1 mL of 10M KOH, 0.44 g K_2HPO_4, 10.45 g MOPS free acid, 2.5 g glycerol, 2.5 g glutamic acid, 0.5 mL amino acids and thiamine concentrate, 0.5 mL micronutrients concentrate, brought to a volume of 500 mL using DI-Water, the mixture is sterile filtered.

3.3.3.1.6. Wash Buffer (Sterile)

225 mL DI-Water, 25 mL of 10X Tbase, 0.25 ml of 1M $MgSO_4$, the mixture was autoclaved for 20 min.

3.3.3.1.7. Treatments Preparation

Treatments containing different concentrations of D-amino acids were prepared by dissolving appropriate amounts of the different D-amino acids in BGM2.

3.3.3.2. Methods

3.3.3.2.1. Bacterial Strain

The *Bacillus sp.* mixture used in this research is known commercially as Unizyme 20X CSB 20 NF SPORE, and it was provided by Germiphene Inc.

3.3.3.2.2. Washing *Bacillus sp.* Spores

Bacillus sp. spore suspension was washed three times. The washing procedure was performed by centrifugation at 10,000 rounds per minute for 1 min, the supernatant was removed and the spore concentrate was resuspended in 1 mL wash buffer using a vortex, this process was repeated twice, in the third time the resuspension was in 1 mL BGM1. This results in 1 mL washed *Bacillus sp.* spores.

3.3.3.2.3. Seed Culture Preparation

A glass flask (150 mL) containing 20mL BGM1 was then inoculated with 0.5 mL of the washed *Bacillus sp.* spores. The glass flask was then incubated at 37°C, stirring at 300 rounds per minute, inside a humidified incubator for approximately 270 min until the optical density measured at 600 nm was between 1 and 1.6.

3.3.3.2.4. Inoculating the Microtiter Plates

The *Bacillus sp.* seed culture was diluted in BGM1 (1:100), while stirring, 100 µL were transferred into each well in the microtiter plates. Controls were prepared by transferring 100 µL of sterile BGM1 into the designated control wells. To reduce evaporation from wells, microtiter plates were covered with a sterile thin film, a single hole was poked in the thin film at the center of each well using a sterile needle (22-gage) for aeration. The microtiter plates were

incubated in static conditions inside a humidified oven at 37 ^{o}C for 32 hrs. At the end of the incubation time, the microtiter plates are washed three times with the wash buffer using the Tecan Hydroflex Plate Washer.

3.3.3.2.5. Fixing Biofilm Growth

Fixed biofilms are presumed to be dead or nonactive. This procedure is performed at two stages, first to the designated wells for initial growth to quantify the initial biofilm growth following the 32 hrs incubation, second to the designated treated wells in the microtiter plates to be able to quantify biofilm formation following the 24 hrs application of treatments (explained below). The BGM1 was removed using the Tecan Hydroflex Plate Washer, and each well was incubated with 200µL methanol for 20 min. Microtiter plates are then placed on the bench top to air dry completely at room temperature (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010).

3.3.3.2.6. Applying Treatments

After the initial biofilm growth, the microtiter plates are washed and treatments were applied to the designated wells by dispensing 125 µL of treatment solutions into wells. The microtiter plates were then incubated for 24 hrs in the humidified oven with the same conditions described above.

3.3.3.2.7. Total Biofilm Quantification Using Crystal Violet Stain

The crystal violet binding assay is a method commonly used to quantify biofilms (O'Toole, Kaplan and Kolter, 2000; Kolodkin-Gal *et al.*, 2010, 2012; Brandenburg *et al.*, 2013; Zhang and Hu, 2013). To quantify the biofilm, 150 µL of 0.1 % (W/V) crystal violet was transferred into

each well in the microtiter plates, plates are covered using aluminum foil and incubated on bench top for 1 hr (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010). Subsequently, the microtiter plates were washed 4 times using the Tecan Hydroflex plate washer by dispensing 200 µL DI-Water in drip mode and aspirating, previous work showed that 4 times was sufficient for removing all excess (nonbinding) crystal violet stain. The microtiter plates are then left on bench top to air dry completely. The biofilm-binding crystal violet was then resolubilized by transferring 200 µL of 30% acetic acid into each well (Stepanović *et al.*, 2000), the microtier plates are then covered with aluminum foil on bench top for 20 min at room temperature. Subsequently,100 µL from each well were transferred to clear flat bottom polystyrene plates for absorbance quantification at OD 570 nm using the plate reader (TECAN infinite M200 Pro).

3.3.4. Results

All treatments in this section were delivered to the biofilm in a solution that contained BGM2. When the treatment was only containing BGM2 without any D-amino acids, it was termed as zero concentration. Following the 32-hr incubation time, the designated wells for initial biofilm growth were treated with methanol, and it is termed as Fixed. Methanol treatment was done to kill the biofilm and preserve its initial growth to be used as a benchmark to compare it later with the treated biofilm and to be further used for biofilm removal calculations. This will allow to evaluate the effectiveness of the D-amino acid treatments in biofilm removal.

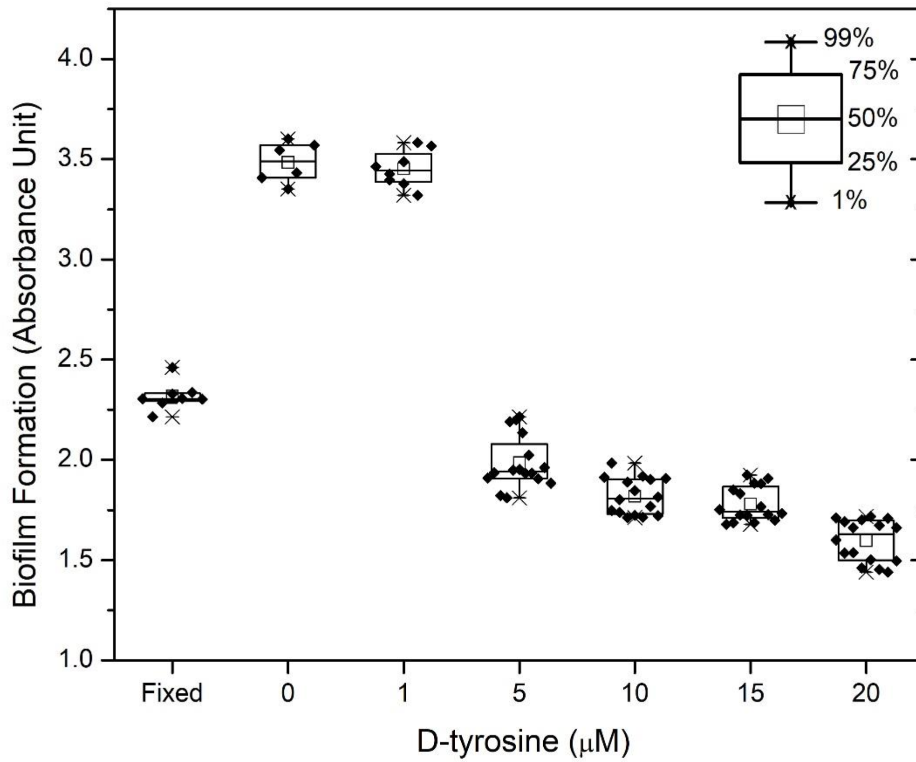

Figure 3-5.The effect of D-tyrosine on *Bacillus sp.* biofilm formation.

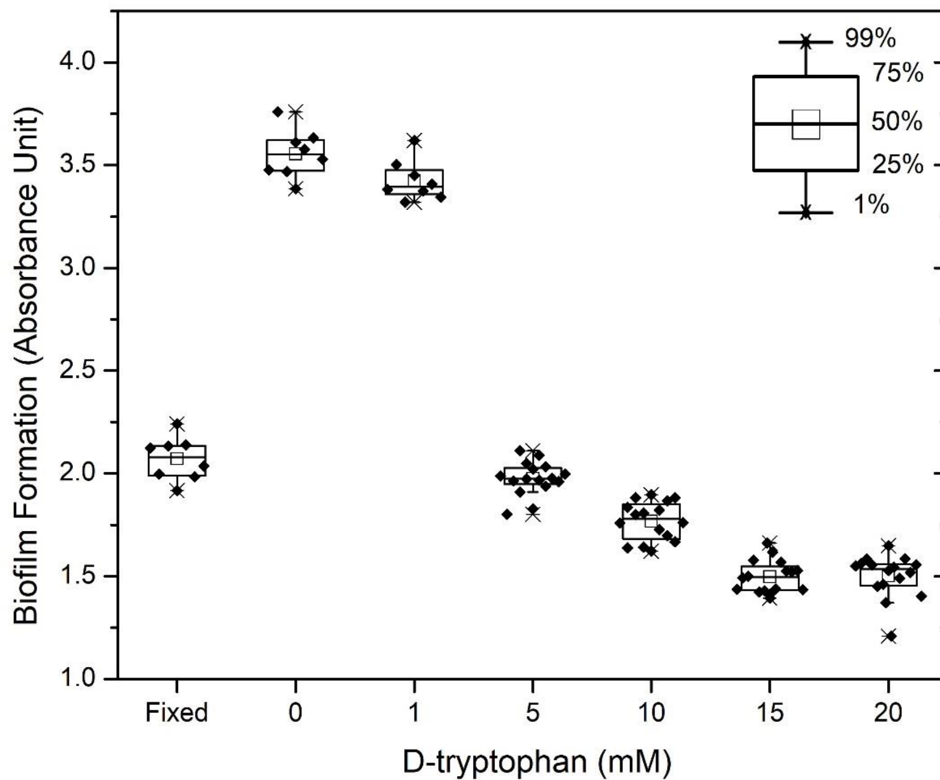

Figure 3-6. The effect of D-tryptophan on *Bacillus sp.* biofilm formation.

It can be seen from *Figure 3-5* and *Figure 3-6* that D-tyrosine and D-tryptophan at concentrations of 5 mM and higher inhibited biofilm formation. The pre-existing biofilm was dispersed at concentrations of 10 mM and higher for both D-tyrosine and D-tryptophan. The increase in the concentration of both D-tyrosine and D-tryptophan was proportional with the amount of biofilm dispersion. For D-tyrosine and D-tryptophan, a concentration less than 5 mM did not show any significant inhibitory effects on biofilm formation. The maximum biofilm removal using D-tyrosine at 20 µM and D-tryptophan at 15 mM was 31% and 28% respectively.

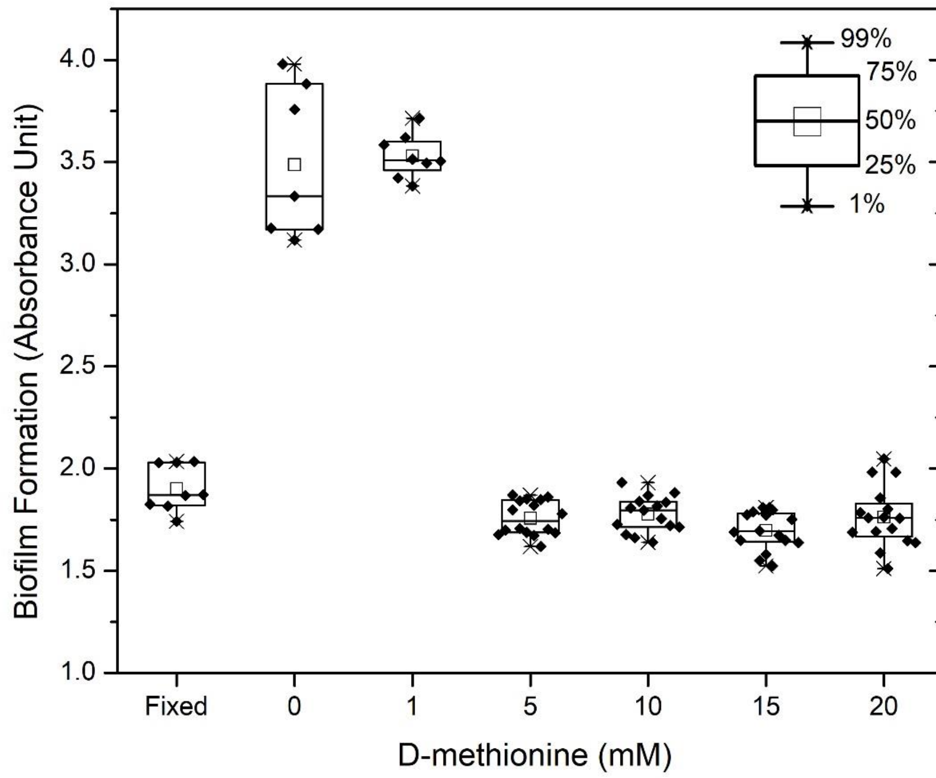

Figure 3-7. The effect of D-methionine on *Bacillus sp.* biofilm formation.

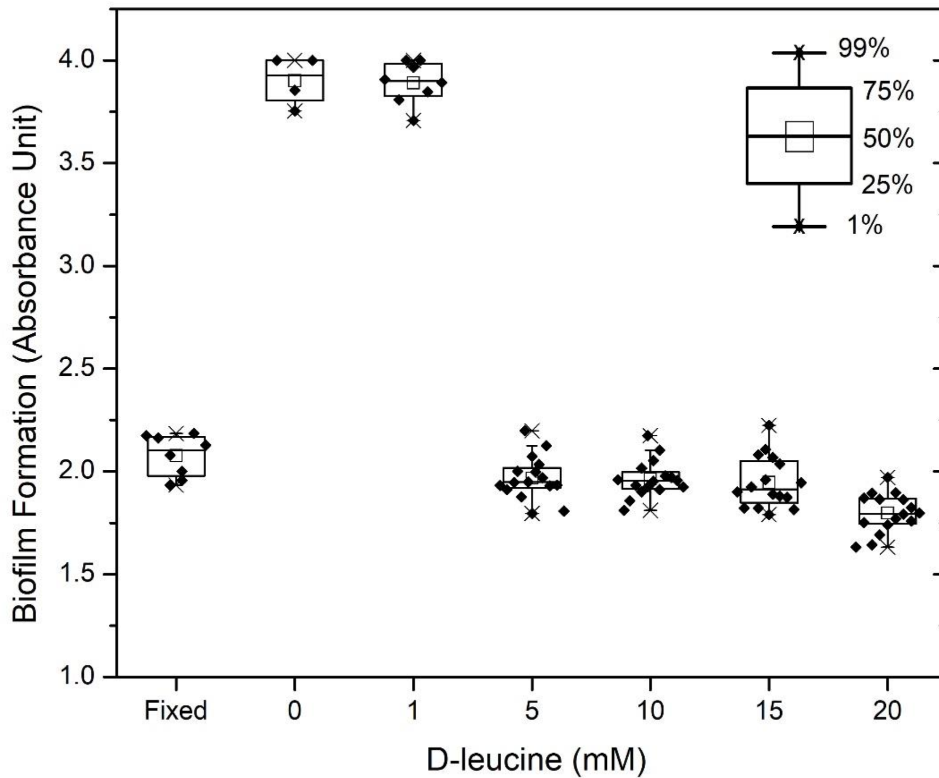

Figure 3-8. The effect of D-leucine on *Bacillus sp.* biofilm formation.

D-methionine and D-leucine were successful in inhibiting biofilm formation (*Figure 3-7* and *Figure 3-8,* respectively) at concentrations of 5 mM and higher. However, no significant reduction in the pre-existing biofilm was noticed. Moreover, the increase in their concentration did not seem to have any significant effect on biofilm control. No significant effects on biofilm formation were noticed for concentrations of 1 mM for both D-methionine and D-leucine.

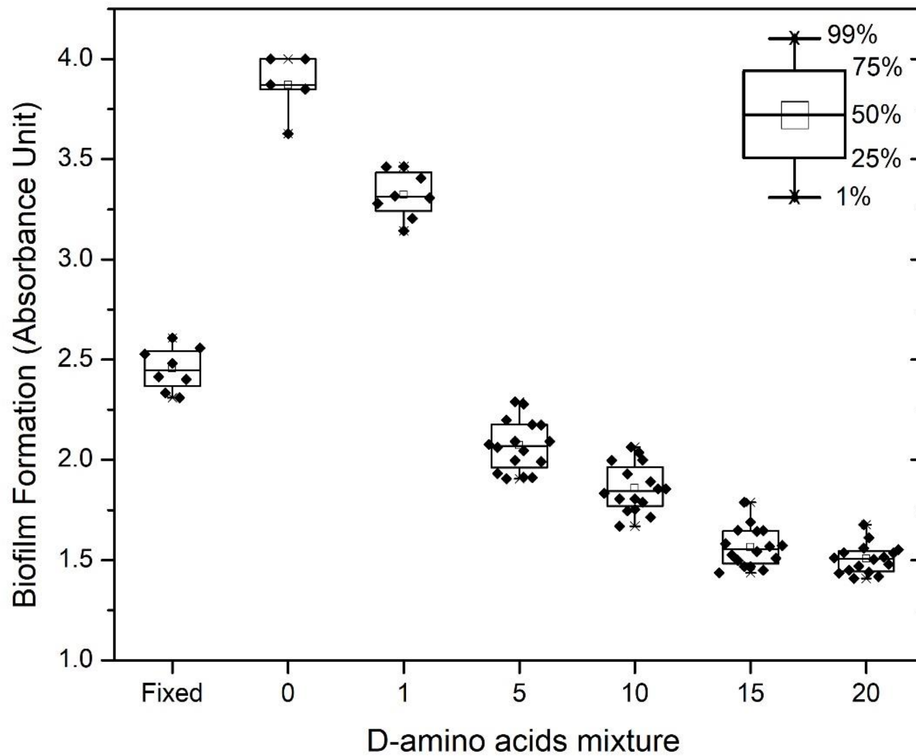

Figure 3-9. The effect of D-amino acids mixture on *Bacillus sp.* biofilm formation. D-tyrosine concentration is in the units of µM while the remaining D-amino acids are in the units of mM.

The results of D-amino acid mixture are presented in *Figure 3-9*. Results show that the D-amino acid mixture was successful in inhibiting biofilm formation for minimum concentrations of 5. Significant reduction in the pre-existing biofilm was noticed for concentrations of 10 mM and higher of each. Moreover, the increase in the mixture concentration had a significant effect on biofilm control. Apparently, the D-amino acids mixture was the most effective treatment for biofilm control, the highest biofilm removal was 39% at concentrations of 20. No significant effect was noticed on the biofilm removal for concentrations less than 5.

3.3.5. Discussion

Biofilm control do not require the development of treatments that can only kill biofilms without removing them. The desired treatments for biofilm control will have the ability to enhance, inhibit, or disperse biofilm formation. The results obtained (*Figure 3-5*) showed that D-tyrosine could inhibit biofilm formation at a concentration of 5 µM. The other three D-amino acids (D-tryptophan, D-methionine, and D-leucine) were also all successful in inhibiting biofilm formation, only at a higher concentration of 5 mM for each (*Figure 3-6*, *Figure 3-7*, and *Figure 3-8*, respectively). Lower concentrations did not show any inhibitory effects on biofilm formation. This inhibition in biofilm formation was obtained through different mechanisms. Bacterial cells are connected within the biofilm by the amyloid protein fibers that anchor to the D-alanine in the peptidoglycan cell wall of the bacterial cell, when D-amino acids are present they can incorporate themselves into the cell wall by replacing the pre-existing D-alanine. This incorporation disengages the protein fibers from the cell wall and consequently causing the inhibition of biofilm formation and the pre-existing biofilm to disperse (Kolodkin-Gal *et al.*, 2010). The D-amino acids tested can also be miss-incorporated into the protein synthesis, which results in the inhibition of the biofilm formation (Leiman *et al.*, 2013). Although all four D-amino acids investigated in this research showed inhibitory effects on biofilm formation, only D-tyrosine and D-tryptophan showed the ability to remove pre-existing biofilms.

It is hypothesised that D-tyrosine inhibits biofilm formation and disperses the pre-existing biofilm by two mechanisms; first by the miss-incorporation of D-tyrosine into the cell wall by replacing the D-alanine in the cell wall, and its consequent disengagement of attached cells and preventing new cells to attach, due to lack of attachment sites. Second due to

metabolic inhibition associated with D-tyrosine (Champney and Jensen, 1969), which inhibit biofilm formation due to lack of access to nutrients, and disperse the pre-existing biofilm due to the unfavorable environment surrounding it, as cells prefer to return to its free planktonic form (Karatan and Watnick, 2009).

D-tryptophan also showed inhibition of biofilm formation and dispersed pre-existing biofilms. One mechanism was the miss-incorporation of D-tryptophan into the cell wall as explained previously. The other mechanism is the significant increase in swimming motility, D-tryptophan has been reported to significantly increase the swimming motility of bacterial cells (Brandenburg *et al.*, 2013), and cells may favor the detachment from the biofilm (Boles, Thoendel and Singh, 2005), consequently dispersing pre-existing biofilm. Since flagellar arrest is important for biofilm formation an increase in flagellar activity will inhibit biofilm formation, and disperse pre-existing biofilms (O'Toole and Kolter, 1998; Brandenburg *et al.*, 2013). The results obtained for D-tryptophan was agreeing with the results obtained by Kolodkin-Gal et al. 2010 and Leiman et al. 2013.

The minimum concentration of D-leucine required to inhibit biofilm formation (5 mM) was lower than that obtained by Kolodkin-Gal et al. 2010 and Leiman et al. 2013 (8.5 mM for both previous studies). While the minimum concentration of D-tyrosine required to inhibit biofilm formation (5 µM) was higher than that obtained by Kolodkin-Gal et al. 2010 (3 µM) and less than that obtained by Leiman et al. 2013 (6 µM). Although some differences existed in the minimum concentration required for the inhibition of biofilm formation, these differences remain limited. The minimum concentration of D-methionine to inhibit biofilm formation was 5 mM, this concentration was higher than that reported by Kolodkin-Gal et al. 2010 (2 mM).

The results for the combined treatment (*Figure 3-9*) suggests that for complete inhibition of biofilm formation a minimum concentration of 5 µM of D-tyrosine and 5 mM each for the other three D-amino acids was required to inhibit biofilm formation. The treatment with a lower concentration of 1 µM of D-tyrosine and 1 mM of the three other D-amino acids showed limited biofilm inhibition, this can be seen when compared with both the treatment containing no D-amino acids (0) and the fixed biofilm. The removal of the pre-existing biofilm was not noticed for combined treatments with concentrations less than 5 µM for D-tyrosine and 5 mM for each of the other three D-amino acids. The amount of biofilm dispersed was proportional to the concentration of the D-amino acids until it levels at concentrations between 15 and 20 mM.

The minimum concentration required for biofilm inhibition and dispersal obtained in this research was higher than that reported by previous researchers. Kolodkin-Gal et al. 2010 reported that a mixture of the four mentioned D-amino acids with a concentration of 10 nM of each D-amino acid was potent to the biofilm growth, and it was successful in dismembering part of the pre-existing biofilms. Following that, Leiman et al. 2013 also reported that a mixture of the four D-amino acids at a concentration of 10 nM of each was not able to inhibit biofilm formation, but rather a higher concentration of 500 nM was required to achieve inhibitory effects.

By comparing the results obtained in *Figure 3-5* and *Figure 3-6* for D-tyrosine and D-tryptophan respectively, with the results obtained from the D-amino acids mixture presented in *Figure 3-9* , no clear synergetic effects can be seen from combining D-amino acids in a mixed treatments. The mixed treatment was more effective in removing pre-existing biofilm (39%)

when compared with the biofilm removal achieved by D-tyrosine (31%) and D-tryptophan (28%), this is hypothesised to be a result of combining the effects of D-tyrosine and D-tryptophan in the mixed treatment containing the four different D-amino acids.

3.3.6. Conclusions

The results obtained suggests that the D-amino acids investigated in this research can be utilized to inhibit growth of pre-existing *Bacillus sp.* biofilm. The use of D-tyrosine and D-tryptophan did result to some extent in partial removal of pre-exiting *Bacillus sp.* biofilm. The D-amino acid mixture although removed pre-existing *Bacillus sp.* biofilm, it did not show clear synergetic effects from combining the amino acids into a mixed treatment. Therefore, the use of D-amino acids can be seen beneficial for inhibiting *Bacillus sp.* biofilm formation, but showed limited abilities in removing *Bacillus sp.* pre-existing biofilms. Since no clear synergetic effects were detected for the combined treatments, it is not recommended to conduct any optimization studies on biofilm removal using D-amino acids.

3.3.7. Acknowledgments

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3.3.8. References

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3.4 Biofilm Control Using Enzymes

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3.4.1. Preface

Section 3.4 presents the experimental work conducted to fulfill the specific objective presented in Section 1.3.2.3. A comprehensive approach was used in the selection of relevant enzymes and evaluate their ability to remove biofilms of different compositions. The results presented in Section 3.4 suggests that the protease Savinase and the polysaccharide-degrading enzyme Pectinex had the highest biofilm removal capabilities among the different tested enzymes. The results obtained in Section 3.4 along with previous recommendations from other researchers suggests promising results from the optimization of biofilm removal using an enzymatic treatment containing proteases and polysaccharide-degrading enzymes.

3.4.2. Introduction

Biofilms are multicellular heterogenous communities formed by the aggregation of bacteria on surfaces. Cells within a biofilm community are encased and connected together by an extracellular matrix that consists mainly from exopolysaccharides and protein fibers (Costerton, Stewart and Greenberg, 1999; Donlan, 2002; Hall-Stoodley, Costerton and Stoodley, 2004). The extracellular matrix encases the biofilm community, it connects the bacterial cells together and to the surface. Bacterial cells living in a biofilm are characterised by an increased synthesis of extracellular matrix, UV light tolerance, the formation of free planktonic cells, the formation of bacterial spores, and higher antibacterial resistance, (Habash and Reid, 1999; O'Toole, Kaplan and Kolter, 2000; Hall-Stoodley and Stoodley, 2002; Garrett, Bhakoo and Zhang, 2008; Vlamakis *et al.*, 2013).

Removal of biofilms using chemical treatments was found to be a challenging process (Ntsama-Essomba *et al.*, 1997; Vickery, Pajkos and Cossart, 2004). Chemical treatments cannot penetrate the biofilm due to the reaction-diffusion barrier it forms between the chemicals and the bacterial cells living within the biofilm (Gilbert and McBain, 2001). This fact makes biofilm communities highly resistant to disinfectants, antibiotics, and biocides. Bacteria living in a biofilm formation was found to be between 100 and 1000-fold more resistant to chemical treatments than bacteria living in free planktonic form (Gilbert and McBain, 2001).

Depending on the bacterial strain and other extrinsic factors (gaseous levels, nutrients fluctuations, and fluid shear) the extracellular matrix composition can vary (Simões, Simões and Vieira, 2010). The composition of the biofilm can contain all or some of the following: phospholipids, carbohydrates, glycoproteins, lipids, nucleic acids, polysaccharides, and amyloid

proteins (Branda *et al.*, 2005; Flemming and Wingender, 2010; Hobley *et al.*, 2015). Regardless of the different biofilm components, the main two components are the polysaccharides and proteins (Sutherland, 2001; Marvasi, Visscher and Casillas Martinez, 2010; Molobela, Cloete and Beukes, 2010).

Bacillus sp. is considered a model microorganism for biofilm related studies and it was employed by different researchers in their studies (K. P. Lemon *et al.*, 2008). *Bacillus sp.* is a gram-positive, biofilm forming, and non-pathogenic bacteria. *Bacillus sp*. can adapt in a wide range of conditions and is usually found in terrestrial and aquatic environments (Grossman, 1995; Maglott *et al.*, 2007; Earl, Losick and Kolter, 2008). The major component of the extracellular structural matrix in biofilms formed by *Bacillus sp.* is the polysaccharides (Branda *et al.*, 2001; Morikawa *et al.*, 2006). Another model bacterium for biofilm related studies is the gram-negative, biofilm forming, and non-pathogenic *Pseudomonas fluorescens* (Rossignol *et al.*, 2008). Due to its high adaptation and its ability to resist a wide range of disinfectants and antibiotics this bacterium is ubiquitous in the environment and hospitals (Spiers, Buckling and Rainey, 2000; Rossignol *et al.*, 2008). It has been reported that biofilms formed by *Pseudomonas fluorescens* are mainly composed of proteins (Molobela, Cloete and Beukes, 2010). Currently, there is an increased interest in the use of enzymes as anti-biofilm agents, this came following their successful use in removing biofilms from industrial surfaces (Taraszkiewicz *et al.*, 2013; Thallinger *et al.*, 2013; Meireles *et al.*, 2016). Enzymes have been described as efficient, affordable, and greener alternative than the harmful and ineffective chemicals used in diminishing problems associated with biofilm formation (Cordeiro, Hippius and Werner, 2011; Cortés, Bonilla and Sinisterra, 2011; Srey, Jahid and Ha, 2013). There is no evidence of any side

effects from using enzymes in the industry and the use of enzymes for cleaning surfaces was approved by regulatory agencies (Schmidt, 2012; Meireles *et al.*, 2016). Biofilms can be degraded by enzymes through different routes, enzymes can degrade the extracellular matrix encasing the biofilm, degrade the components of the biofilm, interfere with quorum sensing, cause cells lysis, and catalyse the production of antimicrobials (Donlan, 2002; Augustin, Ali-Vehmas and Atroshi, 2004; Simões, Simões and Vieira, 2010; Werner, 2011; Thallinger *et al.*, 2013). Enzymes weaken the structural integrity of the biofilm by breaking the components of the extracellular matrix (Molobela, Cloete and Beukes, 2010). Regardless of the different modes of action, for an enzyme to degrade a biofilm the first line of defence it will face is the extracellular matrix that covers and shields the bacterial cells in biofilm community. Enzymes used to remove biofilms are divided into four groups and those are oxidative enzymes, anti-quorum sensing, proteolytic enzymes such as proteases, and polysaccharide-degrading enzymes (Thallinger *et al.*, 2013).

Proteases are a class of proteolytic enzymes that can hydrolyse proteins. This class of enzymes contain a wide range of enzymes that are different in their target substrates and mechanisms (Hedstrom, 2002).

Previous research has shown that proteases have been successful in degrading proteins in pipelines (Augustin, Ali-Vehmas and Atroshi, 2004). Different researchers reported that Savinase was successful in preventing and removing biofilm formations (C Leroy *et al.*, 2008; Molobela, Cloete and Beukes, 2010). Leroy et al. 2008 compared the efficiency of different commercial enzymes (seven polysaccharide-degrading, four proteases, and one lipase) to eradicate biofilms formed by the marine bacterial strain *Pseudoalteromonas sp.* D41. The

biofilm was grown in a 96-well microtiter plate for 24 h using sterile seawater at 20 ^{o}C. The biofilm biomass was quantified by using fluorescent dye DAPI (4[prime]6-diamidino-2-phenylindole). The results presented suggested that Savinase was the most efficient enzyme in removing and preventing biofilms (50% reduction of pre-existing biofilms after a 24 hrs treatment at a concentration of 1.7 mg mL^{-1}, and up to 100% reduction in bacterial adhesion). The authors concluded that depending on the type of enzyme and the used concentrations enzymes might remove biofilms or inversely enhance their formation (C Leroy *et al.*, 2008).

Protamex was used by previous researchers for the hydrolyses of peptide bonds from different sources such as Yellowfin Tuna, Sardine heads, and Soybean (Dumay *et al.*, 2009; Nguyen *et al.*, 2011; Minh, 2015). Cordeiro et al. 2011 investigated the effect of immobilized subtilisin A on the initial attachment of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. The tested enzymes (Subtilisin A and Cellulase) were immobilized on a poly(ethylene-alt-maleic) anhydride copolymer films by covalent binding. Test controls were prepared using heat to inactivate enzymes. Test slides and controls were submerged in bacterial suspension (10^6 CFU/mL) and incubated in sterile conditions for 24 hrs at 37°C. After incubation, the test samples were washed to remove loosely attached bacteria by immersion into a saline solution, the cells that remained attached after the wash step were plated in serial dilutions for quantification of viable cell count. By comparing the active and inactive surfaces, the immobilized subtilisin A had no effect on the attachment of S. epidermidis but reduced the attachment of P. aeruginosa by 44%. The authors concluded that the initial steps of attachment for different bacteria involves different biomolecules, which requires a broad spectrum of enzymatic coatings to control biofilm(Cordeiro, Hippius and Werner, 2011). A study by Tasso et

al. 2009 investigated the control of biofilm by targeting the adhesives used by microorganisms to attach to surfaces. The researchers used the protease Subtilisin A by covalently attaching it to tested surfaces at different concentrations. The immobilized enzymatic coating was tested for its effect on the adhesion strength and settlement of two major species (green alga *Ulva linza*, and the diatom *Navicula perminuta*). The researchers reported that the immobilized Subtilisin A was effective in reducing the adhesion strength and settlement of the tested microorganisms (Tasso *et al.*, 2009).

The protease Trypsin is substrate-specific for the peptide bonds of arginine and lysine (Chaignon *et al.*, 2007). A study by Chaignon et al. 2007 evaluated the ability of trypsin to remove biofilms formed by *S. epidermidis RP62A, S. epidermidis 5, S. epidermidis 444, S. lugdunensis 47, S. lugdunensis 18a, S. aureus 383* in 96-well microtiter plates. The pre-grown biofilms of the different bacterial species were treated with 100 µL of trypsin at 1 mg mL^{-1} in 20 mM Tris buffer, and pH 7.5 for 2 h. Biofilms were stained using 5% safranin and quantified by measuring absorbance at 492 nm. Results obtained showed that Trypsin treatment removed more than 70% of biofilm formed by *S. epidermidis 444, S. lugdunensis 47, S. lugdunensis 18a, S. aureus 383*. While no effect of trypsin was noticed for biofilm formed by *S. epidermidis RP62A, S. epidermidis 5*. The authors stated that Trypsin had no effect on biofilms formed by *S. epidermidis RP62A* and *S. epidermidis 5* due to their polysaccharide-rich composition. The authors also hypothesised that the removal of biofilms formed by S. epidermidis 444 (polysaccharide rich biofilm) by Trypsin was due to the important role of proteins and polysaccharide as well in the stability of the biofilm formed by this bacterial strain (Chaignon *et al.*, 2007). In a study by Rohde et al. 2007, Trypsin was used to remove pre-grown biofilm from

Staphylococcus epidermidis and *Staphylococcus aureus*. Biofilm was cultivated in 96-well microtiter plates, and a treatment containing Trypsin (100 µg/mL) was applied for 16 hrs at 37°C. The Trypsin treatment removed more than 98% of the biofilm formed in the microtiter plates from both tested bacterial species (Rohde *et al.*, 2007).

Polysaccharides molecules and their degrading enzymes are abundant in nature, with many of these polysaccharide-degrading enzymes produced by bacteria, which in turn utilizes these enzymes in their invasions to bypass host defences (Jedrzejas, 2000). The polysaccharide degrading enzyme Cellulase arises among the different enzymes used as a common choice of enzymatic treatments for prevention and removal of bacterial biofilms (Cordeiro, Hippius and Werner, 2011). Following the same procedure discussed previously for Subtilisin A, and by comparing results for active and inactive layers of enzymes, Cordeiro et al. 2011, reported that Cellulase, in contrary to Subtilisin A, did not affect the attachment of *P. aeruginosa* and reduced the attachment of *S. epidermidis* by 67%. (Cordeiro, Hippius and Werner, 2011). Loiselle et al. 2003 conducted a study to investigate the ability of Cellulase to inhibit biofilm formation by *Pseudomonas aeruginosa*. Biofilm was formed on glass slides in a flow chamber for 4 days. Biofilm formation was assessed by quantifying the biomass areal density (mg cm^{-2}) and by viable cell count (CFU). Biofilm was grown in the presence of different Cellulase concentrations at pH 7 and 5. The test controls were prepared using deactivated Cellulase. The results reported suggests that Cellulase was successful in partially inhibiting CFU formation and biomass by *P. aeruginosa*. Areal densities were reduced by 36% and 58% for Cellulase concentrations of 9.4 and 37.6 Unit mL^{-1} at pH 7. While areal density was decreased by 60% and 88% for Cellulase concentrations of 9.4 and 75.2 Unit mL^{-1} at pH 5. Viable cell counts decreased by 60% and 57%

at pH 5 and 7 for Cellulase concentration of 9.4 Unit mL^{-1}. The increase of Cellulase concentration did not have any effect of viable cell counts (CFU) at the tested pH values (Loiselle and Anderson, 2003).

Pectinases (pectinolytic enzymes) are heterogeneous mixtures of enzymes that are abundant in plants and microorganisms for their ability to hydrolyze pectic substances (Jayani, Saxena and Gupta, 2005). Pectic compounds are polysaccharides that are negatively charged, acidic, high molecular weight macromolecules that are abundant in the plant kingdom (Jayani, Saxena and Gupta, 2005). Pectinases play an important role in the plant kingdom as they contribute in the plant cell wall degradation (Ward and Moo-Young, 1989). They also contribute in softening plant tissue at maturation and storage (Sakai, 1992), and have an important ecological role in the decomposition and recycle of plant waste materials (Lang and Dörnenburg, 2000).

Pectinases have shown promising results as an anti-biofilm enzyme (Johansen and Falholt, 1997). In their study, Johansen et al. 1997, evaluated the use of Pectinex on biofilms formed from *Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 10148, and Pseudomonas fluorescens AH2*. Sterile discs made from steel and polypropylene were immerged vertically in a steel tank containing growth medium and biofilm was allowed to develop on both sides of the discs at 26°C for 4 days with stirring (200 rpm). The discs were then rinsed to remove loosely attached cells using phosphate buffer (pH 7) prior to incubating them with Pectinex in phosphate buffer for 15 min at 20 ^{o}C static. Phosphate buffer solution (sterile) was used as a control. The total and respiring number of bacterial cells in the biofilm was determined using fluorescence microscopy. From the results presented Pectinex was

effective in removing biofilms formed by all tested species. Almost two log reductions in the cell number for *S. aureus* and *P. aeruginosa*, and one log reduction in the cell number for *P. fluorescens* was achieved with Pectinex concentration of 180 PSU mL^{-1} (Johansen and Falholt, 1997).

Due to the complexity of the different biofilm compositions among different bacterial species, and the abundant available enzymes in the market coupled with the enzymatic substrate specificity (Simões, Simões and Vieira, 2010), it is of vital importance to evaluate and compare the available related enzymes for their ability to remove biofilms. Although different biofilms compositions are described as complex, the to main components of biofilms are polysaccharides and proteins, hence, the most related enzymatic treatments recommended for degrading different biofilms are the proteases and the polysaccharide-degrading enzymes (Meyer, 2003). Far to the authors knowledge, the efficiency of the industrial enzymatic mixtures CellicCTec2, CellicHTec2, and Protamex have never been evaluated for biofilm removal.

In this research, the efficiency of commercially available polysaccharide-degrading enzymes (Carezyme, CellicCTec2, CellicHTec2, and Pectinex) and proteolytic enzymes (Savinase, Protamex, Trypsin, and Subtilisin A) in the removal of pre-grown biofilms of different compositions formed by *Bacillus sp.* and *Pseudomonas fluorescens* will be evaluated and compared.

3.4.3. Experimental Procedures

3.4.3.1. Materials

3.4.3.1.1. 10X Tbase

To 232 mL DI-Water, add 2.5 g Trisodium citrate (34 mM sodium citrate), 4.95 g $(NH_4)_2SO_4$ (0.15M ammonium sulphate), 14.97 g KH_2PO_4 (0.44M potassium phosphate monobasic), 34.84 g K_2HPO_4 (0.8M potassium phosphate dibasic), autoclave for 20 min.

3.4.3.1.2. Biofilm Growth Media for Bacillus species.

To 900 mL of DI-Water, add 10 g tryptone, 5 g yeast extract, 10 g NaCl, 0.04 g D-glucose), 10 mL of 10X Tbase, 0.012 g $MgSO_4$, autoclave for 20 min.

3.4.3.1.3. Biofilm Growth Media for *Pseudomonas fluorescens*.

To 1000 mL of DI-Water, add 20 g peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 10 gm glycerol, autoclave for 20 min.

3.4.3.1.4. Wash Buffer (Sterile)

900 mL DI-Water, 1 ml of 1M $MgSO_4$, 100 mL of the 10X Tbase solutions, autoclaved for 20 min.

3.4.3.1.5. Sodium Acetate Buffer for Polysaccharide-degrading enzymes (pH 4)

410 mL of 0.2 M acetic acid solution, 2.45 g sodium acetate trihydrate, bring final volume to 1 L using DI-Water, autoclave for 20 min.

3.4.3.1.6. Sodium Phosphate Buffer for Proteases (pH 8)

To 1000 mL of DI-Water, add 0.74 g Sodium Phosphate monobasic monohydrate, 25.23 g sodium phosphate dibasic heptahydrate, autoclave 20 min.

3.4.3.2. *Methods*

3.4.3.2.1. Bacterial Strains

The *Bacillus species* strain used in this research was obtained from Germiphene Inc. It is known commercially as Unizyme 20X CSB 20 NF SPORE. The *Pseudomonas fluorescens* strain used in this research is known as *Pseudomonas fluorescens* Migula 1895 (DSM No. 50090, type strain), and was obtained from DSMZ in Germany.

3.4.3.2.2. Seed Culture Preparation

For *Bacillus sp.*, one mL of the spore suspension was washed via centrifuge at 10,000 rounds per minute for 60 seconds and then re-suspended in 1 mL wash buffer (repeated three times), this was followed by resuspension in 1 mL biofilm growth medium. A 150mL glass flask containing 20mL medium was then inoculated with 0.5 mL of the resuspended cells at 300 rounds per minute inside a 37 ^{o}C humidified incubator oven for approximately 270 min until the optical density at 600 nm was between 1 and 1.6 (using DI-Water as blank).

For *P. fluorescens* strain, the culture was revived in growth media and samples of 1 mL size were sampled and preserved with 20% glycerol in -80°C freezer. Prior to each experiment the frozen samples were revived in growth media until optical density measured at 600 nm was between 1 and 1.6 (using DI-Water as blank).

3.4.3.2.3. Inoculating the Microtiter Plates and Biofilm Growth

The prepared seed culture for each of the two strains was diluted in a 1:100 ratio in the designated biofilm growth media, and each well in the microtiter plates was filled with 100 µL

of the diluted seed culture. Control wells were prepared by filling wells in the microtiter plates with 100 µL of sterile biofilm growth media. Each plate was covered with a sterile thin film to reduce evaporation, a single hole was added for aeration in the center of each well using a 22-gauge sterile needle. The microtiter plates containing *Bacillus sp.* were incubated at 37 ^{o}C in a humidified oven in static conditions for 32 h. While the microtiter plates containing *P. fluorescens* were incubated in a sterile chamber at 21 ^{o}C for 72 h and media was replenished every 24 h. Subsequently the plates are washed (Tecan Hydroflex Plate Washer) gently three times using wash buffer.

3.4.3.2.4. Fixing Biofilm Growth

This step is done to fix initial biofilm growth to be compared with the biofilm remaining in other wells following treatment. It is assumed that fixed biofilms are nonactive or dead. At the end of the treatment duration this process is repeated to treated wells and prior to biofilm quantification. Growth medium was removed, and each well was filled with 200µL methanol for 20 min. Microtiter plates are then left to air dry completely at room temperature (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010).

3.4.3.2.5. Enzymatic Treatments Preparation

The protease treatments (Savinase, Protamex, Subtilisin A, and Trypsin) were prepared in sodium phosphate buffer (pH 8) at five concentrations (1, 5, 10, 15, and 30 U/mL), while the polysaccharide-degrading enzymes (Pectinex, Carezyme, Cellic-CTec2, Cellic-HTec2) were prepared in sodium acetate buffer (pH 4) at five concentrations 1, 5, 10, 15, and 30 U/mL for Carezyme, Cellic-Ctec2, and Cellic-HTec and 5, 15, 25, 50, and 75 U/mL for Pectinex.

The enzymatic activity of the different proteases was quantified before and after conducting the experiments using casein as substrate (Folin and Ciocalteu, 1927; Anson, 1938), no change was noticed in the enzymatic activity of the proteases (data not shown). The protease activity unit (U) is defined as the amount of enzyme required to hydrolyse casein in one minute, pH 7.5, 37°C, to produce color (Folin & Ciocalteau's reagent) equivalent to 1 µmole of tyrosine (Folin and Ciocalteu, 1927; Anson, 1938).

The activity of the polysaccharide degrading enzymes (Carezyme, Cellic-CTec2, and Cellic-HTec2) were quantified before and after conducting the experiments using cellulose as a substrate (Worthington, 1988), no change was noticed in the enzymatic activity of the tested enzymes (results not shown). The enzymes activity unit (U) is defined as the amount of the enzyme required at pH 5 and 37 ^{o}C, to liberate 1 µmole of glucose from cellulase in 1 h (Worthington, 1988). The list of enzymes used in this work are presented in Table 3–1. The activity unit (U) for Pectinex is defined as the amount of enzyme required to liberate 1 µmole of galacturonic acid from poly-galacturonic acid in 1 h at pH 4 and 25 ^{o}C.

Table 3–1. List of enzymes used and their optimum activity conditions.

Enzyme		Optimum Activity Range		Source
Name	**Type**	**pH**	**Temp. (C°)**	
Protamex®	Protease	7.5 - 9.5	40 - 60	Sigma-Adrich Inc., Saint Louis, Mo, USA
Subtilisin A	Protease	7.5 - 10	50 - 65	Sigma-Adrich Inc., Saint Louis, Mo, USA
Trypsin	Protease	6.5 - 10	30 - 44	Sigma-Adrich Inc., Saint Louis, Mo, USA
Savinase	Protease	8.0 - 11	55 - 75	Sigma-Adrich Inc., Saint Louis, Mo, USA
Carezyme	Polysaccharide-degrading	4.0 - 5.5	35 - 60	Sigma-Adrich Inc., Saint Louis, Mo, USA
Pectinex Ultra	Polysaccharide-degrading	3.5 - 6.0	30 - 60	Sigma-Adrich Inc., Saint Louis, Mo, USA
Cellic CTec2	Polysaccharide-degrading	4.0 - 6.5	40 - 60	Novozymes A/S, Bagsværd, Denmark
Cellic HTec2	Polysaccharide-degrading	4.0 - 6.0	60 - 75	Novozymes A/S, Bagsværd, Denmark

3.4.3.2.6. Applying Treatments

After washing the microtiter plates with the buffer solution, the designated treatment was applied by dispensing 125 µL of enzymatic treatment solution into desired wells, that was followed by incubation at 21 °C for 24 h.

3.4.3.2.7. Total Biofilm Quantification Using Crystal Violet Stain

Biofilm formation was quantified using the crystal violet binding assay method. This method is commonly used to quantify biofilms (O'Toole, Kaplan and Kolter, 2000; Kolodkin-Gal *et al.*, 2010, 2012; Brandenburg *et al.*, 2013; Zhang and Hu, 2013). To quantify biofilm formation in the microtiter plates, using a multichannel pipette, 150 µL of 0.1 % (W/V) crystal violet stain was dispensed into each well, this was followed by 60 min incubation (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010). The microtiter plate was washed 4 times by dispensing 200 µL DI-Water in drip mode and then aspirating using the Tecan Hydroflex plate washer without touching the bottom of the wells (previous testing proved that washing the plates 4 times

produce a washing waste liquid that is stain free). Microtiter plates were then air dried completely at room temperature. The crystal violet was then resolubilized by manually dispensing 200 µl 30% acetic acid using a multichannel pipet into each well (Stepanović *et al.*, 2000), the microtitet plate is then incubated static at room temperature for 20 min. Using a multichannel pipet, 100 µL from each well were transferred to clear polystyrene plates with the flat bottom for to be quantified via measuring absorbance at OD 570 nm (TECAN infinite M200 Pro Plate reader).

3.4.4. Results

3.4.4.1. Reproducibility of Biofilm Formation in Different Microtiter Plates

Reproducibility of biofilm growth in the microtiter plates was evaluated first to validate comparisons between different treatments within the same microtiter plate (intra-plate) and between different microplates (inter-plate) prepared from the same batch. Biofilm growth for *P. fluorescens* was quantified in 96 wells per plate in three separate plats following the designated incubation time. A one-way ANOVA test was performed for inter-plate comparison of biofilm formation. The results showed no significant difference (p-value = 0.63) among the three plates. Reproducibility of *P. fluorescens* biofilm within the same plate was also assessed using one-way ANOVA, the results obtained showed no significant difference (p-value = 0.73) among the twelve columns (8 wells/column) within the same microtiter plate. Reproducibility testing for *Bacillus sp.* showed no significant difference for both inter-plate and intra-plate comparisons, for details please see Section 3.2.4.1 .

3.4.4.2. Biofilm Removal Using Proteases

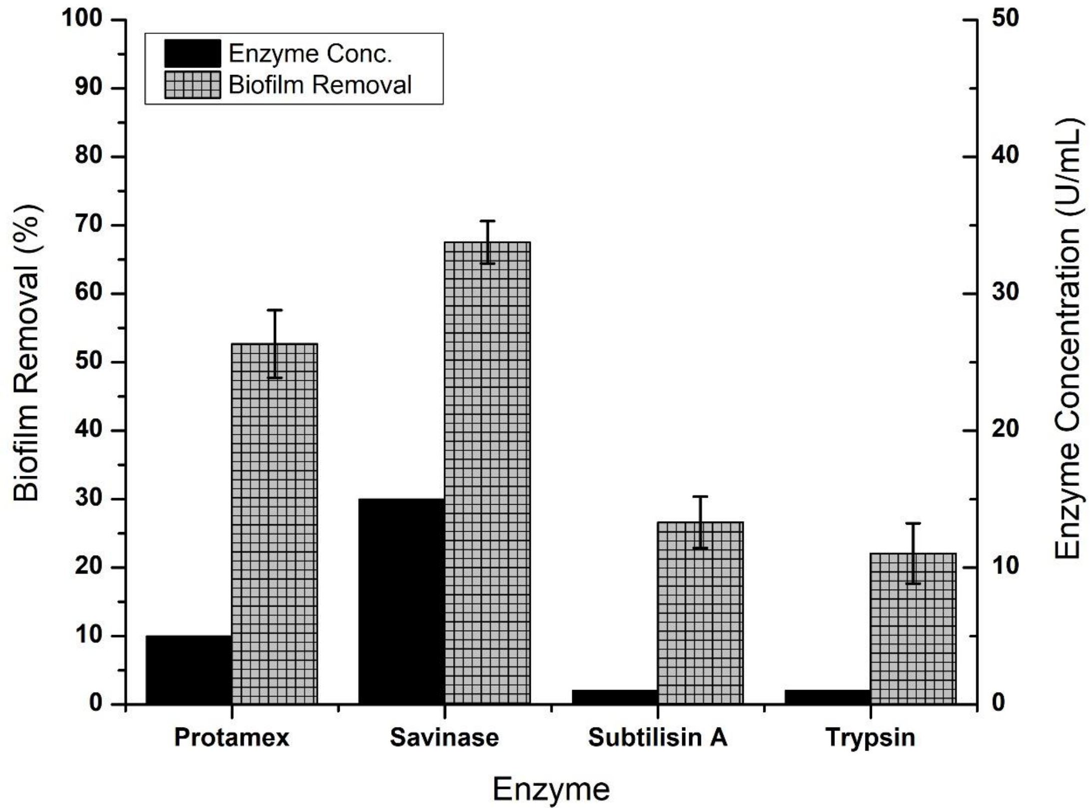

Figure 3-10. *Bacillus sp.* viable biofilm removal using proteases. Each enzyme was tested at 5 concentrations ranging between 1 and 30 U/mL. For each enzyme tested, only the enzyme concentration resulting in the highest biofilm removal is plotted for comparison with other tested enzymes. Each column is an average of 8 replicates. Error bars represent 95% confidence intervals.

The effect of enzyme concentration on biofilm removal was evaluated for four different enzymes. Savinase was the most effective protease in removing *Bacillus sp.* biofilms, as shown in *Figure 3-10*. That was followed by Protamex, Subtilisin A, and Trypsin respectively. The highest *Bacillus sp.* biofilms removal (68%) using proteases was achieved by Savinase (*Figure 3-10*).

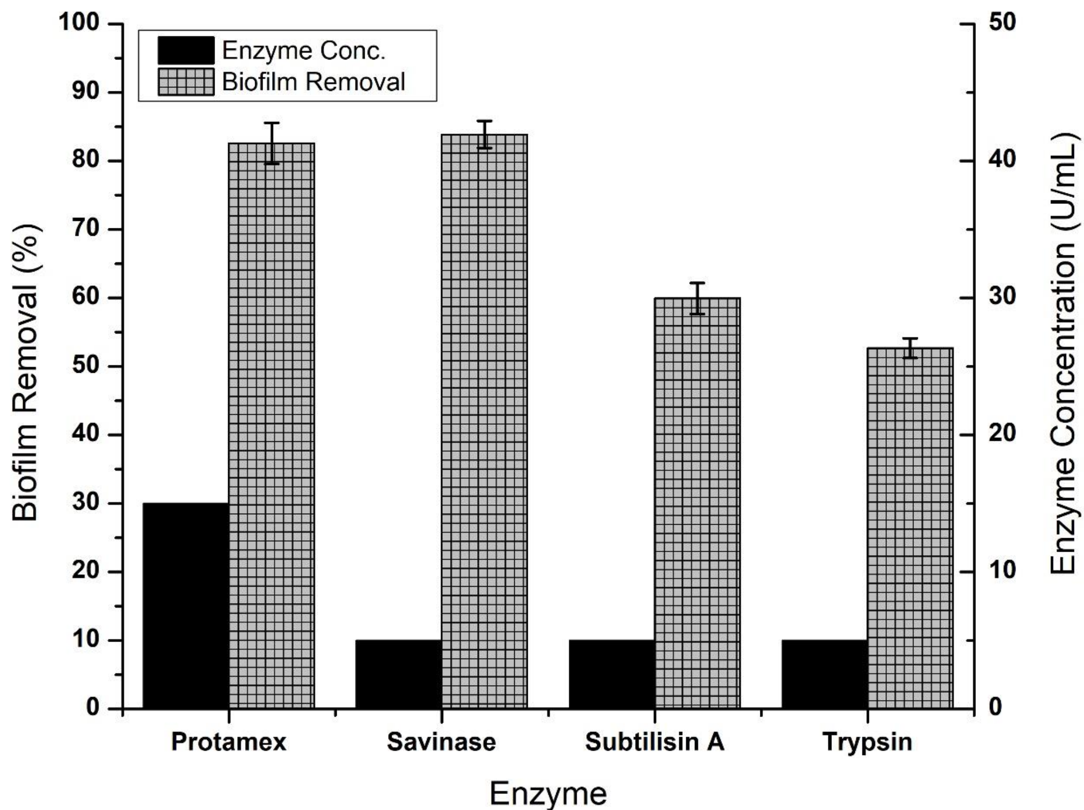

Figure 3-11. *Pseudomonas fluorescens* viable biofilm removal using proteases. Each enzyme was tested at 5 concentrations ranging between 1 and 30 U/mL. For each enzyme tested, only the enzyme concentration resulting in the highest biofilm removal is plotted for comparison with other tested enzymes. Each column is an average of 8 replicates. Error bars represent 95% confidence intervals.

Savinase was the most effective protease in *P. fluorescens* biofilm removal presented in *Figure 3-11*, this was closely followed by Protamex, and then Subtilisin A, and Trypsin respectively. The highest *P. fluorescens* biofilm removal (84%) by proteases was achieved by Savinase (*Figure 3-11*).

3.4.4.3. Biofilm Removal Using Polysaccharides-degrading Enzymes

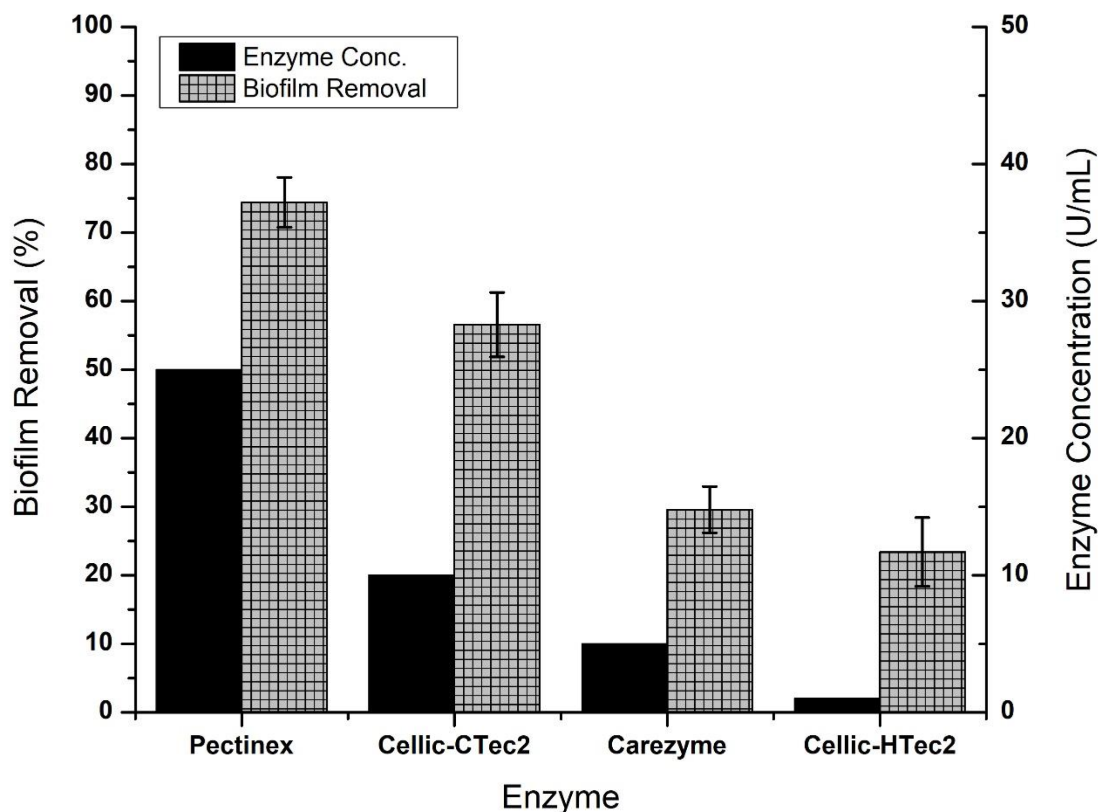

Figure 3-12. *Bacillus Sp.* viable biofilm removal using polysaccharide-degrading enzymes. Each enzyme was tested at 5 concentrations ranging between 1 and 75 U/mL. For each enzyme tested, only the enzyme concentration resulting in the highest biofilm removal is plotted for comparison with other tested enzymes. Each column is an average of 8 replicates. Error bars represent 95% confidence intervals.

The polysaccharide-degrading enzyme Pectinex was the most effective enzyme in removing biofilms formed by *Bacillus sp.* as presented in *Figure 3-12*

, this was followed by Cellic-CTec2, Carezyme, and Cellic-HTec2 respectively (*Figure 3-12*). The highest *Bacillus sp.* biofilm removal (74%) using polysaccharide-degrading enzymes was achieved by Pectinex (*Figure 3-12*).

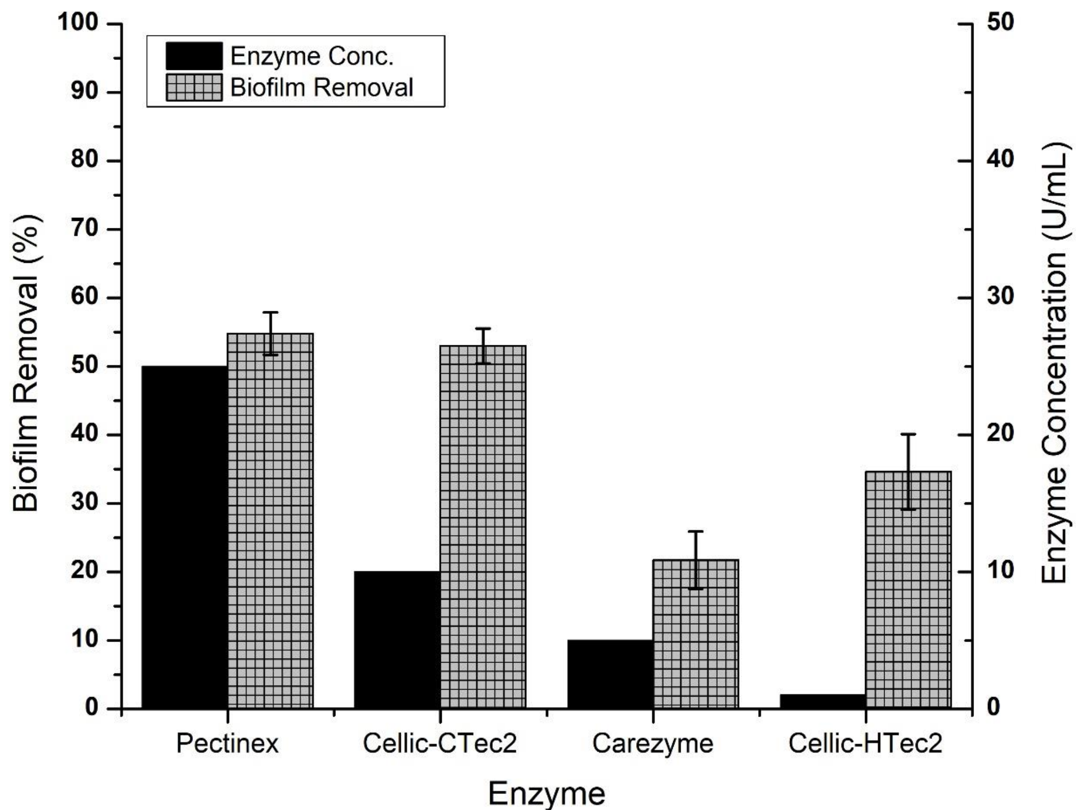

Figure 3-13. *Pseudomonas fluoresces* viable biofilm removal using polysaccharide-degrading enzymes. Each enzyme was tested at 5 concentrations ranging between 1 and 75 U/mL. For each enzyme tested, only the enzyme concentration resulting in the highest biofilm removal is plotted for comparison with other tested enzymes. Each column is an average of 8 replicates. Error bars represent 95% confidence intervals.

Pectinex was the most effective polysaccharide degrading enzyme in removing *P. fluorescens* biofilms as presented in *Figure 3-13*, this was followed closely by Cellic-CTec2, and then Cellic-HTec2, and Carezyme. The highest biofilm removal (55%) of *P. fluorescens* biofilms using polysaccharide degrading enzymes was achieved by Pectinex (*Figure 3-13*).

3.4.5. Discussion

The ability of different enzymatic treatments to remove biofilms formed by two different bacterial strains has been evaluated. The bacterial strains tested in this research are the gram-positive bacteria *Bacillus sp.* and the gram-negative bacteria *P. fluorescens*. Biofilms formed by *Bacillus sp.* are well characterised, which made it a model microorganism for biofilm studies (K. P. Lemon *et al.*, 2008; Dervaux, Magniez and Libchaber, 2014). Biofilms formed by *Bacillus sp.* has been reported to be mainly composed of polysaccharides (Branda *et al.*, 2001; Morikawa *et al.*, 2006), while proteins are reported to be the main component of *P. fluorescens* biofilms (Molobela, Cloete and Beukes, 2010), this variation in their biofilms composition provides more different scenarios for testing the different enzymatic treatments. In addition, it has been reported previously that when compared with the biofilms formed by other disease causing gram-negative microorganisms (*Pseudomonas aeruginosa, Staphylococcus aureus, and Staphylococcus epidermidis*), biofilms formed by *P. fluorescens* has shown the highest resistance to enzymatic treatments (Johansen and Falholt, 1997).

Biofilms formed by *Bacillus sp.* and *P. fluorescens* were effectively removed by both proteases and polysaccharide-degrading enzymes (*Figure 3-10*, *Figure 3-11, Figure 3-12*, and *Figure 3-13*) only at different removal efficiencies. In general, proteases were more effective in removing biofilms formed by *P. fluorescens* (84%) than biofilms formed by *Bacillus sp.* (68%) this can be seen by comparing *Figure 3-10* and *Figure 3-11*, while polysaccharide-degrading enzymes were more effective in removing biofilms formed by *Bacillus sp.* (74 %) than biofilms formed by *P. fluorescens* (55%), this can be seen by comparing *Figure 3-12* and *Figure 3-13*. A possible reason for this is the difference in the specific compositions of the biofilms formed by

the two species. Biofilms formed by *P. fluorescens* are mainly composed from proteins (Molobela, Cloete and Beukes, 2010), which makes it a more suitable substrate for proteases and hence more vulnerable to degradations by proteases, while biofilms formed by *Bacillus sp.* are mainly composed of polysaccharides (Branda *et al.*, 2001; Morikawa *et al.*, 2006), which is a more suitable substrate for the polysaccharide-degrading enzymes, and therefor biofilms formed by *Bacillus sp.* were more effectively removed by polysaccharide-degrading enzymes.

Among the proteases tested in this research, results obtained indicate that Savinase and Protamex have the highest biofilm removals for biofilms formed by both *Bacillus sp.* (*Figure 3-10*) and *P. fluorescens* (*Figure 3-11*), this was followed by Subtilisin A and Trypsin respectively. Possible reasons for that is the variation in the protein composition of the tested biofilms which made them less resistant to Savinase and Protamex (Augustin, Ali-Vehmas and Atroshi, 2004; Chaignon *et al.*, 2007; Lequette *et al.*, 2010; Molobela, Cloete and Beukes, 2010; Cordeiro, Hippius and Werner, 2011). In addition, unlike Subtilisin A and Trypsin, Protamex contains different enzymatic mixtures (bacillolysin, subtilisin, neutral proteases), which might give it an advantage facing the variations in the biofilm protein components (Garcia-Mora *et al.*, 2014; Fernandes, 2016). Previous research has shown that Subtilisin A was effective at inhibiting initial biofilm attachment, and not as much effective at dismembering pre-existing biofilms (C. Leroy *et al.*, 2008). The results obtained for Subtilisin A biofilm removal efficiency was similar to previous research findings by others (Vickery, Pajkos and Cossart, 2004; Cordeiro, Hippius and Werner, 2011). Previous research has also found Trypsin inefficient in removing biofilms formed by *Staphylococcus epidermidis* biofilms, in contrary, they found it efficient in removing biofilms formed by *Staphylococcus aureus*, this variation in efficiency was attributed to the difference in

the protein composition between the two bacterial strains (Chaignon *et al.*, 2007). The results obtained here suggests that Savinase was the most effective protease in removing biofilms formed by both species *Bacillus sp.* (68%) and *P. fluorescenes* (84%), this can be attributed to the broad substrate specificity and the superior stability that Savinase posses when compared to other proteases (Betzel *et al.*, 1992; Georgieva *et al.*, 2001; Marcato-Romain *et al.*, 2012; Garcia-Mora *et al.*, 2014). Moreover, Savinase has been reported to possess bactericidal effects (Smith, Green and Mason, 2003), and it was suggested that Savinase targets proteins involved in bacterial adhesion in biofilm formation (C Leroy *et al.*, 2008). Previous researchers have also reported Savinase to be the most effective enzyme in biofilm removal (C Leroy *et al.*, 2008; Molobela, Cloete and Beukes, 2010; Marcato-Romain *et al.*, 2012).

The results obtained for the polysaccharide degrading enzymes (*Figure 3-12* and *Figure 3-13*) showed that Pectinex and Cellic-CTec2 had the highest removal efficiency for biofilm formed by *Bacillus sp.* and *P. fluorescens*. For *Bacillus sp.* (*Figure 3-12*) this was followed by Carezyme and Cellic-HTec2 respectively, while for *P. fluorescens* (*Figure 3-13*) this was followed by Cellic-HTec2 and Carezyme respectively. The higher biofilm removal results obtained for Pectinex and CellicCTec2 can be a results of specific substrate compatibility which made the polysaccharides in biofilms formed by *Bacillus sp.* and *P. fluorescens* more suitable substrates for enzymatic degradation by Pectinex and CellicCTec2 (Augustin, Ali-Vehmas and Atroshi, 2004; Chaignon *et al.*, 2007; Lequette *et al.*, 2010; Molobela, Cloete and Beukes, 2010). Previous research reported that Cellulase (the main active enzyme in Carezyme, CellicCTec2 and CellicHTec2) was successful in reducing initial attachment of biofilms formed by *S. epidermidis*, contrary results were obtained for Cellulase on biofilms formed by *P. aeruginosa*

(Cordeiro, Hippius and Werner, 2011). Another research reported that Cellulases obtained from two different sources partially inhibited biofilm by *P. aeruginosa* (Loiselle and Anderson, 2003). Results obtained (*Figure 3-12* and *Figure 3-13*) shows that for the exopolysaccharide-degrading enzymes investigated in this research, Pectinex achieved the highest biofilm removal efficiency for biofilms formed by both *Bacillus sp.* (74%) and *P. fluorescens* (55%). The superiority of Pectinex removal efficiency over the other polysaccharide-degrading enzymes tested can be due the fact that Pectinex is a multicomponent enzymatic mixture that contains different enzymes including proteases (Protease, Pectinase, Arabanse, Cellulase, Hemicellulase, β-glucanase, and Xylanase) (Johansen and Falholt, 1997; Olwoch *et al.*, 2014), this wide diversity in its enzymatic components gives it a broad substrate specificity that in addition to the broad polysaccharide-degradation activity includes proteins degradation. Similar results for Pectinex were obtained by previous researchers (Johansen and Falholt, 1997).

3.4.6. Conclusions

It could be concluded from the results presented here that the use of proteases and polysaccharide degrading enzymes was successful in the removal of pre-existing biofilms formed by *Bacillus sp.* and *P. fluorescens*. The proteases Savinase and Protamex, and the polysaccharide-degrading enzymes Pectinex and CellicCTec2, have shown the highest removal efficiency for pre-existing biofilm formed by *Bacillus sp.* and *P. fluorescens.* Proteases were more effective in removing biofilm formed by *P. fluorescens*, while polysaccharide-degrading enzymes were more effective in removing *Bacillus sp.* biofilm. Interestingly, the results obtained emphasise the complexity of the structural stability of the biofilm. The ability of polysaccharide degrading enzymes to remove a substantial amount of *P. fluorescens* biofilm

(which is mainly composed of proteins), and the ability of proteases to remove a substantial amount of *Bacillus sp.* biofilm (which is mainly composed from polysaccharides), reflects the complex interdependent relationship between polysaccharides and proteins in maintaining the stability and structural integrity of the biofilm formation. Future work should be directed toward optimizing biofilm removal using enzymatic treatments to achieve the highest efficiency in biofilm removal treatments that target a broader spectrum of biofilms.

3.4.7. Acknowledgements

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3.4.8. References

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3.5 Optimization of Biofilm Removal Using Enzymatic Treatments

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3.5.1. Preface

Section 3.5 presents the experimental work done to fulfill the specific objective previously presented in Section 1.3.2.4. Based on the results obtained in Section 3.5 the proteases Savinase and Protamex and the polysaccharide-degrading enzymes Pectinex and Cellic-Ctec2 showed the highest biofilm removal among the tested enzymes. Section 3.5 builds on the results previously obtained in Section 3.4 and further optimize biofilm removal using enzymatic treatments. The results of the optimization experiments showed that a treatment containing Savinase and Pectinex had the highest biofilm removal efficiency. The models developed were successful in predicting biofilm removal.

3.5.2. Introduction

Biofilms are multicellular communities formed by bacterial aggregation on surfaces, bacterial cells within a biofilm are connected together and to the surface by an extracellular matrix consisting mainly from polysaccharides and proteins (Costerton *et al.*, 1987; Donlan, 2002; Hall-Stoodley; Kolodkin-Gal *et al.*, 2012). Distinctive characteristic of bacterial cells living in a biofilm community can be summarised by higher antibacterial resistance, the formation of free planktonic cells, UV light tolerance, the formation of bacterial spores, and an increased synthesis of extracellular matrix (Habash and Reid, 1999; O'Toole, Kaplan and Kolter, 2000; Hall-Stoodley and Stoodley, 2002; Rowland and Voorheesville, 2003; Garrett, Bhakoo and Zhang, 2008; Coleman *et al.*, 2010; Vlamakis *et al.*, 2013). Biofilm formation can be problimatic for the healthcare and industrial sectors (Barbeau *et al.*, 1996; Barbeau, Bokum and Gauthier, 1998). Biofilm communities can harbour different undesirable pathogens, biofilm formation can cause clogging of pipes and tubes, in addition, biofilms can interfere with the mechanical properties of dental devices (Barbeau *et al.*, 1996; Coleman *et al.*, 2010; Vasickova *et al.*, 2010).

The use of chemical treatments to remove biofilms was found to be challenging (Ntsama-Essomba *et al.*, 1997; Vickery, Pajkos and Cossart, 2004). Due to the physical barrier between the chemical treatments and the bacterial cells living within the biofilm, chemical treatments cannot access the bacterial cells (Gilbert and McBain, 2001). Lower diffusivity of chemical treatments through biofilms makes them highly resistant to disinfectants, biocides, and antibiotics. When compared to its free planktonic form, bacteria living in a biofilm formation was reported to be up to 1000-fold more resistant to chemical treatments (Gilbert and McBain, 2001). The composition of the extracellular matrix can vary depending on the

bacterial strain and other external factors (such as gaseous levels, and nutrients) (Simões, Simões and Vieira, 2010). Biofilms composition includes carbohydrates, polysaccharides, glycoproteins, nucleic acids, amyloid proteins, phospholipids, lipids, nucleic acids (Branda *et al.*, 2005; Flemming and Wingender, 2010; Hobley *et al.*, 2014)

Enzymatic treatments have been recently suggested as anti-biofilm treatments, previous research has shown their ability in removing biofilm from industrial surfaces (Taraszkiewicz *et al.*, 2013; Thallinger *et al.*, 2013; Meireles *et al.*, 2016). Enzymatic treatments have been described as more affordable, efficient, and eco-friendly alternative than the chemical treatments used in removing biofilms (Cordeiro, Hippius and Werner, 2011; Cortés, Bonilla and Sinisterra, 2011; Srey, Jahid and Ha, 2013). The use of enzymes in industry and for cleaning surfaces has been approved by regulatory agencies and no evidence of side effects has been reported (Schmidt, 2012; Meireles *et al.*, 2016).

Due to the substrate-specific nature of enzymes, it is vital to identify the components of the extracellular matrix prior to the selection of the enzymatic treatment (Molobela, Cloete and Beukes, 2010). Although there is variation in the constituents of biofilms the major two components remains to be proteins and polysaccharides (Stewart and William Costerton, 2001; Sutherland, 2001; Molobela, Cloete and Beukes, 2010), the specific enzymes that degrade these two major components of biofilm are proteases and polysaccharide-degrading enzymes, respectively (Jedrzejas, 2000; Jayani, Saxena and Gupta, 2005; C. Leroy *et al.*, 2008). The ability of proteases and polysaccharide-degrading enzymes in removing biofilm have been investigated previously, the proteases Savinase and Protamex, and the polysaccharide-degrading enzymes Pectinex and Cellic-CTec2, have shown the highest efficiency in removing

biofilms formed by *Bacillus sp.* and *P. fluorescens*, for more details please refer to Sections 3.4.4.2 and 3.4.4.3.

Bacillus species (*Bacillus sp.*) and *Pseudomonas fluorescens* (*P. fluorescens*) are considered model microorganisms for biofilm related studies (K. P. Lemon *et al.*, 2008; Rossignol *et al.*, 2008). *Bacillus sp.* is a non-pathogenic, gram-positive, biofilm forming bacteria, they can be found in abundance in both terrestrial and aquatic environments due to their ability to adapt to different environments (Grossman, 1995; Maglott *et al.*, 2007; Earl, Losick and Kolter, 2008). It has been reported that the major structural component of biofilms formed by *Bacillus sp.* is polysaccharides (Branda *et al.*, 2001; Morikawa *et al.*, 2006). *Pseudomonas fluorescens* is a gram-negative, non-pathogenic, biofilm forming bacteria (Rossignol *et al.*, 2008). This bacterium is ubiquitous in the environment and in hospitals, it is characterised with high adaptation to different environments and its ability to resist a wide range of antibiotics and disinfectants (Spiers, Buckling and Rainey, 2000; Rossignol *et al.*, 2008). Previous research reported that *P. fluorescens* produces biofilms that are mainly composed of proteins (Molobela, Cloete and Beukes, 2010).

Taking into consideration the heterogeneity of biofilms compositions, and the substrate-specific characteristic of enzymes makes it vital to use enzymatic mixtures to achieve a broader spectrum and higher efficiency of biofilm removal (Augustin, Ali-Vehmas and Atroshi, 2004; Torres *et al.*, 2011). The use of enzymatic mixtures containing proteases and polysaccharide-degrading enzymes is expected to achieve higher biofilm removal efficiency (Thallinger *et al.*, 2013). Previous researchers expected promising results from using enzymatic mixtures containing proteases and polysaccharide-degrading enzymes for biofilm removal in different

applications (Orgaz *et al.*, 2006; Torres *et al.*, 2011; Zanaroli *et al.*, 2011). To the best knowledge of the authors, this is the first-of-kind optimization study on the use of proteases and polysaccharide-degrading enzymes mixtures for biofilm removal.

Response surface methodology (RSM) is an effective mathematical and statistical tool used for analysis, modeling, and optimization of a complicated process that include several independent variables (factors) affecting a dependent variable (response) (Montgomery, Runger and Hubele, 2001; Mason, Gunst and Hess, 2003; Montgomery, 2008). The advantage of using this method is the reduction in the number of experiments required to optimize the desired response, this method have been utilized by many researchers for optimization studies in different fields (Sarchami and Rehmann, 2015; Barwal and Chaudhary, 2016; Sarchami, Johnson and Rehmann, 2016).

The objective of this research is to optimize biofilm removal efficiency of pre-existing biofilms formed by *Bacillus sp.* and *P. fluorescens* by using enzymatic mixtures consisting of the proteases Savinase and Protamex, and the polysaccharide-degrading enzymes Pectinex and Cellic-CTec2.

3.5.3. Experimental Procedures

3.5.3.1. Materials

3.5.3.1.1. 10X Tbase

928 mL DI-Water,10 g Trisodium citrate (34 mM sodium citrate), 19.8 g $(NH_4)_2SO_4$ (0.15M ammonium sulphate), 139.36 g K_2HPO_4 (0.8M potassium phosphate dibasic), 59.88 g KH_2PO_4 (0.44M potassium phosphate monobasic), autoclaved for 20 min.

3.5.3.1.2. Biofilm Growth Media for Pseudomonas fluorescens

500 mL of DI-Water, 10 g peptone, 5 gm glycerol, 0.75 g K_2HPO_4, 0.75 g $MgSO_4.7H_2O$, autoclaved for 20 min.

3.5.3.1.3. Biofilm Growth Media for Bacillus species

450 mL of DI-Water, 50 mL of 10X Tbase, 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 0.02 g D-glucose), 0.006 g $MgSO_4$, autoclaved for 20 min.

3.5.3.1.4. Wash Buffer

450 mL DI-Water, 50 mL of the 10X Tbase solution, 0.5 mL of 1M $MgSO_4$, autoclaved for 20 min.

3.5.3.1.5. Sodium Phosphate Buffer for Proteases (pH 8)

500 mL of DI-Water, 12.7 g sodium phosphate dibasic heptahydrate, 0.37 g sodium phosphate monobasic monohydrate, autoclaved 20 min.

3.5.3.1.6. Sodium Phosphate Buffer for Mixed Enzymes Treatments (pH 6)

500 mL of DI-Water, 1.65 g sodium phosphate dibasic heptahydrate, 6.10 g sodium phosphate monobasic monohydrate, autoclaved for 20 min.

3.5.3.1.7. Sodium Acetate Buffer for Polysaccharide-degrading enzymes (pH 4)

2.45 g sodium acetate trihydrate, 410 mL of 0.2 M acetic acid solution, bring final volume to 1 L, autoclaved for 20 min.

3.5.3.1.8. Bacterial Strains

The *Bacillus species* strain used is known commercially as Unizyme 20X CSB 20 NF SPORE was provided by Germiphene Inc. The *Pseudomonas fluorescens* strain used was obtained from DSMZ in Germany. It is known as *Pseudomonas fluorescens* Migula 1895 (DSM No. 50090, type strain).

3.5.3.2. Methods

3.5.3.2.1. Seed Culture Preparation

3.5.3.2.1.1. Bacillus species

One milliliter of the *Bacillus sp.* spore suspension was washed three times. The wash procedure was carried on via centrifuge at 10,000 rounds per minute for 1 min and then resuspended in 1 mL wash buffer using a vortex, the washed spores are then concentrated using the centrifuge (10,000 rpm, 1 minute) and then resuspended in 1 mL biofilm growth medium, half a milliliter of the washed resuspended spores were used to inoculate 20 mL of the growth medium using a 150 mL glass flask inside a 37 ^{o}C humidified incubator oven at 300 rounds per min until optical density was between 1 and 1.6 at 600 nm (using DI-Water as blank).

3.5.3.2.1.2. Pseudomonas fluorescens

The received *P. fluorescens* culture was revived in growth media until optical density was 1.2 at 600 nm. Samples of 1 mL size were taken and preserved with 20% glycerol in -80°C freezer. At the start of each experiment, the frozen culture samples were revived in growth media, by adding 0.5 mL of thawed culture to 20 mL fresh growth media, at 21°C and 300 rounds per minute, until optical density measured was between 1 and 1.6 at 600 nm (using DI-Water as blank).

3.5.3.2.2. Inoculating the Microtiter Plates and Biofilm Growth

For each of the two bacterial strains, the prepared seed culture was diluted in a 1:100 ratio in the appropriate growth media. The wells in the microtiter plates were filled with 100 µL of the diluted seed culture. The control wells were prepared using 100 µL of sterile growth media. The plates were covered with a thin sterile film to reduce evaporation, a hole was poked in the center of the wells for aeration using a sterile 22-gauge needle. The *Bacillus sp.* microtiter plates were then incubated in a humidified oven at 37 °C in static conditions for 38 h. The *P. fluorescens* microtiter plates were incubated at 21 °C for 96 h, the growth media was replenished every 24 h. Subsequently at the end of the incubation time the plates were washed gently three times using wash buffer (Tecan Hydroflex Plate Washer).

3.5.3.2.3. Fixing Biofilm Growth

Fixed biofilms are assumed to be dead or nonviable. This procedure is done to preserve the biofilm that formed at a certain stage to be quantified later. This procedure is repeated twice during the experiment. First time is after initial biofilm growth and just before applying the enzymatic treatments, this is done to specific wells (8 wells) to be able to quantify initial biofilm

growth prior to the application of treatments. The second time is done after the end of the enzymatic treatment duration to the wells containing the enzymatic treatments and prior to final biofilm quantification. The procedure was done by washing the microtiter plates to remove growth medium, and filling each well with 200 µL methanol for 20 minutes at room temperature. The methanol is then removed using the Tecan Hydroflex Plate Washer and microtiter plates are left to air dry at room temperature (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010).

3.5.3.2.4. Enzymatic Treatments Preparation

The protease treatment containing Savinase and Protamex was prepared in sodium phosphate buffer (pH 8), while the polysaccharide-degrading enzymes treatment containing Pectinex and Cellic-CTec2 was prepared in sodium acetate buffer (pH 4). The mixed enzymatic treatment containing Savinase and Pectinex was prepared in three different buffers, sodium phosphate buffer (pH 8), sodium phosphate buffer (pH 6), and sodium acetate buffer (pH 4) as previously explained.

The activities of the enzymes used were quantified before and after conducting the experiments (data not shown), no change was noticed in the enzymatic activity, for more details please refer to Section (3.4.3.2.5). The activity unit (U) of proteases (Savinase and Protamex) is defined as the required amount of enzyme to hydrolyse casein at pH 7.5, 37 ^{o}C, in 1 minute, to produce color equivalent to 1 µmole of tyrosine (Folin and Ciocalteu, 1927; Anson, 1938). The activity unit (U) for Pectinex is defined as the amount of enzyme required to liberate 1 µmole of galacturonic acid from poly-galacturonic acid in 1 h at pH 4 and 25 ^{o}C. The activity

unit (U) for Cellic-CTec2 is defined as the amount of Cellic-CTec2 required at 37°C, and pH 5 to liberate 1 µmole of glucose from cellulase in 1 h (Worthington, 1988).

3.5.3.2.5. Applying Treatments

At the end of initial biofilm growth, the microtiter plates are washed with the wash buffer using Tecan Hydroflex Plate Washer. One column (8 wells) of each plate is fixed with methanol as explained previously. The designated enzymatic treatments were applied by dispensing 125 µL of each enzymatic treatment into their designated wells. The microtiter plates for both bacterial strains were then incubated at 21 °C in a sterile chamber for 24 h.

3.5.3.2.6. Total Biofilm Quantification Using Crystal Violet Stain

Crystal violet binding assay is a widely used method for biofilm quantification (O'Toole, Kaplan and Kolter, 2000; Kolodkin-Gal *et al.*, 2010, 2012; Brandenburg *et al.*, 2013; Zhang and Hu, 2013). At the end of the treatment duration, microtiter plates are washed and fixed, and left to air dry completely, now the microtiter plates are ready for biofilm quantification. By using a manual multichannel pipette, each well was filled with 150 µL of 0.1 % (W/V) crystal violet stain, the plates are then incubated at the bench top and room temperature for 60 minutes (Stepanović *et al.*, 2000; Kwasny and Opperman, 2010). The non-binding crystal violet stain in the wells is then washed 4 times using Tecan Hydroflex plate washer by dispensing 200 µL DI-Water in drip mode and aspirating without touching the bottom of the wells. The microtiter plates were left to air dry on the bench top at room temperature. The biofilm-binding crystal violet was resolubilized by dispensing 200 µl of 30% acetic acid using a manual multichannel pipettor into each well (Stepanović *et al.*, 2000), the microtiter plates are then incubated on bench top at room temperature for 20 min. Using a manual multichannel pipet, a

volume of 100 µL from each well was transferred to polystyrene plates with the flat clear bottom and absorbance is measured at 570 nm optical density using TECAN infinite M200 Pro Plate reader.

3.5.3.2.7. Optimization of Biofilm Removal

3.5.3.2.7.1 Biofilm Removal

Biofilm removal experiments were conducted on pre-grown biofilms in 96-well microtiter plates using the different enzymatic treatments. Biofilm removal was calculated using Equation *(1)* below:

$$\% \, Biofilm \; Removal = \frac{Initial \; Biofilm - Final \; Biofilm}{Initial \; Biofilm} \times 100\% \cdots \quad (1)$$

Where the initial biofilm is the biofilm formed in the wells before the treatments were applied, and the final biofilm is the biofilm remaining in the wells after the enzymatic treatment.

3.5.3.2.7.2 Central Composite Design and Statistical Analysis

A two-factor central composite design (CCD) was developed to find the optimal combination of enzymes concentrations for maximizing the biofilm removal efficiency using enzymatic treatments. This method has been widely used by researchers in optimization experiments in different fields of research (Kumari and Sarkar, 2014; Sarchami and Rehmann, 2015; Barwal and Chaudhary, 2016; Sarchami, Johnson and Rehmann, 2016). The five un-coded values used for the different concentrations of each enzyme were as follows [high star point,

high central point, center point, low central point, low star point]: Savinase, Protamex, and Cellic concentrations (U/mL) [30.27, 28, 16, 4, 1.73]: Pectinex concentrations (U/mL) [97.1, 90, 52.5, 15, 7.9].

For the optimization of the protease enzyme Savinase and the polysaccharide-degrading enzyme Pectinex, the pH, and microorganism type were both set as a categoric factors, the enzymatic combinations were tested at three pH values (4, 6, and 8), and two microorganisms (*Bacillus sp.* and *P. fluorescens*), these conditions result in a total of 6 blocks. The optimization of the two proteases Savinase and Protamex was performed at a single pH value of 8 (optimal pH for proteases), and microorganism type (*Bacillus sp.* and *P. fluorescens*) was set as categoric factor, these conditions result in a total of 2 blocks. The optimization of the polysaccharide-degrading enzymes Pectinex and Cellic were performed at a single pH value of 4 (optimal pH for polysaccharide-degrading enzymes), and microorganism type (*Bacillus sp.* and *P. fluorescens*) was set as categoric factor, these conditions result in a total of 2 blocks. These conditions combined for all the different tested conditions result in a total number of 10 blocks.

The software Design Expert 9.0.6.2 was used to develop the experimental central composite design. Each non-centre point (total of 8 points) was tested in 4 replicates, while centre points (total of 1 point) were tested in 9 replicates, a total of 41 runs for each block. All locations of testing conditions were randomized on the microtiter plate.

Following the application of the different treatment combinations, the biofilm removal was calculated for the different treatments using Equation *(1)*. The treatment conditions (block)

that resulted in the highest biofilm removal for each of *Bacillus sp.* and *P. fluorescens* was chosen for model fitting and analysis (total of 2 models one for each microorganism).

The selection of the fitted model was determined using sequential model probability (p-value), Lack-of-Fit probability (p-value), adjusted R^2, and predicted R^2. The second order model presented in Equation *(2)* was selected and fitted to the experimental data using linear regression analysis.

$$Y = C_0 + \sum_{i=1}^{2} C_i X_i + \sum_{i=1}^{2} C_{ii} X_i^2 + \sum_{1 \leq i \leq j}^{2} C_{ij} X_i X_j + \varepsilon \quad \cdots \quad (2)$$

Where: *Y* is the dependent variable (biofilm removal), *C* is a constant representing the regression coefficient, *X* is the independent variables (enzymes concentrations), and Ɛ is the unobserved random error.

Design expert software 9.0.6.2 was used to analyse the experimental data obtained. Analysis of variance (ANOVA) was used to verify the significance of the models selected and the significance of each model term. The F-test with an alpha value of 0.05 was used to evaluate the significance. The adequacy of the models was verified using model adequate precision and predicted versus actual plots. Normal probability plots were used to evaluate the normal distribution of errors for the fitted models (normally distributed, and insignificant).

Design Expert 9.0.6.2 was used for numerical optimization to find the optimal enzymes concentrations required to achieve the highest biofilm removal efficiency. The developed

model and enzymes optimization results were then validated by conducting experiments with values near the predicted optimal points.

3.5.4. Results

3.5.4.1. Central Composite Design (CCD)

The enzymes used in this research are industrial mixtures that have shown high efficiency in biofilm removal for biofilms formed by *Bacillus sp.* and *P. fluorescens* (please see Sections 3.4.4.2 and 3.4.4.3). The optimal conditions for these enzymes as specified by the manufacturer are listed in *Table 3–2*.

Table 3–2. List of Enzymes and Their Reported Optimum Conditions.

Enzymes		**Optimum Activity Range**		**Source**
Name	**Type**	**pH**	**Temp. (°C)**	
Protamex	Protease	7.5 - 9.5	40 - 60	Sigma-Aldrich Inc., USA
Savinase	Protease	8.0 - 11	55 - 75	Sigma-Aldrich Inc., USA
Pectinex	Polysaccharide-degrading	3.5 - 6.0	30 - 60	Sigma-Aldrich Inc., USA
Cellic	Polysaccharide-degrading	4.0 - 6.5	40 - 60	Novozymes A/S, Denmark

Synergistic effects are expected when using mixtures of the enzymes presented in *Table 3–2*, however conditions suitable for both proteases and polysaccharide-degrading can be outside of the optimum conditions of the individual enzymes, hence an overall optimization using mixtures of two enzymes in each tested block were conducted via a central composite design. The pH value and the microbial strain were treated as categoric factors, and the

enzymes concentrations were optimized, leading to 10 independent CCDs (blocks) as shown in *Table 3–3*.

Table 3–3. List of Optimization Factors Used in CCDs.

Optimized factors	Categoric Factors			Number
Enzymes (U/mL)	pH	Bacterial Strain		of Blocks
Savinase & Pectinex	4, 6, 8	*Bacillus sp.*	*P. fluorescens*	6
Pectinex & Cellic	4	*Bacillus sp.*	*P. fluorescens*	2
Savinase & Protamex	8	*Bacillus sp.*	*P. fluorescens*	2
			Total Blocks	10

After applying the different treatments for 24 hrs, biofilm removal was calculated using Equation *(1)*. The data of the block that resulted in the highest biofilm removal for each of the two microbial strains (*Bacillus* sp. and *P. fluorescens*) are presented in *Table 3–4* (similar data was collected for the 8 other blocks). It can be seen from the experimental results presented in *Table 3–4* that the enzymatic treatment mixture containing Savinase and Pectinex resulted in the highest biofilm removal for both bacterial strains, only at two different pH values (6 and 8 for *Bacillus sp*. and *P. fluorescens, respectively*). The use of Savinase and Pectinex was successful in removing biofilms formed by *Bacillus sp*. and *P. fluorescens* within the range of tested input variables. When compared with biofilms formed by *Bacillus sp.*, results obtained in *Table 3–4* show that a higher biofilm removal was achieved for biofilms formed by *P. fluorescens*.

Table 3–4. Biofilm Removal Corresponding to Conditions (two blocks, one for each microbial strain) That Resulted in The Maximum Biofilm Removal for Bacillus sp. and P. fluorescens Determined for CCDs. Error values represent 95% confidence intervals.

Savinase	Pectinex	Number of	pH		Biofilm Removal (%)	
U/mL	U/mL	replicates	*Bacillus sp.*	*P. fluorescens*	*Bacillus sp.*	*P. fluorescens*
1.7	52.5	4	6	8	48.7 ± 1.7	56.9 ± 4.1
4	15	4	6	8	47.0 ± 3.4	61.0 ± 3.5
4	90	4	6	8	53.0 ± 3.5	48.4 ± 4.1
16.0	7.9	4	6	8	61.6 ± 1.3	76.8 ± 5.1
16.0	52.5	9	6	8	66.2 ± 1.1	81.5 ± 2.9
16.0	97.1	4	6	8	65.4 ± 2.0	65.5 ± 4.4
28.0	15.0	4	6	8	63.4 ± 2.2	76.3 ± 2.5
28.0	90.0	4	6	8	67.5 ± 1.0	77.9 ± 4.5
30.3	52.5	4	6	8	65.2 ± 2.5	81.8 ± 2.5

The two treatment conditions (two blocks) presented in *Table 3–4* were further used for model selection and optimization (for all the following work presented in this research).

3.5.4.2. Models Selection, Fitting, and Statistical Analysis

The data presented in *Table 3–4* was fitted with the quadratic model presented in Equation *(2)*, the fit summary of the tested models are presented in *Table 3–5*. Based on the results obtained for the sequential model sum of squares, the lack of fit test, adjusted R^2 and predicted R^2 for the fitted models, a quadratic model was found to best fit the data and was chosen for both *Bacillus sp.* and *P. fluorescens* (the data was also tested with linear and cubic models, it was found to best fit the quadratic model presented in Equation *(2)*, data not shown for linear and quadratic models).

Table 3–5. Fitted Model Selection Summary

Microbial Strain	Model Order	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2
Bacillus sp.	Quadratic	< 0.0001	0.6159	0.9186	0.8993
P. fluorescens	Quadratic	< 0.0001	0.6266	0.8861	0.8642

The results obtained from the ANOVA test for the response surface of the fitted quadratic models for *Bacillus sp.* and *P. fluorescens* are presented in *Table 3–6* and *Table 3–7*, respectively. The results presented in *Table 3–6* shows all the significant model parameters. The p-value obtained for the model suggests that it is highly significant, while the p-value for the Lack-of-Fit test suggest that it was insignificant, which is desired. Similar results were obtained in *Table 3–7* for *P. fluorescens*. The results obtained for R^2, Adjusted R^2, and predicted R^2 are presented in *Table 3–6* and *Table 3–7*, R^2 and Adjusted R^2 values are close to 1.0 which indicates a high correlation between predicted and observed values, this shows that the models are excellent in explaining the relationship between response (biofilm removal) and the independent variables (enzymes concentrations) (Myers, Raymond H; Montgomery, Douglas C; Anderson-Cook, 2009). From the results presented in *Table 3–6* and *Table 3–7* it can be seen that Savinase was the parameter with the highest influence on the biofilm removal for both microorganisms tested, the results showed that the interaction term (AB) was significant for the removal of biofilm formed by *P. fluorescens* and not significant for *Bacillus sp.* The results show that an optimal biofilm removal exist at a concentration that if exceeded the biofilm removal will be negatively affected.

Table 3–6. Analysis of variance table for response surface fitted quadratic model for Bacillus sp. viable biofilm removal.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	Significant
Model	2207.58	4	551.89	91.27	< 0.0001	Yes
A(Savinase)	*1498.70*	*1*	*1498.70*	*309.30*	*< 0.0001*	*Yes*
B(Pectinex)	*124.71*	*1*	*124.71*	*25.74*	*< 0.0001*	*Yes*
A^2	*573.78*	*1*	*573.78*	*118.42*	*< 0.0001*	*Yes*
B^2	*48.89*	*1*	*48.89*	*10.09*	*0.0031*	*Yes*
Residual	173.20	36	4.81			
Lack of Fit	*12.73*	*4*	*3.18*	*0.63*	*0.6416*	*No*
Pure Error	*160.47*	*32*	*5.01*			
Cor Total	2380.78	40				
R^2						0.93
Adjusted R^2						0.92
Predicted R^2						0.91
Adequate Precision						25.0

Final Equation in Terms of Actual Factors:

$$Biofilm\ Removal\ (\%) = 37.954 + 2.068A + 0.199B - 0.045A^2 - 0.001B^2$$

The goodness of fit was statistically tested for the models and their associated parameters using the F-test for ANOVA, the results obtained are presented in *Table 3–6* and *Table 3–7*. The models fitted to the biofilm removal of *Bacillus sp.* and *P. fluorescens* have an F-value of 91.27 and 63.21, the probability of an F-value this large to occur due to noise is 0.01%. This indicates that the models are highly significant for the prediction of biofilm removal. The p-values presented in *Table 3–6* and *Table 3–7* were used to evaluate the significance of the models' parameters. A p-value less than 0.05% is required for a model parameter to be considered significant (Berthouex and Brown, 1994). From the results presented in *Table 3–6*

and *Table 3–7*, it can be seen that all models' parameters were found to be highly significant. An adequate precision value of 4 is required for the model to be considered adequate. The results obtained for adequate precision for the models in *Table 3–6* and *Table 3–7* are 22.67 and 22.29, which indicates that both models are highly adequate. The results presented in *Table 3–6* and *Table 3–7* indicates that the models selected are suitable for prediction of biofilm removal within the central composite design range.

Table 3–7. Analysis of variance table for response surface fitted quadratic model for P. fluorescens viable biofilm removal.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	Significant
Model	5224.25	5	1044.85	63.21	< 0.0001	Yes
A(Savinase)	3243.76	1	3243.76	196.25	< 0.0001	*Yes*
B(Pectinex)	347.87	1	347.87	21.05	< 0.0001	*Yes*
AB	203.36	1	203.36	12.30	0.0013	*Yes*
A^2	959.45	1	959.45	58.05	< 0.0001	*Yes*
B^2	693.12	1	693.12	41.93	< 0.0001	*Yes*
Residual	578.51	35	16.53			
Lack of Fit	30.28	3	10.09	0.59	0.63	*No*
Pure Error	548.23	32	17.13			
Cor Total	5802.76	40				
R^2						0.90
Adjusted R^2						0.89
Predicted R^2						0.86
Adequate Precision						22.29

Final Equation in Terms of Actual Factors:

$$Biofilm\ Removal\ (\%) = 49.479 + 2.369A + 0.314B + 0.008AB - 0.059A^2 - 0.005B^2$$

The numerical relationship between the independent variables (enzymes concentrations) and the dependent variable (biofilm removal) was described using a second-order polynomial quadratic equation. The quadratic equations developed in terms of actual factors (enzymes concentrations) for the response (biofilm removal) are presented *Table 3–6* and *Table 3–7* for *Bacillus sp.* and *P. fluorescens*, respectively.

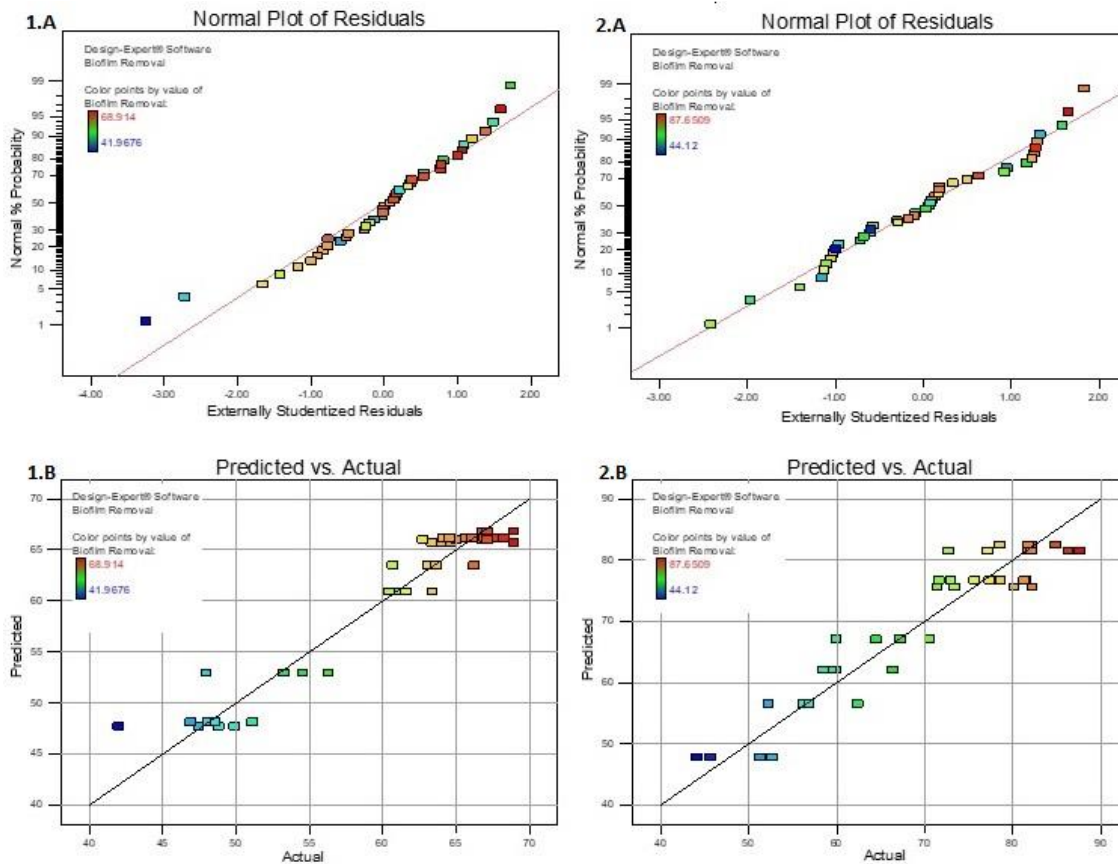


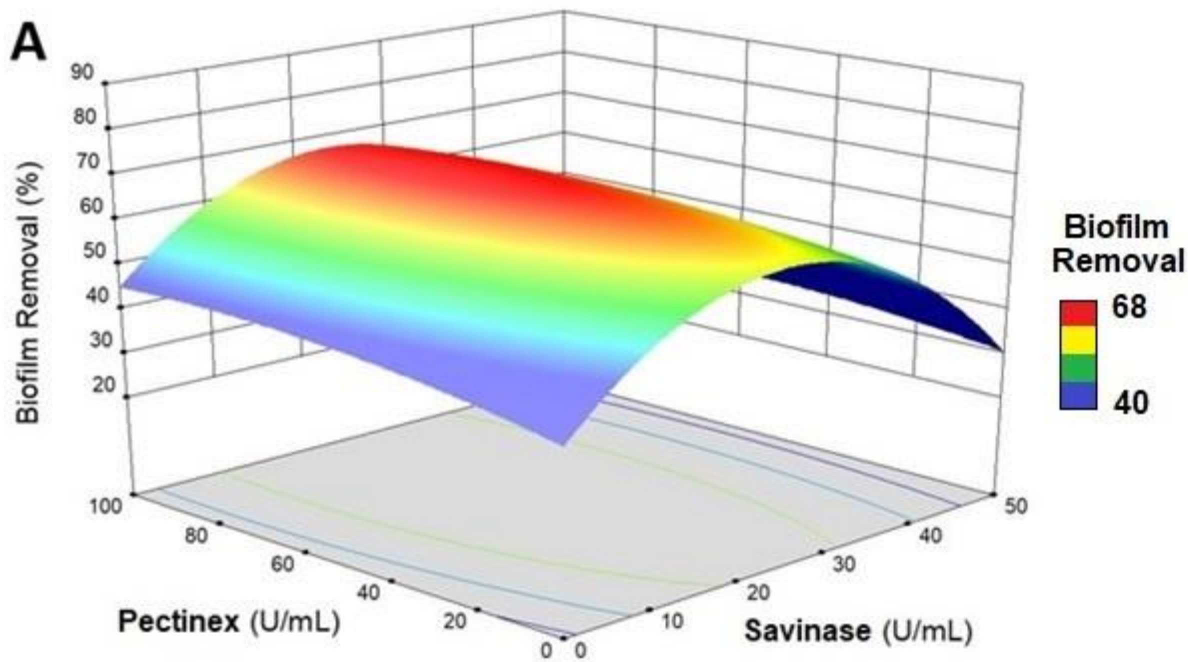
Figure 3-14. Normal % Probability Versus Externally Studentized Residuals for *Bacillus sp.* (1.A) and *P. fluorescens* (2.A) and The Model Predicted Values Versus Actual Experimental Values for *Bacillus sp.* (1.B) and *P. fluorescens* (2.B).

The results of the normal (%) probability plots of the external studentized residuals for the suggested models are presented in *Figure 3-14* (1.A and 2.A, for *Bacillus sp.* and *P. fluorescens* respectively), It can be seen from the plots that the errors were generally

insignificant and normally distributed, which is desired. Residual analysis assures that the analytical data fits the statistical assumptions. The results of the predicted versus actual plots presented in *Figure 3-14* (1.B and 2.B, for *Bacillus sp.* and *P. fluorescens* respectively) shows that the models were satisfactory in predicting real conditions. Good agreement can be seen between predicted and actual and no obvious dispersal can be noticed. Results obtained for predicted versus actual (*Figure 3-14*) suggests that the models were adequate in predicting real conditions. The diagnostic plots presented in *Figure 3-14* can be used to evaluate how satisfactory are the models developed, which was found to be highly satisfactory.

3.5.4.3. Response Surface Analysis and Numerical Optimization

Response surface plots provide a better visualization of the effect of each of the tested factors (enzymes concentration) on the measured response (Biofilm Removal) within the range of the tested factors.



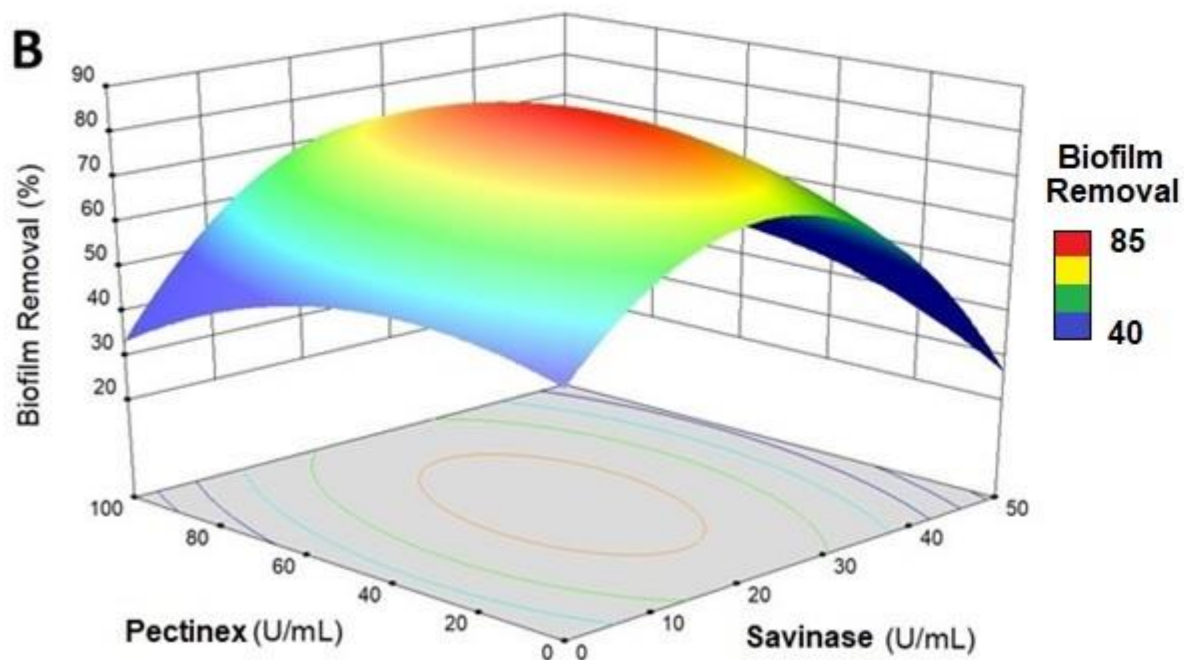


Figure 3-15. Optimal Biofilm Removal for Bacillus sp. (A), and P. fluorescens (B).

The response surface plots of biofilm removal for *Bacillus sp.* and *P. fluorescens* are presented in *Figure 3-15*. It can be clearly seen from the plots that an optimum biofilm removal exists within the observed design space with respect to enzymes concentration for both *Bacillus sp.* and *P. fluorescens*. Increasing the enzymes concentrations close to midranges results in an increase in biofilm removal for both *Bacillus sp.* and *P. fluorescens*. While increasing the concentration of the enzyme beyond midrange results in a decrease in biofilm removal for both *Bacillus sp.* and *P. fluorescens*.

Numerical optimization was conducted on the models developed for Biofilm removal for each bacterial strain. The results of the numerical optimization for the different treatment conditions tested (all 10 blocks) are presented in *Table 3–8*. The highest biofilm removal for *Bacillus sp.* (68.7%) was achieved by Savinase (22.6 U/mL) and Pectinex (70.9 U/mL) at a pH of 6. The highest biofilm removal for *P. fluorescens* (84.5%) was achieved by Savinase (23.7 U/mL) and Pectinex (48.8 U/mL) at a pH value of 8. The results presented in *Table 3–8* suggest that, for both microorganisms tested in this research, generally a higher biofilm removal can be achieved at higher pH values, and when compared with *Bacillus sp.*, a higher biofilm removal was achieved by most treatments for biofilms formed by *P. fluorescens*.

Table 3–8. Numerical Optimization Results of All Initial Treatment Conditions (10 blocks) for Biofilm Removal for Bacillus sp. and P. fluorescens.

Bacterial Species	**pH**	**Enzymes**				**Optimal Biofilm Removal (%)**
		Name		**Conc.* (U/mL)**		
		1	**2**	**1**	**2**	
Bacillus sp.	4	Savinase	Pectinex	18.3	76.9	52.8
P. fluorescens	4	Savinase	Pectinex	18.1	65.2	82
Bacillus sp.	6	Savinase	Pectinex	19.6	63.8	68.1[H]
P. fluorescens	6	Savinase	Pectinex	14.1	57.3	79.3
Bacillus sp.	8	Savinase	Pectinex	23.3	62	50.6
P. fluorescens	8	Savinase	Pectinex	23.7	48.8	84.5[H]
Bacillus sp.	4	Pectinex	Cellic-CTec2	59.1	19	67.3
P. fluorescens	4	Pectinex	Cellic-CTec2	57.6	14.2	55.8
Bacillus sp.	8	Savinase	Protamex	19.7	13.3	38.7
P. fluorescens	8	Savinase	Protamex	20.8	10.3	75.2

[H] represents the highest biofilm removal obtained for the specified bacterial strain

* represents the enzyme concentration corresponding to the optimal biofilm removal

The results presented in *Table 3–8* shows the numerical optimization of all treatment conditions (10 blocks) tested at the beginning of this work. The results of the numerical optimization of all treatment conditions (10 blocks) reassures the proper selection of the treatment conditions (blocks) that resulted in the highest biofilm removal for each bacterial strain (presented in *Table 3–4*). The results of the treatment conditions containing both Savinase and Pectinex (6 blocks) presented in *Table 3–8* showed that the pH value appears to be proportional to the optimal concentration of Savinase and inversely related to the optimal concentration of Pectinex.

3.5.4.4. *Verification of Response Surface Models and Optimal Conditions*

The actual and predicted values of *Bacillus sp.* and *P. fluorescens* near optimal biofilm removal is presented in *Table 3–9*. The results of predicted and actual biofilm removal presented in *Table 3–9* for *Bacillus sp.* and *P. fluorescens* was compared using the statistical T-test at 95% confidence interval and no significant difference was found (data not shown). The results presented in *Table 3–9* shows that the response surface models were successful in predicting optimal biofilm removal.

Table 3–9. Models and Optimal Conditions Verification. Errors for biofilm removal represent 95% confidence intervals.

Microorganism	**Factor 1**	**Factor 2**	**Factor 3**	**Biofilm Removal (%)**	
	Savinase	**Pectinex**	**pH**	**Predicted**	**Actual**
Bacillus sp.	16	52.5	6	66.17±1.42	67.27±1.29
P. fluorescens	16	52.5	8	81.44±2.62	80.09±3.25

3.5.5. Discussion

Treatments tested in this research were applied to pre-existing biofilms of both *Bacillus sp.* and *P. fluorescens* at room temperature (21ºC) for 24 h to mimic actual conditions in treatment application. The enzymes used in this research are presented in *Table 3–2*, these enzymes were chosen due to their reported efficiency in the removal of biofilms formed by *Bacillus sp.* and *P. fluorescens* (please see Sections 3.4.4.2 and 3.4.4.3). The two microorganisms used to produce biofilms, *Bacillus sp.* and *P. fluorescens*, are chosen due to their different biofilm composition (please see Section 3.5.2); to represent more scenarios that might be

encountered in real conditions. The intention of this study is not to discuss the mechanism of biofilm removal using enzymes as this was covered in previous work (please see Section 3.4). Although researchers have previously recommended the use of different enzymatic mixtures for biofilm treatment (Cordeiro, Hippius and Werner, 2011; Torres *et al.*, 2011), to the best knowledge of the authors, this is the first attempt to optimize the use of proteases and polysaccharide-degrading enzymes in a mixed enzymatic treatment containing both enzymes types for biofilm removal.

The experimental conditions and biofilm removal results presented in *Table 3–4* are the actual values of the independent variables (concentrations of enzymes used, pH, and bacterial strain) and their measured responses (biofilm removal). All experimental conditions presented in *Table 3–4* were chosen based on a central composite design. The aim of the central composite design was to present a simple empirical correlation between the concentrations of the two different enzymes used (two independent variables) and biofilm removal (the measured response). As can be seen from *Table 3–4* and *Table 3–8* all tested enzymatic treatments were successful in removing biofilms formed by *Bacillus sp.* and *P. fluorescens* within the ranges of tested input values. This was expected as previous work conducted indicated that optimal biofilm removal existed within these ranges (please see Section 3.4.4). The experimental conditions (enzyme 1, enzyme 2, pH, and bacterial strain) presented in *Table 3–4* (and later confirmed in *Table 3–8*) that resulted in the highest biofilm removal for *Bacillus sp.* and *P. fluorescens* were selected for further analysis.

The generated actual datasets for the treatment condition that resulted in the highest biofilm removal (presented in *Table 3–4*) went through further analysis for model selection,

using the sequential model sum of squares, lack-of-fit test, predicted R^2, adjusted R^2 presented in *Table 3–5* and were found to best fit the quadratic model presented in Equation *(2)*. The p-value under the sequential term in *Table 3–5* represents the probability that the order terms are modelling the noise rather than the trend in the response (biofilm removal). The p-values obtained for the quadratic models of *Bacillus sp.* and *P. fluorescens* were less than 0.0001 for both bacterial strains, the probability that the order terms are modelling the noise rather than the trend in the response is less than 0.01%, which is highly desirable. The p-values obtained for the lack-of-fit test for *Bacillus sp.* and *P. fluorescens* were 0.62 and 0.63, the best model should have an insignificant lack-of-fit with the desired p-value higher than 0.10, the probability that this large lack of fit could occur due to noise is 61.59% and 62.66%, respectively. The obtained p-values for the lack-of-fit test shows that the selected models have highly insignificant lack-of-fit, which is highly desirable. The selected quadratic models for *Bacillus sp.* and *P. fluorescens* have high R^2 and adjusted R^2 values (presented in *Table 3–5*), which is highly desirable. Based on the results presented in *Table 3–5*, a quadratic model was selected for both *Bacillus sp.* and *P. fluorescens*. The response surface representing the selected models is presented in *Figure 3-15* for *Bacillus sp*. and *P. fluorescens*.

The selected quadratic models and their associated parameters for *Bacillus sp.* and *P. fluorescens* were analysed for their significance using their F-value and the results are presented in *Table 3–6* and *Table 3–7*, respectively. The models F-value for *Bacillus sp.* and *P. fluorescens* were found to be 91.27 and 63.21, there is less than 0.01% chance that F-values this large could be due to noise. The associated model p-value is the probability that the data used produced false effects, this value is desired to be less than 0.05 for the model to be significant,

results of model p-value obtained for *Bacillus sp.* and *P. fluorescens* were less than 0.0001 for both bacterial strains, which indicates that both models are highly significant. The significance of models' parameters was determined using their associated p-values, a p-value less than 0.05 is required for a model parameter to be considered significant, as can be seen from p-values presented in *Table 3–6* and *Table 3*–7, all models parameters were found to be highly significant with p-values less than 0.0001. The results presented in *Table 3–6* and *Table 3–7* also suggest that all tested factors have a great effect on biofilm removal.

The points presented in *Figure 3-14* (1.A and 2.A) for the normal (%) probability of the external studentized residuals lay close to the straight line and between -2.0 and 2, which indicates that errors were insignificant and normally distributed (Mason, Gunst and Hess, 2003). This assures that the analytical data presented fits the statistical assumptions made. Another important part of the model statistical analysis is the model adequacy check. Models adequacy was evaluated using actual versus predicted plots presented in *Figure 3-14* (1.B and 2.B), it can be seen that there is good agreement between actual and predicted values with no obvious dispersal, this indicates that the models are adequate. These results confirm the adequate precision results presented in *Table 3–6* and *Table 3*–7.

Bacillus sp. optimal biofilm removal was found to be 68.7% at pH of 6, and concentrations of 22.6 U/mL and 70.9 U/mL for Savinase and Pectinex respectively. Optimal biofilm removal for *P. fluorescens* was found to be 84.5% at pH of 8, and concentrations of 22.6 U/mL and 70.9 U/mL for Savinase and Pectinex respectively. The response surface plots presented in *Figure 3-15* shows that Savinase and Pectinase were effective in removing pre-existing biofilms formed by *Bacillus sp.* and *P. fluorescens*. Generally, the enzymatic treatments

tested showed less biofilm removal (%) for *Bacillus sp.* and higher biofilm removal (%) for biofilms formed by P. fluorescens, possible explanation for that is the different composition of the biofilms formed by the two different bacterial strains (Morikawa *et al.*, 2006; Molobela, Cloete and Beukes, 2010), moreover, biofilms formed by *Bacillus sp.* were more robust (thicker) when compared to biofilms formed by *P. fluorescens* (biofilm formation was measured indirectly using crystal violet stain).

Previous studies that have separately used Pectinex and Savinase reported them to be successful in removing biofilms (Johansen and Falholt, 1997; C. Leroy *et al.*, 2008; Molobela, Cloete and Beukes, 2010). Studies on optimization of enzymatic treatments for biofilm removal are very limited, to the best knowledge of the authors, this is the first study that optimizes the combined treatment of proteases and polysaccharide-degrading enzymes for removal of biofilms. Only one enzymatic optimization study was found by Johansen and Falholt 1997, the authors used two polysaccharide-degrading enzymes, Mutanase and Dextranase, for the removal of plaque. In their study, a plaque was formed on hydroxyapatite discs and the removal of the plaque was measured in terms of log reductions in colony forming units per disc (CFU/disc). The authors reported additive effect (log reduction from 10^8 to 10^6 CFU/disc) from combining both enzymes in one treatment rather than using them separately in treatments (Johansen and Falholt, 1997). The authors also reported that Pectinex alone was able to reduce the cell count for biofilm formed by *P. fluorescens* one log reduction ($2.5x10^{-7}$ to $2.4x10^{-6}$) using a concentration of 180 PSU mL^{-1} for 15 min at pH 7. A study by Leroy et al. 2008 investigated and compared the efficiency of different enzymes (four proteases, seven polysaccharide-degrading, and one lipase) used as anti-biofilm agents against biofilms formed by marine

bacterial strain *Pseudoalteromonas sp.* D41. The biofilm was grown for 24 hrs in a 96-well microtiter plate using sterile seawater at 20 ^{o}C. Biofilm was quantified using fluorescent dye DAPI (4[prime]6-diamidino-2-phenylindole). Results obtained showed that Savinase had the highest efficiency in preventing and removing biofilms (up to 100% reduction in bacterial adhesion and 50% reduction of pre-existing biofilms after a 24 hrs treatment at a concentration of 1.7 mg mL^{-1}). From their results, the authors concluded that depending on the enzymes used and their concentrations, enzymes could remove biofilms or inversely increase their formation (C Leroy *et al.*, 2008).

The validation of the response surface models and optimal conditions was done by performing and independent confirming experiment around the estimated optimal conditions, the results of the predicted and actual values of biofilm removal are presented in *Table 3–9*. The actual and predicted values presented in *Table 3–9* were compared using the statistical T-test at 95% confidence interval and no significant difference was found between them. This indicates that the proposed models can be used for the prediction of biofilm removal using Savinase and Pectinex.

3.5.6. Conclusions

In conclusion, synergetic effects can be noticed by combining the protease Savinase and the polysaccharide-degrading enzyme Pectinex. The enzymatic mixtures containing these two enzymes were more effective than treatments containing two enzymes from the same type (the proteases Savinase and Protamex, and the Polysaccharide-degrading Pectinex and Cellic). Identifying the optimal concentrations of enzymes for biofilm removal is vital not only from an economical point, the results presented suggests that increasing the enzyme concentration

beyond the optimal resulted in adverse effects on biofilm removal, this is hypothesised to be a result of the elevated amounts of sugars that are associated in the enzymatic mixtures preparations during their manufacturing process. The results suggest that the pH can have a high influence on the biofilm removal process, this is due to its influence on both the efficiency of the enzymes and its direct effect on biofilm formation. The use of a mixed (protease and polysaccharide-degrading) enzymatic treatment was successful in removing biofilms formed by *Bacillus sp.* and *P. fluorescens*. The models developed were successful in estimating real conditions for the removal of biofilm formed by *Bacillus sp.* and *P. fluorescens* using the enzymes mixture. The optimal conditions for the highest removal (68.1%) of biofilm formed by *Bacillus sp.* was achieved by Savinase and Pectinex at concentrations of 19.6 U/mL and 63.8 U/mL, and a pH value of 6. The optimal conditions for the highest removal (84.5%) of biofilm formed by *P. fluorescens* was achieved by Savinase and Pectinex at concentrations of 23.7 U/mL and 48.8 U/mL, and a pH value of 8. Higher biofilm removal was achieved for pH values at or above neutral (pH 6-8).

3.5.7. Acknowledgments

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Chapter 4 - Thesis Summary and Conclusions

4.1 Preface

Chapter 4 provides a summary of the experimental work performed in this thesis and the thesis' general conclusions.

4.2 Thesis Summary

The control of biofilm formation is of high interest in medical and industrial areas. Consequently, biofilm control agents are of industrial and academic interest. Therefore, the goal of this thesis is to investigate the ability of potential treatments to control biofilm formation.

4.2.1 Biofilm Control Using Norspermidine

Studies that have previously investigated biofilm removal debated the effect of norspermidine on biofilm formation control. The effect of norspermidine on biofilm formation was of specific interest for this thesis. The results presented in this study suggest that norspermidine enhanced biofilm growth for active biofilm communities by 73%, while it caused disassembly for nonactive (dead) biofilm with 39% biofilm removal. This dual effect is possibly responsible for the apparent contradiction on the role of norspermidine in the previous literature.

4.2.2 Biofilm Control Using D-amino Acids

Amino acids have been described as self-produced triggers by bacteria at late stages of mature biofilm for biofilm disassembly. Varying results have been presented by previous researchers on the effects of D-amino acids on biofilm formation. Currently, the literature is

lacking for quantitative studies that investigate the effect of D-amino acids on biofilm formation. The four D-amino acids investigated in this research (D-tyrosine, D-tryptophan, D-Methionine, and D-leucine) could inhibit biofilm growth for *Bacillus sp.* at concentrations as low as 5 µM for D-tyrosine and 5 mM for D-methionine, D-tryptophan, and D-leucine. The increase in the concentration of D-tyrosine (µM) and D-tryptophan (mM) between 10 and 20 had limited effects on the dispersal (31% and 28%, respectively) of *Bacillus sp.* pre-existing biofilms. The concentration of D-tyrosine and D-tryptophan applied was proportional to biofilm removal. D-leucine and D-methionine did not show the ability to remove *Bacillus sp.* pre-existing biofilm for treatments concentrations up to 20 mM. D-amino acids mixture treatment consisting of 5 µM D-tyrosine and 5 mM of D-tryptophan, and D-leucine, and D-Methionine could inhibit biofilm formation. The increase of D-tryptophan (mM) and D-tyrosine (µM) concentration between 10 and 20 in the D-amino acids mixture resulted in the dispersal (39%) of *Bacillus sp.* pre-existing biofilm. The results obtained for the D-amino acid mixture showed limited synergetic effects.

4.2.3 Biofilm Control Using Enzymes

Previous studies have presented promising results on the use of enzymatic treatments for biofilm removal. Previous studies have approached the problem without a comprehensive approach to the type of biofilm tested and selected enzymes. Current limitations on the use of enzymatic treatments for biofilm removal is due to the substrate-specific nature of enzymes which is met with biofilm composition of high complexity. In this thesis, a comprehensive approach was used in investigating the efficiency of two different enzyme types (polysaccharide-degrading and proteases) as biofilm control agents for the removal of two different composition biofilms formed by *Bacillus species* and *Pseudomonas fluorescens*. The

results obtained suggest that the proteases, Savinase had the highest biofilm removal capabilities when compared to other tested proteases. Savinase removed 68% and 84% of biofilm formed by *Bacillus sp*. and *P. fluorescens* respectively. The highest biofilm removal by polysaccharide-degrading enzymes was achieved by Pectinex. Pectinex removed 74% and 55% of biofilm formed by *Bacillus sp.* and *P. fluorescens* respectively. The results obtained suggest that an optimization of enzymatic treatment containing a mixture of enzymes can be beneficial for higher removal efficiency for biofilms with different compositions.

4.2.4 Optimization of Biofilm Removal Using Enzymatic Treatments

The use of enzymatic mixture treatments as biofilm control agents have been recommended by previous researchers. This is due to the promising results enzymes have shown when used for biofilm removal. The removal of biofilms formed by two different bacterial strains (different biofilm compositions), using enzymatic mixtures formed from polysaccharide-degrading enzymes and proteases was optimized using response surface analysis and central composite design. Ten central composite designs have been used to evaluate the effect of enzymes concentration and pH on the removal of biofilm formed by *Bacillus sp.* and *P. fluorescens*. The experimental data were fitted with a quadratic model. The model developed was successful in predicting the biofilm removal as a function of the concentrations of enzymes used. The quadratic model developed was verified by additional experiments, there was no significant difference between actual and predicted values of the biofilm removed. The results obtained in this thesis suggests that enzymatic treatments containing Savinase and Pectinex were the most effective for biofilm removal. The treatment containing Pectinex and Savinase at concentrations of 63.8 and 19.6 U/mL, respectively,

removed 68.1% of *Bacillus sp.* biofilm at pH 6, and at concentrations of 48.8 and 23.7 U/mL, respectively, removed 84.5% of *P. fluorescens* biofilm at pH 8.

4.3 General Conclusions

The main objective of this thesis was to investigate and develop treatments that can be used for biofilm control. The work presented in Section 3.2 highlights the importance of considering the biological activity of the tested biofilm. The results presented showed that active and nonactive biofilms responded differently to the treatment containing norspermidine. This different response was attributed to the competition between the biological process (biofilm growth) and the chemical interaction between norspermidine and the polysaccharides in the biofilm. When norspermidine was added to active biofilm, the biological process was dominant over the chemical interaction between norspermidine and the polysaccharides in the biofilm matrix and biofilm growth was enhanced. When the biofilm was nonactive (dead) the biological process ceased and the chemical interaction between norspermidine and the polysaccharide was dominant which resulted in biofilm removal.

The results obtained in Section 3.3 suggests that the D-amino acids examined in this work can be used to inhibit the growth of *Bacillus sp.* pre-existing biofilm. The use of D-tyrosine and D-tryptophan treatments did result in partial removal of pre-existing biofilm. The D-amino acid mixture did not show clear synergetic effects in the removal of pre-existing biofilm. Therefore, the use of D-amino acids can be utilized for inhibiting *Bacillus sp.* biofilm formation. Due to the lack of synergetic effects for the combined treatment, an optimization study for biofilm removal using D-amino acids mixtures was not recommended.

The results presented in Section 3.4 suggests that the use of proteases and polysaccharide-degrading enzymes was effective in the removal of biofilms formed by the two bacterial strains *Bacillus sp.* and *P. fluorescens*. The polysaccharide-degrading enzymes Cellic-CTec2 and Pectinex, and the proteases Protamex and Savinase exhibited the highest biofilm removal efficiency for pre-existing biofilms formed by *Bacillus sp.* and *P. fluorescens.* Higher biofilm removal was achieved for biofilm formed by *P. fluorescens* using proteases., while for biofilm formed by *Bacillus sp.* the polysaccharide-degrading enzymes exhibited the highest biofilm removal efficiency. Remarkably, the results obtained in Section 3.4 highlight the interdependent relationship between the proteins and the polysaccharides in maintaining the structural integrity of the biofilm formation. The ability of the polysaccharide-degrading enzymes to remove a considerable amount of biofilm formed by *P. fluorescens* biofilm (composed mainly from proteins), and the ability to remove a significant amount of the biofilm formed by *Bacillus sp.* (composed mainly from polysaccharide) using proteases, emphasises the complex interdependent relation between proteins and polysaccharides in maintaining the integrity of the biofilm structure.

From the results presented in Section 3.5 it could be stated that synergetic effects on biofilm removal were obtained after combining the protease Savinase and the polysaccharide-degrading enzyme Pectinex in a mixed treatment. The treatment containing Savinase and Pectinex were more effective than treatments containing two proteases or two polysaccharide-degrading enzymes. Recognizing the optimal concentrations of enzymes used for biofilm removal is critical. The results presented in Section 3.5 suggests that when the enzyme concentration exceeded the optimal concentration, adverse effects on biofilm removal were

obtained, it is hypothesised that this was a result of the increase in the concentration of sugars and other nutrients that are incorporated in the preparation process of enzymatic mixtures during the manufacturing process. The results obtained highlighted the effect of the pH on biofilm removal using enzymes. The pH affects the biofilm removal by affecting the efficiency of the enzymes used and through its direct effect on biofilm growth. The mixed enzymatic treatments were successful in removing biofilms formed by the two bacterial strains tested, *Bacillus sp.* and *P. fluorescens*. The quadratic models developed were successful in predicting actual conditions for removal of biofilms formed by *P. fluorescens and Bacillus sp.* using the enzymes mixtures.

A suitable biofilm control agent is expected to enhance, inhibit, or eliminate biofilm formation on surfaces. The different treatments investigated in this thesis are norspermidine, D-amino acids, and enzymes. Based on the results presented in chapter 3 and using the evaluation procedure presented in *Figure 1-2*, it was concluded that the use of the treatments containing D-amino acids was found efficient for inhibiting biofilm formation, the ability of D-amino acids to remove biofilm formation was found to be limited. The use of norspermidine was found to be effective in enhancing active biofilm formation which can be utilized in beneficial biofilm applications. The use of the treatment containing norspermidine enhanced the biofilm formation for active biofilm. Norspermidine showed limited ability in removing nonactive biofilm, this required a pre-treatment of the biofilm with methanol to inactivate the biofilm and this rendered the norspermidine treatment nonpractical for biofilm removal. In addition, the biofilm removed using norspermidine was limited. The use of enzymatic treatments was found to be the most efficient method investigated for biofilm removal. In

specific, the use of optimized enzymatic treatments containing the protease Savinase and the polysaccharide-degrading enzyme Pectinex were found to be the most effective treatment for the removal of biofilm. The developed models using the central composite designs and the response surface methodology were successful in predicting biofilm removal, no significant difference was found between predicted and actual values of biofilm removal.

4.4 Future Recommendations

The use of norspermidine and D-amino acids returned limited ability in removing pre-existing biofilm formed by *Bacillus sp.* and *P. fluorescens*, therefore they are not recommended for the removal of pre-existing biofilm. Based on the resulted presented in this thesis, enzymes were the most effective treatment tested for the removal of pre-existing biofilm, therefore, it is recommended that future research on treatments for biofilm removal should be directed toward the use of enzymes. Future research should be directed toward developing a consistent and reproducible biofilm in flexible PVC tubes that are used in dental unit water lines (DUWLs) and other related applications under similar conditions found in DUWLs, this can help in producing results and comparisons that are statistically sound from biofilm removal experiments that are conducted on a biofilm that is more analogous to actual biofilm found in DUWLs. Enzymes are abundant and new enzymes emerge into the market constantly, it is recommended to continue testing new emerging enzymes for their ability to remove biofilm. Future research should be directed toward developing short duration treatments for cleaning DUWLS that can be used in between consecutive patients, this will help in reducing cross-contamination.

Chapter 5 - Appendices

5.1 Biofilm Production Protocol

The biofilm used for testing the different treatments evaluated in this thesis was grown in 96-well microtiter plates. Biofilm production and treatment testing that was used to evaluate the ability of different treatments to remove pre-existing biofilm is presented in the following eight steps:

1- Preparing seed culture.

2- Inoculation of microtiter plates.

3- Washing initial biofilm in microtiter plates.

4- Fixing initial biofilm formation.

5- Applying Treatments.

6- Washing final biofilm in microtiter plates.

7- Fixing final biofilm formation.

8- Quantification of biofilm formation using crystal violet stain.

The aforementioned steps are explained in detail in subsections 5.1.1 – 5.1.8. These steps should be followed in the order they are mentioned.

5.1.1 Prepare Bacterial Seed Culture

1- Add 1 mL of bacterial suspension into a sterile microcentrifuge vial.
2- Centrifuge the vial at 10,000 RPM for 60 seconds.
3- Remove supernatant and add 1 mL wash buffer.
4- Vortex the vial until the bacterial spores are completely dissolved
5- Repeat steps 2-4 twice.

6- Add 1 milliliter of appropriate biofilm growth media to the vial and vortex.

7- Using a 125-mL glass flask with a stir, half a milliliter of the washed bacterial spores are re-suspended in 20 mL growth media.

8- The glass flask in then placed on the magnetic stirrer at 300 RPM and incubated at appropriate temp (37oC for Bacillus sp. And 21 oC for P. fluorescens) until optical density at 600 nm is between 1 and 1.6 when Milli-Q water is used as blank.

5.1.2 Inoculation of Microtiter Plates with Bacterial Seed Culture

1- A 200-mL glass petri dish with a flat bottom is filled with appropriate biofilm growth media and a stir bar.

2- The prepared bacterial seed culture is then diluted in a 1:100 ratio in appropriate biofilm growth media.

3- The petri dish is then placed on a magnetic stirrer at 300 rpm.

4- 100 µL of sterile biofilm growth media are transferred into control wells in the microtiter plates.

5- 100 µL from the petri dish are transferred into wells in the microtiter plates.

6- Each microtiter plate is then covered with a sterile thin film (25 µm) to seal it and reduce evaporation.

7- One hole is poked in the center of each well using a 22-gauge sterile needle (separate needles should be used for each of the inoculated wells and control wells to ensure no cross contamination will occur).

8- The microtiter plates are then incubated at the appropriate conditions (37oC for Bacillus sp. and 21 oC for P. Fluorescens) and time (32 h for Bacillus sp. Abd 72 h for P. Fluorescens, biofilm growth media is replenished daily for P. Fluorescens only) for biofilm to form in the microtiter plate wells.

5.1.3 Washing Initial Biofilm in Microtiter Plates with Wash Buffer

After the incubation time is over and the biofilm has formed in the microtiter plate, the biofilm will be ready for washing.

1- The thin film placed on the microplate is removed and then the microplate is placed in the Tecan Hydroflex Plate Washer. Each well in the plate is filled with 100 µL of wash buffer solution using the drip mode and then wash buffer is aspirated.

2- Following that 150 µL of wash buffer solution is dispensed and aspirated same as the previous step, the process is repeated for a total of three times.

3- The microtiter plate is then gently flicked and the face of the microtiter plate is placed on a paper towel to release any excess liquid.

5.1.4 Fixing Initial Biofilm Formation

This step in performed to fix initial biofilm growth that will be compared with the biofilm remaining in other wells after applying treatment to them.

1- 200 µL of methanol (99%) are dispensed into designated initial growth wells only.

2- The microtiter plate is covered with aluminum foil to prevent evaporation for 20 min.

3- The methanol is removed using the multichannel micropipette.

5.1.5 Applying Treatments

1- 125 µL of treatment solution are dispensed into desired wells.

2- The microtiter plates are then covered with thin film.

3- A single hole is poked in the center of each well using the 22-gauge sterile needle.

4- The microtiter plates are then incubated at 21 oC for 24 h in sterile conditions.

5.1.6 Washing Final Biofilm in Microtiter Plates With DI-Water

After the treatment incubation time (24 h) is over the microtiter plate are ready for washing to remove exhausted media and loosely attached cells.

1- The thin film placed on the microplate is removed and then the microplate is placed in the Tecan Hydroflex Plate Washer. Each well in the plate is filled with 100 µL of DI-Water using the drip mode and then the DI-Water is aspirated.

2- Following that 150 µL of DI-Water is dispensed and aspirated same as the previous step, the process is repeated for a total of three times.

3- The microtiter plate is then gently flicked and the face of the microtiter plate is placed on a paper towel to release any excess liquid.

5.1.7 Fixing Final Biofilm Formation

This step in performed to fix the final biofilm formation that will be compared with the initial biofilm formation in the control wells.

1- 200 µL of methanol (99%) are dispensed into designated treated wells only.

2- The microtiter plate is covered with aluminum foil to prevent evaporation for 20 min.

3- The methanol is removed by aspiration using the Tecan Hydroflex Plate Washer.

4- The microtiter plates are left to air dry completely on bench top.

5.1.8 Quantification of Biofilm Formation Using Crystal Violet Stain

The crystal violet staining method is an indirect method for quantifying biofilm formation in microtiter plates.

1- 150 µL of 0.1 % (W/V) crystal violet is dispensed into each well using the multichannel micropipette.

2- The microtiter plate is covered to prevent evaporation and incubated at room temperature for 60 min.

3- The microtiter plates are washed 4 times by dispensing 200 µL DI-Water in drip mode and aspirating using the Tecan Hydroflex plate washer.

4- The microtiter plates are then left to air dry completely on bench top.

5- The crystal violet is then resolubilized by dispensing 200 µl 30% acetic acid into each well.

6- The microtiter plates are then covered and incubated without shaking at room temperature for 20 min.

7- 100 µL from each well are transferred to new hard polystyrene plate with the clear flat bottom for quantification.

8- Absorbance at OD 570 nm is measured using a TECAN infinite M200 Pro plate reader. The absorbance measured in proportional to the biofilm formed in the plates.

5.2 Raw Data Used in Section 3.4.

The data used in creating the graphs presented in Section 3.4 is presented in Table A and Table B presented below.

Table A. The final biofilm formation following 24 h treatment duration of the tested proteases at different concentrations on biofilm formed by *Bacillus sp.* and *P. fluorescens* with 95% confidence intervals (n=8) (Raw data used in Section 3.4).

Enzyme		*Bacillus sp.*		*P. fluorescens*	
Name	Conc.	Abs. Unit	C.I.	Abs. Unit	C.I.
	U/mL	570 nm	95%	570 nm	95%
Protamex	1	1.18	0.04	0.30	0.03
	5	0.89	0.09	0.20	0.05
	10	1.11	0.03	0.17	0.02
	15	1.50	0.04	0.14	0.02
	30	1.69	0.08	0.25	0.03
	Initial Growth	1.89	0.07	0.81	0.05
Savinase	1	1.11	0.07	0.33	0.02
	5	0.74	0.04	0.16	0.02
	10	0.64	0.06	0.18	0.02
	15	0.61	0.06	0.31	0.02
	30	0.74	0.08	0.42	0.05
	Initial Growth	1.89	0.05	0.98	0.07
Subtilisin A	1	1.29	0.07	0.34	0.02
	5	1.36	0.05	0.30	0.02
	10	1.36	0.04	0.32	0.01
	15	1.41	0.05	0.32	0.03
	30	1.58	0.04	0.39	0.02
	Initial Growth	1.76	0.07	0.76	0.04
Trypsin	1	1.46	0.08	0.42	0.01
	5	1.55	0.08	0.37	0.01
	10	1.60	0.13	0.41	0.01
	15	1.61	0.13	0.45	0.04
	30	1.59	0.09	0.52	0.02
	Initial Growth	1.87	0.09	0.79	0.05

Table B. The final biofilm formation following 24 h treatment duration of the tested polysaccharide degrading enzymes at different concentrations on biofilm formed by *Bacillus sp.* and *P. fluorescens* with 95% confidence intervals (n=8) (Raw data used in Section 3.4).

Enzyme		Bacillus sp.		P. fluorescens	
Name	Conc.	Abs. Unit	C.I.	Abs. Unit	C.I.
	U/mL	570 nm	95%	570 nm	95%
Pectinex	5	1.21	0.07	0.51	0.05
	15	0.92	0.05	0.37	0.03
	25	0.46	0.07	0.34	0.02
	50	0.53	0.08	0.35	0.03
	75	0.88	0.04	0.37	0.02
	Initial Growth	1.81	0.12	0.76	0.05
Cellic-CTec2	1	1.47	0.11	1.11	0.10
	5	0.94	0.08	0.74	0.04
	10	0.86	0.09	0.61	0.03
	15	1.32	0.07	0.65	0.03
	30	1.44	0.12	0.70	0.06
	Initial Growth	1.98	0.07	1.29	0.05
Carezyme	1	1.41	0.10	0.75	0.09
	5	1.22	0.06	0.68	0.04
	10	1.25	0.08	0.69	0.03
	15	1.23	0.06	0.73	0.02
	30	1.34	0.06	0.75	0.04
	Initial Growth	1.74	0.10	0.87	0.05
Cellic-HTec2	1	1.51	0.10	0.61	0.05
	5	1.52	0.09	0.63	0.03
	10	1.58	0.06	0.63	0.03
	15	1.57	0.05	0.68	0.04
	30	1.70	0.06	0.74	0.02
	Initial Growth	1.97	0.09	0.94	0.06

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ARTICLES PREPARED FOR PUBLICATION

- **Bilal Al-Bataina**, Erin Johnson, Lars Rehmann; Biofilm control using norspermidine.
- **Bilal Al-Bataina**, Lars Rehmann; Biofilm control using amino acids.
- **Bilal Al-Bataina**, Lars Rehmann; Biofilm control using enzymes.
- **Bilal Al-Bataina**, Lars Rehmann; Optimization of enzymatic treatments for biofilm control.

CONFERENCE PRESENTATIONS

Bilal AL-Bataina, Erin Johnson, Lars Rehmann. (2016). Biofilm control in dental applications using norspermidine. Oral presentation. Quebec – Ontario Biotechnology Meeting. Waterloo, Ontario, Canada.