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Proteolytic and amylolytic enzymes for bacterial biofilm control

By

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I hereby declare that the thesis entitled **"PROTEOLYTIC AND AMYLOLYTIC ENZYMES FOR BACTERIAL BIOFILM CONTROL"** which I hereby submit for the degree Philosophiae Doctor is my own original work and has not previously in its entirely or part been submitted at any university for a degree.

Signature: _____

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List of abbreviations

- AMG – Amyloglucosidase
- AMP – Ampicillin
- BAN – Bacterial Amylase Novo
- BRR – Biofilm Removal Reactor
- BSA – Bovine Serum Albumin
- CBA – Chlorobenzoic acid
- CF100XNB – Contineous fed with 100 times Nutrient Broth
- CFU – Colony Forming Units
- DNA – Deoxyribonucleic acid
- DS – Distribution System
- EPS – Extracellular Polymeric Substance
- HDPE – High density polyethelene
- HOC – Hydrophobic organic compound
- HUS – Hemolytic uremic syndrome
- kDa – kilodalton
- LPS – Lipopolysaccharide

- MIC – Microbiologically induced corrosion
- MIC – Minimum inhibitory concentration
- NAG – N – acetylglucosamide
- NaOH – Sodium Hydroxide
- OD – Optical density
- OMP – Outer Membrane Protein
- PE – Poly ethelene
- PIA – Polysaccharide intracellular adhesin
- PR – Percentage Reduction
- PVC – Polyvinyl chloride
- QS – Quarum Sensing
- SEM – Scanning Electron Microscopy
- TTP – Thrombocytopenic pupura
- UV – Ultra violet
- WAN – Without additional nutrients

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Quotes

- An aim in life is the only fortune worth finding

(Jacqueline Kennedy Onassis)

- All personal achievements start within the mind of the individual

(W Clement Stone)

- Every great success is an accumulation of thousands of ordinary efforts that no one sees or appreciates

(Brian Tracy)

- Selecting a challenge and meeting it creates a sense of self empowerment that becomes the ground for further successful challenges

(Julia Cameron)

- Treat people as if they were what they ought to be and you will help them become what they are capable of becoming.

(Johann Wolfgang Von Geothe)

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Dedications

This work is dedicated to my son “Thuto” and my late brother “Douglas” This is it brother, it happened! You really wanted to witness the ceremony when I would be receiving my PhD degree certificate, but death destroyed all of your plans. It is well brother and i promise to stick to those rules you have coached me on “HOW TO LIVE A SIMPLE BUT LUXURIOUS LIFE. Till we meet again!!

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Summary

Biofilms are characterized by surface attachment, structural heterogeneity; genetic diversity; complex community interactions and an extracellular matrix of polymeric substances (EPS). Biofilms deposit and adhere to all surfaces that are immersed in aqueous environments. EPS serves many functions including: facilitation of the initial attachment of bacterial cells to a surface; formation and maintenance of the micro colony; enables the bacteria to capture nutrients; causes biofouling; cell-cell communication and enhances bacterial resistance antimicrobial agents. EPS also function as a stabilizer of the biofilm structure and as a barrier against hostile environments. Extracelullar polymeric substances are composed of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid, DNA, lipid and even humid substances.

EPS can be hydrophilic or hydrophobic depending on the structural components making up such EPS and the environmental conditions were the biofilms are developing. The exopolysachharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties within the bacterial strains. Due to variety in the structural components of the bacterial EPS, removal of biofilms by compounds that have no effects on the biofilm EPS would be difficult. Enzymes are proven to be effective in degrading biofilm EPS. The manner in which enzymes degrade the biofilm EPS is through binding and hydrolysis of the EPS components (proteins and carbohydrates) molecules and converting them into smaller units that can be transported through the cell membranes and then be metabolized.

The objectives of this study were to grow *Pseudomonas fluorescens* and mixed bacterial species biofilms in nutrient rich and nutrient limited medium conditions; to determine the EPS, protein and carbohydrate concentrations of the biofilm grown in rich and in limited nutrient conditions and to test the efficiency of protease and amylase enzymes for the degradation of the EPS and biofilm removal. In the results, there was a slight difference in the number of viable cells grown in biofilms that were fed than the cells of the unfed biofilms. As a result, the EPS, protein and carbohydrate concentrations were higher in the

fed biofilms than the unfed biofilms. There are contradictory reports about the composition of EPS especially with the ratio of carbohydrate to protein. Some of these reports indicate that certain biofilms EPS have bigger proportion of proteins and some found polysaccharides to be the dominant composition of the EPS of the biofilms. Nonetheless, the quantity and the composition of the EPS produced by bacterial biofilms depend on a number of factors such as microbial species, growth phase and the type of limiting substrate.

Enzymes were tested individually and in combination for the degradation of biofilm EPS. For efficient removal of biofilm, it is important that the structural components of the biofilm EPS should be known before application of the relevant enzymes. In this study, the test enzymes were effective for the degradation of the biofilm EPS except for the protease Polarzyme which had no activity. The reason for the inefficiency of Polarzyme may be due to its incompatibility with the specific protein structural components of the biofilm EPS tested in this study. The manner in which the enzymes degrade the biofilm EPS is through binding and hydrolysis of the protein and carbohydrate molecules and converting them into smaller units that can be transported through the cell membranes and then be metabolized. In addition, the mode of enzymatic action will depend on the specific EPS components and this in turn will determine its efficacy. The protease enzymes tested individually and in combination were most effective for EPS degradation. The efficiency of the proteases may be due to their broad spectrum activity in degrading a variety of proteins acting partly as the multi structural components of *Pseudomonas fluorescens* and mixed bacterial species biofilm EPS.

On the other hand, amylase enzymes tested individually and in combination was less effective for the EPS degradation. The structures of polysaccharides synthesized by microbial cells vary. Microbial exopolysaccharides are comprised of either homopolysachharides or heteoropolysaccharides. A number of lactic acid bacteria produce heteropolysaccharides and these molecules form from repeating units of monosaccharides including D- glucose, D- galactose, L- fructose, L- rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid. The type of both linkages between monosaccharides units and the branching of the chain determines the physical

properties of the microbial heteropolysaccharides. Due to a wide range of linkages and the complexity of polysaccharides structures, it would therefore be difficult for the amylases to break down the bond linkages and the monomers making up polysaccharides which determine the physical and chemical structure of the EPS. It was therefore not surprising that the amylase enzymes tested for the degradation of *Pseudomonas fluorescens* and mixed bacterial species biofilms, were less effective than the proteases. Hence, when the amylase enzymes were tested in combination with the protease enzymes, efficiency improved. It was therefore concluded that the protease enzymes were the primary remedial compounds and the amylase enzymes were the secondary remedial compounds.

Conclusion

If a compound or compounds capable of destroying all the structural components of different EPS that are produced by different biofilms growing under different conditions is found then the "city of microbes" (biofilms) would be destroyed permanently. If only an enzyme or enzymatic mixture capable of shutting down or deactivating the quorum sensing systems of different biofilm EPS could be found, then there would not be any formation of biofilms. In this study, protease enzymes tested individually and in combination were the most effective in the degradation of biofilm EPS than the amylase enzymes resulting in the reduction of large population of the biofilm cells attached on the substratum.

Recommendation

Amylase enzymes tested individually and in combination were less efficient for the degradation of the biofilm EPS and biofilm removal. This may be due to the complex structure of the exopolysaccharides synthesized by different biofilms. Also, the bond linkages between monosaccharides units and the branching of the chain complex the structures and as a result confer in the physical properties of the microbial biofilms. Hence, when the amylase enzymes were tested in combination with the protease enzymes, activity improved. For efficient degradation of biofilm EPS, it is therefore recommended that, protease and amylase enzymes should be tested in combination. In

addition, the structure of the biofilm EPS should be investigated so that relevant enzymatic mixtures are tested for biofilm removal.

Chapter 1

Introduction

1.1 Microbial biofilms

Biofilms are described as surface associated bacterial communities forming micro colonies surrounded by a matrix of exopolymers (Izano *et al.,* 2007). Microorganisms in biofilms display some particular features that are not shared with the same microorganisms in suspended form. In biofilms, the cells are embedded in a polymer matrix of their own origin that mainly consists of polysaccharides and proteins (Flemming, 1998; Decho, 2000). Biofilms contain mixed populations of bacteria, fungi, protozoa and if conditions allow, they can host even higher organisms in the food chain such as nematodes and larvae (Decho, 2000). All bacteria within a biofilm live together and depend on other microorganisms for energy, carbon and other nutrients (Prakash *et al.,* 2003).

The extracellular matrix contributes to the mechanical stability of the biofilms enabling them to withstand shear forces (Morikawa *et al.,* 2003). Biofilm formation occurs in response to a variety of environmental triggers including high cell density, nutrient deprivation and physical environmental stress (Li *et al.,* 2003). Biofilms are common form of microbial ecosystems associated with surfaces and they are found in an extremely varied environment, from pure water systems to stream beds. In response to varying environmental conditions, biofilms develop different structures expressed in various morphologies (Hermanowicz, 2001).

The EPS matrix is important both in the formation and structure of the biofilm and also protects the cells by preventing the access of the antimicrobial and xenobiotics to the cells in the biofilm and confers protection against environmental stresses such as UV radiation, pH shift, osmotic shock and desiccation (de Carvalho, 2007).

The primary stage for biofilm formation is the attachment of bacteria to a surface followed by proliferation of attached cells which leads to the accumulation of multilayer clusters of cells and glycocalix formation (EPS) (Shakeri *et al.,* 2007). Biofilms are composed of bacteria, extracellular polymeric substances (EPS) of microbial origin and other particulate substances. EPSs are composed of diverse substances including polysaccharides, proteins, nucleic acids (Morikawa *et al.,* 2003), lipids and humic substances (Xavier *et al.,* 2005).

Biofilm formation involves the cell to cell communication quorum sensing (QS) systems. QS is a cell density dependent mechanism through which bacteria coordinate different activities including bioluminescence, plasmid conjugation and the production of different virulence factors (Schaber *et al.,* 2007). *Pseudomonas aeruginosa* is one of the bacteria that possess at least two well defined interrelated QS systems, the *las* and *rhl* which control the production of virulence factors. Each QS system consists of two components, the auto inducer synthases *LasI* and *RhII* and their cognate transcriptional regulators *LasR* and *RhIR*. *LasI* is the synthase for the auto inducer N-3-oxododecanoyl homoserine lactone $3OC_{12}$-HSL, while *RhII* synthesizes the auto inducer N-butyryl homoserine lactone C_4-HSL (Schaber *et al.,* 2007).

Another characteristic of biofims is their heterogeneity, for example, aerobic microorganisms in aerobic system consume oxygen resulting in anaerobic zone within the biofilm (Flemming, 1998). This process provides habitats for anaerobes that could not proliferate under aerobic conditions. A biofilm is mainly composed of water (80-90%), extracellular polymer substances (EPS) that contribute 85-98% of the organic matter, the microorganisms, entrapped organic and inorganic particles, substances sorbed to EPS, cells or particles and substances dissolved in the interstitial water (Flemming, 1998).

Biofilm formation has serious implications in industry, the environmental, public health and medicine due to increased resistance to antibiotics and UV light and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production (Meyer, 2003; Bourne *et al.,* 2006; Giaouris *et al.,* 2006).

Biofilms cause fouling of industrial equipment such as heat exchangers, cooling towers resulting in efficient heat transfer, energy loss, increased fluid frictional resistance and accelerated corrosion and also deteriorates the quality of various chemicals and process additives (Xiong and Liu, 2010). For example, in the paper industry, biofilms cause deterioration of chemicals like starch and calcium carbonate slurries which are added to the pulp slurries in the wet end processes (US Patent 7052614, 2006). In addition, biofilm formation in water distribution systems decreases water quality and increases health risks (Dewanti *et al.,* 1995; Rao *et al.,* 1998, US Patent 7052614, 2006). In the food industry, the occurrence of biofilms in food processing environments can cause post processing contamination leading to lower shelf life of products and transmission of diseases (Poulsen, 1999). Biofilms do not only present a hygiene risk in food industries but also cause economical losses due to technical failures (Meyer, 2003; Giaouris *et al.,* 2006).

Several methods have been proposed to prevent and destroy biofilms including (1) mechanical removal such as scrabing, sonication, freezing and thawing (2) chemical removal using biocides, detergents and surfactants (de Carvalho, 2007). However, it has been difficult to completely remove biofilms by these methods due to protection of the biofilms cells by the extracellular polymeric substances (EPS) which act as barriers protecting the biofilm cells. Therefore there is a need for methods that are capable of removing the biofilms by destroying the extracellular polymerisc substances (EPS).

1.2 Enzymes for biofilm control

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Previous studies have indicated that antimicrobial agents such as chemical biocides were the main strategy to control and prevent the formation of biofilms (Walker *et al.,* 2007). In many industries, it is important that both the inactivation and the removal of biofilms from the surfaces are achieved (Simoes *et al.,* 2003). A wide range of biocides have been used in controlling biofilms, however these cleaning chemical agents have little to no effect at removing an established biofilms (Walker *et al.,* 2007). Therefore, application of enzymes would be an attractive strategy for the control and removal of biofilms. Enzymes remove biofilms by destroying the physical integrity of

the biofilm matrix (EPS) (Xavier *et al.,* 2005). Study made by Loiselle *et al.* (2003) indicated that cellulose from *Penicillium funiculusum* was one of the most effective enzymes in degrading mature biofilms of *Pseudomonas aeruginosa*. Cellulose was also found to be effective in degrading the exopolysaccharides from *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004). Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and two carbohydrates, namely alpha - amylase and beta – glucanase and the enzymatic mixture was found to be effective in digesting slime layers produced by cultures of pure and mixed strains of bacteria. The main objectives of this study were to:

- Standardize the method for biofilm growth.
- Standardize the EPS extraction method and to determine EPS compositions
- Test the effects of proteolytic and amylase enzymes tested individually and in combination for the degradation of EPS and removal of mono (*Pseudomonas fluorescens*) and mixed bacterial species (*Gram negative and positive*) biofilms.

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Chapter 2

Literature review

2.1 Defining biofilms

Biofilms are defined as assemblages of microbial cells which can be formed by single and or a mixture of bacterial species that are irreversibly associated with a surface and enclosed in a matrix of primary polysaccharide materials that allow the growth and survival in sessile environments (Kalmokoff *et al.,* 2001; Prakash *et al.,* 2003; Smith, 2005). Biofilms form when bacteria adhere to surfaces in aqueous environments and excrete extracellular polymeric substances (EPS) that can anchor the cells in all kinds of material such as metals, plastics, soil particles, medical implant materials (Costerton *et al.,* 1995; Decho, 2000; Mah *et al.,* 2001; Chmielewski *et al.,* 2003; Wingender and Flemming, 2004), living tissues, industrial or potable waste-system piping, or natural aquatic systems (Costerton *et al.,* 1995; Giaouris *et al.,* 2006).

A single bacterial species can form a biofilm, but in natural environment biofilms are often formed from various species of bacteria, fungi, algae, and protozoa (Costerton *et al.,* 1995). Biofilms represent a very complex form of microbial life that is mainly characterized by a high degree of interaction between different types of organisms and by a more or less immobilized form of life. This allows the formation of stable aggregates in which synergistic effects can develop (Chen and Sterwart, 2002; Donlan, 2002).

Debris along with corrosion products provides a considerable advantage for the biofilm forming bacteria (Diosi *et al.,* 2003), such as protection from antimicrobial agents, exchange of nutrients, metabolites or genetic material from close proximity to other microorganisms (Costerton *et al.,* 1995, Morikawa, 2000). In biofilms, the cells can tolerate much higher concentrations of biocides in suspension (Flemming, 1998). Another characteristic of biofims is their heterogeneity, for example, aerobic microorganisms in aerobic system consume oxygen resulting in an anaerobic zone within

the biofilm (Flemming, 1998). This process provides habitats for anaerobes that cannot grow under aerobic conditions.

Such symbiotic relationships, although beneficial to the participating bacteria, often damages the surface. This kind of damage is called biofouling and causes dental decay, metal pipeline corrosion, colonization of medical implants, product contamination and equipment failure (Cloete *et al.,* 2003; Prakash *et al.,* 2003; Kumar *et al*., 2006).

2.2 Biofilm formation and stages involving during biofilm development

2.2.1 The primary stage

The primary adhesion stage constitutes the beneficial contact between a conditioned surface and planktonic microorganisms. During the process of attachment, the organism must be brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and motility (Prakash *et al.,* 2003). Once the organism reaches critical proximity to a surface, the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces. These forces include electrostatic and hydrophobic interactions (Melo *et al.,* 1997; Kumar *et al.,* 2006) and van der Waal's attractions (Denyer *et al.,* 1993). This attachment is unstable and reversible and if the environment is not favorable for microbial attachment, cells can detach from the surface (Ghannoum and O'Toole, 2004). The solid-liquid interface between a surface and an aqueous medium (e.g. water, blood etc.) provides an ideal environment (microhabitat) for the attachment and growth of microorganisms (Flemming, 1998; Kerr *et al.,* 1999; Spiers *et al.,* 2003). Attachment will occur mostly on surfaces that are rougher, more hydrophobic (Palmer *et al.,* 1997) and coated by conditioning films (Schwartz *et al.,* 1998; Kalmokoff *et al.,* 2001; Liu *et al.,* 2004).

The primary stage is reversible and it is characterized by a number of physiochemical variables that define the interaction between the bacterial cell surface and the conditioned surface of interest (An *et al.,* 2000; Singh *et al*., 2002; Liu *et al.,* 2004). When a biofilm is composed of heterogeneous species, the metabolic byproducts of one organism might serve to support the growth of another, while the adhesion of one species might provide

ligands which allow the attachment of others (Dunne, 2002). Conversely, the depletion of nutrients and accumulation of toxin byproducts generated by primary colonizers may limit the species diversity within a biofilm (Marsh, 1995).

2.2.2 The secondary stage

The secondary stage involves the anchoring of bacteria to the surface by molecular mediated binding between specific adhesins and the surface (Kumar *et al.,* 2006). In this process loosely bound organisms gather together and produce exopolysaccharides that complex with surface materials (An *et al.,* 2000; Rachid *et al.,* 2000; Li *et al.,* 2007). Once the bacteria have attached irreversibly to the surface they undergo a range of genotypic and phenotypic changes to ensure the development and maturation of the biofilm. All bacteria produce multiple adhesions some of which are regulated at the transcriptional level depending on the genes encoded, permitting organisms to switch from sessile to planktonic forms under different environmental influences (Li *et al.,* 2007). A good example of this phenomenon is that of *Staphylococcus epidermidis*, which produces a polysaccharide intracellular adhesin (PIA) that is essential for cell to cell adhesion and biofilm formation (Dunne, 2002).

The changes described above result in the production of increased amounts of EPS, increased resistance to antibiotics, increased UV resistance, gene exchange events that occur more frequently and higher amounts of secondary metabolites that are produced (O'Toole *et al*., 2000). With certain organisms, several distinct adhesins might be used for surface attachment depending on the environment (O'Toole *et al*., 2000).

Various structures such as flagella, fimbriae, outer membrane proteins (OMPs), curli (a proteinaceous surface structure) and extracellular polymers structure (EPS) are involved in biofilm formation (Watnick *et al.,* 1999). They have distinct roles in different species and under different environmental conditions (Giaouris *et al.,* 2006). Flagella motility is important to overcome the forces that repel bacteria from reaching many abiotic materials. Once it reaches the surface, appendages such as pili, OMPs and curli are required to achieve stable cell-to-cell and cell-to-surface attachments. Flagella apparently

play an important role in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum (Giaouris *et al.,* 2006).

For example, in the case of *Vibrio cholerae* EI Tor, a toxin-coregulated pilus is used as an attachment and colonizes intestinal epithelium during the process of human infection. In contrast, a mannose- sensitive hemagglutinin is the primary adhesin used to anchor to abiotic surfaces in an aquatic environment (Watnick *et al.,* 1999). The acidophilic and iron (II) oxiding bacterium *Acidithiobacillus ferrooxidans* is one of the most important mesophiles for the extraction of metals from sulphidic ores by bioleaching. Attachment of these bacteria to the mineral surfaces seems to enhance bioleaching of pyrite and other minerals (Kinzler *et al.,* 2003).

Studies showed that the EPS of *A. ferooxidans* consist of neutral sugars and lipids (Kinzler *et al.,* 2003; Harneit *et al.,* 2006). In contrast to the cells of *A. ferrooxidans* growing on sulphur, cells growing on pyrite or iron (II) sulphate incorporate uronic acids and iron (II) ions in their EPS, providing the cell surface with a net positive charge under physiological conditions where pyrite is negatively charged (Harneit *et al.,* 2006). Thus the EPS complexed iron (II) ions enable the cells to interact with a pyrite surface through electrostatic forces. The EPS containing complexed iron (II) ions comprise a reaction space in which dissolution process takes place (Harneit *et al.,* 2006).

Korber *et al.* (1989) used motile and nonmotile strains of *Pseudomonas fluorescens* to show that motile cells attach in greater numbers and against the flow more rapidly than do nonmotile strains. Nonmotile strains do not recognize the substratum as evenly as motile strains, resulting in slower biofilm formation by the nonmotile organisms (Prakash *et al.,* 2003). A number of aquatic bacteria possess fimbriae, which have also been shown to be involved in bacterial attachment to animal cells (Meyer, 2003; Prakash *et al.,* 2003, Giaouris *et al.,* 2006).

2.2.3 Micro colony formation

After the adherence of bacteria to the inert surface, the association becomes stable for micro colony formation (Palmer *et al.,* 1997; O'Toole *et al.,* 2000, Bechmann *et al.,*

2006). The bacteria begin to multiply while sending out chemical signals that intercommunicate among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of a micro colony (Prakash *et al.,* 2003).

Microcolonies further develop into macrocolonies which are divided by fluid-filled channels and enclosed in an extacellular polysaccharide matrix (Allison, 2003). Macrocolonies, compared to microcolonies, are composed of more bacteria cells and are enclosed in an extracellular matrix and have a higher metabolic and physiological heterogeneity (Ghannoum and O'Toole, 2004). In the non-motile *Staphylococcus epidermidis,* polysaccharide and protein adhesins were linked for the attachment of this bacterial species, while a novel biofilm-associated protein was found to be involved in attachment and intercellular adhesion of *S. aureus* (Rupp *et al.,* 1991).

2.2.4 Formation of three dimensional structures

During the attachment phase of biofilm development, the transcription of specific genes takes place. These are required for the synthesis of EPS (Prakash *et al.,* 2003). Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded followed by formation of water filled channels in the circulatory system that help in delivering nutrients to and removing waste products from the cell communities in the micro colonies (Prakash *et al.,* 2003).

2.2.5 Biofilm maturation

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The overall density and complexity of the biofilm increases as surface-bound organisms begin to actively replicate and extra cellular components generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to create the glycocalyx (Carpentier *et al.,* 1993). The availability of nutrients in the immediate environment within the biofilm and the removal of waste, limits the growth potential of any bacterial biofilm (O' Toole *et al.,* 1998; O' Toole *et al.,* 2000). In

addition, there is an existence of an optimum hydrodynamic flow across the biofilm that determines the maximum growth (Carpentier *et al.,* 1993). Other factors that control biofilm maturation include the internal pH, oxygen, carbon source, osmolarity, temperature, electrolyte concentration and the flux of materials and surface types. The surface types can be either:

- High surface energy materials that are negatively charged; hydrophilic materials such as glass, metals or minerals
- Low surface energy materials that are either low positively or low negatively charged; hydrophobic materials such as plastic made up of organic polymers (O' Toole *et al.,* 1998).

At some point, the biofilm reaches a critical mass and a dynamic equilibrium is reached at which the outermost layers of growth begin to generate planktonic organisms. These organisms are free to escape the biofilm and colonise other surfaces. Cells nearest the surface become inactive or die due to a lack of nutrients, decrease in pH, pO_2 or an accumulation of toxic metabolic byproducts (Dunne, 2002).

The primary development, maturation and breakdown of a biofilm might be regulated at the level of population density dependent gene expression controlled by cell-to cell signaling molecules such as acylated homoserine lactones (Stickler *et al.,* 1998). Once fully matured, a logical cooperation and metabolic efficiency provides a form of functional communal coordination that mimics primitive eukaryotic tissues (Costerton *et al.,* 1995).

2.2.6 Detachment and dispersal of biofilm cells

As the biofilm gets older, cells detach and disperse and colonise a new niche. This detachment can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni *et al.,* 2007). Some bacteria are shed from the colony and some stop producing EPS and are released into the surrounding environment (Herrera *et al.,* 2007). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells or detachment as a result of nutrient levels (Spiers *et al.,* 2003). The

released microorganisms may be transported to new locations and restart the biofilm process (Prakash *et al.,* 2003).

As the thickness of the EPS increases, anaerobic conditions develop within the biofilm (Spiers *et al.,* 2003). Because of the film thickness and the activity of anaerobic species, the film detaches and sloughs off from the surface of the substrate. Polysaccharides enzymes specific for EPS degradation for different organisms may be produced during different phases of biofilm growth and contribute to detachment. It has been suggested that the escape of *P. aeruginosa* cells from the biofilm matrix involved the action of an enzyme that digests alginate (Prakash *et al.,* 2003).

In previous studies, it was mentioned that several bacterial species can synthesize polymer degrading enzymes to control the production of the EPS. *P. fluorescens* and *P. aeruginosa* were indicated to produce enzymes known as lyases which can degrade their exopolysaccharides and lead to the detachment of the cells from the surface (Boyd and Chakrabarty, 1994). Allison *et al.* (1998) observed that *P. fluorescens* biofilms grown for longer that 50 h, detached from the surface as a result of polymer degrading enzymes.

2.2.7 Summarized life cycle of biofilms

Biofilm formation begins with the attachment of single cell to a substratum. This first step is reversible and may require active swimming motility or may just be caused by random contacts. In the second phase, the attachment if fixed by adherence of the cells to the substratum through surface appendages such as flagella, fimbria, pili or by production of EPS (An *et al.,* 2000; Liu *et al.,* 2004).

The third phase represents growth of firmly attached cells forming micro colonies based on the availability of nutrients on the surface itself or from water column above the substratum. In a hydrodynamic environment, the development of micro colonies depends on cell to cell binding interaction (Palmer *et al.,* 1997; Singh *et al*., 2002).

The fourth stage is often referred to as mature biofilms. At this point the biofilm structure with its distribution of biomass and the presence of water filled channels illustrates the heterogeneity of the biofilms and the rigid properties of the developed structure

(Carpentier *et al.,* 1993; Singh *et al*., 2002). The stability of the biofilm is secured partly by cell-cell interaction and partly by EPS matrix surrounding and intergrated into biomass of the biofilms. Finally, at some point the biofilms may partially dissolve releasing cells that may move away to other locations where a new cycle begins (Singh *et al*., 2002; Prakash *et al.,* 2003). Below is the schematic illustration of biofilm attachment and formation.

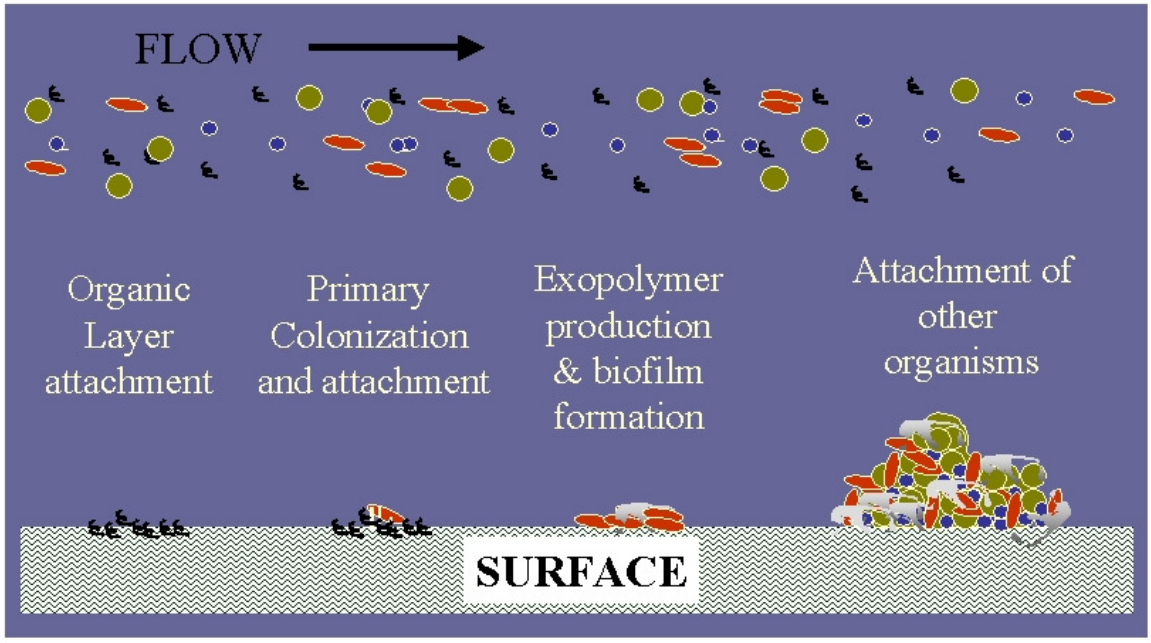

Fig. 2.1 Schematic illustrations of biofilm formation and development. Tracy Hudson (2002).

2.3 Factors affecting the growth and development of biofilms

2.3.1 Nutrients

Biofilms can form under diverse nutrient concentrations, ranging from high to almost non-detectable (Prakash *et al.,* 2003). They are, however, more abundant, densely packed and thicker in environments with high nutrient levels (Allison *et al.,* 2000; Prakash *et al.,* 2003; Rochex and Lebeault, 2007). High nutrient concentrations promote the transition of bacterial cells from the planktonic to biofilm state (O'Toole *et al.,* 2000)

while depletion of these nutrients has shown to cause detachment of biofilm cells from surfaces (Allison *et al.,* 1998; Hunt *et al.,* 2004; Rochex and Lebeault, 2007).

In an open reticulating system, there are abundant nutrients derived from water particularly in cooling towers. Closed systems (i.e. not exposed to the atmosphere) are ideal systems in that the problem of biofouling is either unlikely to be encountered or reduced (Melo *et al.,* 1997). High levels of nutrients appear to produce an open structure in the biofilm whereas lower concentrations tend to give a more compact structure. The structure of the biofilm has an effect on the availability of nutrients to the constituent cells. An open structure facilitates the diffusion of nutrients to the bacteria (Allison, 2003). For aerobic bacteria, the availability of oxygen is necessary unless the particular microorganism can exist under oxygen starved conditions (Melo *et al.,* 1997).

An increase in nutrient concentrations correlated with an increase in the number of attached bacterial cells (Cowan *et al.,* 1991; Dunne, 2002; Prakash *et al.,* 2003). However, nutrient concentrations too low to measure are still sufficient for biofilm growth. Biofilm bacteria acquire nutrients by concentrating trace organics on surfaces by the extracellular polymer, using the waste products from previously attached cells and secondary colonizers and also by pooling the biochemical resources with different enzymes to break down food supplies (Cowan *et al.,* 1991).

Sauer and Camper (2004) and Rochex and Lebeault (2007) demonstrated that *P. aeruginosa* and *P. putida* biofilms detached at high nutrient levels. On the contrary, Oh *et al.* (2007) demonstrated by means of atomic force microscopy (AFM) that *Escherichia coli* O157:H7 biofilms formed faster and as a result more cells attached to a glass surface under low nutrient conditions compared to high nutrient conditions. In addition, under low nutrient conditions, *Serratia marcescens* form biofilms consisting of single microcolonies, while under high nutrients conditions these microcolonies can revert to filamentous biofilms (Rice *et al.,* 2005).

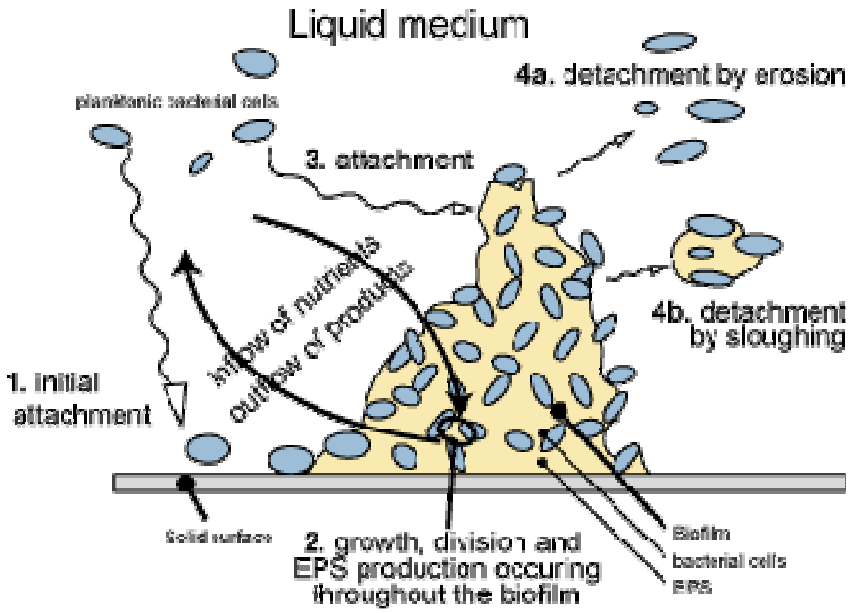

Fig. 2.2 Schematic illustration of processes involved in biofilm formation and development (Picioreanu *et al.,* 1998; Kreft *et al.,* 2001; Picioreanu *et al.,* 2004).

2.3.2 Temperature effects

For many bacteria found in cooling water systems, the optimum temperature for maximum growth is about 40^{O}C (Melo *et al.,* 1997, Timothy and Hansen, 2006). At this temperature small changes in temperature are likely to produce substantial changes in biofilm growth (Dewanti and Wong, 1995), because microbial activity is very sensitive to temperature. For instance, studies have shown that biofilm thickness of *Escherichia coli* increased by 80% by raising the temperature from 30^{O}C to 35^{O}C (Melo *et al.,* 1997).

2.3.3 Surface condition

The surface could be a dead or living tissue or any inert surface. The attachment of microorganisms to surfaces is a complex process with many variables affecting the outcome. Attachment will occur most readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Zacheus *et al.,* 2000; Dunne, 2002). Furthermore, growth requires complex developmental pathways that are regulated in response to environmental and bacterial derived signals. Studies based on the effect of

substratum were made and results found showed that the extent of microbial colonization appears to increase as the surface roughness increases (Prakash *et al.,* 2003).

It has been demonstrated that the surface condition (e.g. whether rough or smooth) affects the ability of bacteria to adhere to a surface. A material surface exposed in an aqueous medium will inevitably become conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment (Prakash *et al.,* 2003). Studies based on the films were made on surfaces exposed in seawater and results found showed that films were organic in nature and they formed within minutes of exposure and continued to grow for several hours (Prakash

et al., 2003).

The surface may have several characteristics that are important in the attachment process (Sauer and Camper, 2001). An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment if these factors do not exceed critical levels (Donlan, 2002). Properties of the cell surface, specifically the presence of fimbriae, flagella and surface associated polysaccharides or proteins, are also important and may provide a competitive advantage for one organism where mixed community is involved (Zobell, 1943; Donlan, 2002).

A material surface exposed in an aqueous medium will become conditioned or coated by polymers from that medium and the resulting chemical modification will affect the rate and extend of microbial attachment (Prakash *et al.,* 2003). A material surface exposed in an aqueous medium will become conditioned or coated by polymers from that medium and the resulting chemical modification will affect the rate and extend of microbial attachment (Prakash *et al.,* 2003). Surfaces cannot be colonized by biofilms unless they have been exposed to organic material from the surrounding environment (Allison *et al.,* 2000). However the effect of surface characteristics like charge, hydrophobicity, roughness and elasticity on microbial attachment cannot be ignored (Allison *et al.,* 2000).

Studies based on the films were made on surfaces exposed in seawater and results obtained showed that films were organic in nature and they form when exposed to

surfaces and continue to grow for several hours. The nature of conditioning films may be quite different for surfaces exposed in the human host (Donlan, 2002). Studies in medical research based on microbial films were involved and results obtained showed that a number of host produce conditioning film such as blood, tears, urine, saliva, intravascular fluid and respiratory secretion influenced the attachment of bacteria to biomaterials and that, the surface energy of the suspending medium may affect hydrodynamic interaction of microbial cells with surfaces by altering the substratum characteristics (Donlan, 2002).

2.3.4 Velocity, turbulence and hydrodynamics

The area from the surface where no turbulent flow is experienced is known as the boundary layer. Within this area, the flow velocity has been shown to be insufficient for biofilm removal (Dreeszen, 2003). The area outside this layer is characterized by high levels of turbulent flow and has an influence on the attachment of cells to the surface (Donlan, 2002). Studies showed that an increase in water flow velocity resulted in an increased bacterial number in biofilms. This is attributable to better mass transfer of growth limiting nutrients at the higher flow velocity of water (Lehtola *et al.,* 2006). Biofilms rely on defensive mechanisms to resist detachment by the higher fluid shear. It has been proposed that the viscoelasticity of biofilms allows them to resist detachment as has been found in *Staphylococcus aureus* biofilms (Lehtola *et al.,* 2006).

The size of the boundary layer is dependant on the flow velocity of the water. At high velocities, the boundary layer decreases in size and the cells are exposed to high turbulence levels (Donlan, 2002). Hydrodynamic conditions can influence the formation, structure, EPS production, and thickness, mass and metabolic activities of biofilms (Stoodley *et al.,* 2002; Liu and Tay, 2002; Simoes *et al.*, 2003).

Studies concerning the hydrodynamic of aqueous medium demonstrated that the flow velocity adjacent to the substratum/liquid interface is negligible. This zone of negligible flow is termed as the hydrodynamic boundary layer (Kumar *et al.,* 2006). Its thickness is dependent on linear velocity, the higher the velocity, the thinner the boundary layer. The region outside the boundary layer is characterized by substantial mixing or turbulence.

For flow regimes characterized as laminar or minimally turbulent, the hydrodynamic boundary layer may affect cell substratum interactions (Kumar *et al.,* 2006).

Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface will depend largely on the velocity characteristics of the liquid. Under very low linear velocities, the cells must transverse the sizeable hydrodynamic boundary layer, and association with the surface will depend on cell size and cell mortality (Donlan, 2002). As the velocity increases, the boundary layer decreases and cells will be subjected to increasingly greater turbulence and mixing. Higher linear velocities would therefore be expected to equate to more rapid association with the surface, at least until velocities become high enough to exert substantial shear forces on the attaching cells resulting in detachment of these cells (Donlan, 2002).

Studies have indicated that biofilms formed under low shear conditions (laminar flow conditions) are characterized by spherical microcolonies divided by water channels (Stoodley *et al*., 2002). Simoes *et al.* (2003) for instance determined the differences between three strains of *P. fluorescens* biofilms which were grown under turbulent and laminar flow. All biofilms grown under turbulent flow were denser, had a higher mass, and were more active, produced similar amounts of matrix proteins and the *P. fluorescens* strains had higher amounts of extracellular polysaccharides (Simoes *et al.,* 2003). It has been demonstrated that biofilms formed under higher detachment forces produced more extracellular polysaccharides in order to stabilize the biofilm structure and to withstand the shear force (Ohashi and Harada, 1994; Chen *et al.* 1996)

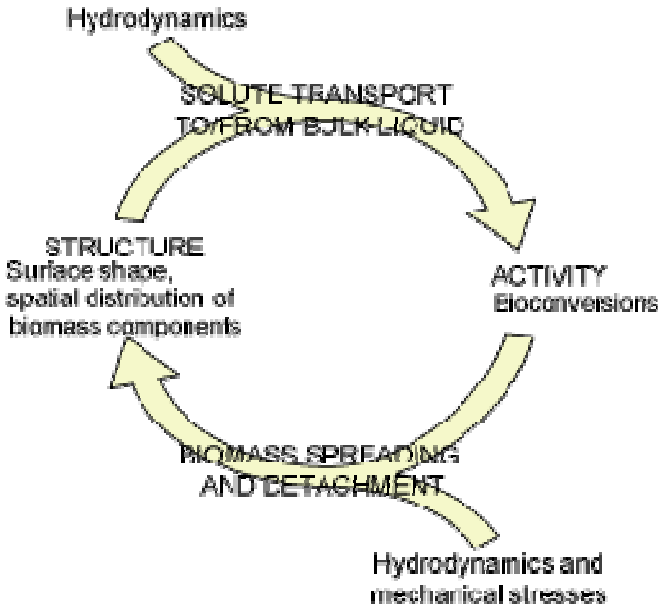

Fig. 2.3 Schematic illustrations of the structure/ activity relationship in biofilms (Picioreanu *et al.,* 1998; Kreft *et al.,* 2001; Picioreanu *et al.,* 2004).

2.3.5 Effects of particles

Biofouling of industrial equipment occur together with other kinds of fouling. The most common being the simultaneous deposition of small particles that are transported with the incoming water and/or those which are formed in the plant as a consequence of metal corrosion (Bouwer, 1987). When the particles are organic in nature, they act as substrate for microorganisms and are being degraded by them contributing to the growth of the biomass (Melo *et al.,* 1997). In most cases, however, the biological matrix incorporates inorganic particles that are relatively inert but may cause changes in the structure and activity of the biofilms (Battin *et al.,* 2003). The adhesion between particles and microorganisms could be facilitated by the electropositive charges developed at the surfaces of some particles depending on the pH of the environment. Metabolic inhibitors and toxic metallic ions could be adsorbed on the particle surface favouring biomass formation and microbial respiration stimulation in the presence of particles (Melo *et al.,* 1997).

2.3.6 Gene regulation

Studies based on gene regulation of microbial biofilms were made and results showed that 22% of the genes were up regulated and 16% down regulated in biofilm forming of *Pseudomonas aeruginosa* (Steyn *et al.,* 2001). Other studies demonstrated that the *algC* was up regulated within minutes of attachment to a surface in a flow cell system. Genes encoding for enzymes involved in glycolysis or fermentation such as phosphoglycerate mutase, triosephosphate isomerase and alcohol dehydrogenase were up regulated in biofilm formation of *Staphylococcus aureus*. A recent genetic study also showed that *algD, algU, rpoS* and genes controlling polyphosphokinase synthesis were up regulated in biofilm formation of *Pseudomonas aeruginosa* (Prakash *et al.,* 2003).

2.3.7 Quorum sensing (QS)

Quorum sensing is dependent on the cell density (Hammer, 2003). The development of biofilms on surfaces is mediated by a density dependent chemical signal released by bacterial cells densely packed within an EPS matrix. Microorganisms can use quorum sensing to coordinate their communal behavior such as biofilm formation, motility and production of EPS (Xiong and Liu, 2010).

Quorum systems make use of a transcriptional activator protein that acts in concert with a small autoinducers (AI) signaling molecule to stimulate expression of target genes (de Kievit *et al.,* 2001). Increasing bacterial density gives rise to an accumulation of autoinducers (AIs) (Xiong and Liu, 2010). Once the critical AIs concentrations are achieved, the regulator of proteins are triggered and further induce target DNA seguence leading to transcription of quorum sensing regulated genes which will then result in changes of bacterial behavior (Decho, 2000; Xiong and Liu, 2010). This form of intercellular communication serves to coordinate gene expression and structures morphological differentiation and development responses of bacterial cells (Fuqua *et al.,* 1996; Dunlap, 1997).

Cell-to-cell communication is essential for biofilm formation and is closely regulated to AIs. There are three types of AIs that have been identified including oligopeptides, *N* - acylhomoserine lactones (AHL) (Davies *et al.,* 1998; Elsri *et al.,* 2001) and autoinducers - 2 (AI-2) synthesized by LuxS (Pierson *et al.,* 2001; Xiong and Liu, 2010). Oligopeptides and AHL are involved in cellular communication of Gram positive and Gram negative bacteria respectively, whereas AI- 2 played a role in the interspecies communication of both Gram positive and Gram negative bacteria. AIs mediated QS systems play an important role in the regulation of microbial attachment and biofilm formation (Xiong and Liu, 2010).

Quorum-sensing signals can also control biofilm detachment by the accumulation of the signal molecules (excreted by bacteria) to a threshold concentration which will eventually trigger the dispersion of the biofilm (Hentzer *et al.,* 2002). Experiments with pure culture *Pseudomonas aeruginosa* biofilms in continuous-flow conditions indicated that these biofilms detached after flow was stopped. When these biofilms were starved from nutrients under continuous-flow conditions, detachment also occurred. These observations suggest that starvation and not the accumulation of signal/metabolic products was responsible for triggering the detachment of these biofilms (Hunt *et al.,* 2004) (Fig 2, 4).

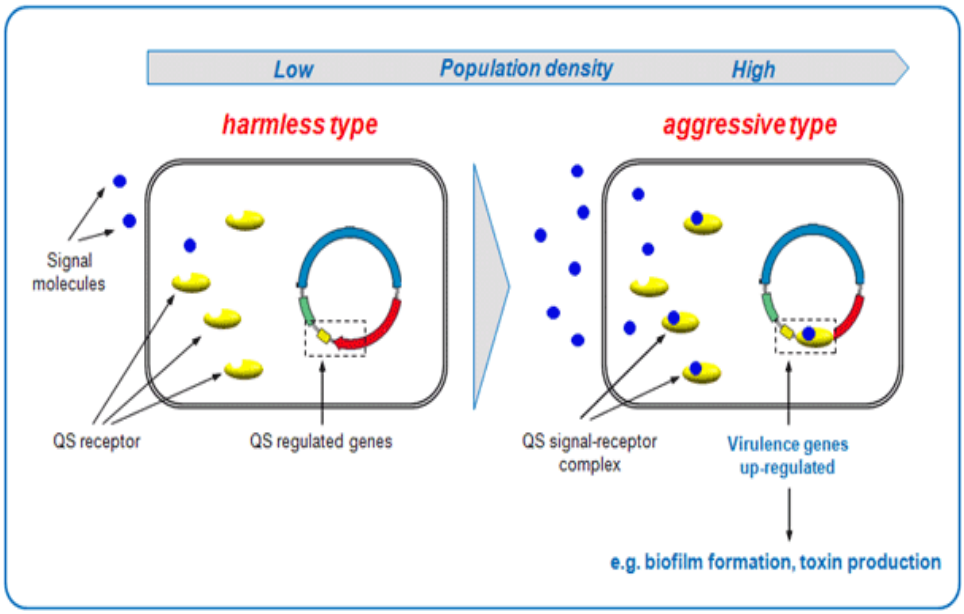

Fig. 2.4 Quorum sensing processes in bacterial biofilms (Nadell *et al.,* 2008).

When the density of bacteria reaches a sufficient level, the concentration of the auto inducer passes a threshold, initiating a positive feedback as more signalling molecule is synthesized, and the receptor becomes fully activated (Nadell *et al.,* 2008). It also induces the up regulation of other specific genes, for example, various virulence genes like proteases and toxins or the formation of biofilms. Thus in an opportunistic bacterium such as *Pseudomonas aeruginosa*, QS-related processes include biofilm development, exopolysaccharide production, and cell aggregation. *P. aeruginosa* can grow within a host without harming it, until they reach a certain population density when they become aggressive, their numbers sufficient to overcome the host's immune system and form a biofilm, leading to disease (Hunt *et al.,* 2004).

2.3.8 Properties of the cells

Cell surface hydrophobicity, presence of fimbriae and flagella, and productions of EPS influence the rate and extend of attachment of microbial cells (Liu *et al.,* 2002). The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non polar nature of one or both surfaces involved (i.e. the microbial cell surface and the substratum surface) Most bacteria are negatively charged but still contain hydrophobic surface components (An *et al.,* 2000).

Fimbrae contribute to cell surface hydrophobicity. Most fimbriae that have been examined contained high proportion of hydrophobic amino acid residues. They play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum. A number of aquatic bacteria possess fimbriae which are involved in bacterial attachment to animal cells (Spiers *et al.,* 2003). Treatment of adsorbed cells with proteolytic enzymes caused a marked release of attached bacteria, providing evidence for the role of protein in attachment (Donlan, 2002).

Mycolic acid containing organisms such as *Corynebacterium, Norcadia* and *Mycobacterium* were more hydrophobic than the non mycolic acid containing bacteria and an increase in mycolic acid chain length coincided with an increase in hydrophobicity (Spiers *et al.,* 2003). For most bacterial strains tested, adhesion was greater on hydrophobic materials. The O antigen component of lipopolysaccharide (LPS) also contributes hydrophilic properties to gram negative bacteria (Spiers *et al.,* 2003).

Mutants of *P. fluorescens* lacking the O antigen adhered in greater numbers to hydrophobic materials (Spiers *et al.,* 2003). Fletcher *et al.* (1991) found that treatment of attached fresh water bacteria with cations resulted in contraction of the initial adhesives (decrease in the cell distance from the substratum), indicating that the material was an anionic polymer. Glucosidase and N-acetylglucosaminidase (NAG) reduced attachment for *P. fluorescens*, while NAG only reduced attachment for *Disulfovibrio desulfuricans*. Lactins preferentially bind to polysaccharides on the cell surface or to the EPS. Binding of lactins by the cells would minimize the attachment sites and affect cell attachment if polysaccharides were involve in attachment. This was demonstrated with *Pseudomonas fragie* confirming the role of polysaccharides in attachment (Dunne, 2002). Cell surface polymers with non polar sites such as fimbriae, other proteins and components of certain gram positive bacteria (mycolic acids) appear to dominate attachment to hydrophobic substrata while EPS and LPS are more important in attachment to hydrophilic materials (Donlan, 2002).

With all these findings, it has been shown that cell surface structures such as fimbriae, other proteins, LPS, EPS and flagella play an important role in the attachment process (Dunne, 2002).

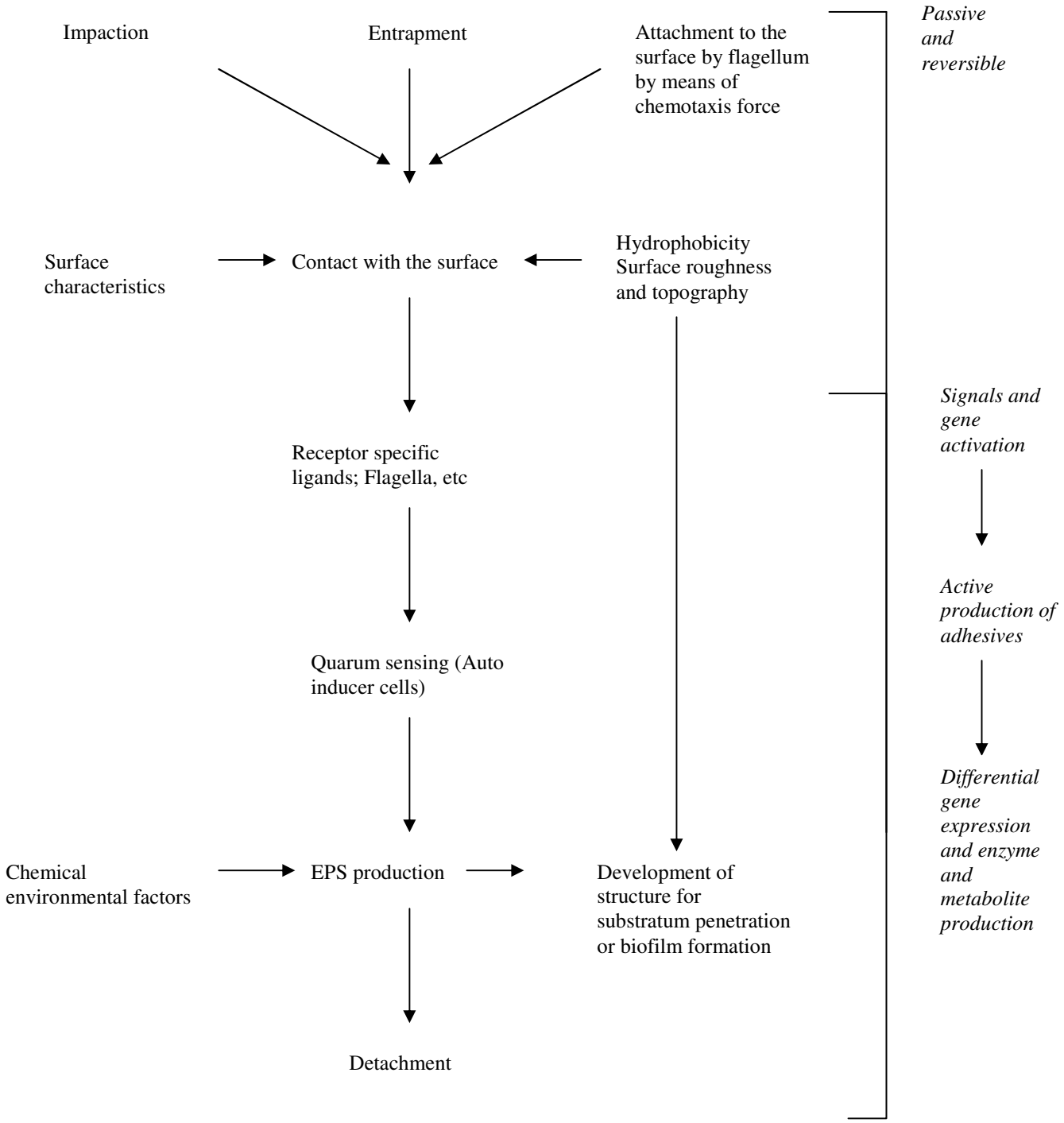

Fig. 2.5 Flow chart of biofilm formation on a surface (Gutierrez- Correa *et al.*, 2003).

2.4 Description of extracellular polymeric structure (EPS)

Flemming (2007) describes extracellular polymeric substances as the "house of biofilm cells". Biofilms are composed of microbial cells and extracellular polysaccharide structures (EPS). EPS may account for 50-90% of the total organic carbon of biofilms and can be considered as the primary matrix material of the biofilms (Flemming 1998). Biofilm- associated EPS is distinct, both chemically and physically from the bacterial capsule, but primarily composed of polysaccharides (Prakash *et al.,* 2003). EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. The EPS formation rate is proportional to the rate of substrate utilization. Cells use electrons from electron donor substrate to build active biomass and they also produced bound and soluble EPS (Sponza, 2003). Different biofilms produce different amount of EPS, and the amount of EPS increases with age of the biofilm (O' Toole *et al.,* 2000).

2.4.1 EPS production

A number of parameters including reactor type, substrate composition, substrate loading rate, hydraulic retention time, hydrodynamic shear force, culture temperature etc have been indicated to facilitate the production of biofilm EPS (Liu *et al.,* 2004). In biological wastewater treatment, biomass generates extracellular polymeric substances (EPS) when consuming organic material present in the wastewater. EPS are complex mixture of high molecular polymer (M_w>10,000) excreted by microorganisms, products from lysis and hydrolysis and adsorbed organic matters from wastewater (Wingender *et al.,* 1999). EPS of bacteria are involved in the formation of microbial aggregates (Geesey, 1982; Davies *et al.,* 1993), adhesion to surfaces and flocculation (Wingender *et al.,* 1999, Bhaskar and Bhosle, 2005). Furthermore, EPS are major components of aggregates for keeping the floc together in a three dimensional matrix due to bridging with multivalent cations and hydrophobic interactions (Wingender *et al.,* 1999).

Such a polymer network has a vast surface area and is capable of absorbing pollutants, nutrients and minerals. EPS plays an important role in the flocculation of bacterial cells and provides energy and carbon when substrates are in short supply (Laspidou and Rittmann, 2002; Sheng *et al.,* 2005). The EPS compostion is also related to the characteristics of waste water. For example, EPS has high protein and DNA concentrations in protein grown granules while high polysaccharide concentrations are found in granules grown in other type of organic substrates. Nitrogen limiting conditions favour the production of EPS which in turn accelerates anaerobic granulation (Liu *et al.,* 2004).

Proteins and carbohydrates are the main constituents of the EPS but other organic substances such as lipid and nucleic acid are also present (Watson *et al.,* 2004). Starkey and Kerr (1984) have proposed that the production of EPS is inhibited as a result of the anaerobic processes taking place. The production of EPS is known to be affected by nutrients status of the growth medium and the availability of carbon. Nielson *et al.* (1998) reported that a significant degradation of the sludge flox matrix occurred during anaerobic storage over a few days and that the reduction in sludge was mainly due to degradation of the sludge proteins and carbohydrates (Watson *et al.,* 2004). The limitation of nitrogen, potassium or phosphate promotes EPS synthesis. Slow bacterial growth will also enhance EPS production (Prakash *et al.,* 2003). EPS produce by the microorganisms exist as tightly bound (capsular), loosely adhered (slime type) to the cells or as free dissolved matter (Bhaskar and Bhosle, 2005).

2.4.2 EPS composition

The composition of extracellular polymeric substances (EPS) may vary amongst bacterial strains (Liu et *al.,* 2004). Extracelullar polymeric substances are composed of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid, DNA, lipid and humid substances (Hoyle, 1992; Dignac *et al.,* 1998; Frøelund *et al.,* 1995; Donlan, 2002; Liu *et al.,* 2004; Cheng *et al.,* 2007). The composition may be the result of active secretion, shedding of cell surface material, cell lysis and adsorption from the environment (Zhang *et al.,* 2001). Chemically, the bacterial EPS is a heterogeneous

polymer containing a number of distinct monosaccharides and non carbohydrate substituents, many of which are strain specific (Liu *et al.,* 2004).

There are contradictory reports in the literature about the composition of EPS especially with the ratio of carbohydrate to protein. Some of these reports indicated that certain EPS from wastewater biofilms (activated sludge) have a higher concentration of proteins than polysaccharides and some instances polysaccharides were found to be dominant in the biofilm (Sutherland, 1994; Nielsen *et al* 1997). Nonetherless, the quantity and the composition of the EPS produced by bacterial biofilms depend on a number of factors among which are, microbial species, growth phase, the type of limiting substrate (carbon, nitrogen and phosphorus), oxygen limitation, ionic strength culture temperature and shear force (Zhang *et al.,* 2001; Fang *et al.,* 2002; Liu *et al.,* 2003; Liu *et al.,* 2004; Bhaskar and Bhosle, 2005; Ratto *et al.,* 2005; Orgaz *et al.,* 2006). This implies that the EPS composition is variable and is related to microbial species, the physiological state of the bacteria and the operating conditions under which biofilms are developed. As a result, the multi structural components of the EPS are the main causes of ineffective removal of biofilms by antimicrobial agents (Cloete, 2003; Sreenivasan *et al.,* 2005; Lequette *et al.,* 2010).

The exopolysachharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties (Nielsen *et al.,* 1997; Sutherland, 2001, Bhaskar and Bhosle, 2005). One of the major components of the bacterial EPS is the uronic acids costituting up to 20 – 50% of the polysaccharide fraction (Bhaskar and Bhosle, 2005). Liu *et al.* (2004) revealed that polysaccharides are the only components that are synthesized extracellularly for a specific function, while proteins, lipid and nucleic acid exist in the extracellular polymer due to excretion of intracellular polymers or as a result of cell lysis.

Some microorganisms can bind ions into their EPS which have an influence on the nature of the biofilm (Poulsen, 1999). The bacterial extracellular polysaccharides are composed of homo and heteropolysaccharides including glucose, fructose, mannose, galactose, puryvate and mannuronic acid or glucoronic acid based complexes. The EPS are held to

the cell wall either by linkage between the carboxyl gropus of the EPS and hydroxyl groups of lipopolysaccharides or by a covalent bonding through phospholipids and glycoproteins (Bhaskar and Bhosle 2005). The exopolymer (EPS) produced by microorganisms also vary depending on whether the microorganisms are Gram negative or Gram positive cells. In addition, the EPS concentration depends on the physiological state of the microorganisms (Poulsen, 1999; Bhaskar and Bhosle, 2005).

Donlan (2002) indicated that EPS may be hydrophilic or hydrophobic depending on the structural components and the environmental conditions were the biofilms are developing. EPS components may also differ among individual members of a single bacterial species (Czaczyk *et al.,* 2007).

The structure of polysaccharides synthesized by microbial cells may vary. Microbial exopolysaccharides are composed of either homopolysachharides or heteoropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type such as D- glucose or L- fructose. Homopolysaccharides belong to three distinct groups including:

α - D - glucan which is produced by *Leuconostoc mesenteroides*

ß- D- glucans which is produced by *Pediococcus spp* and *Streptococcus spp*.

Fructans produced by *Streptococcus salivarius*.

Several groups of lactic acid bacteria produce heteropolysaccharides which are made up of repeating units of monosaccharides including D- glucose, D- galactose, L- fructose, L-rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid (Czaczyk *et al.,* 2007). The type of both linkages between monosaccharides units and the branching of the chain determines the physical properties of the microbial heteropopysaccharides (Bhaskar and Bhosle, 2005). As an example, bacterial alginate is a heteropolysaccharide with irregular structure. In this polymer, 1.4 linked β - D- mannurosyl and 1.4 α - L-guluronosyl residues are found. Alginate is mostly produced by the cells of *Pseudomonas*

aeruginosa and *Azatobacter vinelandii* (Christensen *et al.,* 2001; Sutherland, 2001, Czaczyk *et al.,* 2007).

Bacterial alginates produced by *Pseudomonas spp* differ from algal alginates produced by *Laminaria hyperborean* and *Microcystis pyrifera* (Christensen *et al.,* 2010). The polymer chains of algal alginates contains numerous blocks of L – guluronic acid (G – blocks), thus enabling intermolecular cross linking via selective binding of Ca^{2+} ions to form gels. On the contrary, *Pseudomonas spp* alginates do not contain G- blocks. In this case, the G-risidues (0 to 40%) occur as single residues and such alginates do not form rigid gels in the presence of Ca^{2+}. In addition bacterial alginates are commonly O – acetylated (Czaczyk *et al.,* 2007) which further counteracts gelation with Ca^{2+} (Christensen *et al.,* 2010).

Extracellularly secreted proteins are substances with a molecular weight between 10kDa and 200kDa. These compounds contain 40- 60 % of hydrophobic amino acids. It was observed that the extra cellular proteins synthesized by *Sulfolobus acidocalcidarius* are mainly composed of amino acids with hydroxyl groups. However, the *Bacillus substilis* extracellular protein layer is a composition of L and D glutaminosyl residues (Czaczyk and Myszka, 2007). According to Ton-That *et al.* (2004), the ratio of glutaminosyl isomers in *Bacillus substilis* extracellular protein layer changed significantly in oxygen limited conditions.

2.4.3 EPS chemistry

Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30kDa) and have a heteropolymeric composition. The polysaccharide chain might be branched or unbranched with side chains of other compounds attached to the polymeric chain (Allison, 1998). Polysaccharide chains vary in size from 103-108kDa and contain subunits which may also be both functionally- and species – specific. Furthermore, polysaccharides may be hydrophyllic but can also have hydrophobic properties (Allison, 1998; Flemming *et al.,* 1998). Generally, the polysaccharides are made of monosaccharides with hexose and pentoses forming the bulk of EPS. However, the contribution of different monomers to the total polysaccharide varies with the source and

such variations in the polysaccharide chain composition can alter its physiological chemical properties (Bhaskar and Bhosle, 2005).

EPS are generally rich in hexoses like glucose and galactose wheares planktonic EPS have a higher content of sugar like rhamnose, xylose, and mannose. Such variations in monomer composition can alter the properties of EPS. The presence of sugar like arabinose in EPS helps in cell aggregation in biofilms wheareas deoxy sugars like fucos and rhamnose found in diatom EPS help in foaming and flocculation (Bhaskar and Bhosle, 2005). Apart from polysaccharides, EPS also contain proteins, lipids and nucleic acids. The composition of EPS varies between planktonic and biofilms which in turn reflect on its prpperties. For example, one of the major components of biofilm EPS is uronic acid consisting of up to 20-50% of the polysaccharide fraction. On the other hand, plaktonic EPS are poor in uronic acid concentrations (Allison, 1998; Bhaskar and Bhosle, 2005).

2.4.4 Role of EPS

The production of EPS have several functions such as: facilitation of the initial attachment of bacteria to a surface (Laspidou and Rittmann, 2002); formation and maintenance of micro colony (Flemming *et al.,* 1998); enables the bacteria to capture nutrients; stabilization of the biofilm structure (Laspidou and Rittmann, 2002); organic chemical degradation; biofouling (Cloete *et al.,* 1998; Diosi *et al.,* 2003; Coetser and Cloete, 2005; Gonzalez *et al.,* 2006); cell-cell communication (Zhang *et al.,* 2001) enhances biofilm resistance to environmental stress and antimicrobial agents (Gilbert 1997, Andearl, 2000; Cochran, 2000; Laspidou and Rittmann, 2002; Chapman, 2003; Parkar *et al.,* 2004; Lequette *et al.,* 2010); provides resistance to heavy metal toxicity (Zhang *et al.,* 2001). EPS also function as a stabilizer of the biofilm structure and as a barrier against hostile environments (Desai, 1998; Zhang *et al.,* 2001; Chen and Stewart, 2002; Gomez-Suarez *et al.,* 2002; Stoodley *et al.,* 2002; Arevalo-Ferro *et al.,* 2005; Lapidot *et al.,* 2006; Ploux *et al.,* 2007; Donlan, 2008).

2.4.5 Heterogeneity of EPS structures

The structures which make up a biofilm contain canals through which nutrients circulate in different zones of the biofilms (Comte *et al.,* 2006). The cells express different genes as if they were part of an organized structure (Giaouris *et al.,* 2006). The micro colony continues to grow in volume, and the bacteria in proximity to the surface have difficulties in gaining access to nutrients from the external environments. Only those located in the upper layers of the colony are able to continue multiplying (Giaouris *et al.,* 2006).

Any given cell within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus growing at the different rate. Factors such as gradients of nutrients, waste products and signaling factors contribute to this heterogeneity in biofilms. Heterogeneity has also been shown for protein synthesis and respiratory activity as DNA content remains constant throughout the biofilm (Prakash *et al.,* 2003). This concept of heterogeneity is descriptive not only for mixed-culture biofilms, but also for pure-culture biofilms (Allison, 1998). The matrix will change considerably as equilibrium between the species is established and a balance between competition and commensalism is achieved within the microbial community (Prakash *et al.,* 2003).

2.4.6 Cell structures associated with EPS

While EPS and lipopolysaccharides are more important in attachment to hydrophilic material cell-surfaces, polymers with nonpolar sites such as fimbria and other proteins and components of certain Gram positive bacteria facilitate attachment to the hydrophobic substratum (Arevalo-Ferro *et al.,* 2005). Flagella are important in attachment also, although their main role may be to overcome repulsive force rather than to act as adsorbents and adhesive (Arevalo-Ferro *et al.,* 2005; Donlan, 2008).

2.5 Biofilm producing enzymes

2.5.1 Extracellular enzymes

Hydrolytic enzymes such as glucosidase, lipase and protease have an important function at the hydrolysis stage of complex organic structures in the degradation of biodegradable organic matter. Several reports have indicated that the hydrolases are mainly localized in the extracellular polymeric substances (EPS) matrix of the bacterial aggregates. This debris can be either organic or inorganic (Ayol *et al.,* 2008; Burgess *et al.,* 2008). These exoenzymes (glucosidase, lipase and protease) can originate from the sewage effluent, the activated sludge via cell autolysis or as enzymes that are actively secreted by the cells (Romani *et al.,* 2008). Furthermore, exoenzymes are cell surface bound in free form (exoenzymes in water or absorbed within the extracellular polymeric substances (EPS) of the sludge matrix) (Higuchi *et al.,* 2005).

2.5.2 Enzyme activity in sludge flocs

The activated sludge process has long been employed to treat a wide variety of wastewater (Yan *et al.,* 2008). It has been reported that a number of enzymes such as aminopeptidase, galactosidase, glucosidase, lipase and phosphatase and protease have been extracted from sludge. These enzymes found in sludge may originate from the effluent sewage, from the sludge itself or even as actively secreted extracellular enzymes (de Beer *et al.,* 1996; Watson et *al.,* 2004; Burgess *et al.,* 2008). Proteolytic, lipolytic and cellulolytic enzymes synthesized within bacterial cells are secreted into the extracellular environment and hydrolyse the absorbed macromolecules into small units that can be transported across the cell membrane and then metabolized (Watson *et al.,* 2004; Li and Yang, 2007).

Protease, α- amylase and α glucosidase play important role in the biological waste water treatment. In the bulk solution of activated sludge, the amount of extracellular enzymes are immobilized in flocs. How the extracellular enzymes distribute in sludge flocs determines the contact probability of enzymes with proteins or polysaccharides, hence affecting the process treatment efficiency (Yu *et al.,* 2007). EPS in sludge flocs were

proposed to exhibit a dynamic double layer like structure, composed of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The LB-EPS is considered to easily exchange substances with the bulk solution hence having greater impact to numerous sludge processes such as coagulation and dewatering (Yu *et al.,* 2007).

2.5.3 Enzyme mediated resistance

The resistance of biofilms resistance to antimicrobial agents can be due to enzymes transforming the bactericide to a non-toxic form. The phenomenon is usually investigated from the biodegradation point of view, i.e. the biodegradation of toxic pollutants (Gu, 2007). A host of aromatic, phenolic and other compounds, toxic to many bacteria can be degraded by certain bacteria (Cloete, 2003). Enzyme-mediated resistance mechanism includes heavy metal resistance and formaldehyde resistance. Mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, tellurite, copper, chromate and silver are some of the compounds where biofilms are found to be resistance to due to enzymatic activity (Cloete, 2003; Bhaskar and Bhosle, 2005). Detoxification is usually by enzymatic reduction of the cation to the metal, whereas some heavy metal resistance genes are carried on plasmids, whilst others are chromosomal. The resistant phenotype is usually inducible by the presence of the heavy metal. Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite and antimony, for example, induce resistance to each other in *E. coli* (Cloete, 2003). The diagram below illustrates the multimetal resistance and tolerance in microbial biofilms!

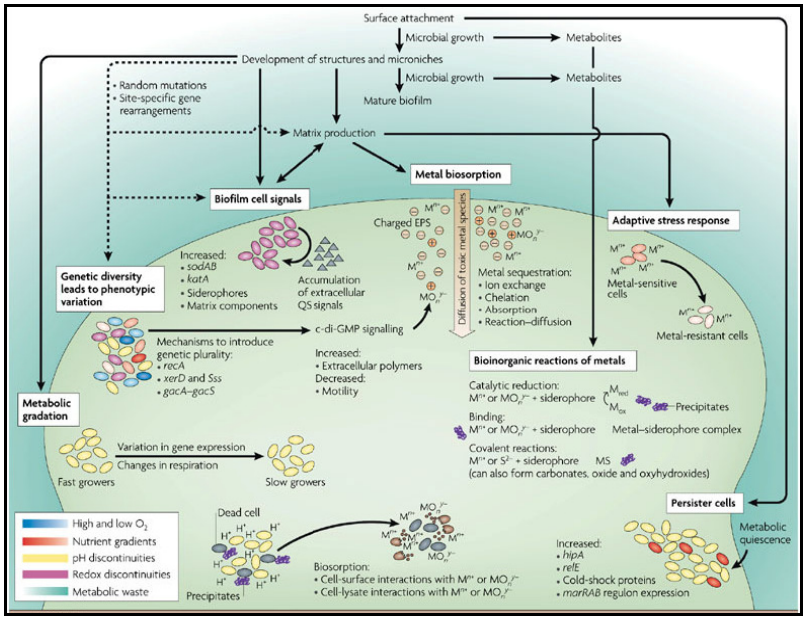

Fig. 2.6 Illustrations of the processes taking place during the multimetal resistance and tolerance in microbial biofilms (Harrison *et al.,* 2007).

2.5.4 Application of enzymes for biofilm control

The polymeric matrix that anchors the cells constitutes a penetration barrier to biocides (Pozos and Pater, 2007), decreasing their potency in comparison to the planktonic cells while promoting microbial resistance (Mah and O'Toole, 2001; O'Toole and Sterwart, 2005). The cells inside the biofilms have lower acess to nutrients and thus a slower growth rate, becoming more protected to the majority of antibiotics biocidal agents (de Carvalho, 2007).

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Studies have shown chemical biocides as the main strategy of biofilms control to kill the attached microorganisms from the surface (Rao *et al.,* 1998; Ramesh *et al* 2002; Smith, 2005). In industrial systems, it is important that both the inactivation of the microorganisms and the removal of biofilms from the surfaces are achieved (Simoes *et al.,* 2003).

A wide range of cleaners and sanitizers are available for use in line cleaning at retail outlets, however these cleaning chemicals are not successful in removing an established biofilms (Walker *et al.,* 2007). Studies have indicated that disinfection with chlorine dioxide and chlorine (der Beer (1994), for example can reduce the concentrations of planktonic bacteria, but have little to no effect on the concentrations of biofilm bacteria (Berry *et al.,* 2006). The mechanism behind the observed resistance of biofilm cells to disinfection is still unknown, although hypotheses include mass transfer resistance, the formation of persister cells and protection due to the production of extracellular polymeric substances (Berry *et al.,* 2006; Walker *et al.,* 2007).

The tolerance of biofilms to antimicrobials combined with their complex architecture and dynamic nature makes them quite difficult to measure, monitor and control and thus reduces the effectiveness of treatment strategies (Vickery *et al.,* 2004). An alternative method could be the mechanical cleaning but the disadvantage of this method is that, it is costly as it involves equipments down time and also significant labour expenditure (Vickery *et al.,* 2004).

Thus, the application of enzymes to degrade the EPS of the biofilms is an attractive method in many industries where complete biofilms removal is essential, but due to the heterogeneity of the extracellular polymeric substances in the biofilms, the application may turn ineffective. However, studies by Augustin *et al.* (2004) showed that the use of enzymes for removal of bacterial biofilms is still limited partly due to the very low prices of biocide chemicals in use. Augustin *et al*. (2004) also indicated that the lack of techniques for quantitative evaluation of the effect of enzymes as well as the commercial accessibility of different enzyme activities limits their use.

Nonetheless, Walker *et al.* (2007) indicated that in order to design enzymes which target the EPS of the biofilms, it is important to have an understanding of the nature of the EPS. Xavier *et al.* (2005) further explained that enzymes remove biofilms directly by destroying the physical integrity of the biofilm matrix (EPS).

The effect of any one enzyme degrading any one EPS will depend on the structure of the EPS in the biofilms. Thus degrading EPS from the biofilms can result in the release of

cells of one species while on the other hand, enhancing the growth of other species present in the biofilm (Walker *et al.,* 2007). This however happens in a mixed culture biofilm in which enzymatic treatment can either strengthen or weaken the EPS structure. This depends on the mode of action on individual EPS and the role of that EPS provides in the biofilms (Walker *et al.,* 2007).

While there are several studies on enzyme degradation of mature biofilms using synthetic polysaccharases, studies investigating the use of enzymes in inhibiting biofilm formation on surfaces are very scarce (Loiselle *et al.,* 2003; Vickery *et al.,* 2004, Xavier *et al.,* 2005). Previous studies have shown that cellulose from *Penicillium funiculusum* is one of the effective enzymes in degrading mature biofilms of *Pseudomonas aeruginosa*. Cellulose was also found to be useful in degrading the exopolysaccharides from *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004).

Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and alpha - amylase and beta – glucanase. This enzymatic mixture was effective in digesting slime layers produced by cultures of pure (*Pseudomonas species*) and mixed strains of bacteria. Among 24 preparations of the enzymes, only three types of enzymes were found to be effective for the removal of slime produced by *Pseudomonas* bacteria which were alpha-amylase, protease and the combination of amylase, glucanase and protease.

Many fungi also degrade complex plant cell wall material by secreting a large variety of enzymes (Orgaz *et al.,* 2006). This versatility makes commercial polysaccharide degrading enzyme mixture to have a wide spread use in various fields such as in fruit processing or wastewater treatment. Fungal enzymes could possibly be used for the degradation of bacterial biofilm matrices as well (Orgaz *et al.,* 2006).

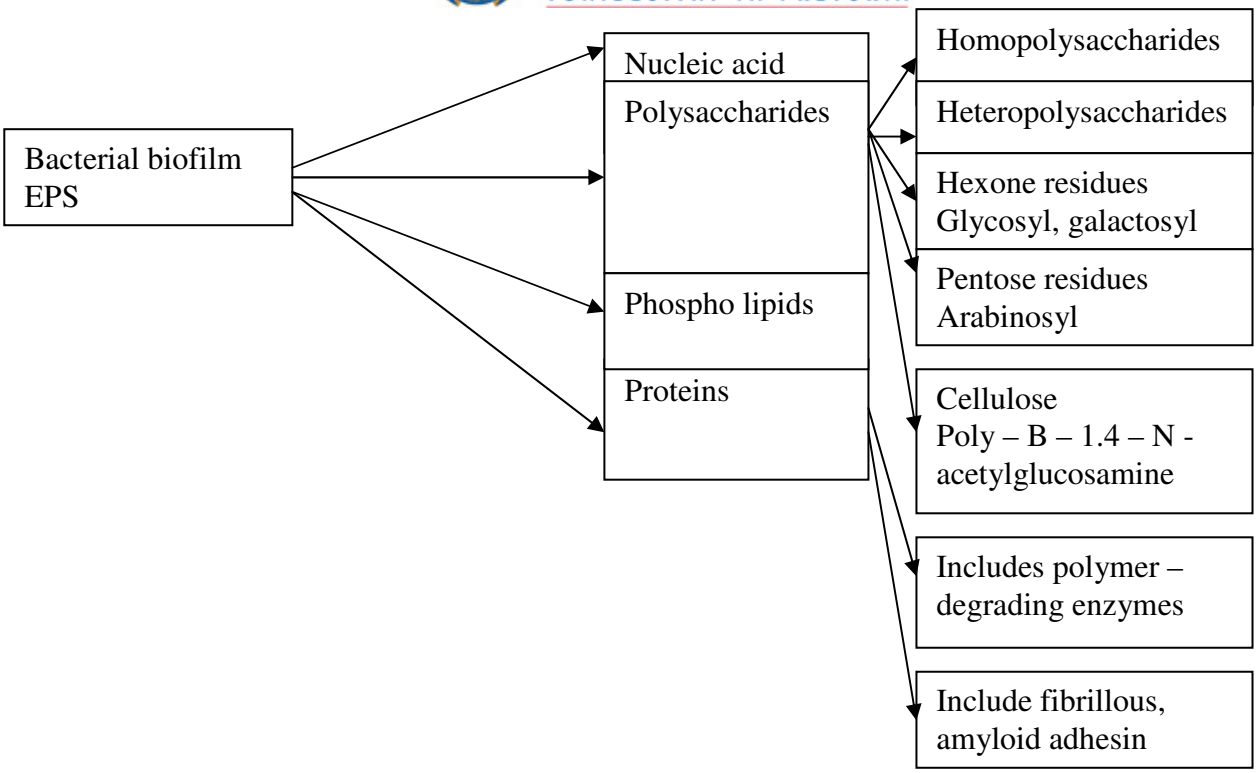

Fig. 2.7 Schematic overview of the structural components of extracellular polymeric substances (EPS) involved in biofilm formation (Kristensen *et al.,* 2008).

Table 2.1 Available enzymes used for the control of biofilms (Xavier *et al.,* 2005)

Agent	**Origin**	**Substrate**	**Action**	**Reference**
Enzymes				
Crude cellulose preparation	*Trichodema viridae* (Maxazyme CL2000)	Dephosphorylared and partially deharmnosylated EPS of *Lactococcus lactis* subsp. Cremoris B40	1. EPS was incubated with various commercial enzymes preparations and analyzed for degradation. In crude enzyme preparation tests, one enzyme acted very specifically.	1. Van Casteren *et al.,* 1998
Polysaccharide depolymerase	Bacteriophage	*Enterobacter agglomerans* GFP in monospecies biofilms and in the dual species biofilms with *Klebsiella pneumoniae* G1	1. Phage glycanase are very specific. Action of enzyme was observed when added to the phage susceptible monospceies biofilms leading to substantial biofilms degradation.	1. Hughes *et al.,* 1998 2. Skillman *et al.,* 1996

			2. A 60 min treatment with a polysaccharase caused a 20% reduction in dual species biofilms adhesion	
Alginate lyase	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* alginate	1. Strains of *P. aeruginosa* overproducing alginate lyase detached at a higher rate than the wild type. 2. However, other studies showed that addition of alginate lyase to established *P. aeruginosa* biofilms caused no observable detachment	1. Boyd and Chakrabarty, 1994 2. Christensen *et al.,* 2001
Disaggregatase enzyme	*Methanosarcina mazei*	*Methanosarcina mazei* heteropolysaccharide capsule mediating cell aggregation	1. Conditions that are generally unfavourable for growth are associated with disaggregatase activity	1. Xun *et al.,* 1990
Esterase with wide	Wide range of bacteria	Acyl residues from	1. Acetyl residues from	1. Sutherland 2001

specificity		bacterial polymers as well as other esters	intracellular carboxylesterase (EC 3.1.1.1) isolated from *Arthrobacter viscosus* removed acetyl residues from xanthan, alginate, glucose, pentaacetate, cellobiose, octaacetate, exopolysaccharide produced by *A. viscosus*. 2. deacetylated p-nitrophenyl propionate, naphthyl acetate, isopropenyl acetate and triacetin. Esterases could alter the physical properties of a biofilms structure	2. Cui *et al.,* 1999
Dispersin B (DspB)	*Actinobacillus actinomycetemcomitans*	Poly-ß-1,6-GlcNAc implicated as an adhesion factor for biofilms of several bacterial species	1. Causes detachment of cells from *A. actinomycetemcomitans* biofilms and disaggregation in solution.	1. Kaplan *et al.,* 2003 2. Kaplan *et a.1,* 2004 3. Itoh *et al.,* 2005

			2. Treatment of *S. epidermidis* biofilms with dispersin B caused dissolution of the EPS matrix and detachment of biofilms cells from the surface. 3. Disrupts biofilms formation by *E.coli, S.* epidermidis, Yersina pestis and Pseudomonas fluorescens.	
DNase 1	Commercial (Sigma-Aldrich)	Extracellular DNA in *Pseudomonas aeruginosa* biofilms	DNase affects the capability of P. aeruginosa to form biofilms when present in the initial deveoplment stages. Established biofilms were only affected to a minor degree by the presence of DNase	Whitchurch *et al,* 2002
Mixture of enzymes	Commercial	*S. aureus*, *S. epidermis*, *P. fluorescens* and *P.*	1. Pectinex UntraSP (Novo Nordisk A/S, a	Johansen *et al.,* 1997

		aeruginosa biofilms on steel and polypropylene substrata	multicomponent enzyme preparation) reduced the number of bacrterial cells in biofilms on stainless steel without any significant bactericidal activity (the activity of Pectinex Ultra is mainly a degradation of extracellular polysaccharides)	

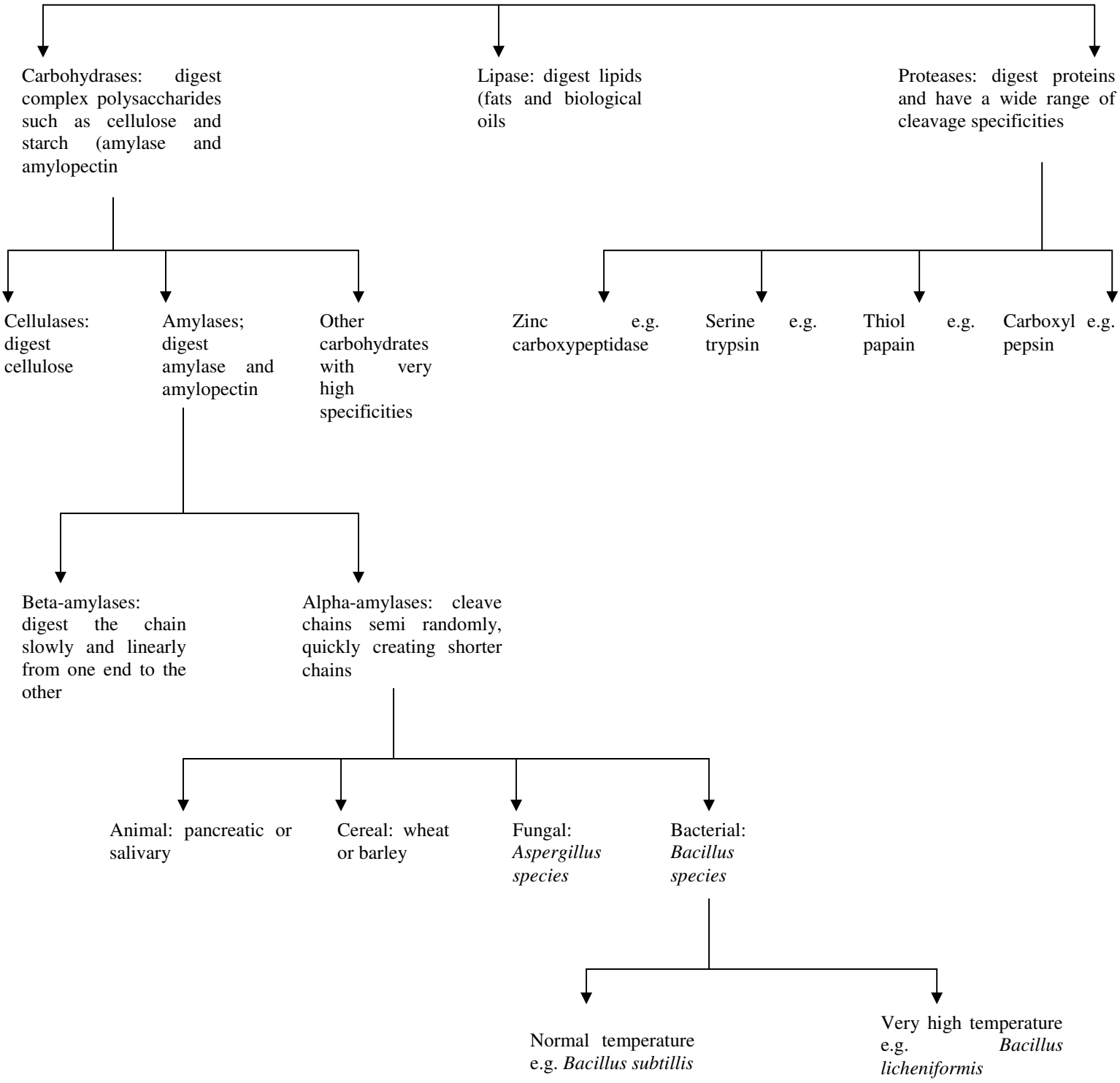

Fig. 2.8 Illustrations of various α-amylases with applications to conservation practise (Harold, 1992).

Table 2.2 The general composition of some bacterial extracellular polymeric substances (EPS) including humic substances (Wingeder *et al.,* 1999; Flemming *et al.,* 2000)

EPS	Principal components (subunits, precursors)	Main type of linkage between subunits	Structure of polymer backbone	Substituents
Polysaccharides	Monosaccharide	Glycosidic bonds	Linear branched side chain	Organic: ✓ O-Acetyl ✓ N-Acetyl ✓ Succynyl ✓ Pyruvyl Inorganic: ✓ Sulphate ✓ Phosphate
Proteins (polypeptide)	Amino acid	Peptide bonds	Linear	Oligosaccharides ✓ glycoproteins Fatty acids ✓ lipoproteins
Nucleic acid	✓ Nucleotides	Phosphodiester	Linear	_
(Phospho)lipids	✓ Fatty acid ✓ Glycerol ✓ Phosphate ✓ Ethanolamine ✓ Serine choline	Ester bonds	Side chains	_
Humic substances	✓ Pholic compounds ✓ Simple sugars ✓ Amino acid	Ester bond- C C- bonds Peptide bonds	Cross liked	_

Summarised literature review

Biofilms are characterized by surface attachment, structural heterogeneity; genetic diversity; complex community interactions and an extracellular matrix of polymeric substances. Biofilms deposit and adhere to all surfaces that are immersed in aqueous environments. A number of parameters including reactor type, substrate composition, substrate loading rate, hydraulic retention time, hydrodynamic shear force, culture temperature etc have been indicated to facilitate the production of biofilm EPS. EPS is highly hydrated and consists of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid and humic substances. The exopolysachharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties. This implies that the EPS composition is variable and is related to microbial species, the physiological state of the bacteria and the operating conditions under which biofilms are developed. The EPS formation rate is proportional to the rate of substrate utilization. Chemically, the bacterial EPS is a heterogeneous polymer containing a number of distinct monosaccharides and non carbohydrate substituents, many of which are strain specific. One of the major components of the bacterial EPS is the uronic acids costituting up to 20 – 50% of the polysaccharide fraction.

EPS are complex mixture of high molecular polymer (M_w>10,000) excreted by microorganisms, products from lysis and hydrolysis and adsorbed organic matters from wastewater. Such a polymer network has a vast surface area and is capable of absorbing pollutants, nutrients and minerals. EPS plays an important role in the flocculation of bacterial cells and provides energy and carbon when substrates are in short supply. The EPS compostion is also related to the characteristics of waste water. For example, EPS has high protein and DNA concentrations in protein grown granules while high polysaccharide concentrations are found in granules grown in other type of organic substrates. Nitrogen limiting conditions favour the production of EPS which in turn accelerates anaerobic granulation. The production of EPS is known to be affected by nutrients status of the growth medium and the availability of carbon. The bacterial extracellular polysaccharides are composed of homo and heteropolysaccharides including glucose, fructose, mannose, galactose, puryvate and mannuronic acid or glucoronic acid

based complexes. The exopolysaccharides are held to the cell wall of the bacteria either by linkage between the carboxyl gropus of the EPS and hydroxyl groups of lipopolysaccharides or by a covalent bonding through phospholipids and glycoproteins.

The activated sludge process has long been employed to treat a wide variety of wastewater and a number of enzymes including aminopeptidase, galactosidase, glucosidase, lipase and phosphatase and protease have been extracted from sludge. These enzymes found in sludge may originate from the effluent sewage, from the sludge itself or even as actively secreted extracellular enzymes. Proteolytic, lipolytic and cellulolytic enzymes synthesized within bacterial cells are secreted into the extracellular environment and hydrolyse the absorbed macromolecules into small units that can be transported across the cell membrane and then metabolized. Protease, α- amylase and α glucosidase play important role in the biological waste water treatment.

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Chemical biocides as the main strategy of biofilms control to kill the attached microorganisms from the surface. However, these antimicrobial agents fail to remove the biofilm cells due to the extracellular polymeric substances (EPS) that prevent the penetration of the antimicrobial agents within the biofilm cells, thus decreasing the potency of the biocides. The tolerance of biofilms to antimicrobials combined with their complex architecture and dynamic nature makes them quite difficult to measure, monitor and remove thus reduces the effectiveness of treatment strategies. Enzymes have been proven to be effective in the degradation of the biofilm EPS. The manner in which enzymes degrade the biofilm EPS is through binding and degradation of the EPS constituents and converting them into smaller units that can be transported through the cell membranes and then be metabolized.

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Chapter 3

Microplate assay for screening of proteolytic and amylase enzymes for biofilm removal

3.1 Abstract

Microbial communities that form biofilm are directly involved in biofouling and biocorrosion phenomena. In this study, the potential of proteolytic and amylolytic enzymes for the removal of biofilms was assessed using the microplate assay. Polarzyme was not effective with the lowest percentage reduction of < 20% for the removal of biofilm. Savinase and Everlase tested individually had the highest percentage reduction of > 80% for the removal of mono species biofilm (*Pseudomonas fluorescens*). A combination of protease enzymes was also evaluated and resulted in a 70 – 80% removal of mixed bacterial species biofilm. The amylase Fungamyl removed 70 – 80% of mono species biofilm. The amylase BAN was the least effective enzyme for the removal of biofilm with a reduction percentage ranging from 40 – 50%.

Keywords: Polarzyme, Everlase, Savinase, Esperase, biofilms, biofouling, biocorrosion,

3.2 Introduction

The development of multicellular aggregates also known as biofilms is a common phenomenon in aqueous environments and occurs through bacterial adhesion at solid-liquid interfaces (Kumar and Prasad, 2006). Biofilm development is widely believed to be initiated by bacteria sensing certain surface associated parameters that trigger the transition from a planktonic to a biofilm mode growth (Stoodley *et al.,* 2002). This involves a number of changes in gene regulation that cause the adhering cells to become phenotypically and metabolically distinct from their planktonic counterparts (Stoodley *et al.,* 2002).

Biofilm bacteria have a greatly enhanced tolerance to stress and antimicrobial agents. Thus, biofilm bacteria are different from planktonic bacteria with relation to gene expression and cellular physiology. Genetic studies involving various Gram negative bacteria have identified genes involved in the formation and development of biofilms (Oosthuizen *et al.,* 2001). In *Pseudomonas aeruginosa,* expression of a number of genes such as *algC*, *algD* and *pilA* are up regulated in biofilm growing cells. Most biofilm regulatory genes have been identified by screening for mutants that could not form biofilms (Steyn *et al.,* 2001). Biofilm formation occurs in response to a variety of environmental signals that lead to the expression of new phenotypes that distinguish the attached cells from their planktonic counterparts (Steyn *et al.,* 2001). The phenotype is believed to be responsible for the distinct properties of bacteria in biofilms, most notably their increased resistance to antimicrobial agents.

Various factors that enhance the initial adhesion processes of bacteria on a surface include; types of finish and surface roughness. Surface roughness may play a significant role under turbulent flow conditions in the initial stage of biofilm formation (Bachmann *et al.,* 2006). The deficiency of certain nutrients may also increase the biofilm formation capacity of some microbes (Wirtanen *et al.,* 1996).

A single bacterial species can form a biofilm but in natural environments, biofilms are formed by various microorganisms such as bacteria, fungi, algae, protozoa and debris along with corrosion products. Adhesion to surfaces provides considerable advantages for the biofilm forming microorganisms such as protection from anti microbial agents, exchange of nutrients, metabolites and/or genetic material from close proximity to other microorganisms. Microbial biofilms can exist as aggregates more or less confluent as a single layer, mat or three dimensional architecture with channels allowing liquid and gas flow and dispersion of nutrients and waste components (Johansen *et al.,* 1997). Such structures can develop on many abiotic and biotic surfaces (Chavant *et al.,* 2007). Once established, sessile bacteria express genes in a pattern that greatly differs from their planktonic counterparts leading to phenotypic changes. One of the most remarkable properties is the increased resistance of sessile cells to biocides (Pitts *et al.,* 2003), antibiotics (Narisawa *et al.,* 2005) and various physicochemical agents (McFeters *et al.,* 1995; Pitts *et al.,* 2003). Thus cells in biofilms can persist and survive even after decontamination procedures and may represent the original source for human and animal infections in foodstuff and in drinking water (Chavant *et al.,* 2007).

Microorganisms are less of a problem in the planktonic phase, due to increased disinfection efficiency. Promoting detachment of sessile cells is the least investigated of the possible strategies to remove unwanted biofilms (Simoes *et al.,* 2007).

In drinking water distribution systems, microbial adhesion will initiate biofilm formation leading to contamination of drinking water, reducing the quality of potable water, increasing corrosion rate of pipes and reducing microbiological safety through increased survival of pathogens (Klarhe and Flemming, 2001; Coetser and Cloete, 2005; Simoes *et al.,* 2007). Bacterial adhesion to surfaces is one of the initial steps leading to biofilm formation and is therefore an important microbiological event in medicine and industrial environments.

Efficient disinfection of microbial biofilms remains an area of significant investigation with a number of studies examining different disinfectants, novel biocides and procedures to enhance the efficacy of biocides for diverse industries such as food, water and medicine (Simoes *et al.,* 2003; Sreenivasan and Chorny, 2005). However, these antimicrobial agents do not completely remove biofilms due to protection by extracellular polymeric substances (EPS) which act as barriers preventing the biofilm cells. The use of substances to remove and / or kill biofilm directly by destroying the physical intergrity of the biofilm would be an attractive alternative for medical, environmental and industrial applications where complete biofilm removal is essential (Xavier *et al.,* 2005).

Therefore there is a need for substances that are capable of killing and removing biofilms (Xavier *et al.,* 2005). Applications of enzymes to control biofilm have been investigated as an alternative method. Walker *et al.* (2007) indicated that in order to design enzymes that target the EPS of a biofilm, it is important to have an understanding of the nature of the EPS. In addition, enzymes remove biofilm directly by destroying the physical integrity of the biofilm matrix (EPS) (Xavier *et al.,* 2005). The enzymatic efficiency of any one enzyme degrading EPS will either strengthen or weaken the EPS structure depending on the EPS composition (Walker *et al.,* 2007). Several studies based on enzymatic removal of biofilm have been investigated (Kaplan *et al.,* 2004), for example synthetic polysaccharases has been found to be effective on the degradation of mature biofilms. Additionally, cellulose from *Penicillium funiculusum* was found to be effective in degrading mature biofilms of *Pseudomonas aeruginosa* and that of *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004).

The availability of sensitive, specific and reproducible methodology for the quantification of biofilms is essential for the evaluation of biofilm formation (Shakeri *et al.,* 2007). Many methods have been developed for assessing and characterizing attached microorganisms from various environmental applications (Burton *et al.,* 2007). The annular reactor for example is frequently used for assessing biofilm accumulation in drinking water, because of its ease of operation and it can approximate growth conditions for a section of a distribution system (Gagnon *et al.,* 1999). Furthermore, a variety of direct (light microscopy, laser scanning confocal microscopy, transmission electron- and

scanning electron microscopy) and indirect methods (plate count, scraping, vortexing and sonication) have been developed for the quantitative and qualitative assessment of biofilms (Burton *et al.,* 2007).

The microtiter plate systems for quantification of biofilm formation have extensively been used (Djordjevic *et al.,* 2002; Pitts *et al.,* 2003). One of the stains that have been extensively used is crystal violet as an indicator of total attached biomass. This technique has been used to distinguish adherent parent strains from adhesion- altered mutants of *Escherichia coli* and *Pseudomonas aeruginosa* and also to differentiate strains of *Staphylococcus epidermidis* and *Vibrio species* by their adhesive properties (Pitts *et al.,* 2003). Crystal violet is suitable for measuring quantitatively the amount of biofilm biomass but not its activity. Therefore, crystal violet can be used to measure biofilm removal, but not disinfection (Pitts *et al.,* 2003).

In this study, the microtiter plate assay was used for screening of proteolytic and amylolytic enzymes to remove *Pseudomonas fluorescens* and mixed bacterial species biofilms. Enzymes were selected in this study because they exhibit broad specificity towards major biomolecules responsible for the physical integrity of biofilms.

3.3 Materials and methods

3.3.1 Bacterial inoculum used for biofilm growth

Pseudomonas fluorescens was used to grow mono species biofilm and mixed bacterial species biofilm was grown from *Pantoea ananatis*, *Proteus vulgaris, Serratia marcescens, Pseudomonas putida, Staphylococcus aeureus*, *and Staphylococcus xylosus.* Bacterial cultures were grown in Nutrient Broth for 24h at 26^{O}C and 30^{O}C for mono and mixed bacterial species respectively with agitation at 100rpm. The bacterial suspensions were adjusted to standard 1 McFarland.

3.3.2 Enzymes tested for biofilm removal

Enzymes used were from Novozymes (Ltd) South Africa and included proteases and amylases. The proteases were Savinase, Everlase, Esperase, and Polarzyme and the amylases were Fungamyl, Amiloglucosidase (AMG) and Bacterial Amylo Novo (BAN). Selected concentrations of 1 and 2 U/ml were tested in this study for the activity of these enzymes for biofilm removal. Protease enzymes were diluted in 0.1M Phosphate buffer, pH 8.3; Fungamyl was diluted in Phosphate buffer, pH 7; the gluco- amylase AMG was diluted in Phosphate buffer, pH 5, and the alpha- amylase BAN in 0.2M Tris- maleate, pH 7.0. Activity of enzymes in combination was also evaluated, neutral pH (7) was selected for the activity and Phosphate buffer was used for the dilution of the enzyme mixture.

Table 3.3.1 Enzymes used for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilm

Single enzymes		**Mixed enzymes**
Proteases	**Amylases**	**Proteases**
Savinase 16L Type EX	Amyloglucosidase (AMG) 300L	Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX
Everlase 16L Type EX	Bacterial Amylase Novo (BAN) 240L	**Amylases**
Esperase 16L Type EX	Fungamyl 800 L	AMG 300L, BAN 240L, Fungamyl 800 L
Polarzyme 6.0T		**Mixed protease and amylase enzymes**
		Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX AMG 300L, BAN 240L, Fungamyl 800 L

3.3.3 Micro plate assay for the evaluation of enzyme efficacy on biofilms

The Microtiter assay was performed according to Pitts *et al.* (2003) with the following modifications; 200µl of standardized bacterial suspensions were inoculated in the wells of a polystyrene micro titer plate (Lasec, S.A.) and incubated with shaking at 100rpm for 48h at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species biofilm without medium replenishment.

After 48h of incubation, the supernatant was discarded and plates were washed three times with 200µl sterile distilled water to remove non adherent bacterial cells. Test concentrations (1 U/ml and 2 U/ml) of proteases and amylases were added. A well with Ringer's solution was used as control. Plates were incubated for 1h at 26^{O}C and 30^{O}C. Following incubation, plates were emptied and washed twice with sterile distilled water. The remaining attached cells were fixed with 200µl of 95% ethanol for 15 min and the ethanol was discarded and plates were allowed to dry. Crystal violet solution (200µl) was added into each well for 30 min. Plates were washed five times with sterile distilled water followed with 30% glacial acetic acid (200µl) (Merck, S.A.). Plates were read at 595nm using a Multiskan Ascent ELISA plate reader (Termo Labsystems). The experiment was repeated twice.

The microtiter screening method was used to quantitatively measure the removal efficacy of enzymes on *Pseudomonas fluorescens* and mixed bacterial species biofilms. A measure of efficacy called Percentage Reduction (%) by Pitts *et al.* (2007) was used to evaluate the efficacy of these enzymes. The equation was calculated from the blank, control and treated absorbance values on a plate (Pitts *et al.,* 2007), i.e.

$$\text{Percentage Reduction} = \left[\frac{(C - B) - (T - B)}{(C - B)} \right] \times 100$$

Where, B denotes, the average absorbance per well for blank (no biofilm, no treatment); C denotes the average absorbance per well for control wells (biofilm, no treatment) and T denotes the average absorbance per well for treated wells (biofilm and treatment).

3.4 Results

Table 3.4.1 Effects of enzymes on biofilms as measured by the mean percentage reduction

Commercial enzymes	**Tested concentrations (U/ml)**			
	1 U/ml (Percentage Reduction) %		2 U/ml (Percentage Reduction) %	
Proteases	*Pseudomonas fluorescens biofilm* Average ± SD	*Mixed bacterial species biofilm* Average ± SD	*Pseudomonas fluorescens biofilm* Average ± SD	*Mixed bacterial species biofilm* Average ± SD
Savinase	84 ± 2.121	64 ± 0.001	85± 0.001	84 ± 0.021
Everlase	85 ± 5.657	61 ± 0.001	85± 0.002	84 ± 0.020
Esperase	74 ± 0.001	38 ± 0.384	74 ± 0.138	67 ± 0.045
Polarzyme	18 ± 1.414	8 ± 0.379	9 ± 0.328	5 ± 0.011
Mixed proteases (MP)	72 ± 0.001	73 ± 0.080	75 ± 0.046	88 ± 0.063
Amylases				
Fungamyl	77 ± 0.001	54 ± 0.131	81 ± 0.059	62 ± 0.001
Amyloglucosidase (AMG)	54 ± 6.364	39 ± 0.018	52 ± 0.107	61± 0.026
Bacterial Amylase Novo (BAN)	42 ± 2.121	39 ± 0.004	45± 0.004	51± 0.089
Mixed amylases (MA)	60 ± 0.001	48 ± 0.014	75 ± 0.001	77± 0.017
Mixed proteases and amylases (MPA)	67 ± 0.001	76 ± 0.089	78 ± 0.001	79± 0.011
Ringers solution (control)	18 ± 0.001	13± 0.036	7 ± 0. 001	8 ± 0.012

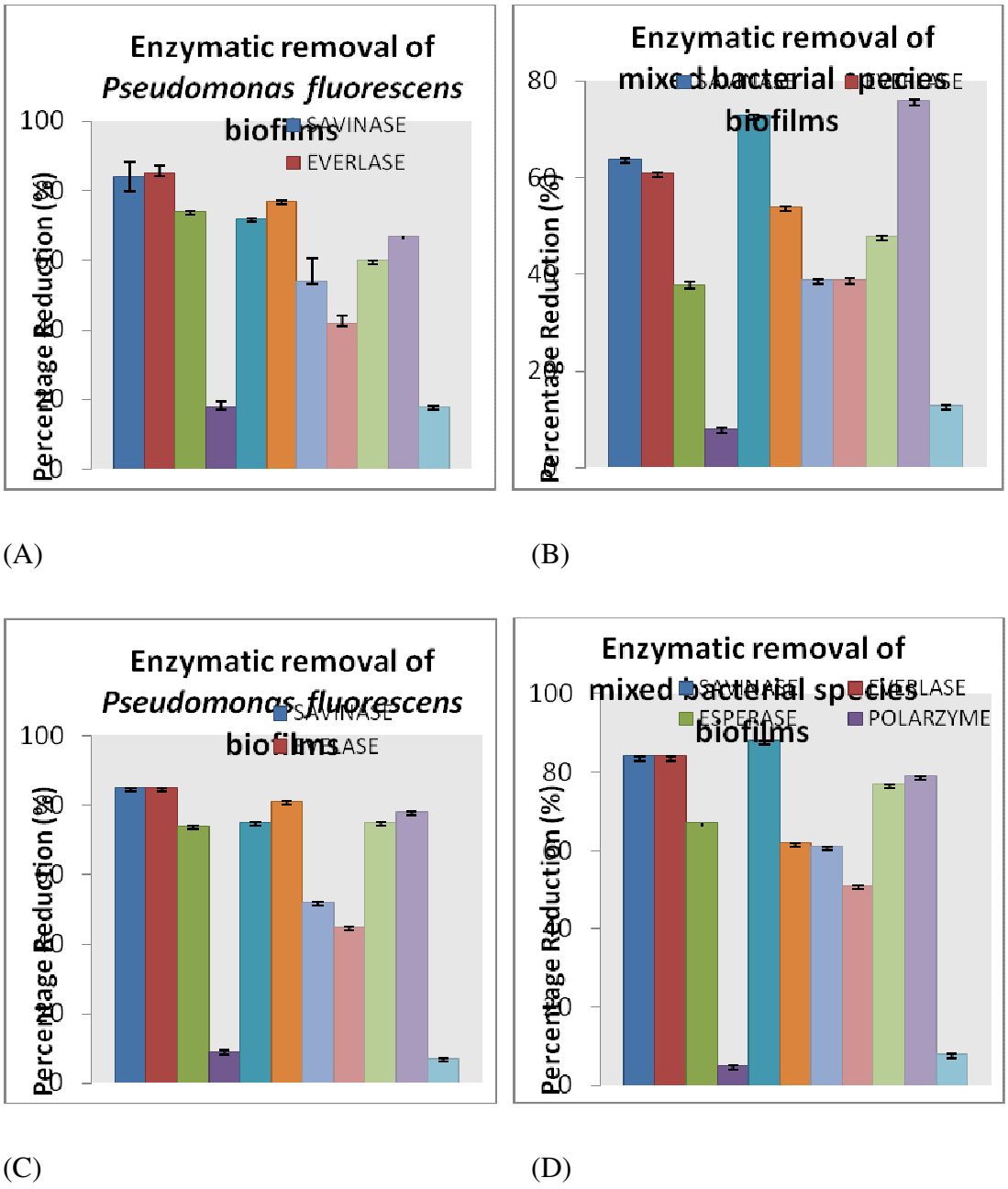

Fig. 3.4.1 Enzyme efficacy for removal of (A, C) *Pseudomonas fluorescens* biofilms and (B, D) mixed bacterial species biofilms treated at (A, B) 1 U/ml and (C, D) 2 U/ml. Bars indicate standard errors.

In this study, the potential of proteolytic and amylolytic enzymes for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilms using the microtiter plate assay was assessed. Polarzyme was not effective for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilms (PR< 40%) and was comparable to the control (biofilm treated with Ringer's solution) (Table 3.4.1, Fig 3.4.1)

Savinase was more effective for the removal of *Pseudomonas fluorescens* biofilm than mixed bacterial species biofilm at 1 and 2 U/ml (Table 4.3.1). At 1 U/ml the percentage reduction was 84% and the activity increased by 1% (85%) at 2 U/ml for the removal of *Pseudomonas fluorescens* biofilm (Table 3.4.1). When this enzyme was tested on mixed bacterial species biofilm, there was a noticeable difference, at the lower concentration (1 U/ml) Savinase resulted in a PR of 64% and the PR increased to 84% when tested at 2 U/ml (Table 4.3.1; Fig 3.4.1).

Activity of Everlase at 1 U/ml in terms of PR was comparable to the activity of Savinase at 2 U/ml, when tested on *Pseudomonas fluorescens* biofilm (Table 4. 3.1). On the other hand, Everlase was less effective at 1 U/ml with PR of 61 and 84% at 2 U/ml when tested for mixed bacterial species biofilm removal. Savinase and Everlase had a PR of 84% at 2 U/ml when tested on mixed species biofilms (Table 4. 3.1; Fig 3.4.1).

The protease Esperase was also effective for the removal of *Pseudomonas fluorescens* biofilm. The activity in terms of Percentage Reduction (PR) remained the same at 1 and 2 U/ml (PR = 74%) when tested for the removal of *Pseudomonas fluorescens* biofilms (Table 3.4.2; Fig 3.4.1). Esperase was less effective (PR of 38%) at 1 U/ml than the higher concentration (PR = 67%) at 2 U/ml for the removal of mixed bacterial species biofilms (Fig 3.4.1).

The activity of combined proteolytic enzymes (Savinase, Everlase and Esperase) was evaluated. There was no noticeable difference in terms of PR at 1 and 2 U/ml when this mixture was tested for the removal of *Pseudomonas fluorescens* biofilm. Percentage reduction (PR) was 72% at 1 U/ml and 75% at 2 U/ml (Table 3.4.1; Fig 3.4.1). However, there was slight difference in terms of activity when this mixture was tested for the removal of mixed bacterial species biofilm. At 1 U/ml, the PR was 73% and as the

concentration was increased to 2 U/ml, the activity increased to a PR of 88% (Table 3.4.1).

Fungamyl (amylase) was effective for the removal of biofilms. The efficacy of Fungamyl for the removal of biofilm was higher on *Pseudomonas fluorescens* biofilm than for mixed bacterial species biofilm. A PR of 77% and 81% were obtained after treatment at 1 and 2 U/ml, respectively when tested for *Pseudomonas fluorescens* biofilm (Fig 3.4.1). When tested on mixed bacterial species biofilm the PR was 54% at 1 U/ml and 62% at 2 U/ml (Table 3.4.1, Fig 3.4.1). There was no noticeable difference in terms of PR when AMG (amylase) was tested for the removal of *Pseudomonas fluorescens* biofilm. At 2 U/ml (PR = 52%) the activity was slightly higher than at 1 U/ml (PR = 54%) (Fig 3.4.1; Table 3.4.1).

The amylase BAN was the least effective enzyme for the removal of biofilms with PR of 42% and 45% at 1 and 2 U/ml, respectively for the removal of *Pseudomonas fluorescens* biofilms. At 1 U/ml with PR of 39% was lower that the PR of 51% at 2 U/ml when trested for the removal of mixed bacterial species biofilms (Table 3.4.1).

The activity of amylase enzymes in combination was assessed. This enzymatic mixture was more effective for the removal of *Pseudomonas fluorescens* biofilm than mixed bacterial species biofilm (Table 3.4.1). A PR of 60 and 75% at 1 and 2 U/ml respectively were obtained for the removal of *Pseudomonas fluorescens* biofilm. On the other hand, the reduction was less (PR = 48%) at 1 U/ml than at 2 U/ml, the PR increased to 77% for the removal of mixed bacterial species biofilms.

The activity of protease and amylase enzymes in combination was evaluated for the removal of biofilms. A PR of 67 and 78% at 1 and 2 U/ml respectively were obtained after treatment for the removal of *Pseudomonas fluorescens* biofilms. A PR of 76 and 79% tested at 1 and 2 U/ml, respectively were obtained for the removal of mixed bacterial species biofilms (Table 3.4 1; Fig 3.4.1).

3.5 Discussion

Biofilms are less sensitive to antimicrobial agents than are the cells growing planktonically. Much of this resistance has been attributed to the production of the extracellular polymeric substances (EPS) produce by the biofilms (Loiselle *et al.,* 2003). The objective of this study was to employ the microtiter plate assay to test the potential of protease and amylase enzymes for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilm. This method is based on the measurement of crystal violet use as an indicator of total attached biomass. Protease and amylase enzymes have been selected for the control of biofilms because proteins and carbohydrates are the main structural components of the EPS.

In the results, variations in terms of enzyme activity for biofilm removal were observed. These variations were as a result of difference in strength and efficacy of the enzymes depending on their formulations. Polarzyme had no effect for the removal of the biofilm tested in this study at both concentrations (1 and 2 U/ml) (Table 3.4.1) (Fig. 3.4.1). The inability of Polarzyme to remove biofilms may be due to poor chemical interaction between this enzyme and the biofilm EPS matrix. Another reason could be that, Polarzyme used in this study was in granular form and during experimental procedures; the coat material used to coat the enzyme could not completely dissolve in the diluents used to dilute the enzymes (phosphate buffer). Therefore, this could have had a negative effect on the activity of Polarzyme on the biofilms.

On the other hand, other enzymes tested in this study were effective, although the effectivity was concentration dependent. In addition, the concentration - dependence response was determined by the type of the EPS of the biofilm. Looking at the results of Savinase, Everlase, Esperase and Fungamyl, these enzymes were more effective for the removal of *P. fluorescens* biofilm than removing mixed bacterial species biofilm at both tested concentrations (1 and 2 U/ml).

Leroy *et al.* (2008) tested the activity of Savinase for the removal of biofilms produced by *Pseudoalteromonas sp*. D41 and in the results 50% of the biofilm cells were reduced at the lowest concentration. Based on the previous and present study on the activity of Savinase for removal of most *Pseudomonas* species, it is therefore suggested that this enzyme should be used as an anti *Pseudomonas* species for removal of biofilms produced by single or multi *Pseudomonas species*. The efficacy of this enzyme relates to the structural components of the EPS of *Pseudomonas* species. In addition, cellulose from *Penicillium funiculusum* was found to be useful in degrading the exopolysaccharides of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004).

When the enzymes were tested for the removal of mixed bacterial species biofilm, the efficacy was less and was affected by change in concentrations. Efficacy was far less at 1 U/ml and improved as the concentration increased to 2 U/ml (Table 3.4.1) (Fig. 3.4.1). Activity of a single enzyme tested for the removal of mixed bacterial species biofilm is unpredictable, due to heterogeneity of the structural components making the EPS. Walker *et al.* (2007) indicated that the enzymatic efficiency of any one enzyme degrading EPS of the biofilms will either strengthen or weaken the EPS structure depending on the EPS composition. Thus, this indicates that efficacy is dependent on the type of the EPS of the biofilm. In addition, the concentrations of the biofilm EPS determines the required dose for better efficiency. For example, Esperase and Everlase tested for the removal of *P. fluorescens* biofilm remained unchanged while the efficacy of these enzymes was concentration dependent when tested for the removal of mixed bacterial species biofilm (Fig. 3.4.1). This again, indicates that the degrading strength of each enzyme depends on its formulation and to the structural composition of the EPS of the biofilm.

A combination of enzymes was assessed for biofilm removal to determine if efficacy will improve or not. A combination of protease enzymes and a combination of protease and amylase seemed more effective for mixed bacterial species biofilm removal. It was expected that these enzyme mixtures would be more effective for the removal of *P. fluorescens* biofilm than mixed bacterial species biofilms since the structural components of the EPS produced by mixed bacterial species would be more complex than that of

Pseudomonas fluorescens and might limit the activity of the enzymes (Fig. 3.4.1). These enzymatic mixture results are in support of Wiatr (1991) who tested five enzymes and also a combination of one protease and two amylase enzymes namely the alpha and beta – glucanase and found this mixture to be effective in digesting slime layers produced by cultures of pure *Pseudomonas species* and mixed strains of bacteria in the biofilm removal reactor (BRR). On the other hand a combination of amylase enzymes was more effective for removal of *P. fluorescens* than mixed bacterial species biofilm. These results show that enzyme efficacy is dependent on the structural components of the EPS matrix produced by the biofilms.

3.6 Conclusion

The microtiter assay has been helpful for screening of proteolytic and amylolytic enzymes for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilms. It has been demonstrated that the enzymes tested individually or in combination were effective for removal of the biofilms, however, activity amongst the enzymes varied. The effects of the enzymes for biofilm removal dependent on the concentration tested. The reduction percentage was higher at 2 U/ml and lower at 1 U/ml. Savinase, Everlase, Esperase and Fungamyl tested individually were most effective for the control of *P. fluorescens* biofilm while enzymatic mixtures of protease enzymes and protease and amylase enzymes were more effective for removal of mixed bacterial species biofilm. The results of this study showed that enzymes differed in terms of activity on the biofilms. The difference in activity was as a result of the difference in structural components of the EPS produced by different biofilms. Therefore, in order to design enzymes which target the EPS of the biofilms, it is important to have an understanding of the structural components of the EPS. The formulation of an enzyme may determine poor or good activity. Savinase has been previously and recently tested for removal of biofilms produced by most *Pseudomonas* species and this enzyme has been reported to be effective in removing biofilms produced by this bacterial species. It was therefore concluded that these enzymes could be used for the removal of biofilms of *Pseudomonas* biofilms as well as multi bacterial species biofilms.

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Chapter 4

Spectrophotometric assay for the evaluation of proteolytic and amylolytic enzymes for biofilm removal

4.1 Abstract

The objective of this study was to determine the removal potential of proteolytic and amylase enzymes on biofilm produced by *Pseudomonas fluorescens* and mixed bacterial species (*Gram negative and positive)*. Biofilms were grown with continuous agitation for 7d in Nutrient broth medium with glass wool used as substratum for biofilm attachment. After incubation, planktonic cells were discarded. The glass wool was vortexed for 1 min to detach loosely bound cells. The density of the remaining cells was determined at OD 595nm. Test concentrations of the enzymes (1 and 2 U/ml) were added to the glass wool and incubated for 24h at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species respectively without agitation. After 24h the effect of enzymes for removal and detachment of the cells on the glasss wool was evaluated spectrophotometrically at OD 595. The density of the non treated cells was lower and the density increased after enzymatic treatment. Hence, the cell density of *Pseudomonas fluorescens* and mixed bacterial species cells after Polarzyme treatment was lower while the non treated cells had higher density. Savinase and Everlase tested individually had the highest *Pseudomonas fluorescens* cell density after treatment. The number of mixed bacterial species cells recovered was higher after treatment with a combination of protease enzymes than when individual enzymes were used. The cell density of *Pseudomonas fluorescens* and mixed bacterial species after treated with Fungamyl was much higher compared to the density of the cells treated with AMG and BAN. In conclusion, Polarzyme did remove the cells attached to the glass wool; it promoted growth and development of the biofilm cells.

Key words: EPS, *Pseudomonas fluorescens*, proteases, amylases, biofilm, glass wool

4.2 Introduction

A biofilm is formed by a community of microorganisms embedded in an extracellular polymeric substance (EPS) attached firmly to a biotic or abiotic surface (Zhang *et al.,* 2001; Orgaz *et al.,* 2006; Leroy *et al.,* 2008). The biofilm matrix is mainly composed of water (97%) and extracellular polymeric substances (EPS). The chemistry of the EPS is complex which includes polysaccharides, nucleic acids and proteins (Stoodley *et al.,* 2002). The EPS serves many functions such as providing an adhesive foundation, structural intergrity (de Carvalho, 2007, Leroy *et al.,* 2008), bacterial protection and intercellular communication (Zhang *et al.,* 2001).

When bacterial cells approach inert surfaces, they first get bound to the substratum by weak forces involving their external structures such a flagella, fimbria or capsular components. When the cells remain attached for some time to the substratum, they secret sticky extracellular substances (EPS) forming a matrix of gel that embeds several layers of the cells as the biofilm mature (Orgaz *et al.,* 2006). This matrix is known to include mainly polysaccharides besides of proteins, nucleic acids, lipids, mineral ions and various debris as well (Orgaz *et al.,* 2006). The polysaccharides are partly responsible for bacterial adhesion and biofilm accumulation on the surface (Loiselle *et al.,* 2003).

Biofilm predominate in nature because attached cells have certain advantages over planktonic cells, such as the ability to metabolize recalcitrant organic compounds and increased resistance to chlorine and other biocides (Xavier *et al.,* 2005). The growth patterns, coverage and the adherence of biofilm depend on the substrate roughness, composition, type of microorganisms and other factors (Augustin *et al.,* 2004).

Removal of biofilm poses considerable difficulties in many areas such as in cooling water systems, in medicine, paper making and in food processing industry (Cloete, 2003; Simoes *et al.,* 2003; Augustin *et al.,* 2004; Sreenivasan *et al.,* 2005). In the manufacturing environments, microbial biofilm contribute to poor efficiencies in the manufacturing processes such as blocked pipes, poor heat transfer and microbiologically induced corrosion (MIC). This adversity may result in significant delays, cost overrun and potential health related concerns to the end product user (Sreenivasan *et al.,* 2005).

Control of biofilm in industrial water systems is an important aspect of any successful water treatment program. In industrial settings, unwanted biofilm are responsible for fouling of cooling water tower, water pipe lines, membrane units or food processing plants (Xavier *et al.,* 2005; Berry *et al.,* 2006).

In previous literature, chemical biocides have been used as the main strategy to control and kill the attached microorganisms from the surface. In industrial systems, it is important that both the inactivation and the removal of biofilm from the surfaces are achieved (Simoes *et al.,* 2003). A wide range of cleaners and sanitizers are available for use in line cleaning at retail outlets, however these cleaning chemicals are not successful at removing an established biofilm (Cloete, 2003; Walker *et al.,* 2007). Studies have indicated that disinfection with chlorine dioxide and chlorine, for example can reduce the concentrations of planktonic bacteria, but have little to no effect on the concentrations of biofilm bacteria (Berry *et al.,* 2006). The mechanism behind the observed resistance of biofilm cells to disinfections is still unknown, although hypotheses include mass transfer resistance, the formation of persister cells (Berry *et al.,* 2006) and protection due to the production of extracellular polymeric substances (Walker *et al.,* 2007).

Augustin *et al.* (2004) indicated several reasons that can account for the reduced sensitivity of bacteria within a biofilm and they are: (a) reduced access of disinfectants to the cells within a biofilm (b) chemical interaction between the disinfectants and the biofilm itself, (c) modulation of the microenvironments, (d) production of degradative enzymes and (e), genetic exchange between the cells in a biofilm.

Recent studies concerning induced detachment by depriving the biofilm of essential nutrients reveal the potential of this strategy which however, will not be applicable in cases where controlling the nutrients in the medium is not possible. On the contrary, Cowan *et al.* (1991) stated that nutrient concentration too low to measure is still sufficient for biofilm growth. In addition, Berry *et al.* (2006) also indicated that lapses in chlorination (served as nutrients) led to regrowth of biofilm communities and increased resistance of biofilm bacteria to chlorine.

Recently, more focus has been on the use of enzymes for the control of biofilm. For example, Walker *et al.* (2007) indicated that in order to design enzymes which target the EPS of the biofilm, it is important to have an understanding of the nature of the EPS while Xavier *et al.* (2005) further explained that enzymes remove biofilm directly by destroying the physical integrity of the biofilm matrix (EPS).

The use of enzymes for biofilm control also has disadvantages. For example, the effect of any one enzyme degrading any one EPS will depend on other EPS in the biofilm. Thus degrading EPS from the biofilm can result in the release of cells of one species while on the other hand, enhancing the growth of other species present in the biofilm (Walker *et al.,* 2007). This however happens in mixed culture biofilm. Thus enzymatic treatment can either strengthen or weaken the EPS structure depending on the mode of action on individual EPS and the role of that EPS provides in the biofilm (Walker *et al.,* 2007).

A number of studies have investigated enzyme degradation of mature biofilm using synthetic polysaccharases and very few studies have investigated the use of enzymes in inhibiting biofilm formation on surfaces (Loiselle *et al.,* 2003; Vickery *et al.,* 2004). Previous studies have shown that cellulose from *Penicillium funiculusum* is one of the effective enzymes in degrading mature biofilm of *Pseudomonas aeruginosa*. Cellulose was also found to be useful in degrading the exopolysaccharides from *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004).

Orgaz *et al.* (2006) indicated that many fungi can degrade complex plant cell wall material by secreting a large variety of enzymes. This versatility makes commercial polysaccharide degrading enzyme mixtures useful for a variety of applications in fields such as fruit processing and wastewater treatment. Fungal enzymes could possibly be used to degrade bacterial biofilm matrices as well (Orgaz *et al.,* 2006).

Therefore, the application of enzymes to remove biofilm by degrading the EPS is an attractive application in many industries where complete biofilm removal is essential. In this study, proteolytic and amylolytic enzymes were assessed for the removal of biofilm produced by *Pseudomonas fluorescens* and mixed bacterial species using a spectrophotometric assay.

4.3 Materials and methods

4.3.1 Bacterial inoculum used to grow biofilms

Pseudomonas fluorescens was used to grow a mono species biofilms. Mixed bacterial species biofilms were grown from *Pantoea ananatis*, *Proteus vulgaris, Serratia marcescens, Pseudomonas putida, Staphylococcus aeureus*, *and Staphylococcus xylosus.* A standardised suspension (100µl) was inoculated in sterile flasks containing Nutrient Broth (Merck) (100ml) and 1g of sterile glass wool and incubated at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species respectively. Incubation was for 7d in a shake incubator at a controlled speed of 100rpm.

4.3.2 Enzymes tested for biofilm removal

The enzymes used in this study were proteases and amylases and were supplied by Novozymes (Ltd) South Africa. The proteases were Savinase, Everlase, Esperase, and Polarzyme and the amylases were Fungamyl, Amiloglucosidase (AMG) and Bacterial Amylase Novo (BAN). Enzyme concentrations tested were 1 and 2 U/ml. Protease enzymes were diluted in 0.1M Phosphate buffer, pH 8.3; Fungamyl was diluted in Phosphate buffer, pH 7; AMG was diluted in Phosphate buffer, pH 5, and BAN in 0.2M Tris- maleate, pH 7.0. The combined enzyme activity was also evaluated. Phosphate buffer was used to adjust the enzyme mixture to a pH of 7.

4.3.4 Spectrophotometric assay for the assessment of enzymes for biofilm removal

A spectrophotometric assay described previously by Ledder *et al.* (2008) was used with modifications to determine the effects of the enzymes for biofilm removal formed by *Pseudomonas fluorescens* and mixed bacterial species. Briefly, following 7d of biofilm growth, planktonic cells were discarded and the glass wool was vortexed for 1 min to detach loosely bound cells. After vortexing, the cell density was measured at OD, 595nm. The enzyme solutions (100ml) were added to the vortexed glass wool and incubated for 24h without agitation at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species biofilm respectively. After incubation, the mixture was vortexed for 1

min and the cell density was measured again at the same wavelength (OD, 595nm). Glass wool without enzyme concentrations was used as control.

4.3.5 Quantitative determination of viable cells

Ten fold series of dilutions were made by inoculating 100µl of the mixture to 900µl of Ringer's solutions and mix. The aliquots (0.1ml) was spread onto sterile Nutrient agar plates (Merck) and incubated for 24- 48h at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species respectively (3 plates for each dilution). Non treated cells were used as control. Viable cells were enumerated and expressed as Colony Forming Units (CFU/ml).

Table 4.3.1 Enzymes used for biofilm removal

Single enzymes		**Mixed enzymes**
Proteases	**Amylases**	**Proteases**
Savinase 16L Type EX	Amyloglucosidase (AMG) 300L	Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX
Everlase 16L Type EX	Bacterial Amylase Novo (BAN) 240L	**Amylases**
Esperase 16L Type EX	Fungamyl 800 L	AMG 300L, BAN 240L, Fungamyl 800 L
Polarzyme 6.0T		**Mixed protease and amylase enzymes**
		Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX AMG 300L, BAN 240L, Fungamyl 800 L

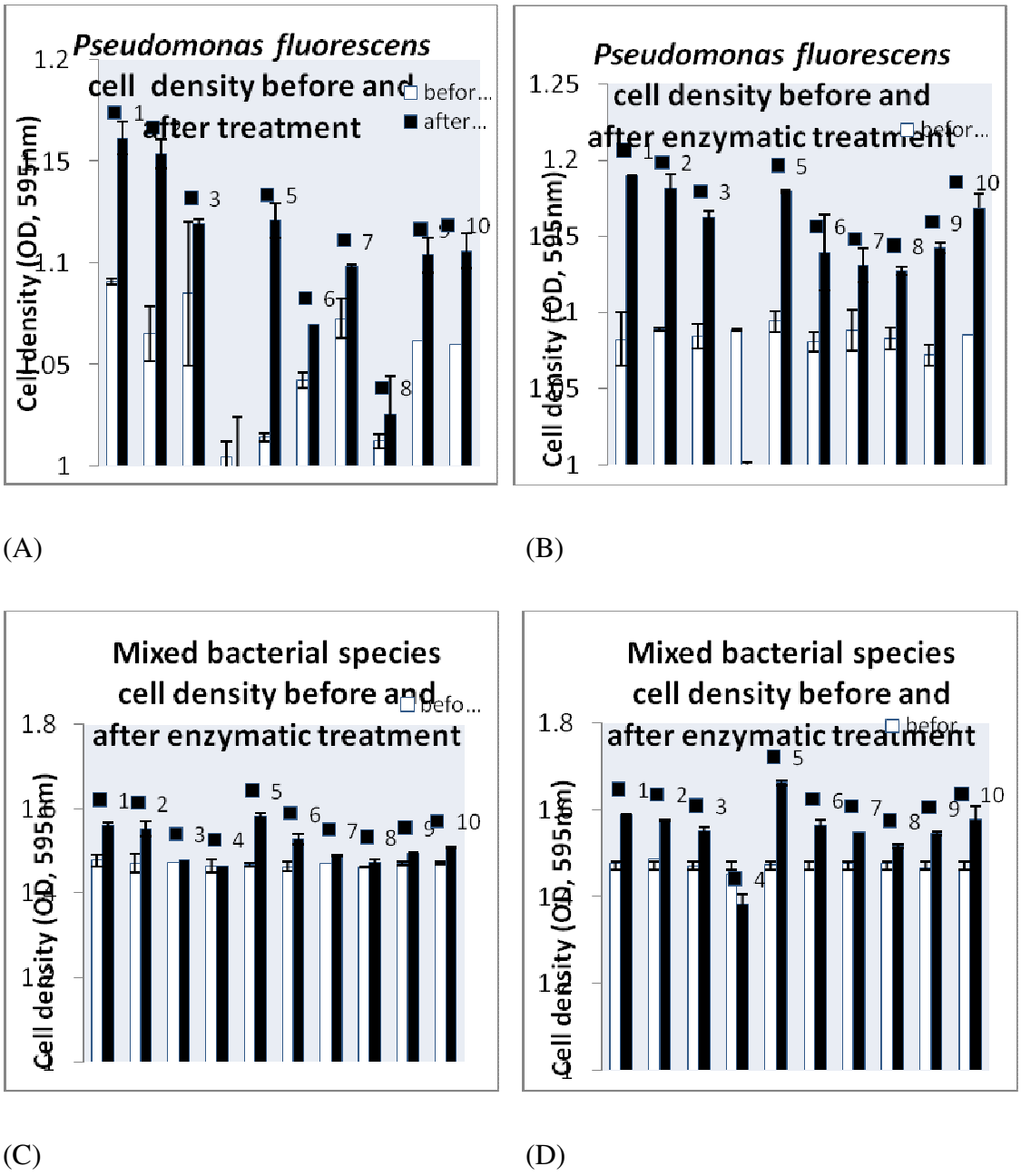
4.4 Results

Table 4.4.1 Spectrophotometric evaluation of cell density before and after enzymatic treatment tested at 1 U/ml

Cell density before enzymatic activity		**Cell density after enzymatic activity**	**Cell density before enzymatic activity**		**Cell density after enzymatic activity**
Concentrations (1 U/ml)			Concentrations (1 U/ml)		
Pseudomonas fluorescens cells Average ± SD	Tested enzymes	*Pseudomonas fluorescens cells* Average ± SD	*Mixed bacterial cells* Average ± SD	Tested enzymes	*Mixed bacterial cells* Average ± SD
	Proteases			**Proteases**	
1.091 ± 0.001	1. Savinase	1.162 ± 0.008	1.447 ± 0.014	Savinase	1.561 ± 0.008
1.083 ± 0.013	2. Everlase	1.154 ± 0.007	1.487 ± 0.023	Everlase	1.553 ± 0.018
1.085 ± 0.035	3. Esperase	1.120 ± 0.002	1.472 ± 0.001	Esperase	1.479 ± 0.001
1.005 ± 0.008	4. Polarzyme	0.938 ± 0.087	1.476 ± 0.016	Polarzyme	1.465 ± 0
1.015 ± 0.008	5. Mixed proteases (MP)	1.121± 0.008	1.468 ± 0.004	Mixed proteases (MP)	1.583 ± 0.008
	Amylases			**Amylases**	
1.043 ± 0.004	6. Fungamyl	1.070 ± 0.000	1.463 ± 0.011	Fungamyl	1.528 ± 0.008
1.073 ± 0.009	7. AMG	1.099 ± 0.001	1.469 ± 0.000	AMG	1..490± 0.012
1.013 ± 0.004	8. BAN	1.026 ± 0.018	1.462 ± 0.002	BAN	1.473 ± 0.001
1.062 ± 0.008	9. Mixed amylases (MA)	1.104 ± 0.008	1.470 ± 0.004	Mixed amylases (MA)	1.494 ± 0.002
1.060 ± 0.008	10. Mixed proteases and amylases (MPA)	1.106 ± 0.008	1.470 ± 0.003	Mixed proteases and amylases (MPA)	1.509 ± 0.001

Table 4.4.2 Spectrophotometric evaluation of cell density before and after enzymatic treatment tested at 2 U/ml

Cell density before enzymatic activity		**Cell density after enzymatic activity**	**Cell density before enzymatic activity**		**Cell density after enzymatic activity**
Concentrations (1U/ml)			Concentrations (1U/ml)		
Pseudomonas fluorescens cells Average ± SD	Tested enzymes	*Pseudomonas fluorescens cells* Average ± SD	*Mixed bacterial cells* Average ± SD	Tested enzymes	*Mixed bacterial cells* Average ± SD
	Proteases			**Proteases**	
1.083 ± 0.018	1. Savinase	1.190 ± 0.001	1.476 ± 0.000	Savinase	1.590 ± 0.001
1.089 ± 0.001	2. Everlase	1.182 ± 0.009	1.489 ± 0.015	Everlase	1.577 ± 0.001
1.085 ± 0.008	3. Esperase	1.163 ± 0.004	1.471 ± 0.000	Esperase	1.552 ± 0.009
1.089± 0.001	4. Polarzyme	0.995 ± 0.007	1.452 ± 0.008	Polarzyme	1.384 ± 0.023
1.094 ± 0.007	5. Mixed proteases (MP)	1.180 ± 0.001	1.474 ± 0.021	Mixed proteases (MP)	1.664 ± 0.006
	Amylases			**Amylases**	
1.081 ± 0.006	6. Fungamyl	1.140 ± 0.025	1.479 ± 0.012	Fungamyl	1.558 ± 0. 013
1.089 ± 0.013	7. AMG	1.131 ± 0.011	1.476 ± 0.014	AMG	1.550 ± 0.000
1.083 ± 0.007	8. BAN	1.127 ± 0.003	1.477 ± 0.006	BAN	1.517 ± 0.004
1.072 ± 0.007	9. Mixed amylases (MA)	1.143 ± 0.004	1.467 ± 0.016	Mixed amylases (MA)	1.547 ± 0.003
1.085 ± 0.0	10. Mixed proteases and amylases (MPA)	1.169 ± 0.009	1.462 ± 0.006	Mixed proteases and amylases (MPA)	1.578 ± 0.033



(A) (B)

(C) (D)

Fig. 4.4.1 Cell density of (A, B) *Pseudomonas fluorescens* and (C, D) mixed bacterial species treated at (A, C) 1 U/ml and (B, D) 2 U/ml. Each number represents activity of each enzyme: **1.** Savinase **2.** Everlase **3.** Esperase **4.** Polarzyme **5.** Fungamyl **6.** AMG **7.** BAN **8.** Mixed protease enzymes **9.** Mixed amylase enzymes **10.** Mixed protease and amylase enzymes. White graphs show cell density before enzymatic treatment and black graphs show cell density after enzymatic treatment. Bars indicate standard errors.

Proteolytic and amylolytic enzymes were tested for removal of *Pseudomonas fluorescens* and mixed bacterial species cells attached on the glass wool. In the results, the optical density of the mixed bacterial species was higher than the optical density of *Pseudomonas fluorescens*. The density of the non treated cells was lower than the density of the cells after enzymatic treatments (Table 4.4.1; 4.4.2; Fig 4.4.1). The density of *Pseudomonas fluorescens* and mixed bacterial species increased at 2 U/ml. On the contrary, the density of *Pseudomonas fluorescens* and mixed bacterial species after treated with Polarzyme at 1 and 2 U/ml was lower than the density of the non treated cells (Table 4.4.1; 4.4.2; Fig 4.4.1).

The cell density of *Pseudomonas fluorescens* and mixed bacterial species before Polarzyme treatment was OD 1.005 and 1.476, respectively and after treatment at 1 U/ml, the cell density decreased to OD 0.938 and 1.456. When *Pseudomonas fluorescens* and mixed bacterial species cells were treated at 2 U/ml with the same enzymes (Polarzyme), cell density decreased from 1.089 and 1.452 to OD 0.995 and 1.384 respectively (Table 4.4.1; 4.4.2; Fig 4.4.1).

Other enzymes tested individually and in combination resulted in an increase in cell density after treatment. Savinase and Everlase treatment at 1 U/ml increased the cell density of *Pseudomonas fluorescens* to OD 1.162 and 1.154 respectively, while the density of the non treated cells were OD 1.091 and 1.083. When Savinase and Everlase were used for the treatment of mixed bacterial species at 1 U/ml, the density of the cells increased to OD 1.561 and 1.553 respectively, and the density of the non treated cells was OD 1.447 and 1.487 (Table 4.4.1; 4.4.2; Fig 4.4.1). When Esperase was tested for removal of *Pseudomonas fluorescens* cells at 1 and 2 U/ml, the cell density increased to OD 1.120 and 1.163 respectively while the density of the non treated *Pseudomonas fluorescens* cells was OD 1.085 (Table 4.4.1; 4.4.2; Fig 4.4.1).

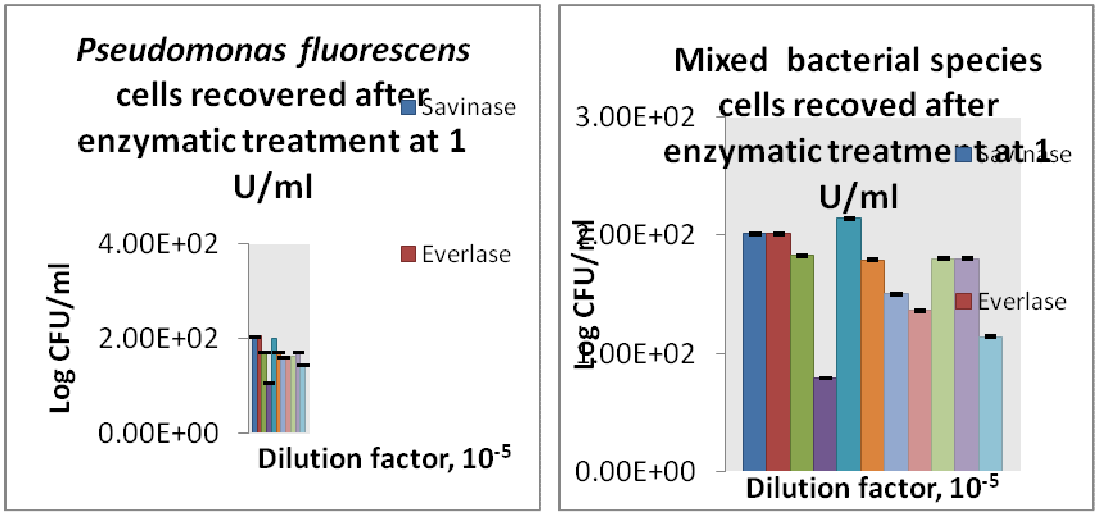
Combination of protease enzymes was assessed. In the results found, the cell density of the non treated *Pseudomonas fluorescens* and mixed bacterial species was OD 1.015 and 1.468, respectively. After treatment at 1 U/ml, the cell density increased to OD 1.121 and 1.583 (Table 4.4.1; Fig 4.4.1). When this enzymatic mixture was tested at 2 U/ml for

removal of *Pseudomonas fluorescens* and mixed bacterial species cells, the cell density further increased to OD 1.180 and 1.664, respectively from OD 1.094 and (Table 4.4.2; Fig 4.4.1).

The density of the non treated *Pseudomonas fluorescens* and mixed bacterial species cells was OD 1.043 and 1.463 respectively. The cell density of *Pseudomonas fluorescens* slightly increased to OD 1.070 while the density of mixed bacterial species cells highly increased to 1.528 after Fungamyl treatment at 1 U/ml (Table 4.4.1; Fig 4.4.1). At 2 U/ml, the density of *Pseudomonas fluorescens* and mixed bacterial species cells was OD 1.140 and 1.558 respectively (Table 4.4.2; Fig 4.4.1).

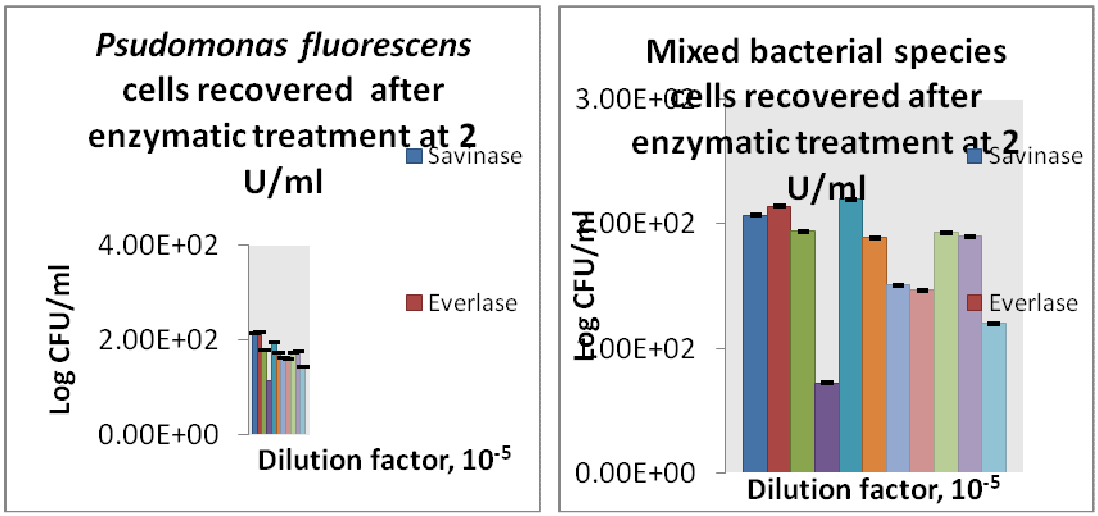
When *Pseudomonas fluorescens* cells were treated with AMG at 1 and 2 U/ml, the cell density slightly increased to OD 1.099 and 1.131 respectively from OD 1.073 and 1.089. On the other hand, the cell density of mixed bacterial species at 1 and 2 U/ml highly increased to OD 1.490 and 1.550 respectively from the density of OD 1.469 and 1.476 (Table 4.4.1; 4.4.2; Fig 4.4.1). The density of *Pseudomonas fluorescens* and mixed bacterial species cells treated with BAN at 1 U/ml was OD 1.026 and 1.473 respectively, and the density of the non treated cells was OD 1.013 and 1.462. At 2 U/ml, the density of *Pseudomonas fluorescens* and mixed bacterial species cells increased to OD 1.127 and 1.517 (Table 4.4.2; Fig 4.4.1).

Combination of amylase enzymes was assessed on *Pseudomonas fluorescens* and mixed bacterial species cells. The cell density of mixed bacterial species treated at 1 and 2 U/ml slightly increased to OD 1.494 and 1.547 respectively and before treatment the density was OD 1.470 and 1.467. On the other hand, the cell density of *Pseudomonas fluorescens* before treatment at 1 and 2 U/ml was OD 1.062 and 1.072 and slightly increased to OD 1.104 and 1.143 after treatment (Table 4.4.1; 4.4.2; Fig 4.4.1). Combination of protease and amylase enzymes was also assessed for removal of *Pseudomonas fluorescens* and mixed bacterial species. An in increased in cell density of *Pseudomonas fluorescens* was observed after treatment at 1 and 2 U/ml (OD 1.106 and 1.169, respectively). The density of mixed bacterial species also increased after treatment at 1 and 2 U/ml (OD 1.509 and 1.578, respectively) while the density of the non treated cells was OD 1.470 and 1.462.



(A) (B)

Fig 4.4.2 (A) *Pseudomonas fluorescens* and (B) mixed bacterial species cells recovered after enzymatic treatment at 1 U/ml. Bars indicates standard errors.



(C) (D)

Fig 4.4.3 (A) *Pseudomonas fluorescens* and (B) mixed bacterial species cells recovered after enzymatic treatment at 2 U/ml. Bars indicates standard errors.

In the results, the number of cells recovered after enzymatic treatments was more than the number of cells without treatment as determined by the plate count assay (Fig 4.4.3). However, cells that recovered after Polarzyme treatment were fewer than the non treated cells (Fig 4.4.3). *Pseudomonas fluorescens* cells treated with Savinase and Everlase at 1 U/ml had the highest number of cells removed from the glass wool (log 2.3 CFU). On the other hand, *Pseudomonas fluorescens* cells recovered after treated with mixed proteases at 1 and 2 U/ml slightly reduced to log 1.8 CFU cells. Conversely, mixed proteases had the highest number of mixed bacterial species cells recovered at 1 and 2 U/ml. Log 2.3 CFU cells were recovered after treatment (Fig 4.2; 4.3). Number of *Pseudomonas fluorescens* cells recovered after Esperase treatment at 1 and 2 U/ml was log 1.8 CFU. The number of mixed species cells recovered after Esperase treatment at 1 and 2 U/ml slightly increased to log 1.9 CFU (Fig 4.2; 4.3).

Pseudomonas fluorescens cells recovered after AMG, BAN and mixed amylases was comparable. Log 1.6 CFU cells recovered after treatment (Fig 4.2; 4.3). However, *Pseudomonas fluorescens* cells recovered after mixed amylases treatment at 2 U/ml was log 1.8 CFU and was comparable to cells recovered after mixed protease and amylase treatment at 2 U/ml (Fig 4.2; 4.3). On the other hand, mixed bacterial species cells recovered after treatment with mixed amaylases at 1 and 2 U/ml was log 1.8 CFU and was comparable to cells recovered after mixed protease and amylase treatment at 1 and 2 U/ml (Fig 4.2; 4.3).

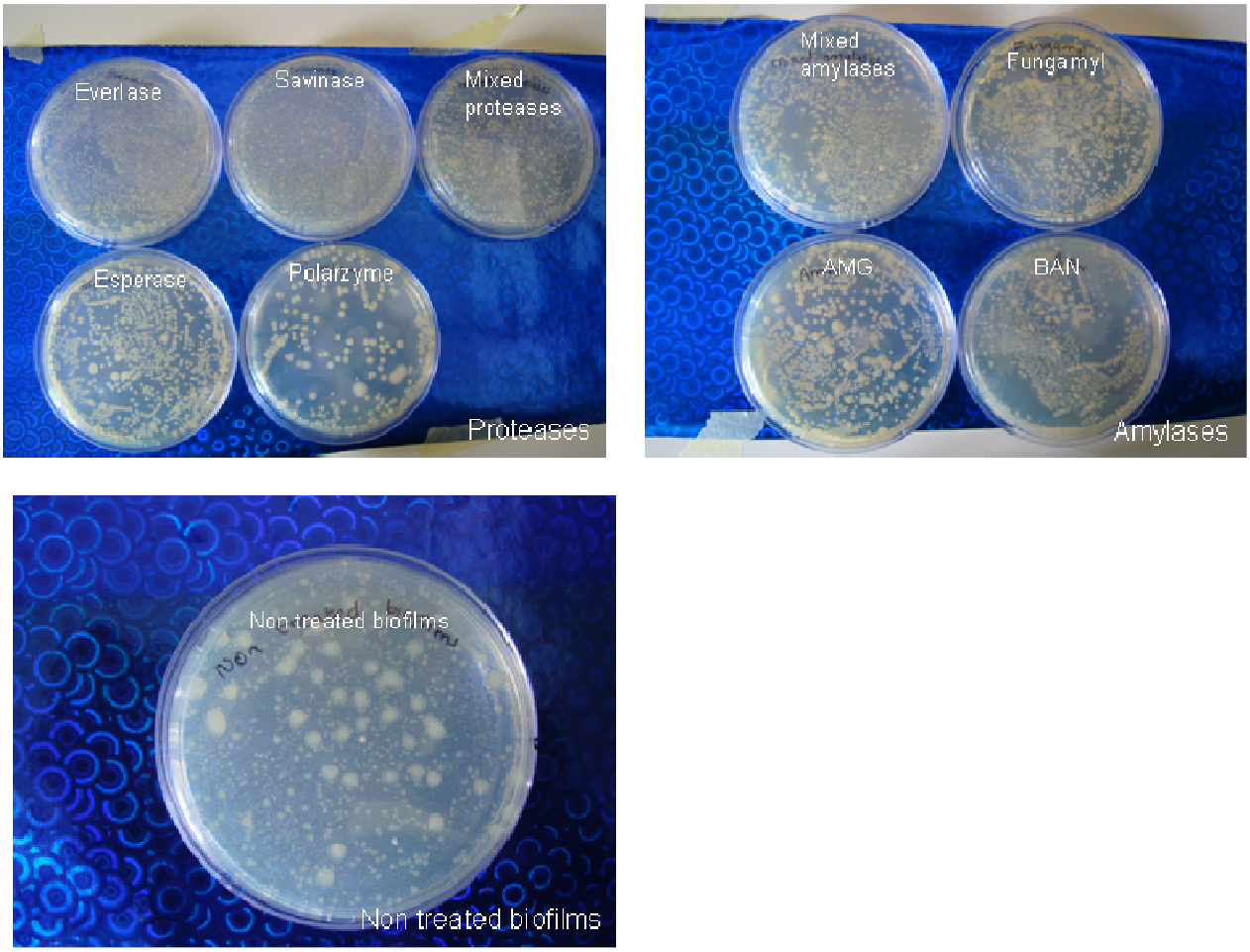


Fig. 4.4.4 Photographs showing cells recovered after enzymatic treatment.

4.5 Discussion

Eradication of biofilm cells is a challenging and a demanding task due to different in the quality and quantity of the EPS produce by different biofilms. Therefore, the main challenge is to destroy the EPS.

In this study, proteolytic and amylolytic enzymes were tested for the removal of *Pseudomonas fluorescens* and mixed bacterial species biofilms. This is because proteins and polysaccharides are the main structural components of the EPS matrix. In the results, the cell density of non treated mixed bacterial species and *Pseudomonas fluorescens* was lower than the density of the treated mixed bacterial species and *Pseudomonas fluorescens* biofilms (Table 4.2; 4.3).

It was observed that the enzymes tested showed different in activity for the biofilm removal. This variation in activity indicates that enzymes are dependent on the structural compositions of the EPS. In addition, the good or poor efficacy determines the strength and the mode of action of the enzymes and the biofilm EPS. Activity of the enzymes was concentration dependent. Activity was higher at 2 U/ml than at 1 U/ml. On the contrary, Polarzyme was not effective at both test concentrations for the degradation of the EPS and biofilm removal. The inefficiency of Polarzyme for poor biofilm removal may be due to poor in the mode of action between this enzyme and the EPS. Another reason that could have resulted in poor efficiency of Polarzyme resulted from the structural heterogeneity of the EPS.

On the other hand, the cell density of *Pseudomonas fluorescens* after treatment with Savinase, Everlase and Esperase was much higher and a large number of *Pseudomonas fluorescens* cells recovered after treatment. Leroy *et al.* (2008) found Savinase to be more effective for the reduction of *Pseudoalteromonas sp*. D41 biofilm cells attached on the surface reaching 50% cell inhibition at the lowest concentration. Ledder *et al*. (2009) also found protease enzyme to be effective for the removal of dental plaque biofilms formed by *P. gingivalis*, *S. oralis*, *A. naeslundii*. In addition, Walker *et al.* (2007) found an increase in released cell number after treatment with single type protease enzyme.

The cell density of the mixed bacterial species was higher than the cell density of *Pseudomonas fluorescens* after treatment with protease enzymes in combination. A combination of protease enzymes seemed more effective for the degradation of mixed bacterial species biofilm EPS than the EPS of *Pseudomonas fluorescens*. It was expected that this enzyme mixture would be more effective for the removal of *P. fluorescens* biofilm than mixed bacterial species biofilm since the structural components of the EPS produced by mixed bacterial species would be more complex than that of *Pseudomonas fluorescens* and might limit the activity of the enzymes

A combination of protease and amylase enzymes was effective for the degradation of *Pseudomonas fluorescens* and mixed bacterial species biofilms EPS. It was expected that these enzyme mixtures would be more effective for the removal of *P. fluorescens* biofilm than mixed bacterial species biofilms since the structural components of the EPS produced by mixed bacterial species would be more complex than that of *Pseudomonas fluorescens* and might limit the activity of the enzymes (Table 4.4.1; 4.4.2). Walker *et al.* (2007) found enzyme treatment containing a mixture of enzymes (α- amylase, β-glucuronidase, glucose oxidase, protease and dextranase) to be effective for reduction in the number of microbial biofilm cells. Hence, an increase in released cell number was also observed with the single type enzyme treatments. Another study by Ledder *et al.* (2009) found combination of amylase, lipase and protease enzymes to be effective in reducing coaggregation formed by different bacterial species from Gram negative and Gram positive.

The results of the present study showed that activity of the enzymes for biofilm removal varied depending on the type structural components of the biofilm EPS. In addition several factors may attribute to the activity of the enzymes including: the type of microorganisms within a biofilm; the mode of action between the enzyme and the biofilm, specificity of the enzyme to the structural components of the EPS; activity of the enzyme is concentration – dependent and the formulation of the enzymes.

4.6 Conclusion

All enzymes (except for Polarzyme) tested in this study were effective for the degradation of *Pseudomonas fluorescens* and mixed bacterial species EPS although activity amongst the enzymes varied. The protease enzymes were most effective for biofilm removal than the amylase enzymes. Savinase, Everlase, Esperase and Fungamyl tested individually were most effective for the control of *P.fluorescens* biofilm while enzymatic mixtures of protease enzymes and protease and amylase enzymes were more effective for removal of mixed bacterial species biofilm. The effects of the enzymes for biofilm removal depended on the concentration tested. The cell density was higher at 2 U/ml and lower at 1 U/ml. The results of this study showed that enzymes differed in terms of activity for biofilm

removal depending on the EPS of the biofilms. Therefore, in order to design enzymes which target the EPS of the biofilms, it is important to have an understanding of the structural components of the EPS. In addition, the mode of action of the enzymes and their formulation will determine poor or good efficacy.

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Chapter 5

The chemical composition of EPS in *Pseudomonas fluorescens* and mixed bacterial species biofilms and application of enzymes for EPS degradation

5.1 Abstract

The structural compositions of the EPS differ from biofilms depending on the microorganisms within the biofilm. Several factors may attribute (nutrients, flow velocity, quorum sensing) to the structural compositions of the EPS. The objectives of this study were to: (1) determine the influence of nutrients on the production of EPS in biofilms grown in nutrient rich medium and nutrient limited medium conditions; (2) to extract EPS and determine protein and carbohydrate concentrations and (3) to test the efficiency of protease and amylase enzymes for the degradation of the EPS and biofilm removal. Viable cell counts showed that the number of biofilm cells grown in enriched medium (fed biofilms) was slightly higher than the number of unfed biofilm cells. The EPS, protein and carbohydrate concentrations of the fed biofilms were higher than that of the unfed biofilms. Enzyme assay results showed that the protease enzymes tested individually and in combination were most effective for the degradation of the biofilm EPS. The protease Polarzyme was not effective for the degradation of the EPS of the test biofilms. Savinase, Esperase and Everlase tested individually were most effective for the degradation of *Pseudomonas fluorescen*s biofilms while a combination of protease enzymes was effective for the degradation of mixed bacterial species biofilm EPS. On the other hand, the amylase enzymes tested individually and in combination were less effective for the degradation of the biofilm EPS. However, when the amylases were tested in combination with the protease enzymes, the efficacy improved. These results showed that enzyme efficacy was dependent on the type and nature of the EPS produced by the biofilms. It is therefore important that the nature of the EPS should be known before selecting the relevant enzymatic mixture for biofilm removal.

Keywords: *Pseudomonas fluorescens*, protease, amylase, EPS, biofilms, degradation.

5.2 Introduction

Flemming *et al.* (2007) describes biofilms as a "city of microbes" and extracellular polymeric substances (EPS) as the "house of the biofilms. Biofilms are characterized by surface attachment, structural heterogeneity; genetic diversity; complex community interactions and an extracellular matrix of polymeric substances (Glaouris, *et al.,* 2006; Or *et al.,* 2007; Ploux *et al*., 2007). Biofilms deposit and adhere to all surfaces that are immersed in aqueous environments (Gomez-Suarez *et al.,* 2002, de Carvalho, 2007). Biofilm- associated EPS is distinct, both chemically and physically from the bacterial capsule (Prakash *et al.,* 2003; Lequette *et al.,* 2010). EPS is highly hydrated and consists of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid and humic substances (Frøelund *et al.,* 1995; Flemming 1998; Chen *et al.,* 2007).

The difference in the quantity of biofilm EPS is as a result of the growing conditions of the biofilms (O' Toole *et al.,* 2000). The architectural structure of the EPS is complex (Flemming *et al.,* 1998) with channels allowing the inflow of water, oxygen and nutrients and out flow of byproducts (Zhang *et al.,* 2001; Arevalo-Ferro *et al.,* 2005; Donlan, 2008).

EPS serves many functions including: facilitation of the initial attachment of bacterial cells to a surface (Stoodley *et al.,* 2002); formation and maintenance of the micro colony (Flemming *et al.,* 1998, Hallam *et al.,* 2001); enables the bacteria to capture nutrients (Gomez-Suarez *et al.,* 2002) causes biofouling (Cowan *et al.,* 1991; Cloete *et al.,* 1998); facilitates cell-cell communication (Zhang *et al.,* 2001) and enhances bacterial resistance antimicrobial agents (Wood *et al.,* 1996; Parkar *et al.,* 2000; Meyer, 2003; Prakash *et al.,* 2003; Pozos and Pater, 2007; Lequette *et al.,* 2010). EPS also function as a stabilizer of the biofilm structure and as a barrier against hostile environments (Zhang *et al.,* 2001; Arevalo-Ferro *et al.,* 2005; Lapidot *et al.,* 2006; Ploux *et al.,* 2007; Donlan, 2008;).

The production of the EPS is influenced by internal and external factors including: quorum sensing (cell to cell communication); surface topography, hydrodynamic shear forces; fluid velocity and nutrient availability (Cloete, 1998; Cloete, 2003; Sreenivasan *et al.,* 2005). EPS is a complex structure made up of different components including

carbohydrates, proteins, lipids and nucleic acid (Flemming 1998, Allison *et al.,* 2000; Liu *et al.,* 2003).

Enzymes have been used and proven to be effective for the degradation of the multistructural EPS of the biofilms (Johansen *et al.,* 1997; Melo *et al.,* 1997; Bockelmann *et al,* 2003; Lequette *et al.,* 2010). The mode in which enzymes destroy the EPS is by degrading the physical integrity of the EPS (Xavier *et al.,* 2005).

Several studies on the application of enzymes have been made and enzymes were effective for biofilm removal whether tested individually or in combination (Johansen *et al.,* 1997; Melo *et al.,* 1997; Leroy *et al.,* 2007; Walker *et al.,* 2007). Leroy *et al.* (2007) found the protease and Savinase to be more effective for the prevention of adhesion and detachment of a *Pseudoalteromonas* sp. D14 biofilm than xylanase, amylase, cellulase and lipase. Ledder *et al.* (2009) also found the activity of the protease enzymes for the removal of *A. naeslundii* and *F. nucleatum* to be effective but neither the amylase nor the lipase enzyme tested was effective for the removal of four human oral bacterial biofilms.

A combination of amylase, lipase and protease enzymes was tested and found to be effective in reducing biofilm formation by different bacterial species of *A. naeslundii*, *N. subflava*, *L. rhamnosus*, *P. gingivalis*, *S. oralis*, *S. mutans*, *V. dispar* and *S. sanguinis* (Ledder *et al.* (2009). Walker *et al.* (2007) showed that a mixture of enzymes (proteases and alpha amylases) was effective for reducing the number of viable cells on stainless steel coupons. Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and alpha - amylase and beta – glucanase. This enzymatic mixture was effective in digesting slime layers produced by cultures of pure (*Pseudomonas species*) and mixed strains of bacteria. Among 24 preparations of the enzymes, only three types of enzymes were found to be effective for the removal of slime produced by *Pseudomonas* bacteria which were alpha-amylase, protease and the combination of amylase, glucanase and protease (Wiatr, 1991). Cellulose from *Penicillium funiculusum* was tested for the removal of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* biofilms and was found to be effective in degrading the EPS of the biofilms (Loiselle *et al.,* 2003; Vickery *et al.,* 2004). Walker *et*

al. (2007) indicated that in order to design enzymes that target the EPS of the biofilms, it is important to have an understanding of the nature and composition of the EPS.

In this study, the main objectives were to:

- To determine the effects of nutrients on biofilm growth (fed and unfed biofilms)
- To evaluate the amount of the EPS produced in fed and unfed biofilms
- To determine the protein and carbohydrate concentration of the EPS
- To test the efficiency of protease and amylase enzymes for the degradation of the biofilm EPS

5.3 Materials and methods

5.3.1 Bacterial inoculum used for biofilm growth

Growth of biofilms was adapted from the method of Rochex and Lebeault (2007) with modification. *Pseudomonas fluorescens* was used to grow a mono species biofilm. Mixed bacterial species biofilm was grown from *Pantoea ananatis*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas putida*, *Staphylococcus aeureus*, and *Staphylococcus xylosus*. Bacterial cultures were grown in Nutrient Broth for 24h at 26^{O}C and 30^{O}C respectively for *Pseudomonas fluorescens* and mixed bacterial species with agitation at 100rpm. The bacterial suspensions were adjusted to standard 1 McFarland. Standardised suspensions (100µl) were inoculated in sterile flasks containing 100X diluted Nutrient Broth (100ml) and 1g of sterile glass wool and incubated at 26^{O}C and 30^{O}C respectively for 7d in a shake incubator at a controlled speed of 100rpm. During the incubation period, one set of samples was daily fed with 2ml of the diluted medium and the control samples were unfed. Bacterial growth was monitored daily by measuring the optical density at 620nm.

5.3.2 Quantitative determination of viable cells

Ten fold series of dilutions were made by inoculating 100µl of the bacterial suspensions to 900µl of Ringer's solutions and mix. The aliquots (0.1ml) were spread onto sterile Nutrient agar plates (Merck) and incubated for 24- 48h at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species respectively (3 plates for each dilution). Viable cells were enumerated and expressed as Colony Forming Units (CFU/ml).

5.3.3 Extraction of extra cellular polymeric substances (EPS)

Flasks containing glass wool with attached biofilms were vortexed vigorously for 5 min to detach the cells. Vortexed aliquots (20ml) were added to 50ml sterile centrifuge tubes (Merck). The contents were homogenized for about 30s using a Cole-Parmer homogenizer at adjusted output of 50% and spun at 3500xg for 5 min at 4^{O}C. The supernatants were transferred to sterile centrifuge tubes and further spun at 9000xg for 30 min; 4^{O}C. Pellets were resuspended in 20ml sterile distilled water, freeze dried and dissolved in Phosphate buffer, pH 8.3 for mixed proteases and pH 7.0 for mixed amylases and pH 7.0 for mixed proteases and amylases for enzyme assays. Dissolved pellets were filtered through 0.8/0.2µm filters (Acrodics PF, PA//Inc). Filtrates were assayed for EPS composition and enzyme activity.

5.3.4 Determination of the carbohydrate concentration in the EPS

The carbohydrate concentrations were determined according to Gaudy's method (1962). Briefly, pellets dissolved in Phosphate buffer (1ml) was added to 10ml sterile test tubes. Freshly prepared Anthrone solution (1ml) was added in each test tube. The mixture was incubated in a water bath at 95^{O}C for 15 min. After incubation, the mixture was allowed to cool to room temperature. Cooled aliquots (200µl) were transferred to micro plate wells (Lasec, SA) and read at 620nm using a plate reader (Multiskan Ascent V1.24, Amersham). Glucose was used as a standard to construct a standard curve.

5.3.5 Determination of the protein concentration in the EPS

Protein concentrations were determined by the modified method of Lowry (Froelund *et al.,* 1996). Dissolved extra cellular polymeric substances (EPS) (10µl) were inoculated into wells of a micro titter plate. Control wells were inoculated with phosphate buffer. Comassie plus reagent (300µl) was added to each well. The plate was incubated at room temperature for 10 min. After incubation, absorbances were read at 595nm using a Multiskan Ascent V1.24 plate reader, (Amersham). Bovine serum albumin (BSA) was used as a standard to construct a standard curve.

5.3.6 Degradation of biofilm EPS

After protein and carbohydrate analysis, 1ml of suspended EPS was added into 50ml centrifuge tubes containing the enzymes diluted in specific buffer solutions (Refer to chapter 4). The samples were incubated at 26^{O}C and 30^{O}C for *Pseudomonas fluorescens* and mixed bacterial species respectively and aliquots were taken at 15 min intervals. For the protease activity, 300µl of sample was transferred to micro plates and analyzed *via* the Bradford assay, while the amylase activity was analyzed using the Anthrone assay.

5.3.8 Testing of enzymes for the removal of biofilm cells on the glass wool

Enzymes tested individually and in combination for the removal and degradation of the extracellular polymeric substances (EPS) was assessed. To ensure activity of the enzymatic mixture, the mixtures were first tested for the degradation of Bovine serum albumin and glucose. Glass wool – attached biofilms from fed and unfed cultures were incubated with enzymatic mixtures at 30^{O}C for mixed bacterial biofilms (*Gram negative and positive*) and at 26^{O}C for *Pseudomonas fluorescens* biofilms for 24h without agitation. A sample with no enzyme solution was used as control. After the incubation period, effects of enzyme in combination on *Pseudomonas fluorescens* and mixed bacterial biofilms were evaluated using Scanning Electron Microscopy (SEM).

5.3.9 Sample preparation for Scanning Electron Microscopy

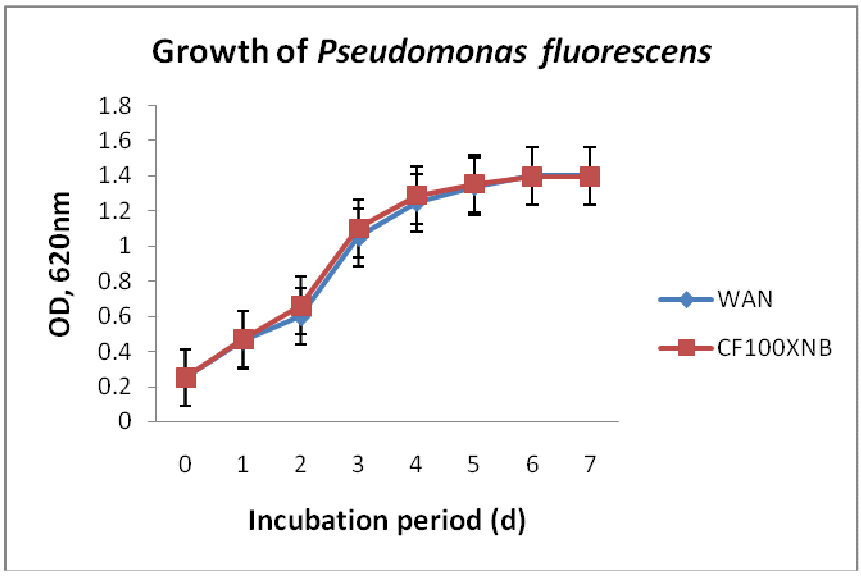
Glass wool samples were fixed in a solution of 2.5% glutaraldehyde in 75mM Phosphate buffer, pH 7.4 for 1h. Samples were rinsed three times for 15 min at a time in 50% 75mM phosphate buffer. After the rinsing step, samples were dehydrated in ethanol at concentrations of 50, 70, 90 and three times 100% each for 15 min respectively. After the drying step samples were critically dried with CO_2 (Martin *et al.,* 2006). Samples were coated with gold and visualized using a Scanning Electron Microscope (JSM-840, JEOL, TOKYO Japan).

Table 5.2.1 Enzymes used for biofilm removal

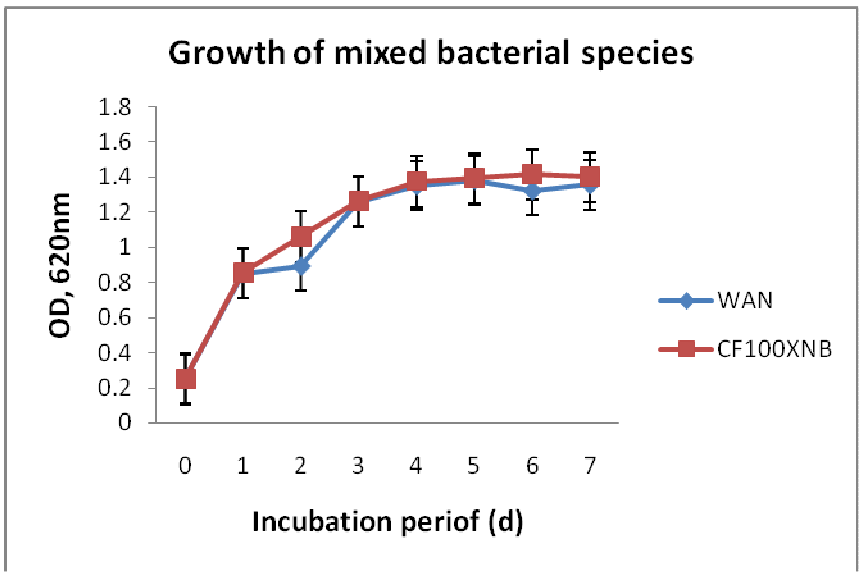
Single enzymes		**Mixed enzymes**
Proteases	**Amylases**	**Proteases**
Savinase 16L Type EX	Amyloglucosidase (AMG) 300L	Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX
Everlase 16L Type EX	Bacterial Amylase Novo (BAN) 240L	**Amylases**
Esperase 16L Type EX	Fungamyl 800 L	AMG 300L, BAN 240L, Fungamyl 800 L
Polarzyme 6.0T		**Mixed protease and amylase enzymes**
		Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX AMG 300L, BAN 240L, Fungamyl 800 L

5.4 Results

Pseudomonas fluorescens and mixed bacterial biofilms in nutrient rich and nutrient poor medium



(A)



(B)

Fig. 5.4.1 Biofilm growth of (A) *Pseudononas fluorescens* and (B) mixed bacterial species. Bars indicate standard errors. CF100XNB – fed growth; WAN – unfed growth

Table 5.4.1 Comparison of viable cells between fed and unfed *Pseudomonas fluorescens* biofilms

Viable biofilm cells (CFU/ml) x 10^5 (Average ± SD)	
Fed biofilms	1. 93 ± 8.485
Unfed biofilms	1.76 ± 5.657

CFU = Colony Forming Units

Table 5.4.2 Comparison of viable biofilm cells between fed and unfed mixed bacterial biofilms

Viable biofilm cells (CFU/ml) x 10^5 (Average ± SD)	
Fed biofilms	2.03 ± 4.9493
Unfed biofilms	1.87 ± 1.414

CFU = Colony Forming Units

The rate of biofilm formation was maximal after the 5th day of incubation and progressively reached a plateau phase thereafter. There was no noticeable difference in the biofilm growth of the daily fed (CF100XNB) and unfed (WAN) although the fed growth biofilm yield was slightly higher than the unfed biofilm (Fig 5.3.1). There were more viable cells of the mixed bacterial species biofilms than the cells of *Pseudomonas fluorescens* biofilms (Table 5.3.1; 5.3.2).

Extraction of EPS and determination of protein and carbohydrate concentrations

Table 5.4.3 Comparison of extracted EPS, protein and carbohydrate concentrations produced from fed and unfed *Pseudomonas fluorescens* biofilm

Fed biofilms (Av ± SD)			Unfed biofilms (Av ± SD)		
EPS mass (g)	Protein concentration (µg/ml)	Carbohydrate concentration (µg/ml)	EPS mass (g/)	Protein concentration (µg/ml)	Carbohydrate concentration (µg/ml)
0.91 (± 0.001)	1835 (± 0.008)	163 (± 0.005)	0.58 (± 0.023)	1725 (± 0.0169)	155 (± 0.001)

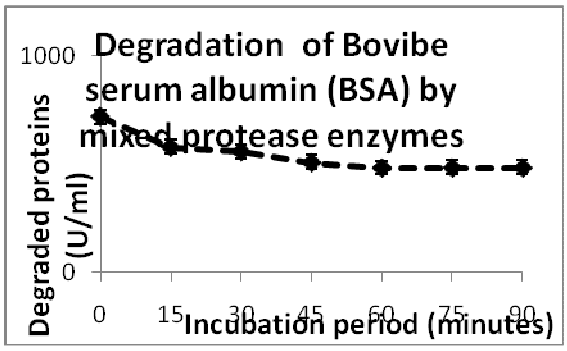
Table 5.4.4 Comparison of extracted EPS, protein and carbohydrate concentrations produced from fed and unfed mixed bacterial species biofilm

Fed biofilms (Av ± SD)			Unfed biofilms (Av ± SD)		
EPS mass (g)	Protein concentration (µg/ml)	Carbohydrate concentration (µg/ml)	EPS mass (g)	Protein concentration (µg/ml)	Carbohydrate concentration (µg/ml)
0.16 (± 0.001)	3345 (± 0.012)	182 (± 0.002)	0.12 (± 0.001)	2660 (± 0.017)	172 (± 0.002)

The EPS concentrations of the *Pseudomonas fluorescens* and mixed bacterial species biofilms that were fed daily with 2 ml of Nutrient Broth were higher than the EPS concentrations in the unfed control (Table 5.3.2; 5.3.3). The protein concentrations in both daily and unfed experiments were higher than the carbohydrate concentrations in both experiments (Table 5.3.2; 5.3.3). The protein concentrations in the EPS of *Pseudomonas fluorescens* (1835 µg/l) and mixed bacterial species fed biofilms (3345µg/l) was higher than the protein concentrations in the unfed EPS *P. fluorescens*

(1725µmg/l) and mixed bacterial species biofilms (2660 µmg/l) (Table 5.3.3; 5.3.4). Carbohydrate concentrations were higher in the daily fed *Pseudomonas fluorescens* (163 µg/ml) and mixed bacterial species biofilms (182 µg/ml) than the unfed biofilm *P.fluorescens* (155 µg/l) and mixed bacterial species EPS (172 µg/l) (Table 5.3.4; 5.3.4).

Effects of enzymes tested individually for the degradation of *Pseudomonas fluorescens* biofilm EPS



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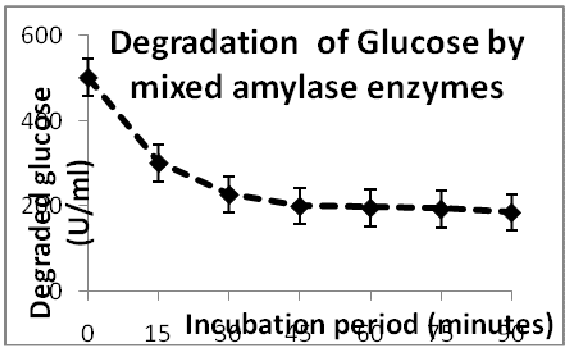

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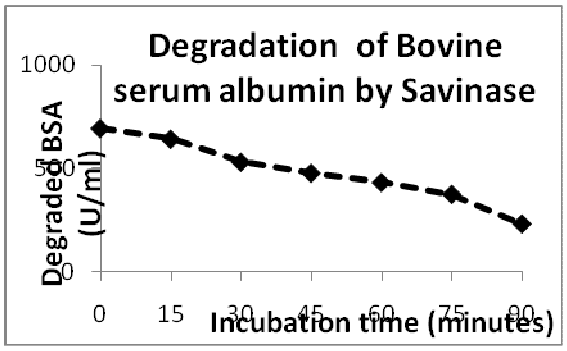

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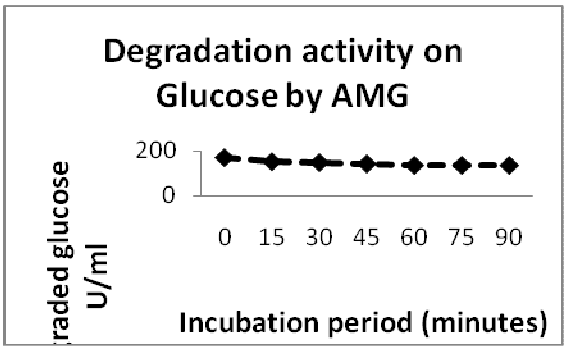

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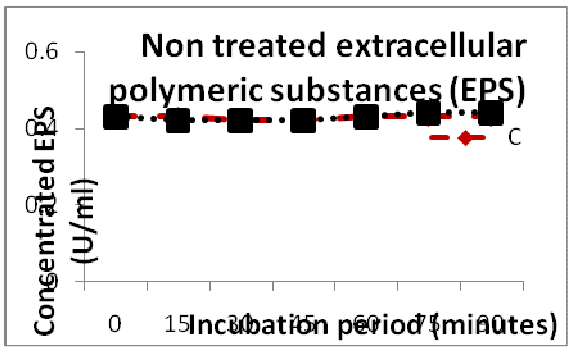
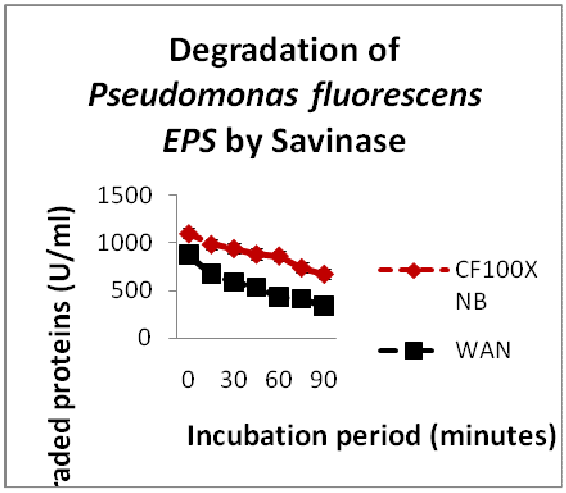
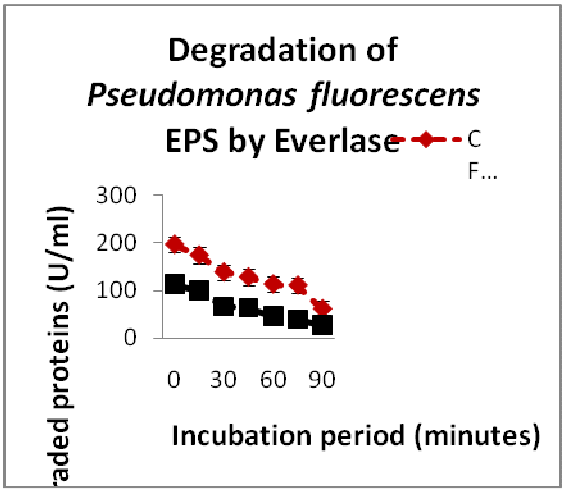


(D)

Fig. 5.4.2 Effects of enzymes on BSA and glucose (D) the non treated extracellular polymeric substance (EPS).



(A)



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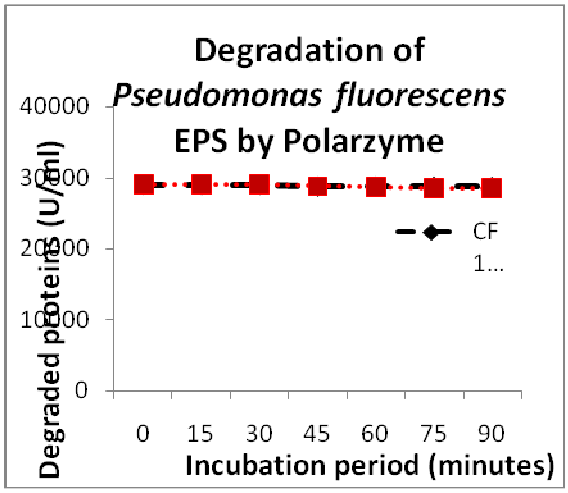
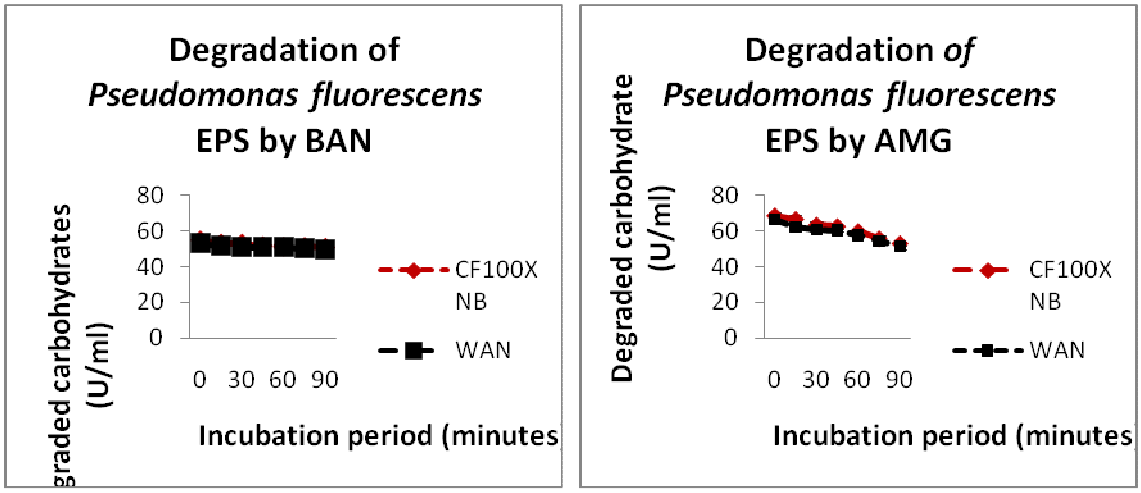


(C)

Fig. 5.4.3 Degradation activity of protease enzymes on *Pseudomonas fluorescens* biofilms EPS.



(A) (B)

Fig. 5.4.4 Degradation activity of amylase enzymes tested individually on *Pseudomonas fluorescens* biofilm EPS.

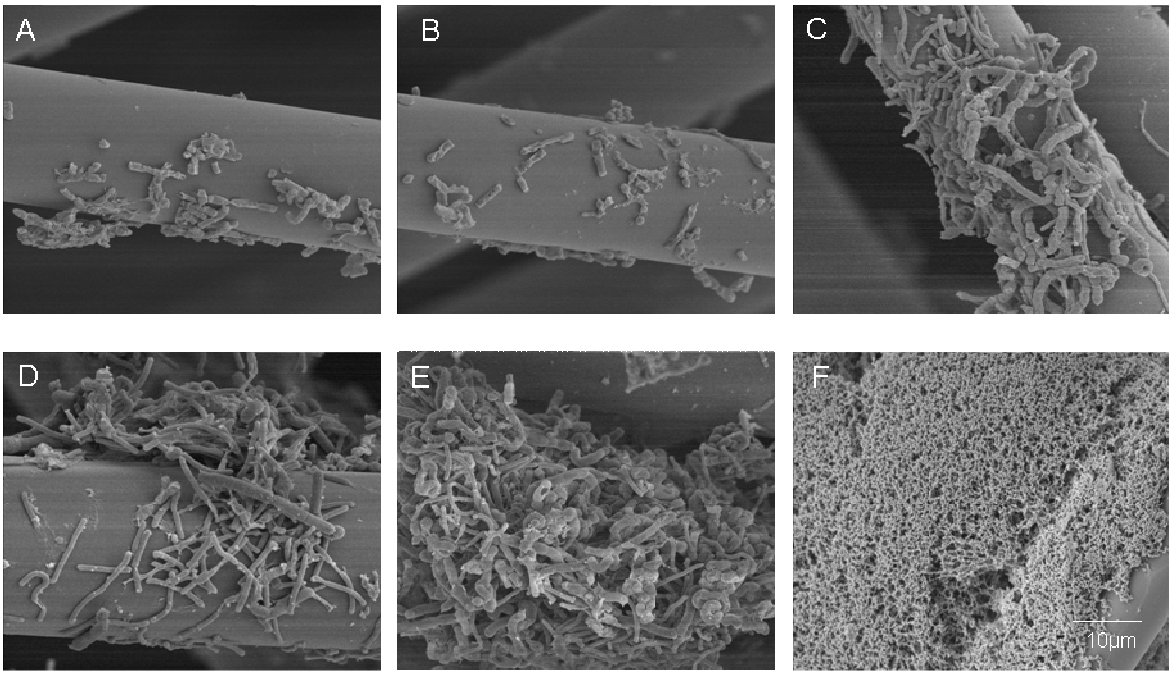
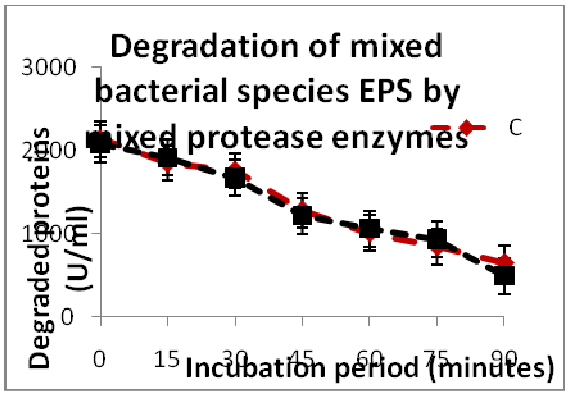


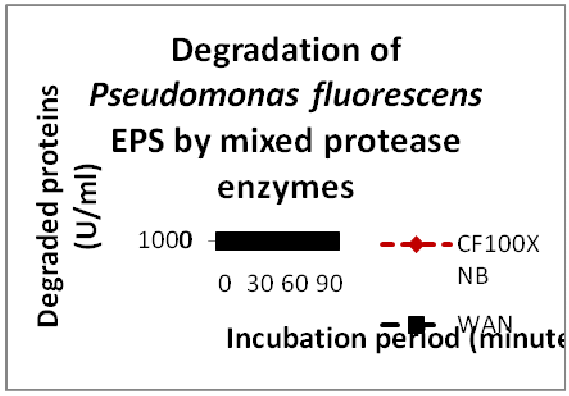
Fig. 5.4 .5 Scanning Electron Microscope analysis of the degradation activity of enzymes on extra cellular polymeric substances (EPS) of 7d old *Pseudomonas fluorecens* biofilm attached to glass wool after 24h incubation at 26^{O}C. E and F show non treated biofilm, (A) Savinase, (B) Everlase, (C) AMG and (D) BAN.

Effects of enzymes tested individually for the degradation of *Pseudomonas fluorescens* biofilm EPS

To ensure activity, the protease Savinase and the amylase AMG were first tested on BSA and glucose respectively for activity and these enzymes were effective for the degradation of the BSA and glucose (Fig. 5.3.2).The proteases Everlase and Savinase were most effective for the degradation of the EPS of *Pseudomonas fluorescens* which allowed the detachment of the cells from the glass wool (Fig. 5.3.5 A and B). The protease Polarzyme did not show any degradation activity in the protein concentrations of the EPS of *Pseudomonas fluorescens* biofilms (Fig. 5.3.3). AMG and BAN were partially effective for the degradation of the EPS (Fig. 5.3.5 C and D). Electron microscopy images revealed that the EPS of the non treated biofilms was dense covering the glass wool and were seen to be having channels (Fig. 5.3.5 E and F).



(A)



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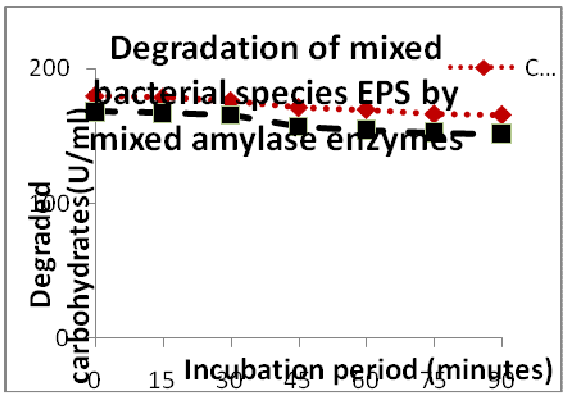

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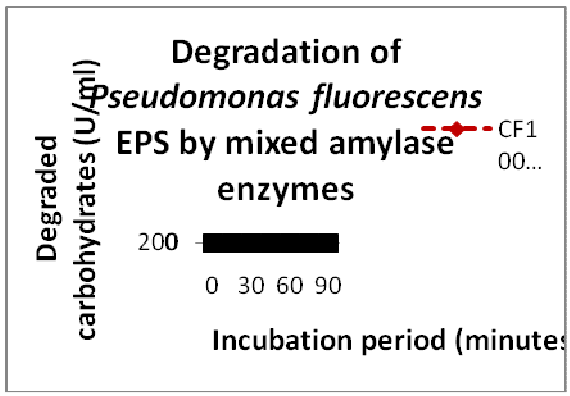

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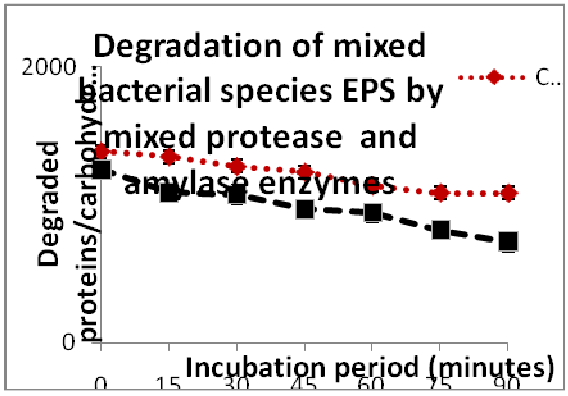

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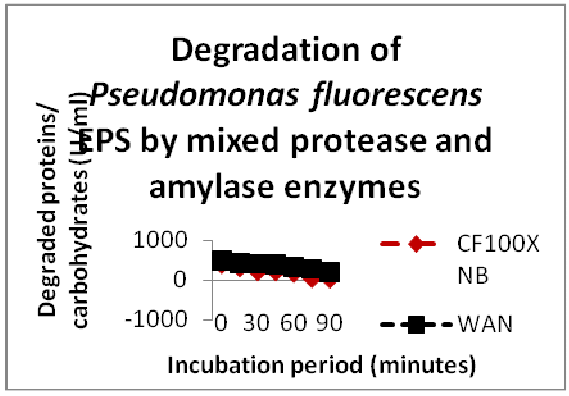

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Fig. 5.4.6 Degradation activity of mixed enzymes on *Pseudomonas fluorescens* and mixed bacterial species biofilm EPS.

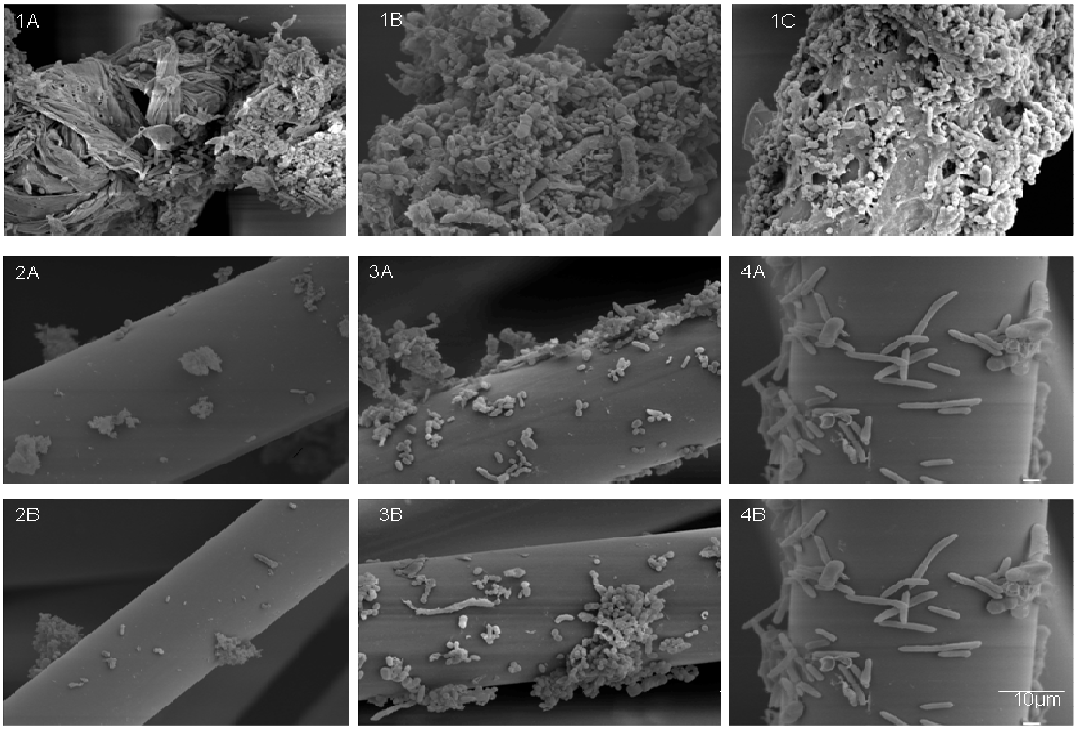

Fig. 5.4.7 Scanning Electron Microscopy analysis of the degradation activity of mixed enzymes on extra cellular polymeric substances (EPS) of 7d old *Pseudomonas fluorescens* biofilms attached on the glass wool after 24h incubation at 26^{O}C. 1 (A, B, C) shows non treated mixed bacterial species biofilms, 2 (A, B) mixed protease treated biofilms, 3 (A, B) mixed amylase treated biofilms and 4 (A, B) mixed protease and amylase treated biofilms.

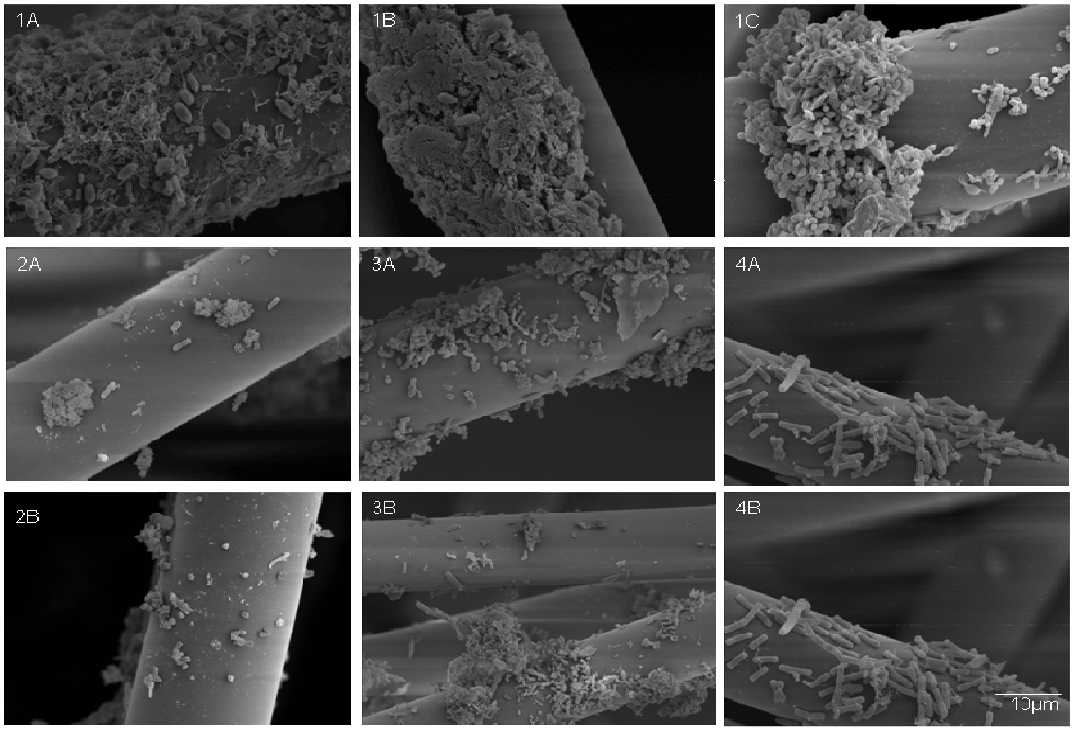

Fig. 5.4.8 Scanning Electron Microscope analysis of the degradation activity of mixed enzymes on extra cellular polymeric substances (EPS) of 7d old mixed bacterial species biofilm (*Gram negative and positive bacteria*) attached on the glass wool after 24h incubation at 30^{O}C. 1 (A, B, C) shows non treated mixed bacterial species biofilms, 2 (A, B) mixed protease treated biofilms, 3 (A, B) mixed amylase treated biofilms and 4 (A, B) mixed protease and amylase treated biofilms.

Effects of enzymes tested in combination for the degradation of biofilm EPS

Enzymes tested in combination for the removal and degradation of the extracellular polymeric substances (EPS) was assessed. All enzymatic treatments were effective in degrading the extracellular polymeric substances and removal of *Pseudomonas fluorescens* and mixed bacterial biofilms attached on the glass wool (EPS) (Fig 5.3.6; 5.3.7; 5.3.8). Protease enzymes in combination were most effective in degrading the EPS of the biofilms resulting in the reduction of a large population of the biofilm cells attached to the glass wool. Activity of protease and amylase enzymes in combination was

also effective in degrading the EPS of the biofilms (Fig. 5.3.7; 5.3.8). On the other hand, the amylase enzymes in combination was less effective for the degradation of the EPS produced by mixed species biofilms but effective in degrading the EPS of *Pseudomonas fluorescens* biofilm. The non treated biofilms were not affected and a large number of cells were still embedded within in the EPS (Fig. 5.3.7; 5.3.8).

5.5 Discussion

The effect of nutrient concentration on biofilm yield

In this study there was a slight difference in the number of viable cells grown in the fed and unfed nutrient medium conditions and there was no noticeable difference in biofilms cells grown in fed and unfed medium but there was a difference in the amount of EPS produced. The fed biofilms had more EPS than the unfed biofilms. Nutrients boosted the biofilm cells growing in rich medium which resulted in more EPS produced. It was indicated in previous studies that biofilms growing in high nutrient medium were more abundant, densely packed and thicker (Allison *et al.,* 2000; Prakash *et al.,* 2003; Rochex and Lebeault, 2007). Rochex and Lebeault (2007) showed that nutrient conditions influenced biofilm formation of bacterial strains isolated from a paper machine. Rochex and Lebeault (2007) also compared biofilms growing in two different medium concentrations and found that the biofilm mass in medium containing 0.1g/l of glucose was 90% lower than the biofilm mass in medium containing 0.5g/l of glucose.

Protein and carbohydrate concentrations in the biofilm EPS

The EPS of *Pseudomonas fluorescens* and mixed bacterial species biofilm grown in fed medium had a higher protein and carbohydrate concentration than in the unfed biofilm EPS. Protein concentrations were higher than the carbohydrate concentration in both fed and unfed biofilms. This indicated that the structural components of the biofilm EPS was dependent on the nutrient status in which the biofilm was grown. These results correspond to the work of Simoes (2003) who found more protein (total protein = *217.7 mg/g*) than carbohydrate (total carbohydrate = *63.3 mg/g*) in the EPS produced by *Pseudomonas fluorescens* biofilms under specific growth conditions. Nielsen and Jahn

(1999) also found proteins to be the largest fraction than the carbohydrates in the EPS of the biofilms. Dignac *et al.* (1998) also found that among activated sludge EPS, proteins predominated.

In some studies, it was indicated that carbohydrates are the main constituents of the EPS while some studies found proteins to dominate (Zhang *et al.,* 2001; Liu *et al.,* 2003; Orgaz *et al.,* 2006). In this study proteins were found to be dominant rather than carbohydrates. Nonetheless, the EPS components of the biofilms differ in quantity; structure or nature depending on the microorganisms within the biofilm.

The structural components of the EPS depend on the type of microorganisms within the biofilm (Ahimou *et al.,* 2007). Allison *et al.* (2000) indicated that the EPS of the biofilms is highly heterogenous even among the same bacterial species and therefore its composition and function within the biofilms will differ. O' Toole *et al.* (2000) indicated that different biofilms produce different amounts of EPS.

In addition, depending on the extraction protocols used, the EPS composition will differ (Liu *et al.,* 2003; Augustus and Ali-Vehmas, 2004). Liu *et al.* (2003) studied mixed cultures in wastewater treatment systems and found that the protein (41.3%) concentration was greater than the carbohydrate concentration (18.7%) in the methanogenic sludge when the formaldehyde–NaOH extraction method was applied. In addition, the formaldehyde–NaOH process extracted the highest concentration of EPS from all the sludges. In this study, EPS of the biofilms was extracted by centrifuging the sample at low and high speed to separate the biomass from the EPS. This method was chosen because of its higher extraction efficiency and lower cell lysis. Then, the Anthrone and Lowry assays were employed for the quantification of glucose and protein concentrations respectively in the EPS. Anthrone and Lowry assays were employed in this study for the quantification of total carbohydrate and proteins in the EPS since enzymes were tested for the degradation of a broad spectrum of carbohydrates and proteins. These assays are based on the colorimetric determination of colour development. The advantage is that these assays can also be performed in a micro plate

format and can be performed at room temperature. In addition standard curves can be constructed to convert the absorbencies into concentrations.

The use of protease and amylase enzymes for the degradation of biofilm EPS

Many antimicrobial agents fail to penetrate the biofilm due to the EPS which acts as a barrier protecting the bacterial cells within. The alternative will be the use of compounds which can degrade the EPS of the biofilm (Loiselle *et al.,* 2003; Walker *et al,* 2007). Enzymes have been proven to be effective for the degradation of the EPS of the biofilms (Johansen *et al.,* 1997; Melo *et al.,* 1997; Lequette *et al.,* 2010). Enzymes remove biofilms directly by destroying the physical integrity of the EPS (Liu *et al.,* 2004; Xavier *et al.,* 2005). The mechanism in which enzymes destroy the physical integrity of the EPS is through weakening the proteins, carbohydrate and lipid components making up the structures of the EPS through the degradation process. For efficient removal of biofilm, it is therefore important that the structural components of the EPS should be known before application of the relevant enzymes.

In the present study, enzymes were tested for the eradication of *Pseudomonas fluorescens* and mixed bacterial species biofilms. Enzymes were tested individually and in combination. All enzymes tested mostly the protease enzymes tested individually and in combination, except for the protease Polarzyme were effective for the degradation of the biofilm EPS. The reason for the inefficiency of Polarzyme may be due to its incompatibility with the specific protein structural components of the biofilm EPS tested in this study. The manner in which the protease enzymes degrade the proteins in the EPS is through binding and hydrolysis of the protein molecules and converting them into smaller units that can be transported through the cell membranes and then be metabolized (Laspidou and Rittmann, 2002; Czaczyk *et al.,* 2007). The mode of enzymatic action will therefore depend on the specific protein structure and this in turn will determine its efficacy.

The multi structural components of the EPS may be derived from proteins, glycoproteins, nucleic acid, glycolipid, phospholipids including humic substances which are non cellular substances (Liu *et al.,* 2004). The efficiency of the proteases may therefore be due to their broad spectrum activity in degrading a variety of proteins acting partly as the multi structural components of *Pseudomonas fluorescens* and mixed bacterial species biofilm EPS. Extracellularly secreted proteins are substances with molecular weight between 10kDa and 200kDa. These compounds contain 40 – 60 % of hydrophobic amino acids. It was observed that the extra cellular proteins synthesized by *Sulfolobus acidocalcidarius* are composed mostly of amino acid with hydroxyl group. However, the *Bacillus subtilis* extracellular protein layer is a composition of L and D glutaminosyl residues (Czaczyk and Myszka, 2007). According to Ton-That and Schneewind (2004) the ratio of glutaminosyl isomers in *Bacillus subtilis* extracellular protein layer changed significantly in oxygen limited conditions.

Leroy *et al.* (2007) also found the protease, Savinase to be more effective for the prevention of adhesion and detachment of a *Pseudoalteromonas* sp. D14 biofilm than xylanase, amylase, cellulase and lipase. Ledder *et al.* (2009) also found protease to be effective for the removal of *A. naeslundii* and *F. nucleatum* biofilm.

Donlan (2002) indicated that EPS may be hydrophilic or hydrophobic depending on the structural components making up such EPS and the environmental conditions were the biofilms are developing. Studies have indicated that among one bacterial species EPS components may differ (Czaczyk *et al.,* 2007). The structure of polysaccharides synthesized by microbial cells may vary. Microbial exopolysaccharides are comprised of either homopolysachharides or heteropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type such as D- glucose or L- fructose. Homopolysaccharides belong to three distinct groups including:

A- D- glucan which is produced by *Leuconostoc mesenteroides*

ß- D- glucans which is produced by *Pediococcus spp* and *Streptococcus spp*.

Fructans are produced by *Streptococcus salivarius*.

A number of lactic acid bacteria produce heteropolysaccharides. These molecules form from repeating units of monosaccharides including D- glucose, D- galactose, L- fructose, L- rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid. The type of both linkages between monosaccharides units and the branching of the chain determines the physical properties of the microbial heteropolysaccharides. As an example, bacterial alginate is a heteropolysaccharide with an irregular structure. In this polymer, D-mannurosyl and L- guluronosyl residues are found. Alginate is mostly produced by the cells of *Pseudomonas aeruginosa* and *Azatobacter vinelandii* (Davies and Geesey, 1995; Czaczyk *et al.,* 2007). Due to a wide range of linkages and the complexity of polysaccharides structures, it would therefore be difficult for most amylase enzymes (including the test amylases) to break down the bond linkages and the monomers making up polysaccharides which determine the physical structure of the EPS.

It was therefore not surprising that the amylase enzymes tested for the degradation of *Pseudomonas fluorescens* and mixed bacterial species biofilms, were less effective than the proteases. This is also in agreement with previous studies, indicating that the activity of most amylase enzymes tested was less effective for the removal of bacterial biofilms than proteases (Ledder *et al.,* 2009). This was attributed to the dominance of proteins in the EPS. In most cases proteins seem to be the main constituents of the biofilms EPS and are found mostly at the outer layer of the biofilms (Liu *et al.,* 2004; Bhaskar and Bhosle, 2005). Therefore, it is unlikely that the amylase enzymes would degrade the protein in the EPS. Since the biofilm EPS was made up of mostly proteins it explains why the amylase enzymes were less efficient for biofilm degradation.

Hence, when the amylase enzymes were tested in combination with the protease enzymes, efficiency improved. It was therefore concluded that the protease enzymes were the primary remedial compounds and the amylase enzymes were the secondary remedial compounds.

These results are in support of that of Ledder *et al.* (2009) were a combination of amylase, lipase and protease enzymes was tested and found to be effective in reducing biofilm formation by different bacterial species of *A. naeslundii*, *N. subflava*, *L.*

rhamnosus, *P. gingivalis*, *S. oralis*, *S. mutans*, *V. dispar* and *S. sanguinis.* Walker *et al.* (2007) also found a mixture of enzymes (proteases and alpha amylases) to be effective for reducing the number of viable cells on stainless steel coupons. Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and alpha - amylase and beta - glucanase. This enzymatic mixture was effective in digesting slime layers produced by pure cultures of *Pseudomonas species* and mixed strains of bacteria. Among 24 preparations of the enzymes, only three types of enzymes were found to be effective for the removal of slime produced by *Pseudomonas* bacteria. These were alpha-amylase, protease and the combination of amylase, glucanase and protease (Wiatr, 1991). It was therefore concluded that the amylase enzymes are less effective for biofilm removal than protease enzymes.

Scanning electron microscopy analysis of enzyme efficiency for biofilm EPS degradation

SEM analysis confirmed that protease enzymes (Savinase and Everlase tested individually) and a combination of the protease enzymes were more effective than the amylase enzymes for degrading the EPS of *Pseudomonas fluorescens* and mixed bacterial species biofilms.

5.6 Conclusion

If a compound or compounds capable of destroying all the structural components of different EPS that are produced by different biofilms growing under different conditions is found then the “city of microbes” (biofilms) would be destroyed permanently. If only an enzyme or enzymatic mixture capable of shutting down or deactivating the quorum sensing systems of different biofilm EPS could be found, then there would not be any formation of biofilms and the name biofilm will undergo extinction. Protease enzymes were capable of destroying the “house of the microbes” (EPS). In this study different biofilms however produce different types EPS depending on the type of microorgansism within the biofilm. Environmental factors favour the formation of biofilms (nutrients, flow velocity, hydrodynamic etc) and these factors cannot be controlled as they are naturally occurring.

The amylase enzymes were less effective but when tested in combination with the protease enzymes, efficacy improved. Enzymes differed in activity. This may be due to the fact that EPS is highly heterogeneous even among the bacteria of the same species and therefore its structural composition will differ. Another reason for the difference in enzyme activity may be the way they were formulated and the mode of action. In conclusion, in order to design enzymes which target the EPS of the biofilms, it is important to have an understanding of the structural composition of the EPS.

5.7 References

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Chapter 6

General discussion

Microplate and spectrophotometric assays for screening of proteolytic and amylolytic enzymes for biofilm removal

Biofilms are less sensitive to antimicrobial agents than are the cells growing planktonically. Much of this resistance has been attributed to the production of the extracellular polymeric substances (EPS) produce by the biofilms. Protease and amylase enzymes were selected for the control and removal of biofilms because proteins and carbohydrates are the main structural components of most bacterial biofilm EPS.

In the results, variations in terms of enzyme activity for biofilm removal were observed. These variations were as a result of difference in strength and efficacy of the enzymes depending on their formulations. Polarzyme was not effective for the removal of the biofilm tested in this study at both concentrations (1 and 2 U/ml). The inability of Polarzyme to remove biofilms was due to poor chemical interaction between this enzyme and the biofilm EPS matrix. Another reason could be that, Polarzyme used in this study was in granular form and during experimental procedures; the coat material used to coat the enzyme did not completely dissolve in the diluents used to dilute the enzymes (phosphate buffer). Therefore, this had a negative effect on the activity of Polarzyme on the biofilms.

On the other hand, other enzymes tested in this study were effective, although the efficiency was concentration dependent. In addition, the concentration - dependence response was determined by the type of the EPS of the biofilm. Looking at the results of Savinase, Everlase, Esperase and Fungamyl, these enzymes were more effective for the removal of *P. fluorescens* biofilm than removing mixed bacterial species biofilm at both tested concentrations (1 and 2 U/ml).

Based on the previous and present study on the activity of Savinase for removal of most *Pseudomonas* species, it was therefore suggested that this enzyme should be used as an anti *Pseudomonas* species for removal of biofilms produced by single or multi *Pseudomonas species*. The efficacy of this enzyme was related to the structural components of the EPS of *Pseudomonas* species.

When the enzymes mentioned above were tested for the removal of mixed bacterial species biofilm, the efficacy was less and was affected by change in concentrations. Efficacy was far less at 1 U/ml and improved as the concentration increased to 2 U/ml.

Activity of a single enzyme tested for the removal of mixed bacterial species biofilm was unpredictable, due to heterogeneity of the structural components making the EPS. This indicated that efficacy was dependent on the type of the EPS of the biofilm. In addition, the concentrations of the biofilm EPS determined the required dose for better efficiency. For example, Esperase and Everlase tested for the removal of *P. fluorescens* biofilm remained unchanged while the efficacy of these enzymes was concentration dependent when tested for the removal of mixed bacterial species biofilm. This again, indicated that the degrading strength of each enzyme was dependent on its formulation and to the structural composition of the EPS of the biofilm.

A combination of enzymes was assessed for biofilm removal to determine if efficacy will improve or not. A combination of protease enzymes and combination of protease and amylase seemed more effective for mixed bacterial species biofilm removal. It was expected that these enzyme mixtures would be more effective for the removal of *P. fluorescens* biofilm than mixed bacterial species biofilms since the structural components of the EPS produced by mixed bacterial species were more complex than that of *Pseudomonas fluorescens* and might limited the activity of the enzymes. On the other hand a combination of amylase enzymes was more effective for removal of *P. fluorescens* than mixed bacterial species biofilm. These results showed that enzyme efficacy was dependent on the structural components of the EPS matrix produced by the biofilms.

The chemical composition of EPS in *Pseudomonas fluorescens* and mixed bacterial species biofilm and application of enzymes for EPS degradation

The structural compositions of the EPS differed from biofilms depending on the microorganisms within the biofilm. The objectives of this study were to: determine the influence of nutrients on the production of EPS in biofilms grown in nutrient rich medium and nutrient limited medium conditions; to extract EPS and determine protein and carbohydrate concentrations and to test the efficiency of protease and amylase enzymes for the degradation of the EPS and biofilm removal.

The effect of nutrient concentration on biofilm yield

In this study there was a slight difference in the number of viable cells grown in the fed and unfed nutrient medium conditions and there was no noticeable difference in biofilms cells grown in fed and unfed medium; but there was a difference in the amount of EPS produced. The fed biofilms had more EPS than the unfed biofilms. Nutrients boosted the biofilm cells growing in rich medium which resulted in more EPS produced. It was indicated in previous studies that biofilms growing in high nutrient medium were more abundant, densely packed and thicker.

Protein and carbohydrate concentrations in the biofilm EPS

The EPS of *Pseudomonas fluorescens* and mixed bacterial species biofilm grown in fed medium had a higher protein and carbohydrate concentration than in the unfed biofilm EPS. Protein concentrations were higher than the carbohydrate concentration in both fed and unfed biofilms. This indicated that the structural components of the biofilm EPS was dependent on the nutrient status in which the biofilm was grown. In some studies, it was indicated that carbohydrates are the main constituents of the EPS while other studies found proteins to dominate. In this study proteins were found to be dominant rather than carbohydrates. Nonetheless, the EPS components of the biofilms differed in quantity and structure depending on the microorganisms within a biofilm.

Furthermore, depending on the extraction protocols used, the amount of EPS composition will differ. In previous study, the EPS of mixed cultures in wastewater treatment systems

was evaluated and found that the protein (41.3%) concentration was greater than the carbohydrate concentration (18.7%) in the methanogenic sludge when the formaldehyde–NaOH extraction method was applied. In addition, the formaldehyde–NaOH process extracted the highest concentration of EPS from all the sludges. In this study, EPS of the biofilms was extracted by centrifuging the sample at low and high speed to separate the biomass from the EPS. This method was chosen because of its higher extraction efficiency and lower cell lysis. Then, the Anthrone and Lowry assays were employed for the quantification of glucose and protein concentrations respectively in the EPS. Anthrone and Lowry assays were employed in this study for the quantification of total carbohydrate and proteins in the EPS since enzymes were tested for the degradation of a broad spectrum of carbohydrates and proteins. These assays are based on the colorimetric determination of colour development. The advantage was that these assays can also be performed in a micro plate format and can be performed at room temperature. In addition standard curves were constructed to convert the absorbencies into protein and carbohydrate concentrations.

The use of protease and amylase enzymes for the degradation of biofilm EPS

Many antimicrobial agents fail to penetrate the biofilm due to the EPS which acts as a barrier protecting the bacterial cells within. The alternative will be the use of compounds which can degrade the EPS of the biofilm. Enzymes have been proven to be effective for the degradation of the EPS of the biofilms. Enzymes remove biofilms directly by destroying the physical integrity of the EPS. The mechanism in which enzymes destroy the physical integrity of the EPS is through weakening the proteins, carbohydrate and lipid components making up the structures of the EPS through the degradation process. For efficient removal of biofilm, it is therefore important that the structural components of the EPS should be known before application of the relevant enzymes.

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biofilm EPS. The reason for the inefficiency of Polarzyme was due to its incompatibility with the specific protein structural components of the biofilm EPS tested in this study. The manner in which the protease enzymes degrade the proteins in the EPS is through binding and hydrolysis of the protein molecules and converting them into smaller units that can be transported through the cell membranes and then be metabolized. The mode of enzymatic action was therefore depended on the specific protein structure and this in turn determined efficacy.

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The EPS may be hydrophilic or hydrophobic depending on the structural components making up such EPS and the environmental conditions were the biofilms are developing. Studies have indicated that among one bacterial species, EPS components may differ. The structure of polysaccharides synthesized by microbial cells may vary. Microbial exopolysaccharides were comprised of either homopolysachharides or heteropolysaccharides. Homopolysaccharides were composed of only one monosaccharide type such as D – glucose or L- fructose. Homopolysaccharides belonged to three distinct groups including α – D – glucan which was produced by *Leuconostoc mesenteroides*; ß- D- glucan which was produced by *Pediococcus spp* and *Streptococcus spp*; and Fructans were produced by *Streptococcus salivarius*.

A number of lactic acid bacteria produce heteropolysaccharides. These molecules form from repeating units of monosaccharides including D- glucose, D- galactose, L- fructose, L- rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid. The type of both linkages between monosaccharides units and the branching of the chain determines the physical properties of the microbial heteropolysaccharides. As an example, bacterial alginate was a heteropolysaccharide with an irregular structure. In this polymer, D-mannurosyl and L- guluronosyl residues were found. Alginate was mostly produced by the cells of *Pseudomonas aeruginosa* and *Azatobacter vinelandii*.

Due to a wide range of linkages and the complexity of polysaccharides structures, it was therefore difficult for most amylase enzymes (including the test amylases) to break down the bond linkages and the monomers making up polysaccharides which determined the physical structure of the EPS.

It was therefore not surprising that the amylase enzymes tested for the degradation of *Pseudomonas fluorescens* and mixed bacterial species biofilms, were less effective than the proteases. This was also in agreement with previous studies, indicating that the activity of most amylase enzymes tested were less effective for the removal of bacterial biofilms than proteases. This was attributed to the dominance of proteins in the EPS. In most cases proteins seemed to be the main constituents of the biofilms EPS and are found mostly at the outer layer of the biofilms. Therefore, it was unlikely that the amylase enzymes would degrade the protein in the EPS. Since the biofilm EPS was made up of mostly proteins it explained why the amylase enzymes were less efficient for biofilm degradation.

Hence, when the amylase enzymes were tested in combination with the protease enzymes, efficiency improved. It was therefore concluded that the protease enzymes were the primary remedial compounds and the amylase enzymes were the secondary remedial compounds.

These results are in support of the studies done previously, were a combination of amylase, lipase and protease enzymes was tested and found to be effective in reducing biofilm formation by different bacterial species of *A. naeslundii*, *N. subflava*, *L.*

rhamnosus, *P. gingivalis*, *S. oralis*, *S. mutans*, *V. dispar* and *S. sanguinis.* Another studies found a mixture of enzymes (proteases and alpha amylases) to be effective for reducing the number of viable cells on stainless steel coupons. Five enzymes were tested previously for the removal of biofilms and among those enzymes was a combination of one protease and alpha - amylase and beta – glucanase. This enzymatic mixture was effective in digesting slime layers produced by pure cultures of *Pseudomonas species* and mixed strains of bacteria. It was therefore concluded that the amylase enzymes were less effective for biofilm removal than protease enzymes.

Scanning electron microscopy analysis of enzyme efficiency for biofilm EPS degradation

SEM analysis confirmed that protease enzymes (Savinase and Everlase tested individually) and a combination of the protease enzymes were more effective than the amylase enzymes for degrading the EPS of *Pseudomonas fluorescens* and mixed bacterial species biofilms.

Conclusion

If enzymatic mixtures capable of destroying all the structural components of different EPS that are produced by different biofilms growing under different conditions are found, then biofilm formation and attachment on the substrata would have been destroyed permanently. If only enzymatic mixtures capable of shutting down or deactivating the quorum sensing systems of different biofilm EPS are found, then there would not be any production of biofilm EPS. In this study, protease enzymes tested individually and in combination were most effective for the degradation of biofilm EPS than the amylase. It was observed in this study that growth medium conditions contributed to the production of the biofilm EPS since the EPS, protein and carbohydrate concentrations were higher in the fed biofilms than the unfed biofilms. EPS is highly heterogeneous even among the bacteria of the same species and therefore its structural composition will differ. Another reason for the difference in enzyme activity may be the way they were formulated and the mode of action. In conclusion, in order to design enzymes which target the EPS of the

biofilms, it is important to have an understanding of the structural composition of the EPS.

Recommendation

Amylase enzymes tested individually and in combination were less efficient for the degradation of the biofilm EPS resulting in biofilm removal. This was due to the complex structure of the exopolysaccharides synthesized by different biofilms. Also, the bond linkages between polysaccharide units and the branching of the chain between the monomers as previously described, complex the structures of the biofilm EPS and as a result confer in the physical properties of the microbial biofilms. Hence, when the amylase enzymes were tested in combination with the protease enzymes, it resulted in an improved activity. It is therefore recommended that, protease and amylase enzymes should be tested in combination to improve the efficiency. In addition, the structure of the biofilm EPS should be investigated so that relevant enzymatic mixtures are tested for biofilm removal.

Appendix

Conversion of absorbances into protein and carbohydrate concentrations

For protein concentrations, BSA standard curve:

Y= MX + C

Where;

Y = the absorbance; M = 0.0002; X = unknown concentrations and C = 0.2

An example; calculating the activity of savinase:

Time interval	Absorbance (Savinase)
0	0.421
15	0.397
30	0.388
45	0.370
60	0.330
75	0.290
90	0.260

Y = MX + C

0.421 = 0.0002X + 0.2

X = 1105 U/ml

For carbohydrate concentrations, glucose standard curve:

Y = the absorbance; M = 0.0006; X = unknown concentrations and C = 0.05

An example; calculating the activity of AMG:

Time interval	Absorbance (AMG)
0	0.120
15	0.118
30	0.110
45	0.090
60	0.088
75	0.071
90	0.060

$Y = MX + C$

$0.120 = 0.0006X + 0.05$

$X = 116$ U/ml

Commercial name	Type of enzyme	Manufacturer	Microorganisms source	Form	Optima		Application
					pH	Temperature	
Savinase 16L EX	Subtilisin	Novozyme	Genetically modified *Bacillus clausii*	Liquid	8-11	15-75^{O}C	Laundry, automatic detergent, industrial and institutional laundry
Everlase 16L EX	Subtilisin	Novozyme	Genetically modified *Bacillus clausii*	Liquid	8-11	15-80^{O}C	Detergent industry
Esperase 6.0 T	Subtilisin	Novozyme	*Bacillus lentus*	Liquid	8-11	20-60	Detergent industry
Polarzyme 6.0T	Subtilisin	Novozyme	Genetically modified *Bacillus spp*	Granules	9-11	20-40^{O}C	Detergent industry
Fungamyl 800	Alpha Amylase	Novozyme	*Aspergillus oryzae*	Liquid	4-5.5	20-60	
AMG (Amyloglucosidase)	Glucoamylase	Novozyme	*Aspergillus niger*	Liquid	4-5	20-60^{O}C	Food industry
BAN (Bacterial Amylase Novo)	Alpha Amylase	Novozyme	*Bacillus amyloliquefaciens*	Liquid	6-7	20-60^{O}C	Food industry