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

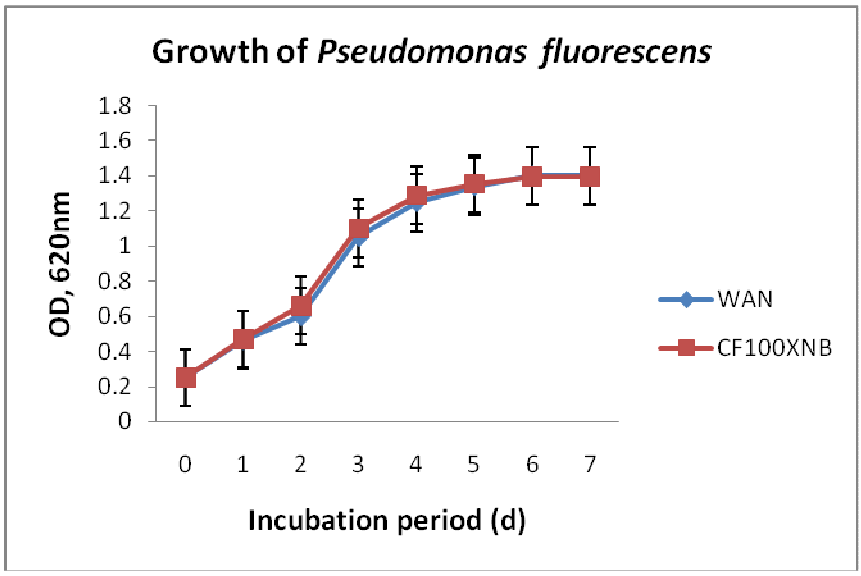
Glass wool samples were fixed in a solution of 2.5% glutaraldehyde in 75mM Phosphate buffer, pH 7.4 for 1h. Samples were rinsed three times for 15 min at a time in 50% 75mM phosphate buffer. After the rinsing step, samples were dehydrated in ethanol at concentrations of 50, 70, 90 and three times 100% 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

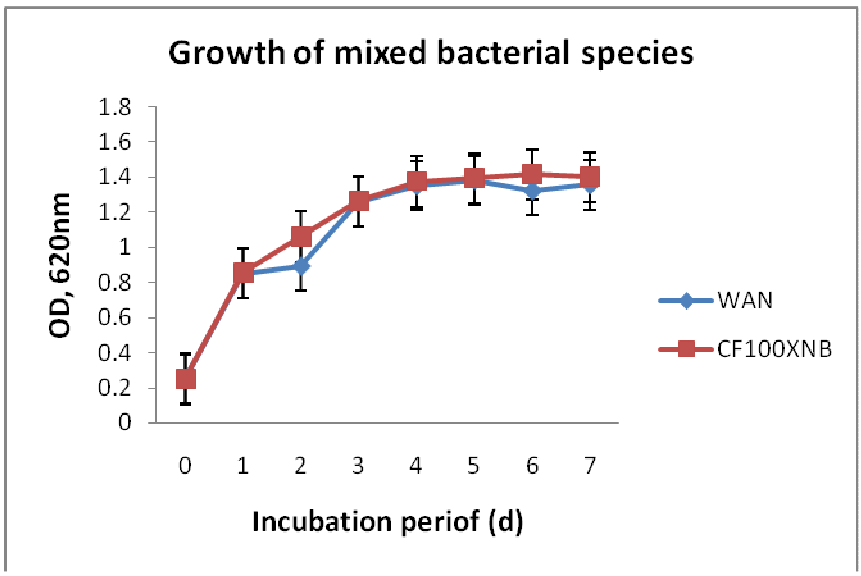
Single enzymes		**Mixed enzymes**
Proteases	**Amylases**	**Proteases**
Savinase 16L Type EX	Amyloglucosidase (AMG) 300L	Savinase 16L Type EX, Everlase 16L Type EX, Esperase 16L Type EX
Everlase 16L Type EX	Bacterial Amylase Novo (BAN) 240L	**Amylases**
Esperase 16L Type EX	Fungamyl 800 L	AMG 300L, BAN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

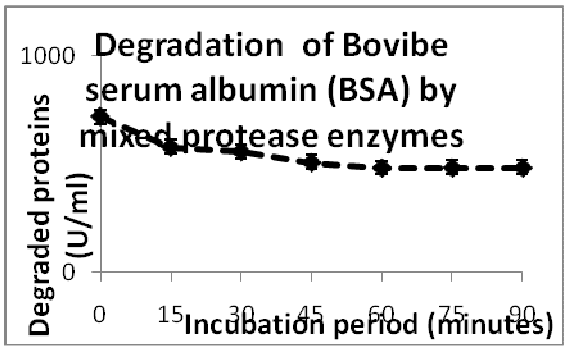
Table 5.4.4 Comparison of extracted EPS, protein and carbohydrate concentrations produced from fed and unfed mixed bacterial species biofilm

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)

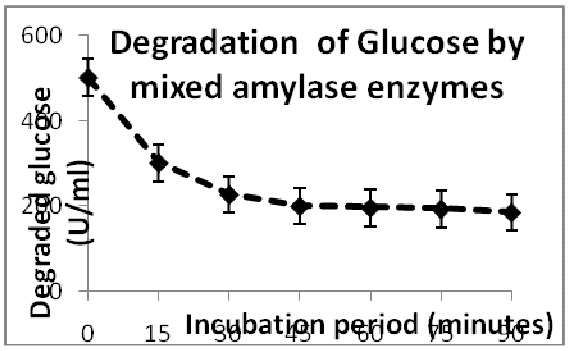
The EPS concentrations of the *Pseudomonas fluorescens* and mixed bacterial species biofilms that were fed daily with 2 ml of Nutrient Broth were higher than the EPS concentrations in the unfed control (Table 5.3.2; 5.3.3). The protein concentrations in both daily and unfed experiments were higher than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

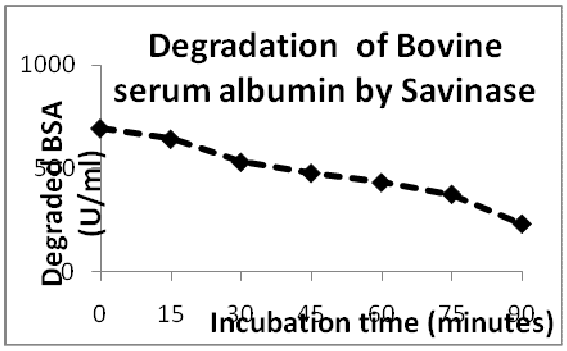
Effects of enzymes tested individually for the degradation of *Pseudomonas fluorescens* biofilm EPS



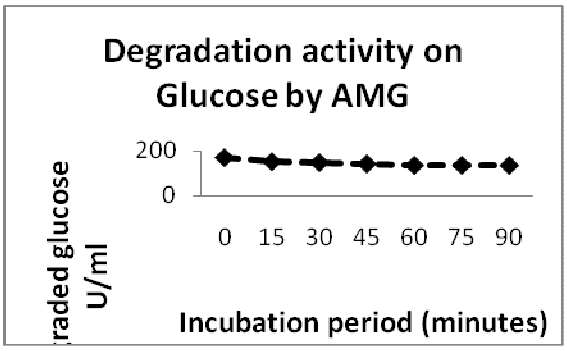
(A)



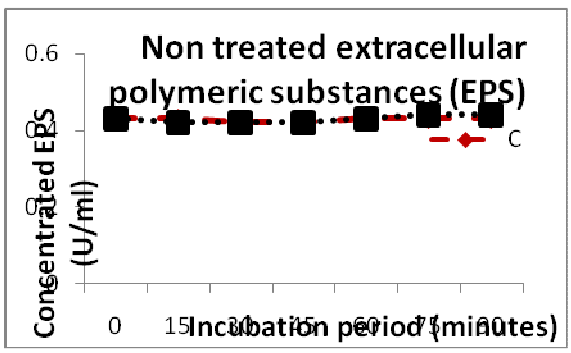
(B)



(C)

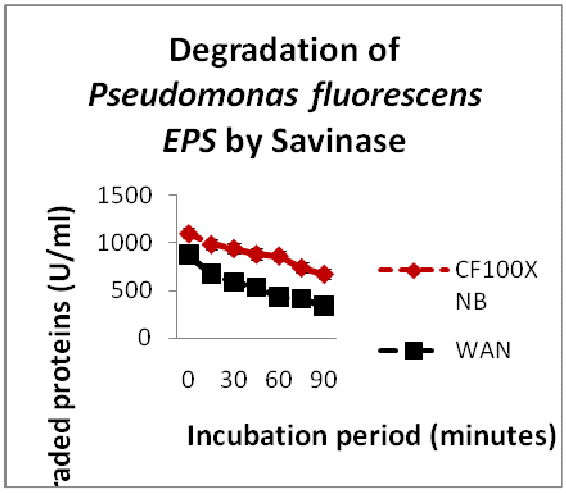


(D)

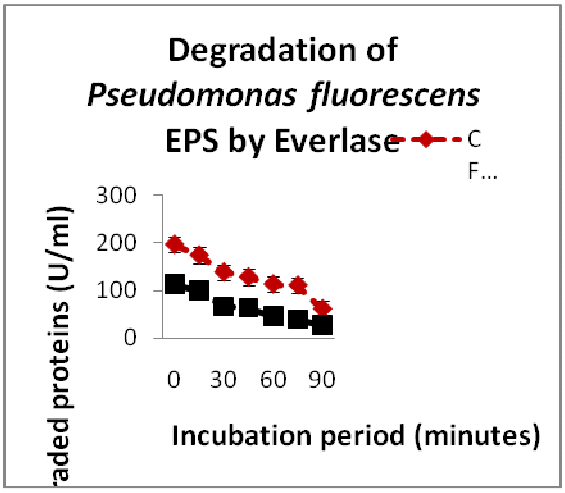


(D)

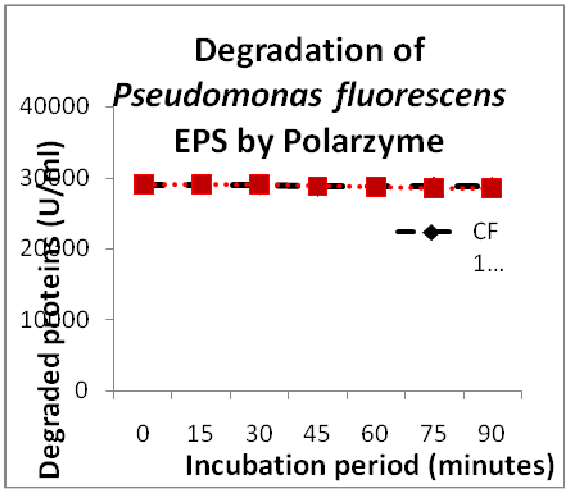
Fig. 5.4.2 Effects of enzymes on BSA and glucose (D) the non treated extracellular polymeric substance (EPS).



(A)

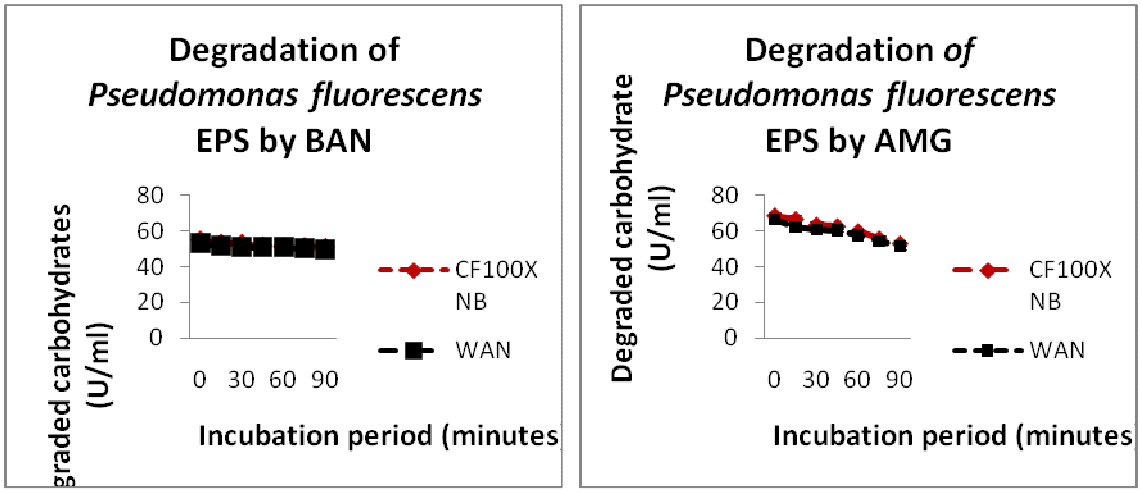


(B)



(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.

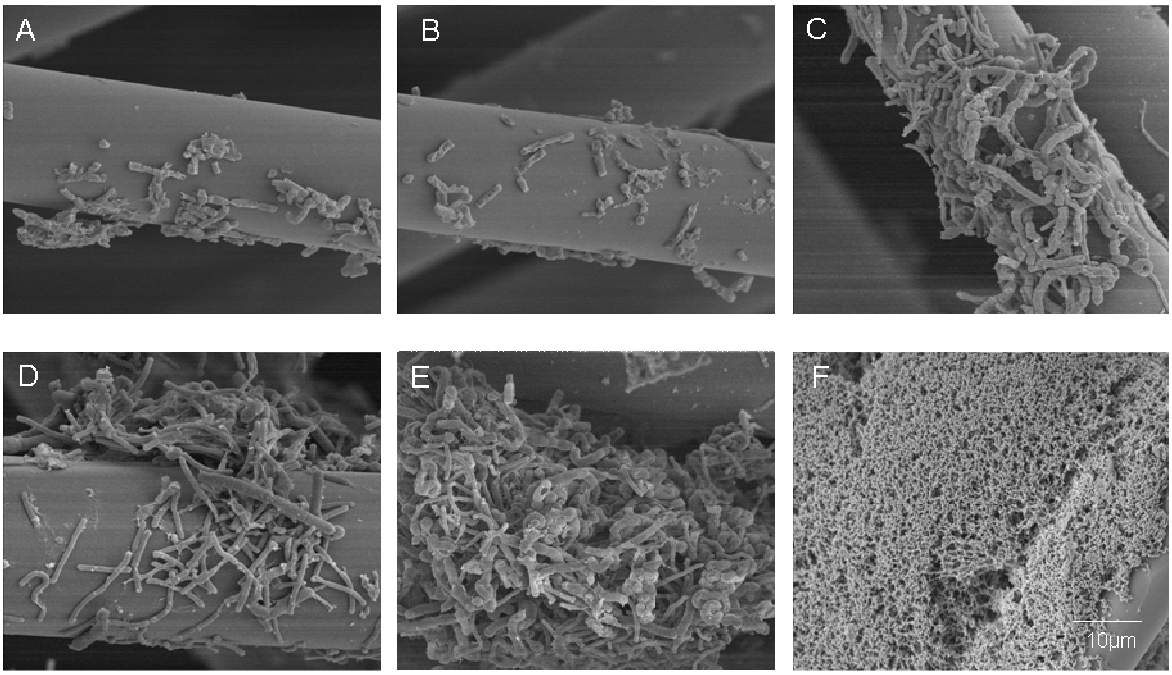
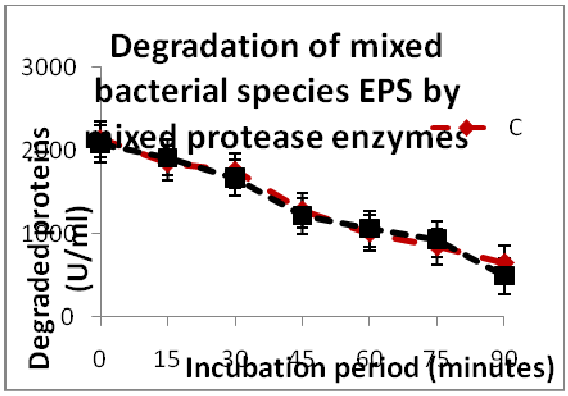


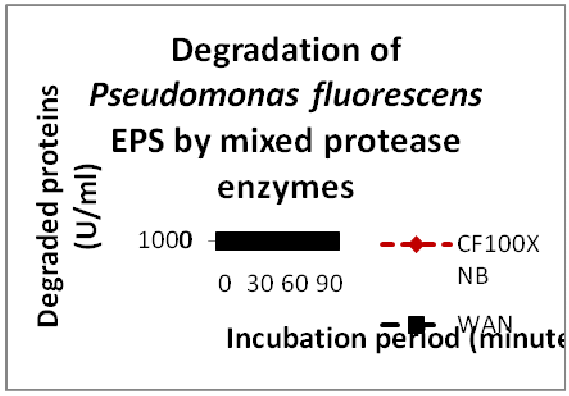
Fig. 5.4 .5 Scanning Electron Microscope analysis of the degradation activity of enzymes on extra cellular polymeric substances (EPS) of 7d old *Pseudomonas fluorecens* biofilm attached to glass wool after 24h incubation at 26^{O}C. E and F show non treated biofilm, (A) Savinase, (B) Everlase, (C) AMG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

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

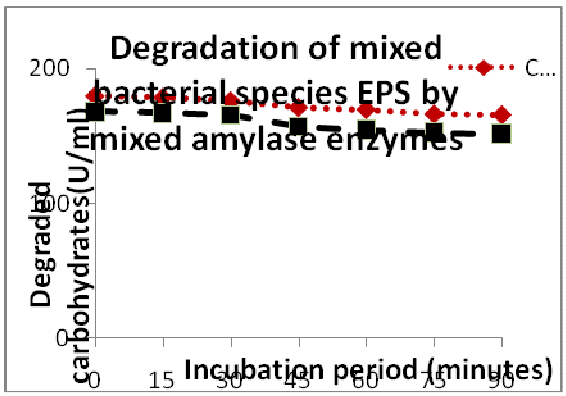
To ensure activity, the protease Savinase and the amylase AMG were first tested on BSA and glucose respectively for activity and these enzymes were effective for the degradation of the BSA and glucose (Fig. 5.3.2).The proteases Everlase and Savinase were most effective for the degradation of the EPS 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).



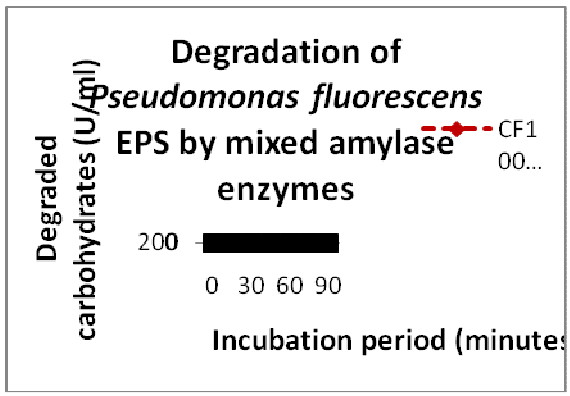
(A)



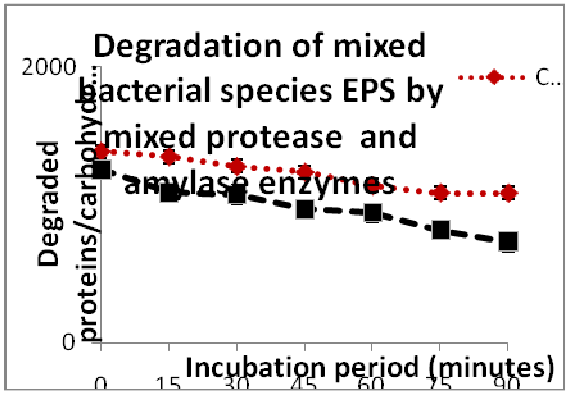
(B)



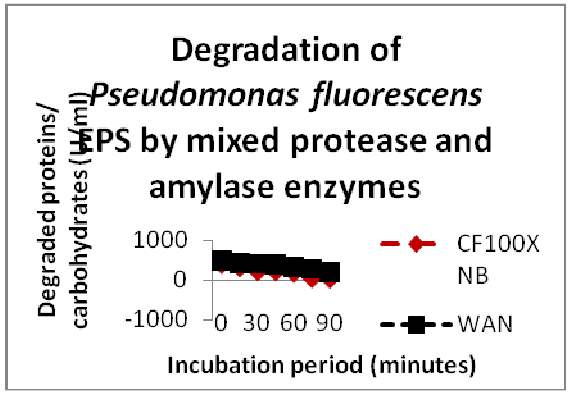
(C)



(D)



(E)



(F)

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

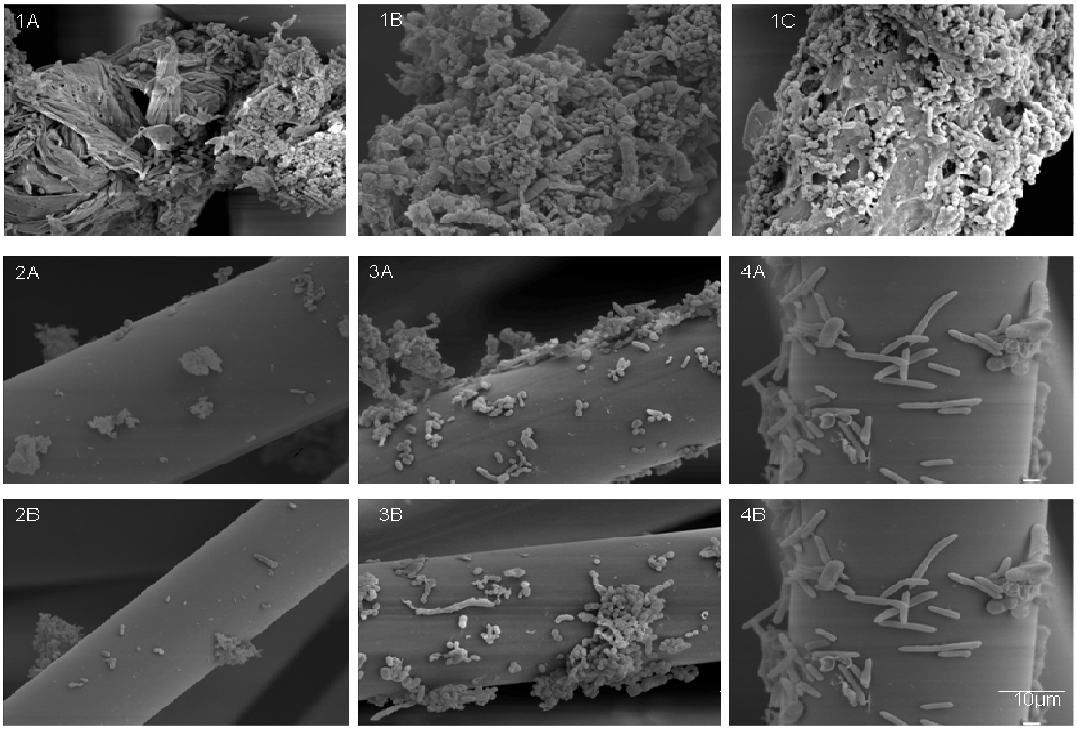


Fig. 5.4.7 Scanning Electron Microscopy analysis of the degradation activity of mixed enzymes on extra cellular polymeric substances (EPS) of 7d old *Pseudomonas fluorescens* biofilms attached on the glass wool after 24h incubation at 26^{O}C. 1 (A, B, C) shows non treated mixed bacterial species biofilms,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

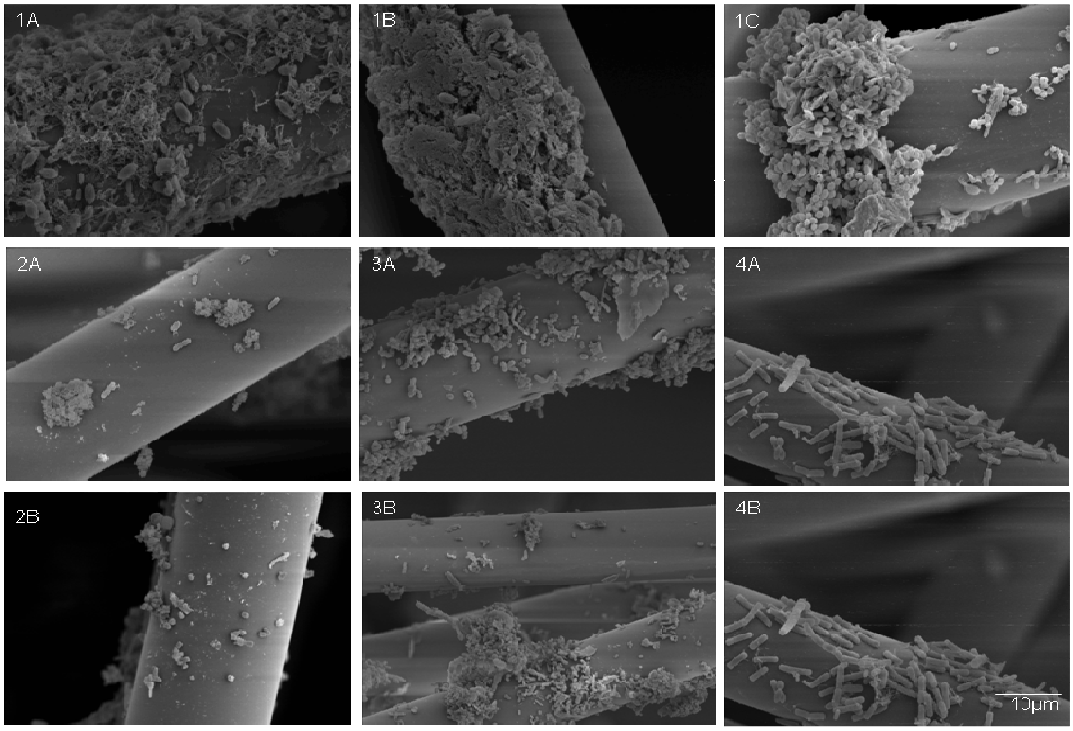


Fig. 5.4.8 Scanning Electron Microscope analysis of the degradation activity of mixed enzymes on extra cellular polymeric substances (EPS) of 7d old mixed bacterial species biofilm (*Gram negative and positive bacteria*) attached on the glass wool after 24h incubation at 30^{O}C. 1 (A, B, C) shows non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

- Ahimou, F. Semmens, M.J. Haugstad, G and Novak, P.J. (2007) Effect of protein, polysaccharide, and oxygen concentration profiles on biofilm cohesiveness. *Appl. Environ. Microbiol.* 73, 2905-2910.
- Allison, D.G. (2000) Microbial biofilms: Problems of control. In: Allison, D.G. Gilbert, P. Lappin – Scott, H. and Wilson, M (Eds) Community structure and cooperation in biofilms. pp, 309-327. Cambridge University Press.
- Arevalo-Ferro, C. Reil, G. Gorg, A. Eberl, L and Riedel, K. (2005) Biofilm formation of *Pseudomonas putida* IsoF: The role of quorum sensing as assessed by proteomics. *J. Sys. Appl. Microbiol*. 28, 87-114.
- Augustin, M. Ali- Vehmas, T. and Atroshi, F. (2004) Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *J. Pharm. Pharmaceut.* 7, 55-64.
- Bhaskar, P.V. and Bhosle, N.B. (2005) Microbial extracellular polymeric substances in marine biogeochemical processes. *Cur. Sci*.88, 47-53.
- Bockelmann, U. Szewzyk, U. and Grohmann, E. (2003) A new enzymatic method for the detachment of particles associated soil bacteria. *J. Microbiolo. Met.* 55, 201-211.
- Chen, M.Y. Lee, D.J. and Tay, J.H. (2007) Distribution of extracellular polymeric substances in aerobic granules. *J. Appl. Microbiol. Biotechnol.* 73, 1463-1469.
- Cloete, T.E. (2003) Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeter. Biodegrad.* 51, 277-282.
- Cloete, T.E. Jacobs, L. and Brozel, V.S. (1998) The chemical control of biofouling in industrial water systems. *J. Biodeter.* 9, 23-37.
- Cowan, M..M. Warren, T.M. and Fletcher, M. (1991) Biofouling. *J. Appl. Environ. Microbiol.* 3, 23-34.
- Czaczyk, K and Myszka, K. (2007) Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilms formation. *Polish J. Environ. Stud.* 16, 799-806.

- Davies, D.G. and Geesey, G.G. (1995) Regulation of the alginate biosynthesis gene algC in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *J. Appl. Environ. Microbiol.* 61, 860-867.
- de Carvalho C.C.C.R. (2007) Biofilms: Recent developments on an old battle. *Recent Patents. Biotechnol.* 1, 49-57.
- Dignac, M.F. Urbain, V. Rybacki, D. Bruchet, A. Snidaro, D. and Scribe, P. (1998) Chemical description of extracellular polymers: implication on activated sludge floc structure. *Water Sci. Technol.* 38, 45-53.
- Donlan, R.M. (2002) Biofilms: Microbial life on surfaces. *Emerg. Infect. Dis.* 8, 1-14.
- Flemming, H.C. (1998) Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. *J. Pol. Degrad. Stab*. 59, 309-315.
- Flemming, H.C. Neu, T.R and Wozniak, D. (2007) The EPS matrix: The "house of biofilms" *J. Bacteriol.* 189, 7945-7947.
- Frøelund, B. Keiding, K. and Nielsen, P. (1995) Enzymatic activity in the activated sludge flocs matrix. *J. Appl. Microbiol. Biotechnol*. 43, 755-761.
- Glaouris, E.D. and Nychas, G.J.E. (2006) The adherence of *Salmonella Enteritidis* PT4 to stainless steel: The importance of the air-liquid interface and nutrient availability. *J. Food Microbiol*. 23, 747-752.
- Gomez-Suarez, C. Pasma, J. van der Borden, A.J. (2002) Influence of extracellular polymeric substances on deposition and redeposition of *Pseudomonas aeruginosa* to surfaces. *J. Microbiol.* 148, 1161-1169
- Hallam, N.B. West, J.R. Forster, C.F and Simms, J. (2001) The potential for biofilms growth in water distribution system. *Wat. Res.* 32, 4063-4071.
- Johansen, C. Falholt, P. and Gram, L. (1997) Enzymatic removal and disinfection of bacterial biofilms. *App. Environ. Microbiol.* 63, 3724-3728.
- Lapidot, A. Romling, U. and Yaron, S. (2006) Biofilm formation and the survival of *Salmonella typhimurium* on parsley. *Int. J. Food Microbiol*. 109, 229-233.
- Laspidou, C.S and Rittmann, B.E. (2002) A unified theory of extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Res.* 36, 2711-2720.

- Ledder, R.G. Timperley, A.S. Friswell, M.K. MacFarlane, S and McBain, A.J. (2009) Coaggregation between and among human intestinal and oral bacteria. *FEMS Microbiol. Ecol.* 66, 630-636.
- Lequette, Y. Boels, G. Clarisse, M. and Faille, C. (2010) Using enzymes to remove biofilms of bacterial isolates samples in the food industry. *Biofoul*. 4, 421-431.
- Leroy, C. Delbarre, C. Gillebaert, F. Compere, C. and Combes, D. (2008) Effect of commercial enzymes on the adhesion of a marine biofilms forming bacterium. *Biofoul.* 24, 11-22.
- Liu, H. and Fang, H.P. (2003) Extraction of extracellular polymeric substances (EPS) of sludge. *J. Biotechnol*. 95, 249-256.
- Liu, Y. Yang, S.F. Li, Y. Xu, H. Qin, L. and Tay, J.H. (2004) The influence of cell substratum surface hydrophobicities on microbial attachment. *J. Biotechnol*. 110, 251-256.
- Loiselle, M. and Anderson, K. (2003) The use of cellulose in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofoul.* 2, 77-85.
- Melo, L.F and Bott, T.R. (1997) Biofouling in water systems. *J. Exp. Therm. Fluid Sci*. 14, 375-381.
- Meyer, B. (2003) Approaches to prevention, removal and killing of biofilms. *Inter. J. Biodeter. Biodegrad*. 51, 249-253.
- Nielsen, P.H. and Jahn. A. (1999) Extraction of EPS. In: Wingender, J. Neu, T.R. and Flemming, H.C., Editors, Microbal extracellular polymeric substances: characterization, structure and function, Springer, Berlin.
- O'Toole, G. Kaplan, H.B. and Kolter, R. (2000) Biofilm formation as microbial development. *Ann. Rev. Microbiol.* 54, 49-79.
- Or, D. Phutane, S. and Dechesne, A. (2007) Extracellular polymeric susbatnces affecting pore- scale hydrologic conditiond for bacterial activity in unsaturated soils. *Vadose Zone J.* 6, 298 – 305.

- Orgaz, B. Neufeld, R.J. and Sanjose, C. (2007) Single – step biofilm removal with delayed release encapsulated pronase mixed with soluble enzymes. *Enz. Microb. Tech.* 40, 1045- 1051.
- Parkar, S.G. Flint, S.H. and Brooks, J.D. (2003) Evalualtion of the effect of cleaning regimes on biofilms of the *thermophillic bacilli* on staineless steel. *J. Appl. Microbiol.* 96, 110-116.
- Ploux, L. Beckendorff, S. Nardin, M and Neunlist, S. (2007) Quantitative and morphological analysis of biofilms formation on self assembled monolayers. *J. Col. Surf.* 57, 174-181.
- Pozos, J.L and Pater, R. (2007) The challenge of treating biofilms associated bacterial infections. *Clin. Pharm. Therapeut.* 82, 204-209.
- Prakash, B. Veeregowda, B.M and Krishnappa, G. (2003) Biofilms: A survival strategy of bacteria. *J. Cur. Sci.* 85, 9-10.
- Rochex, A and Lebeault, J.M (2007) Effects of nutrients on biofilms formation and detachment of *Pseudomonas putida* strain isolated from a paper machine. *Water Res.* 41, 2885-2892.
- Simoes, M. Carvalho, H. Pereira, M.O. and Viera, M.J. (2003) Studies on the behavior of *Pseudomonas fluorescens* biofilms after *Ortho*-phthalaldehyde treatment. *Biofoul.* 3, 151-157.
- Sreenivasan, P.K. and Chorny, R.C. (2005) The effects of disinfectant foam on microbial biofilms. *J. Biofaul.* 21, 141-149.
- Stoodley, L.H. and Stoodley, P. (2002) Developmental regulation of microbial biofilms. *J. Cur. Op. Biotechnol*. 13, 228-233.
- Ton-That, H. Marraffini, L. and Schneewind, O. (2004) Protein sorting to the cell wall envelope of Gram positive bacteria. BBA. 1694, 269.
- Vickery K, Pajkos A, Cossart Y (2004) Removal of biofilms from endoscope: Evaluation of detergent efficacy. *AM J Infect Cont.* 32, 170-176.
- Walker, S.L. Fourgialakis, M. Cerezo, B. and Livens, S. (2007) Removal of microbial biofilms from dispense equipment: The effect of enzymatic pre – digestion and detergent treatment. *J. Inst. Brew.* 113, 61-66.

- Wiatr, C.L. (1991) Application of multiple enzymes blend to control industrial slime on equipments surfaces. United States Patent, Patent No. 5,071,765.
- Wood, P. Jones, M. Bhakoo, M. and Gilbert, P. (1996) A novel strategy for control of microbial biofilms through generation of biocide at the biofilm – surface interface. *Appl. Environ. Microbiol.* 62, 2598-2602.
- Xavier, J.B. Picioreanu, C. Rani, S.A. Van Loosdrecht, M.C.M. and Stewart P.S. (2005) Biofilm control strategies based on enzymatic disruption of the extracellular polymeric substance matrix- a modeling study. *J. Microbiol.* 151, 3817-3832.
- Zhang, T. and Fang, H.P. (2001) Quantification of extracellular polymeric substances in biofilms by confocal laser scanning microscopy. *J. Biotechnol.* 23, 405-409.

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.

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 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.

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. The efficiency of the proteases was 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* were composed mostly of amino acid with hydroxyl group. However, the *Bacillus subtilis* extracellular protein layer was a composition of L and D glutaminosyl residues. In previous studies the protease; Savinase was found to be more effective for the prevention of adhesion and detachment of a *Pseudoalteromonas* sp. D14 biofilm than xylanase, amylase, cellulase and lipase.

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.