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

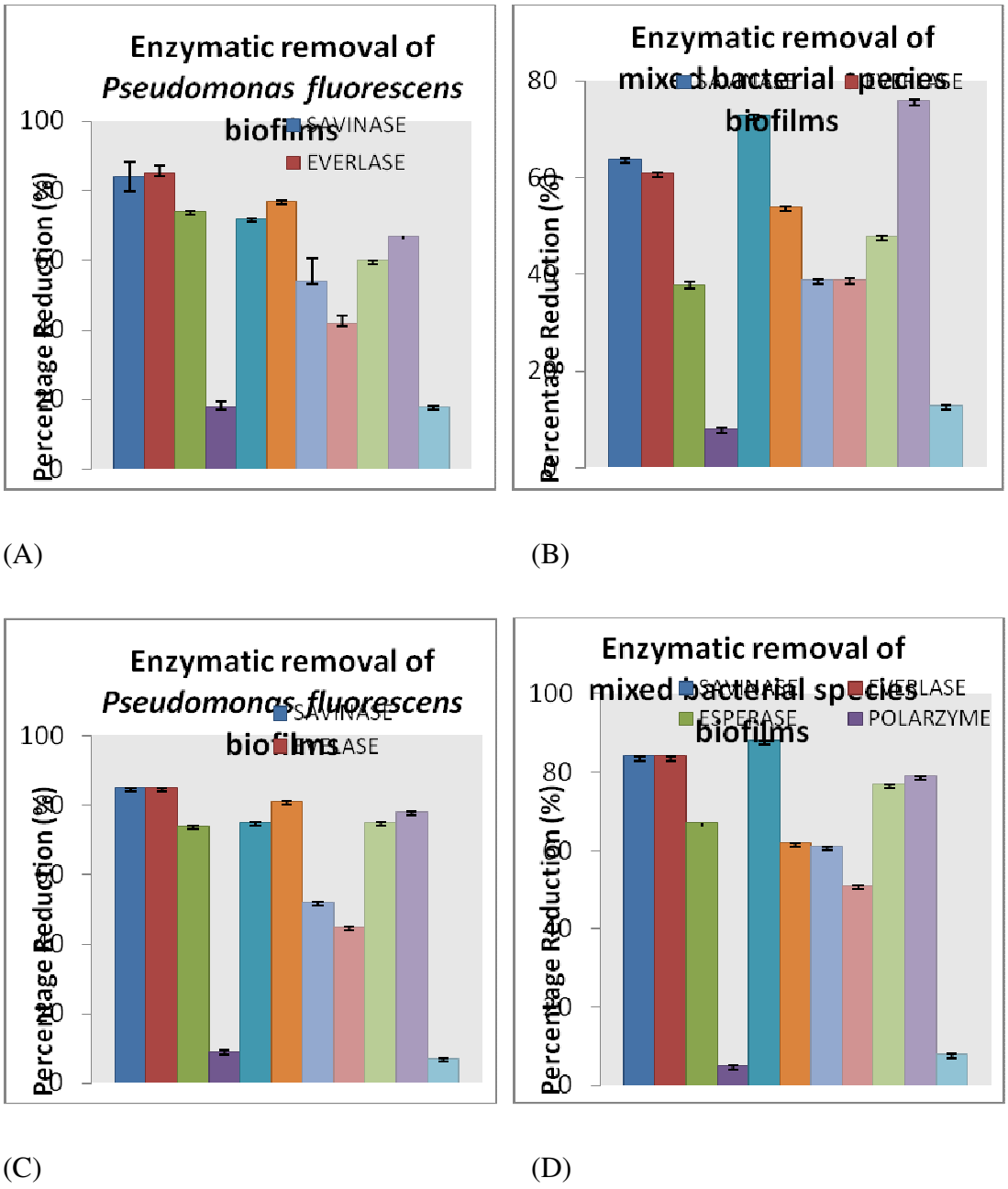

Fig. 3.4.1 Enzyme efficacy for removal of (A, C) *Pseudomonas fluorescens* biofilms and (B, D) mixed bacterial species biofilms treated at (A, B) 1 U/ml and (C, D) 2 U/ml. Bars indicate standard errors.

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.

3.7 References

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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

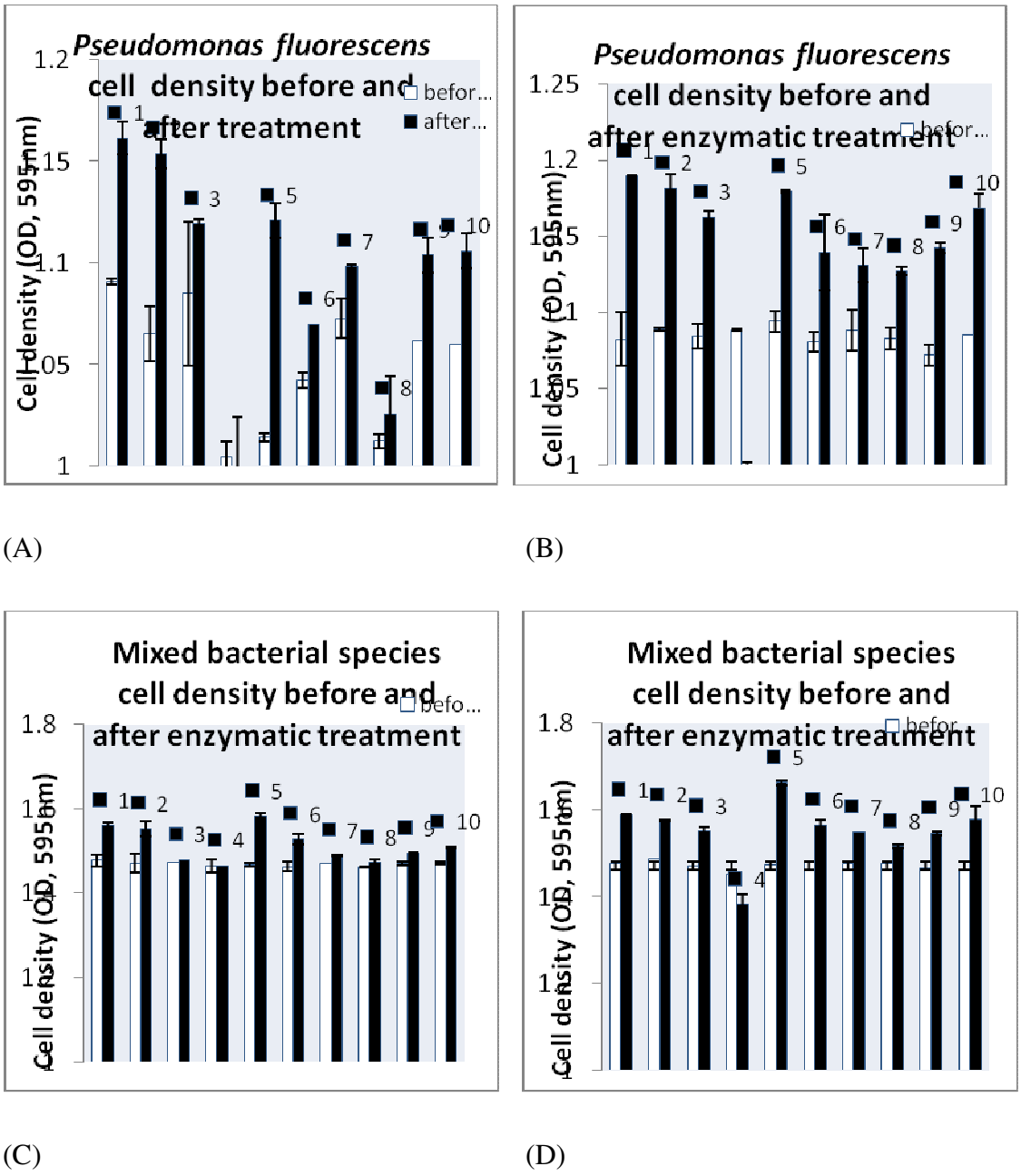
4.4 Results

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).

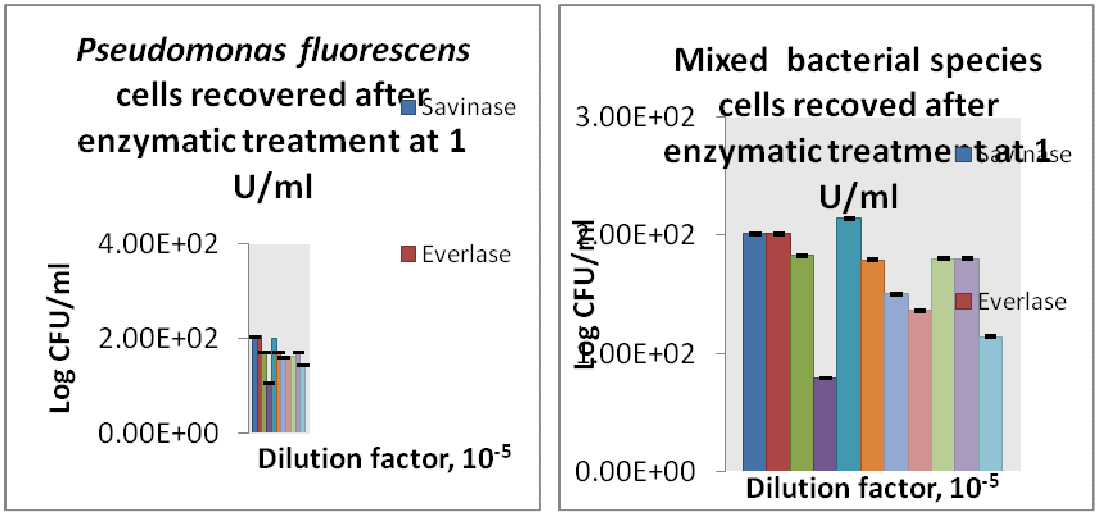
Combination of protease enzymes was assessed. In the results found, the cell density of the non treated *Pseudomonas fluorescens* and mixed bacterial species was OD 1.015 and 1.468, respectively. After treatment at 1 U/ml, the cell density increased to OD 1.121 and 1.583 (Table 4.4.1; Fig 4.4.1). When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

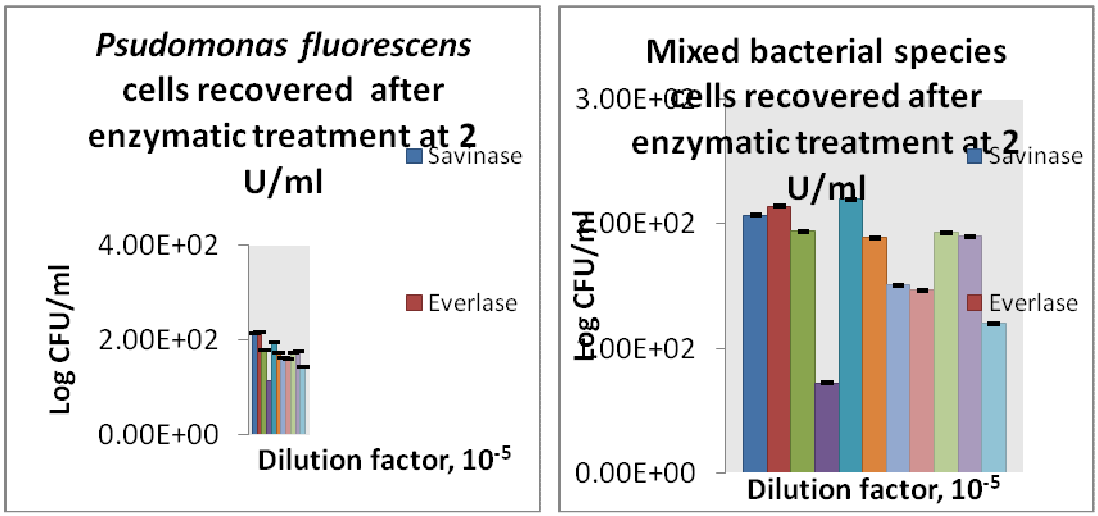
When *Pseudomonas fluorescens* cells were treated with AMG at 1 and 2 U/ml, the cell density slightly increased to OD 1.099 and 1.131 respectively from OD 1.073 and 1.089. On the other hand, the cell density of mixed bacterial species at 1 and 2 U/ml highly increased to OD 1.490 and 1.550 respectively 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).

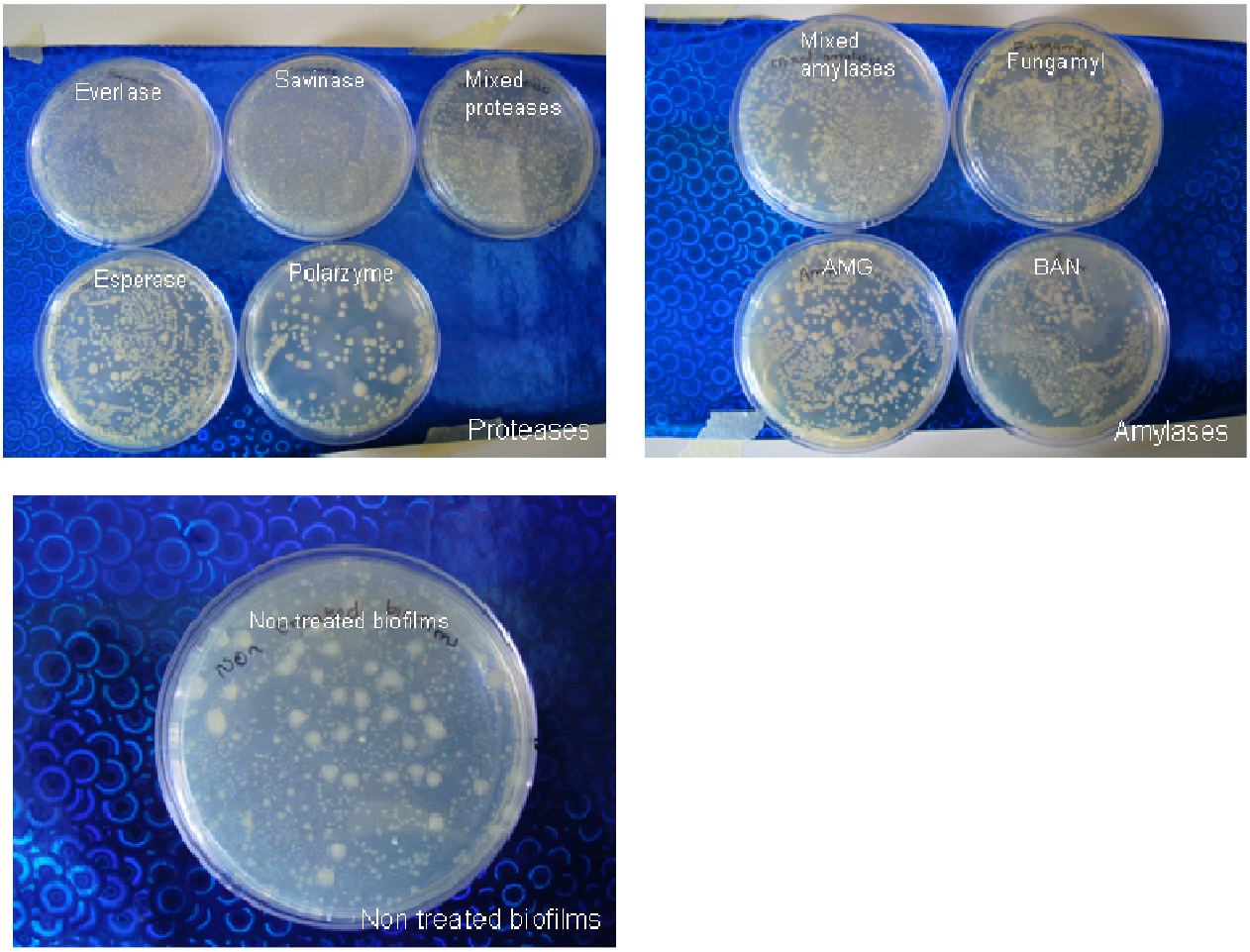


Fig. 4.4.4 Photographs showing cells recovered after enzymatic treatment.

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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