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Full Length Research Paper

Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria

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Removal of biofilms is difficult. In industrial settings, both the inactivation and removal of biofilms are of huge concern. If only disinfection without the removal of attached biofilms occurs, the inactivated biofilm cells may provide an ideal environment for further adhesion and growth, resulting in a complex matrix. Microbial resistance to biocides and their negative environmental impact are the main reasons for finding alternative biofilm control strategies. Enzymes may offer such an alternative. The objective of this study was to determine the effect of commercial proteases and amylases on biofilms formed by *Pseudomonas fluorescens*. Biofilms were grown in diluted medium containing glass wool used as the attachment surface. Extracellular polymeric substances (EPS) were extracted and EPS composition was determined. Protease (savinase, everlase and polarzyme) and amylase (Amyloglucosidase and Bacterial Amylase Novo) activity was tested on both biofilms and on extracted EPS. After testing enzymes, biofilm integrity was evaluated by scanning electron microscopy. EPS composition consisted predominantly of proteins. Everlase and Savinase were the most effective enzymatic treatments on removing biofilms and degrading the EPS.

Key words: Biofilms, extracellular polymeric substances, *Pseudomonas fluorescens*, proteases, amylases.

INTRODUCTION

When bacterial cells approach inert surfaces, they first bound to the substratum by weak forces involving their external structures such as flagella, fimbriae or capsular components (Xavier et al., 2005). As the cells remain attached to the surface for some time, they secret sticky extracellular polymeric substances (EPS) forming a biofilm matrix that embeds several layers of bacterial cells once the biofilms mature (Orgaz et al., 2006; Flemming et al., 2007). EPS are composed of a wide variety of materials including polysaccharides, proteins (Johansen et al., 1997; Leroy et al., 2008), nucleic acid, uronic acid and humic substances (Orgaz et al., 2006). Polysaccharides are partly responsible for bacterial adhesion and biofilm accumulation on the surface (Loiselle et al., 2003). The EPS also serves many other functions such as providing an adhesive foundation, structural integrity, bacterial protection and intercellular communication (Zhang et al., 2005; de Carvalho, 2007; Ploux et al., 2007; Leroy et al., 2008).

The difference in the quantity of biofilm EPS is a result of the growing conditions of the biofilms (O' Toole et al., 2000). EPS has a complex architectural structure (Flemming et al., 1998) containing channels which allow the inflow of water, oxygen and nutrients and outflow of byproducts (Zhang et al., 2001; Arevalo-Ferro et al., 2005; Donlan, 2002) and enhances bacterial resistance to antimicrobial agents(Parkar et al., 2000; Prakash et al., 2003; Lequette et al., 2010).

EPS serves other 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); Enables the bacteria to capture nutrients (Gomez-Suarez et al., 2002) causes

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biofouling (Cloete et al., 1998); Facilitates cell-cell communication (Zhang et al., 2001) and 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, 2002).

The production of 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).

Previous 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 resistance of biofilms to disinfection is through protection of the biofilm cells that are embedded in the extracellular polymeric substances (Xavier et al., 2005; Walker et al., 2007).

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; Augustin et al., 2004; 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). 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 of the EPS. The efficiency of any one enzyme degrading EPS will depend on the EPS composition (Xavier et al., 2005; Walker et al., 2007).

Previous studies have been published regarding enzyme degradation of mature biofilms using synthetic polysaccharides (Loiselle et al., 2003; Vickery et al., 2004). Cellulase from *Penicillium funiculusum* was effective in degrading mature biofilms of *Pseudomonas aeruginosa*; and it was also found to be useful in degrading the exopolysaccharides of *Pseudomonas fluorescens* (Loiselle et al., 2003; Vickery et al., 2004). Therefore, the application of enzymes to degrade EPS is a promising and an attractive option in many industries where complete biofilm removal is essential.

The aim of this study was to test selected commercial proteases and amylases for their effectiveness in the degradation and removal of EPS produced within a *Pseudomonas fluorescens* biofilm. We further establish some standard protocols for the evaluation of enzyme efficiency in degrading EPS and biofilm.

MATERIALS AND METHODS

Pseudomonas fluorescens inoculums used for biofilm growth

P. fluorescens was inoculated into sterile Nutrient Broth and incubated aerobically at 26°C overnight. During the incubation period, one set of *P. fluorescens* samples was daily fed with 2 ml of the diluted medium and the control *P. fluorescens* samples were unfed. Bacterial growth was monitored daily by measuring the optical density at 620 nm. After incubation, the concentration of the bacterial suspension was adjusted to OD, 620 nm.

Biofilm formation and growth

P. fluorescens was grown according to Rochex and Lebeault (2007) with modification. A tandardized *P. fluorescens* suspension (100 µl) was inoculated into flasks containing 100 ml sterile Nutrient Broth (Merck) and 2 g of glass wool used as a surface for the growth of biofilm. Flasks containing the glass wool were incubated at 26°C for 7 days with continuous agitation at 100 rpm. During biofilm growth, flasks marked CF100XNB were daily fed with 2 ml of Nutrient Broth and those marked WAN were unfed. At day 7, bacterial suspensions containing the planktonic cells were discarded from the flasks.

Quantitative determination of viable cells

Ten fold series of dilutions were made by inoculating 100 µl of the bacterial suspensions to 900 µl of Ringer's solutions and mix. The aliquots (0.1 ml) were spread onto sterile Nutrient agar plates (Merck) and incubated for 24 – 48 h at 26°C (3 plates for each dilution) . Viable cells were enumerated and expressed as colony forming units (CFU/ml).

Microtiter assay for efficacy of biofilm removal

The Microtiter assay was performed according to Pitts et al. (2003) with the following modifications; 200 µl of standardized bacterial suspension was added to the wells of a polystyrene microtiter plate (Lasec, S.A.) and incubated at 26°C with shaking at 100rpm for 48 h. Biofilm formation was monitored periodically by visual inspection. After incubation, the supernatant was discarded and plates were washed three times with 200 µl sterile distilled water to remove non adherent bacterial cells. To each well, 1 U/ml and 2 U/ml of proteases and amylases were added. A well without enzymes was used as control. Plates were incubated for 1 h at 26°C. Following incubation, plates were emptied and washed twice with sterile distilled water. The remaining cells were fixed with 200 µl of 95% ethanol for 15 min and allowed to dry. Crystal violet solution (200 µl) was added into each well for 30 min. Plates were washed five times with sterile distilled water. Wells were washed with 30% glacial acetic acid (200 µl) (Merck, S.A.). Plates were read at 595 nm using a Multiskan Ascent ELISA plate reader (Termo Labsystems). The experiment was performed in duplicate.

The micro titer screening method was used to quantitatively measure the removal efficacy of proteases and amylases on biofilms of *P. fluorescens*. A measure of efficacy called Percentage Reduction by Pitts et al. (2003) was used to evaluate the efficacy of these enzymes.

$$\text{Percentage reduction} = [(C - B) - (T - B))/ (C - B)] \times 100\%$$

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).

Table 1. Properties of the commercial enzymes tested in this study.

Name	Enzyme	Manufacturer	Source	Optima conditions		Application
				pH	Temperature (°C)	
Savinase	Protease	Novozyme	Genetically modified *Bacillus clausi*	8 - 11	15 - 75	Laundry
Everlase	Protease	Novozyme	Genetically modified *Bacillus clausii*	8 - 11	15 - 75	Detergent industry
Polarzyme	Protease	Novozyme	Genetically modified *Bacillus* spp	9 - 11	20 - 40	Detergent industry
*BAN	Alphamylase	Novozyme	*Bacillus amyloliquefaciens*	6 - 7	20 - 60	Food industry
¤AMG	Glucoamylase	Novozyme	*Aspergillus niger*	4 - 5	20 - 60	Food industry

*Bacterial Amylase Novo, ¤Amyloglucosidae.

Biofilm detachment and extraction of extracellular polymeric substances (EPS)

Flasks containing glass wool with attached biofilm cells were vortexed vigorously for 5 min to detach loosely bound biofilm cells. Bacterial aliquot (20 ml) was added to 50 ml sterile centrifuge tubes (Merck). The contents were homogenized for about 30 s using a Cole-Parmer homogenizer at an adjusted output of 50% and spun at 3500 xg for 5 min at 4°C. The supernatants were transferred to sterile centrifuge tubes and further spun at 9000 xg for 30 min, 4°C. Pellets were resuspended in 20 ml sterile distilled water, freeze dried and dissolved in Phosphate and Tris-Maleate buffers, respectively for enzyme assays. Dissolved pellets were filtered through 0.8/0.2 µm filters (Acrodics PF, PA//Inc). Filtrates were assayed for EPS composition and enzyme activity.

Determination of the carbohydrate concentration in the EPS

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

Determination of the protein concentration in the EPS

Protein concentration was determined by the modified method of Lowry (Frøelund et al., 1995). Extracellular polymeric substances (EPS) (10 µl) were added into wells of a micro titter plate. Control wells were added with phosphate buffer. Coomassie plus reagent (300 µl) was added to each well. The plate was incubated at room temperature for 10 min. After incubation, absorbances were read at 595 nm using a Multiskan Ascent V1.24 plate reader, (Amersham). Bovine serum albumin (BSA) was used as a standard to construct a standard curve.

Enzymes used in the study

Activity of the enzymes, listed in Table 1, on biofilm removal was evaluated for biofilm removal. The proteases were: (1) Savinase, (2) Everlase and (3) Porlazyme. The Amylases were: (4) Amyloglucosidase (AMG) and (5) Bacterial Amylo Novo (BAN). All enzymes used were purchased from Novozymes (Ltd) South Africa. Proteases were diluted in 0.1 M Phosphate buffer, pH 8.3. Bacterial Amylo Novo (BAN) was dissolved in 0.2 M Tris-maleate, pH 7.0; and Amyloglucosidase (AMG) was diluted in Phosphate buffer, pH 5.

ENZYMATIC TREATMENTS

Degradation of biofilm EPS

Following protein and carbohydrate analysis, 1 ml of suspended EPS was added into 50 ml centrifuge tubes containing the protease or amylase enzymes diluted in specific buffer solutions. The samples were incubated at 26°C and aliquots were taken at 15 min intervals. For the protease activity, 300 µl of sample was transferred to micro plates and analyzed via the Bradford assay, while the amylase activity was analyzed using the Anthrone assay.

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

Glass wool – attached biofilms from fed and unfed cultures were incubated with enzyme solutions (100 ml) at 26°C for 24 h without agitation. Biofilms with no enzymes were used as control. After the incubation period, the effect of enzymatic activity on the biofilms was evaluated using the Scanning Electron Microscope (SEM).

Sample preparation for Scanning Electron Microscopy

Glass wool samples were fixed in a solution of 2.5% glutaraldehyde in 75 mM Phosphate buffer, pH 7.4 for 1 h. Samples were rinsed three times for 15 min at a time in 50% 75 mM 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).

RESULTS

Growth and viable cells of *Pseudomonas fluorescens*

The rate of *P. fluorescens* growth was maximal after the 6^{th} day of incubation and progressively reached a plateau phase thereafter. *P. fluorescens* growing in the daily fed medium (CF100XNB) was slightly higher than unfed

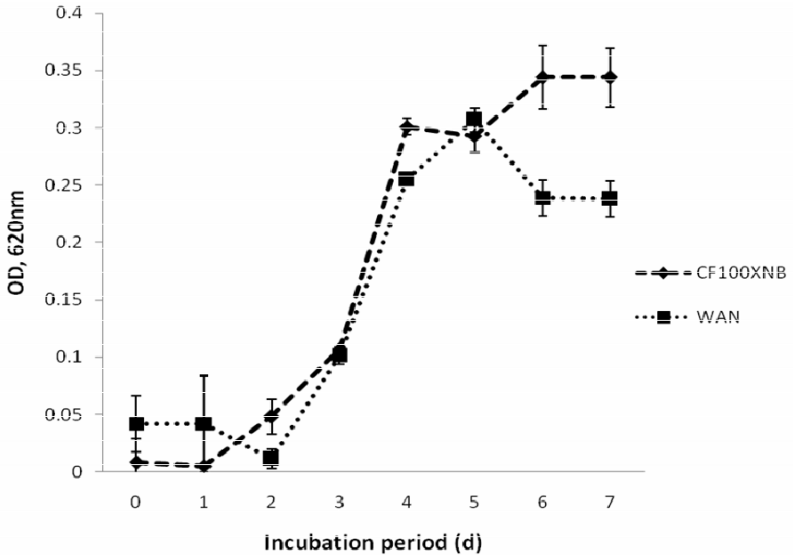

Figure 1. Growth of *Pseudomonas fluorescens* in fed and unfed nutrient medium conditions. Bars indicate standard errors.

Table 2**.** Comparison of viable cells between fed and unfed *Pseudomonas fluorescens* biofilms.

	Viable cells (CFU/ml) ×10^5 Average ± SD *
Fed *P. fluorescens*	1.93 ± 8.485
Unfed *P. fluorescens*	1.76 ± 5.657

*Average ± Standard deviation.

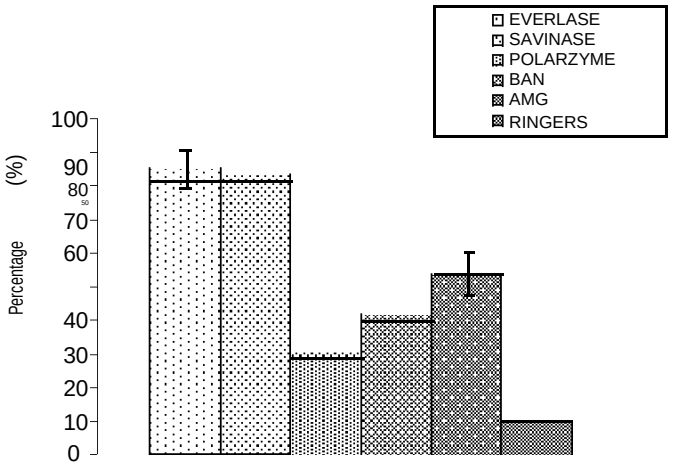

Figure 2. Microtiter assay for the evaluation of enzyme efficacy for the removal of *Pseudomonas fluorescens* biofilms. Bars indicate standard errors.

P. fluorescens (WAN) (Figure 1). Plate count assay results showed slightly more viable cells in the fed *P. fluorescens* growth than the unfed growth of *P. fluorescens* (Table 2).

Microtiter assay for the evaluation of enzyme efficacy for biofilm removal

The micro plate assay was used to determine the activity of enzymes on the reduction of *P. fluorescens* biofilms and on the degradation of extracellular polymeric substances (EPS). Savinase and Everlase showed a highest percentage reduction (Figure 2). This was followed by Amyloglucosidase (AMG) with higher percentage reduction and Bacterial Amylo Novo was less effective with lower percentage reduction. Polarzyme was not effective for removal of *P. fluorescens* biofilms and was comparable to the control (biofilms treated with Ringer's solution (Figure 2).

EPS, proteins and carbohydrate concentrations

The extracellular polymeric substances (EPS) concentration of the biofilm that was fed daily (0.219 g/ml) was slightly higher than the EPS concentration in the unfed biofilms (0.126 g/ml) (Table 3). The protein concentration in both fed and control experiments were higher than carbohydrate concentrations in both experiments (Table 3). However, the protein concentration in

Table 3. Comparison of the production of extracellular polymeric substances (EPS), protein and carbohydrate concentrations of the fed and unfed Pseudomonas fluorescens biofilms

Fed biofilms (Av ± SD)*			Unfed biofilms (Av ± SD)*		
EPS mass (g/OD 620)	**Protein (µg/ml)**	**carbohydrate (µg/ml)**	**EPS mass (g/OD 620)**	**Proteins (µg/ml)**	**Carbohydrate (µg/ml)**
0.219 (± 0.001)	1592 (± 1.989)	119.8 (± 0.004)	0.126 (± 0.023)	1474 (± 1.767)	92.2 (± 0.002)

*Average ± Standard deviation.

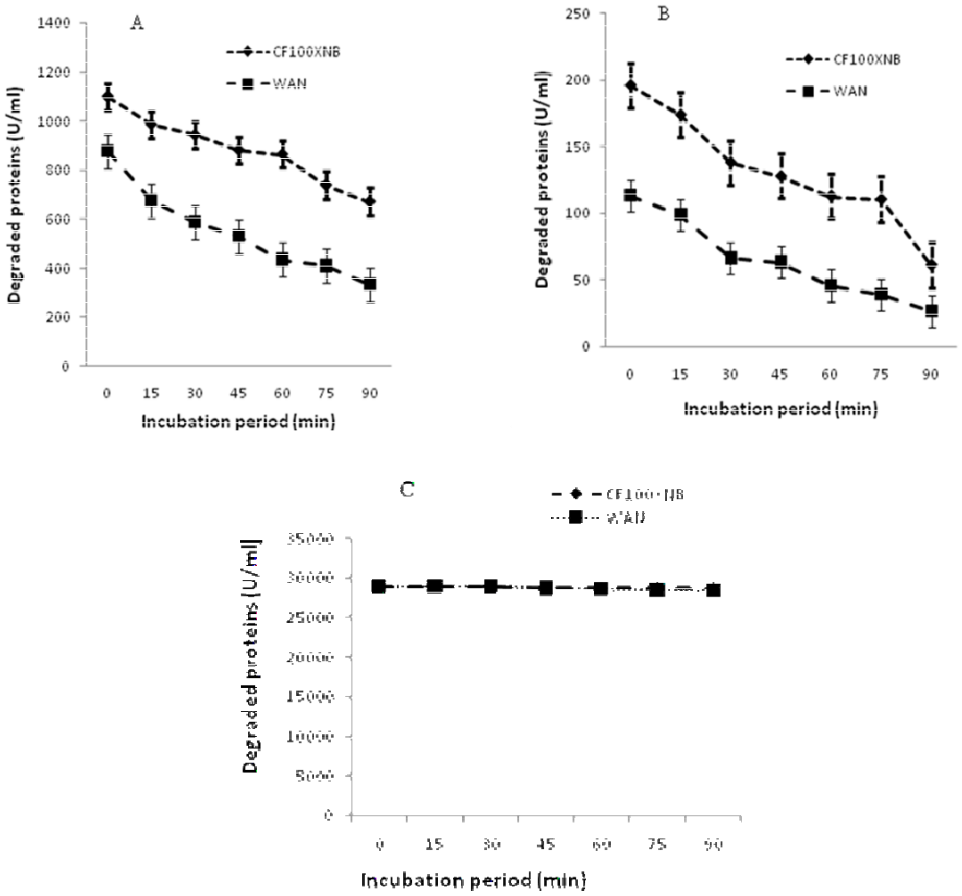

Figure 3. Degradation potential of a. Savinase b**.** Everlase c. Polarzyme on extra cellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* biofilms. Bars indicate standard errors.

the EPS of the biofilm cultured with daily feeding (1592 µg/l) was higher than the protein concentration in the control EPS (1474 µg/l) (Table 3). Similarly, carbohydrate concentrations were higher in the daily fed (119.8 µg/l) than control EPS (92.2 µg/l) (Table 3).

Enzymatic degradation of proteins and carbohydrates in the EPS

The proteases, Savinase and Everlase were the most effective enzymes for the degradation of protein concentration of the extracted EPS (Figures 3A and B). Polarzyme did not show any reduction in the protein concentration (Figure 3C). The amylase

Amiloglucosidase (AMG) was partially effective while the amylase Bacterial Amylase Novo (BAN) was the least effective on carbohydrate degradation in the EPS (Figure 4A and B). The control EPS protein and carbohydrate concentrations remained unaffected (Figure 4C) . Micro-scopic studies of the effect of enzymes on the *P. fluorescens* biofilms revealed that biofilms treated with Savinase and Everlase showed a reduction in biofilm cells and substantial degradation of the extracellular

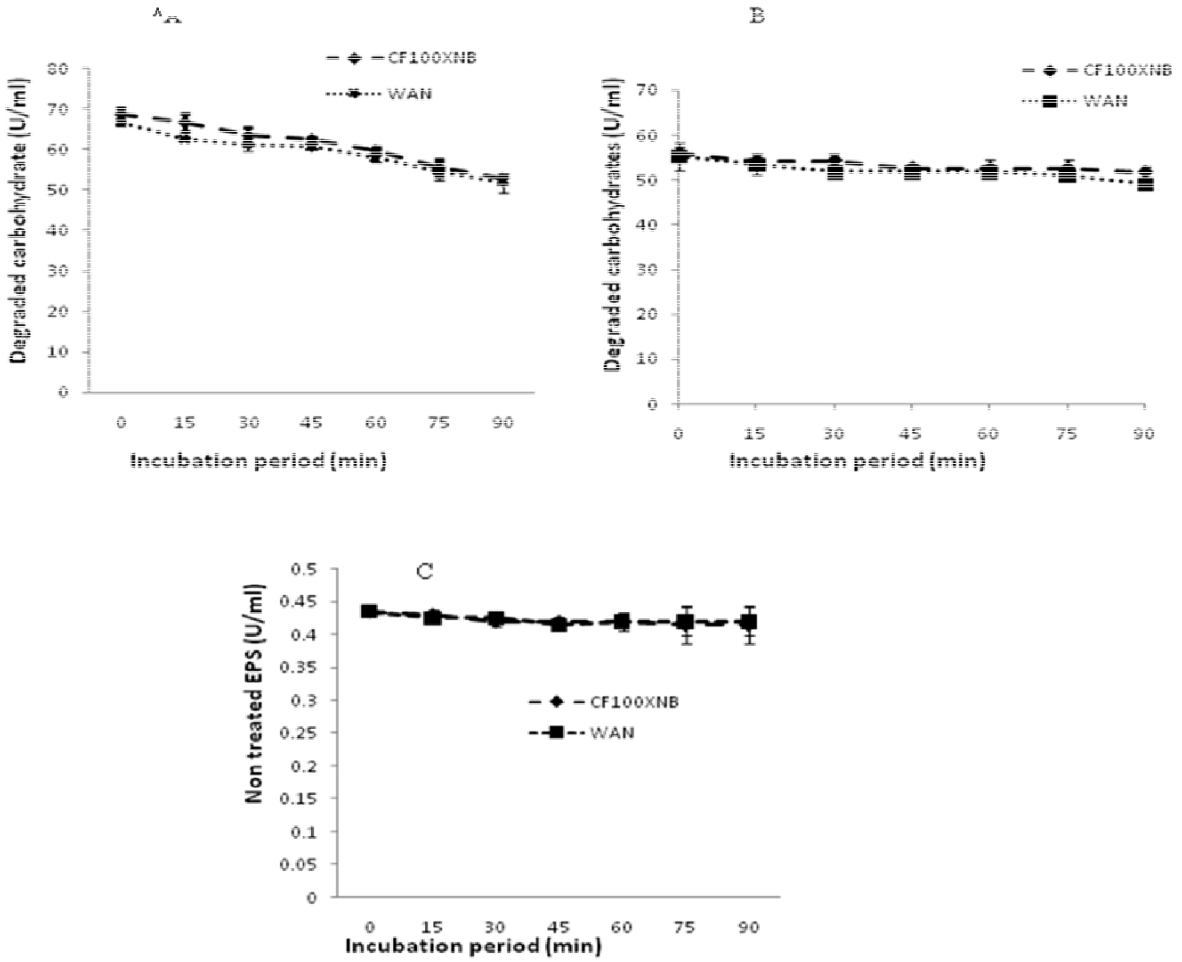

Figure 4. Degradation potential of: A. Amyloglucosidase B. Bacterial Amylase Novo on extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* biofilms C. Non treated EPS. Bars indicate standard errors.

polymeric substances (EPS) (Figures 5A and B) . The amylase Amyloglucosidase and Bacterial Amylase Novo treated biofilms were partially degraded (Figures 5C and D).

DISCUSSION

The effect of nutrient concentration on biofilm yield

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 0.1 g/l of glucose was 90% lower than the biofilm mass in medium containing 0.5 g/l of glucose.

Protein and carbohydrate concentrations in the biofilm EPS

The EPS of *P. fluorescens* 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

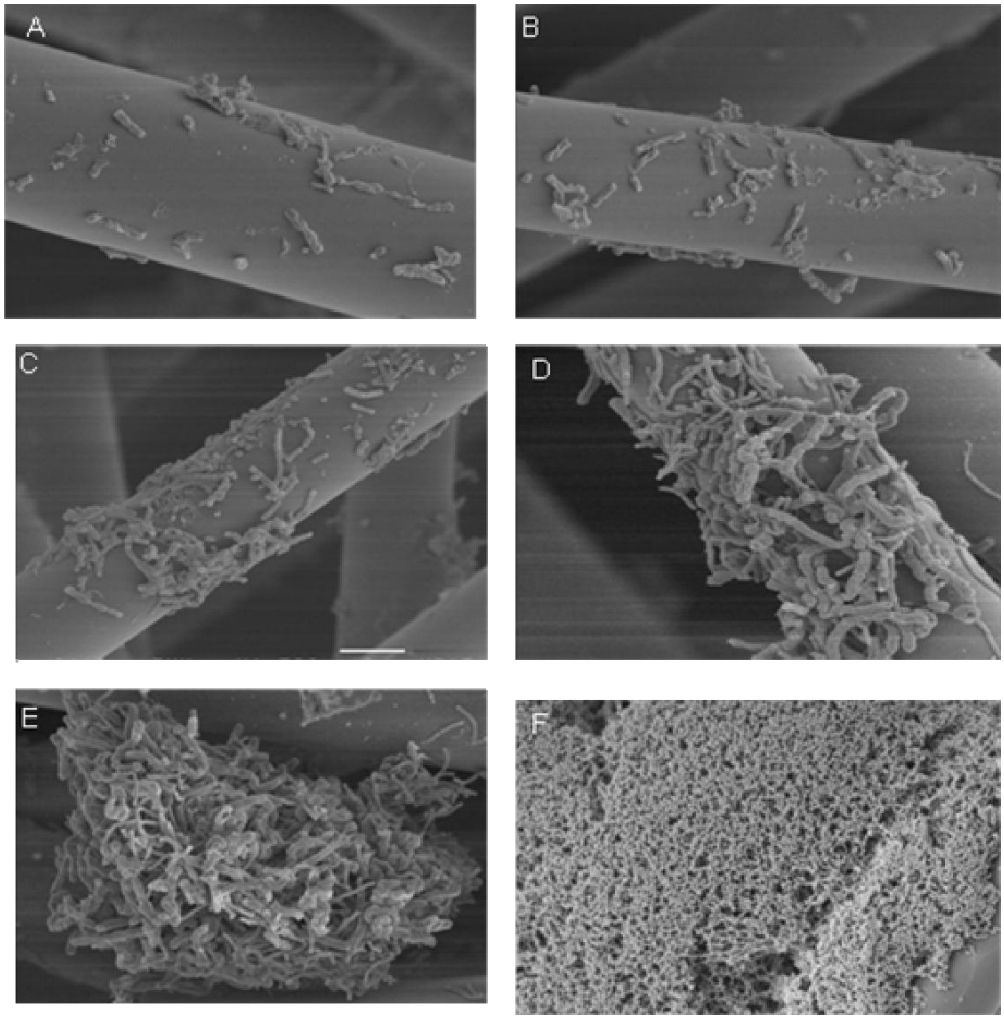

Figure 5. Microscopic analysis of degradation actions of enzymes on extra cellular polymeric substances (EPS) of *Pseudomonas fluorecens* biofilms attached on the glass wool fibers after 24 h incubation at 26˚C. A. Savinase B. Everlase, C. Amyloglucosidase D. Bacterial Amylase Novo E. Polarzyme F. Non treated biofilms.

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 *P. fluorescens* biofilms under specific growth conditions.

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. 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., 2002; Augustus and Ali- Vehmas, 2004). Liu et al. (2002) 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 lyses. 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 con-centrations.

The use of protease and amylase enzymes for the degradation of 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 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 *P. fluorescens* biofilms. All enzymes tested except for the protease Polarzyme were effective for the degradation of the biofilm EPS. Savinase and Everlase were the most effective for the degradation of *P. fluorescens* 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 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 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 *P. fluorescens* and mixed bacterial species biofilm EPS.

Extracellularly secreted proteins are substances with molecular weight between 10 and 200 kDa. 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 et al. (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. (2008) 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 heteoropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type such as D – glucose or L- fructose (Czaczyk et al., 2007). Homopolysaccharides belong to three distinct groups including: – 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 (Sutherland, 2001; Czaczyk et al., 2007). 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* (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 of 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 *P. fluorescens* 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., 2008). 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.

Scanning electron microscopy analysis of enzyme efficiency for EPS degradation

SEM analysis confirmed that protease enzymes (Savinase and Everlase) were more effective than the amylase enzymes for degrading the EPS of *P. fluorescens* (Figure 4).

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. Enzymes differed in activity. Protease enzymes were capable of destroying the "house of the microbes" (EPS). The amylase enzymes were less effective for the degradation of *P. fluorescens* biofilms. 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.

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REFERENCES

Allison DG (2000). Microbial biofilms: Problems of control. In: Allison DG, Gilbert P, Lappin – Scott H Wilson M (Eds) Community structure and cooperation in biofilms.. Cambridge University Press. pp, 309-327

Arevalo-Ferro C, Reil G, Gorg A, Eberl L, Riedel K (2005). Biofilm formation of *Pseudomonas putida* IsoF: The role of quorum sensing as assessed by proteomics. Sys. App. Microbiol., 28: 87-114.

Augustin M, Ali- Vehmas T, Atroshi F (2004). Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. J. Pharm. Pharmaceut., 7: 55-64.

Berry D, Xi C, Raskin L (2006). Microbial ecology of drinking water distribution systems. Cur. Opin. Biotechnol., 17: 297-302.

Bhaskar PV, Bhosle NB (2005). Microbial extracellular polymeric substances in marine biogeochemical processes. Cur. Sci. 88: 47-53.

Cloete TE (2003). Resistance mechanisms of bacteria to antimicrobial compounds. Int. Biodeter.Biodegrad., 51: 272-282.

Cloete TE, Jacobs L, Brozel VS (1998). The chemical control of biofouling in industrial water systems. Biodeter., 9: 23-37.

Czaczyk K, Myszka K (2007). Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilms formation. Pol. J. Environ. Stud., 16: 799-806.

de Carvalho CCCR (2007). Biofilms: Recent developments on an old battle. Patents Biotechnol. 1: 49-57.

Donlan RM (2002). Biofilms: Microbial life on surfaces. Emerg. Infec. Dis., 8: 1-14.

Flemming HC, Neu TR, Wozniak DJ (2007). EPS matrix: The "House of Biofilm cells''. Bacteriol. 189: 7945-7947.

Flemming HC (1998). Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. Pol. Degrad.,Stab. 59: 309-315.

Frøelund B, Keiding K, Nielsen P (1995). Enzymatic activity in the activated sludge flocs matrix. App. Microbiol. Biotechnol., 43: 755-761.

Gaudy AF (1962). Colorimetric determination of protein and carbohydrate. Indus. Water Waste 7: 17-22.

Gomez – Suarez C, van der Borden PJ, Wingender J, Flemming HC (2002). Influence of extracellular polymeric susbatnces deposition and redeposition of *Pseudomonas aeruginosa* to surface. Microbiol., 148: 1161-1169.

Johansen C, Falholt P, Gram L (1997). Enzymatic removal and disinfection of bacterial biofilms. App. Environ. Microbiol., 63: 3724-3728.

Lapidot A, Romling U, Yaron S (2006). Biofilm formation and the survival of *Salmonella typhimurium* on parsley. Int. J. Food Microbiol., 109: 229-233.

Ledder RG, Timperley AS, Friswell MK, MacFarlane S, McBain AJ (2008). Coaggregation between and among human intestinal and oral bacteria. FEMS Microb.Ecol., 66: 630-636.

Lequette Y, Boels G, Clarisse M, 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, Combes D (2008). Effect of commercial enzymes on the adhesion of a marine biofilm forming bacterium. Biofoul. 24: 11-22.

Liu H, Fang HP (2003). Extraction of extracellular polymeric substances (EPS) of sludge. Biotechnol. 95: 249-256.

Liu Y, Yang SF, Li Y, Xu H, Qin L, Tay JH (2004). The influence of cell substratum surface hydrophobicities on microbial attachment. Biotechnol.110: 251-256.

Loiselle M, Anderson KW (2003). The use of cellulose in inhibiting biofilms formation from organisms commonly found on medical implants. Biofoul.19: 77-85.

Melo LF, Bott TR (1997). Biofouling in water systems. Exp. Thermal. Fluid Sci., 14: 375-381.

O'Toole G, Kaplan HB, Kolter R (2000). Biofilm formation as microbial development. An. Rev.Microbiol., 54: 49-79.

Orgaz B, Kives J, Pedregosa AM, Monistrol IF, Laborda F, SanJose C (2006). Bacterial biofilms removal using fungal enzymes. Enz. Microb. Technol., 40: 51-56.

Parkar SG, Flint SH, Brooks JD (2000). Evalualtion of the effect of cleaning regimes on biofilms of the thermophillic bacilli on staineless

steel. App. Microbiol., 96: 110-116.
Pitts B, Hamilton MA, Zelver N, Stewart PS (2003). A microtitter plate screening method for biofilms disinfection and removal. Microbiol. Met., 54: 269-276.
Ploux L, Beckendorff S, Nardin M, Neunlist S (2007). Quantitative and morphological analysis of biofilms formation on self assembled monolayers. Col. Surf., 57: 174-181.
Prakash B, Veeregowda BM, Krishnappa G (2003). Biofilms: A survival strategy of bacteria. Cur. Sci., 85: 9-10.
Rochex A, Lebeault JM (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, de Carvalho H, Pereira MO, Viera MJ (2003). Studies on the behavior of *Pseudomonas fluorescens* biofilms after *Ortho*-phthalaldehyde treatment. Biofoul., 3: 151-157.
Sreenivasan PK, Chorny RC (2005). The effects of disinfectant foam on microbial biofilms. Biofoul., 21: 141-149.
Stoodley LH, Stoodley P (2002). Developmental regulation of microbial biofilms. Cur. Op. Biotechnol., 13: 228-233.
Surtherland IW (2001). Biofilm exopolysaccharides: a strong and sticky framework. Microbiol.147: 3-9.
Ton-That H, Marraffini L, 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. Inf. Con., 32; 170-176.
Walker SM, Fourgialakis M, Cerezo B, Livens S (2007). Removal of microbial biofilms from dispense equipment: Effect of enzymatic pre-digestion and detergent treatment. Inst. Brew., 113: 61-66.
Xavier JB, Picioreanu C, Rani SA, van Loosdrecht MCM, Stewart PS (2005). Biofilm control strategies based on enzymatic disruption of the extracellular polymeric substance matrix- a modeling study. Microbiol., 51: 3817-3832.
Zhang T, Ke SZ, Liu Y, Fang HP (2005). Microbial characteristics of a methanogenic phenol-degrading sludge. Water Sci. Technol., 52: 73-78.