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Proteolytic and amylolytic enzymes for bacterial biofilm control

By

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

Signature: _____

Date: _____

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List of abbreviations

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

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

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Publications

Paper

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Book

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Quotes

- An aim in life is the only fortune worth finding

(Jacqueline Kennedy Onassis)

- All personal achievements start within the mind of the individual

(W Clement Stone)

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

(Brian Tracy)

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

(Julia Cameron)

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

(Johann Wolfgang Von Geothe)

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Dedications

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

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Summary

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

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

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

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

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

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

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

Conclusion

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

Recommendation

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

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