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Detachment of single- and multi-species bacterial biofilms by crude enzymes extracted from wastewater biofilms and bacteria

by

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Submitted in partial fulfilment of the requirements of the degree
Master of Science
in the Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and had not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …………………………………….. Date: …………………………..

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“There is hope in dreams, imagination, and in the courage of those who wish to make those dreams a reality” Jonas Salk

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SUMMARY

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Biofilms are bacterial communities that adhere to biotic and abiotc surfaces, and are embedded in a polymeric matrix composed mainly of polysaccharides and proteins. Not only are biofilms a public health problem, but they are also a hindrance in industrial practices. Due to their intractability by conventional cleaning agents, a number of alternative agents, including enzymes, have been investigated as potential biofilm detachment-promoting agents. Two major types of enzymes, *i.e.* proteases and polysaccharases, have been used for biofilm removal and their use is aimed at degrading or promoting the collapse of the biofilm matrix. Consequently, the aim of this investigation was primarily to assess the use of enzymes originating from a wastewater biofilm to remove biofilms from three *Pseudomonas* species, *viz. P. aeruginosa* PAO1, *P. fluorescens* and *P. putida*.

To investigate, biofilms were sampled from an aerobic reactor at an industrial wastewater treatment plant. Dissolution of the biofilm, as evidenced by reductions in the soluble chemical oxygen demand (COD) and total suspended solids (TSS), coincided with detectable protease and carbohydrate-degrading enzyme activities. Crude extracellular enzyme extracts prepared from the wastewater biofilm were subsequently shown to remove *P. aeruginosa* PAO1 biofilms from a glass surface, suggesting that the wastewater biofilms expressed enzymes that may be used towards the removal of detrimental biofilms. Consequently, representative bacteria were isolated from the wastewater biofilm and, based on 16S rRNA gene sequencing and analyses, were found to represent four major phylogenetic divisions of bacteria, *i.e.* Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. Screening of the bacterial isolates for different enzyme activities indicated that nine isolates produced proteases, while ten isolates produced polysaccharide-degrading enzymes that comprised amylase, xylanase, cellulase, α-glucosidase and β-glucosidase. The ability of these enzymes to degrade proteins and polysaccharides present in purified EPS from *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens* was confirmed by SDS-polyacrylamide gel electrophoresis and an increase in the amount of reducing sugar, respectively, while their efficacy to remove single- and multi-species biofilms cultured in microtiter plates was evaluated using a quantitative spectrophotometric assay.

Proteases produced by four of the strains were effective in degrading the EPS proteins of all three *Pseudomonas* spp., while all bacterial strains that produced polysaccharide-degrading enzymes were capable of degrading the EPS polysaccharides, albeit with different efficiencies. Efficient removal of *P. aeruginosa* PAO1 biofilms was only achieved when mixtures of enzyme extracts, containing protease and different types of polysaccharase activities, were used. Biofilms of *P. putida* and *P. fluorescens* were readily removed with single enzyme extracts prepared from *B. subtilis* and *B. pumilus.* Enzyme combinations showing high biofilm removal for all three *Pseudomonas* species were tested against a mixed species biofilm. These enzyme extracts yielded lower biofilm removal efficiencies than those obtained for mono-species pseudomonad biofilms, possibly due to the heterogenous nature of the EPS. Nevertheless, it may be possible that the enzymes identified in this study could be used in combination with other treatments to increase the biofilm removal effectiveness or in combination with other enzymes to degrade the mixture of proteins and polysaccharides present in the EPS of multi-species biofilms.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Acyl-HSLs	Acyl-homoserine lactones
amu	atomic mass units
bp	base pair
BSA	bovine serum albumin
C	carboxy
ca.	circa approximately
CFU	colony forming units
COD	chemical oxygen demand
CTAB	cetyltrimethylammonium bromide
dH_2O	distilled water
DNA	deoxiribonucleic acid
DNS	dinitrosalicylic acid
dNTP	deoxyribonucleoside-5’-triphosphate
DPAs	detachment-promoting agents
DTT	dithiothreitol
e.g.	for example
EDTA	ethylenediamine-tetra-acetic acid
EGTA	ethyleneglycol-tetra-acetic acid
EPS	extracellular polymeric substances
Fig.	figure
g	gram
Gal	galactose
Glc	glucose
h	hour
kDa	kilodalton
l	litre
LPS	lipopolysaccharide
M	molar
m^3	cubic meter
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimeter

mM	millimolar
N	amino
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppm	parts per million
QACs	quaternary ammonium compounds
QPCs	quaternary phosphonium compounds
Rha	rhamnose
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TCA	trichloroacetic acid
TEMED	N,N,N’,N’-tetramethyl-ethylenediamine
THM	trihalomethane
TSS	total suspended solids
U	unit
V	volts
v.	version
v/v	volume per volume
w/v	weight per volume
α	alpha
β	beta
%	percentage
× *g*	centrifugal force
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometer

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

In the vast majority of ecological niches, bacteria can grow in association with surfaces, which leads to the formation of biofilms (Costerton *et al.*, 1995; Flemming, 2002). Biofilms have been defined as structured communities of bacterial cells that are enclosed in a self-produced polymeric matrix and adhere to biotic and abiotic surfaces, an interface or each other (Costerton *et al.*, 1995). Biofilm formation occurs in response to a variety of environmental signals (Stanley and Lazazzera, 2004) that leads to a number of changes in gene regulation that cause the adhering cells to become phenotypically (Sauer *et al.*, 2002) and metabolically (Davey and O'Toole *et al.*, 2000) distinct from their planktonic counterparts. Indeed, the biofilm mode of growth appears to be the preferred mode of bacterial growth in natural environments, whereas the planktonic mode of growth is seen to play a role in the transport of bacteria to desirable environments (Watnick and Kolter, 2000). The ability of bacteria to form biofilms provides its members with a number of benefits. In addition to increased resistance to environmental changes (Jefferson, 2004) and antibiotics (Drenkard, 2003), the biofilm bacteria may also benefit from a number of properties of communal existence, including division of metabolic burden (Geesey, 2001), gene transfer (Ghigo, 2001) and altruistic behaviour (Kreft, 2004).

It has been suggested that biofilm formation occurs as a sequential development process (O'Toole *et al.*, 2000; Stoodley *et al.*, 2002). Current models, based largely on *Pseudomonas aeruginosa*, depict biofilm formation commencing when planktonic bacterial cells attach irreversibly to a surface. This attachment is followed by growth into a mature complex biofilm and culminates in the dispersion of detached bacterial cells into the bulk fluid. The bacteria within each of the stages of biofilm development are believed to be physiologically distinct from cells in other stages, and the most profound differences have been observed when planktonic cells were compared to biofilm cells (Sauer *et al.*, 2002). Typically, biofilm bacteria differ from their planktonic counterparts in the genes they express (Prigent-Combaret *et al.*, 1999; Sauer and Camper, 2001; Whiteley *et al.*, 2001; Beloin *et al.*, 2004), the rate at which the cells grow (Sternberg *et al.*, 1999; Werner *et al.*, 2004), and their resistance to antimicrobial agents (Mah and O'Toole, 2001; Stewart and Costerton, 2001).

Frequently, biofilms are unwanted and may result in various problems. These may include dental plaque (Rosan and Lamont, 2000), medical implant-associated infections (Schierholz and Beuth, 2001) and corrosion of pipes in the oil and water industries (Videla and Herrera, 2005). In addition, biofilms may harbour pathogens in drinking water distribution systems (Stark *et al.*, 1999), and food spoilage and pathogenic bacteria on food contact surfaces (Kumar and Anand, 1998; Chmielewski and Frank, 2003). Given the medical and economical consequences of biofilms, it is therefore important to develop strategies whereby their formation can be prevented or, once formed, they can be removed.

Several approaches, based primarily on physical or chemical technology, are typically used to control biofilms. Chemical techniques rely on the use of biocide products, oxidant compounds (chlorine, chlorine dioxide and ozone) or synthetic non-oxidant compounds (bactericides and fungicides) (Cloete *et al.*, 1998; Meyer, 2003; Freese and Nozaic, 2004; Dosti *et al.*, 2005). The resistance of biofilms against various biocides and antibiotics (de Beer *et al.*, 1994; Norwood and Gilmour, 2000; Anderl *et al.*, 2000; Kostenko *et al.*, 2007), however, makes their control problematic. In addition, many of the compounds in use have been reported to produce by-products that are toxic or have irritant properties (Augustin and Ali-Vehmas, 2004). In this regard, enzymes may be good anti-biofilm agent candidates due to their biodegradability and weak toxicity (Leroy *et al.*, 2008). Indeed, the potential of enzymatic preparations against biofilm formation associated with pathogenic bacteria has already been shown (Johansen *et al.*, 1997; Kaplan *et al.*, 2004; Itoh *et al.*, 2005), as well as against dental biofilm (Hahn Berg *et al.*, 2001) and industrial biofilms (Walker *et al.*, 2007; Orgaz *et al.*, 2007). The enzyme preparations typically prevent adhesion and remove adhered biofilms by degrading the extracellular polymeric substances (EPS) produced by biofilms. The EPS, which comprises polysaccharides, proteins, nucleic acids, lipids and various cellular debris (Sutherland, 2001; Allison, 2003; Tsuneda *et al.*, 2003), fulfils functions such as providing an adhesive foundation and structural integrity (Hentzer *et al.*, 2001; Allison, 2003), and therefore constitute an important target for the detachment and removal of unwanted biofilms.

The aims of this investigation were therefore the following:

- To determine whether hydrolysed wastewater biofilms contain enzymes that can be used towards removing artificial *P. aeruginosa* PAO1 biofilms.

- To isolate and identify representative bacterial strains from a hydrolysed wastewater biofilm, and to screen the bacterial strains for protease and selected polysaccharase enzymes.
- To assess the effect of crude enzyme extracts produced by the isolated bacteria on the extracellular polymers produced by *P. aeruginosa* PAO1, *P. fluorescens* and *P. putida*.
- To assess the efficacy of crude enzyme extracts produced by the isolated bacteria, singularly and in combination, to remove single-species biofilms (*P. aeruginosa* PAO1, *P. fluorescens* and *P. putida*) and multi-species biofilms.

REFERENCES

- Allison, D.G. 2003. The biofilm matrix. *Biofouling*, **19**: 139-150.
- Anderl, J.N., Franklin, M.J. and Stewart, P.S. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, **44**: 1818-1824.
- Augustin, M. and Ali-Vehmas, T. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmacy and Pharmaceutical Sciences*, **7**: 55-64.
- Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J.A.J., Molin, S., Prensier, G., Arbeille, B. and Ghigo, J-M. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular Microbiology*, **51**: 659-674.
- Chmielewski, R.A.N. and Frank, J.F. 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety*, **2**: 22-32.
- Cloete, T.E., Jacobs, L. and Brözel, V.S. 1998. The chemical control of biofouling in industrial water systems. *Biodegradation*, **9**: 23-37.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual Reviews in Microbiology*, **49**: 711-745.
- Davey, M.E. and O'Toole, G.A. 2000. Microbial biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, **64**: 847-867.
- de Beer, D., Srinivasan, R. and Stewart, P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology*, **60**: 4339-4344.
- Dosti, B., Guzel-Seydim, Z. and Greene, A.K. 2005. Effectiveness of ozone, heat and chlorine for destroying common food spoilage bacteria in synthetic media and biofilms. *International Journal of Dairy Technology*, **58**: 19-24.

- Drenkard, E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes and Infection*, **5**: 1213-1219.
- Flemming, H-C. 2002. Biofouling in water systems – cases, causes and countermeasures. *Applied Microbiology and Biotechnology*, **59**: 629-640.
- Freese, S.D. and Nozaic, D.J. 2004. Chlorine: Is it really so bad and what are the alternatives? *Water SA*, **30**: 18-24.
- Geesey, G.G. 2001. Bacterial behavior at surfaces. *Current Opinion in Microbiology*, **4**: 296-300.
- Ghigo, J-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature*, **412**: 442-445.
- Hahn Berg, I.C., Kalfas, S., Malmsten, M. and Arnebrant, T. 2001. Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: Potential of proteases originating from *Euphausia superba* for plaque control. *European Journal of Oral Science*, **109**: 316-324.
- Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M. and Parsek, M.R. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *Journal of Bacteriology*, **183**: 5395-5401.
- Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F. and Romeo, T. 2005. Depolymerization of β-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology*, **187**: 382-387.
- Jefferson, K.K. 2004. What drives bacteria to produce a biofilm? *FEMS Microbiology Letters*, **236**: 163-173.
- Johansen, C., Falholt, P. and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology*, **63**: 3724-3728.
- Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H. and Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents and Chemotherapy*, **48**: 2633-2636.
- Kostenko, V., Ceri, H. and Martinuzzi, R.J. 2007. Increased tolerance of *Staphylococcus aureus* to vancomycin in viscous media. *FEMS Immunology and Medical Microbiology*, **51**: 277-288.
- Kreft, J-U. 2004. Biofilms promote altruism. *Microbiology*, **150**: 2751-2760.
- Kumar, C.G. and Anand, S.K. 1998. Significance of microbial biofilms in food industry: A review. *International Journal of Food Microbiology*, **42**: 9-27.
- Leroy, C., Delbarre, C., Ghillebaert, F., Compere, C. and Combes, D. 2008. Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. *Biofouling*, **24**: 11-22.

- Mah, T-F. and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, **9**: 34-39.
- Meyer, B. 2003. Approaches to prevention, removal and killing of biofilms. *International Biodeterioration and Biodegradation*, **51**: 249-253.
- Norwood, D.E. and Gilmour, A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology*, **88**: 512-520.
- O'Toole, G.A., Kaplan, H.B. and Kolter, R. 2000. Biofilm formation as microbial development. *Annual Reviews in Microbiology*, **54**: 49-79.
- Orgaz, B., Neufeld, R.J. and SanJose, C. 2007. Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. *Enzyme and Microbial Technology*, **40**: 1045-1051.
- Prigent-Combaret, C., Vidal, O., Dorel, C. and Lejeune, P. 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *Journal of Bacteriology*, **181**: 5993-6002.
- Rosan, B. and Lamont, R.J. 2000. Dental plaque formation. *Microbes and Infection*, **2**: 1599-1607.
- Sauer, K. and Camper, A.K. 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *Journal of Bacteriology*, **183**: 6579-6589.
- Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W. and Davies, D.G. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology*, **184**: 1140-1154.
- Schierholz, J.M. and Beuth, J. 2001. Implant infections: A haven for opportunistic bacteria. *Journal of Hospital Infection*, **49**: 87-93.
- Stanley, N.R. and Lazazzera, B.A. 2004. Environmental signals and regulatory pathways that influence biofilm formation. *Molecular Microbiology*, **52**: 917-924.
- Stark, R.M., Gerwig, G.J., Pitman, R.S., Potts, L.F., Williams, N.A., Greenman, J., Weinzweig, I.P., Hirst, T.R. and Millar, M.R. 1999. Biofilm formation by *Helicobacter pylori*. *Letters in Applied Microbiology*, **28**: 121-126.
- Sternberg, C., Christensen, B.B., Johansen, T., Nielsen, A.T., Andersen, J.B., Givskov, M. and Molin, S. 1999. Distribution of bacterial growth activity in flow-chamber biofilms. *Applied and Environmental Microbiology*, **65**: 4108-4117.
- Stewart, P.S. and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet*, **358**: 135-138.
- Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. 2002. Biofilms as complex differentiated communities. *Annual Reviews in Microbiology*, **56**: 187-209.

- Sutherland, I.W. 2001. Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology*, **147**: 3-9.
- Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiology Letters*, **223**: 287-292.
- Videla, H.A. and Herrera, L.K. 2005. Microbiologically influenced corrosion: Looking to the future. *International Microbiology*, **8**: 169-180.
- Walker, S.L., Fourgialakis, M., Cerezo, B. and Livens, S. 2007. Removal of microbial biofilms from dispense equipment: The effect of enzymatic pre-digestion and detergent treatment. *Journal of the Institute of Brewing*, **113**: 61-66.
- Watnick, P. and Kolter, R. 2000. Biofilm, city of microbes. *Journal of Bacteriology*, **182**: 2675-2679.
- Werner, E., Roe, F., Bugnicourt, A., Franklin, M.J., Heydorn, A., Molin, S., Pitts, B. and Stewart, P.S. 2004. Stratified growth in *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, **70**: 6188-6196.
- Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S. and Greenberg, E.P. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature*, **413**: 860-864.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOFILM FORMATION

Pseudomonas aeruginosa, a motile rod-shaped Gram-negative bacterium that is found in a variety of environments, is one of the most studied biofilm-forming organisms. Due to the analysis of *P. aeruginosa* biofilms using genetic (O'Toole and Kolter, 1998a; Whiteley *et al.*, 2001), proteomic (Sauer and Camper, 2001; Sauer *et al.*, 2002) and molecular biological (Tolker-Nielsen *et al.*, 2000; De Kievit *et al.*, 2001a; Klausen *et al.*, 2003; Lewandowski and Beyenal, 2007) approaches, much information regarding the development of bacterial biofilms has been gained. In addition, biophysical, structural and chemical analysis of the bacterial biofilms has led to a basic model for biofilm structure (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000). The following information regarding biofilm development is therefore based on the information obtained from studies on *P. aeruginosa.* While the exact mechanism may differ from bacterium to bacterium, the stages of biofilm development, as highlighted below, appear to be conserved among a wide range of microbes (O'Toole *et al.*, 2000; Davey and O'Toole, 2000) (Fig. 2.1).

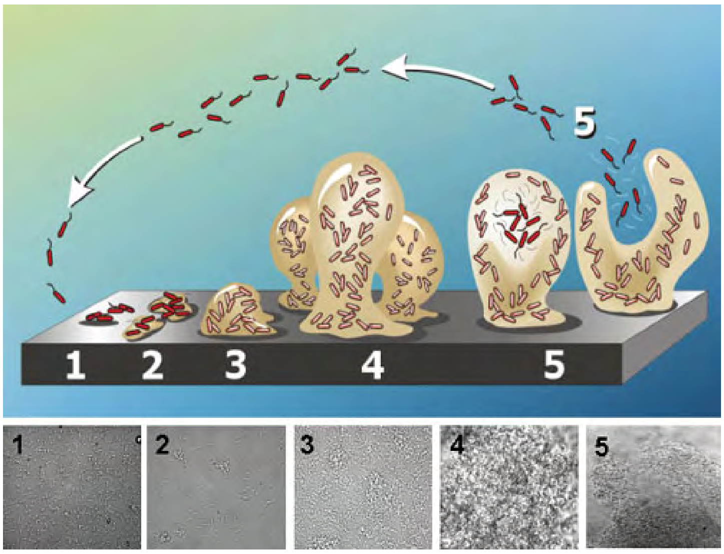


Figure 2.1. Diagram depicting stages involved in the formation of a biofilm. 1, reversible attachment of the bacterial cells to the substratum. 2, irreversible attachment, which is assisted by exopolymeric substances and cells in this stage lose their motility. 3, primary maturation stage, which is associated with the formation of an early biofilm architecture. 4, secondary maturation stage, which leads to fully mature biofilms with complex biofilm architecture. 5, dispersion stage where motile cells (dark cells on the figure) detach from microcolonies. Shown below the diagram are photomicrographs (1-5) illustrating the five stages in biofilm formation of *P. aeruginosa* grown on a glass surface under continuous-flow conditions (Stoodley *et al.*, 2002).

2.1.1 Reversible attachment

Prior to surface colonization, a preconditioning film, composed of polysaccharides, proteins, lipids, humic acids, nucleic acids and aromatic amino acids, is believed to form on the attachment surface, thus resulting in a nutritionally rich zone that is metabolically favourable for bacterial cells (Beveridge *et al.*, 1997; Murga *et al.*, 2001; Siboni *et al.*, 2007). Once a surface has been conditioned, its properties are permanently altered so that the affinity of an organism for a native or conditioned surface can be quite different (Boland *et al.*, 2000). Planktonic bacteria can be brought into close approximation of the conditioned surface by either Brownian motion, active bacterial movement, liquid flow or sedimentation (van Loosdrecht *et al.*, 1990). Initial attachment of the bacteria to the conditioned surface is facilitated by electrostatic and hydrophobic interactions, van der Waals forces and specific interactions, or a combination of these, depending on the proximity of the bacterium to the attachment surface (van Loosdrecht *et al.*, 1990; Habash and Reid, 1999) (Fig. 2.2). Since the initial attachment is unstable, many of the bacterial cells may detach from the surface and resume the planktonic lifestyle (Sauer *et al.*, 2002).

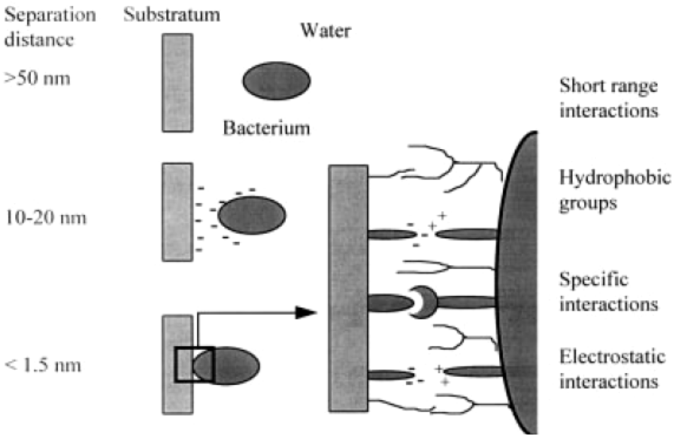


Figure 2.2. Forces involved in attachment of bacteria to a surface. When the bacterium is greater than 50 nm away from the substratum, attractive van der Waals forces arise. At a distance of 10 to 20 nm, attachment is affected by van der Waals forces and electrostatic interactions. Different short-range interactions may occur when the distance from the substratum is less than 1.5 nm (Habash and Reid, 1999).

2.1.2 Irreversible attachment

If environmental conditions are favourable for bacterial attachment, cells may switch from reversible attachment to a more stable irreversible attachment. During this phase, the bacteria produce exopolysaccharides that result in the formation of organic bridges between the cells and substratum (Allison *et al.*, 2000; Lewandowski and Beyenal, 2007). Transition from reversible to irreversible attachment is also mediated by pili and fimbriae (Pratt and Kolter, 1998; O'Toole and Kolter, 1998a; Vidal *et al.*, 1998; Inoue *et al.*, 2003). Whereas flagella are responsible for the movement and initial attachment of bacterial cells to the surface (O'Toole and Kolter, 1998a; Gavín *et al.*, 2003; Lemon *et al.*, 2007), pili (type IV) are required for cell-to-cell interactions and the formation of microcolonies that help strengthen the degree of attachment to a surface (O'Toole and Kolter, 1998a; Semmler *et al.*, 1999). Moreover, based on the observation that *P. fluorescens* WCS365 was unable to attach to surfaces following treatment with proteases (O'Toole and Kolter, 1998b), subsequent studies have shown that membrane proteins may also assist in the attachment of bacteria to surfaces (Espinosa-Urgel *et al.*, 2000; Hinsa *et al.*, 2003; Finelli *et al.*, 2003).

2.1.3 Biofilm maturation

Once bacteria have attached irreversibly to the surface, the process of biofilm maturation begins. During this process, binary division of irreversibly attached cells causes the daughter cells to spread outward and upward from the attachment point to form macrocolonies or cell clusters (Tolker-Nielsen *et al.*, 2000). Alternatively, the attached cells may be redistributed by twitching motility (O'Toole and Kolter, 1998a; Klausen *et al.*, 2003) and/or cells may be recruited from the bulk fluid to the developing biofilm (Tolker-Nielsen *et al.*, 2000). The nature of the surface that is being colonized and the physicochemical conditions of the environment will determine which of the mechanisms of biofilm formation will dominate (Stoodley *et al.*, 2002). The well-developed biofilm (Fig. 2.3a) is characterized by mushroom- or pillar-like structures that are interspersed with fluid-filled channels (Costerton *et al.*, 1995; Tolker-Nielsen *et al.*, 2000), and once fully developed, a biofilm generates altered patterns of bacterial growth, physiological cooperation and metabolic efficiency (Costerton *et al.*, 1995; Geesey, 2001; Werner *et al.*, 2004). Recently, a model for the formation of mushroom-like biofilm structures by *P. aeruginosa* has been proposed by Klausen *et al.* (2003). This model proposes that the bacteria are capable of constructing mushroom-shaped structures. The propagation of non-motile bacteria results in the formation of the mushroom stalk, while motile bacteria use their type IV pili to move up the stalk and form the mushroom cap (Fig. 2.3b).

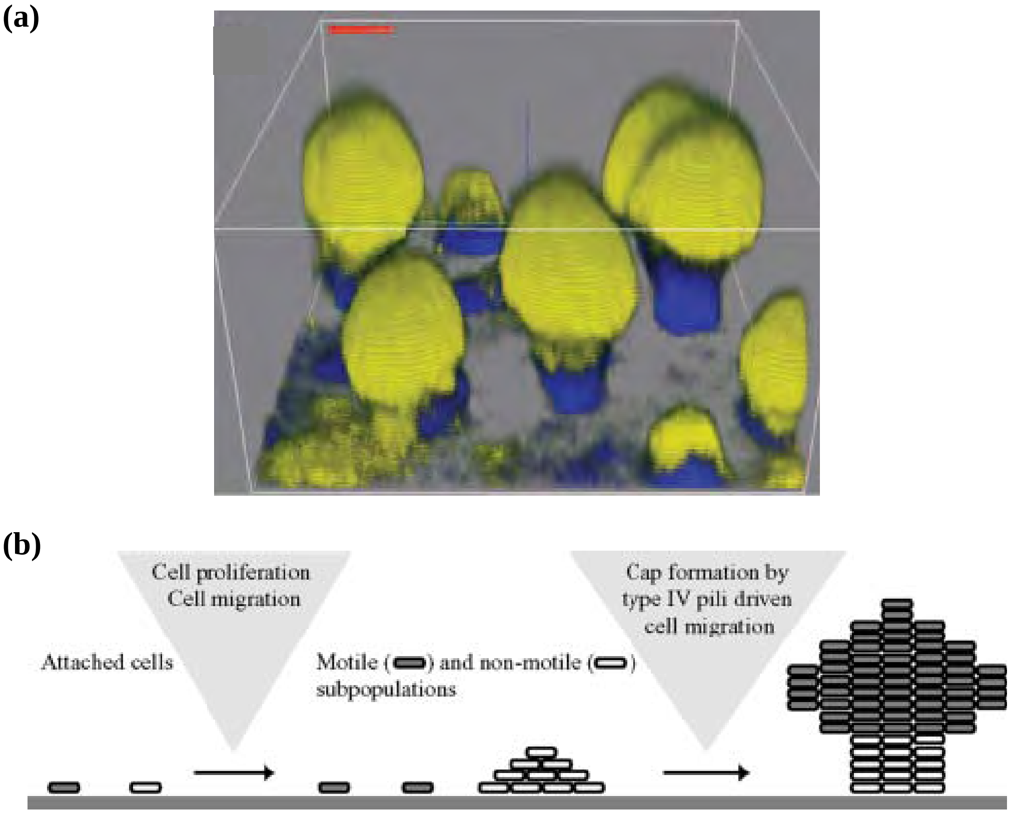


Figure 2.3. Biofilm architecture of *P. aeruginosa*. Confocal laser scanning microscopy (CLSM) images of mushroom-like biofilm structures formed by *P. aeruginosa* are shown in (a), while a model for development of the *P. aeruginosa* biofilm, as proposed by Klausen *et al.* (2003), is presented in (b).

2.1.4 Detachment

The growth potential of the bacterial biofilm is ultimately limited by the availability of nutrients in the immediate environment, the expression of quorum sensing molecules released in response to nutrient limitation, accumulation of toxic by-products and other factors, including pH, oxygen perfusion, carbon source availability and osmolarity (Puskas *et al.*, 1997; Allison *et al.*, 1998; Prigent-Combaret *et al.*, 2001; Sauer *et al.*, 2004; Hunt *et al.*, 2004). Consequently, the biofilm may reach a critical mass and a dynamic equilibrium is reached at which the cells may detach and, together with progeny of other biofilm cells, may colonize other surfaces.

Different mechanisms for biofilm dissolution and consequently, cell dispersal have been proposed. Several recent studies have reported pronounced activity and cellular differentiation localized to the center of mature biofilm structures, which lead to the dispersal of cells from inside the structure, leaving behind large transparent cavities comprised of non-motile cells (Sauer *et al.*, 2002; Tolker-Nielsen *et al.*, 2000). Enzymes such as polysaccharide lyases that degrade the exopolysaccharide matrix have been reported to play a role in biofilm dissolution of *P. fluorescens* (Allison *et al.*, 1998) and *P. aeruginosa* (Boyd and Chakrabarty, 1994), thereby aiding in the dispersal of the bacteria. Moreover, host-specific bacteriophages, which are capable of synthesizing polysaccharide depolymerases that are also responsible for degrading exopolysaccharides, have been reported to aid in the release of bacterial cells from the outer layer of the biofilm matrix (Hughes *et al.*, 1998a; 1998b). In the case of *P. aeruginosa*, death of a subpopulation of cells has been observed to be a normal feature of biofilm development (Webb *et al.*, 2003). Cell death occurred inside microcolony structures, and killed only a subpopulation of the biofilm cells. The cell death was linked to the expression of a Pf1-like filamentous prophage of *P. aeruginosa* and it was suggested that prophage-mediated cell death can assist in the spreading of a subpopulation of surviving cells (Webb *et al.*, 2003).

Biofilms may also be detached from surfaces by physical forces, *i.e.* shearing (continuous removal of small portions of the biofilm), sloughing (rapid removal of large portions of the biofilm) and abrasion (detachment due to collision of particles in the bulk fluid with the biofilm) (Characklis and Marshall, 1990; Donlan, 2002). Sloughing is thought to result from oxygen or nutrient depletion within the biofilm structure and is commonly observed with thick biofilms that have developed in nutrient-rich environments (Characklis and Marshall, 1990; Rice *et al.*, 2005a). In contrast, biofilms growing in fluidized beds, filters and particle-laden environments (surface waters) may be more prone to abrasion (Characklis and Marshall, 1990). Shearing and abrasion cause biofilm removal over the entire biofilm surface, but are not responsible for removal up to the attachment surface. Sloughing, on the other hand, allows for removal of biofilms up to the attachment surface, but does not occur over the entire biofilm surface (Morgenroth and Wilderer, 2000).

2.2 FACTORS INFLUENCING BIOFILM FORMATION

Several diverse factors contribute to bacterial attachment and the subsequent development of mature biofilms. The attachment of bacterial cells to a surface is influenced by the properties of the surface itself, environmental conditions, bacterial cell surface hydrophobicity, presence of surface appendages (*e.g.*, flagella and pili), outer membrane proteins and production of extracellular polymeric substances (EPS) (Allison *et al.*, 2000; Prakash, 2003; Ghannoum and O’Toole, 2004). In addition, evidence suggests that the primary development of a biofilm might be regulated at the level of population density-dependent gene expression controlled by cell-to-cell signaling molecules (Allison *et al.*, 1998; Davies *et al.*, 1998; Parsek and Greenberg, 2005).

2.2.1 Attachment surface

As indicated previously, the formation of a conditioning film can lead to a change in the chemical properties of the surface, thus affecting the number of cells capable of attaching to the surface (Beveridge *et al.*, 1997; Siboni *et al.*, 2007). In addition, the nature of a surface, especially its roughness and physicochemical properties, can also influence the number of cells that may attach to the surface (Characklis and Marshall, 1990; Keevil *et al.*, 1999; Pasmore *et al.*, 2002). Rough surfaces are preferred over smooth surfaces for biofilm formation since they not only present a greater surface area to which bacterial cells can adhere, but the attached bacteria also experience less friction with the bulk fluid (Percival *et al.*, 1999; Carlén *et al.*, 2001; Pasmore *et al.*, 2001; Morgan and Wilson, 2001). It has, however, been noted that a rough surface might only assist in the attachment of the first cells to the surface and that it does not have any further effect on later attachment events (Keevil *et al.*, 1999). Moreover, bacteria are capable of colonizing hydrophobic surfaces (*e.g.*, Teflon and other plastics) more readily than hydrophilic surfaces (*e.g.*, glass and metals) (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Bendinger *et al.*, 1993). The removal of biofilm bacteria from surfaces also appears to be affected by the surface charge. Pasmore *et al.* (2002) demonstrated that surfaces with neutral or low negative charges allowed for easy removal of biofilms, while surfaces with high positive or negative charges harboured biofilms that were difficult to remove.

2.2.2 Environmental conditions

Biofilms can form under diverse nutrient conditions, ranging from high to almost non-detectable nutrient levels (Prakash *et al.*, 2003). Although it has been reported that biofilms are more abundant, densely packed and thicker in environments with high nutrient levels (Cowan *et al.*, 1991), the precise role of nutrient availability in biofilm development has yet to be determined. It has been reported that high nutrient levels promote the transition of bacterial cells from the planktonic to biofilm mode of growth (O'Toole *et al.*, 2000; Murga *et al.*, 2001), while depletion of nutrients causes detachment of biofilm cells from surfaces (Allison *et al.*, 1998; Hunt *et al.*, 2004; Rice *et al.*, 2005a). Other studies, however, have shown that high nutrient levels can lead to the detachment of *P. aeruginosa* (Sauer *et al.*, 2004) and *P. putida* (Rochex and Lebeault, 2007) biofilms from surfaces. Moreover, Oh *et al.* (2007) reported that *Escherichia coli* O157:H7 biofilms formed faster and a higher number of cells attached to a glass surface under low nutrient conditions than under high nutrient conditions.

Hydrodynamic conditions can influence the formation and structure of biofilms, as well as the metabolic activity of biofilm cells (Stoodley *et al.*, 1999; Simões *et al.*, 2007). Biofilms formed under turbulent flow are described as “streamers” and are typically formed by filamentous bacteria. The microcolonies formed under these conditions are stretched out in the direction of flow. In contrast, biofilms formed under low shear conditions (laminar flow conditions) are characterized by spherical microcolonies that are divided by water channels (Stoodley *et al.*, 1999; Purevdorj *et al.*, 2002). Simões *et al.* (2007) investigated the physiological differences between biofilms formed by *P. fluorescens* grown under turbulent and laminar flow conditions. Compared to biofilms grown under laminar flow conditions, biofilms grown under turbulent flow were not only denser, had a higher biomass and were metabolically more active, but they also produced higher amounts of exopolysaccharides (Simões *et al.*, 2007). Similarly, Trinet *et al.* (1991) and Chen *et al.* (1998) also reported that biofilms formed under high flow velocities produced an increased amount of exopolysaccharides that was shown to be required for stabilization of the biofilm structure.

Properties of an aqueous medium such as pH, temperature, oxygen and ionic strength may also play a role in the rate at which microorganisms attach to a surface (Donlan, 2002). The concentrations of cations such as calcium, sodium and ferric iron have been reported to increase the attachment of *P. fluorescens* to glass surfaces, probably by reducing the repulsive forces that exist between the cell surface and attachment surface (Fletcher, 1988).

2.2.3 Microbial cell surface

Cell surface hydrophobicity and charge facilitates the attachment of bacterial cells to surfaces and to each other. During adhesion the hydrophobic nature of the cell surface becomes crucial since hydrophobic interactions are likely to increase when the non-polar properties of the microbial cell surface and/or attachment surface increase (Donlan, 2002). Although most bacterial cell surfaces are negatively charged, bacterial surface structures such as fimbriae, pili and flagella contribute to the hydrophobicity of the cell surface due to their high content of hydrophobic amino acid residues (Rosenberg and Kjelleberg, 1986). In addition, flagella are also involved in the attachment of bacterial cells to the surface by assisting the cell to overcome the initial electrostatic repulsive boundary that exists between the cell and the substratum (Pratt and Kolter, 1998; O'Toole and Kolter, 1998a; Austin *et al.*, 1998), while pili have been shown to effect the formation of microcolonies during biofilm development (O'Toole and Kolter, 1998a). Similarly, the *csgBA* operon of *E. coli*, which is responsible for the production of curli, has been reported to assist in the adhesion of *E. coli* cells to surfaces (Prigent-Combaret *et al.*, 1999).

In addition to bacterial cell surface appendages, lipopolysaccharides (LPS) have also been reported to affect attachment. *P. aeruginosa* produces A- and B-band LPS and studies have shown that mutation of B-band LPS resulted in cells having a higher affinity for hydrophobic than hydrophilic surfaces, while mutation of A-band LPS did not drastically influence attachment to either surfaces (Makin and Beveridge, 1996). Al-Tahhan *et al.* (2000) reported that *P. aeruginosa*, which had been treated with a biosurfactant (rhamnolipid) to remove the LPS, gave rise to a hydrophobic cell surface. These cells were subsequently shown to attach readily to hydrophobic substrates. Williams and Fletcher (1995) constructed mutations resulting in the attenuation or loss of the *P. fluorescens* O antigen (polysaccharide side-chain). Although adhesion of the mutant *P. fluorescens* cells to hydrophobic surfaces was improved, adhesion to hydrophilic surfaces was decreased. It was concluded that the O antigen is partly responsible for the hydrophilic nature of the bacterial cell and that removal of LPS from the bacterial surface lead to a more hydrophobic cell surface (Williams and Fletcher, 1995). Moreover, *E. coli* strains with mutations in core LPS biosynthesis genes (*rfaG*, *rfaP* and *galU*) have also been reported to display reduced attachment to surfaces (Genevaux *et al.*, 1999).

Bacterial exopolysaccharides may also influence attachment and initial biofilm development since they contribute to cell surface charge, which affects electrostatic interactions between bacteria and the attachment surface (van Loosdrecht *et al.*, 1989). In *P. aeruginosa*, expression of the *algC* and *algD* genes has been reported to be up-regulated following bacterial adhesion (Davies *et al.*, 1993; Hoyle *et al.*, 1993; Davies and Geesey, 1995). Both these genes form part of the alginate biosynthetic operon, which controls alginate synthesis, while the *algC* gene is also required for LPS core biosynthesis (Gacesa, 1998; Ramsay and

Wozniak, 2005). The expression of *algC* was shown to be activated as early as 15 min after the bacterial cell attaches to a surface. Cells that did not undergo *algC* up-regulation were less able to remain attached to the surface relative to cells in which expression was activated, suggesting that *algC* may also play a role in maintenance of the biofilm (Davies and Geesey, 1995). In addition to its role in facilitating irreversible attachment, the production of exopolysaccharides also appears to play a role in determining the biofilm structure (Stoodley *et al.*, 2002; Stapper *et al.*, 2004). More detailed information regarding bacterial extracellular polymeric substances and their function in bacterial biofilms is provided in Section 2.4.

Membrane proteins have been reported to have a substantial influence on attachment of bacteria to surfaces and may also play a role in early biofilm development. Mutations in surface and membrane proteins caused defects in the attachment of *P. putida* to corn seeds (Espinosa-Urgel *et al.*, 2000), while the outer membrane protein OprF (Yoon *et al.*, 2002) and the outer porin proteins OpdF (Finelli *et al.*, 2003) and E1 (Sauer *et al.*, 2002) have been reported to play a role in *P. aeruginosa* biofilm development. Similarly, mutagenesis studies have also shown that the large adhesion proteins (Lap) of *P. fluorescens* WCS365 are required for attachment to several abiotic surfaces (Hinsa *et al.*, 2003) and that the large outer membrane protein A (LapA) is involved in the irreversible attachment of the bacterium to surfaces (Hinsa and O'Toole, 2006).

2.2.4 Quorum sensing

Several reports have linked quorum sensing, a process whereby bacteria can communicate with each other via chemical signaling, and all stages of biofilm formation. Acyl-homoserine lactones (Acyl-HSLs), which are quorum sensing signal molecules used by most Gram-negative bacteria (Miller and Bassler, 2001), have been shown to affect the attachment, maturation and detachment of biofilms (Parsek and Greenberg, 2005), as well as their spatial arrangement (Schaber *et al.*, 2007) and maintenance (Kjelleberg and Molin, 2002).

Certain bacteria can produce quorum sensing molecules that repress or enhance their attachment to surfaces. The cyclic peptide-dependant accessory gene regulator *agr* and *luxS* quorum sensing gene of *Staphylococcus aureus* and *Helicobacter pylori*, respectively, suppress the attachment of these bacteria to surfaces (Yarwood and Schlievert, 2003; Cole *et al.*, 2004). In contrast, *luxS* of *Salmonella enterica* serovar Typhimurium has been reported to be necessary for its attachment to human gallstones (Prouty *et al.*, 2002). Quorum sensing systems of *P. aeruginosa* (Davies *et al.*, 1998; De Kievit *et al.*, 2001a; Shih and Huang, 2002), *Serratia liquefaciens* (Labbate *et al.*, 2004), *Burkholderia cepacia* (Huber *et al.*, 2001) and *Aeromonas hydrophila* (Lynch *et al.*, 2002) have all been shown to affect biofilm maturation. Mutations in these systems typically result in a change in the biofilm structure, resulting in biofilms that are thinner and incapable of forming aggregates. Quorum sensing systems of *Rhodobacter sphaeroides* (Puskas *et al.*, 1997) and *Yersinia pseudotuberculosis* (Atkinson *et al.*, 1999) have been shown to be involved in cellular aggregation. More recently, it has been reported that *Vibrio cholerae* makes use of quorum sensing to control the amount of exopolysaccharides in the biofilm matrix by either producing enzymes that can degrade the polysaccharides or by inhibiting their synthesis (Hammer and Bassler, 2003).

2.3 BIOFILM RESISTANCE

Despite the availability of a wide range of antimicrobial agents, most of these compounds are, however, not as effective against biofilms as they are against planktonic cells. Several studies have shown resistance of biofilm cells against an array of antibiotics (Suci *et al.*, 1994; Ishida *et al.*, 1998; Anderl *et al.*, 2000; Stewart and Costerton, 2001; Kostenko *et al.*, 2007) and biocides (de Beer *et al.*, 1994; Norwood and Gilmour, 2000; Grobe *et al.*, 2002). Indeed, it has been reported that bacteria existing in a biofilm can become up to 1000-times more resistant to antimicrobial agents than their planktonic counterparts (Costerton *et al.*, 1999; Mah and O'Toole, 2001). Biofilm resistance does not appear to be dependant on a single survival mechanism, but rather multiple factors have been proposed to play a role.

2.3.1 Biofilm resistance mechanisms

The introduction of a biofilm phenotype has been proposed to lead to the activation of mechanisms that are critical for the development of resistance to antimicrobials (Mah and O'Toole, 2001; Drenkard, 2003). Using DNA microarrays, Whiteley *et al.* (2001) reported a set of 20 genes that were differentially expressed in *P. aeruginosa* biofilms exposed to high concentrations of the antibiotic tobramycin compared to untreated biofilms. Among them, expression of *dnaK* and *groES*, both of which are involved in stress responses, and two putative efflux systems were up-regulated. However, further studies are required to identify the resistance mechanisms associated with the induction of specific genes in *P. aeruginosa* biofilms. Several other mechanisms have also been suggested to account for biofilm resistance to antimicrobial agents. These include slow growth (Gilbert *et al.*, 1990; Evans *et al.*, 1991) owing to nutrient and oxygen limitations (Xu *et al.*, 1998; Xu *et al.*, 2000; Walters *et al.*, 2003) or owing to activation of the general stress response initiated by growth in a biofilm (Brown and Barker, 1999; Cochran *et al.*, 2000), the presence of multidrug efflux pumps (Poole and Srikumar, 2001), and the presence of an exopolysaccharide matrix that can react with antimicrobial agents and thus neutralize their effects and/or slow the diffusion of these agents into the biofilm (Chen and Stewart, 1996; Stewart, 1996; Anderl *et al.*, 2000; de Beer *et al.*, 1994; Kostenko *et al.*, 2007). Several bacteria, including *P. aeruginosa* (Giwercman *et al.*, 1991) and *Klebsiella pneumoniae* (Anderl *et al.*, 2000), produce β-lactamases that are responsible for the inactivation of β-lactam antibiotics. These enzymes have been reported to be immobilized in the exopolysaccharide matrix of the biofilm bacteria, thus restricting penetration of the antibiotics through the biofilm. It has been proposed that the penetration of antimicrobials through the biofilm can furthermore be influenced by (i) the charge of both the exopolysaccharide matrix and the antimicrobial agent, (ii) the dose concentration of the agent used, (iii) the viscosity of the exopolysaccharide matrix, (iv) the thickness and distribution of the biofilm colonies, and (v) the hydrodynamic conditions (de Beer *et al.*, 1994; Stewart *et al.*, 1998; Grobe *et al.*, 2002; Cloete, 2003).

How each of the above-mentioned mechanisms contributes to the overall resistance displayed by bacterial biofilms is not yet clear. For example, with the exception of aminoglycosides, the exopolysaccharide matrix has not been found to notably retard diffusion of fluoroquinones (Ishida *et al.*, 1998; Walters *et al.*, 2003). In addition, since most antibiotics target primarily metabolically active cells it may therefore not be surprising that slow-growing and non-growing bacteria could contribute significantly to a decrease in biofilm susceptibility to antimicrobial agents (Lewis, 2001; Gilbert *et al.*, 2002). Moreover, it has also been reported that different multidrug resistance efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY) do not play a role in *P. aeruginosa* biofilm resistance to antimicrobial agents (Brooun *et al.*, 2000; De Kieviet *et al.*, 2001b). Similarly, the ArcAB efflux pump of *E. coli* was not stimulated during biofilm growth and did not play a significant role in the resistance of *E. coli* to ciprofloxacin (Maira-Literán *et al.*, 2000).

2.3.2 Persistent cells (Persisters)

In a study of *P. aeruginosa* biofilms, Brooun *et al.* (2000) reported that the majority of cells were killed by low concentrations of antibiotics and despite further increases in antibiotic concentration, a small fraction of biofilm cells (1% or less) remained that were invulnerable to killing. It was subsequently shown that these cells, termed persister cells, were largely responsible for the high tolerance of *P. aeruginosa* biofilms to antimicrobial agents (Spoering and Lewis, 2001). According to Keren *et al.* (2004b) persister cells are "phenotypic variants of the wild-type cells that upon re-inoculation produce a culture with a similar amount of persister cells". It has been proposed that the function of persister cells is thus to enable the survival of the population in the presence of lethal factors (Lewis, 2001). Based on the premise that persister cells are not directly responsible for cell death but that they cause cell damage that indirectly triggers programmed cell death, Lewis (2001) proposed that persister cells ensure survival of the biofilm by having a defective programmed cell death program. More recently, the gene expression profile of persister cells of an *E. coli* culture was determined and it was suggested that the formation of persister cells is dependent on chromosomally encoded toxin-antitoxin modules (Keren *et al.*, 2004b). Whilst overexpression of both RelE and HipA toxins caused an increase in multidrug tolerant persister cells, deletion of the *hipBA* module caused a 10- to 100-fold decrease in persister cells in stationary and biofilm cultures. Based on these results, a revised model of persister cell production and antibiotic tolerance was proposed. Due to random fluctuations in the ratio of toxin-antitoxin in a population, it was proposed that some bacterial cells (1% of the population) would express high levels of a toxin, thus giving rise to persister cells. Since bactericidal antibiotics function by binding to a target protein and corrupting its functions, thereby generating a lethal product that results in cell death, it may be that in persister cells the target protein is blocked by binding of a toxin protein and its function is thus inhibited. Although the antibiotic can bind to the blocked target protein, it can no longer corrupt its function and the result is antibiotic tolerance, allowing cells to survive (Keren *et al.*, 2004a; Correia *et al.*, 2006).

In addition to the persister cells reported above, Mah *et al.* (2003) reported the identification of a mutant of *P. aeruginosa* PA14 that was capable of forming mature biofilms but did not develop high level biofilm-specific resistance to antibiotics. The locus identified, designated as *ndvB*, encodes for a glucosyltransferase that is necessary for cyclic glucan synthesis. Based on the physical interaction of these glucans with the antibiotic tobramycin, it was suggested that they may be responsible for entrapping antimicrobial agents in the periplasm and therefore obstruct them from getting to their sites of action in the cytoplasm. Alternatively, it was also proposed that the periplasmic glucans may contribute to antibiotic resistance of biofilm cells by slowing diffusion of antibiotics into the cell, thereby allowing the bacteria additional time to adapt to the antibiotic influx (Mah *et al.*, 2003).

2.4 METHODS USED TO REMOVE BIOFILMS

According to the National Institute of Health (NIH), biofilms are responsible for more than 60% of microbial infections, the most prominent being periodontitis, chronic lung infections (as encountered in cystic fibrosis patients), native valve endocarditis and a range of infections linked to biofilm-invaded medical devices (Schierholz and Beuth, 2001; Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004). More than half of nosocomial infections reported are predicted to be due to biofilms (Mah and O’Toole, 2001) and the treatment of these biofilm-associated infections costs more than $1 billion annually in the United States (Mah and O’Toole, 2001).

In addition to their medical consequences, biofilms also have a major impact on industrial practices like water systems (water distribution systems, pipelines and cooling towers) (Melo and Bott, 1997; Cloete *et al.*, 1998; Ludensky, 2003), paper industries (Blanco *et al.*, 1996; Ludensky, 2003) and food industries (Kumar and Anand, 1998; Chmielewski and Frank, 2003). Financial losses are experienced due to microbial-induced corrosion of water systems, as the equipment need to be replaced. In some cases, cooling water systems need to be stopped in order to clean colonized surfaces and reduce or eliminate health risks posed by biofilm microorganisms (Ludensky, 2003). Biofouling of heat exchangers has been reported to amount in losses of £200 – 400 million per year in the United Kingdom, while chemical treatment of biofilms in a moderate cooling water system could range from £20 – 30 000 per year (Keevil *et al.*, 1999). Biofilms in paper machines can cause paper defects such as stains and holes, and lead to bad odours both in the mill and on the paper. Biofilms are also responsible for the corrosion of surfaces in the paper mill system and for producing explosive gases such as methane (Blanco *et al.*, 1996). Food processing environments are also susceptible to biofilm formation and biofilms can result in losses due to corrosion of equipment, reduction in heat transfer, obstruction of pipelines and spoilage of food products

(Kumar and Anand, 1998; Chmielewski and Frank, 2003; Trachoo, 2003). Moreover, pathogenic microorganisms present in biofilms are difficult to control due to the increased resistance of biofilm bacteria to disinfectants and can therefore represent a potential health risk (Chmielewski and Frank, 2003).

As a consequence of the economic losses associated with the deleterious effects of biofilms on medical and industrial practices, different methods have been used for minimizing the accumulation of biofilms on surfaces. These have included treatment of water to reduce the number of bacteria entering the system, modification of surfaces to prevent their colonization by biofilms, and chemical and mechanical treatments that result in removal of biofilms from surfaces (Characklis and Marshall, 1990).

2.4.1 Chemical treatment

Chemical treatment entails the use of biocides, dispersants and surfactants to decrease the number of microorganisms entering a water system and/or aid in the removal of biofilms from surfaces. Chemicals that are to be used for reducing the number of bacteria should be chosen with care. Such chemicals must not pose health or environmental risks, should not affect the activity of any other chemicals used or cause corrosion of the treated systems, and it must be as cost-effective as possible (Charaklis and Marshall, 1990; Keevil *et al.*, 1999). Also, several factors need to be taken into account when selecting an appropriate chemical for the removal of bacterial biofilms. Not only must the number and diversity of bacteria present at the site be taken into account, but also the minimum inhibitory concentration (MIC) and contact time of the chemical for each of the bacteria. Moreover, to lessen the occurrence of chemical resistance, it is important to alternate the usage of different chemicals, apply the correct MIC and combine the chemicals with surface-active compounds that can detach the bacterial biofilms (Charaklis and Marshall, 1990; Cloete *et al.*, 1998). In order for a biocide to be effective in removing biofilms, it must be able to penetrate through the exopolysaccharide matrix of biofilms. Since the composition of the matrix may differ for different biofilms, the use of biocides that have a non-specific action is favoured (Meyer, 2003; Cloete, 2003).

2.4.1.1 Oxidizing biocides

Oxidizing biocides target the thiol group of cysteine amino acid residues and consequently, result in the oxidation of proteins. Since cysteines play an important role in determining protein structure and function, oxidizing biocides can therefore lead to cellular metabolic inhibition due to the oxidation of structural proteins present in cellular walls, membranes and ribosomes (Cloete *et al.*, 1998; Russel *et al.*, 2003). Several different oxidizing biocides, as discussed below, have been applied to the removal of bacterial biofilms in industrial practices.

Chlorine

Amongst all of the oxidizing and non-oxidizing biocides in use, chlorine is the most frequently used to control biofilms in water distribution systems (Freese and Nozaic, 2004). This is due to the ability of chlorine to destroy the integrity of the biofilm matrix by oxidizing and consequently, depolymerizing the exopolysaccharides present in the matrix (Characklis and Dydek, 1976). The attachment of new bacterial cells to surfaces is therefore more difficult in the presence of chlorine (Meyer, 2003). Chlorine also reacts with organic and inorganic matter present in the bulk fluid (de Beer *et al.*, 1994; Ryu and Beuchat, 2005), therefore necessitating that higher chlorine concentrations be used in order for it to penetrate biofilms (Characklis and Marshall, 1990). A concentration of as high as 1.5 mg/l residual chlorine is sometimes unable to penetrate biofilms (Cloete *et al.*, 1998). Norwood and Gilmour (2000) indicated that sodium hypochlorite at a concentration of 1000 ppm free chlorine was required to cause a substantial reduction in the number of biofilm bacteria, while only 10 ppm free chlorine was enough to totally remove planktonic bacteria. Chlorine can have a detrimental effect on humans and the environment, since its reaction with organic matter in the water may lead to the formation of trihalomethanes (THMs) (Zhao *et al.*, 2006), which have carcinogenic properties (Tokmak *et al.*, 2004). When used at high concentrations, chlorine can also cause corrosion of metal surfaces (Pisigan and Singley, 1987) and it is very sensitive to pH changes, making treatment of biofilms in cooling water systems difficult since an alkaline pH predominates (Keevil *et al.*, 1999). Despite these drawbacks, chlorine is nevertheless an efficient and versatile biocide, its handling and dosing is reasonably effortless and the costs involved in chlorine disinfection are low (Freese and Nozaic, 2004).

Chlorine dioxide

Many of the above-mentioned problems associated with the use of chlorine can be overcome by making use of chlorine dioxide, albeit not as cost-effective as chlorine and its production being more complex (Anderson *et al.*, 1982). Moreover, harmful by-products such as THMs and haloacetic acids are formed at a much lower level when chlorine dioxide is used compared to the use of chlorine (Volk *et al.*, 2002; Zhao *et al.*, 2006). Nevertheless, chlorine dioxide and its by-products (chlorite and chlorate) (Korn *et al.*, 2002) has been reported to cause hemolytic anemia in rats (Abdel-Rahman *et al.*, 1984). However, it has been reported that there appears to be no link between the intake of chlorine dioxide-treated water and human illness (Lubbers *et al.*, 1981). Furthermore, chlorite has also been shown to lessen in quantity as the water is transported through water distribution systems (Baribeau *et al.*, 2002). Chlorine dioxide is applied to water in a gaseous form and has been shown to be successful in penetrating bacterial biofilms. When a chlorine dioxide system (ACTIV-OX$^{®}$) was applied to hot and cold water systems, *Legionella* populations were eliminated without requiring additional chlorine dioxide (Keevil *et al.*, 1999). Gagnon *et al.* (2004) compared the effectiveness of chlorine dioxide, free chlorine and chloramines with regards to their ability to remove bacteria in a laboratory-scale water distribution system and reported that chlorine dioxide was the most effective on biofilm, as well as planktonic bacteria.

Hydrogen peroxide

Hydrogen peroxide is not as cost-effective as chlorine, but it is less harmful to the environment since it is reduced to oxygen and water (Carlsson, 2003; Mara and Horan, 2003). Although hydrogen peroxide has been used to reduce microbial numbers in the cooling water of nuclear reactors, studies have shown that hydrogen peroxide was not very successful in removing biofilms from a laboratory-scale reactor (Characklis and Marshall, 1990). The ineffectiveness of hydrogen peroxide to remove biofilms may be due to the production of catalase, an enzyme responsible for the degradation of hydrogen peroxide. Catalase production has been linked to the increased resistance of bacterial cells to hydrogen peroxide (Mah and O'Toole, 2001). In this regard, Stewart *et al.* (2000) and Elkins *et al.* (1999) reported that, in contrast to biofilms formed by wild-type *P. aeruginosa*, the biofilms formed by a catalase-deficient mutant strain was not protected against hydrogen peroxide penetration and killing.

Ozone

Ozone is a much stronger oxidizing agent than chlorine (Dosti *et al.*, 2005), but its use has been limited due to its low water solubility, especially at high temperatures (Guzel-Seydim *et al.*, 2004). Ozone also readily reacts with organic matter present in water, thus reducing its concentration and making its application in waters with high organic loads uneconomical (Mara and Horan, 2003). Moreover, ozone requires production at the application site since it possesses a high reactivity and unstable nature (Peleg, 1976; Staehelln and Holgné, 1982). Nevertheless, ozone has been shown to be effective in controlling biofilm formation at concentrations of lower than 0.5 g/m^3 (Sugam, 1980). Since ozone does not have a lasting effect, other disinfectants must be used in conjunction with ozone in order to keep water free from microorganisms (Rennecker *et al.*, 2000). Dosti *et al.* (2005) compared the efficacy of ozone and chlorine with regards to killing of food-spoilage bacteria present in biofilms. The number of biofilm bacteria was considerably reduced by treatment with ozone and chlorine, and only a slight difference in their efficacy was noted. Ozone, however, was more effective than chlorine in reducing bacterial numbers when tested against *P. putida* biofilms (Dosti *et al.*, 2005).

2.4.1.2 Non-oxidizing biocides

Non-oxidizing biocides comprise a range of bactericidal organic compounds such as iso-thiazolones, quaternary ammonium compounds (QACs), quaternary phosphonium compounds (QPCs), amines and triazines (Cloete *et al.*, 1998). Since these compounds are not manufactured specifically with the aim of removing biofilm bacteria, major problems are associated with their use as a means to control biofilms (Keevil *et al.*, 1999). Notably, they do not penetrate the biofilm readily (Stewart *et al.*, 1998) and, in some cases, can decrease in efficacy due to repeated application (Sundheim *et al.*, 1998; Langsrud *et al.*, 2003). Non-oxidizing biocides are typically used at high initial concentrations in order to ensure bacterial killing. As soon as the biocide concentration decreases and microbial counts increase, the biocide is re-applied (Keevil *et al.*, 1999) (Fig. 2.4). Of the different non-oxidizing biocides, QACs have been used in a wide range of applications, *e.g.*, as a disinfectant in food industries (Langsrud *et al.*, 2003) and in the medical field (Friedman *et al.*, 1968; Murtough *et al.*, 2000; Ioannou *et al.*, 2007). The positively-charged QACs form an electrostatic bond with negatively-charged bacterial cell walls, which causes stress in the cell wall, denaturation of proteins, disturbance of cell wall-permeability and decreased uptake of nutrients into the cell, thereby resulting in cell lysis (Cloete *et al.*, 1998; Ioannou *et al.*, 2007).

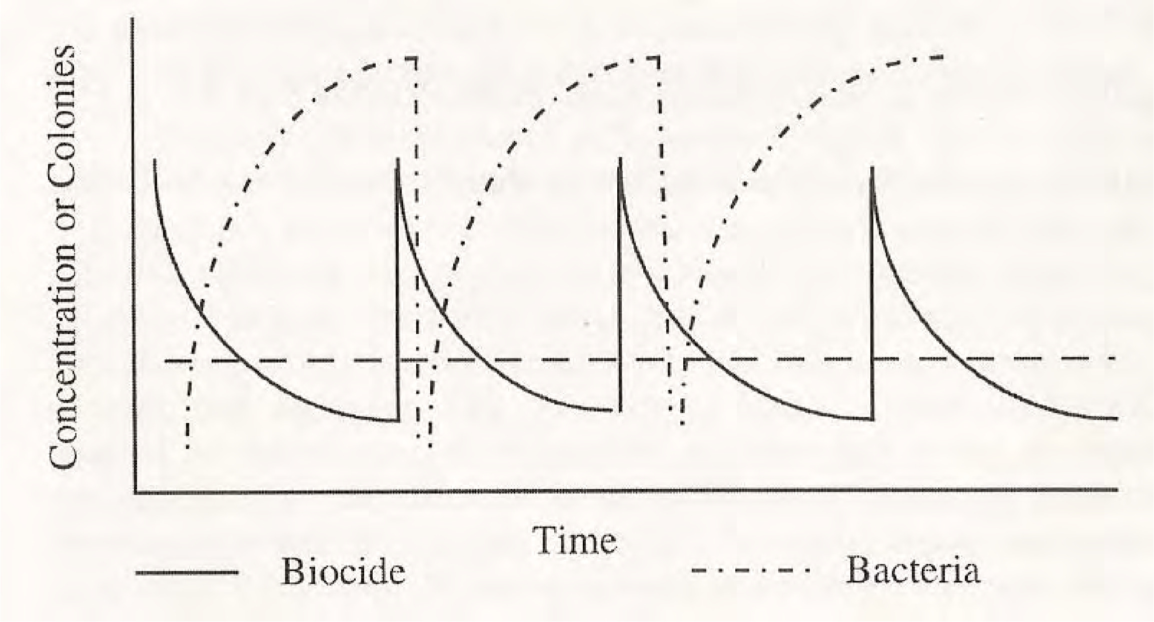


Figure 2.4. Diagram showing the method used for treatment with non-oxidizing biocides. The biocide is applied in order to reduce the microbial numbers. As soon as the concentration of the biocide decreases and microbial numbers increase, the biocide is re-applied (Keevil *et al.*, 1999).

2.4.2 Mechanical treatment

Most biocides do not result in the removal of the entire biofilm and re-growth can therefore occur (Eginton *et al.*, 1998). Physical treatment methods such as mechanical forces (Gibson *et al.*, 1999) and the application of heat (Scher *et al.*, 2005) are typically used to remove bacterial cells that might still be attached to the surface following chemical treatment. The use of mechanical scrubbers and high-pressure cleaners, as well as changes in water flow (laminar to turbulent) can also improve biofilm removal (Meyer, 2003). Gibson *et al.* (1999) reported that high-pressure spraying and mechanical floor scrubbers were successful in removing biofilms prevalent in a food factory. Scher *et al.* (2005) compared the efficacy of chlorine and heat as a means to remove *S. enterica* serovar Typhimurium biofilms. In contrast to the use of chlorine, to which the bacteria showed resistance, heat treatment (10 min at 70°C) resulted in a significant reduction of the biofilms.

2.4.3 Alternative methods for biofilm removal

The use of enzymes as a means to remove biofilms has been investigated in several studies (Johansen *et al.*, 1997; Hahn Berg *et al.*, 2001; Oulahal-Lagsir *et al.*, 2003; Orgaz *et al.*, 2006). Due to the substrate specificity of enzymes and the heterogeneity of the EPS, the removal of biofilms has been shown to be considerably more effective when enzymes were used in combination with detergents (Johansen *et al.*, 1997), sonication (Oulahal-Lagsir *et al.*, 2003) and antibiotics (Selan *et al.*, 1993). Moreover, it has been suggested that the use of different types of enzymes, especially combinations of proteases and polysaccharases, may be effective in removing bacterial biofilms (Meyer, 2003). The enzymatic removal of biofilms will be discussed in greater detail in Section 2.6.

Some research has been carried out regarding the use of bacteriophages to remove bacterial biofilms (Hibma *et al.*, 1997; Hughes *et al.*, 1998a; 1998b; Hanlon *et al.*, 2001; Curtin and Donlan, 2006). Host-specific bacteriophages can infect biofilm cells by first degrading the EPS and then ultimately lysing the bacterial cells (Hughes *et al.*, 1998a). However, since bacteriophages and their bacterial hosts exist together in nature, they are not considered a viable tool for biofilm removal (Tait *et al.*, 2002). Another problem to take into consideration is the ability of bacterial strains to become resistant against bacteriophage attack (Hill *et al.*, 1990; Foley *et al.*, 1998). One way of circumventing these problems is through the use of bacteriophages in combination with sanitizers. In this regard, Tait *et al.* (2002) reported that bacteriophage-treated biofilms displayed a reduction in the amount of exopolysaccharides, making the biofilms more sensitive to subsequent treatment with sanitizers.

Quorum sensing is proposed to affect various different cellular processes, amongst other, biofilm formation (Rice *et al.*, 2005b; Parsek and Greenberg, 2005; Harraghy *et al.*, 2007). Quorum sensing may therefore be a potentially important system to target in order to prevent biofilm formation. Notably, it has been reported that an RNAIII-inhibiting peptide inhibited quorum sensing and subsequently the attachment of *Staphylococcus epidermidis* (Balaban *et al.*, 2003) and *S. aureus* (Yarwood and Schlievert, 2003) to surfaces. Enzymatic degradation of quorum sensing molecules has also been investigated as means to prevent biofilm formation. Acyl-homoserine lactones can be degraded by *N*-Acyl homoserine lactonases from several *Bacillus* species (*B. cereus*, *B. thuringiensis* and *B. mycoides*) (Dong *et al.*, 2002), *Arthrobacter* sp. IBN110 and *K. pneumoniae* (Park *et al.*, 2003), as well as by acylases from *P. aeruginosa* PAO1 (Sio *et al.*, 2006) and *Ralstonia* sp. (Lin *et al.*, 2003). Similarly, Xu *et al.* (2003) reported that formation of biofilms grown from aquarium water decreased by 10-40%, following addition of acylase I from porcine kidney cells. *Delisea pulchra*, an Australian red macroalga, produces furanones that have been reported to interfere with the quorum sensing signaling processes of *S. liquefaciens* (Rasmussen *et al.*, 2000), *V. fischeri* (Manefield *et al.*, 2002) and *P. aeruginosa* (Hentzer *et al.*, 2002). It was subsequently proposed that their use, in combination with antimicrobial agents, may result in biofilm removal at lower doses of the antimicrobial agent (Hentzer *et al.*, 2003).

The removal of nutrients from the bulk fluid has been reported to be successful in reducing biofilm formation (Allison *et al.*, 1998), and also to increase the half-life of disinfectants such as chlorine (Chandy and Angles, 2001). By making use of side-stream biofilters to reduce the amount of nutrients available in cooling water systems, Daamen *et al.* (2000) demonstrated a reduction in the number of bacteria and reported that the need for addition of biocides to remove biofilms was reduced by at least 70%. Hunt *et al.* (2004) investigated the effect of glucose starvation on the detachment of *P. aeruginosa* biofilms. Experimental work, as well as a biofilm model, confirmed that biofilm detachment can occur due to nutrient starvation (Hunt *et al.*, 2004).

Biofilm formation can furthermore be limited or prevented through modification of the attachment surface (Carlén *et al.*, 2001; Pasmore *et al.*, 2002). This approach may not be very successful since many biofilms consist of a range of different bacterial species, each with their own attachment preferences (Palmer and White, 1997). Alternatively, the surface can be made attractive for the attachment of a specific microorganism in order to inhibit attachment of other unwanted bacteria. These methods can, however, only be applied in simple, controlled environments such as medical and dental equipment (Palmer and White, 1997). The covering of medical devices, particularly urinary and vascular catheters, with antibiotics or antimicrobial compounds has showed some success in reducing or completely preventing the attachment of biofilms (Sheng *et al.*, 2000; Donelli *et al.*, 2002). However, covering of these devices with antibiotics has raised concerns regarding the emergence of antibiotic-resistant bacterial strains (Francolini *et al.*, 2003).

2.5 MICROBIAL EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) AND THEIR ROLE IN BIOFILMS

Biofilms are characterized by high levels of EPS and as much as 50-90% of biofilms' total organic matter consists of EPS (Allison, 2000). Geesey (1982) described EPS as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates". Although polysaccharides are believed to dominate the EPS, exceptions do occur. For example, the EPS from wastewater biofilms (activated sludge) have been reported to have a higher proportion of proteins than polysaccharides (Nielsen *et al.*, 1997; Bura *et al.*, 1998).

2.5.1 Composition of EPS

In several bacterial species studied, polysaccharides and proteins have been reported to account for 75-89% of the EPS composition (Tsuneda *et al.*, 2003) (Table 2.1). The main configuration of the polysaccharides present in bacterial EPS is β and rarely α, with a high prevalence of 1,3 or 1,4 linkages in the backbone of the polymer. The polysaccharide compositions in EPS can range from very basic polysaccharides, containing only one type of monomer (homopolysaccharides), to more complex polysaccharides composed of more than two different monosaccharides (heteropolysaccharides). Neutral polysaccharides are scarce, while positively-charged (due to the presence of amino sugars) and negatively-charged (due to the presence of carboxyl, sulfate or phosphate groups) polymers are more abundant (Allison, 2000). The structure and physicochemical properties of polysaccharides can, however, be altered by the presence of non-polymeric organic (acetyl, pyruvyl and succinyl) and inorganic (sulphate and phosphate) compounds (Wingender *et al.*, 1999).

In addition to polysaccharides, proteins also form part of the EPS. The proteins can be glycosylated with oligosaccharides to form glycoproteins, while the oligosaccharides can also be replaced with fatty acids to form lipoproteins (Wingender *et al.*, 1999). EPS assimilate proteins from living and lysed bacterial cells, and these proteins can be absorbed due to the polyanionic properties of the EPS carbohydrates (Ghannoum and O'Toole, 2004). Proteins are typically responsible for the hydrophobic nature of EPS (Dignac *et al.*, 1998; Jorand *et al.*, 1998) and may assist in the attachment of bacteria to negatively-charged surfaces (Characklis and Marshall, 1990). The proteins in EPS do not only play a structural role, but some of the proteins might act as enzymes (Characklis and Marshall, 1990). Polysaccharases are prominent in EPS and are responsible for degradation of EPS and other organic components present in the bulk fluid, thereby supplying the biofilm cells with nutrients (Wingender *et al.*, 1999).

Other substances present in EPS, albeit in smaller amounts, include nucleic acids (DNA and RNA), lipids and other products of cell lysis (Sutherland, 2001a; Whitchurch *et al.*, 2002; Allison, 2003; Tsuneda *et al.*, 2003; Moscoso *et al.*, 2006; Jurcisek and Bakaletz, 2007).

Table 2.1: General composition of bacterial EPS (Nielsen *et al*. 1997; Jahn and Nielsen 1998)

EPS	**Principle component (subunits, precursors)**	**Main type of linkage between subunits**	**Structure of polymer backbone**	**Substituents (examples)**
Polysaccharides	Monosaccharides Uronic acids Amino sugars	Glycosidic bonds	Linear, branched	**Organic:** O-acetyl, N-acetyl, succinyl, pyruvyl **Inorganic:** sulfate, phosphate
Proteins (polypeptides)	Amino acids	Peptide bonds	Linear	Oligosaccharides (glycoproteins), fatty acids (lipoproteins)
Nucleic acids	Nucleotides	Phosphodiester bonds	Linear	-
(Phospho)lipids	Fatty acids Glycerol Phosphate Ethanolamine Serine Choline Sugars	Ester bonds	Side-chains	-
*Humic substances	Phenolic compounds Simple sugars Amino acids	Ether bonds, C-C bonds, peptide bonds	Cross-linked	-

*Humic substances are occasionally seen as being part of the EPS matrix

Cellular material comprises 2-15% of the biofilm matrix, but the amounts of these components can differ (Sutherland, 2001b). Treatment of *P. aeruginosa* biofilms with DNase I was reported to result in the removal of the biofilms from a surface, suggesting a role for DNA in biofilm formation (Whitchurch *et al*., 2002).

2.5.2 Formation of EPS

EPS are situated on or in close proximity to the outer surface of biofilm cells. The exopolymeric substances can be released into the extracellular environment by active secretion, during lysis of bacterial cells or by the detachment of membrane components from the cell surface (Wingender *et al*., 1999). Several cellular systems that actively secrete proteins and polysaccharides have been reported (Rehm and Valla 1997; Jonas and Farah, 1998), while the presence of nucleic acids in the EPS may be due to horizontal gene transfer that occurs between the tightly packed biofilm cells (Christensen *et al*., 1998; Ghigo, 2001) or as consequence of programmed cell lysis (Webb *et al*., 2003). In addition, cell lysis also results in the release of the intracellular bacterial contents that can subsequently be captured within the biofilm matrix (Wingender *et al*., 1999). Moreover, some intracellular components of Gram-negative bacteria have been shown to be released into the extracellular environment by the formation of membrane-derived vesicles (blebs) (Kadurugamuwa and Beveridge, 1995). Cellular components released in this manner include nucleic acids (Dorward and Garon, 1990; Renelli *et al*., 2004) and enzymes (Kadurugamuwa and Beveridge, 1995), as well as membrane components such as phospholipids (Schooling and Beveridge, 2006).

2.5.3 Roles of EPS in biofilms

Due to its high heterogeneity among different bacterial strains, the function of EPS is only partially understood. EPS, however, are involved in the attachment of bacterial cells to a surface and in holding the biofilm cells together (Mack *et al*., 1996; Yildiz and Schoolnik, 1999; Danese *et al*., 2000; Hentzer *et al*., 2001; Lewandowski and Beyenal, 2007). In addition, EPS can change the physicochemical properties of the attachment surface, thereby making it easier for bacterial cells to attach. Tsuneda *et al*. (2003) reported that electrostatic interactions reduced bacterial attachment when low amounts of EPS were produced, while attachment was enhanced by polymeric interactions when the EPS amounts were high. In contrast, Hanna *et al*. (2003) reported that colonic acid, a capsular polysaccharide produced by *E. coli*, obstructed both specific binding and time-dependant interactions between the bacteria and their attachment surfaces.

EPS not only act as a protective layer against antibiotics (Anderl *et al.*, 2000; Kostenko *et al.*, 2007) and biocides (de Beer *et al.*, 1994; Chen and Stewart, 1996; Wai *et al.*, 1998; Stewart and Costerton, 2001; Grobe *et al.*, 2002), but also offer protection against heavy metals (Teitzel and Parsek, 2003) and UV irradiation (Elasri and Miller, 1999), thus reducing their penetration into the bacterial cells. Moreover, EPS also protects biofilm cells against dehydration under water-limited conditions (Ophir and Gutnick, 1994; Schnider-Keel *et al.*, 2001), and other environmental conditions such as temperature fluctuations (Mao *et al.*, 2001) and osmotic shock (Schnider-Keel *et al.*, 2001; Chen *et al.*, 2004). The outer layer of EPS can dry out under water-limited conditions and forms a hard, protective layer that prevents dehydration of the inner cells (Sutherland, 2001a).

In addition to the above, EPS is also considered to be a virulence factor in several different bacterial infections. Bereswill and Geider (1997) reported that production of the acidic EPS, amylovoran, by *Erwinia amylovora* is linked to its virulence, since a strain that was incapable of producing amylovoran was avirulent on its host plants. Several studies concluded that mucoid *P. aeruginosa* initiates chronic lung infection due to the high production of EPS by this strain (Pedersen *et al.*, 1990; Gilligan, 1991; Hentzer *et al.*, 2001). This “mucoid exopolysaccharide” (MEP) is therefore considered a virulence factor in cystic fibrosis infections (Gilligan, 1991). Furthermore, lectins bound to certain carbohydrates present in EPS have been reported to play a role in the pathogenesis of some bacteria, and assist in the attachment of the cell to its host and to other bacterial cells (Sharon and Lis, 2004).

2.6 ENZYMATIC REMOVAL OF BIOFILMS

As highlighted in the previous sections, most biocides and antibiotics are ineffective in biofilm eradication and control. In addition, some of these disinfectants produce by-products that are toxic or have irritant properties (Augustin and Ali-Vehmas, 2004). Therefore, there exists a need to identify agents to which microorganisms cannot acquire resistance and that do not produce harmful by-products. A significant target for detaching and eradicating biofilms may perhaps be the biofilm matrix, since EPS play an important role in the structural integrity and attachment properties of biofilms (Hentzer *et al.*, 2001; Stoodley *et al.*, 2002).

Consequently, various different agents have been investigated for their ability to promote the detachment of bacterial biofilms (Table 2.2). Chemicals (*e.g.*, $NaCl_2$, $MgCl_2$ and $CaCl_2$) have been used to change the ionic strength and composition of the liquid medium which, in turn, influence the electrostatic interactions responsible for the attachment properties of the biofilm matrix (Chen and Stewart, 2000). Chelating agents (*e.g.*, EDTA and EGTA) have been used to cross-link multivalent cations involved in biofilm attachment, while surfactants can interrupt hydrophobic interactions implicated in the maintenance of the matrix structure (Turakhia *et al.*, 1983; Chen and Stewart, 2000). In addition to the above, enzymes capable of degrading extracellular DNA (Whitchurch *et al.*, 2002), as well as polysaccharides and proteins (Johansen *et al.*, 1997; Hughes *et al.*, 1998a; Skillman *et al.*, 1999; Kaplan *et al.*, 2004a; Itoh *et al.*, 2005), the two major components of EPS responsible for its structural stability (Wingender *et al.*, 1999), have also been used to promote the detachment of biofilms.

Combinations of different enzymes, especially proteases and polysaccharases, in the presence or absence of antimicrobial agents and detergents, is generally thought to be useful for degradation of the biofilm EPS. Therefore, the following sections will focus mainly on these two groups of enzymes and their application to the removal of biofilms.

2.6.1 Polysaccharases

Polysaccharide hydrolases and polysaccharide lyases are capable of degrading microbial exopolysaccharides. These enzymes are responsible for controlling the length of polysaccharides during synthesis and can degrade the polymers in order to supply the microorganism with sources of carbon and energy (Sutherland, 1999; Wingender *et al.*, 1999). Polysaccharases capable of degrading EPS have thus attracted considerable attention as a means to remove bacterial biofilms (Bayer *et al.*, 1992; Johansen *et al.*, 1997; Allison *et al.*, 1998; Merritt *et al.*, 2000; Orgaz *et al.*, 2006; Walker *et al.*, 2007). However, in mixed biofilm populations, where a range of different exopolysaccharides is present, EPS degradation may be more challenging (Wingender *et al.*, 1999; Tait *et al.*, 2002). In such instances, a mixture of polysaccharases is typically used. Since the degradation products of one enzyme might serve as a substrate for a different enzyme, it can thus ultimately lead to the degradation of EPS and the removal of biofilms (Wingender *et al.*, 1999).

Table 2.2: Biofilm detachment-promoting agents (DPAs) (Xavier *et al.*, 2005)

Agent	Origin	Substrate	Notes/action
Enzymes			
Crude cellulase preparation	*Trichoderma viride* (Maxazyme CL2000)	Dephosphorylated and partially derhamnosylated EPS of *Lactococcus lactis* subsp. *cremoris* B40	EPS was incubated with various commercial enzyme preparations and analysed for degradation In crude enzyme-preparation tests, one enzyme acted very specifically (van Casteren *et al.*, 1998)
Polysaccharide depolymerase	Bacteriophage	*Enterobacter agglomerans* GFP in monospecies biofilms and in dual-species biofilms with *Klebsiella pneumoniae* G1	Phage glycanases are very specific. Action of enzyme was observed when added to the phage-susceptible monospecies biofilm, leading to substantial biofilm degradation (Hughes *et al.*, 1998) A 60-min treatment with polysaccharase caused a 20% reduction in dual-species biofilm adhesion (Skillman *et al.*, 1999)
Alginate lyase	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* alginate	Strains of *P. aeruginosa* overproducing alginate lyase detached at a higher rate than wild-type (Boyd & Chakrabarty,1994) However, other studies showed that addition of alginate lyase to established *P. aeruginosa* biofilm caused no observable detachment (Christensen *et al.*, 2001)
Disaggregatase enzyme	*Methanosarcina mazei*	*Methanosarcina mazei* heteropolysaccharide capsule mediating cell aggregation	Conditions that are generally unfavourable for growth are associated with disaggregatase activity (Xun *et al.*, 1990)
Esterases with wide specificity	Wide range of bacteria	Acyl residues from bacterial polymers as well as other esters	Acetyl residues from intracellular carboxylesterase (EC 3.1.1.1) isolated from *Arthrobacter viscosus* removed acetyl residues from xanthan, alginate, glucose pentaacetate, cellobiose octaacetate, exopolysaccharide produced by *A. viscosus*, deacetylated *p*-nitrophenyl propionate, naphthyl acetate, isopropenyl acetate and triacetin (Cui *et al.*, 1999) Esterases could alter the physical properties of biofilm structure (Sutherland, 2001b)
Dispersin B (or DspB)	*Actinobacillus actinomycetemcomitans*	Poly-β-1,6-GlcNAc implicated as an adhesion factor for biofilms of several bacterial species	Causes detachment of cells from *A. actinomycetemcomitans* biofilms and disaggregation of clumps of *A. actinomycetemcomitans* in solution (Kaplan *et al.*, 2003) Treatment of *S. epidermidis* biofilms with dispersin B causes dissolution of the EPS matrix and detachment of biofilm cells from the surface (Kaplan *et al.*, 2004) Disrupts biofilm formation by *E. coli*, *S. epidermidis*, *Yersina pestis* and *Pseudomonas fluorescens* (Itoh *et al.*, 2005)
DNase I	Commercial (Sigma-Aldrich)	Extracellular DNA in *Pseudomonas aeruginosa* biofilms	DNase affects the capability of *P. aeruginosa* to form biofilms when present in the initial development stages. Established biofilms were only affected to a minor degree by the presence of DNase I (Whitchurch *et al.*, 2002).

(Table 2.2 continued)

Agent	Origin	Substrate	Notes/action
Mixtures of enzymes	Commercial	*S. aureus, S. epidermidis, P. fluorescens* and *P. aeruginosa* biofilms on steel and polypropylene substrata	Pectinex UltraSP (Novo Nordisk A/S, a multicomponent enzyme preparation) reduced the number of bacterial cells in biofilms on stainless steel without any significant bactericidal activity (the activity of Pectinex Ultra is mainly a degradation of extracellular polysaccharides) (Johansen *et al.,* 1997)
		S. mutans, Actinomyces viscosus and *Fusobacterium nucleatum* biofilms on saliva-coated hydroxyapatite	Mutanase and dextranase were shown to remove oral plaque from hydroxyapatite, but were not bactericidal (Novo Nordisk A/S)
Other agents			
Chelating agents		*Pseudomonas aeruginosa* biofilm or alginate from mucoid *P. aeruginosa*	EGTA, a calcium-specific chelating agent, effected the immediate and substantial detachment of a *P. aeruginosa* biofilm without affecting microbial activity (Turakhia *et al.,* 1983) EDTA and other chelating agents produced major reductions in alginate gel strength (Gordon *et al.,* 1991) EDTA and Dequest 2006 reduced viscosity of a suspension of two-species biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* (Chen and Stewart, 2000)
NaCl, $CaCl_2$ or $MgCl_2$		*Pseudomonas aeruginosa* biofilm or alginate from mucoid *P. aeruginosa*	Tests using several slime dispersants, including sodium salts and chelating agents, determined that alginate gel strength is reduced by sodium salts, albeit to a lesser extent than that observed by using chelating agents (Gordon *et al.,* 1991) Treatment of intact biofilm with NaCl, $CaCl_2$ or $MgCl_2$ resulted in the rapid detachment of a significant percentage of the total biofilm protein (Chen and Stewart, 2000) Increasing the ionic strength of the medium presumably screens out cross-linking electrostatic interactions, diminishing biofilm cohesiveness (Chen and Stewart, 2002)
Surfactants		Two-species biofilms of *P. aeruginosa* and *Klebsiella pneumoniae*	Reduction in total biofilm protein observed, possibly resulting from a disruption of hydrophobic interactions involved in cross-linking the biofilm matrix (Chen and Stewart, 2000)
Urea		Two-species biofilms of *P. aeruginosa* and *Klebsiella pneumoniae*	Treatment with urea caused a 46% reduction in the apparent viscosity of the biofilm suspension, suggesting a role for hydrogen bonding in cross-linking the biofilm (Chen and Stewart, 2000)

2.6.1.1 Enzymatic action

Polysaccharide hydrolases and polysaccharide lyases are both capable of degrading EPS (Sutherland, 1999; Wingender *et al.*, 1999). The substrate specificity of the respective enzymes is determined by the structure of the carbohydrates and, in particular, by the presence of uronic acids. The presence of acyl substituents in the carbohydrate structure has been reported to decrease the activity of certain polysaccharide lyases such as alginate and gellan lyases (Sutherland, 1999). Enzymatic degradation of EPS can also be influenced by the presence of biosurfactants. The biosurfactants associate with the polysaccharides and can either enhance (Eriksson *et al.*, 2002; Helle *et al.*, 2004) or hinder the degradation process (Helle *et al.*, 2004). EPS-degrading enzymes can be classified as either endo- or exo-acting (Sutherland, 1999; Wingender *et al.*, 1999). Whereas endo-acting enzymes can reduce the polysaccharide chain length rapidly by cleaving internal bonds of the polysaccharide, the exo-acting enzymes are responsible for cleaving bonds at the ends of the polysaccharide and therefore its breakdown is slower. The degradation of homopolysaccharides can be facilitated by a range of endo- and exo-acting enzymes (Gilbert and Hazlewood, 1993; Mishra and Robbins, 1995), while heteropolysaccharides are usually degraded by a single, very specific endo-acting polysaccharase (Sutherland, 1999).

2.6.1.2 Production sources

Polysaccharases are produced by polysaccharide-synthesizing microorganisms (endogenous production), other eukaryotic and prokaryotic microorganisms (exogenous production) and bacteriophages (Sutherland, 1999; Wingender *et al.*, 1999).

Endogenous production

Some polysaccharide-producing microorganisms synthesize polysaccharases that are capable of degrading their own polysaccharides to use as a source of carbon and energy (Sutherland, 1999). More commonly, however, the endogenously-produced enzymes do not allow the microorganism to utilize its own EPS as a carbon source, but they can be released into the extracellular environment during cell division and cell lysis (due to autolysis, lytic phages and protozoa) (Conti *et al.*, 1994) (Table 2.3). Endogenously-produced polysaccharases such as glycanases or lyases may often form part of the same gene cassettes that are responsible for exopolysaccharide synthesis, polymerization and excretion (Matthysse *et al.*, 1995; Sutherland and Kennedy, 1996). As yet, it is not clear whether these enzymes are constantly expressed or if they are expressed under the same physiological conditions than genes responsible for EPS synthesis (Sutherland, 1999; Wingender *et al.*, 1999).

Table 2.3: Glycan depolymerases linked to exopolysaccharide production (Sutherland, 1999)

Bacterial species	**Enzymes**
Acetobacter xylinum	Endoglucanase (cellulase)
Agrobacterium tumefaciens	Endoglucanase (cellulase)
Azotobacter chroococcum	Alginate lyase (polymannuronate lyase)
Azotobacter vinelandii	Alginate lyase (polymannuronate lyase)
Cellulomonas flavigena	1,3-β-D-Glucanase
Escherichia coli K5	N-acetyl-heparosan lyase
Pseudomonas aeruginosa	Alginate lyase (polymannuronate lyase)
Pseudomonas fluorescens	Alginate lyase (polymannuronate lyase)
Pseudomonas fluorescens (*marginalis)*	Galactoglucanase
Pseudomonas mendocina	Alginate lyase (polymannuronate lyase)
Pseudomonas putida	Alginate lyase (polymannuronate lyase)
Rhizobium meliloti	Succinoglycan depolymerase
Rhizobium meliloti	Polyglucuronic acid lyase
Sphingomonas spp.	Gellan lyase
Streptococcus equi	Hyaluronidase

Exogenous production

Sources of exogenously-produced polysaccharases may either be a single microbial species (Cadmus *et al.*, 1982; Oyaizu *et al.*, 1982) or, more frequently, mixed bacterial cultures (Hou *et al.*, 1986). These enzymes are produced to enable bacteria to degrade polysaccharides and utilize the monomers as energy and carbon sources. The enzymes are secreted into the extracellular environment and can therefore come into direct contact with the exopolysaccharides produced by biofilm cells of other microbial species. The enzymes usually represent a complex mixture of activities that are capable of degrading polysaccharides to oligosaccharides, while other enzymes are capable of acting on these fragments to reduce them to monosaccharides that can be assimilated by the cells (Wingender *et al.*, 1999). Examples of exogenously-produced enzymes are listed in Table 2.4.

Table 2.4: Polysaccharide depolymerases produced by heterologous microorganisms (Sutherland, 1999)

Polysaccharide	**Enzyme action**	**Linkage cleaved**	**Enzyme source**
Endoglycanases			
Bradyrhizobium sp.	Endorhamnosidase	1,4-β-L-Rha	*Bacillus* sp.
Dextran	Endoglucosidase	1,3- β -D-Glc	*Bacillus* sp.
Dextran	Endoglucosidase	1,2-α-D-Glc	*Flavobacterium* sp.
Dextran	Endoglucosidase	1,2-α-D-Glc	*Streptococcus mutans*
Streptococcus pneumoniae type 3	Endoglucosidase	1,3-β-D-Glc-1,4-β-D-GlcA	*Bacillus palustris*
Lyases			
Streptococcus pneumoniae type 8		1,4-α-D-Gal-β-D-GlcA-	*Bacillus palustris*
Sphingomonas sp. (Gellan)		1,4-β-D-Glc-β-D-GlcA-	Pseudomonad
Sphingomonas sp. (Gellan)		1,4-β-D-Glc-β-D-GlcA-	*Bacillus* sp.

Bacteriophages

A diverse range of virulent bacteriophages is associated with EPS-producing bacteria (Sutherland, 1999). Some of these bacteriophages produce polysaccharases that occur as spikes on the base plate of the phage (Hughes *et al.*, 1998a). Polysaccharase activity has also been detected in the bacterial cell lysates, following release of mature phage particles from infected host cells (Sutherland, 1999). The bacteriophage-produced polysaccharases are very specific and they typically have a single target, except if the polysaccharides are similar in structure. There are, however, a few exceptions where bacteriophage polysaccharases can degrade polysaccharides from different bacterial species (Sutherland, 1999). Removal of biofilms by means of bacteriophages relies greatly on the number of phages present and the activity of the enzyme that they produce. In mixed biofilms, different EPS types are present and these are almost impossible to degrade by a single phage. Phages are thus not very successful when it comes to removing mixed biofilms, but can be used where biofilms of known bacterial strains must be removed (Hughes *et al.*, 1998a).

Degradation of bacterial EPS by bacteriophage-produced polysaccharases occurs in a specific sequence of events (Hughes *et al.*, 1998a) (Fig. 2.5). The bacteriophage first binds to a secondary receptor present in the EPS, whereafter the EPS polymers are degraded enzymatically until the cell surface is exposed. The bacteriophage can then bind to primary receptors present on the bacterial cell wall, allowing the bacteriophage to infect the host cell. Depending on the bacteriophage, it may enter a lytic or lysogenic life cycle that ultimately results in lysis of the host cell when mature phage particles are released (Hughes *et al.*, 1998a).

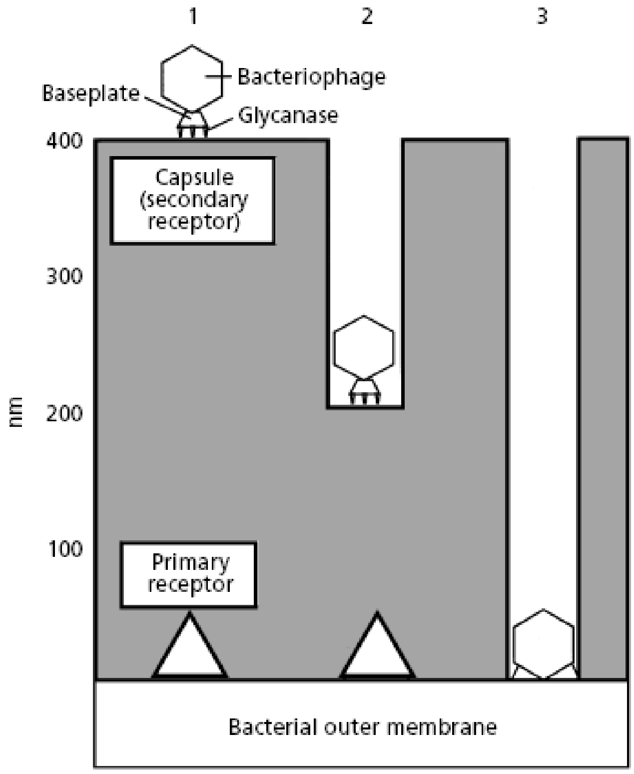


Figure 2.5. Degradation of bacterial extracellular polysaccharides by a bacteriophage producing glycanase. 1, bacteriophage binds to a secondary receptor present in the capsule. 2, the bacteriophage produces glycanases to degrade the exopolysaccharides and reaches the surface of the cell. 3, the bacteriophage binds to the primary receptor on the cell surface and infects the cell (Hughes *et al.*, 1998a).

2.6.2 Proteases

As indicated previously, bacterial surface and membrane proteins assist in the attachment of bacterial cells to surfaces (O’Toole and Kolter, 1998b; Espinosa-Urgel *et al.*, 2000; Finelli *et al.*, 2003; Hinsa and O’Toole, 2006). Moreover, proteins present in the biofilm EPS interact with polysaccharides and aid in maintaining the biofilm structure (Skillman *et al.*, 1999, Sutherland, 2001). Consequently, protein-degrading enzymes may therefore be useful in preventing biofilm formation and in removing biofilms from surfaces.

2.6.2.1 Enzymatic action

Proteases can be placed into two major groups based on the position they cleave in the protein substrate. Exopeptidases cleave peptide bonds close to the termini of the polypeptide, while endopeptidases are responsible for cleaving peptide bonds in the inner region of the polypeptide chain (Table 2.5). Exopeptidases are further classified into amino (N)-peptidases and carboxy (C)-peptidases, depending on whether the enzyme removes amino acids at the N- or C-terminus of the polypeptide. The endopeptidases are divided into four subgroups, *i.e.* serine proteases, cysteine proteases, aspartic proteases and metalloproteases, based on their catalytic mechanism. Moreover, proteases can also be classified as alkaline, acidic or neutral proteases, depending on the pH where they are optimally active (Rao *et al.*, 1998).

Table 2.5: Protease classification, based on mode of action (Rao *et al.*, 1998)

Protease	Mode of action
Exopeptidases	
Aminopeptidases	●↓-○-○-○-○---
Dipeptidyl peptidase	●-●↓-○-○-○---
Tripeptidyl peptidase	●-●-●↓-○-○---
Carboxypeptidase	---○-○-○-○-○↓-●
Serine-type protease	
Metalloprotease	
Cysteine-type protease	
Peptidyl dipeptidase	---○-○-○-○↓-●-●
Dipeptidases	●↓-●
Omega peptidases	*-●↓-○-○---
	---○-○-○↓-●-*
Endopeptidases	---○-○-○↓-○-○-○---
Serine protease	
Cysteine protease	
Aspartic protease	
Metalloprotease	
Endopeptidases of unknown catalytic mechanism	

○ = amino acid residues in polypeptide chain, ● = terminal amino acids, * = blocked termini, ↓ = enzyme action site

2.6.2.2 Production sources

Proteases are produced by an extensive array of plants (Konno *et al.*, 2004), animals (Furihata *et al.*, 1980; Fujinaga *et al.*, 1987) and microorganisms (Leger *et al.*, 1992; Ghorbel *et al.*, 2003). Fungi produce many different proteases that display activities over broad pH ranges and show activity against a wider range of substrates than bacterial proteases. In contrast, bacterial proteases display tolerance over a broader range of temperatures and the reaction rate of these proteases are higher than those of fungal proteases. Indeed, the genus *Bacillus* is responsible for producing the majority of commercial proteases, especially the neutral and alkaline proteases. Viruses are noted for their production of aspartic, cysteine and serine peptidases (Rao *et al.*, 1998).

2.6.3 Application of enzymes to biofilm removal

2.6.3.1 Polysaccharide-degrading enzymes

Loiselle and Anderson (2003) investigated the effect of a fungal cellulase on *P. aeruginosa* biofilms. The cellulase caused a decrease in the biofilm biomass and colony forming units (CFU). The enzyme was also tested against the purified biofilm EPS and it was shown by size exclusion chromatography that the enzyme caused a reduction in the molecular weight of the polymers. The enzyme did, however, not lead to the complete inhibition of biofilm formation and it was therefore suggested that it might be more useful when used in combination with other treatments or enzymes.

Kaplan *et al.* (2004b) reported that *Actinobacillus actinomycetemcomitans* produces an enzyme, known as Dispersin B, which targets polymers that contain *N*-acetylglucosamine (Kaplan *et al.*, 2004a), and is capable of detaching biofilms of *A. actinomycetemcomitans* (Kaplan *et al.*, 2004b) and *Staphylococcus epidermidis* (Kaplan *et al.*, 2004a). More recently, Itoh *et al.* (2005) reported that Dispersin B likewise hydrolyzes the glycosidic linkages in the capsular polysaccharide β-1,6-*N*-acetyl-D-glucosamine (poly-β-1,6-GlcNAc) required for biofilm formation by *E. coli* and staphylococci. The synthesis of this polysaccharide is dependent on the presence of *pgaABCD* and *icaABCD* loci. Dispersin B was capable of detaching biofilms from *E. coli, S. epidermidis, Y. pestis* and *P. fluorescens*, all of which posses either a *pgaABCD* or *icaABCD* locus (Itoh *et al.*, 2005).

2.6.3.2 Proteases

Hahn Berg *et al.* (2001) tested the activity of several proteases obtained from Antarctic krill shrimp against oral biofilms, which can cause dental caries and periodontal diseases if they are not controlled. The enzyme mixture, known as Krillase, is comprised of different endo- and exopeptidases. Krillase not only prevented the attachment of oral bacteria by degrading saliva proteins present on the surface, but it also removed existing biofilms by destroying adhesion receptor interactions involved in the aggregation and attachment of bacterial cells to each other and to the surface. Moreover, Krillase did not exhibit microbicidal activity and therefore is unlikely to disrupt the balance of the oral bacteria.

2.6.3.3 Mixed enzymes

Johansen *et al.* (1997) tested different commercial enzymes for their bactericidal and biofilm removal properties. *Staphylococcus aureus*, *S. epidermidis*, *P. fluorescens* and *P. aeruginosa* biofilms were grown on steel and polypropylene surfaces and subsequently treated with glucose oxidase, lactoperoxidase or Pectinex Ultra SP, an enzyme mixture containing protease activity and several polysaccharide-hydrolyzing enzymes. It was reported that although glucose oxidase and lactoperoxidase each had a bactericidal effect on the biofilm bacteria, they were incapable of removing the biofilms. In contrast, Pectinex Ultra SP was capable of detaching the biofilms, but it did not show bactericidal activity against the biofilm bacteria. However, when both the oxidoreductase enzymes were used together with Pectinex Ultra SP the respective biofilms was not only removed from both types of surfaces but the viable cells were also killed.

As a means to detach bacteria from soil particles, Böckelmann *et al.* (2003) compared a conventional method (dispersal with sodium pyrophosphate buffer) with a method whereby enzymes were used in combination with sonication. An enzyme mixture comprising two polysaccharide-degrading enzymes (β-galactosidase and α-glucosidase) and a lipase were used to assist in weakening of the EPS. The combined use of these enzymes and sonication detached a 22-fold higher number of bacterial cells compared to the conventional method. When the enzymes were used individually, β-galactosidase, α-glucosidase and lipase detached a 2- to 10-fold, 1- to 7-fold and 1- to 3-fold higher amount of bacterial cells, respectively, compared to the conventional method (Böckelmann *et al.*, 2003).

Walker *et al.* (2007) tested a range of commercially available enzymes of various types for their ability to destroy microbial biofilms. Biofilms of *Lactobacillus brevis*, *Flavimonas oryzihabitans*, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* were incubated with an enzyme mixture containing α-amylase, β-glucuronidase, glucose oxidase, dextranase, protease and pectinase. Enzyme mixtures comprising of either single strength (10 U/ml of each enzyme) or double strength enzymes were used. The double strength enzyme mixture was shown to be more effective in removing biofilms of *L. brevis*, *L. mesenteroides* and *S. cerevisiae* compared to the use of a single strength enzyme mixture. The number of *F. oryzihabitans* cells, however, increased when treated with the single strength enzyme mixture, while the double strength enzyme mixture was less effective in removing biofilms of this bacterium compared to the other microorganisms. Based on the results obtained, Walker *et al.* (2007) noted that a range of factors such as biofilm composition, attachment surface, application time and temperature may have a major effect on the efficacy of enzymes to remove biofilms.

Orgaz *et al.* (2006) tested various different enzymes from fungal origin for their ability to remove *P. fluorescens* biofilms from glass coupons. Although each of the enzymes, *i.e.* cellulase, pectin lyase, polygalacturonase, arabinase, alginate lyase and proteinase, showed some degree of biofilm removal, a mixture of these enzymes was less efficient in removing the biofilms. The latter was ascribed to the proteinase degrading the other enzymes present in the mixture. In a subsequent study, Orgaz *et al.* (2007) investigated the usefulness of three polysaccharide-degrading enzymes (cellulase, pectin esterase and pectin lyase) and Pronase® (a mixture of proteinases consisting of an alkaline protease, neutral protease, and amino- and carboxy-peptidases) in the removal of *P. fluorescens* biofilms. These enzymes were tested individually or in a sequential (two-step) application. Of the single polysaccharide-degrading enzymes, pectin esterase was the most efficient in removing *P. fluorescens* biofilms, showing removal of approximately 75% of the biofilm cells. Soluble Pronase® removed 80% of the biofilm cells and 30% of the biofilm biomass. Sequential (two-step) treatment with cellulase, pectin esterase or pectin lyase, followed by soluble Pronase®, obtained a 2-3 $\log_{10}$ count reduction and up to 94% biomass removal. To allow enzyme treatment in a single step, a newly developed form of delayed release encapsulated Pronase (DRE Pro), which only releases proteases after the soluble enzymes have worked for 20-60 min, was also used. Treatment of a *P. fluorescens* biofilm for 2 h with a combination of the DRE Pro and polysaccharide-degrading enzymes resulted in a 4 $\log_{10}$ reduction in biofilm cells and removed 96% of the biofilm from the surface.

Chaignon *et al.* (2007) assessed the susceptibility of biofilms of clinical staphylococcal strains to a range of enzyme preparations, *i.e.* Dispersin B, pancreatin, proteinase K, Pectinex Ultra SP, periodate and trypsin. It was demonstrated that Dispersin B was the most effective against strains that contained *N*-acetylglucosamine as the major component in the extracellular biofilm matrix, whereas the biofilms were not affected by protease treatment. Conversely, strains that lacked *N*-acetylglucosamine were removed efficiently by treatment with proteases. The components making up the matrix of staphylococcal biofilms therefore play an important role in the enzymatic removal of these biofilms, especially since the composition may differ among the clinical strains. Chaignon *et al.* (2007) proposed that by using both Dispersin B and a protease (proteinase K or trypsin), biofilms formed by a range of staphylococcal strains can be removed from inert surfaces.

2.6.3.4 Combined use of enzymes and chemicals

Merritt *et al.* (2000) tested several cleaning agents (tap water, commercial detergents with surfactants, detergents containing enzymes, enzymatic and non-enzymatic contact lens cleaning solutions, commercial mouthwash, peroxide-based bleach, bleach containing 5.25% sodium hypochlorite, Triton X-100) against biofilms of *Staphylococcus epidermidis* and *Candida albicans*, as well as biofilms formed by oral microorganisms grown in 96-well polystyrene plates. The results indicated that biofilms formed by the respective microorganisms were the most successfully removed by detergents containing enzymes and sodium hypochlorite (NaOCl), while the other detergents and surfactants were less effective.

Vickery *et al.* (2004) also tested the effectiveness of several non-enzymatic and enzymatic detergents on the removal of *E. coli* biofilms from medical tubing used in endoscope and infusion set construction. Of the different detergents tested, a non-enzymatic detergent, Matrix, and a high-activity enzymatic detergent, Epizyme Rapid, were the most effective and were capable of detaching 75% and 60% of the biofilm, respectively. Epizyme Rapid, which consists of high amylase and protease activity, removed 30% of the exopolysaccharides, but it did not show more than a 2 $\log_{10}$ reduction in the viable bacterial count. In contrast, the non-enzymatic detergent Matrix reduced the viable bacterial count by more than 4 $\log_{10}$ and the exopolysaccharides were reduced by 80%.

Oulahal *et al.* (2007) investigated the removal of biofilms formed by two meat spoilage microorganisms (*E. coli* and *S. aureus*) by making use of combined ultrasonic and enzymatic and/or chelating agent treatment. The application of ultrasound alone was not effective in removing all biofilms from a stainless steel surface. When ultrasound was used in combination with EDTA and/or enzymes, approximately 75% of *E. coli* and 100% of *S. aureus* biofilms were removed. Similarly, Oulahal-Lagsir *et al.* (2003) reported that ultrasonication combined with enzymatic treatment (proteolytic or glycolytic enzymes) were more effective in removing *E. coli* biofilms from a stainless steel surface than application of ultrasound alone.

2.7 REFERENCES

- Abdel-Rahman, M.S., Couri, D. and Bull, R.J. 1984. Toxicity of chlorine dioxide in drinking water. *International Journal of Toxicology*, **3**: 277-284.
- Allison, D.G. 2003. The biofilm matrix. *Biofouling*, **19**: 139-150.
- Allison, D.G., Gilbert, P., Lappin-Scott, H.M. and Wilson, M. 2000. Community structure and co-operation in biofilms. Cambridge University Press, Cambridge.
- Allison, D.G., Ruiz, B., SanJose, C., Jaspe, A. and Gilbert, P. 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiology Letters*, **167**: 179-184.
- Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A. and Maier, R.M. 2000. Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. *Applied and Environmental Microbiology*, **66**: 3262-3268.
- Anderl, J.N., Franklin, M.J. and Stewart, P.S. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, **44**: 1818-1824.
- Anderson, A.C., Reimers, R.S. and DeKernion, P. 1982. A brief review of the current status of alternatives to chlorine disinfection of water. *American Journal of Public Health*, **72**: 1290-1293.
- Atkinson, S., Throup, J.P., Stewart, G.S.A.B. and Williams, P. 1999. A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Molecular Microbiology*, **33**: 1267-1277.

- Augustin, M. and Ali-Vehmas, T. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmacy and Pharmaceutical Sciences*, **7**: 55-64.
- Austin, J.W., Sanders, G., Kay, W.W. and Collinson, S.K. 1998. Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. *FEMS Microbiology Letters*, **162**: 295-301.
- Balaban, N., Giacometti, A., Cirioni, O., Gov, Y., Ghiselli, R., Mocchegiani, F., Viticchi, C., Prete, M.S.D., Saba, V., Scalise, G. and Dell'Acqua, G. 2003. Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation *in vivo* by drug-resistant *Staphylococcus epidermidis. Journal of Infectious Diseases*, **187**: 625-630.
- Baribeau, H., Prévost, M., Desjardins, R., Lafrence, P. and Gates, D.J. 2002. Chlorite and chlorate ion variability in distribution systems. *Journal of the American Water Works Association*, **94**: 96-105.
- Bayer, A.S., Park, S., Ramos, M.C., Nast, C.C., Eftekhar, F. and Schiller, N.L. 1992. Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa. Infection and Immunity*, **60**: 3979-3985.
- Bendinger, B., Rijnaarts, H.H.M., Altendorf, K. and Zehnder, A.B.J. 1993. Physicochemical cell surface and adhesive properties of Coryneform bacteria related to the presence and chain length of mycolic acids. *Applied and Environmental Microbiology*, **59**: 3973-3977.
- Bereswill, S. and Geider, K. 1997. Characterization of the *rcsB* gene from *Erwinia amylovora* and its influence on exopolysaccharide synthesis and virulence of the fire blight pathogen. *Journal of Bacteriology*, **179**: 1354-1361.
- Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L. and Li, Z. 1997. Interactions between biofilms and the environment. *FEMS Microbiology Reviews*, **20**: 291-303.
- Blanco, M.A., Negro, C., Gaspar, I. and Tijero, J. 1996. Slime problems in the paper and board industry. *Applied Microbiology and Biotechnology*, **45**: 132-136.
- Böckelmann, U., Szewzyk, U. and Grohmann, E. 2003. A new enzymatic method for the detachment of particle associated soil bacteria. *Journal of Microbiological Methods*, **55**: 201-211.
- Boland, T., Dufrêne, Y., Barger, B. and Lee, G. 2000. Molecular basis of cell adhesion to polymers characterized by AFM. *Critical Reviews in Biomedical Engineering*, **28**: 195-196.
- Boyd, A. and Chakrabarty, A.M. 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, **60**: 2355-2359.
- Brooun, A., Liu, S. and Lewis, K. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, **44**: 640-646.
- Brown, M.R.W. and Barker, J. 1999. Unexplored reservoirs of pathogenic bacteria: Protozoa and biofilms. *Trends in Microbiology*, **7**: 46-50.

- Bura, R., Cheung, M., Liao, B., Finlayson, J., Lee, B.C., Droppo, I.G., Leppard, G.G. and Liss, S.N. 1998. Composition of extracellular polymeric substances in the activated sludge floc matrix. *Water Science and Technology*, **37**: 325-333.
- Cadmus, M.C., Jackson, L.K., Burton, K.A., Plattner, R.D. and Slodki, M.E. 1982. Biodegradation of xanthan gum by *Bacillus* sp. *Applied and Environmental Microbiology*, **44**: 5-11.
- Carlén, A., Nikdel, K., Wennerberg, A., Holmberg, K. and Olsson, J. 2001. Surface characteristics and *in vitro* biofilm formation on glass ionomer and composite resin. *Biomaterials*, **22**: 481-487.
- Carlsson, F.H.H. 2003. Elementary handbook of water disinfection. Water Research Commission Report No. TT 205/03, South Africa.
- Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J.B. and Jabbouri, S. 2007. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Applied Microbiology and Biotechnology*, **75**: 125-132.
- Chandy, J.P. and Angles, M.L. 2001. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Research*, **35**: 2677-2682.
- Characklis, W.G. and Dydek, S.T. 1976. Influence of carbon-nitrogen ratio on the chlorination of microbial aggregates. *Water Research*, **10**: 515-522.
- Characklis, W.G. and Marshall, K.C. 1990. Biofilms. Wiley Interscience Publishers, Canada.
- Chen, J., Lee, S.M. and Mao, Y. 2004. Protective effect of exopolysaccharide colonic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *International Journal of Food Microbiology*, **93**: 281-286.
- Chen, M.J., Zhang, Z. and Bott, T.R. 1998. Direct measurement of the adhesive strength of biofilms in pipes by micromanipulation. *Biotechnology Techniques*, **12**: 875-880.
- Chen, X. and Stewart, P.S. 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. *Environmental Science and Technology*, **30**: 2078-2083.
- Chen, X. and Stewart, P.S. 2000. Biofilm removal caused by chemical treatments. *Water Research*, **34**: 4229-4233.
- Chen, X. and Stewart, P.S. 2002. Role of electrostatic interactions in cohesion of bacterial biofilms. *Applied Microbiology and Biotechnology*, **59**: 718-720.
- Chmielewski, R.A.N. and Frank, J.F. 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety*, **2**: 22-32.
- Christensen, B.E., Ertesvag, H., Beyenal, H. and Lewandowski, Z. 2001. Resistance of biofilms containing alginate-producing bacteria to disintegration by an alginate degrading enzyme (AlgL). *Biofouling*, **17**: 203-210.

- Christensen, B.B., Sternberg, C., Andersen, J.B., Eberl, L., Møller, S. and Givskov, M. 1998. Establishment of new genetic traits in a microbial biofilm community. *Applied and Environmental Microbiology*, **64**: 2247-2255.
- Cloete, T.E. 2003. Resistance mechanisms of bacteria to antimicrobial compounds. *International Biodeterioration and Biodegradation*, **51**: 277-282.
- Cloete, T.E., Jacobs, L. and Brözel, V.S. 1998. The chemical control of biofouling in industrial water systems. *Biodegradation*, **9**: 23-37.
- Cochran, W.L., Suh, S-J., McFeters, G.A. and Stewart, P.S. 2000. Role of RpoS and AlgT in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide and monochloramine. *Journal of Applied Microbiology*, **88**: 546-553.
- Cole, S.P., Harwood, J., Lee, R., She, R. and Guiney, D.G. 2004. Characterization of monospecies biofilm formation by *Helicobacter pylori*. *Journal of Bacteriology*, **186**: 3124-3132.
- Conti, E., Flaibani, A., O'Regan, M. and Sutherland, I.W. 1994. Alginate from *Pseudomonas fluorescens* and *P. putida*: Production and properties. *Microbiology*, **140**: 1125-1132.
- Correia, F.F., D'Onofrio, A., Rejtar, T., Li, L., Karger, B.L., Makarova, K., Koonin, E.V. and Lewis, K. 2006. Kinase activity of overexpressed HipA is required for growth arrest and multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology*, **188**: 8360-8367.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual Reviews in Microbiology*, **49**: 711-745.
- Costerton, J.W., Stewart, P.S. and Greenberg, E.P. 1999. Bacterial biofilms: A common cause of persistent infections. *Science*, **284**: 1318-1322.
- Cowan, M.M., Warren, T.M. and Fletcher, M. 1991. Mixed-species colonization of solid surfaces in laboratory biofilms. *Biofouling*, **3**: 23-34.
- Cui, W., Winter, W.T., Tanenbaum, S.W. and Nakas, J.P. 1999. Purification and characterization of an intracellular carboxylesterase from *Arthrobacter viscosus* NRRL B-1973. *Enzyme and Microbial Technology*, **24**: 200-208.
- Curtin, J.J. and Donlan, R.M. 2006. Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy*, **50**: 1268-1275.
- Daamen, E.J., Wouters, J.W. and Savelkoul, J.T.G. 2000. Side stream biofiltration for improved biofouling control in cooling water systems. *Water Science and Technology*, **41**: 445-451.
- Danese, P.N., Pratt, L.A. and Kolter, R. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. *Journal of Bacteriology*, **182**: 3593-3596.

- Davey, M.E. and O'Toole, G.A. 2000. Microbial biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, **64**: 847-867.
- Davies, D.G. and Geesey, G.G. 1995. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Applied and Environmental Microbiology*, **61**: 860-867.
- Davies, D.G., Chakrabarty, A.M. and Geesey, G.G. 1993. Exopolysaccharide production in biofilms: Substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, **59**: 1181-1186.
- Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. and Greenberg, E.P. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, **280**: 295-298.
- de Beer, D., Srinivasan, R. and Stewart, P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology*, **60**: 4339-4344.
- De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B. 2001a. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: Their role and expression patterns. *Applied and Environmental Microbiology*, **67**: 1865-1873.
- De Kievit, T.R., Parkins, M.D., Gillis, R.J., Srikumar, R., Ceri, H., Poole, K., Iglewski, B.H. and Storey, D.G. 2001b. Multidrug efflux pumps: Expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, **45**: 1761-1770.
- Dignac, M-F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D. and Scribe, P. 1998. Chemical description of extracellular polymers: Implication on activated sludge floc structure. *Water Science and Technology*, **38**: 45-53.
- Donelli, G., Francolini, I., Piozzi, A., Di Rosa, R. and Marconi, W. 2002. New polymer-antibiotic systems to inhibit bacterial biofilm formation: A suitable approach to prevent central venous catheter-associated infections. *Journal of Chemotherapy*, **14**: 501-507.
- Dong, Y-H., Gusti, A.R., Zhang, Q., Xu, J-L. and Zhang, L-H. 2002. Identification of quorum-quenching *N*-Acyl Homoserine Lactonases from *Bacillus* species. *Applied and Environmental Microbiology*, **68**: 1754-1759.
- Donlan, R. 2002. Biofilm: Microbial life on surfaces. *Emerging Infectious Diseases*, **8**: 881-890.
- Donlan, R.M. and Costerton, J.W. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, **15**: 167-193.
- Dorward, D.W. and Garon, C.F. 1990. DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria. *Applied and Environmental Microbiology*, **56**: 1960-1962.

- Dosti, B., Guzel-Seydim, Z. and Greene, A.K. 2005. Effectiveness of ozone, heat and chlorine for destroying common food spoilage bacteria in synthetic media and biofilms. *International Journal of Dairy Technology*, **58**: 19-24.
- Drenkard, E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes and Infection*, **5**: 1213-1219.
- Eginton, P.J., Holah, J., Allison, D.G., Handley, P.S. and Gilbert, P. 1998. Changes in the strength of attachment of microorganisms to surfaces following treatment with disinfectants and cleaning agents. *Letters in Applied Microbiology*, **27**: 101-105.
- Elasri, M.O. and Miller, R.V. 1999. Study of the response of a biofilm bacterial community to UV radiation. *Applied and Environmental Microbiology*, **65**: 2025-2031.
- Elkins, J.G., Hassett, D.J., Stewart, P.S., Schweizer, H.P. and McDermott, T.R. 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. *Applied and Environmental Microbiology*, **65**: 4594-4600.
- Eriksson, T., Börjesson, J. and Tjerneld, F. 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology*, **31**: 353-364.
- Espinosa-Urgel, M., Salido, A. and Ramos, J-L. 2000. Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *Journal of Bacteriology*, **182**: 2363-2369.
- Evans, D.J., Allison, D.G., Brown, M.R.W. and Gilbert, P. 1991. Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: Effect of specific growth rate. *Journal of Antimicrobial Chemotherapy*, **27**: 177-184.
- Finelli, A., Gallant, C.V., Jarvi, K. and Burrows, L.L. 2003. Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology*, **185**: 2700-2710.
- Fletcher, M. 1988. Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium-substratum separation distance. *Journal of Bacteriology*, **170**: 2027-2030.
- Fletcher, M. and Loeb, G.I. 1979. Influence of substratum characteristics on the attachment of a marine Pseudomonas to solid surfaces. *Applied and Environmental Microbiology*, **37**: 67-72.
- Foley, S., Lucchini, S., Zwahlen, M-C. and Brüssow, H. 1998. A short non-coding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus. Virology*, **250**: 377-387.
- Francolini, I., Donelli, G. and Stoodley, P. 2004. Polymer designs to control biofilm growth on medical devices. *Reviews in Environmental Science and Bio/Technology*, **2**: 307-319.
- Freese, S.D. and Nozaic, D.J. 2004. Chlorine: Is it really so bad and what are the alternatives? *Water SA*, **30**: 18-24.

- Friedman, H., Volin, E. and Laumann, D. 1968. Terminal disinfection in hospitals with quaternary ammonium compounds by use of a spray-fog technique. *Applied Microbiology*, **16**: 223-227.
- Fujinaga, M., Sielecki, A.R., Read, R.J., Ardelt, W., Laskowski, M. and James, M.N.G. 1987. Crystal and molecular structures of the complex of α-chymotrypsin with its inhibitor turkey ovomucoid third domain at 1.8 Å resolution. *Journal of Molecular Biology*, **195**: 397-418.
- Gacesa, P. 1998. Bacterial alginate biosynthesis – recent progress and future prospects. *Microbiology*, **144**: 1133-1143.
- Gagnon, G.A., O’Leary, K.C., Volk, C.J., Stover, L. and Andrews, R.C. 2004. Comparative analysis of chlorine dioxide, free chlorine and chloramines on bacterial water quality in model distribution systems. *Journal of Environmental Engineering*, **130**: 1269-1279.
- Gavín, R., Merino, S., Altarriba, M., Canals, R., Shaw, J.G. and Tomás, J.M. 2003. Lateral flagella are required for increased cell adherence, invasion and biofilm formation by *Aeromonas* spp. *FEMS Microbiology Letters*, **224**: 77-83.
- Geesey, G.G. 1982. Microbial exopolymers: Ecological and economic considerations. *American Society for Microbiology News*, **48**: 9-14.
- Geesey, G.G. 2001. Bacterial behavior at surfaces. *Current Opinion in Microbiology*, **4**: 296-300.
- Genevaux, P., Bauda, P., DuBow, M.S. and Oudega, B. 1999. Identification of Tn10 insertions in the *rfaG*, *rfaP*, and *galU* genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. *Archives of Microbiology*, **172**: 1-8.
- Ghannoum, M. and O’Toole, G.A. 2004. Microbial biofilms. ASM Press, USA.
- Ghigo, J-M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature*, **412**: 442-445.
- Ghorbel, B., Sellami-Kamoun, A. and Nasri, M. 2003. Stability studies of protease from *Bacillus cereus* BG1. *Enzyme and Microbial Technology*, **32**: 513-518.
- Gibson, H., Taylor, J.H., Hall, K.E. and Holah, J.T. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology*, **87**: 41-48.
- Gilbert, H.J. and Hazlewood, G.P. 1993. Bacterial cellulases and xylanases. *Microbiology*, **139**: 187-194.
- Gilbert, P., Allison, D.G. and McBain, A.J. 2002. Biofilms *in vitro* and *in vivo*: Do singular mechanisms imply cross-resistance? *Journal of Applied Microbiology Symposium Supplement*, **92**: 98S-110S.
- Gilbert, P., Collier, P.J. and Brown, M.R.W. 1990. Influence of growth rate on susceptibility to antimicrobial agents: Biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial Agents and Chemotherapy* **34**: 1865-1868.

- Gilligan, P.H. 1991. Microbiology of airway disease in patients with cystic fibrosis. *Clinical Microbiology Reviews*, **4**: 35-51.
- Giwercman, B., Jensen, E.T., Høiby, N., Kharazmi, A. and Costerton, J.W. 1991. Induction of β-lactamase production in *Pseudomonas aeruginosa* biofilm. *Antimicrobial Agents and Chemotherapy*, **35**: 1008-1010.
- Gordon, C.A., Hodges, N.A. and Marriott, C. 1991. Use of slime dispersants to promote antibiotic penetration through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy*, **35**: 1258-1260.
- Grobe, K., Zahler, J. and Stewart, P. 2002. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Journal of Industrial Microbiology and Biotechnology*, **29**: 10-15.
- Guzel-Seydim, Z., Greene, A.K. and Seydim, A.C. 2004. Use of ozone in the food industry. *Swiss Society of Food Science and Technology*, **37**: 453-460.
- Habash, M. and Reid, G. 1999. Microbial biofilms: Their development and significance for medical device-related infections. *Journal of Clinical Pharmacology*, **39**: 887-898.
- Hahn Berg, I.C., Kalfas, S., Malmsten, M. and Arnebrant, T. 2001. Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: Potential of proteases originating from *Euphausia superba* for plaque control. *European Journal of Oral Science*, **109**: 316-324.
- Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*, **2**: 95-108.
- Hammer, B.K. and Bassler, B.L. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae. Molecular Microbiology*, **50**: 101-114.
- Hanlon, G.W., Denyer, S.P., Olliff, C.J. and Ibrahim, L.J. 2001. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, **67**: 2746-2753.
- Hanna, A., Berg, M., Stout, V. and Razatos, A. 2003. Role of capsular colanic acid in adhesion of uropathogenic *Escherichia coli*. *Applied and Environmental Microbiology*, **69**: 4474-4481.
- Harraghy, N., Kerdudou, S. and Herrmann, M. 2007. Quorum-sensing systems in staphylococci as therapeutic targets. *Analytical and Bioanalytical Chemistry*, **387**: 437-444.
- Helle, S.S., Duff, S.J.B. and Cooper, D.G. 2004. Effect of surfactants on cellulose hydrolysis. *Biotechnology and Bioengineering*, **42**: 611-617.
- Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., Rice, S.A., Eberl, L., Molin, S., Høiby, N., Kjelleberg, S. and Givskov, M. 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology*, **148**: 87-102.

- Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M. and Parsek, M.R. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *Journal of Bacteriology*, **183**: 5395-5401.
- Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N., Kumar, N., Schembri, M.A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J.W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Høiby, N. and Givskov, M. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *European Molecular Biology Organization Journal*, **22**: 3803-3815.
- Hibma, A.M., Jassim, S.A.A. and Griffiths, M.W. 1997. Infection and removal of L-forms of *Listeria monocytogenes* with bred bacteriophage. *International Journal of Food Microbiology*, **34**: 197-207.
- Hill, C., Miller, L.A. and Klaenhammer, T.R. 1990. Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. *Journal of Bacteriology*, **172**: 6419-6426.
- Hinsa, S.M. and O'Toole, G.A. 2006. Biofilm formation by *Pseudomonas fluorescens* WCS365: A role for LapD. *Microbiology*, **152**: 1375-1383.
- Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L. and O'Toole, G.A. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Molecular Microbiology*, **49**: 905-918.
- Hou, C.T., Barnabe, N. and Greaney, K. 1986. Biodegradation of xanthan by salt-tolerant microorganisms. *Journal of Industrial Microbiology and Biotechnology*, **1**: 31-37.
- Hoyle, B.D., Williams, L.J. and Costerton, J.W. 1993. Production of mucoid exopolysaccharide during development of *Pseudomonas aeruginosa* biofilms. *Infection and Immunity*, **61**: 777-780.
- Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S. and Eberl, L. 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology*, **147**: 2517-2528.
- Hughes, K.A., Sutherland, I.W. and Jones, M.V. 1998a. Biofilm susceptibility to bacteriophage attack: The role of phage-borne polysaccharide depolymerase. *Microbiology*, **144**: 3039-3047.
- Hughes, K.A., Sutherland, I.W., Clark, J. and Jones, M.V. 1998b. Bacteriophage and associated polysaccharide depolymerases - novel tools for study of bacterial biofilms. *Journal of Applied Microbiology*, **85**: 583-590.
- Hunt, S.M., Werner, E.M., Huang, B., Hamilton, M.A. and Stewart, P.S. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. *Applied and Environmental Microbiology*, **70**: 7418-7425.

- Inoue, T., Shingaki, R., Sogawa, N., Sogawa, C.A., Asaumi, J-I., Kokeguchi, S. and Fukui, K. 2003. Biofilm formation by a fimbriae-deficient mutant of *Actinobacillus actinomycetemcomitans*. *Journal of Microbiology and Immunology*, **47**: 877-881.
- Ioannou, C.J., Hanlon, G.W. and Denyer, S.P. 2007. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **51**: 296-306.
- Ishida, H., Ishida, Y., Kurosaka, Y., Otani, T., Sato, K. and Kobayashi, H. 1998. *In vitro* and *in vivo* activities of levofloxacin against biofilm-producing *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **42**: 1641-1645.
- Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F. and Romeo, T. 2005. Depolymerization of β-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology*, **187**: 382-387.
- Jahn, A. and Nielsen, P.H. 1998. Cell biomass and exopolymers composition in sewer biofilms. *Water Science and Technology*, **37**: 17-24.
- Johansen, C., Falholt, P. and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology*, **63**: 3724-3728.
- Jonas, R. and Farah, L.F. 1998. Production and application of microbial cellulase. *Polymer Degradation and Stability*, **59**: 101-106.
- Jorand, F., Bouge-Bigne, F., Block, J.C. and Urbain, V. 1998. Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Water Science and Technology*, **37**: 307-316.
- Jurcisek, J.A. and Bakaletz, L.O. 2007. Biofilms formed by non-typeable *Haemophilus influenzae in vivo* contain both double-stranded DNA and type IV pilin protein. *Journal of Bacteriology*, **189**: 3868-3875.
- Kadurugamuwa, J.L. and Beveridge, T.J. 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: A novel mechanism of enzyme secretion. *Journal of Bacteriology*, **177**: 3998-4008.
- Kaplan, J.B., Ragunath, C., Ramasubbu, N. and Fine, D.H. 2003. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous β-hexosaminidase activity. *Journal of Bacteriology*, **185**: 4693-4698.
- Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H. and Ramasubbu, N. 2004a. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents and Chemotherapy*, **48**: 2633-2636.
- Kaplan, J.B., Velliyagounder, K., Ragunath, C., Rohde, H., Mack, D., Knobloch, J.K-M. and Ramasubbu, N. 2004b. Genes involved in the synthesis and degradation of matrix

polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *Journal of Bacteriology*, **186**: 8213-8220.

- Keevil, C.W., Godfree, A., Holt, D. and Dow, C. 1999. Biofilms in the aquatic environment. The Royal Society of Chemistry, UK.
- Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K. 2004a. Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters*, **230**: 13-18.
- Keren, I., Shah, D., Spoering, A., Kaldalu, N. and Lewis, K. 2004b. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology*, **186**: 8172-8180.
- Kjelleberg, S. and Molin, S. 2002. Is there a role for quorum sensing signals in bacterial biofilms? *Current Opinion in Microbiology*, **5**: 254-258.
- Klausen, M., Heydorn, A., Ragas, P.C., Lambertsen, L., Aaes-Jørgensen, A., Molin, S. and Tolker-Nielsen, T. 2003. Biofilm formation by *Pseudomonas aeruginosa* wild-type, flagella and type IV pili mutants. *Molecular Microbiology*, **48**: 1511-1524.
- Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M. and Kohno, K. 2004. Papain protects papaya trees from herbivorous insects: Role of cysteine proteases in latex. *The Plant Journal*, **37**: 370-378.
- Korn, C., Andrews, R.C. and Escobar, M.D. 2002. Development of chlorine dioxide-related by-product models for drinking water treatement. *Water Research*, **36**: 330-342.
- Kostenko, V., Ceri, H. and Martinuzzi, R.J. 2007. Increased tolerance of *Staphylococcus aureus* to vancomycin in viscous media. *FEMS Immunology and Medical Microbiology*, **51**: 277-288.
- Kreft, J-U. 2004. Biofilms promote altruism. *Microbiology*, **150**: 2751-2760.
- Kumar, C.G. and Anand, S.K. 1998. Significance of microbial biofilms in food industry: A review. *International Journal of Food Microbiology*, **42**: 9-27.
- Labbate, M., Queck, S.Y., Koh, K.S., Rice, S.A., Givskov, M. and Kjelleberg, S. 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. *Journal of Bacteriology*, **186**: 692-698.
- Langsrud, S., Sundheim, G. and Borgmann-Strahsen, R. 2003. Intrinsic and acquired resistance to quaternary ammonium compounds in food-related *Pseudomonas* spp. *Journal of Applied Microbiology*, **95**: 874-882.
- Leger, R.J.S.T., Frank, D.C., Roberts, D.W. and Staples, R.C. 1992. Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *European Journal of Biochemistry*, **204**: 991-1001.
- Lemon, K.P., Higgins, D.E. and Kolter, R. 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology*, **189**: 4418-4424.

- Lewandowski, Z. and Beyenal, H. 2007. Fundamentals of biofilm research. CRC Press, Boca Raton.
- Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy*, **45**: 999-1007.
- Lin, Y-H., Xu, J-L., Hu, J., Wang, L-H., Ong, S.L., Leadbetter, J.R. and Zhang, L-H. 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology*, **47**: 849-860.
- Loiselle, M. and Anderson, K.W. 2003. The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling*, **19**: 77-85.
- Lubbers, J.R., Chauhan, S. and Bianchine, J.R. 1981. Controlled clinical evaluations of chlorine dioxide, chlorite and chlorate in man. *Toxicological Sciences*, **1**: 334-338.
- Ludensky, M. 2003. Control and monitoring of biofilms in industrial applications. *International Biodeterioration and Biodegradation*, **51**: 255-263.
- Lynch, M.J., Swift, S., Kirke, D.F., Keevil, C.W., Dodd, C.E.R. and Williams, P. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. *Environmental Microbiology*, **4**: 18-28.
- Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H. and Laufs, R. 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan: Purification and structural analysis. *Journal of Bacteriology*, **178**: 175-183.
- Mah, T-F. and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*, **9**: 34-39.
- Mah, T-F., Pitts, B., Pellock, B. and Walker, G.C. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature*, **426**: 306-310.
- Maira-Litrán, T., Allison, D.G. and Gilbert, P. 2000. An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump *arcAB* to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms. *Journal of Antimicrobial Chemotherapy*, **45**: 789-795.
- Makin, S.A. and Beveridge, T.J. 1996. The influence of A-band and B-band lipopolysaccharides on the surface characteristics and adhesion of *Pseudomonas aeruginosa* to surfaces. *Microbiology*, **142**: 299-307.
- Manefield, M., Rasmussen, T.B., Hentzer, M., Andersen, J.B., Steinberg, P., Kjelleberg, S. and Givskov, M. 2002. Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology*, **148**: 1119-1127.
- Mao, Y., Doyle, M.P. and Chen, J. 2001. Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. *Journal of Bacteriology*, **183**: 3811-3815.

- Mara, D. and Horan, N. 2003. The Handbook of Water and Wastewater Microbiology. Academic Press, UK.
- Matthysse, A.G., White, S. and Lightfoot, R. 1995. Genes required for cellulose synthesis in *Agrobacterium tumefaciens*. *Journal of Bacteriology*, **177**: 1069-1075.
- Melo, L.F. and Bott, T.R. 1997. Biofouling in water systems. *Experimental Thermal and Fluid Science*, **14**: 375-381.
- Merritt, K., Hitchins, V.M. and Brown, S.A. 2000. Safety and cleaning of medical materials and devices. *Journal of Biomedical Materials Research*, **53**: 131-136.
- Meyer, B. 2003. Approaches to prevention, removal and killing of biofilms. *International Biodeterioration and Biodegradation*, **51**: 249-253.
- Miller, M.B. and Bassler, B.L. 2001. Quorum sensing in bacteria. *Annual Reviews in Microbiology*, **55**: 165-199.
- Mishra, C. and Robbins, P.W. 1995. Specific β-glucanases as tools for polysaccharide structure determination. *Glycobiology*, **5**: 645-654.
- Morgan, T.D. and Wilson, M. 2001. The effects of surface roughness and type of denture acrylic on biofilm formation by *Streptococcus oralis* in a constant depth film fermentor. *Journal of Applied Microbiology*, **91**: 47-53.
- Morgenroth, E. and Wilderer, P.A. 2000. Influence of detachment mechanisms on competition in biofilm. *Water Research*, **34**: 417-426.
- Moscoso, M., García, E. and López, R. 2006. Biofilm formation by *Streptococcus pneumoniae*: Role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *Journal of Bacteriology*, **188**: 7785-7795.
- Murga, R., Miller, J.M. and Donlan, R.M. 2001. Biofilm formation by Gram-negative bacteria on central venous catheter connectors: Effect of conditioning films in a laboratory model. *Journal of Clinical Microbiology*, **39**: 2294-2297.
- Murtough, S.M., Hiom, S.J., Palmer, M. and Russell, A.D. 2000. A survey of disinfectant use in hospital pharmacy aseptic preparation areas. *The Pharmaceutical Journal*, **264**: 446-448.
- Nielsen, P.H., Jahn, A. and Palmgren, R. 1997. Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology*, **36**: 11-19.
- Norwood, D.E. and Gilmour, A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology*, **88**: 512-520.
- O’Toole, G., Kaplan, H.B. and Kolter, R. 2000. Biofilm formation as microbial development. *Annual Reviews in Microbiology*, **54**: 49-79.
- O’Toole, G.A. and Kolter, R. 1998a. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, **30**: 295-304.

- O'Toole, G.A. and Kolter, R. 1998b. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: A genetic analysis. *Molecular Microbiology*, **28**: 449-461.
- Oh, Y.J., Jo, W., Yang, Y. and Park, S. 2007. Influence of culture conditions on *Escherichia coli* O157:H7 biofilm formation by atomic force microscopy. *Ultramicroscopy*, **107**: 869-874.
- Ophir, T. and Gutnick, D.L. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Applied and Environmental Microbiology*, **60**: 740-745.
- Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F. and SanJose, C. 2006. Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology*, **40**: 51-56.
- Orgaz, B., Neufeld, R.J. and SanJose, C. 2007. Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. *Enzyme and Microbial Technology*, **40**: 1045-1051.
- Oulahal, N., Martial-Gros, A., Bonneau, M. and Blum, L.J. 2007. Removal of meat biofilms from surfaces by ultrasound combined with enzymes and/or a chelating agent. *Innovative Food Science and Emerging Technologies*, **8**: 192-196.
- Oulahal-Lagsir, N., Martial-Gros, A., Bonneau, M., d'Blum, L.J. 2003. ''*Escherichia coli*-milk'' biofilm removal from stainless steel surfaces: Synergism between ultrasonic waves and enzymes. *Biofouling*, **19**: 159-168.
- Oyaizu, H., Komagata, K., Amemura, A. and Harada, T. 1982. A succinoglycan-decomposing bacterium, *Cytophaga arvensicola* sp. nov. *Journal of General and Applied Microbiology*, **28**: 369-388.
- Palmer, R.J. and White, D.C. 1997. Developmental biology of biofilms: Implications for treatment and control. *Trends in Microbiology*, **5**: 435-440.
- Park, S-Y., Lee, S.J., Oh, T-K., Oh, J-W., Koo, B-T., Yum, D-Y. and Lee, J-K. 2003. AhlD, an *N*-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology*, **149**: 1541-1550.
- Parsek, M.R. and Greenberg, E.P. 2005. Sociomicrobiology: The connections between quorum sensing and biofilms. *Trends in Microbiology*, **13**: 27-33.
- Pasmore, M., Todd, P., Pfiefer, B., Rhodes, M. and Bowman, C. 2002. Effect of polymer surface properties on the reversibility of attachment of *Pseudomonas aeruginosa* in the early stages of biofilm development. *Biofouling*, **18**: 65-71.
- Pasmore, M., Todd, P., Smith, S., Baker, D., Silverstein, J., Coons, D. and Bowman, C.N. 2001. Effects of ultrafiltration membrane surface properties on *Pseudomonas aeruginosa* biofilm initiation for the purpose of reducing biofouling. *Journal of Membrane Science*, **194**: 15-32.

- Pedersen, S.S., Kharazmi, A., Espersen, F. and Hoiby, N. 1990. *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. *Infection and Immunity*, **58**: 3363-3368.
- Peleg, M. 1976. The chemistry of ozone in the treatment of water. *Water Research*, **10**: 361-365.
- Percival, S.L. and Bowler, P.G. 2004. Biofilms and their potential role in wound healing. *Wounds*, **16**: 234-240.
- Percival, S.L., Knapp, J.S., Wales, D.S. and Edyvean, R.G.J. 1999. The effect of turbulent flow and surface roughness on biofilm formation in drinking water. *Journal of Industrial Microbiology and Biotechnology*, **22**: 152-159.
- Pisigan, R.A. and Singley, J.E. 1987. Influence of buffer capacity, chlorine residual, and flow rate on corrosion of mild steel and copper. *Journal of American Water Works Association*, **79**: 62-70.
- Poole, K. and Srikumar, R. 2001. Multidrug efflux in *Pseudomonas aeruginosa*: Components, mechanisms and clinical significance. *Current Topics in Medicinal Chemistry*, **1**: 59-71.
- Prakash, B., Veeregowda, B.M. and Krishnappa, G. 2003. Biofilms: A survival strategy of bacteria. *Current Science*, **85**: 1299-1307.
- Pratt, L.A. and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology*, **30**: 285-293.
- Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P. and Dorel, C. 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. *Journal of Bacteriology*, **183**: 7213-7223.
- Prigent-Combaret, C., Vidal, O., Dorel, C. and Lejeune, P. 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *Journal of Bacteriology*, **181**: 5993-6002.
- Pringle, J.H. and Fletcher, M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Applied and Environmental Microbiology*, **45**: 811-817.
- Prouty, A.M., Schwesinger, W.H. and Gunn, J.S. 2002. Biofilm formation and interaction with the surfaces of gallstones by *Salmonella* spp. *Infection and Immunity*, **70**: 2640-2649.
- Purevdorj, B., Costerton, J.W. and Stoodley, P. 2002. Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, **68**: 4457-4464.
- Puskas, A., Greenberg, E.P., Kaplan, S. and Schaefer, A.L. 1997. A quorum- sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides.* *Journal of Bacteriology*, **179**: 7530-7537.

- Ramsay, D.M. and Wozniak, D.J. 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Molecular Microbiology*, **56**: 309-322.
- Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, **62**: 597-635.
- Rasmussen, T.B., Manefield, M., Andersen, J.B., Eberl, L., Anthoni, U., Christophersen, C., Steinberg, P., Kjelleberg, S. and Givskov, M. 2000. How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1. *Microbiology*, **146**: 3237-3244.
- Rehm, B.H. and Valla, S. 1997. Bacterial alginates: Biosynthesis and applications. *Applied Microbiology and Biotechnology*, **48**: 281-288.
- Renelli, M., Matias, V., Lo, R.Y. and Beveridge, T.J. 2004. DNA-containing membrane vesicles of *Pseudomonas aeruginosa* PAO1 and their genetic transformation potential. *Microbiology*, **150**: 2161-2169.
- Rennecker, J.L., Driedger, A.M., Rubin, S.A. and Mariñas, B.J. 2000. Synergy in sequential inactivation of *Cryptosporidium parvum* with ozone/free chlorine and ozone/monochloramine. *Water Research*, **34**: 4121-4130.
- Rice, S.A., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W. and Kjellerberg, S. 2005a. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *Journal of Bacteriology*, **187**: 3477-3485.
- Rice, S.A., McDougald, D., Kumar, N. and Kjelleberg, S. 2005b. The use of quorum-sensing blockers as therapeutic agents for the control of biofilm-associated infections. *Current Opinion in Investigative Drugs*, **6**: 178-184.
- Rochex, A. and Lebeault, J-M. 2007. Effects of nutrients on biofilm formation and detachment of a *Pseudomonas putida* strain isolated from a paper machine. *Water Research*, **41**: 2885-2892.
- Rosenberg, M. and Kjellerberg, S. 1986. Hydrophobic interactions: Role in bacterial adhesion. *Advances in Microbial Ecology*, **9**: 353-393.
- Russell, A.D. 2003. Similarities and differences in the responses of microorganisms to biocides. *Journal of Antimicrobial Chemotherapy*, **52**: 750-763.
- Ryu, J.-H. and Beuchat, L.R. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: Effect of exopolysaccharide and curli production on its resistance to chlorine. *Applied and Environmental Microbiology*, **71**: 247-254.
- Sauer, K. and Camper, A.K. 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *Journal of Bacteriology*, **183**: 6579-6589.

- Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W. and Davies, D.G. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology*, **184**: 1140-1154.
- Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A.H., Davies, D.G. and Gilbert, P. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *Journal of Bacteriology*, **186**: 7312-7326.
- Schaber, J.A., Hammond, A., Carty, N.L., Williams, S.C., Colmer-Hamood, J.A., Burrowes, B.H., Dhevan, V., Griswold, J.A. and Hamood, A.N. 2007. Diversity of biofilms produced by quorum-sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*, **56**: 738-748.
- Scher, K., Romling, U. and Yaron, S. 2005. Effect of heat, acidification and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Applied and Environmental Microbiology*, **71**: 1163-1168.
- Schierholz, J.M. and Beuth, J. 2001. Implant infections: A haven for opportunistic bacteria. *Journal of Hospital Infection*, **49**: 87-93.
- Schnider-Keel, U., Lejbølle, K.B., Baehler, E., Haas, D. and Keel, C. 2001. The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. *Applied and Environmental Microbiology*, **67**: 5683-5693.
- Schooling, S.R. and Beveridge, T.J. 2006. Membrane vesicles: An overlooked component of the matrices of biofilms. *Journal of Bacteriology*, **188**: 5945-5957.
- Selan, L., Berlutti, F., Passariello, C., Comodi-Ballanti, M.R. and Thaller, M.C. 1993. Proteolytic enzymes: A new treatment strategy for prosthetic infections? *Antimicrobial Agents and Chemotherapy*, **37**: 2618-2621.
- Semmler, A.B.T., Whitchurch, C.B. and Mattick, J.S. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology*, **145**: 2863-2873.
- Sharon, N. and Lis, H. 2004. History of lectins: From hemagglutinins to biological recognition molecules. *Glycobiology*, **14**: 53R-62R.
- Sheng, W-H., Ko, W-J., Wang, J-T., Chang, S-C., Hsueh, P-R. and Luh, K-T. 2000. Evaluation of antiseptic-impregnated central venous catheters for prevention of catheter-related infection in intensive care unit patients. *Diagnostic Microbiology and Infectious Disease*, **38**: 1-5.
- Shih, P-C. and Huang, C-T. 2002. Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, **49**: 309-314.

- Siboni, N., Lidor, M., Kramarsky-Winter, E. and Kushmaro, A. 2007. Conditioning film and initial biofilm formation on ceramics tiles in the marine environment. *FEMS Microbiology Letters*, **274**: 24-29.
- Simões, M., Pereira, M.O., Silankorva, S., Azeredo, J. and Vieira, M.J. 2007. The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms. *Biofouling*, **23**: 249-258.
- Sio, C.F., Otten, L.G., Cool, R.H., Diggle, S.P., Braun, P.G., Bos, R., Daykin, M., Cámara, M., Williams, P. and Quax, W.J. 2006. Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infection and Immunity*, **74**: 1673-1682.
- Skillman, L.C., Sutherland, I.W. and Jones, M.V. 1999. The role of exopolysaccharides in dual species biofilm development. *Journal of Applied Microbiology*, **85**: 13S-18S.
- Spoering, A.L. and Lewis, K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology*, **183**: 6746-6751.
- Staehelln, J. and Holgné, J. 1982. Decomposition of ozone in water: Rate of initiation by hydroxide ions and hydrogen peroxide. *Environmental Science and Technology*, **16**: 676-681.
- Stapper, A.P., Narasimhan, G., Ohman, D.E., Barakat, J., Hentzer, M., Molin, S., Kharazmi, A., Høiby, N. and Mathee, K. 2004. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. *Journal of Medical Microbiology*, **53**: 679-690.
- Stewart, P.S. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy*, **40**: 2517-2522.
- Stewart, P.S. and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet*, **358**: 135-138.
- Stewart, P.S., Grab, L. and Diemer, J.A. 1998. Analysis of biocide transport limitation in an artificial biofilm system. *Journal of Applied Microbiology*, **85**: 495-500.
- Stewart, P.S., Roe, F., Rayner, J., Elkins, J.G., Lewandowski, Z., Ochsner, U.A. and Hassett, D.J. 2000. Effect of catalase on hydrogen peroxide penetration into *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, **66**: 836-838.
- Stoodley, P., Lewandowski, Z., Boyle, J.D. and Lappin-Scott, H.M. 1999. Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: An *in situ* investigation of biofilm rheology. *Biotechnology and Bioengineering*, **65**: 83-92.
- Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. 2002. Biofilms as complex differentiated communities. *Annual Reviews in Microbiology*, **56**: 187-209.
- Suci, P.A., Mittelman, M.W., Yu, F.P. and Geesey, G.G. 1994. Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, **38**: 2125-2133.

- Sugam, R. 1980. Biofouling control with ozone at the Bergen generation station. Report no. CS-1629, EPRI project no. 733-1, Electric Power Research Institute, Palo Alto, CA.
- Sundheim, G., Langsrud, S., Heir, E. and Holck, A.L. 1998. Bacterial resistance to disinfectants containing quaternary ammonium compounds. *International Biodeterioration and Biodegradation*, **41**: 235-239.
- Sutherland, I.W. 1999. Polysaccharases for microbial exopolysaccharides. *Carbohydrate Polymers*, **38**: 319-328.
- Sutherland, I.W. 2001a. Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology*, **147**: 3-9.
- Sutherland, I.W. 2001b. The biofilm matrix – an immobilized but dynamic microbial environment. *Trends in Microbiology*, **9**: 222-227.
- Sutherland, I.W. and Kennedy, L. 1996. Polysaccharide lyases from gellan-producing *Sphingomonas* spp. *Microbiology*, **142**: 867-872.
- Tait, K., Skillman, L.C. and Sutherland, I.W. 2002. The efficacy of bacteriophage as a method of biofilm eradication. *Biofouling*, **18**: 305-311.
- Teitzel, G.M. and Parsek, M.R. 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*, **69**: 2313-2320.
- Tokmak, B., Capar, G., Dilek, F.B. and Yetis, U. 2004. Trihalomethanes and associated potential cancer risks in the water supply in Ankara, Turkey. *Environmental Research*, **96**: 345-352.
- Tolker-Nielsen, T., Brinch, U.C., Ragas, P.C., Andersen, J.B., Jacobsen, C.S. and Molin, S. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. *Journal of Bacteriology*, **182**: 6482-6489.
- Trachoo, N. 2003. Biofilms and the food industry. *Songklanakarin Journal of Science and Technology*, **25**: 807-815.
- Trinet, F., Heim, R., Amar, D., Chang, H.T. and Rittmann, B.E. 1991. Study of biofilm and fluidization of bioparticles in a three-phase liquid-fluidized bed reactor. *Water Science and Technology*, **23**: 1347-1354.
- Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiology Letters*, **223**: 287-292.
- Turakhia, M.H., Cooksey, K.E. and Characklis, W.G. 1983. Influence of a calcium-specific chelant on biofilm removal. *Applied and Environmental Microbiology*, **46**: 1236-1238.
- van Casteren, W.H.M., Kabel, M.A., Dijkema, C., Schols, H.A., Beldman, G. and Voragen, A.G.J. 1998. Endoglucanase V and a phosphatase from *Trichoderma viride* are able to act on modified exopolysaccharide from *Lactococcus lactis* subsp. *cremoris* B40. *Carbohydrate Research*, **317**: 131-144.

- van Loosdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.J.B. 1990. Influence of interfaces on microbial activity. *Microbiological Reviews*, **54**: 75-87.
- van Loosdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.J.B. 1989. Bacterial adhesion: A physicochemical approach. *Microbial Ecology*, **17**: 1-15.
- Vickery, K., Pajkos, A. and Cossart, Y. 2004. Removal of biofilm from endoscopes: Evaluation of detergent efficiency. *American Journal of Infection Control*, **32**: 170-176.
- Vidal, O., Longin, R., Prigent-Combaret, C., Dorel, C., Hooreman, M. and Lejeune, P. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new *ompR* allele that increases curli expression. *Journal of Bacteriology*, **180**: 2442-2449.
- Volk, C.J., Hofmann, R., Chauret, C., Gagnon, G.A., Ranger, G. and Andrews, R.C. 2002. Implementation of chlorine dioxide disinfection: Effects of the treatment change on drinking water quality in a full-scale distribution system. *Journal of Environmental Engineering and Science*, **1**: 323-330.
- Wai, S.N., Mizunoe, Y., Takade, A., Kawabata, S-I. and Yoshida, S-I. 1998. *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance and biofilm formation. *Applied and Environmental Microbiology*, **64**: 3648-3655.
- Walker, S.L., Fourgialakis, M., Cerezo, B. and Livens, S. 2007. Removal of microbial biofilms from dispense equipment: The effect of enzymatic pre-digestion and detergent treatment. *Journal of the Institute of Brewing*, **113**: 61-66.
- Walters, M.C., Roe, F., Bugnicourt, A., Franklin, M.J. and Stewart, P.S. 2003. Contributions of antibiotic penetration, oxygen limitation and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrobial Agents and Chemotherapy*, **47**: 317-323.
- Webb, J.S., Thompson, L.S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., Givskov, M. and Kjelleberg, S. 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology*, **185**: 4585-4592.
- Werner, E., Roe, F., Bugnicourt, A., Franklin, M.J., Heydorn, A., Molin, S., Pitts, B. and Stewart, P.S. 2004. Stratified growth in *Pseudomonas aeruginosa* biofilms. *Applied and Environmental Microbiology*, **70**: 6188-6196.
- Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and Mattick, J.S. 2002. Extracellular DNA required for bacterial biofilm formation. *Nature*, **295**: 1487-1487.
- Whiteley, M., Bangera, M.G., Bumgarner, R.E., Parsek, M.R., Teitzel, G.M., Lory, S. and Greenberg, E.P. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature*, **413**: 860-864.

- Williams, V. and Fletcher, M. 1995. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Applied and Environmental Microbiology*, **62**: 100-104.
- Wingender, J., Nue, T.R. and Flemming, H-C. 1999. Microbial extracellular polymeric substances: Characterization, structure and function. Springer, Gemany.
- Xavier, J.B., Picioreanu, C., Rani, S.A., van Loosdrecht, M.C.M. and Stewart, P.S. 2005. Biofilm-control strategies based on enzymatic disruption of the extracellular polymeric substance matrix – a modelling study. *Microbiology*, **151**: 3817-3832.
- Xu, K.D., McFeters, G.A. and Stewart, P.S. 2000. Biofilm resistance to antimicrobial agents. *Microbiology*, **146**: 547-549.
- Xu, K.D., Stewart, P.S., Xia, F., Huang, C-T. and McFeters, G.A. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Applied and Environmental Microbiology*, **64**: 4035-4039.
- Xun, L., Mah, R.A. and Boone, D.R. 1990. Isolation and characterization of disaggregatase from *Methanosarcina mazei* LYC. *Applied and Environmental Microbiology*, **56**: 3693-3698.
- Yarwood, J.M. and Schlievert, P.M. 2003. Quorum sensing in *Staphylococcus* infections. *Journal of Clinical Investigation*, **112**: 1620-1625.
- Yildiz, F.H. and Schoolnik, G.K. 1999. *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance and biofilm formation. *Proceedings of the National Acadamy of Sciences USA*, **96**: 4028-4033.
- Yoon, S.S., Hennigan, R.F., Hilliard, G.M., Ochsner, U.A., Parvatiyar, K., Kamani, M.C., Allen, H.L., DeKievit, T.R., Gardner, P.R., Schwab, U., Rowe, J.J., Iglewski, B.H., McDermott, T.R., Mason, R.P., Wozniak, D.J., Hancock, R.E.W., Parsek, M.R., Noah, T.L., Boucher, R.C. and Hassett, D.J. 2002. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: Relationships to cystic fibrosis pathogenesis. *Developmental Cell*, **3**: 593-603.
- Zhao, Z-Y., Gu, J-D., Fan, X-J. and Li, H-B. 2006. Molecular size distribution of dissolved organic matter in water of the Pearl river and trihalomethane formation characteristics with chlorine and chlorine dioxide treatments. *Journal of Hazardous Materials*, **B134**: 60-66.

CHAPTER 3

CRUDE ENZYMES EXTRACTED AFTER THE AUTO-HYDROLYSIS OF WASTEWATER BIOFILMS AND THEIR ABILITY TO REMOVE BIOFILMS

Abstract

The auto-hydrolysis of wastewater biofilms supplemented with or without a mixture of peptone and polysaccharides was investigated over a period of 24 days at room temperature. Measurements of the biofilm viscosity, chemical oxygen demand (COD) and total suspended solids (TSS) indicated a reduction in these parameters over the incubation period, which, in turn, resulted in improved settling. Moreover, a decrease in the protein and carbohydrate contents of the wastewater biofilm samples over the incubation period coincided with detectable protein- and polysaccharide-degrading enzyme activities. Subsequent incubation of *Pseudomonas aeruginosa* PAO1 biofilms with a crude extracellular enzyme extract prepared from the auto-hydrolyzed wastewater biofilm resulted in efficient removal of the *P. aeruginosa* biofilms from a glass surface. This could be ascribed to enzymatic hydrolysis of both the protein and polysaccharide components present in the biofilm matrix of *P. aeruginosa* PAO1. These results suggested that the wastewater biofilm contained enzymes that could be used to remove detrimental biofilms.

3.1 INTRODUCTION

Biological wastewater treatment entails the degradation of organic compounds present in the incoming wastewater by microorganisms (Droste, 1997; Vesilind, 2003). Although the organic composition of wastewater is site-specific, wastewaters typically contain a complex mixture of single carbohydrates, amino acids, alcohols and volatile fatty acids mixed with polymers and heteropolymers, including proteins (*ca*. 30% of the chemical oxygen demand, COD), polysaccharides (*ca*. 20% of COD) and lipids (*ca*. 30% of COD) (Raunkjaer *et al*., 1994; Confer and Logan, 1998; Goel *et al*., 1998). A large fraction of the wastewater organic matter (60%) consists of particles of which the size exceeds 1000 atomic mass units (amu) (Logan and Jiang, 1990; Confer and Logan, 1991; 1998). The large size of these particles precludes them from being directly assimilated by microorganisms (Cadoret *et al*., 2002). Consequently, these high-molecular-weight compounds need to be hydrolyzed into smaller polymers or monomers by extracellular enzymes before they can be transported across the bacterial cell wall. Confer and Logan (1998) suggested a general model for macromolecule degradation in wastewater, which was applicable for both polysaccharide and protein biodegradation (Confer and Logan, 1997a; 1997b). According to this model, macromolecules make contact with bacterial cells by diffusion and are subsequently hydrolyzed into smaller polymers that are returned to the surrounding environment. This process occurs repeatedly until the macromolecules are degraded into small enough units (<1000 amu) that can be taken up directly into the bacterial cells.

The high-molecular-weight organic matter is hydrolyzed by extracellular enzymes produced in sewage biofilms (Confer and Logan, 1998) and enzymes localized in activated sludge flocs (Confer and Logan, 1998; Sanders *et al.*, 2000; Gessesse *et al.*, 2003), while complex organic matter can be degraded by several enzymes expressed by mixed-species microbial biofilms (Boczar *et al.*, 1992). The extracellular enzymes involved in biodegradation of the macromolecules are either associated with the cell surface (ecto-enzymes) or are released into the surrounding environment (exo-enzymes) (Vetter and Deming, 1999). Several studies have, however, demonstrated that these extracellular enzymes are mostly cell surface-associated and/or associated to the extracellular polymeric matrix in activated sludge flocs, while very low enzyme activity is found in the bulk fluid (Confer and Logan, 1998; Goel *et al.*, 1998; Boczar *et al.*, 2001; Cadoret *et al.*, 2002; Xia *et al.*, 2007). According to Confer and Logan (1998), there are two possible reasons for why most of the enzyme activity is located on the bacterial cell surface or within the EPS of wastewater biofilms. Firstly, the enzymes produced are soluble proteins and can be easily degraded by other bacteria when released into the surrounding environment. Secondly, if enzymes are released they can easily be lost from the reactor therefore requiring the bacteria to produce more, a process which is energetically costly (Confer and Logan, 1998).

Considering that the organic composition of wastewater typically comprises of 40-60% proteins and 25-50% carbohydrates (Raunkjaer *et al.*, 1994), it therefore follows that wastewater microorganisms and biofilms may constitute a potentially useful source of enzymes capable of degrading these organic macromolecules. Indeed, several recent reports have demonstrated that biofilm EPS, which is comprised mostly of polysaccharides, proteins, lipids and nucleic acids (Wingender *et al.*, 1999), is also biodegradable and that mixed bacterial cultures can degrade the EPS (Zhang and Bishop, 2003; Wang *et al.*, 2007; Ceyhan and Ozdemir, 2008). Consequently, in this part of the study, a wastewater biofilm was investigated for its ability to produce protein- and polysaccharide-degrading enzymes that may find application in the enzymatic removal of biofilms. For these studies, *Pseudomonas aeruginosa* PAO1, a well characterized biofilm-forming organism, was used to assess the ability of a crude enzyme extract prepared from an auto-hydrolyzed wastewater biofilm to remove *P. aeruginosa* biofilms from a surface and to degrade EPS produced by the bacterium.

3.2 MATERIALS AND METHODS

3.2.1 Sampling site

Wastewater biofilm was obtained from an aeration basin at the Leeuwkuil water purification plant in Vereeniging, Gauteng, South Africa (Fig. 3.1). The water purification plant receives water from approximately 18 000 domestic residences, 500 industrial sites (mainly iron and other heavy metal processing and casting industries), a number of hospitals, an abattoir, several butcheries and restaurants, a pharmaceutical industry and a tannery. The water purification plant makes use of a biological nutrient removal (BNR) process, also known as the modified Bardenpho process. Since no microorganisms are added to the process, nutrient removal is achieved by making use of activated sludge return.

Figure 3.1. Aeration basin at the Leeuwkuil water purification plant. Biofilms were removed from the side of the aeration basin, 60 cm below the water level.

3.2.2 Sampling and storage of wastewater biofilm

Wastewater biofilms were collected in acid-washed flasks from the sides of an aeration basin, approximately 60 cm below the water surface to exclude the sampling of algae. Acid-washed flasks were used to ensure that metals and other contaminants would not inhibit the enzymes produced. The temperature of the water in the aeration tank varied between 20 and 26°C and therefore samples were stored at ambient temperatures. The same biofilm sample was used throughout this investigation.

3.2.3 Viscosity measurements

A biofilm sample was placed in a flask and incubated at ambient temperature. The viscosity of the sample was measured daily for a period of seven days with an Epprecht viscometer (Spindle 2).

3.2.4 Biofilm auto-hydrolysis studies

3.2.4.1 Experimental setup

For biofilm auto-hydrolysis studies, 500-ml biofilm samples were placed into 1 l acid-washed flasks. The biofilm biomass in one flask, designated supplemented, was supplemented with 0.1% (w/v) peptone and a mixture of polysaccharides (0.1% [w/v] of each hydroxyethyl-cellulose, xylan and amylose) in order to induce enzyme production (Parmar *et al.*, 2001; Ten *et al.*, 2004). These compounds were added during the first day of incubation only. As a control, the biofilm sample in the second flask was not supplemented with nutrients. The flasks were left at room temperature and auto-hydrolysis of the biofilms was monitored over a period of 24 days. Samples were removed from each flask every fourth day and used for total suspended solids (TSS) and chemical oxygen demand (COD) measurements, as well as settleability tests.

3.2.4.2 Analytical methods

To determine total suspended solids (TSS), a well-mixed biofilm sample (20 ml) was centrifuged at 7 000 × *g* for 10 min. After discarding the supernatant, the pellet was washed with 20 ml water and centrifuged again at 7 000 × *g* for 10 min. The solids were transferred to a pre-weighed glass dish and dried overnight at 100°C. The TSS was calculated as follows

(Clesceri *et al.*, 1998): TSS (mg/l) = [(A-B) × 1000] / C, where A = weight of dish + solids after drying (mg), B = weight of dish (mg), and C = volume of sample centrifuged (ml). Settleability was determined, according to the method of Parmar *et al.* (2001), by placing 25 ml of a well-mixed biofilm sample in a measuring cylinder to settle at room temperature for 2 h. The volume of the settled solids was recorded and is presented as ml settled solids per 25 ml total sample. Chemical oxygen demand (COD) was determined with a spectrophotometric test kit (Spectroquant test 14541, Merck) according to the manufacturer's instructions. The biofilm sample was sonicated for 30 s, centrifuged at 10 000 × *g* for 15 min and the COD of the supernatant was subsequently determined.

3.2.5 Enzyme activity assays

Based on reports indicating that proteases and polysaccharases can degrade biofilm EPS (Johansen *et al.*, 1997; Loiselle and Anderson, 2003; Itoh *et al.*, 2005; Orgaz *et al.*, 2006; 2007), the presence of these enzyme activities within the wastewater biofilm biomass was specifically investigated.

3.2.5.1 Preparation of crude enzyme extracts from wastewater biofilm

A crude enzyme extract was prepared from the sampled wastewater biofilm, following incubation at room temperature for seven days. For this purpose, the method of Gessesse *et al.* (2003) with the following minor modifications, was used. The pH of the biofilm sample was adjusted to 7 by adding 10 mM Tris buffer (pH 7) to the biofilm sample in a ratio of 1:2. Following addition of Triton X-100 to yield a final concentration of 0.1% (v/v), the biofilm-buffer mixture was transferred to an acid-washed beaker and stirred for 1 h on ice. After stirring, the sample was centrifuged at 10 000 × *g* for 15 min (4°C) and the supernatant was used as the crude enzyme source.

3.2.5.2 Hydrolysis of proteins

Protease activity was detected using azocasein as substrate according to the method of Olajuyigbe *et al.* (2005) with a few modifications. The azocasein (Sigma-Aldrich) was dissolved in 5 mM Tris-HCl buffer (pH 7) to yield a final concentration of 0.5% (w/v). The protease assay was performed by incubating 100 µl of the crude enzyme preparation, prepared as described above, with 500 µl of the azocasein substrate. Following incubation for 1 h at 30ºC, the reaction was terminated by addition of 500 µl of an ice-cold 10% (w/v) trichloroacetic acid (TCA) solution and incubating on ice for 15 min. After centrifugation at 500 × *g* for 15 min (4ºC), the supernatant (1 ml) was added to an equal volume of 1 M NaOH and the absorbance was measured at 440 nm against a reagent blank. One unit (U) of enzyme activity was defined as the amount of enzyme which results in an absorbance increase of 0.01 at 440 nm in 60 min at 30ºC (Secades and Guijarro, 1999). All enzyme assays were performed in triplicate on three independent samples.

3.2.5.3 Hydrolysis of polysaccharides

Hydrolysis of polysaccharides was determined by measuring reducing sugars by the dinitrosalicyclic (DNS) colorimetric method. The assay was performed as described by Ghose (1987) with minor modifications. The biofilm sample (3 ml) was sonicated for 30 s, followed by centrifugation at 10 000 × *g* for 15 min. The supernatant (1 ml) was added to 1 ml of a 1% DNS reagent (dinitrosalicylic acid, 10 g; sodium sulfite, 0.5 g; sodium hydroxide, 10 g; dH_2O, 1 l) and the mixture was heated at 90°C for 15 min. Following addition of 335 µl of potassium sodium tartrate (40% [w/v]) to allow colour stabilization, the solution was cooled to room temperature and the absorbance was measured at 575 nm. Glucose was used to generate a standard curve. The absorbance values were subsequently converted to mg/ml reducing sugars released by making use of the glucose standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme which released 1 µmol of glucose equivalents under the experimental setting (Ten *et al.*, 2004). All enzyme assays were performed in triplicate on three independent samples.

3.2.6 Protein and carbohydrate concentration determination

Prior to these determinations, biofilm samples were sonicated for 30 s, followed by centrifugation at 10 000 × *g* for 15 min and the supernatant was retained. The protein concentration was determined by the method of Bradford (Bradford, 1976) and with bovine serum albumin (BSA) as standard, while the carbohydrate concentration was determined using anthrone reagent with glucose as standard (Roe, 1954). All assays were performed in triplicate.

3.2.7 Enzymatic removal of *Pseudomonas aeruginosa* PAO1 biofilms

3.2.7.1 Bacterial strain and culturing conditions

Pseudomonas aeruginosa PAO1 was obtained from the culture collection of the University of Pretoria, Pretoria, South Africa. The culture was grown in Tryptone Soy Broth (Merck) or on Tryptone Soy Agar (Merck) at 37°C, and maintained at -70°C as a glycerol culture. The identity of the bacterial culture was verified with the API 20E test kit (bioMérieux, France) prior to its use in experiments.

3.2.7.2 Growth of *P. aeruginosa* PAO1 biofilms on glass slides

A culture of *P. aeruginosa* PAO1, grown overnight at 37°C on Tryptone Soy Agar, was flooded with 10 ml of sterile Ringer’s solution to dislodge the cells and the bacterial suspension was then inoculated into 200 ml of Tryptone Soy Broth in a 500-ml glass beaker. To obtain biofilms, sterile glass slides were partially submerged in the broth and held in a vertical position by clamps fixed to the side of the glass beaker. The culture was incubated at 37°C for 96 h with gentle agitation.

3.2.7.3 Enzyme treatment of *P. aeruginosa* PAO1 biofilms

Following incubation, glass slides containing *P. aeruginosa* PAO1 biofilms were removed and immersed in a solution containing 10 ml of 0.067 M phosphate buffer (pH 7) and 20 ml of crude enzyme extract, prepared as described in Section 3.2.5.1. Following incubation at room temperature for 1, 5, 10 and 22 h the glass slides were processed for scanning electron microscopy, as described below. As a control, one of the *P. aeruginosa* biofilm-containing glass slides was incubated for 22 h in 30 ml of the phosphate buffer only.

3.2.7.4 Scanning electron microscopy (SEM)

The *P. aeruginosa* PAO1 cells were fixed by placing the glass slides for at least 1 h in separate Petri dishes containing 20 ml of fixing solution (2.5% gluteraldehyde in 0.075 M phosphate buffer). The cells were washed three times for 15 min each with 0.0375 M phosphate buffer, before being dehydrated by sequential treatment for 15 min each in 50%, 70%, 90% and 100% ethanol. The treatment with 100% ethanol was repeated twice more to ensure complete dehydration of the samples. The glass slides were critical point-dried, scatter-coated with gold and observed in a JEOL 5800LV scanning electron microscope at 5 kV.

3.2.8 Extraction of EPS from *P. aeruginosa* PAO1

P. aeruginosa PAO1 was inoculated into Tryptone Soy Broth and incubated at 37ºC for 72 h with agitation (200 rpm). The culture was homogenized by stirring thoroughly, after which the EPS was extracted according to Zhang *et al.* (1999). Briefly, 10-ml aliquots of the culture were centrifuged at 6 000 × *g* for 5 min. The supernatant was recovered and stored for later use. The pellet was suspended in 10 ml of dH_2O and sonicated for 15 s on ice at an output of 50%. The supernatant was added to the sonicated pellet and the suspension was centrifuged at 11 227 × *g* for 30 min (4ºC). The recovered supernatant was filtered through a 0.2-µm syringe filter (Acrodisc® PF syringe filters, low protein binding) and the filtrate was used as the crude EPS source.

3.2.9 Hydrolysis of *P. aeruginosa* PAO1 EPS proteins

3.2.9.1 Enzyme reactions

A crude enzyme extract (10 ml), prepared from the auto-hydrolyzed wastewater biofilm, was mixed with 10 ml of the EPS extract from *P. aeruginosa* PAO1. The reaction mixture was incubated at 30ºC for 22 h with slow shaking (100 rpm). As a control, an inactivated crude enzyme extract (boiled for 15 min, followed by rapid cooling) was also added to the EPS extract. Following incubation, proteins were precipitated from the reaction mixture and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.9.2 Protein precipitation

Prior to SDS-PAGE, the proteins were concentrated from the reaction mixtures using the method of Wessel and Flugge (1984), which was adapted as follows for larger sample volumes. The samples (3 ml) were mixed with 12 ml of methanol in 50-ml Greiner tubes and then centrifuged at 9 000 × *g* for 2 min (4°C). After centrifugation, 3 ml of chloroform was added to each tube and briefly vortexed before centrifugation at 9 000 × *g* for 2 min (4°C). The pellets were rinsed with 9 ml of dH_2O by vigorous vortexing of the tubes, followed by brief centrifugation (9 000 × *g*, 30 s). The supernatants were carefully aspirated and the tubes were incubated at room temperature for 3 min to allow all traces of the chloroform to evaporate. Following incubation, 9 ml of methanol was added to the tubes and the samples were centrifuged at 9 000 × *g* for 4 min to collect the white protein pellet. The supernatants were discarded and the pellets were left to dry in a flow cabinet before being solubilized in 50 µl of 2 × sample buffer (0.125 M Tris-HCl, 4% [w/v] SDS, 20% [v/v] glycerol, 0.2 M DTT, 0.02% [w/v] bromophenol blue; pH 6.8).

3.2.9.3 SDS-PAGE

Protein samples were analyzed by electrophoresis in a discontinuous gel system, as described by Laemmli (1970), using a 4% stacking gel and 8% separating gel. Electrophoresis was performed in a BioRad Protean II electrophoresis unit for 30 min at 60 V and then for 12.5 h at 100 V in 1 × TGS buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS; pH 8.3). Following electrophoresis, the gels were stained for 30 min with 0.1% (w/v) Coomassie brilliant blue (prepared in 45% [v/v] methanol; 10% [w/v] acetic acid), and then destained in a solution containing 10% (v/v) methanol and 10% (v/v) glacial acetic acid until the proteins were visible. An unstained protein molecular weight marker (Precision Plus; BioRad), covering the 10-250 kDa range, was included in the SDS-polyacrylamide gel.

3.2.10 Hydrolysis of *P. aeruginosa* PAO1 EPS polysaccharides

The ability of the crude enzyme extract prepared from the auto-hydrolyzed wastewater biofilm to hydrolyze polysaccharides present in the EPS of *P. aeruginosa* PAO1 was assessed according to a slightly modified method of Loiselle and Anderson (2003). For this purpose, 0.1 g of lyophilized EPS was dissolved in 5 ml of 0.05 M citrate-phosphate buffer (pH 6) and incubated with 10 ml of the crude enzyme extract. Following incubation at 30°C for 22 h, the reducing sugars was measured by the dinitrosalicyclic (DNS) colorimetric method, as described previously (Section 3.2.5.3). The assays were performed in triplicate.

3.3 RESULTS AND DISCUSSION

3.3.1 Biofilm auto-hydrolysis studies

In a preliminary experiment, the auto-hydrolysis of wastewater biofilms was evaluated by measuring the viscosity of the biofilm biomass over a period of seven days at room temperature. The results indicated that the viscosity of the wastewater biofilm biomass was reduced by 60% after seven days (Fig. 3.2), suggesting that hydrolysis of the biofilm had occurred.

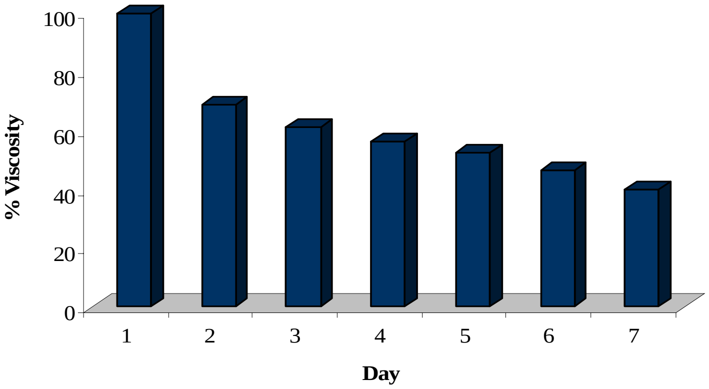


Figure 3.2. Percentage reduction in viscosity of a wastewater biofilm, following incubation at room temperature for seven days.

The reduction in viscosity of the wastewater biofilm was subsequently investigated in greater detail. At regular time intervals, chemical oxygen demand (COD) and total suspended solids (TSS) measurements, as well as settleability tests of an untreated biofilm sample and of a biofilm sample supplemented with peptone and a mixture of different polysaccharides were carried out. The addition of peptone and polysaccharides has previously been shown to induce production of protease- and polysaccharide-degrading enzymes in different bacterial cultures (Parmar *et al.*, 2001; Ten *et al.*, 2004).

Chemical oxygen demand (COD) can be viewed as an indirect measurement of the total organic compounds present in a sample (Clesceri *et al.*, 1998), while total suspended solids (TSS) represents the total biomass (Zhang and Bishop, 2003). COD measurements indicated an initial increase in the COD concentration over the first four days, but were followed by a gradual decrease in the COD concentration up to day 24 (Fig. 3.3a). This was also observed by Roman *et al.* (2006) and the initial increase was explained to be due to the solubilization of organic matter present in sewage sludge due to enzymatic treatment. The COD was reduced by 51.69% and 55.06% in the control and supplemented biofilm samples, respectively, at the end of the 24-day incubation period. Likewise, a reduction in TSS was observed over the incubation period, indicating that solids were biodegraded (Fig. 3.3b). The TSS was reduced by 16.18% in the control biofilm sample and by 21.3% in the supplemented biofilm sample at the end of the 24-day incubation period. Despite an apparent difference in the rate of biodegradation, the concentrations of TSS after 24 days were similar in both biofilm samples (26.79-26.94 mg/ml). In accordance with the reduction in COD and TSS, the reducing volume of settled solids indicated that the settleability of the respective biofilm samples was slightly improved over the incubation period (Fig. 3.3c). Although the initial reduction in settled matter was notably faster in the supplemented biofilm sample, the amount of settled matter remaining after 24 days were similar in both biofilm samples (18.5-17.5 ml solids/25 ml total volume). The pH of the biofilm samples remained fairly constant throughout the 24-day incubation period, increasing from pH 7 on day one to pH 7.76 (control biofilm sample) and 7.99 (supplemented biofilm sample) at the end of the incubation period (data not shown).

It is tempting to speculate that the reduction in viscosity of the wastewater biofilm may have been due to enzymatic hydrolysis of the biofilm EPS (proteins and polysaccharides) and possibly other organic compounds entrapped in the EPS (originating from the wastewater). In this regard, Zhang and Bishop (2003) reported that biofilm bacteria can degrade their own EPS when they were in a starved state. Similarly, Ceyhan and Ozdemir (2008) reported that several bacterial strains from biofilms found in cooling water towers were able to degrade their own EPS and that of other bacteria. Based on these reports, it therefore appears likely that microorganisms present in the wastewater biofilm produced proteases and polysaccharases that were capable of degrading the protein and polysaccharide components of the biofilm EPS.

3.3.2 Hydrolysis of proteins and polysaccharides present in the wastewater biofilm

Since the hydrolysis of macromolecules present in wastewater was reported to be mostly cell-associated (Confer and Logan, 1998), it was subsequently determined whether extracellular enzymes expressed by the wastewater biofilm may have contributed to the hydrolysis of the wastewater biofilm. Consequently, crude enzyme extracts were prepared from the auto-hydrolyzed wastewater biofilm according to the method of Gessesse *et al.* (2003). This method was specifically chosen as it allows for the gentle and efficient extraction of enzymes immobilized on the cell surface and in the EPS matrix. Use of the non-ionic detergent Triton X-100 was proposed to detach the enzymes bound on the surface of cells or adsorbed in the EPS matrix with little or no cell lysis. Moreover, the inclusion of a low shear strength stirring step also assists in dispersion of the biofilm biomass and the release of enzymes.

Determination of the protein concentration in the control and supplemented biofilm samples indicated that the concentration remained constant for the first eight days and then gradually decreased. The protein content in the control and supplemented biofilm samples was reduced

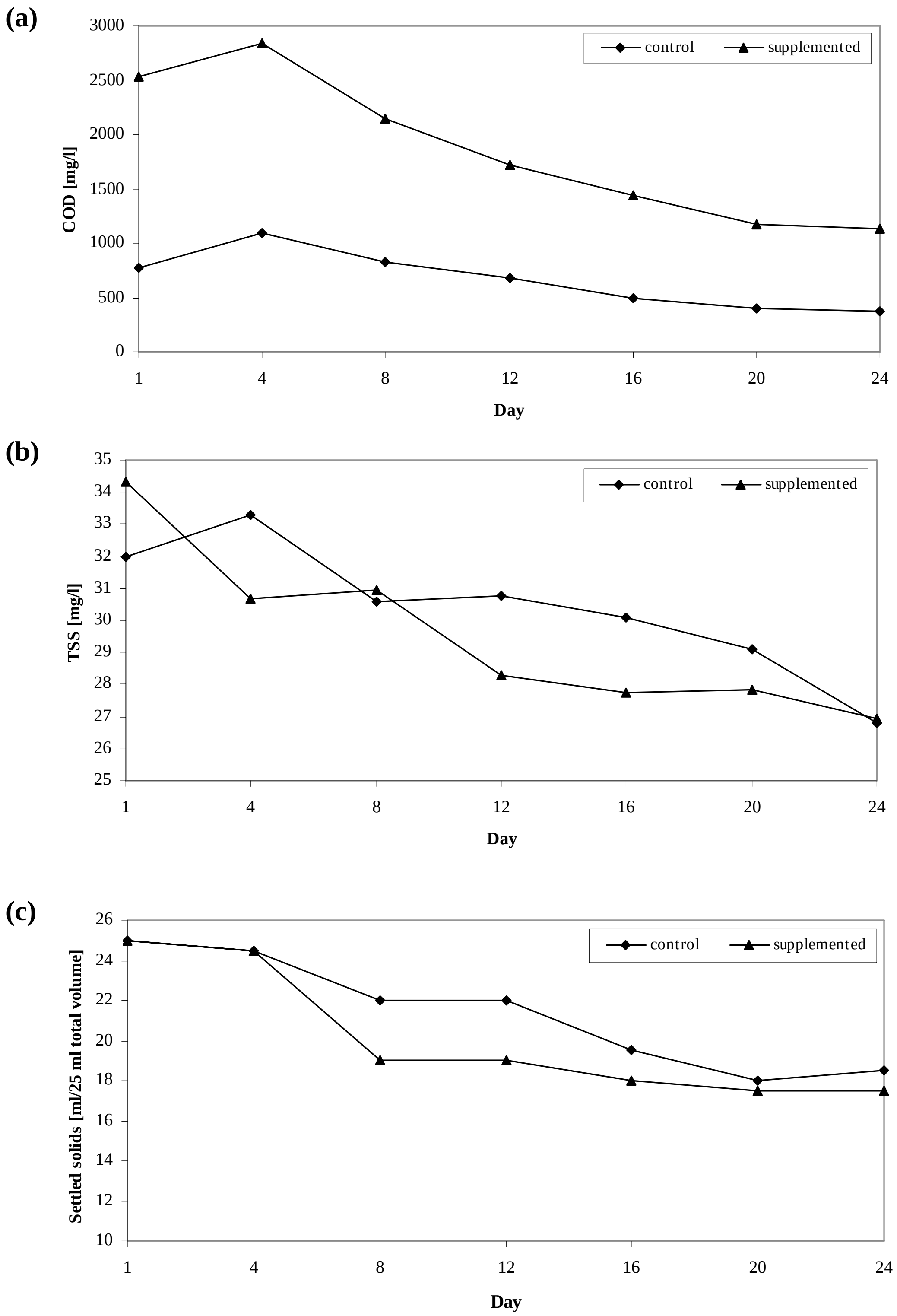


Figure 3.3. Chemical oxygen demand (COD) (a), total suspended solids (TSS) (b), and volume of settled matter (c) measured during a 24-day incubation period of wastewater biofilms. The biofilm samples were either supplemented with peptone and polysaccharides (supplemented) or left unsupplemented (control).

by 50% and 41.03%, respectively, at the end of the 24-day incubation period (Fig. 3.4). To determine whether the observed reduction in protein concentration was due to proteolytic activity, protease enzyme activity assays were subsequently performed using a crude extracellular enzyme extract prepared from the wastewater biofilm and azocasein as substrate. The results indicated that there was an increase in protease activity in both the control and supplemented biofilm samples over the first eight days, which decreased rapidly after 16 days of incubation (Fig. 3.4).

In contrast to the protein concentration, the carbohydrate concentration in the control and supplemented biofilm samples decreased over the first 12 days. This was followed by a slight increase in the concentration between days 12 and 16, after which the carbohydrate concentration decreased and remained at the same concentration until the end of the incubation period. Nevertheless, the carbohydrate content in the control and supplemented biofilm samples was reduced by 30.19% and 56.93%, respectively, at the end of the 24-day incubation period (Fig. 3.5). Measurement of reducing sugars, resulting from the degradation of carbohydrates, can be considered to be indicative of the presence of polysaccharide-degrading enzymes (Dashek, 1997). In accordance with the low concentration of carbohydrates present in the wastewater biofilm samples, very low amounts of released reducing sugars were measured, therefore suggesting a low level of polysaccharide-degrading enzyme activity in the biofilm samples (Fig. 3.5).

The results obtained suggested that a reduction in the wastewater biofilm organic matter previously observed was accompanied by degradation of the protein and carbohydrate contents (Fig. 3.3). Notably, the carbohydrate content of the wastewater biofilm was much less than the protein content, as evidenced from the results obtained for the control, unsupplemented wastewater biofilm sample. The higher initial protein and carbohydrate concentrations observed in the supplemented biofilm sample compared to the control biofilm sample can be ascribed to the addition of peptone and different polysaccharides (carboxymethyl cellulose, xylan and amylose) to the wastewater biofilm sample. The predominance of proteins in the wastewater biofilm samples is in agreement with studies performed by Jahn and Nielsen (1995), Nielsen *et al.* (1997) and Sponza (2003). The high level of proteins and proteolytic activity observed during the course of this study may be indicative of the degradation of readily degradable organic compounds by microorganisms

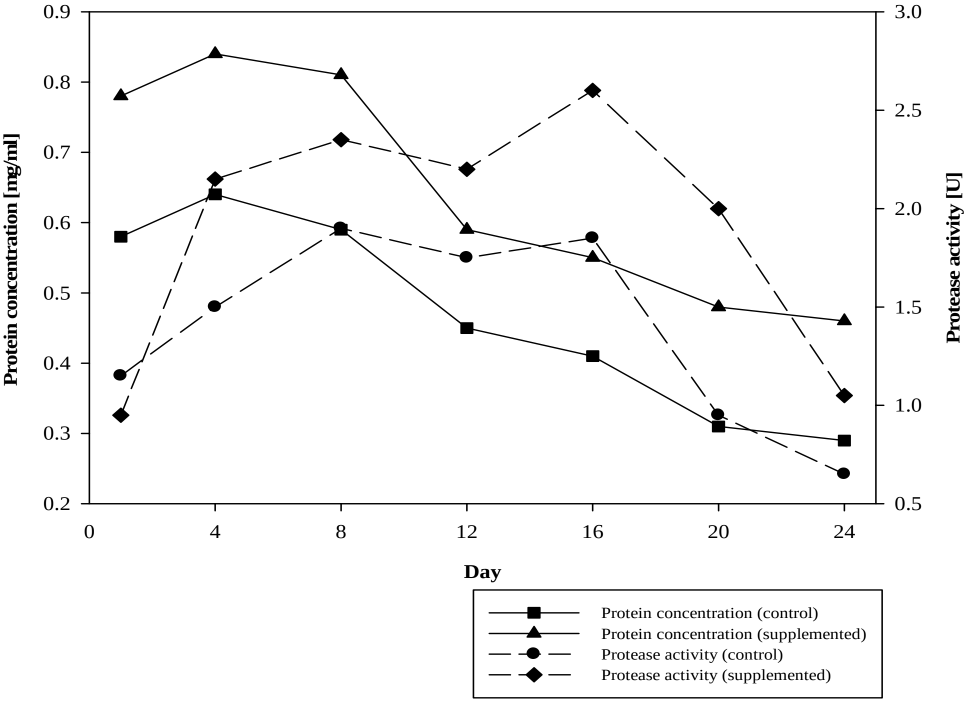


Figure 3.4. Soluble protein concentration and protease activity measured during a 24-day incubation period of wastewater biofilms. The biofilm samples were either supplemented with peptone and polysaccharides (supplemented) or left unsupplemented (control). Values are the means of results of triplicate experiments.

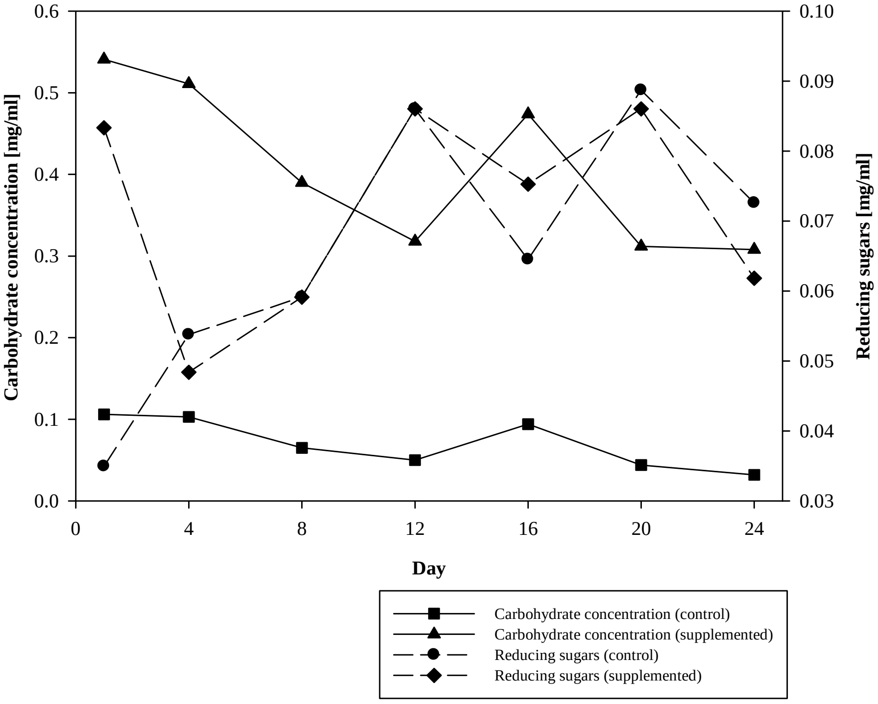


Figure 3.5. Carbohydrate concentration and reducing sugars measured during a 24-day incubation period of wastewater biofilms. The biofilm samples were either supplemented with peptone and polysaccharides (supplemented) or left unsupplemented (control). Values are the means of results of triplicate experiments.

present in the wastewater biofilm. Nevertheless, the addition of polysaccharides to the wastewater biofilm clearly indicated the presence of polysaccharide-degrading enzymes in the wastewater biofilm samples, albeit low. The low enzymatic activity may be due to the extracellular matrix of the biofilm that assisted in the retention, the buildup or stabilization of the enzymes, as suggested by Wingender *et al.* (1999), therefore making their extraction inefficient with the method adopted in this study. Alternatively, since Gram-positive bacteria, *e.g.*, *Micrococcus* spp. and *Bacillus* spp., contain a high amount of polysaccharides compared to Gram-negative bacteria (Cloete and Atlas, 2006), the low amount of polysaccharides measured in the biofilm samples may indicate that the biofilm is composed mostly of Gram-negative bacteria.

3.3.3 Enzymatic treatment of *P. aeruginosa* PAO1 biofilms

Having established that the crude enzyme extract prepared from the wastewater biofilm contained both protein- and polysaccharide-degrading enzyme activities, it was subsequently investigated whether these enzymatic activities could facilitate biofilm removal. For this purpose, *P. aeruginsa* PAO1 was chosen as model organism since it is amongst the best-studied biofilm formers (O'Toole *et al.*, 2000). To investigate, *P. aeruginosa* PAO1 biofilms grown on glass slides for 96 h were treated with the crude enzyme extract for different times and the glass slides were then examined by scanning electron microscopy (SEM).

The results obtained indicated that *P. aeruginosa* PAO1 was capable of attaching to the glass surface and formed copious amounts of biofilm. This was evidenced by the glass slide being densely covered by biofilm and the cells were embedded in an exopolymeric matrix (Fig. 3.6a). For *P. aeruginosa* PAO1 biofilm samples incubated with the crude enzyme extract, a reduction in biofilm and EPS could be observed (Fig. 3.6c-f). Notably, after enzyme treatment for 22 h, only a few individual *P. aeruginosa* PAO1 cells remained attached to the surface and no EPS was visualized (Fig. 3.6f). In contrast, the *P. aeruginosa* PAO1 biofilm was not removed from the glass surface when the biofilm was incubated for 22 h in the presence of phosphate buffer lacking the crude enzyme extract (Fig. 3.6b). The results therefore suggested that the crude enzyme extract prepared from the wastewater biofilm contained different enzymes that were capable of degrading different components present in the EPS of *P. aeruginosa* PAO1 biofilms, thus resulting in their removal from the glass surface.

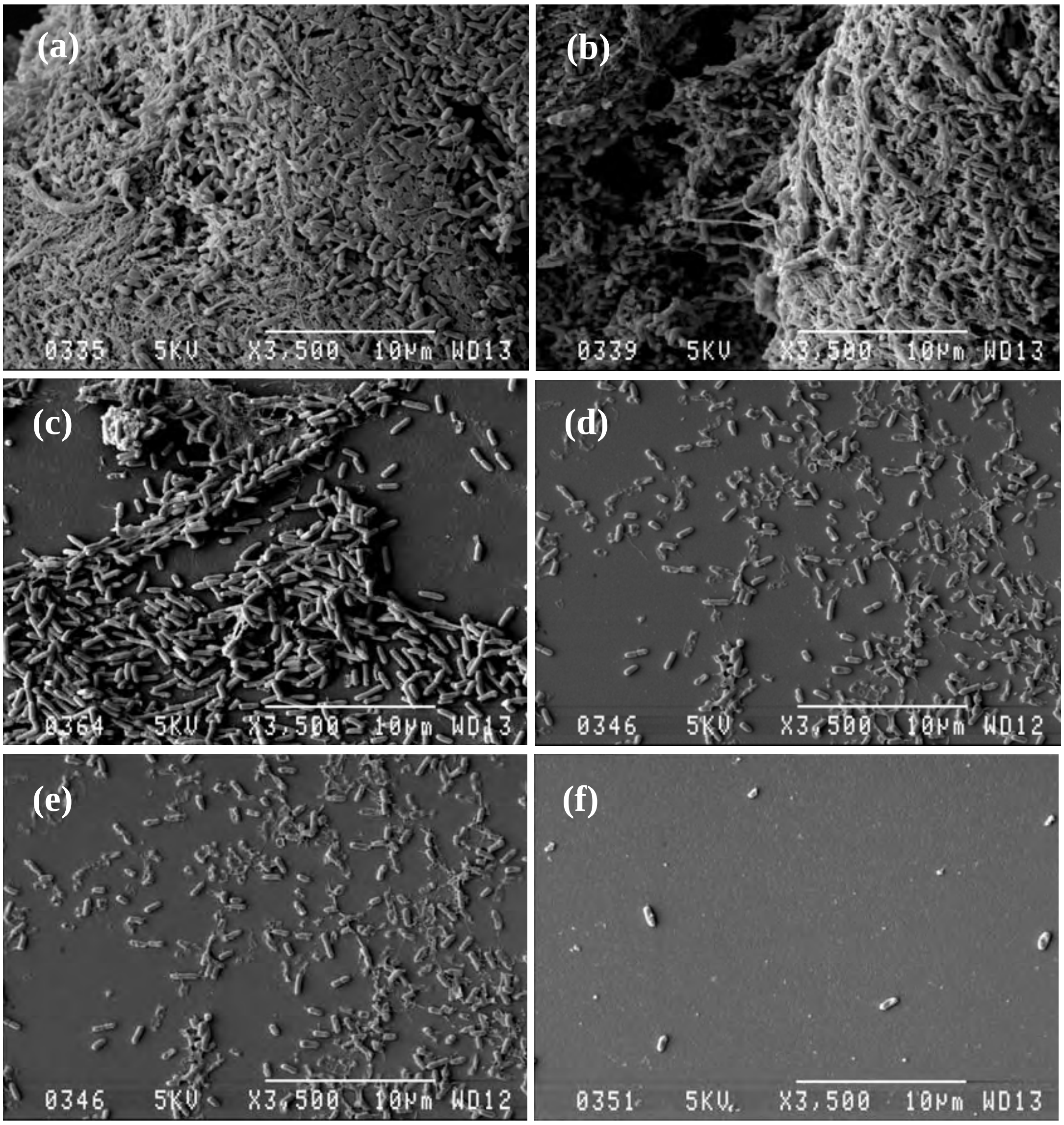


Figure 3.6. Scanning electron microscopy (SEM) micrographs of *P. aeruginosa* PAO1 biofilms treated with a crude enzyme extract prepared from a wastewater biofilm. *P. aeruginosa* PAO1 biofilms were cultured for 96 h in the presence of glass slides as attachment surface. Following incubation, the biofilms were treated with a crude enzyme extract prepared from an auto-hydrolyzed wastewater biofilm for 1 h (c), 5 h (d), 10 h (e) and 22 h (f). As controls, the *P. aeruginosa* PAO1 biofilm was left untreated (a) or incubated in phosphate buffer only for 22 h (b).

3.3.4 Enzymatic hydrolysis of *P. aeruginosa* PAO1 EPS components

To determine whether removal of *P. aeruginosa* PAO1 biofilms from the glass surface was indeed due to degradation of the protein and polysaccharide components present in the biofilm EPS, the EPS of *P. aeruginosa* PAO1 was extracted and incubated for 22 h with a crude enzyme extract prepared from the auto-hydrolyzed wastewater biofilm.

The effect of the crude enzyme extract on the amount of reducing sugar released from *P. aeruginosa* PAO1 EPS is shown in Fig. 3.7. An untreated sample showed no increase in the amount of reducing sugar. However, the amount of reducing sugar increased by 37.79% when the EPS was treated with the crude enzyme extract. The increase in reducing sugar concentration is in agreement with the previous results (Fig. 3.5), indicating that the wastewater biofilm displayed a low level of polysaccharide-degrading enzyme activity.

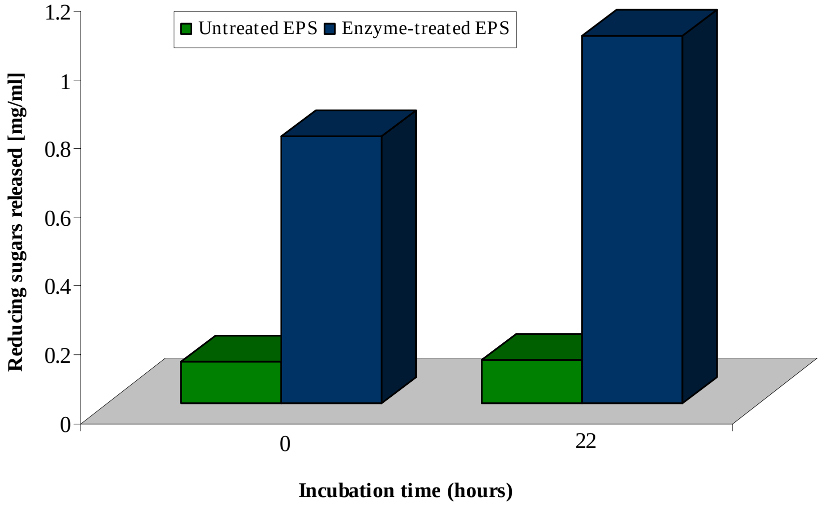


Figure 3.7. Reducing sugars released from the extracted EPS of *P. aeruginosa* PAO1, following incubation for 22 h with a crude enzyme extract prepared from an auto-hydrolyzed wastewater biofilm.

The extent of protein degradation, following incubation of *P. aeruginosa* PAO1 EPS with the crude enzyme extract, was assessed by SDS-PAGE analysis of the samples following protein extraction. The results are shown in Fig. 3.8. The results indicated a marked difference in the protein profiles of samples treated with the crude enzyme extract (lanes 3 and 6) compared to untreated samples (lanes 2 and 5) and samples treated with an inactivated enzyme extract (lanes 4 and 7). Whereas the latter two control samples contained multiple protein bands that were similar in size, the enzyme-treated *P. aeruginosa* EPS extracts contained very few bands. These results therefore indicated that the crude enzyme extract contained proteolytic enzymes that were capable of efficiently degrading proteins present in the EPS of *P. aeruginosa* PAO1.

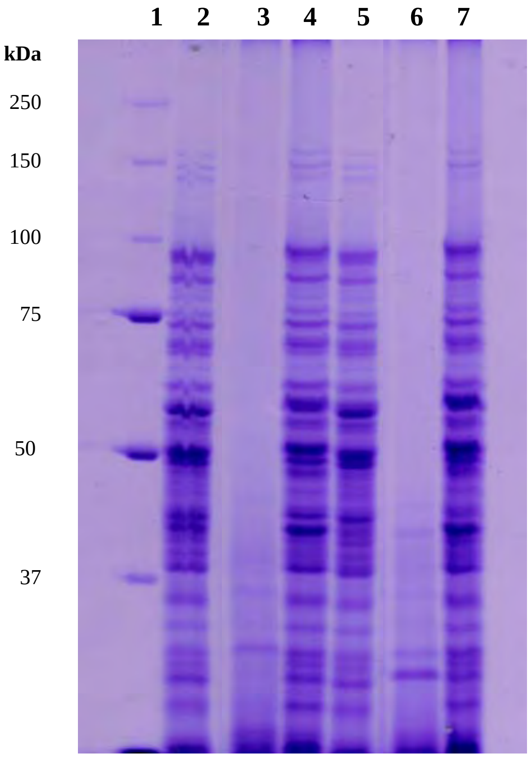


Figure 3.8. SDS-PAGE analysis of precipitated proteins, following incubation of EPS extracted from *P. aeruginosa* PAO1 with a crude enzyme extract prepared from an auto-hydrolyzed wastewater biofilm. Lanes: 1, Protein Molecular Weight Marker; 2 and 5, *P. aeruginosa* PAO1 proteins precipitated from an untreated EPS extract; 3 and 6, *P. aeruginosa* PAO1 proteins precipitated from the reaction mixture following enzymatic treatment of the EPS extract for 22 h; 4 and 7, *P. aeruginosa* PAO1 proteins precipitated from the reaction mixture following treatment of the EPS extract with a heat-inactivated crude enzyme extract for 22 h.

3.4 CONCLUSIONS

Analytical measurements of COD, settled matter and TSS indicated that auto-hydrolysis of a wastewater biofilm occurred over a 24-day incubation period. The hydrolytic capacity of the wastewater biofilm was also characterized and reductions in protein and carbohydrate concentrations were noted, suggesting that proteases and polysaccharases degraded proteins and polysaccharides present in the biofilm exopolymeric matrix. Since proteins and polysaccharides are major components of the biofilm EPS (Wingender *et al.*, 1999), their degradation may have compromised the structural integrity of the biofilm and thus resulted in its hydrolysis. Moreover, a crude enzyme extract prepared from the auto-hydrolyzed wastewater biofilm was shown to be effective in detaching *P. aeruginosa* PAO1 biofilms from a glass surface and this could be ascribed to the degradation of EPS proteins and polysaccharides. Further characterization of the wastewater biofilm bacteria and the enzymes that they produce may thus lead to the identification of enzymes that could potentially be used to remove detrimental biofilms.

3.5 REFERENCES

- Boczar, B.A., Begley, W.M. and Larson, R.J. 1992. Characterization of enzyme activity in activated sludge using rapid analyses for specific hydrolases. *Water Environment Research*, **64**: 792-797.
- Boczar, B.A., Rorney, L.J., Begley, W.M., Larson, R.J. and Federle, T.W. 2001. Characterization and distribution of esterase activity in activated sludge. *Water Research*, **35**: 4208-4216.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**: 248-254.
- Cadoret, A., Conrad, A. and Block, J-C. 2002. Availability of low and high molecular weight substrates to extracellular enzymes in whole and dispersed activated sludges. *Enzymes and Microbial Technology*, **31**: 179-186.
- Ceyhan, N. and Ozdemir, G. 2008. Extracellular polysaccharides produced by cooling water tower biofilm bacteria and their possible degradation. *Biofouling*, **24**: 129-135.
- Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. 1998. Standard methods for the examination of water and wastewater. 20th Edition. American Public Health Association, American Water Works Association, Water Environment Federation, USA.
- Cloete, T.E. and Atlas, R. 2006. Basic and Applied Microbiology. Van Schaik Publishers, South Africa.
- Confer, D.R. and Logan, B.E. 1991. Increased bacterial uptake of macromolecular substrates with fluid shear. *Applied and Environmental Microbiology*, **57**: 3093-3100.
- Confer, D.R. and Logan, B.E. 1997a. Molecular weight distribution of hydrolysis products during the biodegradation of model macromolecules in suspended culture and wastewater biofilms. I. Bovine serum albumin. *Water Research*, **31**: 2127-2136.
- Confer, D.R. and Logan, B.E. 1997b. Molecular weight distribution of hydrolysis products during the biodegradation of model macromolecules in suspended culture and wastewater biofilms. II. Dextran and dextrin. *Water Research* **31**: 2137-2145.

- Confer, D.R. and Logan, B.E. 1998. Location of protein and polysaccharide hydrolytic activity in suspended and biofilm wastewater cultures. *Water Research*, **32**: 31-38.
- Dashek, W.V. 1997. Methods in plant biochemistry and molecular biology. CRC Press, USA.
- Droste, R.L. 1997. Theory and practice of water and wastewater treatment. John Wiley and Sons, Inc. Canada.
- Gessesse, A., Dueholm, T., Petersen, S.B. and Nielsen, P.H. 2003. Lipase and protease extraction from activated sludge. *Water Research*, **37**: 3652-3657.
- Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*, **59**: 257-268.
- Goel, R., Mino, T., Satoh, H. and Matsuo, T. 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. *Water Research*, **32**: 2081-2088.
- Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F. and Romeo, T. 2005. Depolymerization of β-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology*, **187**: 382-387.
- Jahn, A. and Nielsen, P.H. 1995. Extraction of extracellular polymeric substances (EPS) from biofilms using a cation exchange resin. *Water Science and Technology*, **32**:157–64.
- Johansen, C., Falholt, P. and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology*, **63**: 3724-3728.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.
- Logan, B.E. and Jiang, Q. 1990. A model for determining molecular size distributions of DOM. *Journal of Environmental Engineering*, **116**: 1046-1062.
- Loiselle, M. and Anderson, K.W. 2003. The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling*, **19**: 77-85.
- Nielsen, P.H., Jahn, A. and Palmgren, R. 1997. Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology*, **36**: 11-19.
- O'Toole, G., Kaplan, H.B. and Kolter, R. 2000. Biofilm formation as microbial development. *Annual Reviews in Microbiology*, **54**: 49-79.
- Olajuyigbe, F.M. and Ajele, J.O. 2005. Production dynamics of extracellular protease from *Bacillus* species. *African Journal of Biotechnology*, **4**: 776-779.
- Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F. and SanJose, C. 2006. Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology*, **40**: 51-56.

- Orgaz, B., Neufeld, R.J. and SanJose, C. 2007. Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. *Enzyme and Microbial Technology*, **40**: 1045-1051.
- Parmar, N., Singh, A. and Ward, O.P. 2001. Enzyme treatment to reduce solids and improve settling of sewage sludge. *Journal of Industrial Microbiology and Biotechnology*, **26**: 383-386.
- Raunkjær, K., Hvitved-Jacobsen, T. and Nielsen, R.H. 1994. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. *Water Research*, **28**: 251-262.
- Roe, J.H. 1954. The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological Chemistry*, **208**: 889-896.
- Sanders, W.T.M., Geering, M., Zeeman, G. and Lettinga, G. 2000. Anaerobic hydrolysis kinetics of particulates substrates. *Water Science and Technology*, **41**: 17-24.
- Secades, P. and Guijarro, J.A. 1999. Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Applied and Environmental Microbiology*, **65**: 3969-3975.
- Sponza, D.T. 2003. Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions. *Enzyme and Microbial Technology*, **32**: 375-385.
- Ten, L.N., Im, W-T., Kim, M-K., Kang, M.S. and Lee, S-T. 2004. Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *Journal of Microbiological Methods*, **56**: 375-382.
- Vesilind, P.A. 2003. Wastewater treatment plant design. Water Environment Federation, Alexandria, USA.
- Vetter, Y.A. and Deming, J.W. 1999. Growth rates of marine bacterial isolates on particulate organic substrates solubilized by freely released extracellular enzymes. *Microbial Ecology*, **37**: 86-94.
- Wang, Z-W., Liu, Y. and Tay, J-H. 2007. Biodegradability of extracellular polymeric substances produced by aerobic granules. *Applied Microbiology and Biotechnology*, **74**: 462-466.
- Wessel, D. and Flugge, U.I. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry*, **138**: 141-143
- Wingender, J., Nue, T.R. and Flemming, H-C. 1999. Microbial extracellular polymeric substances: Characterization, structure and function. Springer, Germany.
- Xia, Y., Kong, Y. and Nielsen, P.H. 2007. *In situ* detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbial Ecology*, **60**: 156-165.
- Zhang, X. and Bishop, P.L. 2003. Biodegradability of biofilm extracellular polymeric substances. *Chemosphere*, **50**: 63-69.

- Zhang, X., Bishop, P.L. and Kinkle, B.K. 1999. Comparison of extraction methods for quantifying extracellular polymers in biofilms. *Water Science and Technology*, **39**: 211-218.

CHAPTER 4

IDENTIFICATION OF WASTEWATER BIOFILM BACTERIA CAPABLE OF DEGRADING PROTEINS AND POLYSACCHARIDES

Abstract

Bacteria were isolated from a wastewater biofilm previously shown to produce protein- and polysaccharide-degrading enzymes. The bacteria were isolated on nutrient agar medium and 21 representative isolates, based on colony morphology, were purified and identified. The identity of the cultured bacterial isolates was determined by extracting their chromosomal DNA, followed by PCR amplification and sequencing of the 16S rRNA genes. The isolates represented four major phylogenetic divisions of bacteria, *i.e.* Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. Screening of the bacterial isolates for their ability to degrade proteins indicated that nine isolates produced protein-degrading enzymes, while ten isolates produced polysaccharide-degrading enzymes. Based on the use of culture media supplemented with different polysaccharide substrates, bacterial isolates capable of producing amylase, xylanase, cellulase, α-glucosidase and β-glucosidase were identified.

4.1 INTRODUCTION

Microbial biofilms are populations of microorganisms that are concentrated at an interface and typically surrounded by an extracellular polymeric matrix (Costerton *et al.*, 1995; Watnick and Kolter, 2000; Donlan, 2002). Biofilms are responsible for causing problems such as increased frictional resistance to fluids in technical water systems (Characklis and Marshall, 1990; Flemming, 2002), biofouling in engineered systems (White *et al.*, 1996; Flemming, 2002), decreased heat transfer from heat exchangers (Ludensky, 2003), corrosion of metallic substrata (LeChevallier *et al.*, 1993), and contamination in food processing and drinking water distribution systems (Kumar and Anand, 1998; Flemming *et al.*, 2002). Biofilms are also a severe problem in medical science and industry, causing prosthetic device colonization and biofilm formation on medical implants (Sihorkar and Vyas, 2001; Hall-Stoodley *et al.*, 2004). Most treatment systems for controlling biofilms include the use of antibiotics, biocides and disinfectants (Stewart and Costerton, 2001; Ludensky, 2003; Meyer, 2003). However, their efficacy may be limited due to the reduced susceptibility of microorganisms in biofilms to such antimicrobial agents (Ishida *et al.*, 1998; Anderl *et al.*, 2000; Norwood and Gilmour, 2000; Stewart and Costerton, 2001; Kostenko *et al.*, 2007), while some disinfectants have been reported to produce by-products that are toxic or have irritant properties (Augustin and Ali-Vehmas, 2004). This therefore necessitates that alternative approaches to control unwanted biofilms be investigated.

The main structural and physical component of microbial biofilms is the extracellular matrix (Allison *et al.*, 1998; Allison, 2003; Danese *et al.*, 2000; Hentzer *et al.*, 2001; Sutherland, 2001), which is comprised mostly of proteins and polysaccharides (Wingender *et al.*, 1999; Tsuneda *et al.*, 2003). The exopolysaccharides present in the matrix are partly responsible for bacterial adhesion and biofilm accumulation on surfaces (Allison *et al.*, 1998; Cammarota and Sant'Anna, 1998; Hentzer *et al.*, 2001), and thus represent a potentially significant target for biofilm removal and control. Several reports have indicated that enzymes can be used for degradation of biofilms and for their removal from surfaces. Enzymes reported to be capable of removing bacterial biofilms from different surfaces include: cellulase, α-amylase, protease, esterase, glucose oxidase, arabinase, β-glucuronidase, α-glucosidase, lipase, xylanase, β-glucanase, lactoperoxidase, polygalacturonase, lyases and Dispersin B (Selan *et al.*, 1993; Boyd and Chakrabarty, 1994; Johansen *et al.*, 1997; Allison *et al.*, 1998; Hahn Berg *et al.*, 2001; Loiselle and Anderson, 2003; Kaplan *et al.*, 2004; Itoh *et al.*, 2005 Orgaz *et al.*, 2006; Walker *et al.*, 2007). However, due to the heterogeneity of the polysaccharides and proteins present in the biofilm EPS, it has been suggested that a mixture of enzymes with different specificities should be used in order to degrade bacterial biofilms effectively (Meyer, 2003).

In the previous Chapter it was shown that a biofilm from an aeration basin at an industrial wastewater treatment plant produced enzymes that were capable of degrading the protein and polysaccharide components of the EPS from *Pseudomonas aeruginosa* PAO1, thus resulting in detachment of its biofilms from a glass surface. These findings suggested that the wastewater biofilm-expressed enzymes might prove useful disinfectants or cleaning agents for detrimental biofilms. Therefore, this study aimed to isolate and characterize the biofilm bacteria. Although the microbial populations in wastewater treatment bioreactors have received little attention, studies have shown that wastewater bioreactors are similar to many natural environments, in that the number of culturable bacteria is 5-15% of the total number of bacteria (Wagner *et al.*, 1993; Kämpfer *et al.*, 1996; Amann *et al.*, 1998). Microbes in wastewater treatment reactors may be identified using either traditional or molecular methods. Using traditional methods, bacterial cultures are isolated and then identified by their morphological, physiological and biochemical properties. However, these traditional identification methods have a number of intrinsic limitations (Amann *et al.*, 1995; Theron and Cloete, 2000). Not only may the growth medium selectively favour certain populations, but many bacteria may share similar morphological, physiological and biochemical properties making it difficult to differentiate them. However, 16S rRNA-based molecular methods with high degrees of precision and specificity have been widely used for environmental microbial studies (Wagner *et al.*, 1994; Bond *et al.*, 1995; Theron and Cloete, 2000; Bramucci *et al.*, 2003; Liu *et al.*, 2005). Using these methods, the microbial identities may be revealed from their 16S rRNA sequences, following amplification of the 16S rRNA gene using polymerase chain reaction (PCR).

Since the aim of this research project was to identify enzymes that may prove useful in biofilm removal, pure cultures of the wastewater biofilm bacteria were required. Therefore, the objectives of this part of the study were to isolate bacteria from the wastewater biofilm using a culture-based approach and to identify representative isolates based on their 16S rRNA sequences. Furthermore, the bacterial isolates were screened for production of enzymes capable of degrading proteins and polysaccharides.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of bacteria from wastewater biofilm

A thoroughly-mixed wastewater biofilm sample, previously obtained from an aeration basin at the Leeuwkuil water purification plant in Vereeniging, Gauteng, South Africa (Chapter 3, Section 3.2.2), was serially diluted (10-fold) in quarter strength Ringer’s solution (Merck). Aliquots of 1 ml of each dilution were aseptically plated onto nutrient agar medium (Merck). The agar plates were incubated at 25°C for 48 h. Following incubation, single colonies were characterized with regard to their colony morphology, pigmentation and Gram-staining reaction. The bacterial isolates were also screened for catalase and oxidase production, as previously described by Harrigan and McCance (1966). Representative colonies were purified by repeated sub-culturing onto nutrient agar and the pure cultures were then maintained at -70°C as glycerol cultures.

4.2.2 PCR amplification and sequencing of the 16S rRNA genes from bacterial isolates

4.2.2.1 Genomic DNA extraction

Genomic DNA was extracted from the bacterial isolates with cetyltrimethylammonium bromide (CTAB), as described by Ausubel *et al.* (2002). The cells from 1.5 ml of an overnight culture were pelleted by centrifugation at 11 000 × *g* for 5 min and suspended in 567 µl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8). The cells were lysed by addition of SDS to a final concentration of 0.5% (v/v) and proteins were digested by addition of Proteinase K to a final concentration of 100 µg/ml in a total volume of 600 µl. Following incubation at 37°C for 1 h, 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl) was added, mixed thoroughly and incubated at 65°C for 10 min. The suspension was extracted with an equal volume of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 6 000 × *g* for 5 min. The supernatant, containing the genomic DNA, was transferred to a clean microfuge tube. The residual CTAB was removed by addition of an equal volume of phenol:choroform:isoamyl alcohol (25:24:1), followed by centrifugation at 6 000 × *g* for 5 min. The genomic DNA was precipitated from the recovered aqueous supernatant by addition of 0.6 volume isopropanol. The precipitated genomic DNA was pelleted by centrifugation at 11 000 × *g* for 2 min, washed with 70% ethanol, dried under vacuum and suspended in 30 µl of 1 × TE buffer. An aliquot of the extracted genomic DNA was analyzed by agarose electrophoresis, as described by Sambrook *et al.* (1989).

4.2.2.2 Polymerase chain reaction (PCR) amplification of 16S rRNA genes

The 16S rRNA genes were PCR-amplified using the oligonucleotides PRUN518r (5’-ATTACCGCGGCTGCTGG-3’) (Øvreås *et al.*, 1997) and pA8f-GC (5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGAGTTTGATCCTGGCTCAG-3’) (Fjellbirkeland *et al.*, 2001). Each PCR reaction mixture (20 µl) contained approximately 60 ng of genomic DNA as template, 50 µM of each oligonucleotide, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 0.25 U Supertherm *Taq* DNA polymerase (Southern Cross Biotechnology). The tubes were placed in a Perkin-Elmer GeneAmp® 2700 thermal cycler. Following incubation at 95°C for 10 min, the reaction mixtures were subjected to 35 cycles of denaturation at 94ºC for 30 s, annealing at 53ºC for 30 s and elongation at 72ºC for 1 min. After the last cycle, a final elongation step was performed at 72°C for 10 min to complete synthesis of all DNA strands. For control purposes, a reaction mixture containing all reagents except template DNA was included. Aliquots of the PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker (Gene Ruler™ 100-bp DNA Ladder Plus; Fermentas), and amplicons were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.

4.2.2.3 Nucleotide sequencing and sequence analyses

The nucleotide sequence of the amplicons was determined with the ABI-PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. The oligonucleotides used in the PCR reaction (PRUN518r and pA8f-GC), were used as the sequencing primers. The sequencing reactions comprised approximately 70 ng of DNA, 2 µl of Terminator Ready Reaction Mix, 10 pmol of the sequencing primer and UHQ water to a final volume of 5 µl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp® 2700 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The purified, denatured extension products were loaded onto a Hitachi 3100 capillary array DNA sequencer (Sequencing facility of the University of Pretoria). Nucleotide sequences were analyzed with BioEdit v.5.0.9.1 (Hall, 1999) and their identity were verified by BLASTN searches (Altschul *et al.*, 1997) against the GenBank database (available at http://www.ncbi.nlm.nih.gov/BLAST).

4.2.3 Detection of protein-degrading bacterial isolates

Bacterial isolates were screened for protease activity according to the method of Harrigan and McCance (1966). The isolates were streaked onto 10% skim-milk agar medium and the agar plates were incubated at 25°C for 48 h. Protein degradation is visualized as clear zones surrounding the bacterial growth.

4.2.4 Detection of polysaccharide-degrading bacterial isolates

4.2.4.1 Screening of bacterial isolates for amylase and xylanase activity

All chemicals used in the preparation of dye-labeled polysaccharide substrates were obtained from Sigma-Aldrich. The chromogenic substrates were prepared, as described by Ten *et al.* (2004), with the following minor modifications. Xylan was cross-linked to Cibacron Brilliant Red 3B-A by adding 5 ml of 2 M NaOH, 0.6 ml of 1,4-butanediol diglycidyl ether and 0.95 g of Cibacron Brilliant Red 3B-A to a xylan suspension, which had been prepared by dissolving 1 g of xylan in 15 ml of dH_2O. The suspension was stirred for 5 min and incubated at room temperature for 48 h to solidify in a gel. Subsequently, 50 ml of dH_2O was added to the gel and it was ground with a mortar and pestle until fine particles were produced. To remove the unbound dye, the particles were washed with boiling water and filtered through Whatman Type 1 filter paper until the filtrate was colourless. The xylan-red substrate was scraped from the surface of the filter paper and used as a supplement to nutrient agar medium. Amylose-blue was similarly prepared, except that amylose was cross-linked to Cibacron Blue 3GA using 1.5 ml of 1,4-butanediol diglycidyl ether in the reaction mixture. Nutrient agar medium was subsequently supplemented with 25 g of xylan-red and 75 g of amylose-blue per 1 l of nutrient agar medium. To keep the particles dispersed, the autoclaved (121°C; 20 min) nutrient agar was agitated gently while being poured into plates. Bacterial isolates were streaked onto the supplemented nutrient agar media and the agar plates were incubated at 25°C for 2-5 days. Following incubation, plates were screened for solubilization of the chromogenic substrates and halo formation around the bacterial growth.

4.2.4.2 Screening of bacterial isolates for cellulase activity

Bacterial isolates were screened for cellulase activity according to a slightly modified method of Teather and Wood (1982). Nutrient agar medium, supplemented with 8 g/l hydroxyethyl-cellulose (Sigma-Aldrich), was autoclaved (121°C; 20 min) and thoroughly mixed before pouring of plates. The agar medium was inoculated with the bacterial isolates and the agar plates were incubated at 25°C for 72 h. The surface of the agar medium was rinsed with 0.85% (w/v) saline and then flooded with an aqueous solution of Congo red (1 mg/ml prepared in dH_2O). Following incubation at room temperature for 30 min, the Congo red solution was poured off and the plates were flooded sequentially with 1 M NaCl and 1 M HCl for 15 min each. Cellulose degradation is visualized as halo formation around the bacterial growth.

4.2.4.3 Screening of bacterial isolates for lyase activity

Bacterial isolates were screened for gellan lyase activity, as described by Kennedy and Sutherland (1994), by supplementing nutrient agar medium with 15 g/l Phytagel (Sigma-Aldrich). The isolates were streaked onto the medium and the agar plates were incubated at 25°C for 5 days. The agar plates were examined for the formation of depressions in the gel due to enzymatic degradation of the gellan. The bacterial isolates were also screened for alginate lyase activity, as described by Chavagnat *et al.* (1996). The isolates were streaked onto "black medium" containing 0.5% (w/v) bacteriological agar, 0.5% (w/v) yeast extract, 1% (w/v) sodium alginate and 0.5% (w/v) activated charcoal. Following incubation of the agar plates at 25°C for 5 days, the agar plates were flooded with 10% cetylpyridinium chloride and incubated at room temperature for 20 min. Alginate degradation is visualized as clear zones surrounding the bacterial growth.

4.2.4.4 Screening of bacterial isolates for glucosidase activity

Bacterial isolates were screened for α- and β-glucosidase activity according to the method of Arellano-Carbajal and Olmos-Soto (2002). The isolates were inoculated into nutrient broth and incubated at 25°C for 48 h. To screen for α-glucosidase activity, 1 ml of the liquid culture was mixed with 3 ml of 0.01 M sodium phosphate buffer (pH 7) and 1 ml of 0.1% (w/v) *p*-nitrophenyl α-D-glucopyranoside (*p*NPG; Calbiochem, Merck). Following incubation at 30°C for 30 min, the reaction was terminated by addition of 1 ml of 1 M Na_2CO_3. A change in the colour of the reaction mixture to yellow indicated the presence of α-glucosidase activity. The same methodology was used to screen the bacterial isolates for β-glucosidase activity, except that *p*-nitrophenyl β-D-glucopyranoside (Calbiochem, Merck) was used as the substrate in the enzyme assays.

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation of biofilm bacteria

To isolate biofilm bacteria, serial dilutions of a wastewater biofilm were plated onto nutrient agar medium and representative colonies, based on colony morphology, were selected for further characterization. The bacterial isolates were purified by repeated streaking onto nutrient agar and designated isolates 1 through 21. Of the 21 bacterial isolates selected, eleven were Gram-negative, eight were Gram-positive and two were visualized as Gram-variable. The majority of the bacterial isolates displayed a rod-shaped morphology and only six of the isolates produced coloured pigments following growth on nutrient agar. All of the bacterial isolates produced catalase, and only seven of the isolates did not produce oxidase. The morphological and physiological characteristics of the 21 cultured bacterial isolates are summarized in Table 4.1.

Table 4.1: Morphological and physiological properties of bacterial isolates from wastewater biofilms

Isolate nr.	Gram straining	Cell morphology	Pigmentation	Colony morphology	Oxidase*	Catalase*
1	+	Rods	None	Beige, spreading, margin is undulate, frosted appearance, slimy center	++	++
2	+	Rods	None	Beige, irregular, undulate margin, frosted appearance	++	++
3	-	Rods	None	Dark beige, smooth, circular, mucoid	+	+
4	+	Rods	None	Beige, undulate and translucent margin, impregnated into media	+	++
5	+	Rods	None	Beige, large, irregular, undulate margin, frosted appearance	++	++
6	+	Rods	None	Beige, flat, border around center	+	++
7	+	Rods	Yellow	Circular, iridescent, smooth, entire margin	-	+
8	-	Rods	None	Beige, smooth, mucoid, entire margin, quickly becoming confluent	+	++
9	-	Rods	None	Beige, smooth, translucent, entire margin	++	+
10	Gram variable	Pleomorphic	None	Beige, translucent, smooth, entire margin	-	++
11	-	Rods	None	Beige, spreading margin, translucent	++	++
12	-	Rods	None	Beige, small, circular, smooth to slightly granular, translucent, entire margin	+	+
13	-	Rods	None	Beige, smooth to slightly granular, entire margin, translucent	++	+
14	-	Rods	Bright red	Circular, iridescent with entire margin, slightly convex, mucoid	-	++
15	-	Rods	Bright yellow	Mucoid, entire margin, translucent	-	++
16	-	Rods	Pale yellow	Mucoid, translucent	+	+
17	Gram variable	Pleomorphic	Pale yellow	Circular, entire margin, opaque, elevated center	-	+
18	+	Rods	None	Beige, circular, entire margin, iridescent	-	++
19	-	Rods	None	Beige, very small, circular, entire margin, translucent	+	+
20	-	Rods	None	Beige, circular, translucent	++	++
21	+	Cocci	Bright orange	Circular, entire margin, iridescent, convex	-	++

*: ++, strong positive; +, weakly positive; -, negative

4.3.2 Identification of wastewater biofilm bacteria based on 16S rRNA gene sequence analysis

Genomic DNA was extracted from the individual bacterial isolates and the 16S rRNA gene was amplified by PCR using a set of oligonucleotides specific for eubacteria. The amplicons were approximately 500 bp in length and both DNA strands of the amplicons were sequenced. The 16S rRNA sequences were subsequently compared with the sequences in GenBank by BLAST. Table 4.2 summarizes the isolate number, the closest sequence found in GenBank and their similarity. The results presented in Table 4.2 indicated that the 21 cultured isolates represented four major phylogenetic divisions of bacteria, *i.e.* Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes.

Table 4.2: Identity of cultured bacterial isolates from a wastewater biofilm based on their 16S rRNA sequences

Isolate no.	GenBank accession number/Organism	Percentage similarity	Phylogenetic group
1	DQ520955 *Bacillus subtilis*	99%	Firmicutes
2	AB284820 *Bacillus cereus*	99%	Firmicutes
3	DQ298025 *Rheinheimera chironomi*	97%	Proteobacteria
4	EF523475 *Bacillus pumilus*	97%	Firmicutes
5	AY795568 *Bacillus cereus*	99%	Firmicutes
6	EF433406 *Bacillus amyloliquefaciens*	99%	Firmicutes
7	DQ350825 *Microbacterium oxydans*	98%	Actinobacteria
8	AJ242584 *Ochrobactrum tritici*	98%	Proteobacteria
9	AM490636 *Ochrobactrum tritici*	87%	Proteobacteria
10	AJ243423 *Arthrobacter oxydans*	99%	Actinobacteria
11	DQ229315 *Pseudomonas putida*	99%	Proteobacteria
12	AF078764 *Acidovorax delafieldii*	96%	Proteobacteria
13	DQ857897 *Brevundimonas diminuta*	96%	Proteobacteria
14	EF114344 *Serratia marcescens*	99%	Proteobacteria
15	AY275475 *Chryseobacterium indotheticum*	96%	Bacteroidetes
16	AB166885 *Stenotrophomonas koreensis*	99%	Proteobacteria
17	X80745 *Arthrobacter protophormiae*	99%	Actinobacteria
18	AJ781046 *Leucobacter chromiireducens*	96%	Actinobacteria
19	AF078764 *Acidovorax delafieldii*	96%	Proteobacteria
20	AJ518818 *Acidovorax delafieldii*	97%	Proteobacteria
21	AY211171 *Kocuria rosea*	97%	Actinobacteria

Proteobacteria

Ten of the bacterial isolates, representing seven different genera, were closely affiliated with Proteobacteria. Isolate 3 is related to *Rheinheimera chironomi* of little known characteristics, but has been isolated from the egg mass of a chironomid (non-biting midge) that lays its eggs in freshwater (Halpern *et al.*, 2007). Both isolates 8 and 9 are related to *Ochrobactrum tritici*, which has been isolated frequently from wastewater (Goris *et al.*, 2003; Branco *et al.*, 2004; You *et al.*, 2006). However, the isolates also displayed 97% and 87% similarity, respectively, with *O. anthropi*, an opportunistic human pathogen (Ozdemir *et al*., 2006; Aly *et al*., 2007), that has also been isolated from sewage (Swings *et al*., 2006) and activated sludge (Ozdemir *et al*., 2003). Isolate 11 is most closely related to *Pseudomonas putida*. Members of the *Pseudomonas* genus, including *P. putida*, have been isolated from activated sludge and municipal wastewater (Leung *et al*., 2000; Lim *et al*., 2002). The three isolates 12, 19 and 20 are related to *Acidovorax delafieldii*, which has a flexible metabolism and is commonly found in wastewater treatment plants (Schulze *et al*., 1999; Zhao and Ward, 1999; Khan *et al*., 2002). Isolate 13 is closely related to *Brevundimonas diminuta*, which has been isolated previously from activated sludge in a sequence batch reactor (Liu *et al*., 2005). Isolate 14 is most closely related to *Serratia marcescens*, an opportunistic human pathogen (van Ogtrop *et al*., 1997; Johnson *et al*., 1998), that occurs in soil, water and food (Vignolo *et al*., 1993; Montpas *et al*., 1997). *S. marcescens* has also been isolated from wastewater (Filipkowska, 2003; Ajithkumar *et al*., 2003; Li *et al*., 2008) and tannery effluents (Campos *et al*., 2005). Isolate 16 is most closely related to *Stenotrophomonas koreensis*, a newly described bacterial species that was recently isolated from compost (Yang *et al*., 2006). However, the isolate also displayed 96% similarity with *S. maltophila*, an opportunistic human pathogen (Denton and Kerr, 1998). This bacterial species occurs in various natural environments and has been isolated from several water sources (rivers, wells, hypereutrophic lake, bottled water and wastewater), soil environments, plant rhizosphere environments and food sources (Denton and Kerr, 1998; Brenner *et al*., 2005).

Actinobacteria

Five of the bacterial isolates, representing four different genera, were closely affiliated with Actinobacteria. Isolate 7 is closely related to *Microbacterium oxydans*. Members of this genus have been isolated from variety of environmental sources, including dairy products, plants, soil and sewage (Funke *et al*., 1995), while *M. oxydans* has been isolated from petroleum-contaminated soil (Supaphol *et al*., 2006) and from hydrocarbon-polluted soil (Menendez-Vega *et al*., 2007), as well as from activated sludge (Chen *et al*., 2007). Isolates 10 and 17 are most closely related to *Arthrobacter oxydans* and *A. protophormiae*, respectively. Members of this genus mostly inhabit soil environments (Jones and Keddie, 2006), but *A. oxydans* has been isolated from livestock farming wastewater (Chung *et al*., 1997) and activated sludge (Song *et al*., 2005). Isolate 18 is related to *Leucobacter chromiireducens*, which has been isolated from activated sludge (Morais *et al*., 2004), while isolate 21 is related to *Kocuria rosea*, an opportunistic human pathogen (Altuntas *et al*., 2004). *Kocuria* spp. have been isolated from wastewater and activated sludge (Leung *et al.*, 2000; Yasuhira *et al.*, 2007).

Firmicutes

All five bacterial isolates affiliated with the Firmicutis group comprised species of the genus *Bacillus*. Members of the genus *Bacillus* are ubiquitous and can be isolated from nearly all natural environments, soil, freshwater, foods, milk and pharmaceuticals (Sneath *et al.*, 1986). Isolates 1 and 4 are closely related to *B. subtilis* and *B. pumilus*, respectively. Both these bacterial species have been isolated from activated sludge and municipal wastewater (Leung *et al.*, 2000). Isolates 2 and 5 are most closely related to *B. cereus*, which has been isolated from activated sludge (Sidat *et al.*, 1999) and fish industry wastewater (Ghorbel *et al.*, 2003). Isolate 6 is most closely related to *B. amyloliquefaciens*, which has been previously isolated from soil and water samples (Breccia *et al.*, 1998).

Bacteroidetes

Only one of the cultured bacterial isolates was affiliated with the Bacteroidetes group. Isolate 15 is related to *Chryseobacterium indotheticum*, a member of the family Flavobacteriaceae, which are found widespread in aquatic environments where they are responsible for the degradation of complex organic material (Bernardet *et al.*, 2002). Species within the genus *Chryseobacterium*, including *C. indotheticum*, have also frequently been isolated from wastewater and activated sludge (Kämpfer *et al.*, 2003; Quan *et al.*, 2007; Yoon *et al.*, 2007).

The wastewater treatment plant from which the biofilm was sampled receives its main effluents from domestic residences, several metal processing and casting industries, an abattoir and hospitals. The latter may explain the isolation of several opportunistic human pathogens during the course of this investigation. Many of the cultured bacterial isolates identified in this study have been reported to occur in wastewater and activated sludge, but the presence of *Rheinheimera chironomi* and *Stenotrophomonas koreensis* in this ecological niche is reported here for the first time. Although not an objective of this study, investigations regarding the microbial composition and functionality would be of importance to gain a better understanding of the wastewater treatment process and to optimize the process. However, culture-based methods cannot be directly used to analyze the microbial diversity because it is widely recognized that only a small portion of the total microbiota in environmental samples can be cultured (Wagner *et al.*, 1993; Kämpfer *et al.*, 1996; Amann *et al.*, 1998).

Nevertheless, the results that were obtained in this study are in agreement with several other studies indicating that culture-dependant methods typically show that Proteobacteria dominate the microbial populations in activated sludge from municipal wastewater bioreactors (Kämpfer *et al.*, 1996; Amann *et al.*, 1998; Bramucci and Nagarjan, 2000; Bramucci *et al.*, 2003). Since it was previously shown that a crude enzyme extract containing the wastewater biofilm-expressed enzymes removed *P. aeruginosa* PAO1 biofilms from a glass surface (Chapter 3), the cultured bacterial isolates were subsequently investigated for their ability to produce enzymes that have previously been reported to aid biofilm removal.

4.3.3 Bacterial isolates with protein-degrading capabilities

The bacterial isolates were screened for proteolytic activity on skim-milk agar, and hydrolysis zones were visualized as clear zones against an opaque background (Fig. 4.1). The diameter of the hydrolysis zones served as a semi-quantitative measure of the protease activity. Of the 21 bacterial isolates, nine were identified as being able to produce proteases. Isolates 3 and 14 displayed the most potent protease activity with hydrolysis zones of 7 mm each, followed by isolate 6 (5 mm) and isolate 1 (4 mm). Less potent protease activity was observed for isolates 2, 4 and 15 (3 mm), while isolates 16 and 5 displayed the weakest proteolytic activity with hydrolysis zones of 2 and 1 mm, respectively (Fig. 4.2).

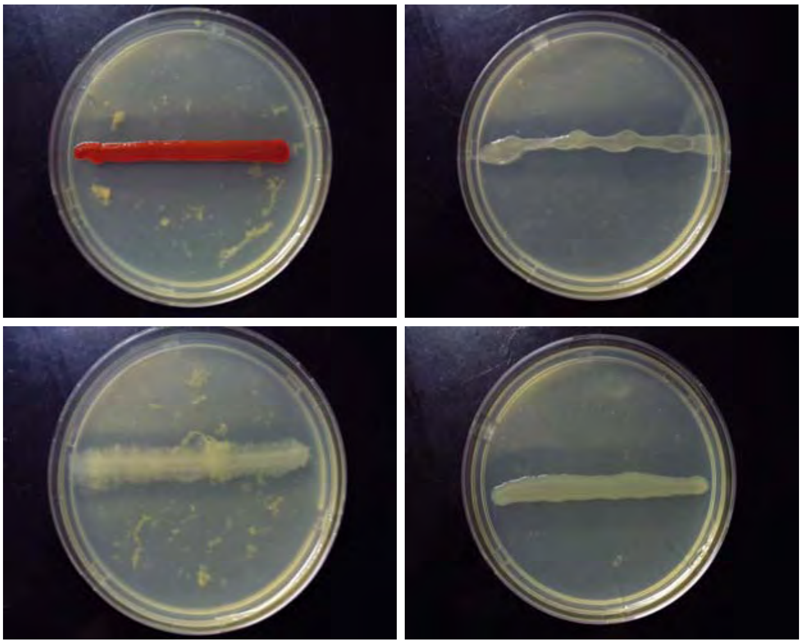


Figure 4.1. Detection of protein-degrading bacteria on skim-milk agar medium. Hydrolysis zones surrounding the bacterial growth are indicative of protease production.

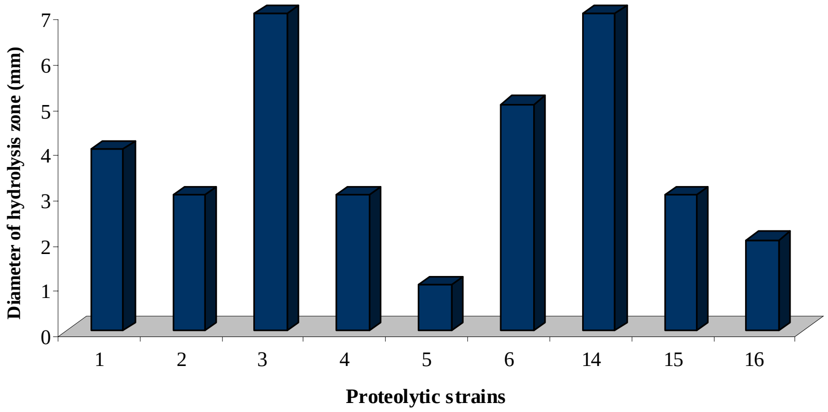


Figure 4.2. Diameter of hydrolysis zones formed on skim-milk agar by bacterial isolates capable of producing proteases. Values are the means of results of triplicate experiments.

4.3.4 Bacterial isolates with polysaccharide-degrading capabilities

4.3.4.1 Xylan- and amylose-degrading bacteria

To identify bacterial isolates with xylanase and/or amylase activity, nutrient agar medium was amended with a mixture of two chromogenic substrates, *i.e.* amylose-blue and xylan-red. Several bacterial isolates were capable of degrading xylan and amylose, as evidenced by solubilization of the substrate particles and halo formation around the bacterial growth. By using this approach, three isolates were identified that produced both xylan- and amylose-degrading enzymes (isolates 1, 6 and 21), while three isolates exhibited amylose-degrading activity (isolates 2, 3 and 5) and one isolate displayed xylan-degrading activity (isolate 4). Representative photographs of the agar plates are presented in Fig. 4.3. Bacterial isolates capable of degrading both xylan and amylose are shown in Figs. 4.3a and 4.3b, while isolates capable of degrading only amylose or xylan is shown in Figs. 4.3c and 4.3d, respectively.

4.3.4.2 Cellulose-degrading bacteria

The bacterial isolates were screened for cellulase activity by streaking the isolates onto nutrient agar medium that was supplemented with hydroxyethyl-cellulose. Following incubation, the plates were flooded with Congo red and the solution was then replaced with NaCl to maximize binding of the dye to the polysaccharide. The plates were then flooded with HCl, resulting in a colour change from red to blue, and thereby increasing the contrast between degradation zones and the blue background. Using this approach, three bacterial isolates (1, 4 and 5) were identified that were capable of degrading hydroxyethyl-cellulose (Fig. 4.4).

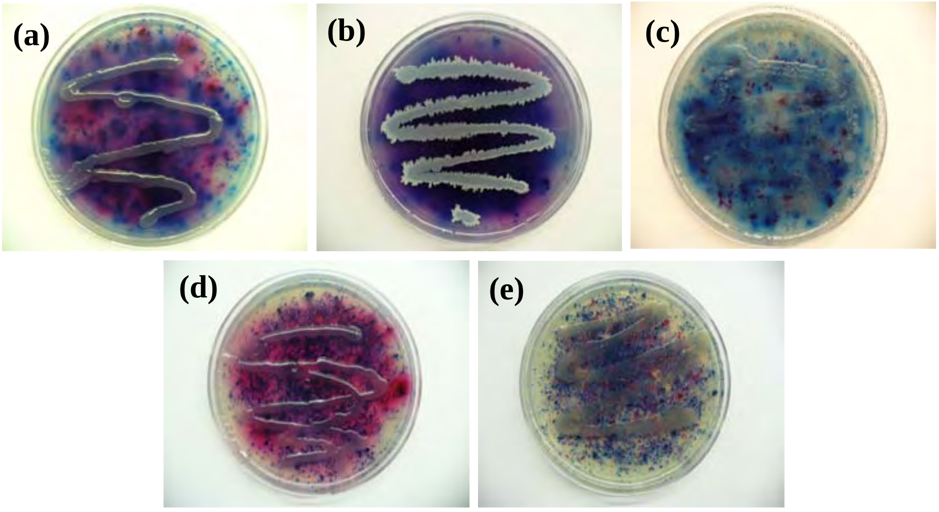


Figure 4.3. Detection of xylan- and amylose-degrading bacteria on nutrient agar supplemented with xylan-red and amylose-blue. (a) and (b) Bacterial isolates were capable of degrading both xylan and amylose, as evidenced by the solubilization of both red and blue chromogenic substances around the bacterial growth. (c) A visible blue halo was observed surrounding the bacterial growth, indicating that the bacterial isolate is capable of degrading amylose only. (d) A visible red halo was observed surrounding the bacterial growth, indicating that the bacterial isolate is capable of degrading xylan only. (e) No visible halo was observed surrounding the bacterial growth, indicating that the bacterial isolate is not capable of degrading amylose and/or xylan.

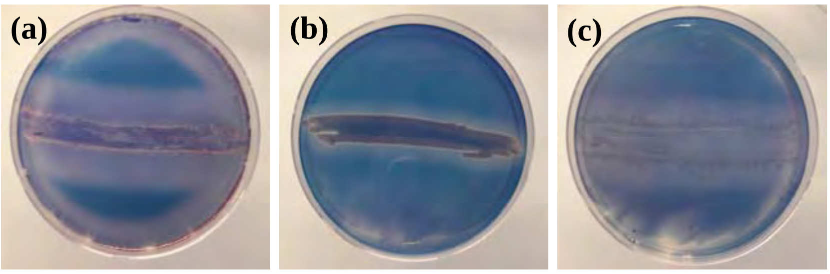


Figure 4.4. Detection of cellulose-degrading bacteria on nutrient agar supplemented with hydroxyethyl-cellulose. Haloes surrounding the bacterial growth are indicative of cellulose degradation. Results are shown for isolates 1 (a), 4 (b), and 5 (c).

4.3.4.3 Alginate- and gellan-degrading bacteria

The bacterial isolates were screened for both alginate and gellan lyase activity. However, the bacterial isolates were unable to degrade the alginate present in the agar medium, as was evidenced by a lack of haloes surrounding the bacterial growth. In addition, the bacterial isolates also did not cause depressions in the nutrient agar medium supplemented with gellan. These results therefore indicated that none of the bacterial isolates were capable of producing alginate or gellan lyase (results not shown).

4.3.4.4 Glucosidase-producing bacteria

The bacterial isolates were screened for α- and β-glucosidase using the synthetic substrates *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside, respectively (Fig. 4.5). The assays are based on the determination of the hydrolytic release of *p*-nitrophenol from the respective *p*NPG substrates. The *p*-nitrophenol is a yellow-coloured compound, the colouration of which deepens in Na_2CO_3, thus providing a semi-quantitative measure of enzyme activity (Chapdelaine *et al.*, 1978). Of the 21 bacterial isolates screened, eight isolates were capable of producing α-glucosidase (isolates 1, 2, 4-7, 10 and 15), while three isolates produced β-glucosidase (isolates 4, 6 and 7).

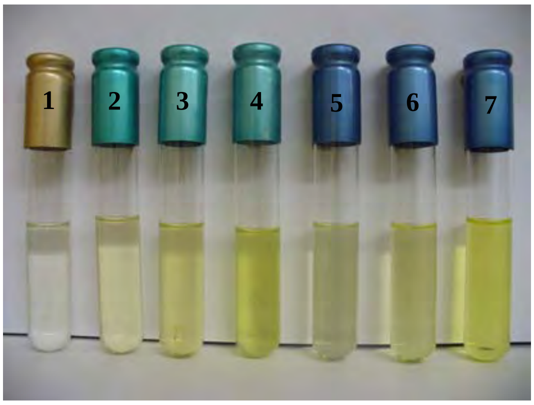


Figure 4.5. Detection of α-glucosidase activity using the chromogenic substrate *p*-nitrophenyl α-D-glucopyranoside (tubes 2-4), and detection of β-glucosidase activity using the chromogenic substrate *p*-nitrophenyl β-D-glucopyranoside (tubes 5-7). The control, uninoculated tube showed no colour reaction (tube 1).

The enzymatic activities displayed by the cultured wastewater biofilm bacteria are summarized in Table 4.3. Of the 21 bacterial isolates, 12 isolates displayed protease- and/or polysaccharide-degrading enzyme activities. Among bacteria, *Bacillus* spp. produces a number of extracellular proteases and polysaccharide-degrading enzymes (Priest, 1977;

Schallmey *et al.*, 2004; Saeki *et al.*, 2007). Indeed, most of the bacterial isolates identified in this study that revealed both proteolytic and polysaccharide-degrading activities belonged to this genus. In particular, *B. subtilis* (isolate 1), *B. pumilis* (isolate 4) and *B. amyloliquefaciens* (isolate 6) displayed a wide range of enzyme activities, followed by *B. cereus* (isolates 2 and 5), albeit that these two strains differed in their ability to produce cellulase (Table 4.3). In addition, *M. oxydans* (isolate 7), *A. oxydans* (isolate 10) and *K. rosea* (isolate 21) produced polysaccharide-degrading enzymes, while *S. marcescens* (isolate 14) and *S. koreensis* (isolate 16) displayed proteolytic activity. Moreover, *R. chironomoni* (isolate 3) diplayed both amylase and protease activities, while *C. indotheticum* (isolate 15) displayed both α-glucosidase and protease activities. These results are in agreement with previous reports regarding enzymes produced by bacterial strains isolated during the course of this study (Table 4.4).

Table 4.3: Enzyme activities displayed by 21 cultured bacterial isolates from a wastewater biofilm

Isolate	Protease	Amylase	Xylanase	Cellulase	α-glucosidase	β-glucosidase	Alginate lyase	Gellan lyase
1	+	+	+	+	+	-	-	-
2	+	+	-	-	+	-	-	-
3	+	+	-	-	-	-	-	-
4	+	-	+	+	+	+	-	-
5	+	+	-	+	+	-	-	-
6	+	+	+	-	+	+	-	-
7	-	-	-	-	+	+	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	+	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	+	-	-	-	-	-	-	-
15	+	-	-	-	+	-	-	-
16	+	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
21	-	+	+	-	-	-	-	-

Table 4.4: Enzymes produced by several of the isolated bacteria, as indicated in previous studies

Bacterial strain	Enzymes produced	References
Bacillus subtilis	- Protease	- Tran *et al.*, 1991; Sloma *et al.*, 1991; Cheggour *et al.*, 2000
	- Amylase	- Yang *et al.*, 1984
	- Xylanase	- Sá-Pereira *et al.*, 2002
	- Cellulase	- Park *et al.*, 1991
	- α-glucosidase	- Schönert *et al.*, 1998
Bacillus cereus	- Protease	- Stark *et al.*, 1992
	- Amylase	- Bogas *et al.*, 2007
	- Cellulase	- Thayer, 1978
	- α-glucosidase	- Suzuki *et al.*, 1982
Rheinheimera chironomi	- Protease	- Halpern *et al.*, 2007
Bacillus pumilus	- Protease	- Huang *et al.*, 2003
	- Xylanase	- El Ahwany and Youssef, 2007
	- Cellulase	- Kotchoni and Shonukan, 2002
	- α-glucosidase	- Ouoba *et al.*, 2004
	- β-glucosidase	- Ouoba *et al.*, 2004
Bacillus amyloliquefaciens	- Protease	- Vasantha *et al.*, 1984; Balaban *et al.*, 2007
	- Amylase	- Takkinen *et al.*, 1983
	- Xylanase	- Breccia *et al.*, 1998
	- α-glucosidase	- Fogarty *et al.*, 1985
Microbacterium oxydans	- β-glucosidase	- Laffineur *et al.*, 2003
Arthrobacter oxydans	- α-glucosidase	- Wauters *et al.*, 2000
Serratia marcescens	- Protease	-Lyerly and Kreger, 1979; Matsumoto *et al.*, 1984; Molla *et al.*, 1987

4.4 CONCLUSIONS

Bacteria from a wastewater biofilm were isolated, identified and subsequently screened using plate screening methods to detect bacterial strains with protein- and polysaccharide-degrading enzyme activities. The enzyme activities screened for in this study were selected based on previous reports indicating their efficacy with regards to bacterial biofilm removal (Selan *et al.*, 1993; Loiselle and Anderson, 2003; Orgaz *et al.*, 2006; Walker *et al.*, 2007). With the exception of lyase enzymes, bacterial strains were identified that produced protease, amylase, xylanase, cellulase, α-glucosidase and β-glucosidase enzymes. Several bacterial strains belonging to the genus *Bacillus* were identified that produced both proteases and different types of polysaccharide-degrading enzymes. Since the EPS of bacterial biofilms is heterogenous (Sutherland, 2001; Allison, 2003), enzyme mixtures comprising different activities have been favoured for the efficient removal of biofilms (Meyer, 2003). The identification of bacterial strains producing different types of enzymes may thus hold promise as anti-biofilm agents.

4.5 REFERENCES

- El Ahwany, A.M.D. and Youssef, A.S. 2007. Xylanase production by *Bacillus pumilis*: Optimization by statistical and immobilization methods. *Research Journal of Agriculture and Biological Sciences*, **3**: 727-732.
- Ajithkumar, B., Ajithkumar, V.P., Iriye, R., Doi, Y. and Sakai, T. 2003. Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank. *International Journal of Systematic and Evolutionary Microbiology*, **53**: 253-258.
- Allison, D.G. 2003. The biofilm matrix. *Biofouling*, **19**: 139-150.
- Allison, D.G., Ruiz, B., SanJose, C., Jaspe, A. and Gilbert, P. 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiology Letters*, **167**: 179-184.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acid Research*, **25**: 3389-3402.
- Altuntas, F., Yildiz, O., Eser, B., Gündogan, K., Sumerkan, B. and Çetin, M. 2004. Catheter-related bacteremia due to *Kocuria rosea* in a patient undergoing peripheral blood stem cell transplantation. *BMC Infectious Diseases*, **4**: 1-3.
- Aly, N.Y.A., Salmeen, H.N. and Joshi, R.M. 2007. *Ochrobactrum anthropi* bacteremia in a child with inborn error of mitochondrial fatty acid oxidation. *Medical Principles and Practices*, **16**: 436-465.
- Amann, R., Lemmer, H. and Wagner, M. 1998. Monitoring the community structure of wastewater treatment plants: A comparison of old and new techniques. *FEMS Microbiology Ecology*, **25**: 205-215.
- Amann, R.I., Ludwig, W. and Schleifer, K.H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiology Reviews*, **59**, 143-169.
- Anderl, J.N., Franklin, M.J. and Stewart, P.S. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, **44**: 1818-1824.

- Arellano-Carbajal, F. and Olmos-Soto, J. 2002. Thermostable α-1,4- and α-1,6-glucosidase enzymes from *Bacillus* sp. isolated from a marine environment. *World Journal of Microbiology and Biotechnology*, **18**: 791-795.
- Augustin, M. and Ali-Vehmas, T. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmacy and Pharmaceutical Sciences,* **7**: 55-64.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 2002. Short Protocols in Molecular Biology. Fifth Edition, Volume One. Wiley-Interscience, Canada.
- Balaban, N.P., Malikova, L.A., Mardanova, A.M., Rudenskaya, G.N. and Sharipova, M.R. 2007. Purification and characterization of a subtilisin-like proteinases secreted in the stationary growth phase of *Bacillus amyloliquefaciens* H2. *Biochemistry*, **72**: 459-465.
- Bernardet, J-F., Nakagawa, Y. and Holmes, B. 2002. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and amended description of the family. *International Journal of Systematic and Evolutionary Microbiology*, **52**: 1049-1070.
- Bogas, A.C., Watanabe, M.A.E., Barbosa, A.V-B., Laurival, A., Bonatto, A.C., Dekker, R., Souza, E.M. and Fungaro, M.H.P. 2007. Structural characterization of the *bglH* gene encoding a β-glucosidase-like enzyme in an endophytic *Bacillus pumilis* strain. *Genetics and Molecular Biology*, **30**: 100-104.
- Bond, P.L., Hugenholtz, P., Keller, J. and Blackall, L.L. 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. *Applied and Environmental Microbiology*, **61**: 1910-1916.
- Boyd, A. and Chakrabarty, A.M. 1995. *Pseudomonas aeruginosa* biofilms: Role of the alginate exopolysaccharide. *Journal of Industrial Microbiology*, **15**: 162-168.
- Bramucci, M., Kane, H., Chen, M. and Nagarajan, V. 2003. Bacterial diversity in an industrial wastewater bioreactor. *Applied Microbiology and Biotechnology,* **65**: 594-600.
- Bramucci, M.G. and Nagarajan, V. 2000. Industrial wastewater bioreactors: Sources of novel microorganisms for biotechnology. *TIBTECH*, **18**: 501-505.
- Branco, R., Alpoim, M.C. and Morais, P.V. 2004. *Ochrobactrum tritici* strain 5bvl1 – characterization of a Cr(VI)-resistant and Cr(VI)-reducing strain. *Canadian Journal of Microbiology*, **50**: 679-703.
- Breccia, J.D., Siñeriz, F., Baigori, M.D., Castro, G.R. and Hatti-Kaul, R. 1998. Purification and characterization of a thermostable xylanase from *Bacillus amyloliquefaciens*. *Enzyme and Microbial Technology*, **22**: 42-49.
- Brenner, D.J., Krieg, N.R. and Staley, J.T. 2005. Bergey's Manual of Systematic Bacteriology. Second Edition, Volume Two, Part B. Springer, USA.

- Cammarota, M.C. and Sant'Anna, G.L. 1998. Metabolic blocking of exopolysaccharides synthesis effects on microbial adhesion and biofilm accumulation. *Biotechnology Letters*, **20**: 1-4.
- Campos, V.L., Moraga, R., Yánez, J., Zaror, C.A. and Mondaca, M.A. 2005. Chromate reduction by *Serratia marcescens* isolated from tannery effluent. *Bulletin of Environmental Contamination and Toxicology*, **75**: 400-406.
- Chapdelaine, P., Tremblay, R.R. and Dube, J.Y. 1978. *p*-nitrophenol-alpha-D-glucopyranoside as substrate for measurement of maltase activity in human semen. *Clinical Chemistry*, **24**: 208-211.
- Characklis, W.G. and Marshall, K.C. 1990. Biofilms. Wiley-Interscience, Canada.
- Chavagnat, F., Duez, C., Guinand, M., Potin, P., Barbeyron, T., Henrissat, B., Wallach, J. and Ghuysen, J-M. 1996. Cloning, sequencing and overexpression in *Escherichia coli* of the alginate lyase-encoding *aly* gene of *Pseudomonas alginovora*: Identification of three classes of alginate lyases. *Biochemical Journal*, **319**: 575-583.
- Cheggour, A., Fanuel, L., Duez, C., Joris, B., Bouillenne, F., Devreese, B., Van Driessche, G., Van Beeumen, J., Frère, J-M. and Goffin, C. 2000. The *dppA* gene of *Bacillus subtilis* encodes a new D-aminopeptidase. *Molecular Microbiology*, **38**: 504-513.
- Chen, J-A., Li, X., Li, J., Cao, J., Qiu, Z., Zhao, Q., Xu, C. and Shu, W. 2007. Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium* sp. strain CQ0110Y. *Applied Microbiology and Biotechnology*, **74**: 676-682
- Chung, Y.-C., Huang, C. and Tseng, C-P. 1997. Biotreatment of ammonia from air by an immobilized *Arthrobacter oxydans* CH8 biofilter. *Biotechnology Progress*, **13**: 794-798.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual Reviews in Microbiology*, **49**: 711-745.
- Danese, P.N., Pratt, L.A. and Kolter, R. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architechture. *Journal of Bacteriology*, **182**: 3593-3596.
- Denton, M. and Kerr, K.G. 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clinical Microbiology Reviews*, **11**: 57-80.
- Donlan, R. 2002. Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases*, **8**: 881-890.
- Filipkowska, Z. 2003. Sanitary and bacteriological aspects of sewage treatment. *Acta Microbiologica Polonica*, **52**: 57-66.
- Fjellbirkeland, A., Torsvik, V. and Øvreås, L. 2001. Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF* and 16S rDNA sequences. *Antonie van Leeuwenhoek*, **79**: 209-217.

- Flemming, H-C. 2002. Biofouling in water systems – cases, causes and countermeasures. *Applied Microbiology and Biotechnology*, **59**: 629-640.
- Flemming H-C., Percival, S.I. and Walker, J.T. 2002. Contamination potential of biofilms in water distribution systems. *Water Science and Technology: Water Supply*, **2**: 271-280.
- Fogarty, W.M., Kelly, C.T. and Kadam, S.K. 1985. Separation and characterization of an α-glucosidase and maltase from *Bacillus amyloliquefaciens*. *Canadian Journal of Microbiology*, **31**: 670-674.
- Funke, G., Falsen, E. and Barreau, C. 1995. Primary identification of *Microbacterium* spp. encountered in clinical specimens as CDC Coryneform group A-4 and A-5 bacteria. *Journal of Clinical Microbiology*, **33**: 188-192.
- Ghorbel, B., Sellami-Kamoun, A. and Nasri, M. 2003. Stability studies of protease from *Bacillus cereus* BG1. *Enzyme and Microbial Technology*, **32**: 513-518.
- Goris, J., Boon, N., Lebbe, L., Verstraete, W. and De Vos, P. 2003. Diversity of activated sludge bacteria receiving the 3-chloroaniline-degradative plasmid pC1gfp. *FEMS Microbiology Ecology*, **46**: 221-230.
- Hahn Berg, I.C., Kalfas, S., Malmsten, M. and Arnebrant, T. 2001. Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: Potential of proteases originating from *Euphausia superba* for plaque control. *European Journal of Oral Science*, **109**: 316-324.
- Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.
- Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*, **2**: 95-108.
- Halpern, M., Senderovich, Y. and Snir, S. 2007. *Rheinheimera chironomi* sp. nov., isolated from a chironomid (Diptera; Chironomidae) egg mass. *International Journal of Systematic and Evolutionary Microbiology*, **57**: 1872-1875.
- Harrigan, W.F. and McCance, M.E. 1966. Laboratory Methods in Microbiology. Academic Press, London.
- Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M. and Parsek, M.R. 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *Journal of Bacteriology*, **183**: 5395-5401.
- Huang, Q., Peng, Y., Li, X., Wang, H. and Zhang, Y. 2003. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Current Microbiology*, **46**: 169-173.
- Ishida, H., Ishida, Y., Kurosaka, Y., Otani, T., Sato, K. and Kobayashi, H. 1998. *In vitro* and *in vivo* activities of levofloxacin against biofilm-producing *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **42**: 1641-1645.

- Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F. and Romeo, T. 2005. Depolymerization of β-1,6-*N*-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology*, **187**: 382-387.
- Johansen, C., Falholt, P. and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology*, **63**: 3724-3728.
- Johnson, S., Croall, J., Power, J.S.F. and Armstrong, G.R. 1998. Fatal *Serratia marcescens* meningitis and myocarditis in a patient with an indwelling urinary catheter. *Journal of Clinical Pathology*, **51**: 789-790.
- Jones, D. and Keddie, R.M. 2006. The genus *Arthrobacter*. *Prokaryotes*, **3**: 945-960.
- Kämpfer, P., Dreyer, U., Neef, A., Dott, W. and Busse, H-J. 2003. *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *International Journal of Systematic and Evolutionary Microbiology*, **53**: 93-97.
- Kämpfer, P., Erhart, R., Beimfohr, C., Böhringer, J., Wagner, M. and Amann, R. 1996. Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus *in situ* identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microbial Ecology*, **32**: 101-121.
- Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H. and Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents and Chemotherapy*, **48**: 2633-2636.
- Kennedy, L. and Sutherland, I.W. 1994. Gellan lyases – novel polysaccharide lyases. *Microbiology*, **140**: 3007-3013.
- Khan, S.T., Horiba, Y., Yamamoto, M. and Hiraishi, A. 2002. Members of the family *Comamonadaceae* as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. *Applied and Environmental Microbiology*, **68**: 3206-3214.
- Kostenko, V., Ceri, H. and Martinuzzi, R.J. 2007. Increased tolerance of *Staphylococcus aureus* to vancomycin in viscous media. *FEMS Immunology and Medical Microbiology*, **51**: 277-288.
- Kotchoni, S.O. and Shonukan, O.O. 2002. Regulatory mutations affecting the synthesis of cellulase in *Bacillus pumilus*. *World Journal of Microbiology and Biotechnology*, **18**: 487-491.
- Kumar, C.G. and Anand, S.K. 1998. Significance of microbial biofilms in food industry: A review. *International Journal of Food Microbiology*, **42**: 9-27.
- Laffineur, K., Avesani, V., Cornu, G., Charlier, J., Janssens, M., Wauters, G. and Delmée, M. 2003. Bacteremia due to a novel *Microbacterium* species in a patient with leukemia and description of *Microbacterium paraoxydans* sp. nov. *Journal of Clinical Microbiology*, **41**: 2242-2246.

- LeChevalier, M.W., Lowry, C.D., Lee, R.G. and Gibbon, D.L. 1993. Examining the relationship between iron corrosion and the disinfection of biofilm bacteria. *Journal of American Water Works Association*, **85**:111-123.
- Leung, W.C., Wong, M-F., Chua, H., Lo, W., Yu, P.H.F. and Leung, C.K. 2000. Removal and recovery of heavy metals by bacteria isolated from activated sludge treating industrial effluents and municipal wastewater. *Water Science and Technology*, **41**: 233-240.
- Li, M.T., Hao, L.L., Sheng, L.X. and Xu, J.B. 2008. Identification and degradation characterization of hexachlorobutadiene-degrading strain *Serratia marcescens* HL1. *BioResource Technology*, **99**: 6878-6884.
- Lim, B-R., Hu, H-Y. and Fujie, K. 2002. Biological degradation and chemical oxidation characteristics of coke-oven wastewater. *Water, Air and Soil Pollution*, **146**: 23-33.
- Liu, Y., Zhang, T. and Fang, H.H.P. 2005. Microbial community analysis and performance of a phosphate-removing activated sludge. *BioResource Technology*, **96**: 1205-1214.
- Loiselle, M. and Anderson, K.W. 2003. The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling*, **19**: 77-85.
- Ludensky, M. 2003. Control and monitoring of biofilms in industrial applications. *International Biodeterioration and Biodegradation*, **51**: 255-263.
- Lyerly, D. and Kreger, A. 1979. Purification and characterization of a *Serratia marcescens* metalloprotease. *Infection and Immunity*, **24**: 411-421.
- Matsumoto, K., Maeda, H., Takata, K., Kamata, R. and Okamura, R. 1984. Purification and characterization of four proteases from a clinical isolate of *Serratia marcescens* kums 3958. *Journal of Bacteriology*, **157**: 225-232.
- Menendez-Vega, D., Gallego, J.L.R., Pelaez, A.I., de Dordoba, G.F., Moreno, J., Muñoz, D. and Sanchez, J. 2007. Engineered *in situ* bioremediation of soil and groundwater polluted with weathered hydrocarbons. *European Journal of Soil Biology*, **43**: 310-321.
- Meyer, B. 2003. Approaches to prevention, removal and killing of biofilms. *International Biodeterioration and Biodegradation*, **51**: 249-253.
- Molla, A., Matsumura, Y., Yamamoto, T., Okamura, R. and Maeda, H. 1987. Pathogenic capacity of proteases from *Serratia marcescens* and *Pseudomonas aeruginosa* and their suppression by chicken egg white ovomacroglobulin. *Infection and Immunity*, **55**: 2509-2517.
- Montpas, S., Samson, J., Langlois, E., Lei, J., Piché, Y. and Chênevert, R. 1997. Degradation of 2,4,6-trinitrotoluene by *Serratia marcescens*. *Biotechnology Letters*, **19**: 291-294.
- Morais, P.V., Francisco, R., Branco, R., Chung, A.P. and da Costa, M.S. 2004. *Leucobacter chromiireducens* sp. nov, and *Leucobacter aridicollis* sp. nov., two new species isolated from a chromium contaminated environment. *Systematic and Applied Microbiology*, **27**: 646-652.

- Norwood, D.E. and Gilmour, A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology*, **88**: 512-520.
- Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F. and SanJose, C. 2006. Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology*, **40**: 51-56.
- Ouoba, L.I.I., Diawara, B., Amoa-Awua, W.K., Traoré, A.S. and Møller, P.L. 2004. Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce Soumbala. *International Journal of Food Microbiology*, **90**: 197-205.
- Øvreås, L., Forney, L., Daae, F.L. and Torsvik, V. 1997. Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, **63**: 3367-3373.
- Ozdemir, D., Soypacaci, Z., Sahin, I., Bicik, Z. and Sencan, I. 2006. *Ochrobactrum anthropi* endocarditis and septic shock in a patient with no prosthetic valve or rheumatic heart disease: Case report and review of the literature. *Japanese Journal of Infectious Diseases*, **59**: 264-265.
- Ozdemir, G., Ozturk, T., Ceyhan, N., Isler, R. and Cosar, T. 2003. Heavy metal biosorption by biomass of *Ochrobactrum anthropi* producing exopolysaccharide in activated sludge. *BioResource Tachnology*, **90**: 71-74.
- Park, S.H., Kim, H.K. and Pack, M.Y. 1991. Characterization and structure of the cellulase gene of *Bacillus subtilis* BSE616. *Agricultural and Biological Chemistry*, **55**: 441-448.
- Priest, F.G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriological Reviews*, **41**: 711-753.
- Saeki, K., Ozaki, K., Kobayashi, T. and Ito, S. 2007. Detergent alkaline proteases: Enzymatic properties, genes, and crystal structures. *Journal of Biosciences and Bioengineering*, **103**: 501-508.
- Sambrook, J., Fritsch, E.F. and Maniates, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA.
- Schallmey, M., Singh, A. and Ward, O.P. 2004. Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, **50**:1-17.
- Quan, Z-X., Kim, K.K., Kim, M-K., Jin, L. and Lee, S.-T. 2007. *Chryseobacterium caeni* sp. nov., isolated from bioreactor sludge. *International Journal of Systematic and Evolutionary Microbiology*, **57**: 141-145.

- Sá-Pereira, P., Costa-Ferreira, M. and Aires-Barros, M.R. 2002. Enzymatic proterties of a neutral endo-1,3(4)-β-xylanase Xyl II from *Bacillus subtilis*. *Journal of Biotechnology*, **94**: 265-275.
- Schönert, S., Buder, T. and Dahl, M.K. 1998. Identification and enzymatic characterization of the maltose-inducible α-glucosidase MalL (sucrase-isomaltase-maltase) of *Bacillus subtilis*. *Journal of Bacteriology*, **180**: 2574-2578.
- Schulze, R., Spring, S., Amann, R., Huber, I., Ludwig, W., Schleifer, K.H. and Kämpfer, P. 1999. Genotypic diversity of *Acidovorax* strains isolated from activated sludge and description of *Acidovorax defluvii* sp. nov. *Systematics and Applied Microbiology*, **22**: 205-214.
- Selan, L., Berlutti, F., Passariello, C., Comodi-Ballanti, M.R. and Thaller, M.C. 1993. Proteolytic enzymes: A new treatment strategy for prosthetic infections? *Antimicrobial Agents and Chemotherapy*, **37**: 2618-2621.
- Sidat, M., Bux, F. and Kasan, H.C. 1999. Polyphosphate accumulation by bacteria isolated from activated sludge. *Water SA*, **25**: 175-180.
- Sihorkar, V. and Vyas, S.P. 2001. Biofilm consortia on biomedical and biological surfaces: Delivery and targeting strategies. *Pharmaceutical Research*, **18**: 1247-1254
- Sloma, A., Rufo, G.A., Theriault, K.A., Dwyer, M., Wilson, S.W. and Pero, J. 1991. Cloning and characterization of the gene for an additional extracellular serine protease of *Bacillus subtilis*. *Journal of Bacteriology*, **173**: 6889-6895.
- Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. 1986. Bergey's Manual of Systematic Bacteriology. Volume 2. Williams and Wilkins, USA.
- Song, Z., Edwards, S.R. and Burns, R.G. 2005. Biodegradation of naphthalene-2-sulfonic acid present in tannery wastewater by bacterial isolates *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC. *Biodegradation*, **16:** 237-252.
- Stark, W., Pauptit, R.A., Wilson, K.S. and Jansonius, J.N. 1992. The structure of neutral protease from *Bacillus cereus* at 0.2-nm resolution. *European Journal of Biochemistry*, **207**: 781-791.
- Stewart, P.S. and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet*, **358**: 135-138.
- Supaphol, S., Panichsakpatana, S., Trakulnaleamsai, S., Tungkananuruk, N., Roughjanajirapa, P. and O'Donnell, A.G. 2006. The selection of mixed microbial inocula in environmental biotechnology: Example using petroleum-contaminated tropical soils. *Journal of Microbiological Methods*, **65**: 432-441.
- Sutherland, I.W. 2001. Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology*, **147**: 3-9.

- Suzuki, Y., Aoki, R. and Hayashi, H. 1982. Assignment of a *p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing α-glucosidase of *Bacillus cereus* ATCC 7064 to an exo-oligo-1,6-glucosidase. *Biochimica et Biophysica Acta*, **704**: 476-483.
- Swings, J., Lambert, B., Kersters, K. and Holmes, B. 2006. The genera *Phyllobacterium* and *Ochrobactrum*. *Prokaryotes*, **5**: 747-750.
- Takkinen, K., Pettersson, R.F., Kalkkinen, N., Palve, I., Soderlund, H. and Kaariainen, L. 1983. Amino acid sequence of α-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. *Journal of Biological Chemistry*, **258**: 1007-1013.
- Teather, R.M. and Wood, P.J. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology*, **43**: 777-780.
- Ten, L.N., Im, W-T., Kim, M-K., Kang, M.S. and Lee, S-T. 2004. Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *Journal of Microbiological Methods*, **56**: 375-382.
- Thayer, D.W. 1978. Carboxymethylcellulase produced by facultative bacteria from the hind-gut of the termite *Reticulitermes Hesperus*. *Journal of General Microbiology*, **106**: 13-18.
- Theron, J. and Cloete, T.E. 2000. Molecular techniques for determining microbial diversity and community structure in natural environments. *Critical Reviews in Microbiology*, **26**: 37-57.
- Tran, L., Wu, X.C. and Wong, S.L. 1991. Cloning and expression of a novel protease gene encoding an extracellular neutral protease from *Bacillus subtilis*. *Journal of Bacteriology*, **173**: 6364-6372.
- Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiology Letters*, **223**: 287-292.
- Van Ogtrop, M.L., van Zoeren-Grobben, D., Verbakel-Salomons, E.M.A. and van Boven, C.P.A. 1997. *Serratia marcescens* infections in neonatal departments: Description of an outbreak and review of the literature. *Journal of Hospital Infections*, **36**: 95-103.
- Vasantha, N., Thompson, L.D., Rhodes, C., Banner, C., Nagle, J. and Filpula, D. 1984. Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. *Journal of Bacteriology*, **159**: 811-819.
- Vignolo, G.M., Suriani, F., de Ruiz Holgado, A.P. and Oliver, G. 1993. Antibacterial activity of *Lactobacillus* strains isolated from dry fermented sausages. *Journal of Applied Bacteriology*, **75**: 344-349.
- Wagner, M., Amann, R., Lemmer, H. and Schleifer, K-H. 1993. Probing activated sludge with oligonucleotides specific for Proteobacteria: Inadequacy of culture-dependent methods

for describing microbial community structure. *Applied and Environmental Microbiology*, **59**: 1520-1525.

- Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K-H. 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. *Applied and Environmental Microbiology*, **60**, 792-800.
- Walker, S.L., Fourgialakis, M., Cerezo, B. and Livens, S. 2007. Removal of microbial biofilms from dispense equipment: The effect of enzymatic pre-digestion and detergent treatment. *Journal of the Institute of Brewing*, **113**: 61-66.
- Wauters, G., Charlier, J., Janssens, M. and Delmée, M. 2000. Identification of *Arthrobacter oxydans*, *Arthrobacter luteolus* sp. nov., and *Arthrobacter albus* sp. nov., isolated from human clinical specimens. *Journal of Clinical Microbiology*, **38**: 2412-2415.
- Watnick, P. and Kolter, R. 2000. Biofilm, city of microbes. *Journal of Bacteriology*, **182**: 2675-2679.
- White, D.C., Arrage, A.A., Nivens, D.E., Palmer, R.J., Rice, J.F. and Sayler, G.S. 1996. Biofilm ecology: On-line methods bring new insights into MIC and microbial biofouling. *Biofouling*, **10**: 1-3
- Wingender, J., Nue, T.R. and Flemming, H-C. 1999. Microbial extracellular polymeric substances: Characterization, structure and function. Springer, Germany.
- Yang, M.Y., Ferrari, E. and Henner, D.J. 1984. Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an *in vitro*-derived deletion mutation. *Journal of Bacteriology*, **160**: 15-21.
- Yang, H-C., Im, W-T., Kang, M.S., Shin, D-Y. and Lee, S-T. 2006. *Stenotrophomonas koreensis* sp. nov., isolated from compost in South Korea. *International Journal of Systematic and Evolutionary Microbiology*, **56**: 81-84.
- Yasuhira, K., Tanaka, Y., Shibata, H., Kawashima, Y., Ohara, A., Kato, D., Takeo, M. and Negoro, S. 2007. 6-Aminohexanoate oligomer hydrolases from the alkalophilic bacteria *Agromyces* sp. strain KY5R and *Kocuria* sp. strain KY2. *Applied and Environmental Microbiology*, **73**: 7099-7102.
- Yoon, J-H., Kang, S-J. and Oh, T-K. 2007. *Chryseobacterium daeguense* sp. nov., isolated from wastewater of a textile dye works. *International Journal of Systematic and Evolutionary Microbiology*, **57**: 1355-1359.
- You, S.J., Tseng, D.H., Liu, C.C., Ou, S.H. and Chien, H.M. 2006. The performance and microbial diversity of a membrane bioreactor treating real textile dyeing wastewater. *Water Practice and Technology*, **1**: 1-8.

- Zhao, J-S. and Ward, O.P. 1999. Microbial degradation of nitrobenzene and mono-nitrophenol by bacteria enriched from municipal activated sludge. *Canadian Journal of Microbiology*, **45**: 427-432.

CHAPTER 5

ENZYMATIC REMOVAL OF PSEUDOMONAD AND MULTI-SPECIES BIOFILMS

Abstract

The biofilm removal potential of proteases and different polysaccharide-degrading enzymes that are produced by bacterial strains previously isolated from a wastewater biofilm was evaluated. Incubation of crude extracellular enzyme extracts prepared from the respective bacterial strains with the EPS extracted from *Pseudomonas aeruginosa* PAO1, *P. putida* and *P. fluorescens* indicated that proteases produced by four of the strains were effective in degrading the EPS proteins of all three *Pseudomonas* spp., while all bacterial strains that produced polysaccharide-degrading enzymes were capable of degrading the EPS polysaccharides, albeit with different efficiencies. The experiments were subsequently extended to screen single and combined crude enzyme extracts for their ability to remove biofilms of the *Pseudomonas* spp. grown in 96-well microtiter plates. Efficient removal of *P. aeruginosa* PAO1 biofilms was only achieved when mixtures of enzyme extracts, containing protease and different types of polysaccharase activities, were used. Biofilms of *P. putida* and *P. fluorescens* were readily removed with single enzyme extracts prepared from *B. subtilis* and *B. pumilus*. Treatment of a multi-species biofilm, grown from dam water, with mixtures of enzyme extracts yielded lower biofilm removal efficiencies than those obtained for mono-species pseudomonad biofilms, possibly due to the heterogenous nature of the EPS.

5.1 INTRODUCTION

In natural and artificial habitats, most bacteria have a strong tendency to adhere to surfaces within microbial consortia called biofilms (Costerton *et al.*, 1995). When bacterial cells approach inert surfaces, they first bind to the substratum by relatively weak forces involving their surface appendages (flagella, fimbriae and/or capsular components) (O'Toole and Kolter, 1998; Vallet *et al.*, 2001; Finelli *et al.*, 2003). However, irreversible attachment of the cells to the substratum coincides with the production of extracellular polymeric substances (EPS), which forms a matrix embedding several layers of the cells, as the biofilm matures. The EPS, which can make up between 50-90% of the total organic matter of biofilms, comprises mainly of polysaccharides and proteins, but nucleic acids, lipids and humic substances have also been identified (Sutherland, 2001; Allison, 2003; Tsuneda *et al.*, 2003). The EPS not only determine the structural and functional integrity of microbial biofilms, but also contribute significantly to the organization of the biofilm community (Allison, 2003; Branda *et al.*, 2005). Furthermore, the EPS matrix acts as an anchor, securing the cells to a surface and preventing their detachment under flow conditions (Donlan and Costerton, 2002).

In industrial settings, unwanted biofilms are responsible for, amongst other, the fouling of water cooling towers, water pipes and membranes, while biofilms forming on indwelling medical devices are a leading cause of human infections (Melo and Bott, 1997; Ludensky, 2003; Hall-Stoodley *et al.*, 2004). There exist several methods to remove such unwanted biofilms, which include: mechanical cleaning, the use of antimicrobial agents, stopping biofilm growth by removal of nutrients, inhibition of microbial attachment to a surface and promotion of biofilm detachment (Xavier *et al.*, 2005). Of these, mechanical cleaning and antimicrobial agents are the most frequently used methods. However, mechanical cleaning can be expensive since systems need to be stopped and additional labour is required. Moreover, a fouled surface may be inaccessible for cleaning (Xavier *et al.*, 2005). In addition to the reduced susceptibility of biofilm bacteria to antimicrobial agents, many of these agents do not remove biofilms from surfaces and a clean surface may therefore not be obtained (Gibson *et al.*, 1999; Anderl *et al.*, 2000; Norwood and Gilmour, 2000; Grobe *et al.*, 2002; Kostenko *et al.*, 2007). In many industrial and medical settings, it may also not be possible to control the nutrients and therefore application of this method as a means to stop biofilm growth is limited (Xavier *et al.*, 2005). However, promoting detachment of unwanted biofilms by the use of substances that destroy the integrity of the biofilm matrix could represent a viable alternative strategy whereby the complete removal of biofilms from surfaces can be achieved.

Methods for promoting biofilm detachment have included the use of enzymes and chemical agents such as chelating agents, surfactants and urea (Turakhia *et al.*, 1983; Gordon *et al.*, 1991; Johansen *et al.*, 1997; Chen and Stewart, 2000; Kaplan *et al.*, 2004). Of these, enzymes have attracted considerable attention as good anti-biofilm agents due to their biodegradability, their weak toxicity, and they are readily available and can easily be produced at an industrial scale (Karam and Nicell, 1997; Schmid *et al.*, 2001). Special consideration has been given to proteases and polysaccharases since they can degrade the primary constituents of the biofilm EPS matrix, although nucleases have also been reported to prevent biofilm formation through the degradation of extracellular DNA (Whitchurch *et al.*, 2002). Due to the heterogeneity of the biofilm matrix and the complex composition of multi-species biofilm EPS (Sutherland, 2001; Allison, 2003), different combinations of enzymes are usually required to degrade these biofilms effectively (Meyer, 2003). In this regard, Pectinex Ultra SP, a multicomponent enzyme preparation containing protease activity and a wide range of polysaccharases, has been reported to remove biofilms from both Gram-positive and Gram-negative bacteria (Johansen *et al.*, 1997). More recently, Orgaz *et al.* (2007) similarly reported that a mixture of different proteases and polysaccharases removed biofilms from *Pseudomonas fluorescens* efficiently.

The objectives of this part of the study were to assess the effect of different protein- and polysaccharide-degrading enzyme preparations on the extracted EPS of three *Pseudomonas* species (*P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*), and to assess the susceptibility of mono-species and multi-species biofilms to these enzyme preparations. The three pseudomonads were specifically chosen for this study as they are of clinical and ecological importance (Fett *et al.*, 1989). These three bacterial strains have also frequently been used in previous studies to determine the efficacy of enzymes to remove biofilms (Johansen *et al.*, 1997; Böckelmann *et al.*, 2003; Orgaz *et al.*, 2006).

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and culturing conditions

Pseudomonas aeruginosa PAO1, *P. fluorescens* and *P. putida* were obtained from the culture collection of the University of Pretoria, Pretoria, South Africa. *P. aeruginosa* PAO1 was grown in Tryptone Soy Broth (Merck) at 37°C with shaking (200 rpm), while *P. fluorescens* and *P. putida* were grown in nutrient broth at 30°C with shaking (150 rpm). The cultures were maintained on Tryptone Soy Agar (Merck) and nutrient agar (Merck) at 4°C, or as glycerol cultures at -70°C. Bacterial strains, previously isolated from wastewater biofilm and shown to produce protease- and/or polysaccharide-degrading enzymes, are listed in Table 5.1, together with their enzymatic activities. Bacterial strains capable of producing proteases were grown for up to five days at 30°C with shaking (150 rpm) in 150 ml of nutrient broth supplemented with 0.25% (w/v) peptone (Parmar *et al.*, 2001). Bacterial strains capable of producing amylase, xylanase or cellulase were grown for up to five days at 30ºC with shaking (150 rpm) in 150 ml of nutrient broth supplemented with amylose (0.06% [w/v]), xylan from oats spelt (0.03% [w/v]) or hydroxyethyl-cellulose (0.07% [w/v]) (Ten *et al.*, 2004). Bacterial strains capable of producing α- and β-glucosidase were grown for up to five days at 30°C with shaking (150 rpm) in 150 ml of nutrient broth supplemented with 0.1% (w/v) starch (Ten *et al.*, 2005). All liquid cultures prepared in this study were standardized by making use of a McFarland standard 1 solution (corresponding to approximately 3×10^8 bacterial cells).

5.2.2 Preparation of crude enzyme extracts

Crude extracellular enzyme extracts were prepared from bacterial cultures by centrifugation of the cultures at 10 000 × *g* for 15 min at 4ºC. The cell-free culture supernatant was used as the crude enzyme source.

5.2.3 *In vitro* enzyme activity assays

5.2.3.1 Protease activity assay

Protease assays were performed using azocasein as substrate according to the method of Olajuyigbe and Ajele (2005) with a few modifications. The azocasein (Sigma-Aldrich) was dissolved in 5 mM Tris-HCl buffer (pH 7) to yield a final concentration of 0.5% (w/v). The protease activity assays were performed by incubating 100 µl of crude enzyme extract with 500 µl of the azocasein substrate. Following incubation at 30ºC for 1 h, the reaction was terminated by addition of 500 µl of an ice-cold 10% trichloroacetic acid (TCA) solution and incubating on ice for 15 min. After centrifugation at 500 × *g* for 15 min (4ºC), the supernatant (1 ml) was added to an equal volume of 1 M NaOH and the absorbance was measured at 440 nm against a reagent blank. One unit (U) of enzyme activity was defined as the amount of enzyme which results in an absorbance increase of 0.01 at 440 nm in 60 min at 30ºC (Secades and Guijarro, 1999). The assays were performed in triplicate.

5.2.3.2 Amylase, cellulase and xylanase activity assays

Amylase, xylanase and cellulase activities were determined by measuring the release of reducing sugars from amylose, oat spelt xylan and hydroxyethyl-cellulose, respectively. The respective enzyme activity assays were performed by incubating 500 µl the crude enzyme extract with 500 µl of amylose, xylan or hydroxyethyl-cellulose (1% [w/v] solution prepared in 0.05 M citrate-phosphate buffer; pH 6). Following incubation at 30ºC for 30 min with slow shaking (100 rpm), the release of reducing sugars from the substrates was measured by the

DNS method, as described below (Section 5.2.6). One unit (U) of amylase, xylanase and cellulase activity was defined as the amount of enzyme that produced reducing sugars corresponding to 1 µmol of glucose equivalents from amylase, xylan or hydroxyethyl-cellulose, respectively, under the assay conditions (Ten *et al.*, 2004). The assays were performed in triplicate.

5.2.3.3 α- and β-glucosidase activity assays

α- and β-glucosidase activity assays were performed according to the method of Arellano-Carbajal and Olmos-Soto (2002). One (1) ml of the crude enzyme extract was mixed with 5 ml of 0.01 M sodium phosphate buffer (pH 7) and 1 ml of a 1% (w/v) solution of *p*-nitrophenyl-α-D-glucopyranoside or *p*-nitrophenyl-β-D-glucopyranoside. Following incubation at 30°C for 30 min, the reaction was terminated by addition of 1 ml of 1 M Na_2CO_3 and then centrifuged at 1 300 × *g* for 5 min (4°C). The absorbance of the supernatant was measured at 405 nm against a reagent blank. One unit of α- and β-glucosidase activity (U) was defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol under the assay condition (Alcalde *et al.*, 1999). The assays were performed in triplicate.

5.2.4 Extraction of EPS from *Pseudomonas* spp.

EPS were extracted from cultures of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens* according to the method of Zhang *et al.* (1999). The cultures were homogenized by stirring thoroughly, after which a 10-ml aliquot of each culture was centrifuged at 6 000 × *g* for 5 min. The supernatants were recovered and stored for later use. The pellets were suspended in 10 ml of dH_2O and sonicated for 15 s on ice at an output of 50%. The supernatants were added to the sonicated pellets and the suspensions were centrifuged at 11 227 × *g* for 30 min (4ºC). The recovered supernatants were filtered through 0.2-µm syringe filters (Acrodisc® PF syringe filters, low protein binding) and the filtrates were used as the crude EPS sources. A 10-ml volume of each EPS extract was also freeze-dried and the dry weight of the EPS was determined. The protein concentration of each EPS extract was determined by the method of Bradford (1976) and with bovine serum albumin (BSA) as standard, while the carbohydrate concentration was determined using anthrone reagent with glucose as standard (Roe, 1954). All assays were performed in triplicate.

5.2.5 Enzymatic hydrolysis of proteins in the EPS of *Pseudomonas* spp.

5.2.5.1 Enzyme reactions

The crude enzyme extracts (1.5 ml), prepared from bacterial strains with protease activity, was mixed with 1.5 ml of the EPS extract from *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*. The reaction mixtures were incubated at 30ºC for 22 h with slow shaking (100 rpm). As a control, inactivated crude enzyme extracts (boiled for 15 min, followed by rapid cooling) were added to the EPS extracts. Following incubation, proteins were precipitated from the reaction mixture and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.5.2 Protein precipitation

Prior to SDS-PAGE, the proteins were concentrated from the reaction mixtures using the method of Wessel and Flugge (1984), which was adapted as follows for larger sample volumes. The samples (3 ml) were mixed with 12 ml of methanol in 50-ml Greiner tubes and then centrifuged at 9 000 × *g* for 2 min (4°C). After centrifugation, 3 ml of chloroform was added to each tube and briefly vortexed before centrifugation at 9 000 × *g* for 2 min (4°C). The pellets were rinsed with 9 ml of dH_2O by vigorous vortexing of the tubes, followed by brief centrifugation (9 000 × *g*, 30 s). The supernatants were carefully aspirated and the tubes were incubated at room temperature for 3 min to allow all traces of the chloroform to evaporate. Following incubation, 9 ml of methanol was added to the tubes and the samples were centrifuged at 9 000 × *g* for 4 min to collect the white protein pellet. The supernatants were discarded and the pellets were left to dry in a flow cabinet before being solubilized in 50 µl of 2 × sample buffer (0.125 M Tris-HCl, 4% [w/v] SDS, 20% [v/v] glycerol, 0.2 M DTT, 0.02% [w/v] bromophenol blue; pH 6.8).

5.2.5.3 SDS-PAGE

Protein samples were analyzed by electrophoresis in a discontinuous gel system, as described by Laemmli (1970), using a 4% stacking gel and 8% separating gel. Electrophoresis was performed in a BioRad Protean II electrophoresis unit for 30 min at 60 V and then for 12.5 h at 100 V in 1 × TGS buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS; pH 8.3). Following electrophoresis, the gels were stained for 30 min with 0.125% (w/v) Coomassie brilliant blue (prepared in 50% [v/v] methanol; 10% [w/v] acetic acid), and then destained in a solution containing 25% (v/v) methanol and 10% (v/v) glacial acetic acid until the proteins were visible. An unstained protein molecular weight marker (Fermentas, SM0431), covering the 14.4-116 kDa range, was included in each SDS-polyacrylamide gel.

5.2.6 Enzymatic hydrolysis of polysaccharides in the EPS of *Pseudomonas* spp.

EPS were extracted from *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*, as described above (Section 5.2.4), and incubated with crude enzyme extracts prepared from bacterial strains producing polysaccharide-degrading enzymes. For this purpose, 0.1 g of lyophilized EPS from the respective *Pseudomonas* spp. was dissolved in 5 ml of 0.05 M citrate-phosphate buffer (pH 6) and incubated with 10 ml of the crude enzyme extract. Following incubation at 30°C for 22 h, the reducing sugars was measured by the dinitrosalicyclic (DNS) colorimetric method, as described by Ghose (1987). The supernatant (1 ml) was added to 1 ml of the DNS reagent and the mixture was heated at 90°C for 15 min. Following addition of 335 µl of potassium sodium tartrate (40% [w/v]), the solution was cooled to room temperature and the absorbance was measured at 575 nm. Glucose was used to generate a standard curve. The absorbance values were subsequently converted to mg/ml reducing sugars released by making use of the glucose standard curve. The assays were performed in triplicate.

5.2.7 Enzymatic removal of biofilms of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*

Biofilm removal, following treatment with different crude enzyme extracts, was determined using a quantitative microtiter plate assay, as described by Stepanović *et al.* (2000) and Pitts *et al.* (2003). The wells of a 96-well polystyrene microtiter plate (Nunc) were filled with 200 µl of standardized cultures of the respective *Pseudomonas* spp. and the plates were incubated for 24 h at 37ºC (*P. aeruginosa* PAO1) or 30ºC (*P. putida* and *P. fluorescens*) with slow shaking (100 rpm). Following incubation, the cultures were aspirated and crude enzyme extracts were applied to the wells. When a single enzyme was tested, 300 µl of the enzyme extract was added to the well, when combinations of two enzymes were tested, 150 µl of each enzyme extract was added to the well, and when combinations of three enzymes were tested, 100 µl of each enzyme extract was added to the well. The plates were incubated at 30ºC for 30 min. Following incubation, the enzyme extract was aspirated and the wells were rinsed three times with sterile Ringer's solution. The plates were left to dry and the wells were stained with 300 µl of a 0.4% (w/v) solution of crystal violet (Sigma-Aldrich). Following incubation at room temperature for 15 min, the wells were rinsed three times with Ringer's solution to remove excess stain and then filled with 250 µl of acetic acid (33% [v/v]) prior to measuring the absorbance at 620 nm. Two controls were included on each plate. These comprised wells containing sterile broth only (to ensure that the crystal violet did not non-specifically adsorb to the microtiter surface) and wells containing bacterial biofilm treated with sterile broth (to verify that biofilm removal was due to added enzyme extracts). The assays were performed in triplicate. The percentage biofilm removal was calculated as follows: Percentage biofilm removal = [(A-B) – (C-B)] / (A-B) × 100, where A represents the average absorbance in the control well (untreated biofilm), B represents the average absorbance in the blank wells (no biofilm) and C represents the average of the test samples (treated biofilm) (Pitts *et al.*, 2003).

5.2.8 Enzymatic removal of a multi-species biofilm

To determine the efficacy of enzyme extracts on the removal of a multi-species biofilm, water was obtained from the Paul van Zyl dam, located on the sport grounds of the University of Pretoria. The water was supplemented with nutrient broth (16 g/l) in a ratio of 7:1 (water:broth) in order to supply the bacteria with nutrients. The wells of six-well plates (Nunc) were filled with 7 ml of the nutrient-supplemented water and the plates were incubated at 30°C for 48 h with slow shaking (50 rpm). Following incubation, the water was aspirated and the crude enzyme extracts were applied to the wells. When single enzymes were tested, 10 ml of the enzyme extract was added to the well, while for combinations of two and three enzymes, 5 ml and 3.3 ml, respectively, of each crude enzyme extract was added to the well. As controls, wells filled with sterile distilled water, and wells filled with nutrient-supplemented water and treated with sterile nutrient broth, were included in each plate. The plates were incubated at 30°C for 1 h. Following incubation, the enzyme extract was aspirated and the plates were processed as described above to determine the percentage biofilm removal. For these experiments, the wells were stained with 10 ml of the crystal violet solution and 10 ml of acetic acid was added to the wells prior to measuring the absorbency at 620 nm. The assays were performed in triplicate.

5.3 RESULTS AND DISCUSSION

5.3.1 Enzyme production by bacterial strains isolated from a wastewater biofilm

It was previously established with semi-quantitative plate screen assays that the bacterial strains isolated from a wastewater biofilm produced extracellular enzymes that may find application in biofilm removal. To quantify the production levels of the different enzymes, crude enzyme extracts were prepared daily over five days from cell-free culture supernatants, and assayed by *in vitro* enzyme activity assays with enzyme-specific substrates. The results of these assays are summarized in Table 5.1.

Of the different bacterial strains capable of producing proteases, the highest enzyme activity (1 324 U/ml) was produced by *S. marcescens* after two days of culture, followed by activities obtained for *B. subtilis* (984 U/ml) and *B. pumilus* (975 U/ml) after three and five days of culture, respectively. High enzyme activity (573 U/ml) was also measured for *B. amyloliquefaciens* after three days of culture. Maximal levels of protease activity was measured for *S. koreensis* (20 U/ml) and *C. indotheticum* (26 U/ml) after two days of culture, but was much lower when compared to the activity obtained for *R. chironomi* (123 U/ml) after five days of culture. Although both *B. cereus* strains were capable of producing proteases, their production levels were also low. Maximal enzyme activities of 52 U/ml for *B. cereus* strain 2 and 17 U/ml for *B. cereus* strain 5 was measured after three and one day of culture.

Compared to the protease activities, much lower levels of polysaccharide-degrading enzyme activities were measured. Of the six bacterial strains capable of producing amylase, *B. subtilis* produced the highest enzyme activity (3.894 U/ml) after two days of culture, followed by *B. amyloliquefaciens* (3.322 U/ml) and *B. cereus* strain 2 (3.199 U/ml) after three and two days of culture, respectively. Lower levels of amylase activity was measured for *R. chironomi* (0.223 U/ml), followed by *Bacillus cereus* strain 5 (0.136 U/ml) and *K. rosea* (0.063 U/ml) after culturing for two and three days, respectively. With regards to xylanase activity, the highest enzyme activity was obtained for *B. subtilis* (2.675 U/ml) after two days of culture, followed by activities measured for *B. pumilus* (1.356 U/ml), *B. amyloliquefaciens* (1.161 U/ml) and *K. rosea* (0.373 U/ml) after three days of culture. Only three *Bacillus* spp. were able to produce cellulase. Of these, *B. subtilis* produced the highest cellulase activity (0.314 U/ml) after two days of culture, while much lower cellulase activities was measured for *B. cereus* strain 5 (0.096 U/ml) and *B. pumilus* (0.029 U/ml) after four days of culture.

Table 5.1: Enzyme activities of bacterial strains isolated from a wastewater biofilm

Isolate	Name	Enzyme	Enzyme activity (U/ml) *				
			Day 1	Day 2	Day 3	Day 4	Day 5
1	*B. subtilis*	Amylase	0.508±0.011	3.894±0.007	3.702±0.057	2.521±0.040	1.843±0.005
		Cellulase	0.092±0.005	0.314±0.110	0.156±0.015	0.111±0.006	0.085±0.004
		Xylanase	1.294±0.020	2.675±0.318	1.359±0.012	0.754±0.005	0.516±0.006
		α-glucosidase	1.721±0.196	1.782±0.013	2.921±0.087	2.355±0.058	2.111±0.061
		Protease	226±4	671±3	984±4	660±7	630±2
2	*B. cereus*	Amylase	3.106±0.099	3.199±0.566	2.490±0.031	1.358±0.031	0.958±0.015
		α-glucosidase	1.843±0.081	4.191±0.048	17.391±0.255	28.403±0.210	34.475±0.322
		Protease	17±3.5	40±3	52±3	28±7	18±6
3	*R. chironomi*	Amylase	0.087±0.013	0.223±0.028	0.084±0.012	0.056±0.022	0.021±0.008
		Protease	4±3	2±0.577	18±0.577	38±3	123±2
4	*B. pumilus*	Cellulase	0±0	0.018±0.011	0.025±0.002	0.029±0.002	0.013±0.009
		Xylanase	0.158±0.011	0.260±0.010	1.356±0.028	1.209±0.042	0.654±0.023
		α-glucosidase	3.732±0.074	4.451±0.083	5.093±0.140	5.407±0.351	4.176±0.092
		β-glucosidase	5.086±0.134	4.497±0.069	6.072±0.312	5.300±0.105	3.984±0.093
		Protease	4±3	280±5	620±2	680±4	975±6
5	*B. cereus*	Amylase	0.096±0.010	0.114±0.011	0.136±0.034	0.040±0.005	0.003±0
		Cellulase	0±0	0.045±0.002	0.070±0.004	0.096±0.019	0.046±0.012
		α-glucosidase	1.514±0.046	3.602±0.100	7.823±0.265	4.680±0.121	2.745±0.187
		Protease	17.3±1.155	12.3±2.517	13.6±1.155	8.6±0.577	7.3±0.577
6	*B. amyloliquefaciens*	Amylase	0.643±0.144	2.684±0.075	3.322±0.154	2.814±0.076	2.033±0.012
		Xylanase	0.613±0.067	0.662±0.010	1.161±0.090	0.871±0.065	0.549±0.013
		α-glucosidase	1.277±0.066	1.392±0.058	1.744±0.040	1.973±0.040	1.950±0.023
		β-glucosidase	0.543±0.058	1.101±0.040	1.422±0.1000	1.805±0.096	1.828±0.096
		Protease	443±4	516±5	573±2	427±4	304±3.512
7	*M. oxydans*	α-glucosidase	2.355±0.070	4.351±0.253	7.204±0.331	10.003±0.080	8.114±0.230
		β-glucosidase	1.262±0.061	2.914±0.315	4.887±0.403	7.655±0.081	7.227±0.161
10	*A. oxydans*	α-glucosidase	1.698±0.096	2.264±0.096	2.562±0.035	3.273±0.058	2.768±0.048
14	*S. marcescens*	Protease	816.6±14.012	1324±39.509	1145.6±17.786	960±44.800	832.3±29.670
15	*C. indotheticum*	α-glucosidase	1.254±0.048	1.690±0.081	2.455±0.293	2.409±0.069	2.478±0.061
		Protease	11±2.646	26±1	10.3±0.577	8.3±1.155	6.6±1.528
16	*S. koreensis*	Protease	7.3±0.577	20±3	16±3	15.6±3.512	13.3±0.577
21	*K. rosea*	Amylase	0.036±0.008	0.024±0.010	0.063±0.047	0.051±0.012	0.022±0.012
		Xylanase	0±0	0.036±0.006	0.373±0.056	0.316±0.055	0.196±0.003

*Crude enzymes were extracted from each strain when the combination of the enzymes produced by each strain was at its highest activity. Enzymes were extracted from strains 1, 2, 14, 15 and 16 after 2 days of culturing, strains 3, 5, 6 and 21 after 3 days of culturing and from strains 4, 7 and 10 after 4 days of culturing

Several of the bacterial strains were capable of producing α- and/or β-glucosidase. *M. oxydans* (7.655 U/ml) and *B. pumilus* (6.072 U/ml) produced high levels of β-glucosidase activity after culturing for four and three days, respectively, while 1.828 U/ml enzyme activity was measured for *B. amyloliquefaciens* after five days of culture. The highest α-glucosidase activity (34.475 U/ml) was obtained for *B. cereus* strain 2 after five days of culture, followed by activities obtained for *M. oxydans* (10.003 U/ml), *B. cereus* strain 5 (7.823 U/ml) and *B. pumilus* (5.407 U/ml) after culturing for three to four days. Lower levels of α-glucosidase activity were measured for *B. subtilis* (2.921 U/ml) and *C. indotheticum* (2.478 U/ml) after three days of culture, as well as for *A. oxydans* (3.273 U/ml) and *B. amyloliquefaciens* (1.973 U/ml) after four days of culture.

5.3.2 Characterization of the EPS extracted from different *Pseudomonas* spp.

Prior to assessing the ability of the proteases and polysaccharases to degrade EPS of different *Pseudomonas* spp., the EPS composition of the respective *Pseudomonas* spp. was characterized. The EPS were extracted from liquid bacterial cultures, since sufficient amounts of EPS for analyses could not be obtained from biofilms. It has previously been reported that extracellular polymers present in biofilms EPS show high similarity to those present in the EPS of planktonic cultures, although they are sometimes present in higher amounts (Sutherland, 2001). Consequently, *P. aeruginosa* PAO1 was cultured in Tryptone Soy Broth, while *P. putida* and *P. fluorescens* were both cultured in nutrient broth. The cultures were grown for five days and the EPS was extracted daily from each bacterial culture, and then characterized with regards to dry weight, as well as protein and carbohydrate contents.

Results obtained for *P. aeruginosa* PAO1 indicated that the EPS contained a higher concentration of carbohydrates compared to proteins (Fig. 5.1a). The highest protein content was observed in the EPS of cultures grown for two days, while similar high concentrations of carbohydrates were present in the EPS of cultures grown for one to four days. In contrast, the EPS of both *P. putida* (Fig. 5.1b) and *P. fluorescens* (Fig. 5.1c) contained low concentrations of carbohydrates and high concentrations of proteins. For both these bacterial cultures, the highest protein content was observed in the EPS of cultures grown for one day, while a similar high concentration of carbohydrates was observed in the EPS of cultures grown for three and four days. The results obtained in this part of the study are in agreement with several other reports indicating that extracellular proteins dominate the biofilm EPS of both

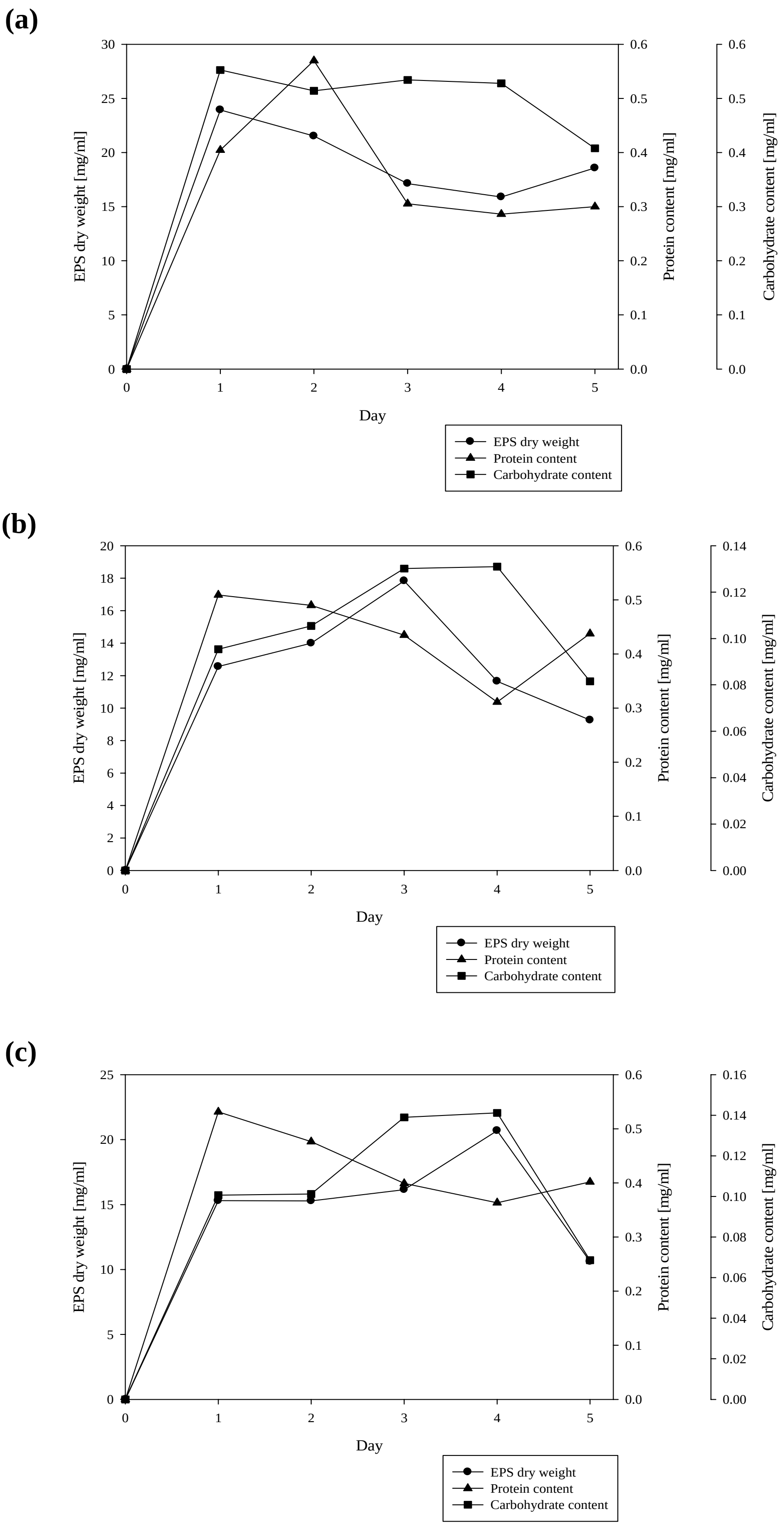


Figure 5.1. Dry weight, as well as protein and carbohydrate contents of EPS extracted from *P. aeruginosa* PAO1 (a), *P. putida* (b) and *P. fluorescens* (c) over a 5-day incubation period. Values are the means of results of triplicate experiments.

P. putida (Jahn *et al*., 2000; Kachlany *et al*., 2001; Priester *et al*., 2006) and *P. fluorescens* (Pereira and Vieira, 2001), while the EPS of *P. aeruginosa* PAO1 biofilms contain a higher concentration of polysaccharides than proteins (Steinberger and Holden, 2004).

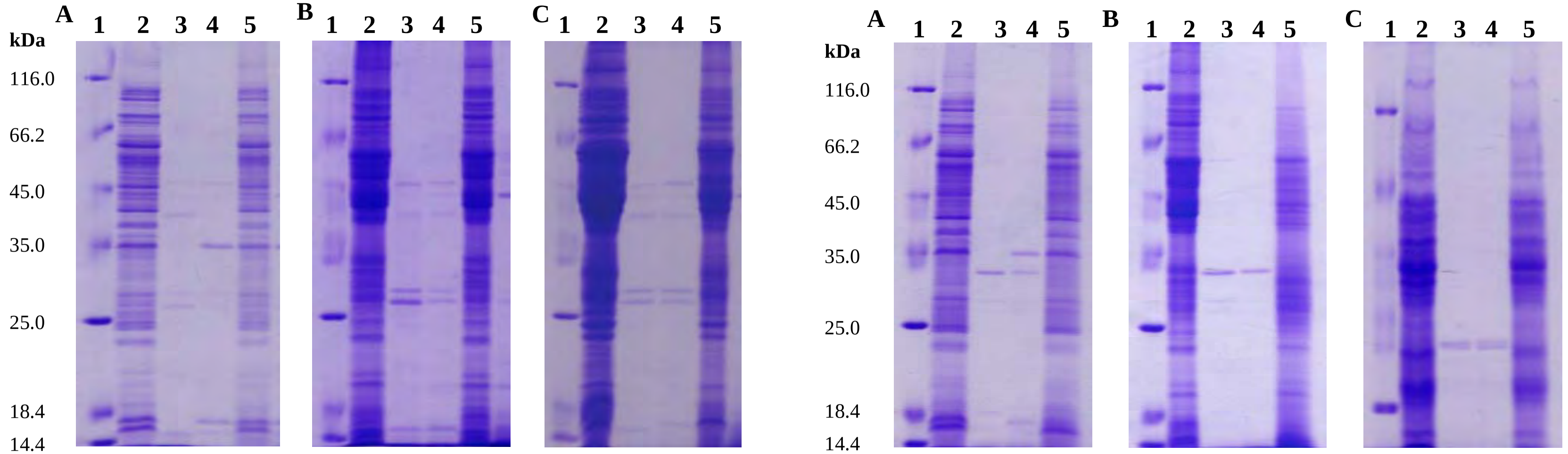
5.3.3 Enzymatic degradation of the EPS of different *Pseudomonas* spp.

5.3.3.1 Protein hydrolysis

To investigate whether the proteases produced by the bacterial strains isolated from a wastewater biofilm was capable of degrading proteins present in the EPS of different *Pseudomonas* spp., the EPS was extracted from liquid cultures of *P. aeruginosa* PAO1 grown for two days, and of *P. putida* and *P. fluorescens* that had been grown for three days. Crude enzyme extracts, prepared from the cell-free culture supernatants of protease-producing bacterial strains when their production levels were at their highest (Table 5.1), were subsequently incubated for 22 h with the extracted EPS. Following incubation, the proteins were precipitated from the reaction mixtures and analyzed by SDS-PAGE in order to determine the extent of EPS protein hydrolysis.

Despite having identified nine bacterial strains with an associated protease activity, not all of the extracellular proteases were capable of degrading the EPS proteins of the respective *Pseudomonas* spp. Extracellular proteases produced by *B. cereus* strains 2 and 5, *R. chironomi*, *C. indotheticum* and *S. marcescens* did not show any visible degradation of EPS proteins from any of the *Pseudomonas* spp. (results not shown). However, proteases produced by *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens* and *S. koreensis* degraded the EPS proteins of the different *Pseudomonas* spp. efficiently (Fig. 5.2). These results suggest that the proteases produced by the respective bacterial strains possess a broad substrate specificity or, alternatively, that proteins degraded by these proteases were present in the EPS of all three *Pseudomonas* spp. In all cases the EPS proteins of *P. putida* and *P. fluorescens* were completely degraded by the proteases, while only a single polypeptide of approximately 35 kDa in the EPS extract of *P. aeruginosa* PAO1 appeared to be recalcitrant to degradation. In contrast, incubation of the inactivated enzyme extracts with the respective EPS extracts did not result in degradation of the proteins. Based on the presence of multiple protein bands precipitated from the crude enzyme extracts of the different protease-producing bacterial strains, it appeared likely that the bacterial strains, excepting *B. pumilus*, produced several different proteases. Whether each of the observed proteins in fact represents distinct protease

B. subtilis **extracellular proteases** ***B. pumilus*** **extracellular proteases**



B. amyloliquefaciens **extracellular proteases** ***S. koreensis*** **extracellular proteases**

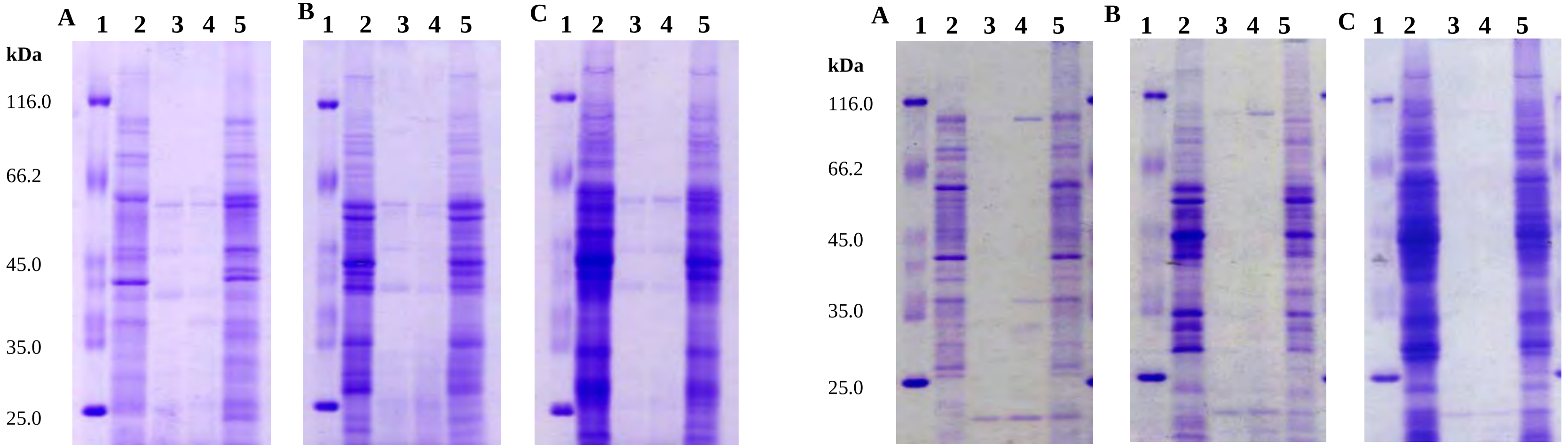


Figure 5.2. SDS-PAGE analyses of precipitated proteins, following incubation of EPS extracted from *P. aeruginosa* PAO1 (A), *P. putida* (B) and *P. fluorescens* (C), with crude enzyme extracts prepared from the bacterial strains as indicated in the figure. Lanes: 1, Protein Molecular Weight Marker; 2, Untreated EPS extract; 3, Proteins precipitated from cell-free culture supernatants of protease-producing bacterial strains; 4, Proteins precipitated from the reaction mixture following enzymatic treatment of the EPS extract for 22 h; 5, Proteins precipitated from the reaction mixture following treatment of the EPS extract with a heat-inactivated crude enzyme extract for 22 h.

species or whether some of these may represent unprocessed forms of other proteases must, however, await further analysis and primary amino acid sequence comparisons.

5.3.3.2 Polysaccharide hydrolysis

Using an approach similar to that described above, the effect of crude enzyme extracts, prepared from polysaccharase-producing bacterial strains, on the amount of reducing sugar released from the EPS of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens* was subsequently examined. All of the polysaccharase-containing enzyme extracts were capable of degrading the polysaccharides present in the EPS extract of the respective *Pseudomonas* spp., albeit with different efficiencies (Fig. 5.3).

The results indicated that the amount of reducing sugar increased by more than 50% and 45%, respectively, when the EPS extracts of all three *Pseudomonas* spp. were treated with crude enzyme extracts prepared from *B. subtilis* (amylase, xylanase, cellulase and α-glucosidase activities) and *B. pumilus* (xylanase, cellulase, α- and β-glucosidase activities). Indeed, treatment of the EPS extracts of *P. aeruginosa* PAO1 and *P. putida* with an enzyme extract prepared from *B. subtilis* resulted in the highest increase in the amount of reducing sugars (53.12 and 62.31%, respectively) compared to the use of other enzyme extracts. For *P. fluorescens*, the highest increase in the amount of reducing sugar (74.12%) was obtained when the EPS extract was treated with an enzyme extract prepared from *B. cereus* strain 5 (amylase, cellulase and α-glucosidase activities). Notably, the amount of reducing sugar increased by 59.78% when the EPS extract of *P. putida* was also treated with this crude enzyme extract, albeit much more inefficient against the EPS extract of *P. aeruginosa* PAO1 (24.14%). The presence of cellulase activity in this enzyme extract, as also observed for enzyme extracts derived from *B. subtilis* and *B. pumilus,* suggests that it may play an important role in degrading the EPS of especially *P. putida* and *P. fluorescens* (Fig. 5.3). This is also in accordance with the presence of cellulose in the EPS of these two *Pseudomonas* spp. (Table 5.2).

Other differences in the efficacy of crude enzyme extracts to degrade the EPS polysaccharides of the respective *Pseudomonas* spp. were also noted. Crude enzyme extracts prepared from *R. chironomi* (amylase activity), *B. amyloliquefaciens* (amylase, xylanase, α- and β-glucosidase activities), *M. oxydans* (α- and β-glucosidase activities) and *A. oxydans* (α-glucosidase activity) were able to degrade the EPS polysaccharides of both *P. putida* and *P. fluorescens* more efficiently compared to that of *P. aeruginosa* PAO1. In contrast, a crude enzyme extract from *K. rosea* (amylase and xylanase activities) showed a higher release of reducing sugars from the EPS of *P. aeruginosa* PAO1 than from the other two *Pseudomonas* spp. Crude enzyme extracts prepared from *B. cereus* strain 2 (amylase and α-glucosidase activities) and *C. indotheticum* (α-glucosidase activity) were the most inefficient at degrading the EPS polysaccharides of the respective *Pseudomonas* spp. (Fig. 5.3). The differences in EPS polysaccharide hydrolysis, as highlighted above, may reflect on differences in the EPS composition of the *Pseudomonas* spp. (Table 5.2).

5.3.4 Enzymatic removal of different *Pseudomonas* spp. biofilms

The capability of selected crude enzyme extracts, singularly and in combination, to remove biofilms of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens* from the surface of microtiter plates was subsequently tested. In contrast to the use of viable plate count techniques, the quantitative microtiter plate screening method adopted in this study is not only rapid and more readily suited to the screening of a large number of anti-biofilm agents, but it also allows for measurement of the removal of biofilms independently of the killing efficiency. Being able to measure biofilm removal independently of killing is attractive, since it represents an environmentally friendly approach that obviates the requirement for inherently toxic disinfectants and biofilm removal is aimed directly at obtaining a clean (biofilm-free) surface, which is the ultimate objective of a cleaning agent (Pitts *et al.*, 2003).

Based on results obtained in the preceding sections, bacterial strains producing proteases, which were capable of degrading the EPS proteins of the three *Pseudomonas* spp., were included in the biofilm removal assays. Moreover, based on differences in the concentration of polysaccharides compared to proteins in the EPS of the *Pseudomonas* spp., bacterial strains that produced polysaccharide-degrading enzymes, resulting in increases of at least 20% (for *P. aeruginosa* PAO1) and 50% (for both *P. putida* and *P. fluorescens*) in the amount of reducing sugar released from the EPS, were also included in the biofilm removal assays.

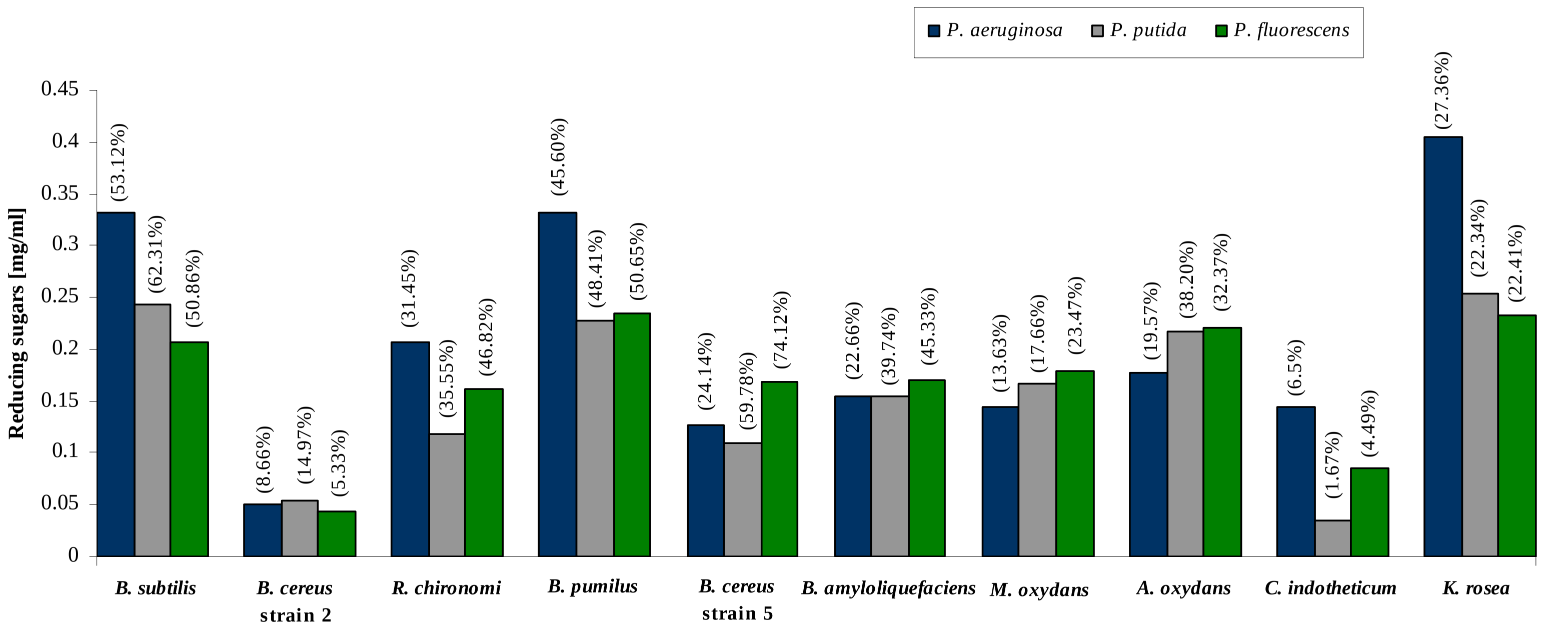


Figure 5.3. Reducing sugars released from the extracted EPS of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*, following incubation for 22 h with crude enzyme extracts prepared from different bacterial strains. Values are the means of results of triplicate experiments.

Table 5.2. Exopolysaccharides produced by *P. aeruginosa* PAO1, *P. fluorescens* and *P. putida*

***Pseudomonas* species**	**Exopolysaccharides produced**	**Composition**	**Reference**
P. aeruginosa PAO1	▪ Alginate (acidic)	▪ Guluronate, mannuronate	▪ Schürks *et al.*, 2002
	▪ -	▪ Glucose, rhamnose, mannose, xylose, ketodeoxyoctulosonate (KDO), *N*-acetyl galactosamine, *N*-acetyl fucosamine, *N*-acetyl glucosamine	▪ Wozniak *et al.*, 2003
P. fluorescens	▪ Alginate (acidic)[a]	▪ Guluronate, mannuronate	▪ Fett *et al.*, 1989
	▪ Marginalan (acidic)[a]	▪ Pyruvate, succinate, glucose, galactose	▪ Fett *et al.*, 1989
	▪ Levan (neutral)[b]	▪ -	▪ Fett *et al.*, 1989
	▪ Novel EPS (acidic)[a]	▪ Pyruvate, acetate, rhamnose, mannose, glucose	▪ Fett *et al.*, 1989
	▪ -	▪ D-glucose, 2-acetamido-2-deoxy-D-gluocose, 4-*O*-acetyl-2-acetamido-2-deoxy-D-mannuronic acid	▪ Osman *et al.*, 1997
	▪ Cellulose	▪ -	▪ Spiers and Rainey, 2005
	▪ -	▪ Neutral monosaccharides: Rhamnose, fucoes, ribose, arabinose, xylose, mannose, galactose, glucose Acidic groups: carboxylic acid	▪ Hung *et al.*, 2005
P. putida	▪ Alginate (acidic)[a]	▪ Guluronate, mannuronate	▪ Fett *et al.*, 1989
	▪ Marginalan (acidic)[a]	▪ Pyruvate, succinate/ glucose, galactose	▪ Fett *et al.*, 1989
	▪ -	▪ Glucose, *N*-acetylgalactosamine, rhamnose, glucuronic acid, ribose	▪ Kachlany *et al.*, 2001
	▪ Cellulose	▪ -	▪ Camesano and Abu-Lail, 2002; Chang and Halverson, 2003

[a] Only one acidic exopolysaccharide can be produced by a single *P. fluorescens* and *P. putida* strain

[b] Levan was only produced in strains that produced either alginate or the novel acidic EPS

5.3.4.1 *P. aeruginosa* PAO1 biofilm removal

Treatment of *P. aeruginosa* PAO1 biofilms with crude enzyme extracts prepared from bacterial strains that produced proteases capable of degrading EPS proteins (*B. subtilis*, *B. pumilus*, *B. amyloliquefaciens* and *S. koreensis*) resulted in a biofilm removal efficiency of 63-70% (Fig. 5.4). In contrast to the *Bacillus* spp., *S. koreensis* did not produce any of the polysaccharide-degrading enzymes that were tested for in this study. Nevertheless, treatment of *P. aeruginosa* PAO1 biofilms with a crude enzyme extract from *S. koreensis* resulted in 67% biofilm removal efficiency, suggesting that the proteases produced by this strain may be particularly useful.

Results obtained for crude enzyme extracts, prepared from bacterial strains unable to degrade *P. aeruginosa* PAO1 EPS proteins but producing enzymes capable of degrading the EPS polysaccharides, indicated that enzyme extracts from *R. chironomi* and *B. cereus* strain 5 were less efficient in removing the *P. aeruginosa* PAO1 biofilms (42% in both instances) compared to that from *K. rosea* (82%). When crude enzyme extracts of either *R. chironomi*, *B. cereus* strain 5 or *K. rosea* were combined with that of *S. koreensis*, the biofilm removal efficiencies were much lower compared to treatment with the single enzyme extracts only. This may be ascribed to the degradation of enzymes present in the extracts by the proteases produced by *S. koreensis*. Similar observations have been noted by Johansen *et al.* (1997), who demonstrated that some enzyme combinations do not work optimally together due to the inhibition of one enzyme by another. Moreover, Orgaz *et al.* (2006) reported that the relative inefficiency of a combination of proteases and polysaccharases to remove *P. fluorescens* biofilms was due to the degradation of the other enzymes by the proteinases.

Analysis of the results obtained when mixtures of different crude enzyme extracts were used to remove the *P. aeruginosa* PAO1 biofilms, indicated that maximal biofilm removal efficiency was obtained when a crude enzyme extract of *B. subtilis* was combined with enzyme extracts derived from either *B. pumilus* (89%) or *B. cereus* strain 5 (90%). When mixtures of three different enzyme extracts were tested, the most efficient biofilm removal was obtained with a mixture comprising enzyme extracts of *B. pumilus*, *B. amyloliquefaciens* and *K. rosea* (98%). Significant biofilm removal was also obtained with a mixture of enzyme extracts of *B. pumilus*, *S. koreensis* and *K. rosea* (90%), and of *B. cereus* strain 5, *B. amyloliquefaciens* and *S. koreensis* (89%).

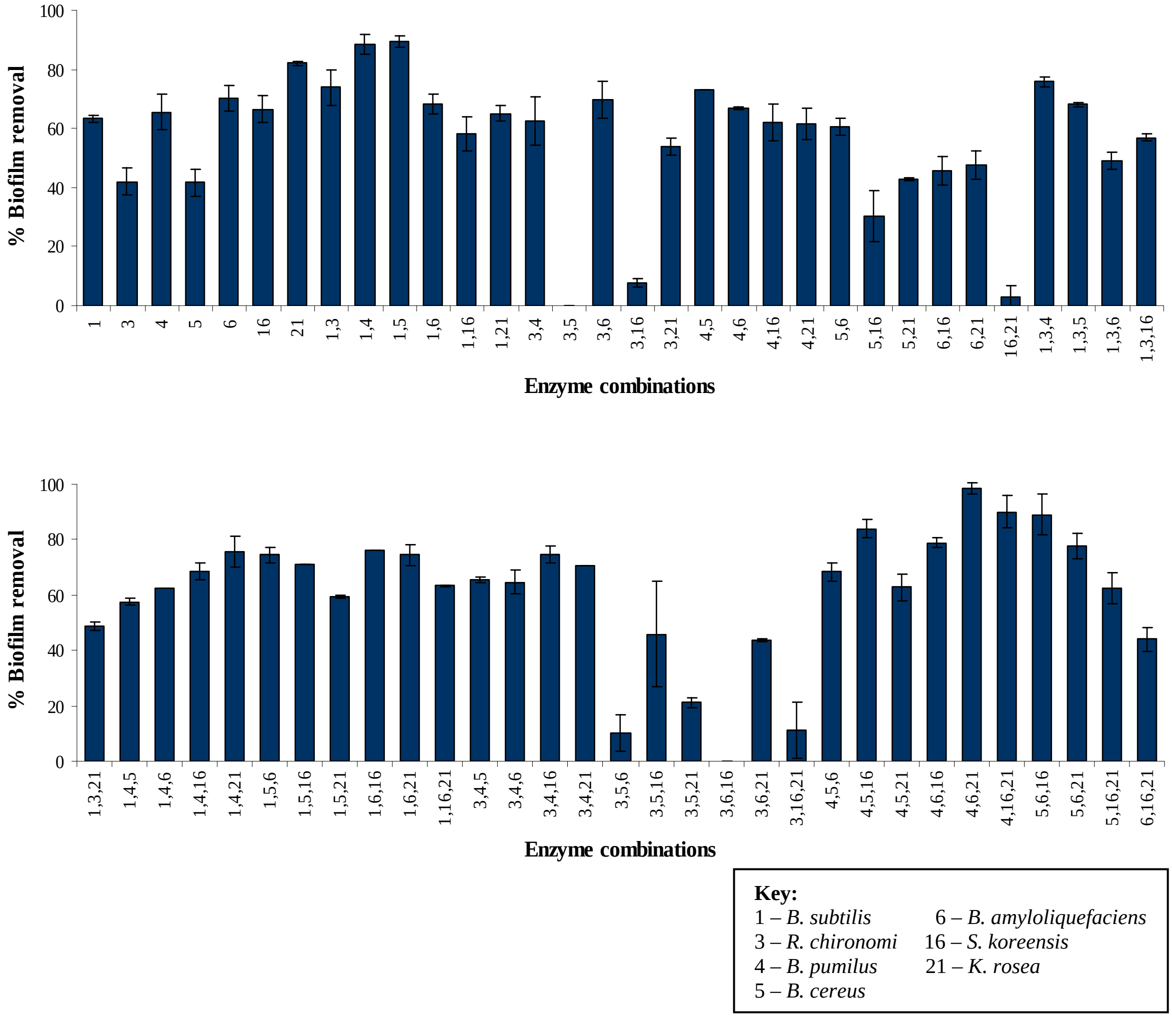


Figure 5.4. Removal of *P. aeruginosa* PAO1 biofilm by treatment with enzyme extracts containing protease and/or polysaccharase enzyme activity. Biofilm removal was assayed by crystal violet staining of biofilm remaining in the wells of microtiter plates, following treatment with individual or combinations of crude enzyme extracts. In the figure, bars represent the mean values and error bars represent the standard deviations of three replicates of each treatment.

5.3.4.2 *P. putida* biofilm removal

Treatment of *P. putida* biofilms with crude enzyme extracts prepared from bacterial strains producing proteases capable of degrading the EPS proteins, indicated that proteases produced by *B. subtilis* and *B. pumilus* were more efficient (95-98%) than enzyme extracts from *B. amyloliquefaciens* and *S. koreensis* (55-56%) in removing the attached biofilms. In contrast to the *Bacillus* spp., *S. koreensis* displayed none of the polysaccharide-degrading enzyme activities tested for in this study. All three *Bacillus* spp. produced polysaccharide-degrading enzymes, but *B. amyloliquefaciens*, in contrast to *B. subtilis* and *B. pumilus*, did not show detectable cellulase activity. These results therefore suggest that both proteases and polysaccharases, particularly cellulases, are required for efficient removal of *P. putida* biofilms. In this regard, not only does the genomic sequence of *P. putida* KT2440 contain a putative operon for cellulose biosynthesis (Chang and Halverson, 2003), but exopolysaccharides produced by *P. putida* KT2442 have been reported to comprise of cellulose-like polymers (Camesano and Abu-Lail, 2002). Based on the high biofilm removal efficiency of crude enzyme extracts prepared from *B. subtilis* and *B. pumilus*, it is therefore not surprising that mixtures of enzyme extracts that included either of the two afore-mentioned enzyme extracts were very efficient in removing *P. putida* biofilms. In all of these cases the biofilm removal efficiency was in excess of 88%.

Treatment of *P. putida* biofilms with a crude enzyme extract prepared from *B. cereus* strain 5, which displayed appreciable polysaccharase activity but did not produce proteases capable of degrading the *P. putida* EPS proteins, yielded a biofilm removal efficiency of 33% (Fig. 5.5). In accordance with their low biofilm removal efficiency, mixtures of crude enzyme extracts derived from *B. cereus* strain 5 and *B. amyloliquefaciens* and/or *S. koreensis* were comparatively inefficient in removing *P. putida* biofilms (38-59%).

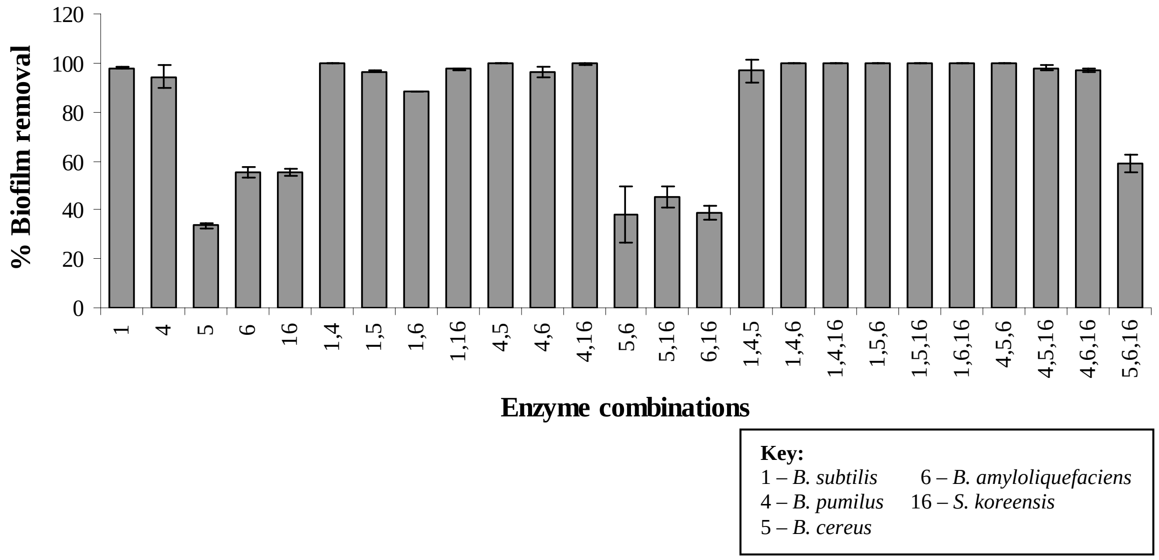


Figure 5.5. Removal of *P. putida* biofilm by treatment with enzyme extracts containing protease and/or polysaccharase enzyme activity. Biofilm removal was assayed as described in the legend to Fig. 5.4. In the figure, bars represent the mean values and error bars represent the standard deviations of three replicates of each treatment.

5.3.4.3 *P. fluorescens* biofilm removal

Similar to the results obtained for *P. putida*, crude enzyme extracts prepared from *B. subtilis* and *B. pumilus* were the most efficient in removing *P. fluorescens* biofilms, yielding biofilm removal efficiencies of 100%. Crude enzyme extracts prepared from *B. amyloliquefaciens* and *S. koreensis* was less efficient and yielded biofilm removal efficiencies of 68 and 60%, respectively (Fig. 5.6). Since all of these bacterial strains produced proteases that were capable of degrading *P. fluorescens* EPS proteins, these results support the use of a protease to remove biofilms of this bacterium. A notable difference in the polysaccharide-degrading enzyme activities of these bacterial strains is, however, the lack of detectable cellulase activity in the crude enzyme extracts of *B. amyloliquefaciens* and *S. koreensis*. These results suggest that cellulase activity, in addition to protease activity, is also important for removal of *P. fluorescens* biofilms. In this regard, it has previously been reported that cellulose in the EPS of *P. fluorescens* wrinkly spreader biofilms is involved in the development, strengthening and integrity of the *P. fluorescens* biofilms (Spiers *et al.*, 2003; Spiers and Rianey, 2005) The importance of using both a protease and cellulase is furthermore supported by the observation that *B. cereus* strain 5, which does not produce proteases capable of degrading the EPS proteins of *P. fluorescens* but was the most proficient at degrading the EPS polysaccharides, was comparatively inefficient in removing the biofilm (25%), despite the presence of cellulase activity (Fig. 5.6). Excepting for a mixture of crude enzyme extracts derived from *B. cereus* strain 5 and *B. amyloliquefaciens*, as well as combinations of these respective enzyme extracts with that of *S. koreensis*, all other mixtures of the enzyme extracts yielded near 100% biofilm removal efficiency.

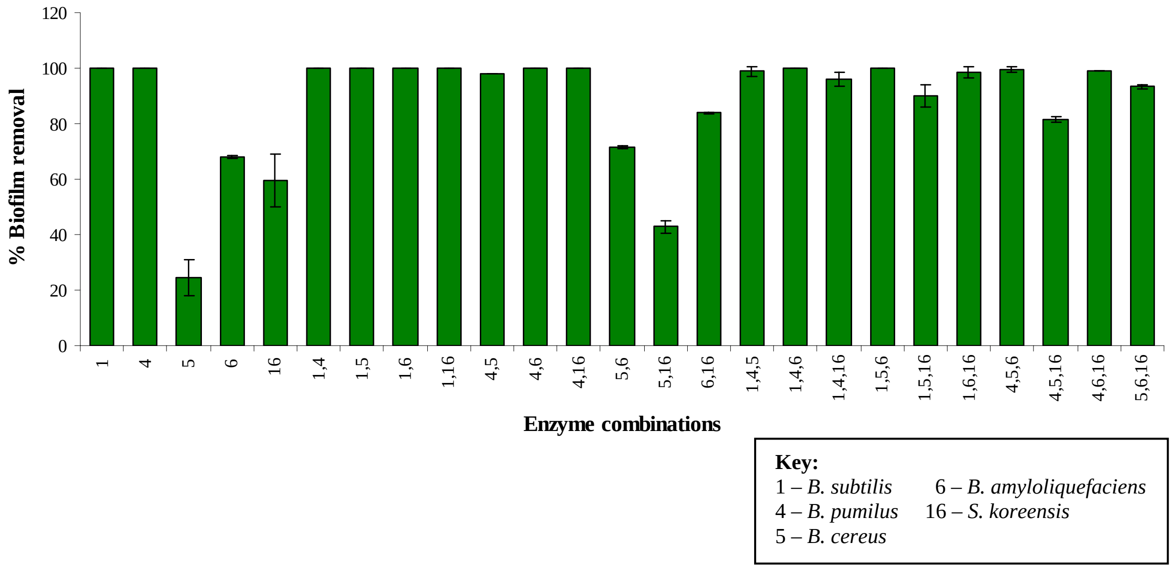


Figure 5.6. Removal of *P. fluorescens* biofilm by treatment with enzyme extracts containing protease and/or polysaccharase enzyme activity. Biofilm removal was assayed as described in the legend to Fig. 5.4. In the figure, bars represent the mean values and error bars represent the standard deviations of three replicates of each treatment.

5.3.5 Enzymatic removal of a multi-species biofilm

Having established that crude enzyme extracts of selected wastewater biofilm bacteria were capable of removing the biofilms of different *Pseudomonas* spp., it was next investigated whether these enzyme extracts could also be used to remove multi-species biofilms. For this purpose, multi-species biofilms were grown in six-well plates from dam water. Based on the heterogeneity and complexity of the EPS of multi-species biofilms (Sutherland, 2001; Allison, 2003), it has been recommended that mixtures of enzymes with different activities be used in order to degrade a broader range of substrates (Meyer, 2003). Consequently, in this part of the study, only mixtures of the enzyme extracts previously shown to remove mono-species pseudomonad biofilms were used.

Of the 29 different mixtures of enzyme extracts tested, only 11 yielded biofilm removal efficiencies in excess of 50%. The highest biofilm removal efficiencies were obtained with enzyme extracts of *B. subtilis* and *B. amyloliquefaciens* combined with that of either *K. rosea* (66%) or *S. koreensis* (62%), as well as with enzyme extracts of *B. cereus* strain 5 and *S. koreensis* combined with that of either *B. pumilus* (61%) or *B. subtilis* (60%). Although the EPS composition of the multi-species biofilm was not determined, the results nevertheless suggest that the EPS comprised of constituents that served as substrates for one or more of the different polysaccharases produced by the *Bacillus* spp. and *K. rosea*, in addition to the proteases produced by the *Bacillus* spp. and *S. koreensis* (Table 5.1).

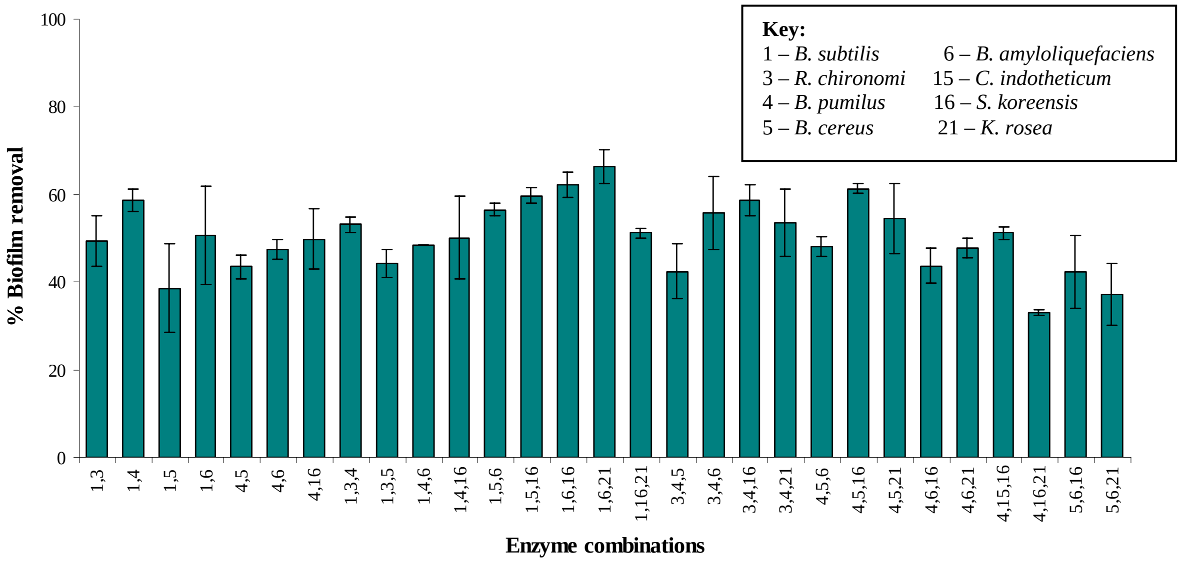


Figure 5.7. Removal of multi-species biofilm grown from dam water by treatment with combinations of enzyme extracts containing protease and/or polysaccharase enzyme activity. In the figure, bars represent the mean values and error bars represent the standard deviations of three replicates of each treatment.

5.4 CONCLUSIONS

Interest in the removal or degradation of bacterial biofilms has attracted great interest due to the problems caused by biofilm formation in industrial and medical settings (Melo and Bott, 1997; Hall-Stoodley *et al.*, 2004). One method that has been proposed for control of biofilms is using enzymes to degrade bacterial EPS, which promote interaction with the surface (Donlan and Costerton, 2002; Branda *et al.*, 2005). Results obtained in this study indicated that crude enzyme extracts containing protease and/or polysaccharase activity could remove adhered biofilms of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*. The polysaccharide-degrading enzymes that showed biofilm removal potential, included xylanase, amylase, cellulase, as well α- and β-glucosidases. These enzymes were capable of hydrolyzing the EPS of the respective *Pseudomonas* spp., as was evidenced by an increase in production of reducing sugar when the EPS was exposed to the crude enzyme extracts. The effectiveness of the polysaccharases may be due to the presence of various different sugar molecules, *e.g.*, glucose and xylose, in the EPS of the different *Pseudomonas* spp. (Frett *et al.*, 1989; Wozniak *et al.*, 2003; Hung *et al.*, 2005). Moreover, cellulase (produced by *B. subtilis*, *B. pumilus* and *B. cereus* strain 5), in combination with proteases, may be particularly useful as a biofilm detachment-promoting agent since the EPS of *P. putida* (Chang and Halverson, 2003) and *P. fluorescens* (Spiers and Rainey, 2005) contains cellulose, while cellulase has also been reported to inhibit biofilm formation by *P. aeruginosa* (Loiselle and Anderson, 2003).

The greater heterogeneity of multi-species biofilm matrix has been reported to limit the potential of monocompound enzymes and consequently, the use of enzyme mixtures containing different enzyme activities has been recommended for their elimination (Johansen *et al.*, 1997; Hahn Berg *et al.*, 2001; Walker *et al.*, 2007). In this study, lower biofilm removal efficiency was obtained for the multi-species biofilms compared to the mono-species pseudomonad biofilms, despite having used enzyme extracts effective in removing the different *Pseudomonas* spp. biofilms. Nevertheless, it may be possible that the enzymes identified in this study could be used in combination with other treatments to increase the biofilm removal effectiveness or in combination with other enzymes to degrade the mixture of proteins and polysaccharides present in the EPS of multi-species biofilms. Based on the presence of nucleic acids and lipids in the EPS of biofilm bacteria (Sutherland, 2001; Tsuneda *et al.*, 2003), other enzymes such as nucleases and lipases can also be considered for use since previous studies have shown that DNaseI (Whitchurch *et al.*, 2002) and lipase (Böckelmann *et al.*, 2003) can assist in biofilm removal.

Note should, however, be taken that crude enzyme extracts were used in this study to remove the mono- and multi-species biofilms. Therefore, the possibility of biofilm removal not being actually (or at least not singularly) caused by the enzyme activities assayed for in the study can not be excluded. Consequently, future studies should aim to purify the individual proteases and polysaccharide-degrading enzymes, and the purified enzymes should then be tested individually or in combination for their biofilm removal capability, using a strategy similar to that described in this study. In addition to measuring their biofilm removal efficacy, viable cell counts could also be determined since it would be desirable if these enzymes have bactericidal activity in order to prevent re-growth of the biofilms and/or colonization of the surface by planktonic bacteria (Johansen *et al.*, 1997).

5.5 REFERENCES

- Alcalde, M., Plou, F.J., Andersen, C., Martin, M.T., Pedersen, S. and Ballesteros, A. 1999. Chemical modification of lysine side chains of cyclodextrin glycosyltransferase from *Thermoanaerobacter* causes a shift from cyclodextrin glycosyltransferase to α-amylase specificity. *FEBS Letters*, **445**: 333-337.
- Allison, D.G. 2003. The biofilm matrix. *Biofouling*, **19**: 139-150.
- Anderl, J.N., Franklin, M.J. and Stewart, P.S. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, **44**: 1818-1824.
- Arellano-Carbajal, F. and Olmos-Soto, J. 2002. Thermostable α-1,4- and α-1,6-glucosidase enzymes from *Bacillus* sp. isolated from a marine environment. *World Journal of Microbiology and Biotechnology*, **18**: 791-795.
- Böckelmann, U., Szewzyk, U. and Grohmann, E. 2003. A new enzymatic method for the detachment of particle-associated soil bacteria. *Journal of Microbiological Methods*, **55**: 201-211.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**: 248-254.
- Branda, S.S., Vik, A., Friedman, L. and Kolter, R. 2005. Biofilms: the matrix revisited. *Trends in Microbiology*, **13**: 20-26.

- Camesano, T.A. and Abu-Lail, N.I. 2002. Heterogeneity in bacterial surface polysaccharides, probed on a singe-molecule basis. *Biomacromolecules*, **3**: 661-667.
- Chang, W-S. and Halverson, L.J. 2003. Reduced water availability influences the dynamics, development, and ultrastructural properties of *Pseudomonas putida* biofilms. *Journal of Bacteriology*, **185**: 6199-6204.
- Chen, X. and Stewart, P.S. 2000. Biofilm removal caused by chemical treatments. *Water Research*, **34**: 4229-4233.
- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. *Annual Reviews in Microbiology*, **49**: 711-745.
- Donlan, R.M. and Costerton, J.W. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, **15**: 167-193.
- Fett, W.F., Osman, S.F. and Dunn, M.F. 1989. Characterization of exopolysaccharides produced by plant-associated fluorescent Pseudomonads. *Applied and Environmental Microbiology*, **55**: 579-583.
- Finelli, A., Gallant, C.V., Jarvi, K. and Burrows, L.L. 2003. Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology*, **185**: 2700-2710.
- Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*, **59**: 257-268.
- Gibson, H., Taylor, J.H., Hall, K.E. and Holah, J.T. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology*, **87**: 41-48.
- Gordon, C.A., Hodges, N.A. and Marriott, C. 1991. Use of slime dispersants to promote antibiotic penetration through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **35**: 1258-1260.
- Grobe, K., Zahler, J. and Stewart, P. 2002. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. *Journal of Industrial Microbiology and Biotechnology*, **29**: 10-15.
- Hahn Berg, I.C., Kalfas, S., Malmsten, M. and Arnebrant, T. 2001. Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: Potential of proteases originating from *Euphausia superba* for plaque control. *European Journal of Oral Science*, **109**: 316-324.
- Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology*, **2**: 95-108.
- Hames, B.D. and Rickwood, D. 1990. Gel electrophoresis of proteins: A practical approach. Second Edition. Oxford University Press, New York. pp. 245.

- Hung, C-C., Santschi, P.H. and Gillow, J.B. 2005. Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* biovar II. *Carbohydrate Polymers*, **61**: 141-147.
- Jahn, A., Griebe, T. and Nielsen, P.H. 2000. Composition of *Pseudomonas putida* biofilms: Accumulation of protein in the biofilm matrix. *Biofouling* **14**: 49–57.
- Johansen, C., Falholt, P. and Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. *Applied and Environmental Microbiology*, **63**: 3724-3728.
- Kachlany, S.C., Levery, S.B., Kim, J.S., Reuhs, B.L., Lion, L.W. and Ghiorse, W.C. 2001. Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. *Environmental Microbiology*, **3**: 774-784.
- Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H. and Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrobial Agents and Chemotherapy*, **48**: 2633-2636.
- Karam, J. and Nicell, J.A. 1997. Potential application of enzymes in waste treatment. *Journal of Chemical Technology and Biotechnology*, **69**: 141-153.
- Kostenko, V., Ceri, H. and Martinuzzi, R.J. 2007. Increased tolerance of *Staphylococcus aureus* to vancomycin in viscous media. *FEMS Immunology and Medical Microbiology*, **51**: 277-288.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.
- Loiselle, M. and Anderson, K.W. 2003. The use of cellulase in inhibiting biofilm formation from organisms commonly found on medical implants. *Biofouling*, **19**: 77-85.
- Ludensky, M. 2003. Control and monitoring of biofilms in industrial applications. *International Biodeterioration and Biodegradation*, **51**: 255-263.
- Melo, L.F. and Bott, T.R. 1997. Biofouling in water systems. *Experimental Thermal and Fluid Science*, **14**: 375-381.
- Meyer, B. 2003. Approaches to prevention, removal and killing of biofilms. *International Biodeterioration and Biodegradation*, **51**: 249-253.
- Norwood, D.E. and Gilmour, A. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multi-species biofilm. *Journal of Applied Microbiology*, **88**: 512-520.
- O’Toole, G.A. and Kolter, R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology*, **30**: 295-304.
- Olajuyigbe, F.M. and Ajele, J.O. 2005. Production dynamics of extracellular protease from *Bacillus* species. *African Journal of Biotechnology*, **4**: 776-779.

- Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F. and SanJose, C. 2006. Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology*, **40**: 51-56.
- Orgaz, B., Neufeld, R.J. and SanJose, C. 2007. Single-step biofilm removal with delayed release encapsulated Pronase mixed with soluble enzymes. *Enzyme and Microbial Technology*, **40**: 1045-1051.
- Osman, S.F., Fett, W.F., Irwin, P., Cescutti, P., Brouillette, J.N. and Connor, J.V. 1997. The structure of the exopolysaccharide of *Pseudomonas fluorescens* strain H13. *Carbohydrate Research*, **300**: 323-327.
- Parmar, N., Singh, A. and Ward, O.P. 2001. Enzyme treatment to reduce solids and improve settling of sewage sludge. *Journal of Industrial Microbiology and Biotechnology*, **26**: 383-386.
- Pereira, M.O. and Vieira, M.J. 2001. Effects of the interactions between glutaraldehyde and the polymeric matrix on the efficacy of the biocide against *Pseudomonas fluorescens* biofilms. *Biofouling*, **17**: 93-101.
- Pitts, B., Hamilton, M.A., Zelver, N. and Stewart, P.S. 2003. A microtiter-plate screening method for biofilm disinfection and removal. *Journal of Microbiological Methods*, **54**: 269-276.
- Priester, J.H., Olson, S.G., Webb, S.M., Neu, M.P., Hersman, L.E. and Holden, P.A. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Applied and Environmental Microbiology*, **72**: 1988-1996.
- Roe, J.H. 1954. The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological Chemistry*, **208**: 889-896.
- Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. 2001. Industrial biocatalysis today and tomorrow. *Nature*, **409**: 258-268.
- Schürks, N., Wingender, J., Flemming, H-C. and Mayer, C. 2002. Monomer composition and sequence of alginates from *Pseudomonas aeruginosa. International Journal of Biological Macromolecules*, **30**: 105-111.
- Secades, P. and Guijarro, J.A. 1999. Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Applied and Environmental Microbiology*, **65**: 3969-3975.
- Spiers, A.J. and Rainey, P.B. 2005. The *Pseudomonas fluorescens* SBW25 wrinkly spreader biofilm required attachment factor, cellulose fibre and LPS interactions to maintain strength and integrity. *Microbiology*, **151**: 2829-2839.
- Spiers, A.J., Bohannon, J., Ghrig, S.M. and Rainey, P.B. 2003. Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. *Molecular Microbiology*, **50**: 15-27.

- Steinberger, R.E. and Holden, P.A. 2004. Macromolecular composition of unsaturated *Pseudomonas aeruginosa* biofilms with time and carbon source. *Biofilms*, **1**: 37-47.
- Stepanović, S., Vuković, D., Dakić, I., Savić, B. and Švabić-Vlahovi, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, **40**: 175-179.
- Sutherland, I.W. 2001. The biofilm matrix – an immobilized but dynamic microbial environment. *Trends in Microbiology*, **9**: 222-227.
- Ten, L.N., Im, W-T., Kim, M-K. and Lee, S-T. 2005. A plate assay for simultaneous screening of polysaccharide- and protein-degrading microorganisms. *Letters in Applied Microbiology*, **40**: 92-98.
- Ten, L.N., Im, W-T., Kim, M-K., Kang, M.S. and Lee, S-T. 2004. Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *Journal of Microbiological Methods*, **56**: 375-382.
- Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiology Letters*, **223**: 287-292.
- Turakhia, M.H., Cooksey, K.E. and Characklis, W.G. 1983. Influence of a calcium-specific chelant on biofilm removal. *Applied and Environmental Microbiology*, **46**: 1236-1238.
- Vallet, I., Olson, J.W., Lory, S., Lazdunski, A. and Filloux, A. 2001. The chaperone/usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *Proceedings of the National Academy of Sciences USA*, **98**: 6911-6916.
- Walker, S.L., Fourgialakis, M., Cerezo, B. and Livens, S. 2007. Removal of microbial biofilms from dispense equipment: The effect of enzymatic pre-digestion and detergent treatment. *Journal of the Institute of Brewing*, **113**: 61-66.
- Wessel, D. and Flugge, U.I. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry*, **138**: 141-143.
- Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and Mattick, J.S. 2002. Extracellular DNA required for bacterial biofilm formation. *Science*, **295**: 1487-1487.
- Wozniak, D.J., Wyckoff, T.J.O., Starkey, M., Keyser, R., Azadi, P., O'Toole, G.A. and Parsek, M.R. 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proceedings of the National Academy of Sciences USA*, **100**: 7907-7912.
- Xavier, J.B., Picioreanu, C., Rani, S.A., van Loosdrecht, M.C.M. and Stewart, P.S. 2005. Biofilm-control strategies based on enzymatic disruption of the extracellular polymeric substance matrix – a modelling study. *Microbiology*, **151**: 3817-3832.

- Zhang, X., Bishop, P.L. and Kinkle, B.K. 1999. Comparison of extraction methods for quantifying extracellular polymers in biofilms. *Water Science and Technology*, **39**: 211-218.

CHAPTER 6

GENERAL CONCLUSIONS

- Wastewater biofilms serve as a habitat for diverse bacteria that encompass different enzyme activities.
- Crude enzyme extracts from wastewater biofilms were capable of degrading proteins and polysaccharides present in the EPS of *P. aeruginosa* PAO1, and were capable of removing biofilms of this bacterium from a glass surface. This could be ascribed to presence of a wide variety of enzymes produced by the wastewater biofilms.
- Proteases and polysaccharases produced by several bacterial strains isolated from the wastewater biofilms showed degradation of proteins and polysaccharides present in the EPS of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*.
- Crude enzyme extracts from individual bacterial strains and mixtures of enzyme extracts from up to three different bacterial strains were capable of removing biofilms of *P. aeruginosa* PAO1, *P. putida* and *P. fluorescens*. The presence of protein- and polysaccharide-degrading enzymes in the crude enzyme extracts indicates that both groups of enzymes are required for detachment of the biofilms.
- Multi-species biofilms are more difficult to remove than single-species biofilms, possibly due to the complex nature of their EPS.