Chapter 1

Introduction

1.1 Microbial biofilms

Biofilms are described as surface associated bacterial communities forming micro colonies surrounded by a matrix of exopolymers (Izano *et al.,* 2007). Microorganisms in biofilms display some particular features that are not shared with the same microorganisms in suspended form. In biofilms, the cells are embedded in a polymer matrix of their own origin that mainly consists of polysaccharides and proteins (Flemming, 1998; Decho, 2000). Biofilms contain mixed populations of bacteria, fungi, protozoa and if conditions allow, they can host even higher organisms in the food chain such as nematodes and larvae (Decho, 2000). All bacteria within a biofilm live together and depend on other microorganisms for energy, carbon and other nutrients (Prakash *et al.,* 2003).

The extracellular matrix contributes to the mechanical stability of the biofilms enabling them to withstand shear forces (Morikawa *et al.,* 2003). Biofilm formation occurs in response to a variety of environmental triggers including high cell density, nutrient deprivation and physical environmental stress (Li *et al.,* 2003). Biofilms are common form of microbial ecosystems associated with surfaces and they are found in an extremely varied environment, from pure water systems to stream beds. In response to varying environmental conditions, biofilms develop different structures expressed in various morphologies (Hermanowicz, 2001).

The EPS matrix is important both in the formation and structure of the biofilm and also protects the cells by preventing the access of the antimicrobial and xenobiotics to the cells in the biofilm and confers protection against environmental stresses such as UV radiation, pH shift, osmotic shock and desiccation (de Carvalho, 2007).

The primary stage for biofilm formation is the attachment of bacteria to a surface followed by proliferation of attached cells which leads to the accumulation of multilayer clusters of cells and glycocalix formation (EPS) (Shakeri *et al.,* 2007). Biofilms are composed of bacteria, extracellular polymeric substances (EPS) of microbial origin and other particulate substances. EPSs are composed of diverse substances including polysaccharides, proteins, nucleic acids (Morikawa *et al.,* 2003), lipids and humic substances (Xavier *et al.,* 2005).

Biofilm formation involves the cell to cell communication quorum sensing (QS) systems. QS is a cell density dependent mechanism through which bacteria coordinate different activities including bioluminescence, plasmid conjugation and the production of different virulence factors (Schaber *et al.,* 2007). *Pseudomonas aeruginosa* is one of the bacteria that possess at least two well defined interrelated QS systems, the *las* and *rhl* which control the production of virulence factors. Each QS system consists of two components, the auto inducer synthases *LasI* and *RhII* and their cognate transcriptional regulators *LasR* and *RhIR*. *LasI* is the synthase for the auto inducer N-3-oxododecanoyl homoserine lactone $3OC_{12}$-HSL, while *RhII* synthesizes the auto inducer N-butyryl homoserine lactone C_4-HSL (Schaber *et al.,* 2007).

Another characteristic of biofims is their heterogeneity, for example, aerobic microorganisms in aerobic system consume oxygen resulting in anaerobic zone within the biofilm (Flemming, 1998). This process provides habitats for anaerobes that could not proliferate under aerobic conditions. A biofilm is mainly composed of water (80-90%), extracellular polymer substances (EPS) that contribute 85-98% of the organic matter, the microorganisms, entrapped organic and inorganic particles, substances sorbed to EPS, cells or particles and substances dissolved in the interstitial water (Flemming, 1998).

Biofilm formation has serious implications in industry, the environmental, public health and medicine due to increased resistance to antibiotics and UV light and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production (Meyer, 2003; Bourne *et al.,* 2006; Giaouris *et al.,* 2006).

Biofilms cause fouling of industrial equipment such as heat exchangers, cooling towers resulting in efficient heat transfer, energy loss, increased fluid frictional resistance and accelerated corrosion and also deteriorates the quality of various chemicals and process additives (Xiong and Liu, 2010). For example, in the paper industry, biofilms cause deterioration of chemicals like starch and calcium carbonate slurries which are added to the pulp slurries in the wet end processes (US Patent 7052614, 2006). In addition, biofilm formation in water distribution systems decreases water quality and increases health risks (Dewanti *et al.,* 1995; Rao *et al.,* 1998, US Patent 7052614, 2006). In the food industry, the occurrence of biofilms in food processing environments can cause post processing contamination leading to lower shelf life of products and transmission of diseases (Poulsen, 1999). Biofilms do not only present a hygiene risk in food industries but also cause economical losses due to technical failures (Meyer, 2003; Giaouris *et al.,* 2006).

Several methods have been proposed to prevent and destroy biofilms including (1) mechanical removal such as scrabing, sonication, freezing and thawing (2) chemical removal using biocides, detergents and surfactants (de Carvalho, 2007). However, it has been difficult to completely remove biofilms by these methods due to protection of the biofilms cells by the extracellular polymeric substances (EPS) which act as barriers protecting the biofilm cells. Therefore there is a need for methods that are capable of removing the biofilms by destroying the extracellular polymerisc substances (EPS).

1.2 Enzymes for biofilm control

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Previous studies have indicated that antimicrobial agents such as chemical biocides were the main strategy to control and prevent the formation of biofilms (Walker *et al.,* 2007). In many industries, it is important that both the inactivation and the removal of biofilms from the surfaces are achieved (Simoes *et al.,* 2003). A wide range of biocides have been used in controlling biofilms, however these cleaning chemical agents have little to no effect at removing an established biofilms (Walker *et al.,* 2007). Therefore, application of enzymes would be an attractive strategy for the control and removal of biofilms. Enzymes remove biofilms by destroying the physical integrity of

the biofilm matrix (EPS) (Xavier *et al.,* 2005). Study made by Loiselle *et al.* (2003) indicated that cellulose from *Penicillium funiculusum* was one of the most effective enzymes in degrading mature biofilms of *Pseudomonas aeruginosa*. Cellulose was also found to be effective in degrading the exopolysaccharides from *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004). Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and two carbohydrates, namely alpha - amylase and beta – glucanase and the enzymatic mixture was found to be effective in digesting slime layers produced by cultures of pure and mixed strains of bacteria. The main objectives of this study were to:

- Standardize the method for biofilm growth.
- Standardize the EPS extraction method and to determine EPS compositions
- Test the effects of proteolytic and amylase enzymes tested individually and in combination for the degradation of EPS and removal of mono (*Pseudomonas fluorescens*) and mixed bacterial species (*Gram negative and positive*) biofilms.

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Chapter 2

Literature review

2.1 Defining biofilms

Biofilms are defined as assemblages of microbial cells which can be formed by single and or a mixture of bacterial species that are irreversibly associated with a surface and enclosed in a matrix of primary polysaccharide materials that allow the growth and survival in sessile environments (Kalmokoff *et al.,* 2001; Prakash *et al.,* 2003; Smith, 2005). Biofilms form when bacteria adhere to surfaces in aqueous environments and excrete extracellular polymeric substances (EPS) that can anchor the cells in all kinds of material such as metals, plastics, soil particles, medical implant materials (Costerton *et al.,* 1995; Decho, 2000; Mah *et al.,* 2001; Chmielewski *et al.,* 2003; Wingender and Flemming, 2004), living tissues, industrial or potable waste-system piping, or natural aquatic systems (Costerton *et al.,* 1995; Giaouris *et al.,* 2006).

A single bacterial species can form a biofilm, but in natural environment biofilms are often formed from various species of bacteria, fungi, algae, and protozoa (Costerton *et al.,* 1995). Biofilms represent a very complex form of microbial life that is mainly characterized by a high degree of interaction between different types of organisms and by a more or less immobilized form of life. This allows the formation of stable aggregates in which synergistic effects can develop (Chen and Sterwart, 2002; Donlan, 2002).

Debris along with corrosion products provides a considerable advantage for the biofilm forming bacteria (Diosi *et al.,* 2003), such as protection from antimicrobial agents, exchange of nutrients, metabolites or genetic material from close proximity to other microorganisms (Costerton *et al.,* 1995, Morikawa, 2000). In biofilms, the cells can tolerate much higher concentrations of biocides in suspension (Flemming, 1998). Another characteristic of biofims is their heterogeneity, for example, aerobic microorganisms in aerobic system consume oxygen resulting in an anaerobic zone within

the biofilm (Flemming, 1998). This process provides habitats for anaerobes that cannot grow under aerobic conditions.

Such symbiotic relationships, although beneficial to the participating bacteria, often damages the surface. This kind of damage is called biofouling and causes dental decay, metal pipeline corrosion, colonization of medical implants, product contamination and equipment failure (Cloete *et al.,* 2003; Prakash *et al.,* 2003; Kumar *et al*., 2006).

2.2 Biofilm formation and stages involving during biofilm development

2.2.1 The primary stage

The primary adhesion stage constitutes the beneficial contact between a conditioned surface and planktonic microorganisms. During the process of attachment, the organism must be brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and motility (Prakash *et al.,* 2003). Once the organism reaches critical proximity to a surface, the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces. These forces include electrostatic and hydrophobic interactions (Melo *et al.,* 1997; Kumar *et al.,* 2006) and van der Waal's attractions (Denyer *et al.,* 1993). This attachment is unstable and reversible and if the environment is not favorable for microbial attachment, cells can detach from the surface (Ghannoum and O'Toole, 2004). The solid-liquid interface between a surface and an aqueous medium (e.g. water, blood etc.) provides an ideal environment (microhabitat) for the attachment and growth of microorganisms (Flemming, 1998; Kerr *et al.,* 1999; Spiers *et al.,* 2003). Attachment will occur mostly on surfaces that are rougher, more hydrophobic (Palmer *et al.,* 1997) and coated by conditioning films (Schwartz *et al.,* 1998; Kalmokoff *et al.,* 2001; Liu *et al.,* 2004).

The primary stage is reversible and it is characterized by a number of physiochemical variables that define the interaction between the bacterial cell surface and the conditioned surface of interest (An *et al.,* 2000; Singh *et al*., 2002; Liu *et al.,* 2004). When a biofilm is composed of heterogeneous species, the metabolic byproducts of one organism might serve to support the growth of another, while the adhesion of one species might provide

ligands which allow the attachment of others (Dunne, 2002). Conversely, the depletion of nutrients and accumulation of toxin byproducts generated by primary colonizers may limit the species diversity within a biofilm (Marsh, 1995).

2.2.2 The secondary stage

The secondary stage involves the anchoring of bacteria to the surface by molecular mediated binding between specific adhesins and the surface (Kumar *et al.,* 2006). In this process loosely bound organisms gather together and produce exopolysaccharides that complex with surface materials (An *et al.,* 2000; Rachid *et al.,* 2000; Li *et al.,* 2007). Once the bacteria have attached irreversibly to the surface they undergo a range of genotypic and phenotypic changes to ensure the development and maturation of the biofilm. All bacteria produce multiple adhesions some of which are regulated at the transcriptional level depending on the genes encoded, permitting organisms to switch from sessile to planktonic forms under different environmental influences (Li *et al.,* 2007). A good example of this phenomenon is that of *Staphylococcus epidermidis*, which produces a polysaccharide intracellular adhesin (PIA) that is essential for cell to cell adhesion and biofilm formation (Dunne, 2002).

The changes described above result in the production of increased amounts of EPS, increased resistance to antibiotics, increased UV resistance, gene exchange events that occur more frequently and higher amounts of secondary metabolites that are produced (O'Toole *et al*., 2000). With certain organisms, several distinct adhesins might be used for surface attachment depending on the environment (O'Toole *et al*., 2000).

Various structures such as flagella, fimbriae, outer membrane proteins (OMPs), curli (a proteinaceous surface structure) and extracellular polymers structure (EPS) are involved in biofilm formation (Watnick *et al.,* 1999). They have distinct roles in different species and under different environmental conditions (Giaouris *et al.,* 2006). Flagella motility is important to overcome the forces that repel bacteria from reaching many abiotic materials. Once it reaches the surface, appendages such as pili, OMPs and curli are required to achieve stable cell-to-cell and cell-to-surface attachments. Flagella apparently

play an important role in the early stages of bacterial attachment by overcoming the repulsive forces associated with the substratum (Giaouris *et al.,* 2006).

For example, in the case of *Vibrio cholerae* EI Tor, a toxin-coregulated pilus is used as an attachment and colonizes intestinal epithelium during the process of human infection. In contrast, a mannose- sensitive hemagglutinin is the primary adhesin used to anchor to abiotic surfaces in an aquatic environment (Watnick *et al.,* 1999). The acidophilic and iron (II) oxiding bacterium *Acidithiobacillus ferrooxidans* is one of the most important mesophiles for the extraction of metals from sulphidic ores by bioleaching. Attachment of these bacteria to the mineral surfaces seems to enhance bioleaching of pyrite and other minerals (Kinzler *et al.,* 2003).

Studies showed that the EPS of *A. ferooxidans* consist of neutral sugars and lipids (Kinzler *et al.,* 2003; Harneit *et al.,* 2006). In contrast to the cells of *A. ferrooxidans* growing on sulphur, cells growing on pyrite or iron (II) sulphate incorporate uronic acids and iron (II) ions in their EPS, providing the cell surface with a net positive charge under physiological conditions where pyrite is negatively charged (Harneit *et al.,* 2006). Thus the EPS complexed iron (II) ions enable the cells to interact with a pyrite surface through electrostatic forces. The EPS containing complexed iron (II) ions comprise a reaction space in which dissolution process takes place (Harneit *et al.,* 2006).

Korber *et al.* (1989) used motile and nonmotile strains of *Pseudomonas fluorescens* to show that motile cells attach in greater numbers and against the flow more rapidly than do nonmotile strains. Nonmotile strains do not recognize the substratum as evenly as motile strains, resulting in slower biofilm formation by the nonmotile organisms (Prakash *et al.,* 2003). A number of aquatic bacteria possess fimbriae, which have also been shown to be involved in bacterial attachment to animal cells (Meyer, 2003; Prakash *et al.,* 2003, Giaouris *et al.,* 2006).

2.2.3 Micro colony formation

After the adherence of bacteria to the inert surface, the association becomes stable for micro colony formation (Palmer *et al.,* 1997; O'Toole *et al.,* 2000, Bechmann *et al.,*

2006). The bacteria begin to multiply while sending out chemical signals that intercommunicate among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of a micro colony (Prakash *et al.,* 2003).

Microcolonies further develop into macrocolonies which are divided by fluid-filled channels and enclosed in an extacellular polysaccharide matrix (Allison, 2003). Macrocolonies, compared to microcolonies, are composed of more bacteria cells and are enclosed in an extracellular matrix and have a higher metabolic and physiological heterogeneity (Ghannoum and O'Toole, 2004). In the non-motile *Staphylococcus epidermidis,* polysaccharide and protein adhesins were linked for the attachment of this bacterial species, while a novel biofilm-associated protein was found to be involved in attachment and intercellular adhesion of *S. aureus* (Rupp *et al.,* 1991).

2.2.4 Formation of three dimensional structures

During the attachment phase of biofilm development, the transcription of specific genes takes place. These are required for the synthesis of EPS (Prakash *et al.,* 2003). Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded followed by formation of water filled channels in the circulatory system that help in delivering nutrients to and removing waste products from the cell communities in the micro colonies (Prakash *et al.,* 2003).

2.2.5 Biofilm maturation

Once bacteria have irreversibly attached to a surface, the process of biofilm maturation begins. The overall density and complexity of the biofilm increases as surface-bound organisms begin to actively replicate and extra cellular components generated by attached bacteria interact with organic and inorganic molecules in the immediate environment to create the glycocalyx (Carpentier *et al.,* 1993). The availability of nutrients in the immediate environment within the biofilm and the removal of waste, limits the growth potential of any bacterial biofilm (O' Toole *et al.,* 1998; O' Toole *et al.,* 2000). In

addition, there is an existence of an optimum hydrodynamic flow across the biofilm that determines the maximum growth (Carpentier *et al.,* 1993). Other factors that control biofilm maturation include the internal pH, oxygen, carbon source, osmolarity, temperature, electrolyte concentration and the flux of materials and surface types. The surface types can be either:

- High surface energy materials that are negatively charged; hydrophilic materials such as glass, metals or minerals
- Low surface energy materials that are either low positively or low negatively charged; hydrophobic materials such as plastic made up of organic polymers (O' Toole *et al.,* 1998).

At some point, the biofilm reaches a critical mass and a dynamic equilibrium is reached at which the outermost layers of growth begin to generate planktonic organisms. These organisms are free to escape the biofilm and colonise other surfaces. Cells nearest the surface become inactive or die due to a lack of nutrients, decrease in pH, pO_2 or an accumulation of toxic metabolic byproducts (Dunne, 2002).

The primary development, maturation and breakdown of a biofilm might be regulated at the level of population density dependent gene expression controlled by cell-to cell signaling molecules such as acylated homoserine lactones (Stickler *et al.,* 1998). Once fully matured, a logical cooperation and metabolic efficiency provides a form of functional communal coordination that mimics primitive eukaryotic tissues (Costerton *et al.,* 1995).

2.2.6 Detachment and dispersal of biofilm cells

As the biofilm gets older, cells detach and disperse and colonise a new niche. This detachment can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni *et al.,* 2007). Some bacteria are shed from the colony and some stop producing EPS and are released into the surrounding environment (Herrera *et al.,* 2007). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells or detachment as a result of nutrient levels (Spiers *et al.,* 2003). The

released microorganisms may be transported to new locations and restart the biofilm process (Prakash *et al.,* 2003).

As the thickness of the EPS increases, anaerobic conditions develop within the biofilm (Spiers *et al.,* 2003). Because of the film thickness and the activity of anaerobic species, the film detaches and sloughs off from the surface of the substrate. Polysaccharides enzymes specific for EPS degradation for different organisms may be produced during different phases of biofilm growth and contribute to detachment. It has been suggested that the escape of *P. aeruginosa* cells from the biofilm matrix involved the action of an enzyme that digests alginate (Prakash *et al.,* 2003).

In previous studies, it was mentioned that several bacterial species can synthesize polymer degrading enzymes to control the production of the EPS. *P. fluorescens* and *P. aeruginosa* were indicated to produce enzymes known as lyases which can degrade their exopolysaccharides and lead to the detachment of the cells from the surface (Boyd and Chakrabarty, 1994). Allison *et al.* (1998) observed that *P. fluorescens* biofilms grown for longer that 50 h, detached from the surface as a result of polymer degrading enzymes.

2.2.7 Summarized life cycle of biofilms

Biofilm formation begins with the attachment of single cell to a substratum. This first step is reversible and may require active swimming motility or may just be caused by random contacts. In the second phase, the attachment if fixed by adherence of the cells to the substratum through surface appendages such as flagella, fimbria, pili or by production of EPS (An *et al.,* 2000; Liu *et al.,* 2004).

The third phase represents growth of firmly attached cells forming micro colonies based on the availability of nutrients on the surface itself or from water column above the substratum. In a hydrodynamic environment, the development of micro colonies depends on cell to cell binding interaction (Palmer *et al.,* 1997; Singh *et al*., 2002).

The fourth stage is often referred to as mature biofilms. At this point the biofilm structure with its distribution of biomass and the presence of water filled channels illustrates the heterogeneity of the biofilms and the rigid properties of the developed structure

(Carpentier *et al.,* 1993; Singh *et al*., 2002). The stability of the biofilm is secured partly by cell-cell interaction and partly by EPS matrix surrounding and intergrated into biomass of the biofilms. Finally, at some point the biofilms may partially dissolve releasing cells that may move away to other locations where a new cycle begins (Singh *et al*., 2002; Prakash *et al.,* 2003). Below is the schematic illustration of biofilm attachment and formation.

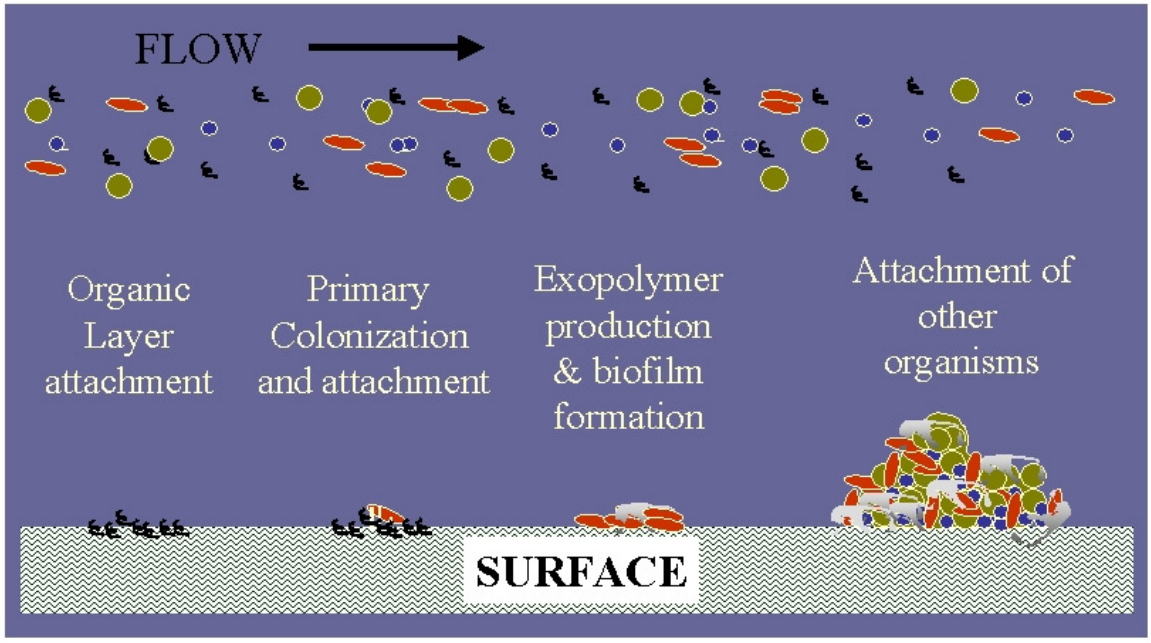

Fig. 2.1 Schematic illustrations of biofilm formation and development. Tracy Hudson (2002).

2.3 Factors affecting the growth and development of biofilms

2.3.1 Nutrients

Biofilms can form under diverse nutrient concentrations, ranging from high to almost non-detectable (Prakash *et al.,* 2003). They are, however, more abundant, densely packed and thicker in environments with high nutrient levels (Allison *et al.,* 2000; Prakash *et al.,* 2003; Rochex and Lebeault, 2007). High nutrient concentrations promote the transition of bacterial cells from the planktonic to biofilm state (O'Toole *et al.,* 2000)

while depletion of these nutrients has shown to cause detachment of biofilm cells from surfaces (Allison *et al.,* 1998; Hunt *et al.,* 2004; Rochex and Lebeault, 2007).

In an open reticulating system, there are abundant nutrients derived from water particularly in cooling towers. Closed systems (i.e. not exposed to the atmosphere) are ideal systems in that the problem of biofouling is either unlikely to be encountered or reduced (Melo *et al.,* 1997). High levels of nutrients appear to produce an open structure in the biofilm whereas lower concentrations tend to give a more compact structure. The structure of the biofilm has an effect on the availability of nutrients to the constituent cells. An open structure facilitates the diffusion of nutrients to the bacteria (Allison, 2003). For aerobic bacteria, the availability of oxygen is necessary unless the particular microorganism can exist under oxygen starved conditions (Melo *et al.,* 1997).

An increase in nutrient concentrations correlated with an increase in the number of attached bacterial cells (Cowan *et al.,* 1991; Dunne, 2002; Prakash *et al.,* 2003). However, nutrient concentrations too low to measure are still sufficient for biofilm growth. Biofilm bacteria acquire nutrients by concentrating trace organics on surfaces by the extracellular polymer, using the waste products from previously attached cells and secondary colonizers and also by pooling the biochemical resources with different enzymes to break down food supplies (Cowan *et al.,* 1991).

Sauer and Camper (2004) and Rochex and Lebeault (2007) demonstrated that *P. aeruginosa* and *P. putida* biofilms detached at high nutrient levels. On the contrary, Oh *et al.* (2007) demonstrated by means of atomic force microscopy (AFM) that *Escherichia coli* O157:H7 biofilms formed faster and as a result more cells attached to a glass surface under low nutrient conditions compared to high nutrient conditions. In addition, under low nutrient conditions, *Serratia marcescens* form biofilms consisting of single microcolonies, while under high nutrients conditions these microcolonies can revert to filamentous biofilms (Rice *et al.,* 2005).

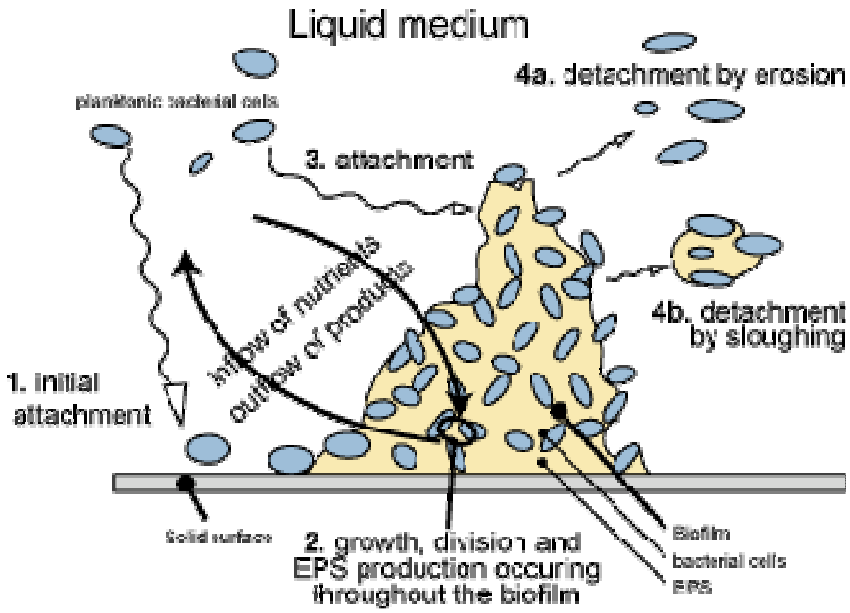

Fig. 2.2 Schematic illustration of processes involved in biofilm formation and development (Picioreanu *et al.,* 1998; Kreft *et al.,* 2001; Picioreanu *et al.,* 2004).

2.3.2 Temperature effects

For many bacteria found in cooling water systems, the optimum temperature for maximum growth is about 40^{O}C (Melo *et al.,* 1997, Timothy and Hansen, 2006). At this temperature small changes in temperature are likely to produce substantial changes in biofilm growth (Dewanti and Wong, 1995), because microbial activity is very sensitive to temperature. For instance, studies have shown that biofilm thickness of *Escherichia coli* increased by 80% by raising the temperature from 30^{O}C to 35^{O}C (Melo *et al.,* 1997).

2.3.3 Surface condition

The surface could be a dead or living tissue or any inert surface. The attachment of microorganisms to surfaces is a complex process with many variables affecting the outcome. Attachment will occur most readily on surfaces that are rougher, more hydrophobic and coated by surface conditioning films (Zacheus *et al.,* 2000; Dunne, 2002). Furthermore, growth requires complex developmental pathways that are regulated in response to environmental and bacterial derived signals. Studies based on the effect of

substratum were made and results found showed that the extent of microbial colonization appears to increase as the surface roughness increases (Prakash *et al.,* 2003).

It has been demonstrated that the surface condition (e.g. whether rough or smooth) affects the ability of bacteria to adhere to a surface. A material surface exposed in an aqueous medium will inevitably become conditioned or coated by polymers from that medium, and the resulting chemical modification will affect the rate and extent of microbial attachment (Prakash *et al.,* 2003). Studies based on the films were made on surfaces exposed in seawater and results found showed that films were organic in nature and they formed within minutes of exposure and continued to grow for several hours (Prakash

et al., 2003).

The surface may have several characteristics that are important in the attachment process (Sauer and Camper, 2001). An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment if these factors do not exceed critical levels (Donlan, 2002). Properties of the cell surface, specifically the presence of fimbriae, flagella and surface associated polysaccharides or proteins, are also important and may provide a competitive advantage for one organism where mixed community is involved (Zobell, 1943; Donlan, 2002).

A material surface exposed in an aqueous medium will become conditioned or coated by polymers from that medium and the resulting chemical modification will affect the rate and extend of microbial attachment (Prakash *et al.,* 2003). A material surface exposed in an aqueous medium will become conditioned or coated by polymers from that medium and the resulting chemical modification will affect the rate and extend of microbial attachment (Prakash *et al.,* 2003). Surfaces cannot be colonized by biofilms unless they have been exposed to organic material from the surrounding environment (Allison *et al.,* 2000). However the effect of surface characteristics like charge, hydrophobicity, roughness and elasticity on microbial attachment cannot be ignored (Allison *et al.,* 2000).

Studies based on the films were made on surfaces exposed in seawater and results obtained showed that films were organic in nature and they form when exposed to

surfaces and continue to grow for several hours. The nature of conditioning films may be quite different for surfaces exposed in the human host (Donlan, 2002). Studies in medical research based on microbial films were involved and results obtained showed that a number of host produce conditioning film such as blood, tears, urine, saliva, intravascular fluid and respiratory secretion influenced the attachment of bacteria to biomaterials and that, the surface energy of the suspending medium may affect hydrodynamic interaction of microbial cells with surfaces by altering the substratum characteristics (Donlan, 2002).

2.3.4 Velocity, turbulence and hydrodynamics

The area from the surface where no turbulent flow is experienced is known as the boundary layer. Within this area, the flow velocity has been shown to be insufficient for biofilm removal (Dreeszen, 2003). The area outside this layer is characterized by high levels of turbulent flow and has an influence on the attachment of cells to the surface (Donlan, 2002). Studies showed that an increase in water flow velocity resulted in an increased bacterial number in biofilms. This is attributable to better mass transfer of growth limiting nutrients at the higher flow velocity of water (Lehtola *et al.,* 2006). Biofilms rely on defensive mechanisms to resist detachment by the higher fluid shear. It has been proposed that the viscoelasticity of biofilms allows them to resist detachment as has been found in *Staphylococcus aureus* biofilms (Lehtola *et al.,* 2006).

The size of the boundary layer is dependant on the flow velocity of the water. At high velocities, the boundary layer decreases in size and the cells are exposed to high turbulence levels (Donlan, 2002). Hydrodynamic conditions can influence the formation, structure, EPS production, and thickness, mass and metabolic activities of biofilms (Stoodley *et al.,* 2002; Liu and Tay, 2002; Simoes *et al.*, 2003).

Studies concerning the hydrodynamic of aqueous medium demonstrated that the flow velocity adjacent to the substratum/liquid interface is negligible. This zone of negligible flow is termed as the hydrodynamic boundary layer (Kumar *et al.,* 2006). Its thickness is dependent on linear velocity, the higher the velocity, the thinner the boundary layer. The region outside the boundary layer is characterized by substantial mixing or turbulence.

For flow regimes characterized as laminar or minimally turbulent, the hydrodynamic boundary layer may affect cell substratum interactions (Kumar *et al.,* 2006).

Cells behave as particles in a liquid, and the rate of settling and association with a submerged surface will depend largely on the velocity characteristics of the liquid. Under very low linear velocities, the cells must transverse the sizeable hydrodynamic boundary layer, and association with the surface will depend on cell size and cell mortality (Donlan, 2002). As the velocity increases, the boundary layer decreases and cells will be subjected to increasingly greater turbulence and mixing. Higher linear velocities would therefore be expected to equate to more rapid association with the surface, at least until velocities become high enough to exert substantial shear forces on the attaching cells resulting in detachment of these cells (Donlan, 2002).

Studies have indicated that biofilms formed under low shear conditions (laminar flow conditions) are characterized by spherical microcolonies divided by water channels (Stoodley *et al*., 2002). Simoes *et al.* (2003) for instance determined the differences between three strains of *P. fluorescens* biofilms which were grown under turbulent and laminar flow. All biofilms grown under turbulent flow were denser, had a higher mass, and were more active, produced similar amounts of matrix proteins and the *P. fluorescens* strains had higher amounts of extracellular polysaccharides (Simoes *et al.,* 2003). It has been demonstrated that biofilms formed under higher detachment forces produced more extracellular polysaccharides in order to stabilize the biofilm structure and to withstand the shear force (Ohashi and Harada, 1994; Chen *et al.* 1996)

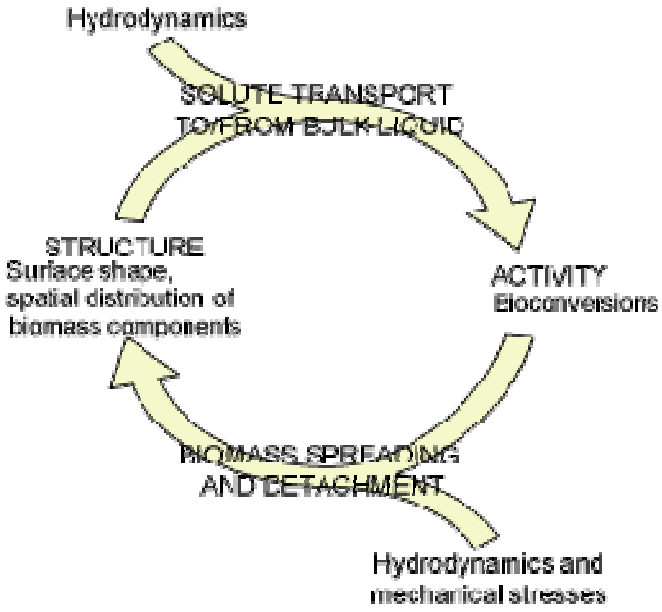

Fig. 2.3 Schematic illustrations of the structure/ activity relationship in biofilms (Picioreanu *et al.,* 1998; Kreft *et al.,* 2001; Picioreanu *et al.,* 2004).

2.3.5 Effects of particles

Biofouling of industrial equipment occur together with other kinds of fouling. The most common being the simultaneous deposition of small particles that are transported with the incoming water and/or those which are formed in the plant as a consequence of metal corrosion (Bouwer, 1987). When the particles are organic in nature, they act as substrate for microorganisms and are being degraded by them contributing to the growth of the biomass (Melo *et al.,* 1997). In most cases, however, the biological matrix incorporates inorganic particles that are relatively inert but may cause changes in the structure and activity of the biofilms (Battin *et al.,* 2003). The adhesion between particles and microorganisms could be facilitated by the electropositive charges developed at the surfaces of some particles depending on the pH of the environment. Metabolic inhibitors and toxic metallic ions could be adsorbed on the particle surface favouring biomass formation and microbial respiration stimulation in the presence of particles (Melo *et al.,* 1997).

2.3.6 Gene regulation

Studies based on gene regulation of microbial biofilms were made and results showed that 22% of the genes were up regulated and 16% down regulated in biofilm forming of *Pseudomonas aeruginosa* (Steyn *et al.,* 2001). Other studies demonstrated that the *algC* was up regulated within minutes of attachment to a surface in a flow cell system. Genes encoding for enzymes involved in glycolysis or fermentation such as phosphoglycerate mutase, triosephosphate isomerase and alcohol dehydrogenase were up regulated in biofilm formation of *Staphylococcus aureus*. A recent genetic study also showed that *algD, algU, rpoS* and genes controlling polyphosphokinase synthesis were up regulated in biofilm formation of *Pseudomonas aeruginosa* (Prakash *et al.,* 2003).

2.3.7 Quorum sensing (QS)

Quorum sensing is dependent on the cell density (Hammer, 2003). The development of biofilms on surfaces is mediated by a density dependent chemical signal released by bacterial cells densely packed within an EPS matrix. Microorganisms can use quorum sensing to coordinate their communal behavior such as biofilm formation, motility and production of EPS (Xiong and Liu, 2010).

Quorum systems make use of a transcriptional activator protein that acts in concert with a small autoinducers (AI) signaling molecule to stimulate expression of target genes (de Kievit *et al.,* 2001). Increasing bacterial density gives rise to an accumulation of autoinducers (AIs) (Xiong and Liu, 2010). Once the critical AIs concentrations are achieved, the regulator of proteins are triggered and further induce target DNA seguence leading to transcription of quorum sensing regulated genes which will then result in changes of bacterial behavior (Decho, 2000; Xiong and Liu, 2010). This form of intercellular communication serves to coordinate gene expression and structures morphological differentiation and development responses of bacterial cells (Fuqua *et al.,* 1996; Dunlap, 1997).

Cell-to-cell communication is essential for biofilm formation and is closely regulated to AIs. There are three types of AIs that have been identified including oligopeptides, *N* - acylhomoserine lactones (AHL) (Davies *et al.,* 1998; Elsri *et al.,* 2001) and autoinducers - 2 (AI-2) synthesized by LuxS (Pierson *et al.,* 2001; Xiong and Liu, 2010). Oligopeptides and AHL are involved in cellular communication of Gram positive and Gram negative bacteria respectively, whereas AI- 2 played a role in the interspecies communication of both Gram positive and Gram negative bacteria. AIs mediated QS systems play an important role in the regulation of microbial attachment and biofilm formation (Xiong and Liu, 2010).

Quorum-sensing signals can also control biofilm detachment by the accumulation of the signal molecules (excreted by bacteria) to a threshold concentration which will eventually trigger the dispersion of the biofilm (Hentzer *et al.,* 2002). Experiments with pure culture *Pseudomonas aeruginosa* biofilms in continuous-flow conditions indicated that these biofilms detached after flow was stopped. When these biofilms were starved from nutrients under continuous-flow conditions, detachment also occurred. These observations suggest that starvation and not the accumulation of signal/metabolic products was responsible for triggering the detachment of these biofilms (Hunt *et al.,* 2004) (Fig 2, 4).

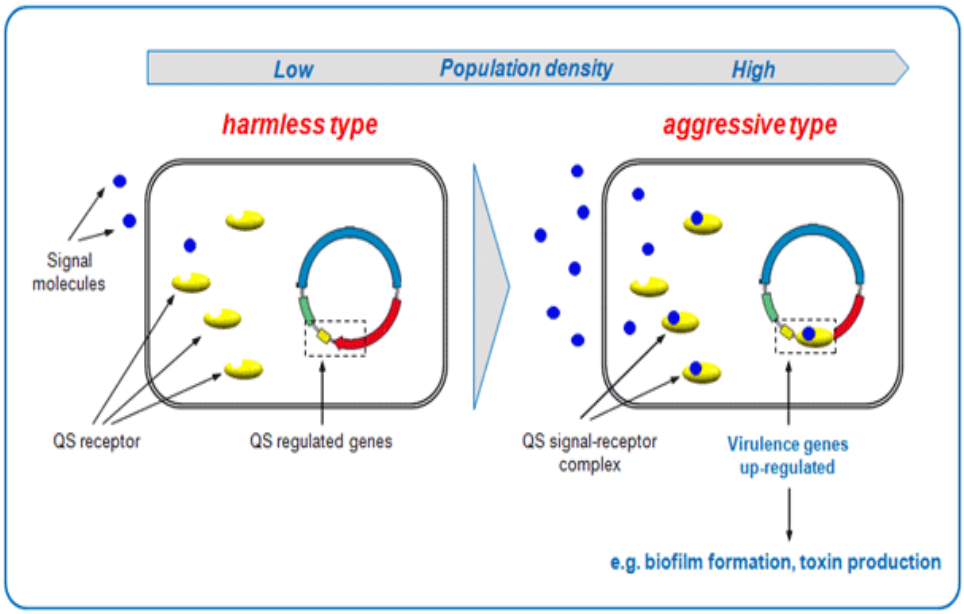

Fig. 2.4 Quorum sensing processes in bacterial biofilms (Nadell *et al.,* 2008).

When the density of bacteria reaches a sufficient level, the concentration of the auto inducer passes a threshold, initiating a positive feedback as more signalling molecule is synthesized, and the receptor becomes fully activated (Nadell *et al.,* 2008). It also induces the up regulation of other specific genes, for example, various virulence genes like proteases and toxins or the formation of biofilms. Thus in an opportunistic bacterium such as *Pseudomonas aeruginosa*, QS-related processes include biofilm development, exopolysaccharide production, and cell aggregation. *P. aeruginosa* can grow within a host without harming it, until they reach a certain population density when they become aggressive, their numbers sufficient to overcome the host's immune system and form a biofilm, leading to disease (Hunt *et al.,* 2004).

2.3.8 Properties of the cells

Cell surface hydrophobicity, presence of fimbriae and flagella, and productions of EPS influence the rate and extend of attachment of microbial cells (Liu *et al.,* 2002). The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non polar nature of one or both surfaces involved (i.e. the microbial cell surface and the substratum surface) Most bacteria are negatively charged but still contain hydrophobic surface components (An *et al.,* 2000).

Fimbrae contribute to cell surface hydrophobicity. Most fimbriae that have been examined contained high proportion of hydrophobic amino acid residues. They play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum. A number of aquatic bacteria possess fimbriae which are involved in bacterial attachment to animal cells (Spiers *et al.,* 2003). Treatment of adsorbed cells with proteolytic enzymes caused a marked release of attached bacteria, providing evidence for the role of protein in attachment (Donlan, 2002).

Mycolic acid containing organisms such as *Corynebacterium, Norcadia* and *Mycobacterium* were more hydrophobic than the non mycolic acid containing bacteria and an increase in mycolic acid chain length coincided with an increase in hydrophobicity (Spiers *et al.,* 2003). For most bacterial strains tested, adhesion was greater on hydrophobic materials. The O antigen component of lipopolysaccharide (LPS) also contributes hydrophilic properties to gram negative bacteria (Spiers *et al.,* 2003).

Mutants of *P. fluorescens* lacking the O antigen adhered in greater numbers to hydrophobic materials (Spiers *et al.,* 2003). Fletcher *et al.* (1991) found that treatment of attached fresh water bacteria with cations resulted in contraction of the initial adhesives (decrease in the cell distance from the substratum), indicating that the material was an anionic polymer. Glucosidase and N-acetylglucosaminidase (NAG) reduced attachment for *P. fluorescens*, while NAG only reduced attachment for *Disulfovibrio desulfuricans*. Lactins preferentially bind to polysaccharides on the cell surface or to the EPS. Binding of lactins by the cells would minimize the attachment sites and affect cell attachment if polysaccharides were involve in attachment. This was demonstrated with *Pseudomonas fragie* confirming the role of polysaccharides in attachment (Dunne, 2002). Cell surface polymers with non polar sites such as fimbriae, other proteins and components of certain gram positive bacteria (mycolic acids) appear to dominate attachment to hydrophobic substrata while EPS and LPS are more important in attachment to hydrophilic materials (Donlan, 2002).

With all these findings, it has been shown that cell surface structures such as fimbriae, other proteins, LPS, EPS and flagella play an important role in the attachment process (Dunne, 2002).

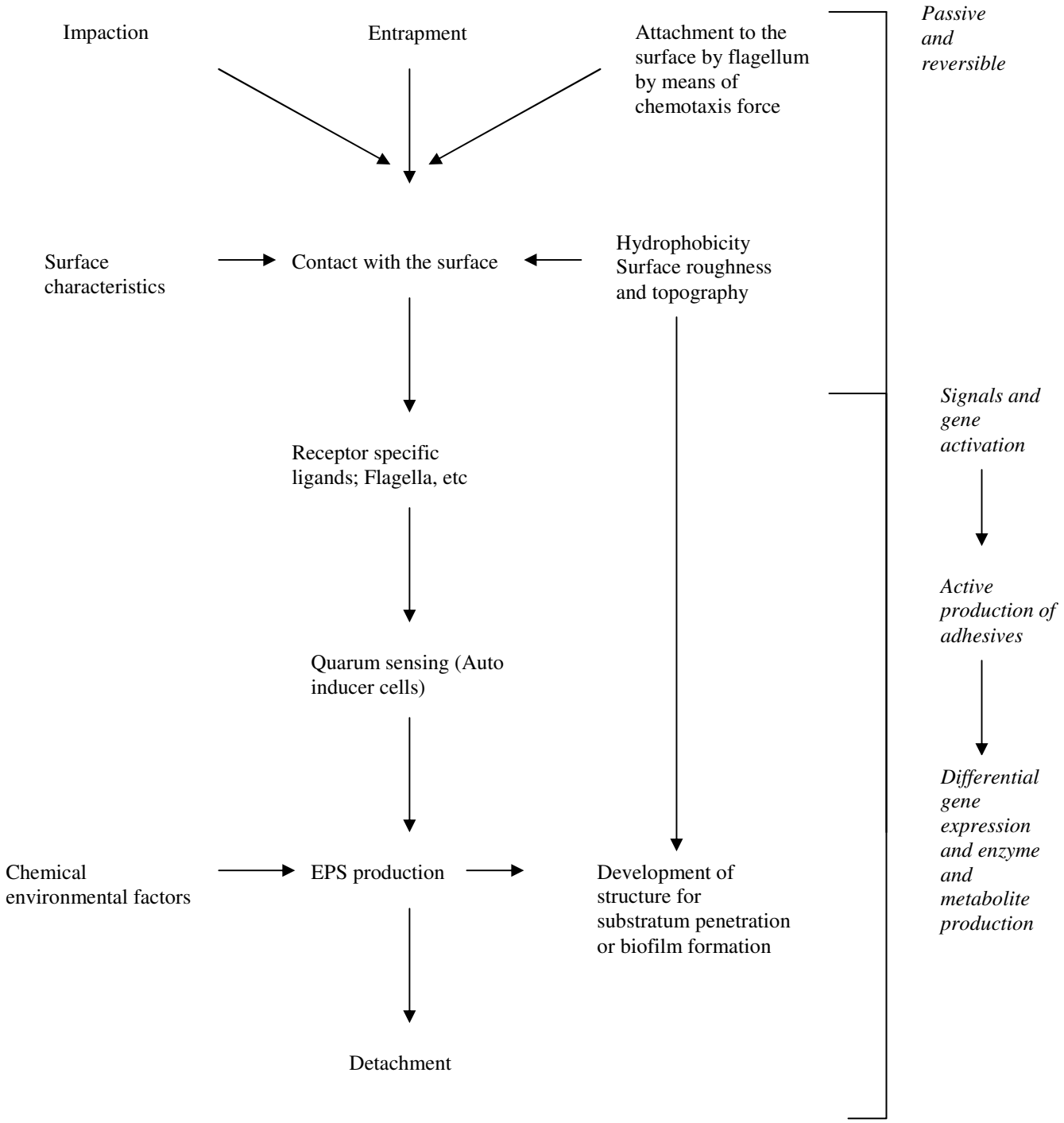

Fig. 2.5 Flow chart of biofilm formation on a surface (Gutierrez- Correa *et al.*, 2003).

2.4 Description of extracellular polymeric structure (EPS)

Flemming (2007) describes extracellular polymeric substances as the "house of biofilm cells". Biofilms are composed of microbial cells and extracellular polysaccharide structures (EPS). EPS may account for 50-90% of the total organic carbon of biofilms and can be considered as the primary matrix material of the biofilms (Flemming 1998). Biofilm- associated EPS is distinct, both chemically and physically from the bacterial capsule, but primarily composed of polysaccharides (Prakash *et al.,* 2003). EPS is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. The EPS formation rate is proportional to the rate of substrate utilization. Cells use electrons from electron donor substrate to build active biomass and they also produced bound and soluble EPS (Sponza, 2003). Different biofilms produce different amount of EPS, and the amount of EPS increases with age of the biofilm (O' Toole *et al.,* 2000).

2.4.1 EPS production

A number of parameters including reactor type, substrate composition, substrate loading rate, hydraulic retention time, hydrodynamic shear force, culture temperature etc have been indicated to facilitate the production of biofilm EPS (Liu *et al.,* 2004). In biological wastewater treatment, biomass generates extracellular polymeric substances (EPS) when consuming organic material present in the wastewater. EPS are complex mixture of high molecular polymer (M_w>10,000) excreted by microorganisms, products from lysis and hydrolysis and adsorbed organic matters from wastewater (Wingender *et al.,* 1999). EPS of bacteria are involved in the formation of microbial aggregates (Geesey, 1982; Davies *et al.,* 1993), adhesion to surfaces and flocculation (Wingender *et al.,* 1999, Bhaskar and Bhosle, 2005). Furthermore, EPS are major components of aggregates for keeping the floc together in a three dimensional matrix due to bridging with multivalent cations and hydrophobic interactions (Wingender *et al.,* 1999).

Such a polymer network has a vast surface area and is capable of absorbing pollutants, nutrients and minerals. EPS plays an important role in the flocculation of bacterial cells and provides energy and carbon when substrates are in short supply (Laspidou and Rittmann, 2002; Sheng *et al.,* 2005). The EPS compostion is also related to the characteristics of waste water. For example, EPS has high protein and DNA concentrations in protein grown granules while high polysaccharide concentrations are found in granules grown in other type of organic substrates. Nitrogen limiting conditions favour the production of EPS which in turn accelerates anaerobic granulation (Liu *et al.,* 2004).

Proteins and carbohydrates are the main constituents of the EPS but other organic substances such as lipid and nucleic acid are also present (Watson *et al.,* 2004). Starkey and Kerr (1984) have proposed that the production of EPS is inhibited as a result of the anaerobic processes taking place. The production of EPS is known to be affected by nutrients status of the growth medium and the availability of carbon. Nielson *et al.* (1998) reported that a significant degradation of the sludge flox matrix occurred during anaerobic storage over a few days and that the reduction in sludge was mainly due to degradation of the sludge proteins and carbohydrates (Watson *et al.,* 2004). The limitation of nitrogen, potassium or phosphate promotes EPS synthesis. Slow bacterial growth will also enhance EPS production (Prakash *et al.,* 2003). EPS produce by the microorganisms exist as tightly bound (capsular), loosely adhered (slime type) to the cells or as free dissolved matter (Bhaskar and Bhosle, 2005).

2.4.2 EPS composition

The composition of extracellular polymeric substances (EPS) may vary amongst bacterial strains (Liu et *al.,* 2004). Extracelullar polymeric substances are composed of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid, DNA, lipid and humid substances (Hoyle, 1992; Dignac *et al.,* 1998; Frøelund *et al.,* 1995; Donlan, 2002; Liu *et al.,* 2004; Cheng *et al.,* 2007). The composition may be the result of active secretion, shedding of cell surface material, cell lysis and adsorption from the environment (Zhang *et al.,* 2001). Chemically, the bacterial EPS is a heterogeneous

polymer containing a number of distinct monosaccharides and non carbohydrate substituents, many of which are strain specific (Liu *et al.,* 2004).

There are contradictory reports in the literature about the composition of EPS especially with the ratio of carbohydrate to protein. Some of these reports indicated that certain EPS from wastewater biofilms (activated sludge) have a higher concentration of proteins than polysaccharides and some instances polysaccharides were found to be dominant in the biofilm (Sutherland, 1994; Nielsen *et al* 1997). Nonetherless, the quantity and the composition of the EPS produced by bacterial biofilms depend on a number of factors among which are, microbial species, growth phase, the type of limiting substrate (carbon, nitrogen and phosphorus), oxygen limitation, ionic strength culture temperature and shear force (Zhang *et al.,* 2001; Fang *et al.,* 2002; Liu *et al.,* 2003; Liu *et al.,* 2004; Bhaskar and Bhosle, 2005; Ratto *et al.,* 2005; Orgaz *et al.,* 2006). This implies that the EPS composition is variable and is related to microbial species, the physiological state of the bacteria and the operating conditions under which biofilms are developed. As a result, the multi structural components of the EPS are the main causes of ineffective removal of biofilms by antimicrobial agents (Cloete, 2003; Sreenivasan *et al.,* 2005; Lequette *et al.,* 2010).

The exopolysachharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties (Nielsen *et al.,* 1997; Sutherland, 2001, Bhaskar and Bhosle, 2005). One of the major components of the bacterial EPS is the uronic acids costituting up to 20 – 50% of the polysaccharide fraction (Bhaskar and Bhosle, 2005). Liu *et al.* (2004) revealed that polysaccharides are the only components that are synthesized extracellularly for a specific function, while proteins, lipid and nucleic acid exist in the extracellular polymer due to excretion of intracellular polymers or as a result of cell lysis.

Some microorganisms can bind ions into their EPS which have an influence on the nature of the biofilm (Poulsen, 1999). The bacterial extracellular polysaccharides are composed of homo and heteropolysaccharides including glucose, fructose, mannose, galactose, puryvate and mannuronic acid or glucoronic acid based complexes. The EPS are held to

the cell wall either by linkage between the carboxyl gropus of the EPS and hydroxyl groups of lipopolysaccharides or by a covalent bonding through phospholipids and glycoproteins (Bhaskar and Bhosle 2005). The exopolymer (EPS) produced by microorganisms also vary depending on whether the microorganisms are Gram negative or Gram positive cells. In addition, the EPS concentration depends on the physiological state of the microorganisms (Poulsen, 1999; Bhaskar and Bhosle, 2005).

Donlan (2002) indicated that EPS may be hydrophilic or hydrophobic depending on the structural components and the environmental conditions were the biofilms are developing. EPS components may also differ among individual members of a single bacterial species (Czaczyk *et al.,* 2007).

The structure of polysaccharides synthesized by microbial cells may vary. Microbial exopolysaccharides are composed of either homopolysachharides or heteoropolysaccharides. Homopolysaccharides are composed of only one monosaccharide type such as D- glucose or L- fructose. Homopolysaccharides belong to three distinct groups including:

α - D - glucan which is produced by *Leuconostoc mesenteroides*

ß- D- glucans which is produced by *Pediococcus spp* and *Streptococcus spp*.

Fructans produced by *Streptococcus salivarius*.

Several groups of lactic acid bacteria produce heteropolysaccharides which are made up of repeating units of monosaccharides including D- glucose, D- galactose, L- fructose, L-rhamnose, D- glucuronic acid, L- guluronic acid and D- mannuronic acid (Czaczyk *et al.,* 2007). The type of both linkages between monosaccharides units and the branching of the chain determines the physical properties of the microbial heteropopysaccharides (Bhaskar and Bhosle, 2005). As an example, bacterial alginate is a heteropolysaccharide with irregular structure. In this polymer, 1.4 linked β - D- mannurosyl and 1.4 α - L-guluronosyl residues are found. Alginate is mostly produced by the cells of *Pseudomonas*

aeruginosa and *Azatobacter vinelandii* (Christensen *et al.,* 2001; Sutherland, 2001, Czaczyk *et al.,* 2007).

Bacterial alginates produced by *Pseudomonas spp* differ from algal alginates produced by *Laminaria hyperborean* and *Microcystis pyrifera* (Christensen *et al.,* 2010). The polymer chains of algal alginates contains numerous blocks of L – guluronic acid (G – blocks), thus enabling intermolecular cross linking via selective binding of Ca^{2+} ions to form gels. On the contrary, *Pseudomonas spp* alginates do not contain G- blocks. In this case, the G-risidues (0 to 40%) occur as single residues and such alginates do not form rigid gels in the presence of Ca^{2+}. In addition bacterial alginates are commonly O – acetylated (Czaczyk *et al.,* 2007) which further counteracts gelation with Ca^{2+} (Christensen *et al.,* 2010).

Extracellularly secreted proteins are substances with a molecular weight between 10kDa and 200kDa. These compounds contain 40- 60 % of hydrophobic amino acids. It was observed that the extra cellular proteins synthesized by *Sulfolobus acidocalcidarius* are mainly composed of amino acids with hydroxyl groups. However, the *Bacillus substilis* extracellular protein layer is a composition of L and D glutaminosyl residues (Czaczyk and Myszka, 2007). According to Ton-That *et al.* (2004), the ratio of glutaminosyl isomers in *Bacillus substilis* extracellular protein layer changed significantly in oxygen limited conditions.

2.4.3 EPS chemistry

Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30kDa) and have a heteropolymeric composition. The polysaccharide chain might be branched or unbranched with side chains of other compounds attached to the polymeric chain (Allison, 1998). Polysaccharide chains vary in size from 103-108kDa and contain subunits which may also be both functionally- and species – specific. Furthermore, polysaccharides may be hydrophyllic but can also have hydrophobic properties (Allison, 1998; Flemming *et al.,* 1998). Generally, the polysaccharides are made of monosaccharides with hexose and pentoses forming the bulk of EPS. However, the contribution of different monomers to the total polysaccharide varies with the source and

such variations in the polysaccharide chain composition can alter its physiological chemical properties (Bhaskar and Bhosle, 2005).

EPS are generally rich in hexoses like glucose and galactose wheares planktonic EPS have a higher content of sugar like rhamnose, xylose, and mannose. Such variations in monomer composition can alter the properties of EPS. The presence of sugar like arabinose in EPS helps in cell aggregation in biofilms wheareas deoxy sugars like fucos and rhamnose found in diatom EPS help in foaming and flocculation (Bhaskar and Bhosle, 2005). Apart from polysaccharides, EPS also contain proteins, lipids and nucleic acids. The composition of EPS varies between planktonic and biofilms which in turn reflect on its prpperties. For example, one of the major components of biofilm EPS is uronic acid consisting of up to 20-50% of the polysaccharide fraction. On the other hand, plaktonic EPS are poor in uronic acid concentrations (Allison, 1998; Bhaskar and Bhosle, 2005).

2.4.4 Role of EPS

The production of EPS have several functions such as: facilitation of the initial attachment of bacteria to a surface (Laspidou and Rittmann, 2002); formation and maintenance of micro colony (Flemming *et al.,* 1998); enables the bacteria to capture nutrients; stabilization of the biofilm structure (Laspidou and Rittmann, 2002); organic chemical degradation; biofouling (Cloete *et al.,* 1998; Diosi *et al.,* 2003; Coetser and Cloete, 2005; Gonzalez *et al.,* 2006); cell-cell communication (Zhang *et al.,* 2001) enhances biofilm resistance to environmental stress and antimicrobial agents (Gilbert 1997, Andearl, 2000; Cochran, 2000; Laspidou and Rittmann, 2002; Chapman, 2003; Parkar *et al.,* 2004; Lequette *et al.,* 2010); provides resistance to heavy metal toxicity (Zhang *et al.,* 2001). EPS also function as a stabilizer of the biofilm structure and as a barrier against hostile environments (Desai, 1998; Zhang *et al.,* 2001; Chen and Stewart, 2002; Gomez-Suarez *et al.,* 2002; Stoodley *et al.,* 2002; Arevalo-Ferro *et al.,* 2005; Lapidot *et al.,* 2006; Ploux *et al.,* 2007; Donlan, 2008).

2.4.5 Heterogeneity of EPS structures

The structures which make up a biofilm contain canals through which nutrients circulate in different zones of the biofilms (Comte *et al.,* 2006). The cells express different genes as if they were part of an organized structure (Giaouris *et al.,* 2006). The micro colony continues to grow in volume, and the bacteria in proximity to the surface have difficulties in gaining access to nutrients from the external environments. Only those located in the upper layers of the colony are able to continue multiplying (Giaouris *et al.,* 2006).

Any given cell within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus growing at the different rate. Factors such as gradients of nutrients, waste products and signaling factors contribute to this heterogeneity in biofilms. Heterogeneity has also been shown for protein synthesis and respiratory activity as DNA content remains constant throughout the biofilm (Prakash *et al.,* 2003). This concept of heterogeneity is descriptive not only for mixed-culture biofilms, but also for pure-culture biofilms (Allison, 1998). The matrix will change considerably as equilibrium between the species is established and a balance between competition and commensalism is achieved within the microbial community (Prakash *et al.,* 2003).

2.4.6 Cell structures associated with EPS

While EPS and lipopolysaccharides are more important in attachment to hydrophilic material cell-surfaces, polymers with nonpolar sites such as fimbria and other proteins and components of certain Gram positive bacteria facilitate attachment to the hydrophobic substratum (Arevalo-Ferro *et al.,* 2005). Flagella are important in attachment also, although their main role may be to overcome repulsive force rather than to act as adsorbents and adhesive (Arevalo-Ferro *et al.,* 2005; Donlan, 2008).

2.5 Biofilm producing enzymes

2.5.1 Extracellular enzymes

Hydrolytic enzymes such as glucosidase, lipase and protease have an important function at the hydrolysis stage of complex organic structures in the degradation of biodegradable organic matter. Several reports have indicated that the hydrolases are mainly localized in the extracellular polymeric substances (EPS) matrix of the bacterial aggregates. This debris can be either organic or inorganic (Ayol *et al.,* 2008; Burgess *et al.,* 2008). These exoenzymes (glucosidase, lipase and protease) can originate from the sewage effluent, the activated sludge via cell autolysis or as enzymes that are actively secreted by the cells (Romani *et al.,* 2008). Furthermore, exoenzymes are cell surface bound in free form (exoenzymes in water or absorbed within the extracellular polymeric substances (EPS) of the sludge matrix) (Higuchi *et al.,* 2005).

2.5.2 Enzyme activity in sludge flocs

The activated sludge process has long been employed to treat a wide variety of wastewater (Yan *et al.,* 2008). It has been reported that a number of enzymes such as aminopeptidase, galactosidase, glucosidase, lipase and phosphatase and protease have been extracted from sludge. These enzymes found in sludge may originate from the effluent sewage, from the sludge itself or even as actively secreted extracellular enzymes (de Beer *et al.,* 1996; Watson et *al.,* 2004; Burgess *et al.,* 2008). Proteolytic, lipolytic and cellulolytic enzymes synthesized within bacterial cells are secreted into the extracellular environment and hydrolyse the absorbed macromolecules into small units that can be transported across the cell membrane and then metabolized (Watson *et al.,* 2004; Li and Yang, 2007).

Protease, α- amylase and α glucosidase play important role in the biological waste water treatment. In the bulk solution of activated sludge, the amount of extracellular enzymes are immobilized in flocs. How the extracellular enzymes distribute in sludge flocs determines the contact probability of enzymes with proteins or polysaccharides, hence affecting the process treatment efficiency (Yu *et al.,* 2007). EPS in sludge flocs were

proposed to exhibit a dynamic double layer like structure, composed of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The LB-EPS is considered to easily exchange substances with the bulk solution hence having greater impact to numerous sludge processes such as coagulation and dewatering (Yu *et al.,* 2007).

2.5.3 Enzyme mediated resistance

The resistance of biofilms resistance to antimicrobial agents can be due to enzymes transforming the bactericide to a non-toxic form. The phenomenon is usually investigated from the biodegradation point of view, i.e. the biodegradation of toxic pollutants (Gu, 2007). A host of aromatic, phenolic and other compounds, toxic to many bacteria can be degraded by certain bacteria (Cloete, 2003). Enzyme-mediated resistance mechanism includes heavy metal resistance and formaldehyde resistance. Mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, tellurite, copper, chromate and silver are some of the compounds where biofilms are found to be resistance to due to enzymatic activity (Cloete, 2003; Bhaskar and Bhosle, 2005). Detoxification is usually by enzymatic reduction of the cation to the metal, whereas some heavy metal resistance genes are carried on plasmids, whilst others are chromosomal. The resistant phenotype is usually inducible by the presence of the heavy metal. Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite and antimony, for example, induce resistance to each other in *E. coli* (Cloete, 2003). The diagram below illustrates the multimetal resistance and tolerance in microbial biofilms!

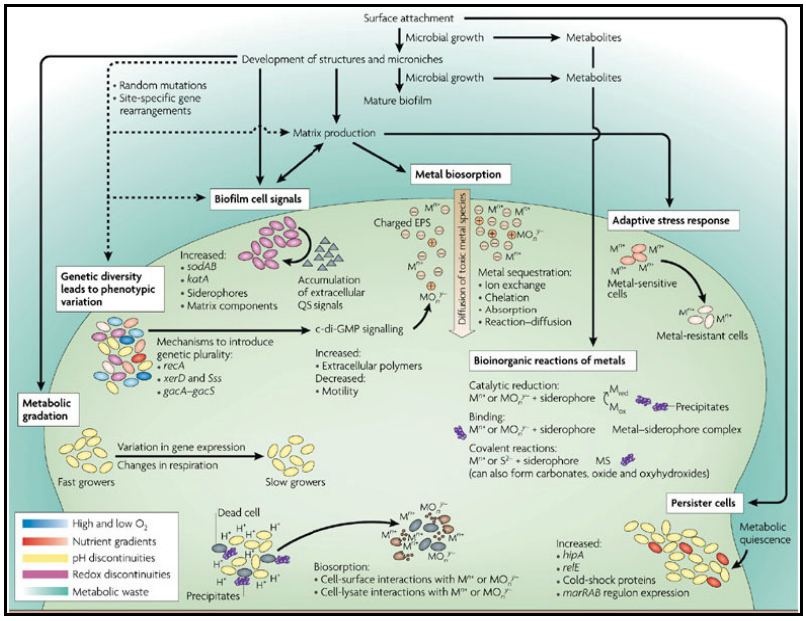

Fig. 2.6 Illustrations of the processes taking place during the multimetal resistance and tolerance in microbial biofilms (Harrison *et al.,* 2007).

2.5.4 Application of enzymes for biofilm control

The polymeric matrix that anchors the cells constitutes a penetration barrier to biocides (Pozos and Pater, 2007), decreasing their potency in comparison to the planktonic cells while promoting microbial resistance (Mah and O'Toole, 2001; O'Toole and Sterwart, 2005). The cells inside the biofilms have lower acess to nutrients and thus a slower growth rate, becoming more protected to the majority of antibiotics biocidal agents (de Carvalho, 2007).

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Studies have shown chemical biocides as the main strategy of biofilms control to kill the attached microorganisms from the surface (Rao *et al.,* 1998; Ramesh *et al* 2002; Smith, 2005). In industrial systems, it is important that both the inactivation of the microorganisms and the removal of biofilms from the surfaces are achieved (Simoes *et al.,* 2003).

A wide range of cleaners and sanitizers are available for use in line cleaning at retail outlets, however these cleaning chemicals are not successful in removing an established biofilms (Walker *et al.,* 2007). Studies have indicated that disinfection with chlorine dioxide and chlorine (der Beer (1994), for example can reduce the concentrations of planktonic bacteria, but have little to no effect on the concentrations of biofilm bacteria (Berry *et al.,* 2006). The mechanism behind the observed resistance of biofilm cells to disinfection is still unknown, although hypotheses include mass transfer resistance, the formation of persister cells and protection due to the production of extracellular polymeric substances (Berry *et al.,* 2006; Walker *et al.,* 2007).

The tolerance of biofilms to antimicrobials combined with their complex architecture and dynamic nature makes them quite difficult to measure, monitor and control and thus reduces the effectiveness of treatment strategies (Vickery *et al.,* 2004). An alternative method could be the mechanical cleaning but the disadvantage of this method is that, it is costly as it involves equipments down time and also significant labour expenditure (Vickery *et al.,* 2004).

Thus, the application of enzymes to degrade the EPS of the biofilms is an attractive method in many industries where complete biofilms removal is essential, but due to the heterogeneity of the extracellular polymeric substances in the biofilms, the application may turn ineffective. However, studies by Augustin *et al.* (2004) showed that the use of enzymes for removal of bacterial biofilms is still limited partly due to the very low prices of biocide chemicals in use. Augustin *et al*. (2004) also indicated that the lack of techniques for quantitative evaluation of the effect of enzymes as well as the commercial accessibility of different enzyme activities limits their use.

Nonetheless, Walker *et al.* (2007) indicated that in order to design enzymes which target the EPS of the biofilms, it is important to have an understanding of the nature of the EPS. Xavier *et al.* (2005) further explained that enzymes remove biofilms directly by destroying the physical integrity of the biofilm matrix (EPS).

The effect of any one enzyme degrading any one EPS will depend on the structure of the EPS in the biofilms. Thus degrading EPS from the biofilms can result in the release of

cells of one species while on the other hand, enhancing the growth of other species present in the biofilm (Walker *et al.,* 2007). This however happens in a mixed culture biofilm in which enzymatic treatment can either strengthen or weaken the EPS structure. This depends on the mode of action on individual EPS and the role of that EPS provides in the biofilms (Walker *et al.,* 2007).

While there are several studies on enzyme degradation of mature biofilms using synthetic polysaccharases, studies investigating the use of enzymes in inhibiting biofilm formation on surfaces are very scarce (Loiselle *et al.,* 2003; Vickery *et al.,* 2004, Xavier *et al.,* 2005). Previous studies have shown that cellulose from *Penicillium funiculusum* is one of the effective enzymes in degrading mature biofilms of *Pseudomonas aeruginosa*. Cellulose was also found to be useful in degrading the exopolysaccharides from *Pseudomonas fluorescens* (Loiselle *et al.,* 2003; Vickery *et al.,* 2004).

Wiatr (1991) tested five enzymes in the biofilm removal reactor (BRR) and among those enzymes was a combination of one protease and alpha - amylase and beta – glucanase. This enzymatic mixture was effective in digesting slime layers produced by cultures of pure (*Pseudomonas species*) and mixed strains of bacteria. Among 24 preparations of the enzymes, only three types of enzymes were found to be effective for the removal of slime produced by *Pseudomonas* bacteria which were alpha-amylase, protease and the combination of amylase, glucanase and protease.

Many fungi also degrade complex plant cell wall material by secreting a large variety of enzymes (Orgaz *et al.,* 2006). This versatility makes commercial polysaccharide degrading enzyme mixture to have a wide spread use in various fields such as in fruit processing or wastewater treatment. Fungal enzymes could possibly be used for the degradation of bacterial biofilm matrices as well (Orgaz *et al.,* 2006).

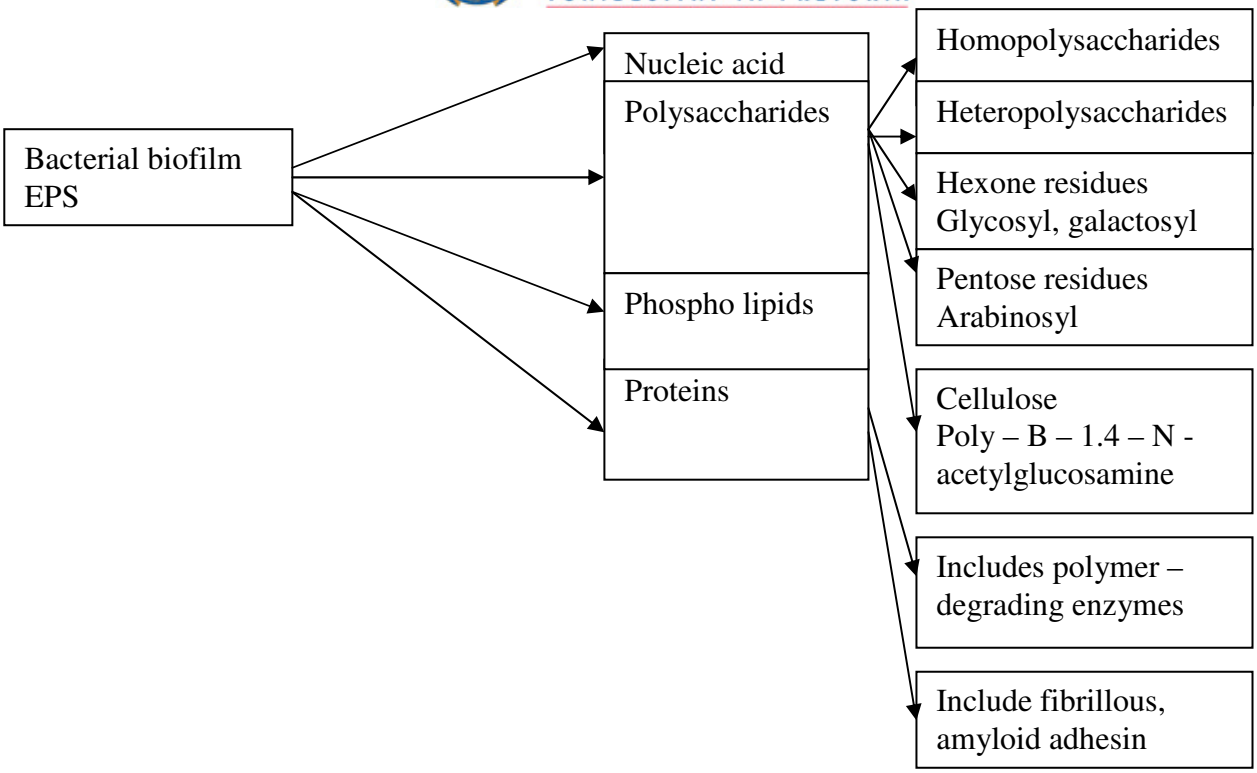

Fig. 2.7 Schematic overview of the structural components of extracellular polymeric substances (EPS) involved in biofilm formation (Kristensen *et al.,* 2008).

Table 2.1 Available enzymes used for the control of biofilms (Xavier *et al.,* 2005)

Agent	**Origin**	**Substrate**	**Action**	**Reference**
Enzymes				
Crude cellulose preparation	*Trichodema viridae* (Maxazyme CL2000)	Dephosphorylared and partially deharmnosylated EPS of *Lactococcus lactis* subsp. Cremoris B40	1. EPS was incubated with various commercial enzymes preparations and analyzed for degradation. In crude enzyme preparation tests, one enzyme acted very specifically.	1. Van Casteren *et al.,* 1998
Polysaccharide depolymerase	Bacteriophage	*Enterobacter agglomerans* GFP in monospecies biofilms and in the dual species biofilms with *Klebsiella pneumoniae* G1	1. Phage glycanase are very specific. Action of enzyme was observed when added to the phage susceptible monospceies biofilms leading to substantial biofilms degradation.	1. Hughes *et al.,* 1998 2. Skillman *et al.,* 1996

			2. A 60 min treatment with a polysaccharase caused a 20% reduction in dual species biofilms adhesion	
Alginate lyase	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* alginate	1. Strains of *P. aeruginosa* overproducing alginate lyase detached at a higher rate than the wild type. 2. However, other studies showed that addition of alginate lyase to established *P. aeruginosa* biofilms caused no observable detachment	1. Boyd and Chakrabarty, 1994 2. Christensen *et al.,* 2001
Disaggregatase enzyme	*Methanosarcina mazei*	*Methanosarcina mazei* heteropolysaccharide capsule mediating cell aggregation	1. Conditions that are generally unfavourable for growth are associated with disaggregatase activity	1. Xun *et al.,* 1990
Esterase with wide	Wide range of bacteria	Acyl residues from	1. Acetyl residues from	1. Sutherland 2001

specificity		bacterial polymers as well as other esters	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*. 2. deacetylated p-nitrophenyl propionate, naphthyl acetate, isopropenyl acetate and triacetin. Esterases could alter the physical properties of a biofilms structure	2. Cui *et al.,* 1999
Dispersin B (DspB)	*Actinobacillus actinomycetemcomitans*	Poly-ß-1,6-GlcNAc implicated as an adhesion factor for biofilms of several bacterial species	1. Causes detachment of cells from *A. actinomycetemcomitans* biofilms and disaggregation in solution.	1. Kaplan *et al.,* 2003 2. Kaplan *et a.1,* 2004 3. Itoh *et al.,* 2005

			2. Treatment of *S. epidermidis* biofilms with dispersin B caused dissolution of the EPS matrix and detachment of biofilms cells from the surface. 3. Disrupts biofilms formation by *E.coli, S.* epidermidis, Yersina pestis and Pseudomonas fluorescens.	
DNase 1	Commercial (Sigma-Aldrich)	Extracellular DNA in *Pseudomonas aeruginosa* biofilms	DNase affects the capability of P. aeruginosa to form biofilms when present in the initial deveoplment stages. Established biofilms were only affected to a minor degree by the presence of DNase	Whitchurch *et al,* 2002
Mixture of enzymes	Commercial	*S. aureus*, *S. epidermis*, *P. fluorescens* and *P.*	1. Pectinex UntraSP (Novo Nordisk A/S, a	Johansen *et al.,* 1997

		aeruginosa biofilms on steel and polypropylene substrata	multicomponent enzyme preparation) reduced the number of bacrterial cells in biofilms on stainless steel without any significant bactericidal activity (the activity of Pectinex Ultra is mainly a degradation of extracellular polysaccharides)	

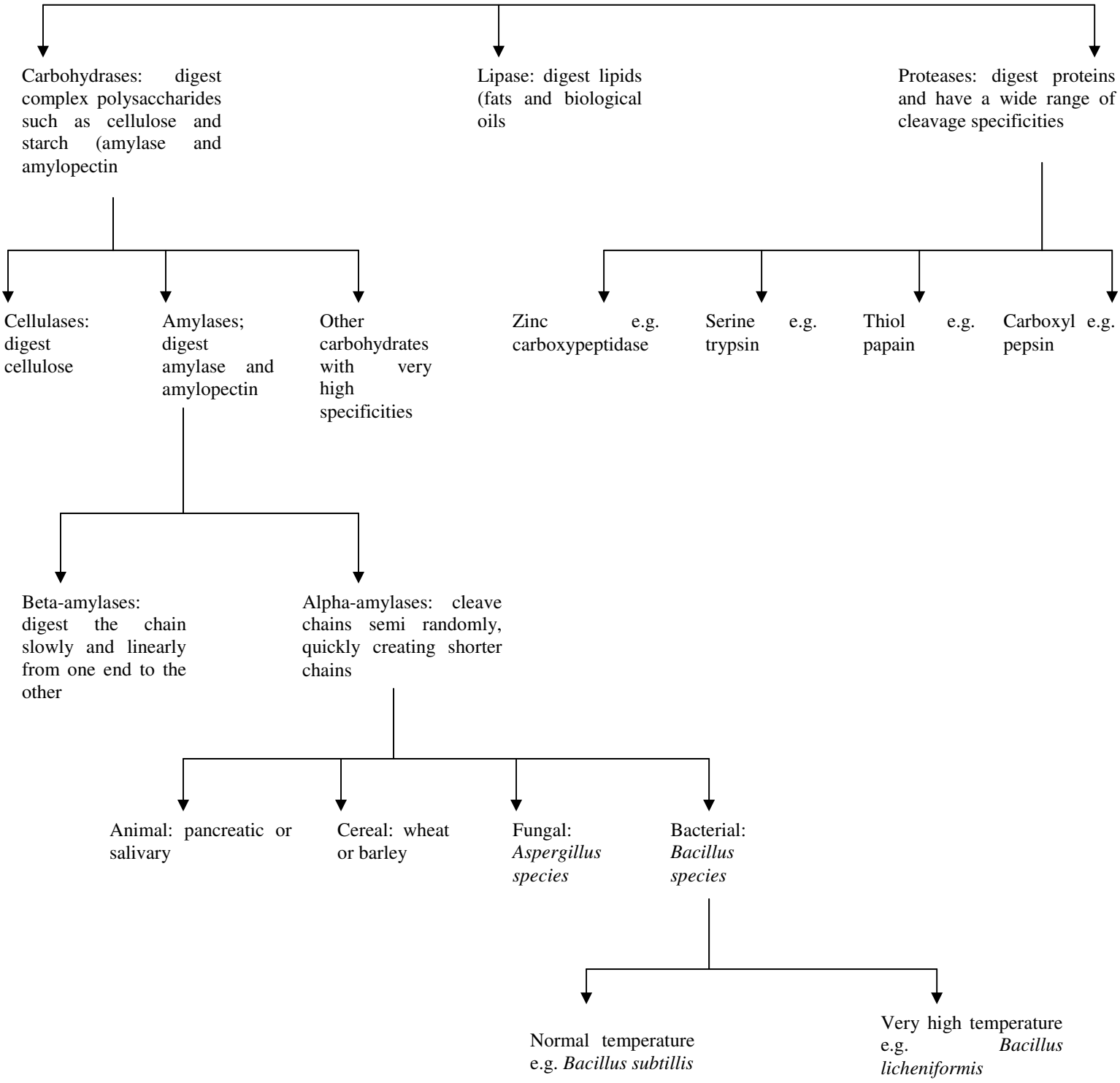

Fig. 2.8 Illustrations of various α-amylases with applications to conservation practise (Harold, 1992).

Table 2.2 The general composition of some bacterial extracellular polymeric substances (EPS) including humic substances (Wingeder *et al.,* 1999; Flemming *et al.,* 2000)

EPS	Principal components (subunits, precursors)	Main type of linkage between subunits	Structure of polymer backbone	Substituents
Polysaccharides	Monosaccharide	Glycosidic bonds	Linear branched side chain	Organic: ✓ O-Acetyl ✓ N-Acetyl ✓ Succynyl ✓ Pyruvyl Inorganic: ✓ Sulphate ✓ Phosphate
Proteins (polypeptide)	Amino acid	Peptide bonds	Linear	Oligosaccharides ✓ glycoproteins Fatty acids ✓ lipoproteins
Nucleic acid	✓ Nucleotides	Phosphodiester	Linear	_
(Phospho)lipids	✓ Fatty acid ✓ Glycerol ✓ Phosphate ✓ Ethanolamine ✓ Serine choline	Ester bonds	Side chains	_
Humic substances	✓ Pholic compounds ✓ Simple sugars ✓ Amino acid	Ester bond- C C- bonds Peptide bonds	Cross liked	_

Summarised literature review

Biofilms are characterized by surface attachment, structural heterogeneity; genetic diversity; complex community interactions and an extracellular matrix of polymeric substances. Biofilms deposit and adhere to all surfaces that are immersed in aqueous environments. A number of parameters including reactor type, substrate composition, substrate loading rate, hydraulic retention time, hydrodynamic shear force, culture temperature etc have been indicated to facilitate the production of biofilm EPS. EPS is highly hydrated and consists of a wide variety of materials including polysaccharides, proteins, nucleic acid, uronic acid and humic substances. The exopolysachharides (EPS) synthesized by microbial cells vary greatly in their composition and in their chemical and physical properties. This implies that the EPS composition is variable and is related to microbial species, the physiological state of the bacteria and the operating conditions under which biofilms are developed. The EPS formation rate is proportional to the rate of substrate utilization. Chemically, the bacterial EPS is a heterogeneous polymer containing a number of distinct monosaccharides and non carbohydrate substituents, many of which are strain specific. One of the major components of the bacterial EPS is the uronic acids costituting up to 20 – 50% of the polysaccharide fraction.

EPS are complex mixture of high molecular polymer (M_w>10,000) excreted by microorganisms, products from lysis and hydrolysis and adsorbed organic matters from wastewater. Such a polymer network has a vast surface area and is capable of absorbing pollutants, nutrients and minerals. EPS plays an important role in the flocculation of bacterial cells and provides energy and carbon when substrates are in short supply. The EPS compostion is also related to the characteristics of waste water. For example, EPS has high protein and DNA concentrations in protein grown granules while high polysaccharide concentrations are found in granules grown in other type of organic substrates. Nitrogen limiting conditions favour the production of EPS which in turn accelerates anaerobic granulation. The production of EPS is known to be affected by nutrients status of the growth medium and the availability of carbon. The bacterial extracellular polysaccharides are composed of homo and heteropolysaccharides including glucose, fructose, mannose, galactose, puryvate and mannuronic acid or glucoronic acid

based complexes. The exopolysaccharides are held to the cell wall of the bacteria either by linkage between the carboxyl gropus of the EPS and hydroxyl groups of lipopolysaccharides or by a covalent bonding through phospholipids and glycoproteins.

The activated sludge process has long been employed to treat a wide variety of wastewater and a number of enzymes including aminopeptidase, galactosidase, glucosidase, lipase and phosphatase and protease have been extracted from sludge. These enzymes found in sludge may originate from the effluent sewage, from the sludge itself or even as actively secreted extracellular enzymes. Proteolytic, lipolytic and cellulolytic enzymes synthesized within bacterial cells are secreted into the extracellular environment and hydrolyse the absorbed macromolecules into small units that can be transported across the cell membrane and then metabolized. Protease, α- amylase and α glucosidase play important role in the biological waste water treatment.

Monitoring and control of biofilms accumulation remains the challenging task to many industries. Chemical biocides as the main strategy of biofilms control to kill the attached microorganisms from the surface. However, these antimicrobial agents fail to remove the biofilm cells due to the extracellular polymeric substances (EPS) that prevent the penetration of the antimicrobial agents within the biofilm cells, thus decreasing the potency of the biocides. The tolerance of biofilms to antimicrobials combined with their complex architecture and dynamic nature makes them quite difficult to measure, monitor and remove thus reduces the effectiveness of treatment strategies. Enzymes have been proven to be effective in the degradation of the biofilm EPS. The manner in which enzymes degrade the biofilm EPS is through binding and degradation of the EPS constituents and converting them into smaller units that can be transported through the cell membranes and then be metabolized.

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