The Importance of Lipids to Biofilm Formation

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Thesis to obtain the Master of Science Degree in

Biotechnology

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November 2014
Acknowledgements

I would like to thank all those who directly or indirectly contributed to this thesis and supported me during the last year.

First, I would like to express my thanks to my supervisor Dr. Carla de Carvalho for the proposal of this thesis, the support and guidance along the last year. For sure, the knowledge acquired with her in the last year will contribute for my future career.

I would like to say thank you to the BEBL group and the department of Bioengineering for receiving me in their laboratories and for support in the thesis.

I would like to acknowledge Fundação para a Ciência e a Tecnologia (FCT) for partially funding this studies through the project “Defects Detection in Microfabrication With Bacterial Cells” (PTDC/EME-TME/118678/2010).

I would like also to say thank you to my colleagues of the Master degree in Biotechnology that helped me in this journey. Especially to Fábio Gonçalves that shared the laboratory with me.

At last but no less important, I would like to express my gratitude to my family and friends that were indispensable to achieve my final thesis work.

Thank you!
Abstract

In nature, most of the microorganisms grow attached to surfaces as biofilms, which are communities of cells encased in an extracellular matrix conferring protection against environmental hazards. Biofilms are responsible for industrial problems and are related to hospital infections. The *Rhodococcus* and *Mycobacterium* genera, unlike other microorganisms, produce an extracellular matrix rich in lipids. The aim of this work was the elucidation of the role of the lipids of *Rhodococcus erythropolis* DCL14 during cell attachment to surfaces and biofilm formation. Adhesion assays were performed to identify the variations in physicochemical cell surface properties. The type of surface, the medium and the carbon source used for biofilm growth influenced its architecture. On tubular surfaces, the decrease of cell hydrophobicity was connected to the contact angle of the surface tested. Also, the biofilm formation was influenced by the growth phase of the initial cells, as well, by the presence of limonene. In a biofilm reactor, the cells showed surface charge and membrane composition similar to planktonic cells along time. The development of a flow cell system allowed the visualization of biofilm behaviour and architecture *in situ* and in real time. Moreover, strongly adherent cells increased the saturation of the membrane and the surface charge compared to weakly adherent cells. Under specific conditions, the increase of 10-methyl branched fatty acids on cell membrane correlated with the increase of biofilm formed on several surfaces. The results obtained in this study may contribute to a greater understanding of the complexity of biofilms.

**Keywords**: biofilm, lipids, surface charge, adhesion, flow cell
Resumo

Na natureza, a maioria dos microorganismos crescem aderidos a superfícies como biofilmes, que são comunidades de células envoltas numa matriz extracelular que confere proteção contra agressões ambientais. Os biofilmes são responsáveis por problemas industriais e estão relacionados com infeções hospitalares. Os géneros *Rhodococcus* e *Mycobacterium*, ao contrário de outros microorganismos produzem uma matriz extracelular rica em lípidos. O objectivo deste trabalho foi elucidar o papel dos lípidos de *Rhodococcus erythropolis* DCL14 durante a adesão celular e a formação de biofilmes em superfícies. Ensaios de adesão foram realizados para identificar variações nas propriedades físico-químicas da superfície celular. O tipo de superfície, o meio e a fonte de carbono onde os biofilmes cresceram influenciaram a sua arquitetura. Nas superfícies tubulares, o decréscimo da hidrofobicidade celular foi relacionada com o ângulo de contacto da superfície. A formação de biofilme foi também influenciada pela fase de crescimento das células iniciais, e pela presença de limoneno. Num reactor, as células do biofilme apresentaram carga superficial e composição de membrana semelhantes às células plantónicas ao longo do tempo. A construção de um sistema de *flow cell* permitiu a visualização do comportamento do biofilme e a sua arquitetura *in situ* e em tempo real. Além disso, células fortemente aderidas aumentaram a saturação da membrana celular e a carga da superfície comparativamente a células fracamente aderidas. Em condições específicas, o aumento na membrana celular de ácidos gordos 10-metil ramificados correlacionou-se com o aumento do biofilme formado em várias superfícies. Os resultados obtidos neste estudo poderão contribuir para compreender melhor a complexidade dos biofilmes.

**Palavras-chave:** biofilme, lípidos, carga da superfície, adesão, *flow cell*
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## Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>10MBFAs</td>
<td>10-methyl branched fatty acids</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminium</td>
</tr>
<tr>
<td>AMPs</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>Bap</td>
<td>Biofilm associated surface proteins</td>
</tr>
<tr>
<td>BCFAs</td>
<td>Saturated iso- and anteiso-methyl branched fatty acids</td>
</tr>
<tr>
<td>CaP</td>
<td>Calcium phosphate</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CSTR</td>
<td>Continuous flow stirred-tank reactor</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DFO-Ga</td>
<td>Desferrioxamine-gallium</td>
</tr>
<tr>
<td>DMAFAs</td>
<td>Dimethylacetals fatty acids</td>
</tr>
<tr>
<td>DNA</td>
<td>Extracellular deoxyribonucleic acid</td>
</tr>
<tr>
<td>eDNA</td>
<td>Extracellular deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>FA(s)</td>
<td>Fatty acid(s)</td>
</tr>
<tr>
<td>FAMEs</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatograph</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HSFAs</td>
<td>Hydroxy substituted fatty acids</td>
</tr>
<tr>
<td>MH</td>
<td>Muller-Hinton medium</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>MM</td>
<td>Mineral medium</td>
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<tr>
<td>MUFAs</td>
<td>Mono-unsaturated fatty acids</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Pb</td>
<td>Lead</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal components analysis</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Poly-unsaturated fatty acids</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxide species</td>
</tr>
<tr>
<td>SCBFAs</td>
<td>Saturated cyclopropyl-branched fatty acids</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SSFAs</td>
<td>Saturated straight chain fatty acids</td>
</tr>
<tr>
<td>SWNTs</td>
<td>Single-walled carbon nanotubes</td>
</tr>
<tr>
<td>TAGs</td>
<td>Triacylglycerols</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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Chapter 1

General Introduction

Context of the Work

Objectives and Thesis Outline

Literature Review

1. The Importance of Actinomycetes
2. Biofilms
   2.1. The Discover of Biofilms
   2.2. Biofilm Development and Life Cycle
   2.3. The Biofilm Extracellular Matrix
   2.4. Factors Affecting Cell Attachment and Biofilm Formation
3. Combating Biofilms
   3.1. The Problem and Challenges
   3.2. New Solutions and Strategies
4. Studying Biofilms in Laboratory
   4.1. Growing Biofilms
   4.2. Monitoring Biofilms
5. References
Context of the Work

Microorganisms are found in most of the surfaces of the world, from the most remote areas, such as deserts and volcanoes, to our food and body. It is estimated that 99% of microbes are on the surfaces in biofilms [1]. Biofilms are characterized by communities of cells that are encased in an extracellular matrix that protect the cells against environmental aggressions and are among the most successful forms of life in nature.

Only a small number of studies regarding biofilms are focused on gram positive bacteria. One of the genus of interest to study is the *Rhodococcus* genus which comprise strains (e.g. *Rhodococcus erythropolis*) that could be used in bioremediation processes to degrade xenobiotics and which have recently be related to hospital infections (e.g. *Rhodococcus equi*) because of their natural tolerance against antibiotics and disinfectants. These bacteria belong to the actinomycete group, and to the mycolata taxon which contains bacterial species with mycolic acids on their cell walls and produce an extracellular matrix rich in lipids. Few studies have addressed the importance and role of lipids on cell adhesion and consequently on the formation and development of a mature biofilm.

Previous work conducted at the Institute for Biotechnology and Bioengineering (IBB), showed the capacity of *R. erythropolis* DCL14 to adapt the fatty acid composition of their cell membrane to different carbon sources [2]. In other study, the use of carveol and carvone to influence the fatty acid composition of the cell membrane was shown to reduce *R. erythropolis* biofilm [3]. In 2009, the comparison of surface membrane properties in planktonic and biofilm cells was assessed using different alkanes as carbon source [4]. Different alterations between the two life styles were observed, including production of surface active compounds and substrate-dependent alterations of physicochemical cell surface properties.

The studies proposed in this work derived from the need to further understand the role and importance of lipids in biofilms. The elucidation of their role could contribute to better bioremediation processes and to the control of biofilm related medical infections.
Objectives and Thesis Outline

In order to elucidate the role of lipids during cell attachment to surfaces and biofilm formation, the objectives of the master studies were the following:

i. to develop a system allowing in vivo visualization of lipids during cell adhesion to surfaces (including design of a microreactor allowing fluorescence microscopy imaging and testing of adequate fluorophores);

ii. to understand the molecular events that determine the attachment of previously planktonic cells;

iii. to determine the identity of the lipids produced by the cells during the formation of the matrix;

To achieve such goals, the work included the following tasks: the development of flow chamber and microscale reactors for biofilm development and monitoring; the assessment of cell morphology and lipid localization by fluorescence microscopy and image analysis; evaluation of cell surface properties; and, lipid extraction and fatty acid analysis by gas chromatography.

This thesis is divided in 5 Chapters:

Chapter 1 - the context of this work, the objectives and the literature review, focusing the current knowledge of biofilms, the strategies to demote their formation and techniques to study bacterial biofilms are presented.

Chapter 2 - the general methodologies used in this thesis are listed, including the experiments for studying bacterial adhesion and biofilm formation on different surfaces and the effect of the fatty acid composition of the cell membrane. The experiments used included a 6-well plate assay, a recirculation tube system, biofilm reactors and flow cell systems.

Chapter 3 - the results from the experiments conducted during the master thesis studies and their discussion are presented.

Chapter 4 – summary of the principle conclusions derived from the thesis and presentation of suggestions for future work.

Chapter 5 – supplementary information is presented.
Chapter 1

General Introduction

Literature Review

1. The Importance of Actinomycetes

Actinobacteria is a phylum comprising gram-positive bacteria able to cause important infections (e.g. Mycobacterium tuberculosis, M. leprae, Corynebacterium diphtheria) but also able to produce secondary metabolites industrially important (e.g. antibiotics) and to convert and degrade recalcitrant xenobiotics.

The principal contribution to the drug discovery and development in the last seven decades came from microbial natural products [5]. The foundation for development of microbial natural products as drugs started with the discovery of penicillin in 1928 by Fleming [6] and its progressive introduction into medicine in the 1940s [7]. The first antibiotics from actinomycetes were reported in the same decade (1940, actinomycin [8]; 1942, streptothricin [9]; 1943, streptomycin [10]). In the end of the last century, numerous compounds have been reported with medical applications, acting as antibacterial, antifungal, antiparasitic, and/or anticancer agents [11].

Of the total bioactive secondary metabolites reported, 45% are produced by actinomycetes and from these, the principal producer are the species of the genus Streptomyces (75%) [5, 12]. This data reveal the importance of actinomycetes in combating infectious diseases.

Despite the problematic cases of antibiotic-resistance bacteria, it is believed that the utility of natural products as sources of novel structures is still alive and the anti-infective area depends on natural products and their structures [13]. Indeed, in the last years new efforts have been done to found new and unexploited actinomycetes. Scientists are looking for rare actinomycetes in remote areas, such as in marine environments, volcanic zones, glaciers, extreme arid and salted areas, with the objective of finding new therapeutical agents [14].

In addition to antibiotic production, actinomycetes have great potential for bioremediation processes. For instance, Alvarez et al. described a strategy using three actinomycete strains to clean up soils contaminated with organic pollutants, such as crude oil [15]. In other study, a Streptomyces sp. M7 strain was used to degrade an organochlorine pesticide (γ-HCH) with carcinogen potential [16]. The potential of Rhodococcus erythropolis for bioremediation applications has been described in several studies [2, 17, 18], including a study by de Carvalho et al. describing the capability of Rhodococcus erythropolis.
**Erythropolis** to utilize C5 to C16 n-alkane hydrocarbons as sole source of carbon and energy [4].

In nature, most cells grow as biofilms which are communities of cells embedded in a “sticky” matrix (discussed further in this work) and not in planktonic state as normally studied in laboratory [19]. In fact, Morikawa [20] drew attention to the need of new cultivation strategies, particularly strategies mimicking the natural habitats of microorganisms, with niche-mimicking bioreactors that can be used to elicit the production of secondary metabolites by apparent nonproducers.

Actinomycetes, in particular *Rhodococcus* and *Mycobacterium*, unlike other microorganisms, produce an extracellular matrix rich in lipids including long chain fatty acids and mycolic acids [21]. As Ojha *et al.* [21] showed, the influence of lipids in *Mycobacterium tuberculosis* biofilm formation can lead to the development of new strategies against the infections caused by this microorganism. In the same direction, understanding the influence of lipids in adhesion and biofilm formation on actinomycete producers of antibiotics or enrolled in bioremediation processes, such as *R. erythropolis*, can lead to new discoveries and applications in these areas.

2. **Biofilms**

2.1. **The Discover of Biofilms**

In the seventeenth century, Antonie van Leeuwenhoek observed in his primitive microscope some kind of life that he called “animalcules”. The isolates, removed from his own teeth that contained these “animalcules” are what we know today as bacteria of the dental plaque. This first documented study of dental plaque can be considered the first evidence of the existence of microbial biofilms [22].

After the brilliant work of Antonie van Leeuwenhoek, only in 1940 some advances were carried out in biofilm research. Heukelekian and Heller [23] observed that bacterial growth and activity were substantially enhanced by the incorporation of a surface to which these organisms could attach: the called “bottle effect” for marine microorganisms was observed. Also, Zobell noted that aquatic bacteria were more numerous on the solid surfaces of sample containers than as single suspended cells [24].

Despite these two documented studies on biofilms, the interest in the physical and chemical properties of biofilm only began in 1960s and early 1970s. Using electron microscopy Jones *et al.* [25] showed that biofilms are composed of different...
microorganisms and also concluded that the extracellular matrix was mainly composed by polysaccharides. Characklis referred to biofilms as tenacious and highly resistant after his studies on microbial slimes present in industrial water [26]. In 1978, Costerton et al. showed that many bacteria spent the majority of their life in sessile communities attached to surfaces and proposed some mechanisms of microbial adherence [27]. Further studies of diverse areas in biofilms focused on the composition of the polymer matrix [28, 29].

Researchers have been defining biofilms in various ways. Characklis and Marshall (1990) defined a biofilm as consisting of “cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin” [30]. In 1995, Costerton et al. defined biofilms as “matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” and presented the “basic model” for biofilms [31]. In this model, microorganisms form microcolonies that are surrounded by an extracellular matrix interspersed with open water channels. Other way of defining biofilms is in terms of what they are not: single cells homogeneously dispersed in fluids [32].

In the last decade, significant advances in the understanding of the behaviour of biofilms have been achieved and will be addressed further in this work.

2.2. Biofilm Development and Life Cycle

Most studies regarding bacteria were made using planktonic cells. However, bacteria are predominantly attached to surfaces in an extracellular matrix that we refer as biofilms.

The formation of a biofilm is divided in the following steps: migration of planktonic cells into a surface proximity, attachment of cells to a surface, growth of biofilm and detachment of portions of the biofilm for colonization of new surfaces (Figure 1.1) [30, 33-35].

Figure 1.1 – Steps of biofilm development.
1 – Interaction of planktonic cells with surface.
2 - attachment of cells to the surface.
3- Growth of biofilm.
4- Detachment of cells/portions of biofilm.
(Adapted from Ref [36])
During the first step, cells interact with surfaces by Vander Waals, hydrophobic and electrostatic forces that are dependent on the nature and the distance between cells and surfaces [37]. When cells adhere to surfaces (step 2) they start to grow and to produce an extracellular polymeric substance (EPS) which will form a matrix. The biofilm structure further increases with the attachment of cells and other particulate matter leading to a complex and dynamic structure originating a mature biofilm (step 3). The detachment of portions of biofilm and of daughter cells into the bulk water allow the colonization of new surfaces (step 4) [30]. Depending on environmental conditions or strain, biofilms can have a "mushroom shape" or a "carpet-like" form [35].

The heterogeneous structure of biofilms include some characteristics such as cell clusters, void spaces, water channels to supply nutrients and remove wastes, and slime streamers that are affected by flow of the fluid surrounding the biofilm [38].

### 2.3. The Biofilm Extracellular Matrix

The principal characteristic of biofilms is the production of sticky polymeric substances in which microbial cells are embedded. When compared planktonic cells, this is the most obvious difference. EPS protects the cells against several environment aggressions such as fluctuating pH [39], exposure to UV light [40], dehydration [41] and antimicrobial agents [42, 43]. EPS is difficult to characterize because their composition varies depending on the organisms present in the biofilm and on environmental parameters [35]. The principal composition of EPS in most of the bacterial species are polysaccharides, but they may also contain proteins, nucleic acids, lipids and other biopolymers such as humic substances [44]. In most biofilms, the matrix can account for over 90% of the dry mass, whereas the microbial cells account for less than 10% [44]. The EPS determines a life style very different from planktonic cells. In terms of architecture, the biofilms can be smooth and flat, rough, fluffy or filamentous [44].

**Water**

Water is by far the largest component of the biofilm matrix, reaching up to 97% of the mass [44, 45]. The EPS matrix provides a highly hydrated environment that dries slower than its surroundings and therefore protects the biofilm cells against fluctuations in water potential and desiccation [46]. In fact, desiccation seems to be one of the environmental conditions under which EPS provides evident global benefits to both EPS producers and other members of the biofilm community. Besides, the EPS matrix can also act as a
molecular sieve, sequestering cations, anions, apolar compounds and particles from the water phase [47].

**Exopolysaccharides**

Polysaccharides are the most studied components of the EPS matrix since they constitute the major fraction in most bacterial species [48]. Most of them are characterized by long molecules, linear or branched, with a molecular mass of $0.5 \times 10^6$ Daltons to $2 \times 10^6$ Daltons [44]. Exopolysaccharides can be classified as homopolysaccharide or heteropolysaccharide depending on their structure. The homopolysaccharides are composed by sucrose-derived glucans and fructans, produced by the *Streptococci* in oral biofilms and cellulose produced by *Gluconacetobacter xylinus*, *Agrobacterium tumefaciens*, *Rhizobium spp.* and other species [44]. Nevertheless, a big percentage of exopolysaccharides are heteropolysaccharides composed by a mixture of neutral and charged sugar residues. Some of the most well-known, such as alginate or xanthan are negatively charged (polyanionic) [44]. Few are positively charged (polycationic), such as intercellular adhesin, which is composed of $\beta$-1,6-linked N-acetylglucosamine with partly deacetylated residues, produced by *Staphylococcus aureus* and *S. epidermidis* and related to medical infections [49].

Different exopolysaccharides can be found between strains of a single species. For example, *P. aeruginosa* strains produce at least three different expolysaccharides (alginate, Pel and Psl) that contribute for biofilm development and architecture [50].

Several polysaccharides are attached to the cell surface and form complex networks as fine strands. Also, polymer strands can interact with each other, forming a viscous aqueous solution with gel-like properties [35].

**Extracellular Proteins**

The percentage of extracellular proteins in the matrix can vary widely relatively to other biopolymers. Enzymatic proteins are involved in the degradation of biopolymers that include water-soluble polymers (e.g. polysaccharides, proteins and nucleic acids), water-insoluble compounds (e.g. cellulose, chitin and lipids) and organic particles trapped in biofilms [51]. According to Flemming et al. [44] the low-molecular-mass products may be used as carbon source by cells. Other enzymes are involved in the detachment of bacteria from biofilms, through the degradation of structural EPS, contributing to cell
dispersion and colonisation of new surfaces [44]. Moreover, some enzymes are involved in infectious processes actuating as virulence factors. On the other hand, lectins (non-enzimatic proteins) are essential for the formation and stabilization of the matrix and constitute a bridge between the EPS and bacterial surface [44]. Some studies described these binding proteins in dental biofilms of *Streptococcus mutans* [52], matrices of activated flocs [53] and on outer membrane of *Azospirillum brasiliense* [54].

Biofilm associated surface proteins (Bap) are other type of extracellular proteins. These high-molecular-mass proteins are present on the cell surface of many species promoting biofilm formation [55].

**Extracellular DNA (eDNA)**

Biofilms of various origins have been found to contain eDNA as an integral part of EPS, although the amount produced, its origin, and its localization can vary even between closely related species [44]. In genus *Rhodovulum*, eDNA is important for microbial aggregation, allowing flocculation of bacteria. When this marine photosynthetic bacteria was treated with nucleolytic enzymes the result was deflocculation, but with enzymes directed to the degradation of proteins and polysaccharides the bacteria kept the flocculation ability [56]. In *P. aeruginosa*, eDNA is fundamental for biofilm formation, acting as intercellular connector, stabilizing the biofilm. Once grown in the presence of DNase I, biofilm formation was inhibited [57]. The eDNA has also antimicrobial activity, causing cell lysis by chelating cations that stabilize lipopolysaccharide and the bacterial outer membrane [58].

The origin of eDNA comes mainly from lysed cells, although the excretion of DNA is also an hypothesis. In an aquatic bacterium, biofilm eDNA presents major similarities in banding patterns with genomic DNA but also some significant differences suggesting that eDNA is not the result of autolysis but obviously of some active production and transport system. [59]. In a study aimed at evaluating eDNA in single and multispecies biofilms (involving *Pseudomonas aeruginosa*, *P. putida*, *R. erythropolis* and *Variovorax paradoxus*), the authors concluded that eDNA production was species dependent and the phylogenetic information present in the DNA pool was distinct from either total or cellular DNA [60].
Lipids and Biosurfactants

Some species produce EPS with hydrophobic properties. For example, a *Rhodococcus* sp. strain, that possesses a capsule but no fimbriae, can adhere to Teflon and colonizes waxy leaf surfaces using EPS with hydrophobic properties [61].

In activated sludge, lipids were found in the matrix (around 1.8%) of yeasts, fungi, sulfate-reducing bacteria, gram-positive and gram-negative bacteria, and, in lesser quantities, of mycobacteria [62]. Lipopolysaccharides are involved in microbial adherence of *Thiobacillus ferrooxidans* to pyrite surfaces [63], and *Serratia marcescens* that produces extracellular lipids with surface-active properties [64]. Other lipids with surface-active EPS include surfactin, viscosin and emulsan, which can disperse hydrophobic substances and make them bioavailable. They may be useful for microbial enhanced oil recovery and for bioremediation of oil spills [44]. In *Rhodococcus erythropolis* DCL14, the surface tension of the culture supernatant decreased with increasing chain length of the alkane that cells were using as sole carbon and energy source, reaching 23 and 37 mN/m when the cells used *n*-hexadecane in planktonic and biofilm state, respectively [4].

Ron and Rosenberg [65] referred that biosurfactants are produced by diverse microorganisms with different characteristics and therefore may have different roles during the growth of the producers. The same author suggested numerous roles of biosurfactants, including antimicrobial activity, virulence factors and binding of heavy metals.

2.4. Factors Affecting Cell Attachment and Biofilm Formation

The adhesion of bacterial cells to a surface in a solid-liquid interface is a key stage during biofilm formation. The adhesion is affected by some factors such as: substratum, conditioning films forming on substratum, hydrodynamics, and various properties of both cell and material surfaces [22].

Substratum Effects

Busscher and Weerkamp [37] described the process of interaction between cells and solid surfaces. According to him, at large (>50nm) and intermediated separation distances (10-20nm), adhesion is mediated by macroscopic surface properties as surface free energy and surface charge, respectively. At large separation distances, only van der Waals forces operate because the distance between cells and surface is too
large to opposite surfaces be recognized. At intermiated distances in addition to Wan der Waals forces, electrostatic repulsion is also present. In this state the adhesion is reversible. Changes in bacteria, such as production of adhesion probes and hydrophobic groups repel water and exposed surfaces. The same authors suggested that the “major role of hydrophobicity in bacterial adhesion is therefore its capacity to remove water between contacting areas, enabling dehydrated parts to interact directly through short-
range interactions”. This enables the overcome of the potential energy barrier and therefore molecular interactions (<1.5nm) (e.g. ionic, hydrogen and chemical bonds) to take place. These specific interactions lead to irreversible adhesion [37].

Characteristics of the solid surface are important for the cell attachment process. The roughness of a surface influences the biofilm formation [30]. In rough surfaces, the area of contact with cells is larger and shear forces are diminished, so microbial colonization increase as the roughness of the solid surface increases [22]. The physicochemical properties of the surface may also exert a strong influence on the rate and extent of attachment. Hydrophobic, nonpolar surfaces such as Teflon and other plastics have been shown to be more rapidly colonized by microorganism than other hydrophilic materials such as glass or metals [66-68]. In fact, the interaction between two hydrophobic entities is favoured because they can enter into closer contact through the decrease of the water in between [69].

**Conditioning Films**

Conditioning films occur mainly in nature when a material surface is exposed in an aqueous medium. The surface becomes conditioned or coated by polymers from the medium, affecting the cell attachment [22]. The role of the conditioning film in biofilm development relies on its ability to modify the physico-chemical properties of the substratum, as well as providing a concentrated nutrient source and important trace elements. This will affect the rate and extent of microbial attachment [34]. In aquatic or terrestrial environments, the conditioning layer has been shown to consist of complex polysaccharides, glycoproteins and humic compounds [70, 71]. In Humans, a well-studied case of conditioning biofilm is the dental plaque in oral diseases. The enamel of teeth is conditioned by a “pellicle” composed of albumin, glycoproteins, lipids, lysozyme, phosphoproteins and other components of saliva and gingival crevicular fluids [72]. In biomaterials for human use, a number of host-produced conditioning films such as blood, tears, urine, saliva, intravascular fluid, and respiratory secretions can be used to influence the attachment of bacteria [73].
**Hydrodynamics**

The cell attachment is affected by the flow velocity of the medium in which they are. The area immediately adjacent to the substratum/liquid interface (boundary layer) is dependent on the linear velocity of the fluid. The higher the linear velocity, the thinner is the boundary layer. Thus, cell attachment is facilitated at higher linear velocities (up to a certain value) because cells, as particles in a liquid, have to travel a minor distance [22]. Whereas at very low linear velocities, the boundary layer is larger, affecting the contact of cells with the surface. In this case, the association with the surface will depend in large part on cell size and cell motility [22]. 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 [22].

**Characteristics of the Aqueous Medium**

The characteristics of the aqueous medium that influence the rate of microbial attachment to a substratum are the pH, nutrient concentration, ionic strength, and temperature. Several studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems [74, 75]. Fletcher and Loeb [76] suggested that the increase of cations affect the attachment of *Pseudomonas flourescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces. In other study, Cowan *et al.* [77] noted the influence of different nutrient concentrations on the adhesion of multiple species biofilms.

**Cell Properties**

As previously stated, hydrophobicity play an important role in cell attachment. Bacterial membranes have structures such as fimbriae, proteins, and flagella that contribute for hydrophobicity of cell surface as reported by Rosenberg and Kjelleberg [69]. The same authors showed that fimbriae contain a high proportion of hydrophobic amino acid residues playing a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum [78]. Mycolic acids of organisms (*Corynebacterium, Nocardia, Rhodococcus* and *Mycobacterium*) contribute to the high hydrophobicity of cells, which increases with the increase of mycolic acid chain length as stated by Bending *et al.* [68]. de Carvalho and de Fonseca [3] showed that hydrophobicity increases linearly with the percentage
of fatty acids with more than 16 carbons in *R. erythropolis* cell membrane. The use of terpenes (carvone and carveol) promoted a decrease in the percentage of fatty acids with more than 16 carbons and subsequently a decrease in cell hydrophobicity. Which resulted in the dispersion of cells previously in an aggregated form. In other study, de Carvalho *et al.* studied the physicochemical cell surface properties of *R. erythropolis* demonstrating that biofilm cells are more hydrophobic compared with planktonic cells utilizing the same carbon source [4].

By comparing motile and non-motile strains of *Pseudomonas fluorescens*, Korber *et al.* [79] found that motility increases the success of attachment in both numbers and speed, suggesting an important role of flagella in the early stages of bacterial attachment, by overcoming the repulsive forces associated with the substratum. Another important factor for cell attachment is the production of EPS and lipopolysaccharides that are associated to the bacterial cell surface and serve as adhesive or adsorbents to cells and seem to be more useful in colonization of hydrophilic surfaces [22].

The process of cell attachment is complex. In general, attachment will occur most readily on surfaces that are rough, hydrophobic, and coated by surface “conditioning” films. An increase in flow velocity, water temperature, or nutrient concentration may also contribute to increased attachment, if these factors do not exceed critical values. Important variables affecting the three most important parameters in cell attachment (surface, bulk fluid and cells) are presented in Table I.

<table>
<thead>
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<th>Properties of substratum</th>
<th>Properties of the bulk fluid</th>
<th>Properties of the cell</th>
</tr>
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<td>Cell surface hydrophobicity</td>
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<td>Hydrophobicity</td>
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<td>Conditioning film</td>
<td>Temperature</td>
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<td>Presence of ionic species</td>
<td>Flagella</td>
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<td></td>
<td>Presence of antimicrobial agents</td>
<td>Extracellular polymeric substances</td>
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3. Combating Biofilms
3.1. The Problem and Challenges

Biofilms are one of the most successful modes of life on Earth. Their existence is documented from 3.5-billion-year-old fossil records [80]. Biofilms may form on a wide variety of surfaces from glaciers to hot vents, from living tissues to medical devices (e.g. catheters, endotracheal tubes, mechanical cardiac valves, prosthetic joints, surgical sutures), even in highly irradiated areas of nuclear power plants. Industrial processes (e.g. paper, food, cosmetic and pharmaceutical industries) have serious problems with biofilms, because they can cause corrosion and limit mass and heat transfer in pipes and tubes [81, 82]. More concerning is the infections that they may cause in water distribution systems and healthcare environments threatening public health.

An estimated 80% of bacterial infections in humans are related to microbes living in biofilms and are now responsible for more deaths annually in the United States than emphysema, AIDS, Parkinson’s disease, and homicide combined [83]. This cost the U.S. health care system over $20 billion annually and led the Center for Disease Control to declare biofilms among the most pressing clinical impediments of the century [84]. Part of these infections is associated to the medical devices used. Over 5 million medical devices or implants are used annually, being associated with 60-70% of hospital-acquired infections [85]. Data from European hospitals revealed that of the patients staying more than two days in intensive care units, 6.2% acquired pneumonia (of which 89.3% were intubation associated) and 3% acquired bloodstream infections (of which 56% were catheter-associated) [86].

Conventional methods that would otherwise lead to eradication of non-attached, non-aggregated (planktonic) microbes are often ineffective to the microbial populations inside the protective biofilms, with the susceptibility decreasing up to 1000-fold [87]. EPS is particularly valuable after the initial phase of adhesion in organisms, conferring protection against phagocytosis, interference with the cellular immune response and reduction of antibiotic potency. The slow growth rate observed in biofilms and/or transport limitations of nutrients, metabolites and oxygen between the surface and the interior of the biofilm could be responsible for an increased antibiotic resistance over planktonic cells [43]. Also, horizontal gene transfer, which improves the exchange of genes between resistant and non-resistant strains, and the communication between cells (quorum sensing), which up-regulate expression of genes contributing to antibiotic resistance, are among several mechanisms that make biofilm difficult to eradicate [81, 82, 88].

Microbial biofilms are responsible for equipment damage, product contamination, energy losses and infections having an impact in the world economy at the level of billions of
dollars [88]. Therefore, novel strategies based on a more fulfilling understanding of the biofilm phenomenon are urgently needed.

### 3.2. New Solutions and Strategies

At the present, different approaches are being developed to prevent and destroy biofilms. Investigators have focused their knowledge in the different phases of formation of biofilm to propose combating strategies. Some of the strategies addressed in this work are based on the reviews of Yang et al. [88] and de Carvalho [81, 82].

**Modification of Surfaces**

Some examples of strategies directed to the process of adhesion with the objective of prevent microbial biofilm colonization, invasion and formation on surfaces will be discussed below.

Antimicrobial agents can be immobilized on surfaces, through non-covalently binding, covalently immobilization and entrapment on polymer matrices to kill attaching organisms [88]. For example, peptides (oligopeptides, polypeptides or peptidomimetics), metallic compounds (metals and alloys based), composite ceramic materials (including oxides, carbides or nitrides of transition elements) could be applied safely in medical implants and catheters, surgical tools and garments, and in bandages to make efficient antimicrobial coatings [89]. In this regard, Gobin [90] invented a coating material that inhibit biofilm formation from catheters with the application of an antimicrobial agent and biofilm-degrading enzyme embedded in a matrix material. In other study, Kazemzadeh Narbat et al. presented a study were antimicrobial peptides (AMPs) were loaded in a matrix of calcium phosphate (CaP) coated with titanium, using a simple soaking technique, and antimicrobial activity against both Gram-positive (Staphylococcus aureus) and Gram-negative (P. aeruginosa) was revealed [91].

Other approaches try to use non-biocidal agents (e.g. peptides, aptamers and bovine lactoferrin) to block microbial attachment to human cells. These agents interact with binding structures of microbes (e.g. pili) and efficiently inhibit invasion [88]. For example, Wu et al. [92] described the use of a 12-mer peptide (RQERSSLSKPVV), which binds to the structural protein PilS of the type IVB pili of Salmonella typhi, resulting in inhibition of adhesion or invasion of human monocyte THP-1 cells.
In the area of physico-chemistry, modified surfaces with anti-adhesive properties have been developed. For example, electropolished stainless steel was shown to significantly reduce attachment and biofilm formation by bacterial cells than the sand-blasted and sanded stainless steel surfaces [93]. Also, adhesion and biofilm formation by *Staphylococcus epidermidis*, *Deinococcus geothermalis*, *Meiothermus silvanus* and *Pseudoxanthomonas taiwanensis* species were almost inhibited in hydrophilic or hydrophobic coated stainless steel surfaces by adding diamond-like carbon or certain fluoropolymers [94].

Materials produced by nanotechnology have gained attention in recent years. New coatings, such as single-walled carbon nanotubes (SWNTs) have been studied to enable permanent, non-leaching antibacterial surfaces [95]. Moreover, carbon nanotubes can be used as nanocomposites, e.g. polymers and biomolecules that may be used for preventing biofouling or to promote the formation of desired biofilms [96].

Microbial species coexist and interact extensively with each other in natural biofilms. So, biological approaches have great potential in promoting cell detachment. Many bacteria are capable of synthesizing and excreting chemicals that inhibit biofilm formation by other species [84, 88]. For example, some studies showed the influence of biosurfactants that are synthesized and excreted by many bacteria, which inhibit attachment of their competitors [97, 98]. Also, the secretion of enzymes (amylases, hydrolases, glycosidases, lipases, proteases and deoxyribonucleases) by some bacteria inhibit the colonization of the same surface by other bacteria [99]. Microorganisms, metabolites and enzymes can thus be added to surfaces to prevent biofilm formation.

**Strategies Targeting the Biofilm**

Since quorum sensing controls bacterial biofilm differentiation and maturation, inhibiting quorum sensing will difficult or prevent biofilm establishment. Therapies involving antibiotics, quorum sensing vaccines and inhibitors have been studied and may prove efficient in i. decreasing the translation of quorum sensing-directed toxins or ii. prematurely activate the quorum sensing response to alert the host immune system of the presence of low cell density bacteria [100]. For example, some methods were developed to control and treat infections in patients using immunoglobulin or similar receptor molecules that have affinity and specificity for acyl-homoserine lactone signalling molecules decreasing the extra-cellular concentrations of the quorum sensing molecules [101].
Phages are an alternative to antibiotics for the treatment of biofilms. They are inexpensive and specific against a host or against a narrow host range, and will not affect the normal microflora of the environment where they are applied [85, 88]. Many phages produce depolymerises able to hydrolyse the extracellular polymers of the biofilms [82]. For instance, Ahiwale et al. [102] showed the ability of a T7-like lytic phage to prevent and disperse biofilms of *P. aeruginosa*. Nevertheless, the use of phage therapy is limited by some disadvantages, including the specificity to infect the bacterial targets, the possibility of virulence genes to incorporate the genome of bacteria, and the possibility of bacteria acquire resistance to phages [103].

Siderophore-mediated iron uptake and signalling are required by some biofilms for structure development and maturation. Siderophore-antibiotic conjugates may be used as ‘Trojan Horses’ to combat pathogenic bacteria as explained by de Carvalho and Fernandes [104]. Banin et al. [105] used the combination of a siderophore with an antibiotic that caused a massive destruction of the mature biofilm. In other study, the use of a conjugate desferrioxamine-gallium (DFO-Ga) killed planktonic cells and blocked biofilm formation by *P. aeruginosa* [106].

Several strategies are being studied around the world with the single objective of developing new ways to control biofilms in industrial and clinical settings. However, dealing with natural biofilms formed by multi-species is more complicated than when biofilms are formed by single-species in model systems. The need of a good method to combat biofilms remains. As stated previously in this work, biofilm is very problematic in hospital infections and cause several losses in industrial processes. Nevertheless, several victories have been achieved as our knowledge about these cell aggregates improved, as described above. The biofilms are present in many and unpredictable surfaces on Earth and certainly they will stay for many years. The objective is not to completely eradicate biofilms, because they are useful in many applications (e.g. bioremediation, waste water treatment), but instead to control biofilm in problematic areas while using them to solve other problems.

4. Studying Biofilms in Laboratory
The importance of biofilms in hospital infections and the biofouling problems in industry led to the development of several methods to study biofilm adhesion and formation. In the last decades our knowledge of biofilms has developed and today there are a number of methods for growing and analysing biofilms in laboratory [107-110]. The use of each method will be dependent of the goal of the study. For instance, to study the first events of biofilm adherence to surfaces, the use of microtiter plate may be a good method. If the objective is to study the effect of hydrodynamics on biofilm formation, it is more appropriate to use a flow cell system mounted on a microscope. Different methods may be used simultaneously. Normally, microscopes are used with other techniques for the study of growing biofilms.

Some of the techniques/apparatus used for in vitro growing and monitoring biofilms are described below.

### 4.1. Growing Biofilms

**Multi-well Plates**

Assays performed on microtiter plates (comprising 6, 24, 96 or more wells) permit the study of biofilm formation. Bacteria are incubated and adhere to the bottom of the wells. After incubation, the medium is removed so that only adherent bacteria (in the biofilm) remain in the well. Afterwards, a dye such as crystal violet is added for visualization or/and quantification of the biofilm [111]. This method could be used in different plates for the study of different surfaces. For example, Bonsaglia et al. has shown recently the production of biofilm by *Listeria monocytogenes* in different materials (polystyrene, glass and stainless steel) and temperatures using such cheap technique [112].

The microtiter plate assay is also useful in molecular genetic studies in order to screen large libraries to find mutants with the ability to form biofilms. For example, Friedman and Kolter [113] used a similar assay that led to the identification of two loci, *pel* and *psl*, that are involved in the production of carbohydrate-rich components of the *Pseudomonas aeruginosa* biofilm matrix. The system has also been variously adapted to measure other parameters of biofilm growth, including the time taken for re-growth following the application of biocides, which permit the comparison of MICs for planktonic and adherent populations against a wide range of antimicrobials [114].

**Robbins Device**
The Robbins device (Figure 1.2-RD) is a simple, widely-used, successful method to establish and analyse surface associated biofilms in tubes or pipes with a reasonably rapid and turbulent fluid flow. It is a multiport sampling device with evenly spaced sampling ports, the samples being mounted on removable plugs to fit flush with the inside surface of the pipe. Multiple biofilm samples cultivated during varying periods of time can be removed and analysed independently without disturbing the balance of the system. A popular modification of the Robbins device is to use a rectangular flow chamber section with a removable top containing sample plugs with experimental substrata glued onto the end for studying adhesion and biofilm growth on catheter sections (Figure 1.2 – MRD) [115].

![Figure 1.2 – The original Robbins’ device (RD) and the modified RD (MRD).](image)

**Roto-Torque (Rotating Annular reactor)**

The Roto-Torque is a CSTR reactor, essentially a chemostat, having an internal cylinder with angled holes which rotates at controlled speeds creating very turbulent flow and hence generating considerable and uniform shear forces in the annular fluid volume (Figure 1.3).

The outside wall has up to 12 removable slides attached which enable analysis of biofilm growth and properties. The effect of shear forces on biofilm growth and properties can be examined, and used to limit biofilm thickness [115].

![Figure 1.3 – Scheme of a Rotating annular reactor (from Ref[115])](image)

**Rotating Disk Reactor**
The rotating disk reactor consists of a rotating disk placed in a nutrient medium (Figure 1.4). The reactor has a container in which one or more disks are inserted or surfaces are mounted on the disk. The disk can be rotated at a desire speed and the influence of fluid shear stress created in biofilm formation can be study. The reactor can operate in continuous, batch or fed-batch mode [30, 35].

*Figure 1.4 – Scheme of a Rotating Disk Reactor (from Ref [30]).*

**Flow cells**

Development of non-invasive methods for the real-time observation of growing biofilms, based mainly on various forms of microscopy, has led to the development of a range of arrangements where a biofilm is cultured in a chamber between two parallel plates, usually microscope slides or coverslips, for study *in situ*. The liquid medium passes through the chamber and biofilms colonize a glass coverslip which can be observed by confocal laser scanning microscopy (CLSM) combined with fluorescent probes or by optical microscopy. Often multiple channels are present to increase replication. Two basic strategies are usually used [35]:

(i) **Once flow through system** – cells are added upstream (in context of liquid fluid direction) from the flow cell and allowed to attach to the tubing and flow cell. Liquid is then pumped from a reservoir, through the flow cell, and into a waste receptacle.

(ii) **Recirculating culture** – involves the pumping of a culture from a chemostat (or other culture vessel) through the flow cell and returning it to the culture vessel. The biofilm formed in the flow cell is analysed.
Flow cells can be used in versatile systems for study of cell attachment and biofilm formation. They can be operated in different configurations, allowing the study of different variables, such as the influence of flow rate, nutrients or time of colonization (i.e. time to colonize a new surface). At the same time, they allow the use of a microscope for real time monitoring of the process (Figure 1.5).

![Figure 1.5 - Flow cell under optical microscope with recirculating flow system. Developed at IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal.](image)

### 4.2. Monitoring Biofilms

**Optical Microscopes**

Optical microscopy is a fundamental tool for both bacterial enumeration and biofilm observation. A standard laboratory microscope with ×10, ×25, ×40, and ×100 objectives is ideal for bacterial adhesion studies. Transmitted light microscopy and fluorescence microscopy may be used to observe bacteria directly or through a histologic section, as biofilm can be embedded in paraffin and cut into conventional histologic sections. The substratum surfaces have to be translucent for transmitted light microscopy but fluorescence microscopy allows the observation of cells on non-fluorescent opaque surfaces. Normally bacteria are stained with dyes such as Gram stain, crystal violet, or Nile red. Microscopy has been used with bacterial flow chambers or slide cultures to observe living attached bacterial in real time [115].
**Electron Microscopy**

Scanning and transmission electron microscopy (SEM and TEM) have proved invaluable for examining the adhesion pattern of bacteria and the structure of biofilms. They offer the advantage of high-magnification spatial images of how the single bacterium is located and interact within the biofilm [116]. However, because these microscopes operate in vacuum and generally require samples to be coated with metals, samples have to be previously fixed and dehydrated. Also, the procedure requires expensive electron microscopes which are not present in all laboratories or departments of investigation. Furthermore, the sample preparation procedure is more time consuming comparing to other methods. Most of the scientists use electron microscope for analysis and characterization of biofilms in medical devices [117]. Despite the disadvantages of electron microscopy, and because of its excellent resolution properties, the electron microscope will continue to be an important tool for the biofilm scientist [107].

**Confocal Laser Scanning Microscopy**

Confocal laser scanning microscopy (CLSM) has revolutionized the morphological observation of adherent bacteria and biofilm (1980s) permitting the examination of wet biofilms *in situ* [118]. The equipment combines laser illumination, confocal imaging, plan-apochromatic objectives, and computer based image processing to generate high-resolution, three-dimensional images of the specimen. The basis of confocal microscopy is showed in Figure 1.6. Illumination from a light source is reflected by a dichroic reflector into an objective lens and brought to a focus in the sample. This focused point is scanned in a horizontal (XY) plane. Emitted fluorescent light is collected by the same lens, passes back through the dichroic reflector and on to the confocal aperture. Only light from the plane of focus passes through the aperture into the detector [119]. Thus, out of focus light is rejected and an optical section is generated.

The use of CLSM requires that the organisms in the biofilms be stained with fluorescent stains or express genes encoding fluorescent proteins (e.g., GFP). The stains are designed to emit light at specific wavelengths and can be used to probe specific cellular functions. For example, nucleic acid stains such as DAPI (4,6-diamidino2-phenylindole), acridine orange, and Syto 9 will stain the DNA and RNA of all cells regardless of their viability. Other stains, such as Nile red can be used to stain lipids.

Despite resolution being lower than SEM and TEM, CLSM permits the visualization of the biofilm structure. The distribution of bacteria or the thickness of the biofilm can be
examined \emph{in situ} and under the original hydrated conditions. Also as a feature of CLSM, samples can be sectioned optically to reveal the three-dimensional structure. Moreover, with various types of time-lapse photomicroscopy the dynamics of a biofilm can be study in real-time.

Other advantage is the use of programs created specifically for CLSM (e.g. COMSTAT) that enables statistical comparisons of biofilms and reproducibility between laboratories [35].

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{clsm_diagram.png}
\caption{The basis of confocal laser scanning microscopy. (Adapted from ref [120])}
\end{figure}
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General Introduction


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Chapter 2
Materials and Methods

1. General methods

1.1. Growth of cells

*Rhodococcus erythropolis* DCL14 cells were grown in 100 mL Erlenmeyer flasks closed with rubber bungs, containing 20 mL of mineral medium and 0.25% (v/v) of absolute ethanol (Panreac) or hexadecane (Sigma-Aldrich, 99%) as carbon source. The mineral medium contained the following compounds per litre of demineralised water: 0.01 g EDTA, 0.002 g ZnSO$_4$ · 7H$_2$O, 0.001 g CaCl$_2$ · 2H$_2$O, 0.005 g FeSO$_4$ · 7H$_2$O, 0.0002 g Na$_2$MoO$_4$ · 2H$_2$O, 0.0002 g CuSO$_4$ · 5H$_2$O, 0.0004 g CoCl$_2$ · 6H$_2$O, 0.001 g MnCl$_2$ · 4H$_2$O, 0.1 g MgCl$_2$ · 6H$_2$O, 2 g (NH$_4$)$_2$SO$_4$, and 1.55 g K$_2$HPO$_4$ and 0.85 g NaH$_2$PO$_4$ · H$_2$O for buffering (to obtain 50 mM, pH 7.0, in the final medium). All chemicals were from Sigma-Aldrich. The cultures were incubated at 28°C and 200 rpm overnight in a refrigerator Agitorb incubator (Aralab, Portugal).

1.1. Extraction, methylation and analysis of bacterial fatty acids

The cell culture samples were centrifuged at 10,000 rpm for 5 min. The supernatant was discharged and the pellet was resuspended in 1 mL of distilled water, to wash the cells, and centrifuged again under same conditions. The supernatant was removed and the pellet was stored at -20°C when not used immediately. The fatty acids from the cell pellets were extracted and simultaneously methylated using the Instant FAME kit from MIDI, Inc. A 6890N gas chromatograph (GC) from Agilent Technologies (Palo Alto, CA, USA), with a FID detector and a 7683 B series injector, equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent was used to analyse the fatty acid methyl esters (FAMEs). The MIDI Sherlock software package (version 6.2) was used to program and control the GC. The same software identified the FAMEs using MIDI calibration standards. The identity of the peaks was confirmed by using a qualitative standard of bacterial fatty acid methyl esters and one of polyunsaturated fatty acids, both from Supelco, and a methyl cis-11-octadecenoate standard solution from Sigma-Aldrich. To better understand the differences observed in the lipid composition of the cells, the fatty acids (FA) were grouped into the different classes according to their chemical structure: saturated straight chain (SSFAs), mono-unsaturated (MUFAs), poly-unsaturated (PUFAs), saturated *iso-* and *anteiso*-methyl branched (BCFAs) and 10-methyl branched (10MBFAs), saturated cyclopropyl-branched (SCBFAs), dimethylacetals (DMAFAs) and hydroxy substituted (HSFAs) fatty acids. The degree of saturation of the fatty acids of
the cell membrane was defined as the ratio between the total straight chain and mono-unsaturated fatty acids.

1.2. Zeta Potential

Concomitantly to sampling for FAMEs (section 1.2), 40 µL of cells were collected after washing with distilled water and suspended in 2 mL of KNO₃ (10 mM). The electrophoretic mobility of the *R. erythropolis* cell suspensions was determined in a Doppler electrophoretic light scattering analyser (Zetasizer Nano ZS, Malvern Instruments Ltd.) using a clear disposable zeta cell. The zeta potential was calculated using the electrophoretic mobility as an indirect measure of cell surface charge, according to the method of Helmholtz and Smoluchowski [1]. Calculations were automatically made using the Zetasizer software version 7.03, from Malvern Instruments Ltd.

1.3. Enzymatic Assay

The enzymatic activities of cell samples recovered at the end of operation of the biofilm reactor were assessed using the API ZYM® kit from BioMérieux (France), according to the kit's instructions.

1.4. Principal Components Analysis (PCA)

The fatty acid composition of the phospholipids of the cells that adhered to metallic (Al, Fe, Zn, Cu and Pb) and non-metallic (PS, PE, PET, Butyl rubber, PTFE, Glass, Glass mosaic, Gas tubing, Marprene, Silicone, Soft silicone, Teflon) surfaces was used to build a data matrix. The lipids were added as percentage of saturated straight, unsaturated, 10-methyl branched, branched and polyunsaturated fatty acids. The data was normalised to mean 0 and variance 1, as suggested by Everitt and Dunn [2] and the principal components were calculated by single value decomposition (SVD) using the statistical software MINITAB® Release 14.1 (Minitab Inc., USA).
1.5. Partial Least Squares (PLS) coefficients determination

The percentages of each FA type (SSFAs, MUFAs, PUFA, BCFAs, 10MBFAs, SCBFAs, DMAFAs and HSFAs) in the phospholipids of cells that had adhered to metallic and non-metallic surfaces were used to build a matrix containing the predictors, whilst the biofilm formed (normalized to vary between 0 and 1) was used as the response vector. Data points were standardized to mean 0 and variance 1 [2]. The PLS coefficients regarding the analysis of the relation between the FA composition of the cell phospholipids and the biofilm formed were determined using the statistical software MINITAB® Release 14.1 (Minitab Inc., USA).

2. Adhesion Experiments

2.1. Growth of Biofilms on 6-well plates

2.1.1. Growth of biofilms

Biofilms were grown in 6-well plates containing 3 mL of Mueller-Hinton (Fluka) or mineral medium. In the latter case, 0.5% (v/v) of absolute ethanol (Panreac) was added as carbon source. Different surfaces were added to the bottom of the wells to study the adhesion of cells to them. The surfaces to be tested were the following: i. the metals aluminium (Al), iron (Fe), zinc (Zn), copper (Cu) and lead (Pb), and ii. the non-metals butyl rubber, polytetrafluoroethylene (PTFE), polyethylene (PE), polyethylene terephthalate (PET), glass and glass mosaic. For each medium, a well containing cell suspension but no additional material was used as reference (blank). The plates were inoculated with 1ml of a previously grown cell suspension of R. erythropolis, and were incubated at 28°C and 40 rpm for 48 hours.

2.1.2. Fatty acid analysis

After 48 h, 1 mL of cell suspension from the middle of the well (referred to as planktonic cells further on in the text) was collected and placed in a 1.5 mL eppendorf tube (Eppendorf, Germany). Simultaneously, 1 mL of cells adherent to the surfaces (referred as biofilm) were collected by scrapping with a micropipette and added to microtubes (1.5 mL from Eppendorf). FAMEs were produced from the samples as described in section 1.2.
2.1.3. Visualization of lipids using Nile Red

The lipids present in the cells and in extrapolymeric substances were stained using Nile Red (Molecular Probes, Life Technologies) as follows: 5 µL of a stock solution of 1.3 mg.mL⁻¹ of Nile red in acetone was prepared and deposited on the wet surfaces studied and incubated for 5 min. The samples were observed by fluorescence microscopy using an Olympus CX40 microscope, with an Olympus U-RFL-T burner and an U-MWG mirror cube unit (excitation filter: BP510-550; barrier filter: BA590). Images were captured by an EvolutionTM MP5.1 CCD colour camera using the software Image-Pro Plus, both from Media Cybernetics, Inc. (USA). At least 4 images were taken of each surface with a magnification of 30x, unless stated otherwise.

2.1.4. Image processing

The software Image-Pro Plus (from Media Cybernetics, Inc., USA) was used to create the surface-plots, which are three-dimensional representations created from the two-dimensional images acquired using intensity data. The software ImageJ (National Institutes of Health, USA) was used to calculate the area occupied by biofilm and the number and size of aggregates in biofilms. The procedure used was the following: Image colour was inverted, the RGB image was transformed to 8-bits type and the threshold (B&W) was adjusted. Finally, the objects were measured using the command “Analyze Particles” and the data was exported to Microsoft Excel for treatment and calculations.

2.2. Recirculation Tube System

A schematic view of the recirculating tube system is shown in Figure 2.1. Five tubes with different properties (Teflon, soft silicone, Marprene, gas tubing and silicone) were cut to present 5 cm in length and connected to each other. A recirculating tube placed on both sides of the five-tube system. The recirculation of the culture medium was carried out by the action of a peristaltic pump (Pharmacia LKB-Pump P-1) at a flow rate of 1 mL.min⁻¹. An Erlenmeyer flask containing mineral medium was inoculated with cells grown overnight (as described in section 1.1) to a final volume of 30 mL. Also, 0.25% (v/v) of carbon source (absolute ethanol; or propionic acid with and without limonene, Sigma-Aldrich) was added. The culture was stirred by magnetic agitation and the system was operated at room temperature. At the end of the experiment, the pump was turned-off and each tube was removed from the system. Each one was cut in half and placed into 1.5 mL microtubes (Eppendorf). The fatty acids from the cells attached to the tube walls were simultaneously extracted and methylated using the Instant FAME kit from MIDI, Inc. inside the microtube as described in section 1.2. For dry weight measurements, the
tubes from the system using propionic acid (Acros Organics) as carbon source were weighed at the end of the system operation. Then, fatty acids were extracted from tubes and dried at 65°C in a Memmert GmbH oven (Model 400) until constant weight.

![Schematic illustration of the recirculation tube system.](image)

**Figure 2.1** – Schematic illustration of the recirculation tube system. Tubes: 1 – Teflon, 2 – Soft silicone, 3 – Marprene, 4 – Gas tubing, 5 – Silicone; black – recirculation tube.

### 2.2.1. Contact angle measurement

The contact angles of each tube tested in the recirculation tube system are presented in Table II. The tubes were open and fixed to the laboratory bench with clamps in order to obtain a flat surface. A 5 µL droplet of distilled water was deposited on top of the flat tube and a photograph in the droplet plane was taken with a Coolpix P5100 camera (Nikon, Japan). The measurement of the contact angle, between the water droplet and the flat tube surface was calculated with Image-Pro Plus. At least 2 different pictures were taken for calculation of the average contact angle in each tube.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas tubing</td>
<td>56.2</td>
</tr>
<tr>
<td>Silicone</td>
<td>57.7</td>
</tr>
<tr>
<td>Soft silicone</td>
<td>72.5</td>
</tr>
<tr>
<td>Teflon</td>
<td>94.5</td>
</tr>
<tr>
<td>Marprene</td>
<td>101.3</td>
</tr>
</tbody>
</table>

*Table II – Contact angles of the tubes used in the recirculation tube system.*
2.3. Biofilm Reactor

Four microscope slides were cleaned with ethanol 70%, attached at the top by a wire and dipped in a beaker with 400 mL of mineral medium and 40 mL of inoculum (grown as described in section 1.1). The carbon source used was 0.25 % (v/v) of \( n \)-hexadecane (Sigma-Aldrich\textsuperscript{®}, 99%) or absolute ethanol (Panreac). The reactors were closed in the top with aluminium paper and stirred by magnetic agitation (see Figure 2.2). The glass slides were removed from the reactor along time and placed inside a 50 mL centrifuge tube. 3 mL of \( \text{H}_2\text{O} \) MiliQ was poured over the slide and the tube was vortexed (Heidolph Reax 2000) to remove the cells attached to the slide. Once the glass was cell free, the liquid containing the cells was transferred to 1.5 mL microtubes (Eppendorf). Also, 1 mL of culture sample were collected from the reactor to 1.5 mL microtubes (Eppendorf). In both cases fatty acids analysis were proceeded as described in section 1.2. Also, at the end of reactor operation, samples of the material that had formed on the top of the culture (see Figure 2.2) were collected and its FAMES analysed (as described in section 1.2). Moreover, due to the high biomass observed at the end of operation, samples from culture, glass surface and supernatant were collected and used for lipid extraction and fractioning as described by de Carvalho and Caramujo [3]. Briefly, the total lipids were extracted to chloroform, added to a 500 mg silica gel column, and fractioned by serial elution with chloroform (apolar lipids), acetone (neutral lipids) and methanol (phospholipids). Other samples were used for semiquantification of enzymatic activity as described in section 1.5.

![Figure 2.2 – Biofilm reactors in operation. Left – hexadecane used as carbon source, Right – ethanol used as carbon source.](image-url)
2.4. Flow cell system

2.4.1. Fabrication and Design

The commercially available elastomer polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) was used to construct two flow cells using the replica molding technique [4, 5] and liquid PDMS was used to seal the flow cells [6] (see figure 2.3). The two flow cells were constructed using the same design (see figure 2.4) but different materials were used for covers. In one flow cell, a glass slide was applied as cover whilst on a second one, a thin layer of PDMS was used. The flow cells had a length of 7 cm, width of 2.5 cm, and a height of 1.2 cm. A central chamber (diameter of 1.1 cm) was made to provide better visualization of biofilm formation. Liquid was directed into and out of the growth chamber by round channels with a diameter of 3 mm. The cover slip provided a flat and transparent surface suitable for imaging. The two different covers surfaces used (PDMS and Glass) allowed the study of hydrophobicity influence in the biofilm formation. The mold was constructed using simple and cheap materials available at the laboratory, such as, hard plastics, stick, lids and tape.

Figure 2.3 – Schematic illustration of the flow cell construction and cover bonding with liquid PDMS.

Figure 2.4 – Final flow cell with adapters installed.
1 – PDMS body; 2 – Cover slip (glass slide or PDMS slide); 3 – plastic adapters for system connection, 4 – channels, 5 – cylindrical centre for cell adhesion observation.
2.4.2. System operation

To monitor cell adhesion in real time, each of the flow cells described above was placed under a microscope as illustrated in Figure 2.5. The culture of cells grown as described in section 1.1 was recirculated through the flow cell using a peristaltic pump (Pharmacia LKB-Pump P-1). Magnetic stirring was used for oxygenation and mixture of the culture. The lipids present in the biofilms were stained using Nile Red (Molecular Probes, Life Technologies) as follows: a stock solution of 1.3 mg/mL of Nile red in acetone was prepared, 5 µL were added to the cell culture and the system was left for incubation 5 min, just before the initiation of the system operation. The samples were observed by fluorescence microscopy as described in section 2.1.2.

2.4.3. Biofilm analysis

At the end of operation, the flow cell was disconnected from the system and the biofilm formed inside the flow cell was removed as follows: 1 mL of distilled water was applied with a micropipette (Finnpipette model 4500 Labsystems) in one of the entries, the liquid being recovered at the other side of the flow cell in a microtube (1.5 mL, Eppendorf®). The process was repeated to collect biofilm samples in different sequential fractions. The FAMEs production and analysis was proceeded as described in section 1.2.

Figure 2.5 - Schematic illustration of the flow cell system setup.
2.4.4. Surface charge density

The surface charge density was determined, according to [7], by the equation: \( \sigma = \eta u / d \)
in which \( u \) was the electrophoretic mobility, obtained from the zeta Potential measurements (as described in section 1.3), \( \eta \) was the viscosity of the solution, and \( d \) was the diffuse layer thickness [8]. The diffuse layer thickness was determined from the formula [9] \( d = \sqrt{\frac{\varepsilon \varepsilon_0 R T}{2 F^2 I}} \), where \( R \) was the gas constant, \( T \) was the temperature, \( F \) was the Faraday number, \( I \) was the ionic strength of KNO\(_3\) (10 mM), and \( \varepsilon \varepsilon_0 \) was the dielectric permittivity of the medium.

3. References

Chapter 3

Results and Discussion

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Chapter 3

Results and Discussion

The process of bacterial adhesion and growth on biofilms is still not very well understood although the many studies performed. In this complex process many factors have to be taken into consideration, including the physicochemical characteristics of bacteria (hydrophobicity, surface charge), the properties of the substratum (chemical composition, surface charge, hydrophobicity and roughness) and the environmental conditions (e.g. temperature, pH, time of exposure, nutrients, the presence of antimicrobials and fluid flow conditions). Some of these parameters were addressed in the present study and the results and their discussion are detailed in this chapter.

1. Influence of Surface Material and Medium

The biofilm growth of *R. erythropolis* cells on 6-well plates was promoted for 48 hours on different surfaces immersed in liquid medium to evaluate the changes occurring in lipid composition of the cells during adhesion. The samples collected from the bottom of the 6 well-plates were expected to be composed of cells and aggregates from the biofilm and were designated as biofilm cells. On the other hand, samples collected from the cell suspension were expected to contain cells in planktonic state or cells that have detached from the biofilm and were called planktonic cells.

The biofilm and planktonic cells were mainly composed of saturated straight fatty acids (SSFAs) at least 34.8% and mono-unsaturated fatty acids (MUFAs) with a minimum of 27.7% (Figure 3.1). These fatty acids (FAs) are the main regulators of membrane dynamic composition and fluidity [1]. In gram-positive bacteria, as *R. erythropolis*, 10-methyl branched fatty acids (10MBFAs) occur in large percentage [2] and in this case were 17.4% of total lipids. In Muller-Hinton (MH) medium, the percentage of 10MBFAs is higher in cells attached (biofilm) to non-metallic surfaces (minimum of 21.3% with the exception of the butyl rubber) than in those adherent to metallic surfaces (maximum of 18.8%) (Figure 3.1). These results suggest that bacteria have changed their lipid composition as response to the surface. Metals have anti-microbial properties known since ancient times [3]. By decreasing the 10MBFAs, the biofilm cells are probably trying to stabilize the membrane upon aggression of metal ions. In mineral medium (MM), the variation of FAs composition in biofilm cells was minimum when compared to those in planktonic state. The exception were observed in the case of planktonic cells in contact with Zn and Al where the 10MBFAs were not detect (0%) or were present at very low percentage (0.5%), respectively.
Figure 3.1 – Fatty acid composition of cells collected from biofilm and liquid medium. MH, Mueller-Hinton. Butyl r., butyl rubber. Glass m., glass mosaic. SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, 10MBFAs – 10-methyl branched fatty acids, PUFAs – polyunsaturated fatty acids.
The degree of saturation of the fatty acids of the membrane phospholipids of cells in biofilm was on average higher in the presence of non-metal surfaces (1.23) than those of cells contacting metal surfaces (0.93; Figure 3.2). In general, the cells in contact with metals in MH presented a slight increased degree of saturation when compared to those in MM and the opposite was observed for non-metallic surfaces with the exception for polyethylene (PE) and glass mosaic biofilm. Similarly, it is possible to observe that planktonic cells presented a higher degree of saturation in non-metals surfaces (average of 1.3) than metal surfaces (average of 0.95). Nevertheless, both planktonic and biofilm cells presented similar responses to both surface and media used for growth.

![Figure 3.2 – Degree of saturation of cells collected from the biofilm and planktonic cells. MM – mineral medium. MH – Mueller-Hinton medium.](image)

To visualize the lipids of cells attached to the surfaces studied, lipids were stained with Nile Red and cells were observed using an optical microscope with fluorescence light. The images were taken with a CCD camera and the corresponding calculated surface plots are present in Figure S1 and S3 of Chapter 5.

**Metallic surfaces**

Although some metals are essential to biologic life, such as the studied Cu, Zn and Fe, in higher concentrations, metals are toxic to cells. The principal cause of toxicity is the induction of Reactive Oxide Species (ROS) that are prejudicial to cells [4]. Nevertheless, some bacterial cells are able to overcome metal toxicity through several mechanisms [4].

In the images taken in the present study, it was possible to observe that cell aggregates attached to every metal surface tested. Also, it was visible that the architecture of the biofilms formed changed, depending on surface and medium used for cell growth. For example, the aggregates of cells on Zn are smaller than the aggregates formed on the Fe surface in MH medium. Indeed, on average about 25.9% of the cell aggregates in Zn
had a size between 25 and 50 pixels and 24.9% of the cell aggregates in Fe had 100 to 200 pixels in size. The majority of aggregates had a size between 25 and 50 pixels in MM and between 50 and 200 pixels in size on MH medium (see Figure S2 of Chapter 5).

Furthermore, the percentage of area occupied by biofilm was on average larger in MM (25%) than in MH medium (14.6%) (Figure 3.3). This observation suggests that the different medium and carbon source influences the composition of the cell membranes and biofilm content in lipids. In a study presented by de Carvalho et al. it was shown that *R. erythropolis* DCL14 adapt their membrane fatty acids according to the carbon sources used for growth, which that also affected the growth rate [5]. An exception, where the MH broth promoted a larger biofilm, was the case of biofilm formed in a Fe surface: the area occupied by cells reaching a percentage of 27.2% of the total image area in MH medium compared to the 9.6% in MM (Figure 3.3). Besides, it was notorious the change in the shape of the biofilm formed in the Fe surface in the two media (Figure S1 of Chapter 5). Despite the abundance of carbon source in MH medium, the *R. erythropolis* cells preferred MM and ethanol as carbon source, contrarily to what has been observed with other species [6].

Interestingly, the percentage of biofilm formed on the Zn surface (57.3%) was higher than even that of the blank assay (42.2%) where the cells adhered to the polystyrene of the well (Figure 3.3). On the other hand, the smallest area of biofilm was achieved on a Cu surface. This result was expected because of the antimicrobial properties of copper [7, 8]. Warnes et al. [9] proposed that the mechanisms involved in copper toxicity for enterococci are the direct or indirect action of copper ions released from the surface and the generation of superoxide species. In the same study biofilms were able to develop in

![Figure 3.3](image)

*Figure 3.3:* Percentage of area occupied by the biofilm in the images presented in Figure S1 of Chapter 5. MH – Mueller-Hinton medium. MM – mineral medium.
copper surfaces mainly if they were wet, emphasizing the importance of maintaining hospital copper surfaces dry to increase the success of biofilm eradication.

During the experiments made in the present study, it was possible to observe that *R. erythropolis* was able to grow on different metal surfaces although they are known to inhibit bacterial growth. This was not a surprise because of the large array of enzymes they contain and their capacity of adaptation to extreme conditions. In several studies de Carvalho and co-workers described the extraordinary capacity of *R. erythropolis* to make numerous bioconversions and degradations and adapt to extreme conditions [5, 7, 10].

**Non-metallic surfaces**

The infections caused by biofilms formed in medical devices are very problematic, resulting in financial losses and human fatalities annually [11-13]. Part of these medical devices is made of different plastics and glass. To assess if *R. erythropolis* form biofilm in these types of surfaces, different materials were tested, namely butyl rubber, teflon, PE, polyethylene terephthalate (PET), glass and glass mosaic (Figure S3 of the Chapter 5). As seen previously with metals, it is noticeable the changes in biofilm area and shape according to the non-metallic surfaces and to the medium used.

Butyl rubber (5.1%, MH; 4.3%, MM) and glass mosaic (1.3%, MH; 2.2%, MM) in both media promoted only small areas of biofilm (Figure 3.4). The plastic surfaces PET (12.2%, MH; 9.2%, MM) and PE (11.8%, MH; 13.9%, MM) promoted a similar percentage of occupied area in both two media. This is also observable in the images taken, although for MH medium the size of biofilm aggregates on PET (between 50 to 200 pixels and 300 to 500 pixels) are larger when compared to those formed on PE (25 to 100 pixels) (Figure S4 of the Chapter 5). Besides, it is possible to verify that the biofilm aggregates in MM had between 0 to 25 pixels and between 50 to 100 pixels in contrast to those formed in

![Figure 3.4](image-url) – Percentage of area occupied by the biofilm in the images presented in Figure S3 of the Chapter 5. MH – Mueller-Hinton medium. MM – mineral medium.
MH medium which had 25 to 200 pixels, indicating a larger diversity in size of the cell aggregates.

After the 48h of the experiment, the surfaces with larger areas occupied by biofilm were the glass (32.3%) and teflon (25%) in MM. Teflon is known for its high hydrophobicity and appears that *R. erythropolis* cells are able to grow and develop biofilms on this material only when in the MM. On glass (hydrophilic material), the biofilm area was significantly larger (32.5%) when the cells were in MM than in MH medium (4.0%) (Figure 3.4). Although the biofilm formed on glass in MH broth occupied a reduced area, the cell aggregates were large in size (Figure S4 of the Chapter 5). These results show the ability of *R. erythropolis* to colonize different types of surface and to develop diverse biofilm structures, although is clear the effect of the medium in this process. Tomaras et al. also showed the ability of *Acinetobacter baumannii* for biofilm formation in different surfaces and postulated that the mechanism by which the strain forms biofilms in plastic, glass and teflon depends on the multiplication and growth of cells and microcolonies already attached to the solid surface [14].

A relationship between the percentage of 10MBFAs and the area occupied by the biofilm on the surface of study is present in Figure 3.5. In MH medium the values are disperse and no clear relationship was found (Figure 3.5 MH). Nevertheless, it is possible to observe that the non-metals have a larger percentage of 10MBFAs (exception for the butyl rubber surface) and a lower percentage of area occupied by biofilm. On the other hand, metals have a diverse range of biofilm areas but the cells presented a lower percentage of 10MBFAs compared to the majority of non-metals.

In MM an influence of 10MBFAs on the area occupied by biofilm in both metal and non-metal surfaces could be observed (Figure 3.5 MM). As indicated by the trendline, in both media an increase of the percentage of 10MBFAs led to an increase in the area occupied

![Figure 3.5](image) – Influence of the percentage of 10MBFAs on the area occupied by biofilm. MH – Mueller-Hilton medium; MM – mineral medium; Metals – Al, Fe, Cu, Zn and Pb; Non-metals – butyl rubber, Teflon, PET, PE, glass and glass mosaic.
by the biofilm in the solid surface studied. MH medium is a rich medium, and cells have a lot of nutrients at their disposal. On the contrary, mineral medium is an elementary medium and cells are expected to present lower metabolic activity. This behaviour could be responsible for the data presented in Figure 3.5, where biofilm area and the percentage of 10MBFAs are more correlated in mineral medium and not so scattered as in MH medium. Cells in MM do not have many nutrients available, and one of the best ways to overcome the lack of nutrients is to develop a community of cells that can cooperate for the benefit of the group [15]. This is probably one of the reasons for a larger area of biofilm formed by *R. erythropolis* cells in most of the surfaces in MM when compared to the MH medium, contrarily to what is observed in most species.

Other important factor for cell adhesion is the chemical nature of the substratum to which the cells adhere. On metals, the highest percentage of area occupied by the *R. erythropolis* biofilm was achieved in a Zn surface (57.3%) and in non-metals on a glass surface (32.3%). Other characteristic observed in the growth of biofilms was their architecture. In some surfaces the change in architecture was notable as in the case of the iron surface (see Figure S1 of the Chapter 5). Moreover, the size of the aggregates also indicated the influence of surface on biofilm architecture. For instance, two plastic surfaces could promote similar areas occupied by biofilm in both media (Figure 3.4) but the cells formed aggregates of different sizes (PET and PE, Figure S4 of the Chapter 5).

2. **Influence of surface hydrophobicity and cell growth phase**

The contact angle of a liquid drop resting on a flat, horizontal solid surface is defined as the angle formed by the intersection of the liquid-solid interface and the liquid-vapour interface ([16]; Figure 3.6). When the contact angle is less than 90° the liquid drop spreads along the surface indicating that the surface is wettable and hydrophilic. Contact angles larger than 90° mean that the surface is unfavourable to wetting and is hydrophobic; on these surfaces the fluid will minimize its contact with the surface and form a compact liquid droplet. The contact angle of the surfaces is correlated with the formation and adhesion of microbial biofilms to them [17, 18]
Figure 3.6 – Contact angles formed by sessile liquid drops on a smooth homogeneous solid surface. Hydrophilic surfaces present contact angles lower than 90º whilst hydrophobic surfaces form angles larger than 90º. Adapted from [16].

Table III presents the contact angles of the 5 tubes with different compositions used in a recirculating system to test the correlation between the surface hydrophobicity and biofilm formation. The contact angle measurements showed three hydrophilic tubes (gas tubing, silicone and soft silicone) and two hydrophobic tubes (teflon and marprene). The FA composition of cells in biofilms attached to the inside of the tube was analysed to unveil their influence on the interaction between cells and the surface of the tubes.

Table III – Tubes used in the recirculation biofilm reactor system and their respective contact angle measured in the laboratory.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Contact angle (°)</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas tubing</td>
<td>56.2</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Marprene</td>
<td>57.7</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Soft silicone</td>
<td>72.5</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Silicone</td>
<td>94.5</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Teflon</td>
<td>101.3</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>

a – classification according to Audrey Wipret [19].

The recirculation biofilm reactor system with the 5 different tubes, indicated in Table III, was operated under different conditions. Ethanol was used as carbon source and the system was inoculated with cells collected in early exponential phase and with cells collected in late exponential phase in order to test the effect of the growth phase on cell adherence capabilities and biofilm development. To study the effect of certain fatty acids on cell adhesion, propionic acid was used as carbon source because it was previously observed that cells grown in this carbon source contain more branched-chain fatty acids (BCFAs, data not shown). Limonene was also tested as carbon source, since it was previously shown that terpenes, such as carveol and carvone, reduce significantly the volume of biofilms by decreasing the production of fatty acids with more than 16 carbon atoms (responsible for cell hydrophobicity) and by influencing the composition and physical properties of exopolymeric substances.

From the FA profile of the cells it was possible to verify a correlation between the contact angle of the tube where cells adhered and the percentage of FAs with more than 16 carbons, as displayed in Figure 3.7. On the four cases, adherent cells responded by
increasing the percentage of FAs with less than 16 carbon atoms in their chain on the cell membrane with the increase of the contact angle in the hydrophilic tubes. The opposite was observed in the hydrophobic tubes: the cells decreased the percentage of these FAs with the increase of the contact angle of the tubes. Obviously, the percentage of FAs with more than 16 carbon atoms decreased in the hydrophilic surfaces and increased in the hydrophobic surfaces.

![Graphs](image-url)

**Figure 3.7** – Correlation between the contact angle and the FAs with more (>C16) or less (<C16) than sixteen carbons in their structure. Contact angles are correspondent to the five tubes used in the recirculating tube system presented in Table III. The percentage of these FAs was obtained from FAMES analysis of cells adherent to the tubes at the end of the experiment. A: left – the system was inoculated with a cell suspension collected in the early exponential phase (O.D. = 0.1), the time of operation was 20.5 h. Right - the system started with an inoculum collected during the late exponential growth phase (O.D. = 1.2), the time of operation was 19.5 h. B: left – propionic acid (0.25 v/v) was used as carbon source. Right - propionic acid (0.25 v/v) and limonene (0.25% v/v) were used as carbon sources. The time of operation was 92 hours in both cases.

de Carvalho et al. [20] shown that hydrophobicity of *R. erythropolis* cells increases linearly with increasing percentage of fatty acids with a number of carbons higher than 16 in the cell membrane. The results obtained in the present study suggest that cells decrease their hydrophobicity with increasing contact angles in hydrophilic surfaces, whilst in hydrophobic surfaces cells increased the percentage of FA with more than 16 carbons in their membrane with increasing contact angle. The changes on membrane
cell hydrophobicity influence the production, the composition and thus the physical properties of the EPS as pointed by Liao et al. [21] and de Carvalho et al. [20], and consequently, the formation of biofilms in solid surfaces.

As mentioned, the adhesion and biofilm formation of *R. erythropolis* inside the tubes in the recirculation reactor system using ethanol as carbon source was tested with an inoculum of cells with an optical density of 0.1 (collected during early exponential growth phase) and an inoculum with an optical density of 1.2 (from the late exponential growth phase). Comparing the two situations, it is possible to observe that the percentage of FAs with less than 16 carbon atoms was higher in cells collected during late exponential phase than in those collected during the exponential phase (Figure 3.7 - A). The older cells should thus present a less hydrophobic character.

Moreover, the same effect was observed in the system that used the terpene limonene additionally to the propionic acid as carbon sources: the cells produced larger amounts of short chain FA except for the most hydrophobic surface (Figure 3.7 – B). As mentioned, terpenes were reported to decrease the percentage of fatty acids with more than 16 carbon atoms in *R. eythropolis* [20].

Regarding the fatty acid composition of cells collected in different growth stages, differences were more noticeable in cell adherent to the marprene tube surface (Figure 3.8). On these cells, SSFAs and MUFAs increased 11.0% and 19.8%, respectively, when the system was inoculated with cells collected in the early exponential phase comparing to cells in the late exponential phase. On the other hand, BCFAs and polyunsaturated fatty acids (PUFAs) decreased 19% and 16.9%, respectively, when the system was inoculated with cell collected during the early exponential phase. Significant variations were also observed in the other surfaces, as in the case of the gas tubing, teflon and soft silicone. On the latter, the percentage of SSFAs increased by 20.2% when late exponential cells where used. In the cells from the silicone tube surface, the variations were less significant. Surprisingly, in these cells the highest variation was in the content of hydroxyl-substituted fatty acids (HSFAs).
The effect of growth phase of bacteria is important to the process of adhesion and infection. During a similar time of system operation, the cells with more capacity to adhere to the tube surfaces will promote larger biofilms on their surfaces. Figure 3.9 demonstrates that more biomass was adherent at the end of the experiment when the system was inoculated with cells from an early exponential growth culture. The exception was observed in the amount of cells which adhered to the silicone tube. These observations are in concordance with the work of Cronin and Wilkinson who studied the

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**Figure 3.8** – Fatty acid composition of the cells attached to the inside surface of tubes in the recirculation reactor system with ethanol (0.25% v/v) as carbon source.

A: left – the system was inoculated with a cell suspension collected during the early exponential phase (O.D. = 0.1), the time of operation was 20.5 h. Right - the system started with a cell suspension collected in the late exponential growth phase (O.D. = 1.2), the time of operation was 19.5 h. Tubes are ordered by increasing contact angle. B – Differences between fatty acid composition of adherent cells, at the end of system operation, when the system was inoculated with cells from early and late exponential growth phases. The “Culture” sample (control) refers to the planktonic cells in the shake flask that fed the system. SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.
adhesion of *Bacillus cereus* and suggested that cells in late exponential phase appeared to have reduced adherence properties compared to cells in early exponential or lag phase [22]. Nevertheless, the chemical nature of the substratum could have a strong influence, as observed on soft silicone tubes, which allowed the highest adherence of cells. On both cases, inoculation with early and late exponential cells, an increase of adherence was observed with increasing contact angle on hydrophilic surfaces (gas tubing, marprene and soft silicone). Contrary, the adherence decreased with increasing contact angle on hydrophobic surfaces (silicone and teflon). These results are in concordance with the results regarding the percentage of FAs with more or less than 16 carbon atoms where the soft silicone was also the inflection point of the “curve”. This suggests a different response of cells when facing hydrophobic or hydrophilic surfaces. Moreover, the results indicate that a decrease in cell hydrophobicity as result of a decrease in the percentage of FA with more than 16 carbon atoms (Figure 3.7), leads to higher adherence of cells to hydrophilic surfaces (Figure 3.9).

![Figure 3.9](image)

*Figure 3.9 – Lipid weight of cells adherent to the tube surfaces at the end of system operation. Ethanol (0.25% v/v) was the carbon source and the system was inoculated with cells from early and late exponential growth phases. Data was normalised using the biomass in the culture at the end of system operation.*

Similar profiles of FA were obtained in cells from the system in the presence and absence of limonene (Figure 3.10). In fact, the variation between the two cases was less than 7% which is low when compared to the differences found in the system using ethanol and cells from early or late exponential phases (Figure 3.8). Although terpenes may be considered toxic compounds that destabilize the cell membrane and to which the microorganisms have to adapt their membranes, *R. erythropolis* cells are able to use limonene as carbon source [5]. Besides, the percentage of each lipid class may be similar but the composition in the individual FA in each class may vary, as shown regarding the composition in FA with 16 carbon atoms (Figure 3.7).
As expected, in the presence of limonene less cells adhered to the tubes (inferred from the percentage of lipids extracted from the cells on the tube surface) than in the absence of this terpene (Figure 3.11). The exception was observed in the silicone tube. The results are in concordance with de Carvalho et al. that showed the ability of terpenes to disperse cell aggregates and demote established biofilms [20].

Figure 3.10 – Fatty acid composition of the cells attached to the inside surface of tubes in the recirculation reactor system with propionic acid (0.25% v/v, A–left) or propionic acid with limonene (0.25% v/v, A – right) as carbon sources, the time of operation was 92h in both cases. B – Differences between fatty acid composition of adherent cells, at the end of system operation, between cells on propionic acid and propionic acid with limonene. The “Culture” sample (control) refers to the planktonic cells in the shake flask that fed the system. SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids. monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.
When comparing the effect of early versus late exponential growth phase cells to inoculate the system and the effect of limonene to the amount of cells that adhered to each tube, the results suggest a very complex array of variables that have to be taken into consideration, such as composition of the substratum, carbon source and presence of toxic compounds to predict cell behaviour. The results obtained thus suggest that no simple, linear correlation between the increase of contact angle and the amount of adhered cells exists. These observations are in concordance with Qian et al. [23] that studied the biofilm formation on a 7 type tube system.

3. Biofilm reactor

To study cell adherence and the growth of biofilms of *R. erythropolis* on glass surfaces (hydrophilic), two biofilm reactors were constructed. To assess the influence of the lipids to adhesion, two carbon sources were used: ethanol and hexadecane at 0.25% v/v in mineral medium.

In the Figure 3.12, the percentage of lipids of the cells samples attached to the glass (biofilm) and in culture (planktonic cells) is presented along the time of operation of the biofilm reactor using ethanol and hexadecane as carbon source. The FA composition of the cells used as inoculum of the reactor was considered at the initial time of operation. At the end of operation it was observed, in both reactors, a considerable deposition of biomass (probably embedded in glycolipids) at the top of the reactor that was also
recovered and analysed (called “supernatant” in the results). In the same figure, the calculated degree of saturation of each sample is depicted in the second axis.

![Graph showing fatty acid composition of R. erythropolis cells grown on ethanol or hexadecane as carbon source and corresponding calculated degree of saturation (Ds). SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.]

**Figure 3.12** - Fatty acid composition of R. erythropolis cells grown on ethanol or hexadecane as carbon source and corresponding calculated degree of saturation (Ds). SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.

When the cells used ethanol as carbon source, the percentage of SSFAs and 10MBFAs increased with time in both biofilm and culture cells, after the initial 6h (Figure 3.12, Ethanol). In other hand, the BCFAs decreased with the time whilst the MUFAs remained constant. The supernatant was clearly visible at the end of the reactor operation and its lipid composition presented and elevated percentage of MUFAs (43.3%) when compared...
to the cells in biofilm biofilm (26%) and planktonic (28.9%) at the end of operation. The percentage of SSFAs was lower and consequently the degree of saturation in these samples was only 0.44, suggesting a very low saturation degree of cell membrane of possible cells in the supernatant. By analysing the degree of saturation of biofilm and planktonic cells, is perceptible that in both cases the degree of saturation decreased until 26.6 hours but at the end the cells presented membranes with higher percentage of saturated FAs than at the beginning. Such behaviour could result from carbon source depletion or/and lack of oxygen at the end of the experiment. In fact, the deposition of a thick mass layer on the top the medium may have hindered oxygen transfer. Supporting this affirmation is the fact that cells in the supernatant decreased their saturation degree possible due to an easier access to oxygen.

The fatty acid profile of cells grown as biofilm on hexadecane increased the SSFAs percentage on cell membranes along the time. In contrast, planktonic cells decrease the percentage of SSFAs (Figure 3.12, Hexadecane). The percentage of MUFAs decreased during the initial hours but increased in both cases till the end. Similarly, 10MBFAs decreased in cells in the biofilm reactor when compared to the cells at time zero but their percentage remained constant until the end of operation. In biofilm cells, the BCFAs were constant but in planktonic cells their percentage was higher than at the beginning of the experiment. The degree of saturation on both cases increased until 26.6 hours and decreased towards the end of the experiment (94.1 h), with planktonic cells presenting a degree of saturation lower than biofilm cells on the glass surface. The supernatant presented a FA profile similar to the biofilm and planktonic cells at the end of the experiment, although it’s noticeable the presence of PUFAs (8.7%) that are almost non-existent in the other two.

Comparing the composition of the cells grown on the two carbon sources, some differences are observed. In ethanol, the samples collected from the established biofilm presented in general more BCFAs, 10MBFAs and decreased degree of saturation. On the other hand, hexadecane grown cells presented HSFAs which in the ethanol grown cells are almost absent. Moreover, the 10MBFAs decreased with the time when compared to ethanol samples.

**Zeta potential**

The net surface charge of the cells may influence the process of interaction and attachment to the substratum, since van der Waals, hydrophobic and electrostatic
interactions are known to be involved in the process of cell adhesion [24]. In order to test the existence of differences in the charge between planktonic and biofilm cells, the zeta potential was measured in samples collected simultaneously to those for the FAMES analyses.

![Graph](image)

**Figure 3.13** – Net surface charge of biofilm and planktonic cells grown in the biofilm reactor with 0.25 % v/v of ethanol (left) or hexadecane (right). At the end of operation, a sample of the supernatant formed was collected.

The net surface charge of cells, represented by the zeta potential, grown in ethanol presented an increase during the first 6 hours (Figure 3.13 - left), this increase being higher in biofilm attached on glass (-16.4 mV) than in planktonic cells (-29 mV). Nevertheless, at 26 hours the charge dropped to values lower than at the beginning of the experiment. At the end (94 hours) the surface charge increased slight in the two cases, although more significantly in planktonic cells than in those in the biofilm. The supernatant formed at the end of operation presented cells with a net charge of -37.55 mV, which is similar to the -36.95 mV of cells attached to the glass surface. Cells in the supernatant might, in fact, grow as a biofilm (a thick layer could be observed at the liquid-gas interphase) similar the cells attached to the glass, which could explain the comparable net surface charge of the cells.

On the other hand, when hexadecane was used as carbon source (Figure 3.13 – right), cells presented behaviour different from those cells grown on ethanol. Planktonic cells changed their surface charge along the time of operation and becoming less negative. The cells in biofilm maintained the charge constant during the first 6-26 h (-31.55 mV), but increased their charge afterwards, reaching up to -15.75 mV at 94.1 h. In both cases is notorious the increase of the membrane charge. The cells at the supernatant at the end of the biofilm reactor operation had a net charge similar to the planktonic cells. In fact, at the end, the cells from the three different situations presented a similar membrane charge.
de Carvalho et al. [24] studied cell wall properties of *R. erythropolis* cells on different *n*-alkane hydrocarbons and demonstrated that the zeta potential decreases with the increase of the chain length of the carbon source and that biofilm and planktonic cells have similar behaviour in terms of the cell surface charge. In the present study it was also observed that planktonic and biofilm cells present similar surface charge during the time of operation and that cells grown on a short chain alkanol (ethanol) presented surface charges lower than those grown on a long chain alkane (hexadecane), as observed in Figure 3.13.

Although the net charge represent the overall charge of a certain sample/population of cells, in some cases different subpopulations, composed of cells with different surface may be present. These subpopulations may be studied by analysis of the zeta potential distribution of each sample (Figure 3.14). At 6.1 h, the “biofilm” sample presented two different populations. The largest percentage of the adherent cells (61.2%) had a charge of -9.19 mV whilst the remaining (38.8%) presented a charge of -28.3 mV, which is in the range of the surface charge of planktonic cells. Similarly at 94.1 hours, cells in culture presented 10% of the population with -10.8 mV of surface charge whilst the remaining

**Figure 3.14** – Zeta potential distribution of cells in planktonic state, biofilm and in the supernatant along the time of operation of the biofilm reactor with ethanol as carbon source (0.25% v/v). Replicates are indicated with numbers one and two. The surface charge of the inoculum cells (culture) was used at time zero.
results indicate that cells presented a surface charge between -40 mV and -30 mV and only one kind of population.

In hexadecane the presence of subpopulations with different surface charges are even more notorious (Figure 3.15). For instance, at 26 hours, planktonic cells presented clearly two different populations: one with an average zeta potential of -27.5 mV (67.4 %) and a second one with -10 mV (27.5 %). At the end of operation, planktonic cells presented a surface charge less negative (Figure 3.13 – right). In fact, 73.7 % of the planktonic cell population had a membrane charge of -13.5 mV and 26.6% of -37.6 mV (Figure 3.15). So, compared to what was observed at 26.6 hours, most of the population became less negative but a small part remained as negative as those at the beginning (0 h). Similar behaviour was observable in cells from the biofilm, which had a small population at 6.1 h with zeta potentials of -15 mV but at 94.1 h this population represented 63% of the cells. The cells in the supernatant at the end presented a distribution similar to the cell on the biofilm, suggesting, as expected that this supernatant formed was a biofilm on top of the culture medium, and so, had comparable characteristics with the biofilm formed on the glass slide.

![Figure 3.15](image_url)

**Figure 3.15** – Zeta potential distribution of cells in planktonic state, biofilm and in the supernatant along the time of operation of the biofilm reactor with hexadecane as carbon source (0.25% v/v). Replicates are indicated with numbers one and two. The surface charge of the inoculum cells (culture) was used at time zero.
Lipid Fractioning

The lipid composition presented in the previous sections represents the total fatty acids in the cells. Since the cells were collected during exponential phases in most of the cases, the large majority of the fatty acids should had been present in the cellular membrane phospholipids. However, after establishment of the biofilm or during periods of nutrient shortage, the cells may accumulate storage lipids and/or produce exopolymERIC glycolipIDS. To study the origin of the fatty acids accurately, the lipIDS were fractioned into apolar lipIDS and neutral lipIDS, which contain storage lipIDS, and phospholipIDS.

Samples from the ethanol and hexadecane biofilm reactors were collected from culture (planktonic cells), glass surface (biofilm) and supernatant and used for the fractioning of the different lipIDS in the cells. The phospholipIDS are present in cell membranes and their percentage is also representative of the quantity of cells present in the sample. The glycolipIDS may act as storage lipIDS but also as biosurfactants that some bacteria, such as *R. erythropolis*, produce and excrete to decrease the superficial tension of the medium [139] or to act as antimicrobial agents [140]. The apolar lipIDS are also storage lipIDS which, in the case of *R. erythropolis*, are mainly triacylglycerols (TAGs). These lipIDS are more common in eukaryotic organisms but are also found in the mycolata taxon, which contains species such as *Mycobacterium*, *Streptomyces*, *Rhodococcus* and *Nocardia*. The function of TAGs are mainly of carbon and energy storage but other functions have been described [27].

The percentage of phospholipIDS was similar in culture and glass samples in both carbon sources (between 25 and 31%) and increased in the supernatant to 40% in hexadecane and 46% in ethanol (Figure 3.16). This result suggests the existence of more cells in the samples collected from the supernatant than in those from the glass (biofilm) and culture (planktonic cells). Accordingly, the percentage of glycolipIDS is higher in cells collected from the glass surface and culture samples (more than 50%) rather than from cells in the supernatant (around 15%). The apolar lipIDS present in supernatant samples represented 45% of total lipIDS in hexadecane grown cells and 38% in ethanol, whilst it represented around 17.8% in planktonic cells and in those attached to the glass. Indeed the samples collected from the glass surface and culture had similar compositions. Cells from the supernatant samples presented less storage lipIDS (53.7% in ethanol and 60.1% in hexadecane) when compared to cells from the glass surface (70.5% in ethanol and 75.1% in hexadecane) and planktonic cells in culture (71.3% in ethanol and 68.9% in hexadecane). This suggests that cells at the surface, with easy access to oxygen, should
have been kept growing while those in the biofilm and in the culture suspension may have been under oxygen depleting conditions because of the thick layer formed by the supernatant at the top of the reactor.

**Enzymatic Profile**

Semiquantification of the enzymatic activities of cells from biofilm and supernatant samples recovered at the end of the biofilm reactor operation was accessed using an API ZYM® kit from BioMérieux (France). The enzymes present in the kit are listed in Table IV.

**Table IV – Enzymes present in the enzymatic kit used (API ZYM®, BioMérieux).**

<table>
<thead>
<tr>
<th>Number</th>
<th>Enzyme</th>
<th>Number</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>11</td>
<td>Acid phosphatase</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline phosphatase</td>
<td>12</td>
<td>Naftol-AS-Bl.phosphohydrolase</td>
</tr>
<tr>
<td>3</td>
<td>Esterase (C 4)</td>
<td>13</td>
<td>α-galactosidase</td>
</tr>
<tr>
<td>4</td>
<td>Esterase Lipase (C 8)</td>
<td>14</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>5</td>
<td>Lipase (C 14)</td>
<td>15</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>6</td>
<td>Leucine arylamidase</td>
<td>16</td>
<td>α-glucosidase</td>
</tr>
<tr>
<td>7</td>
<td>Valine arylamidase</td>
<td>17</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>8</td>
<td>Cystine arylamidase</td>
<td>18</td>
<td>N-acetyl-β-glucosaminidase</td>
</tr>
<tr>
<td>9</td>
<td>Trypsin</td>
<td>19</td>
<td>α-mannosidase</td>
</tr>
<tr>
<td>10</td>
<td>α-quimotrypsin</td>
<td>20</td>
<td>α-fucosidase</td>
</tr>
</tbody>
</table>

The activities of the enzymes were detected by colorimetric reaction and the intensity of the signal was graded in a scale from 0, meaning no enzymatic activity, to 5 representing...
a high enzymatic activity, as suggested by BioMérieux. Regarding cells grown on ethanol, the enzymatic activity was higher, in all cases, in supernatant cells when compared to those in biofilm. Besides, cells from the supernatant presented activity on five different enzymes (Alkaline phosphatase, Valine arylamidase, Trypsin, β-galactosidase, α-glucosidase) that were not detected in biofilm cells.

On other hand, cells grown on hexadecane in the biofilm reactor presented an enzymatic activity was much higher in comparison to those grown on ethanol (Figure 3.17). Besides, the enzymatic activities in biofilm and supernatant cells were similar, with cells from the supernatant presenting higher or equal activity to those from the biofilm (exception observed for the enzyme esterase). The higher enzymatic activity in supernatant cells was expected because, as mentioned before, the deposition of a thick layer of cells on the top of the reactor may facilitate the access to oxygen of cells in the supernatant while difficulting the access to oxygen by the cells attached to the glass. Both the lipid fractioning results and the enzymatic activities test suggest that cells on the supernatant were more active, probably because of the availability of oxygen. In the case of the biofilm cells the access to oxygen could be limited due to a poor superficial mass transfer, although the system was magnetically stirred.

Comparing the results obtained for cells grown on ethanol and hexadecane, it is possible to observe that the metabolism of hexadecane should be more complex than the uptake of ethanol, forcing the cells to produce more and different enzymes (Figure 3.17).
4. Flow Cell System

Flow cells are excellent for non-destructive, in situ imaging of biofilms allowing the study of interactions between biofilms and the surrounding fluid environment in real time. Biofilms may grow in aqueous medium and on a transparent substratum with microscopy providing spatially information about biofilm architecture and physiology if cells are labelled with appropriate fluorescent probes [28].

In this study, Nile red was used to stain both the lipids inside the cells and extracellular lipids. Two different flow cells were constructed, allowing the study of cell adherence and biofilm development on glass (hydrophilic) and PDMS (hydrophobic) surfaces. To
promote the production of different lipids in the cells, ethanol and hexadecane were used as carbon sources. During the system operation, images were captured at certain time periods to compare the architecture of biofilm that was being formed and the positioning of both cells and extracellular lipids. At the end of the experiment, the flow cell was disconnected from the system and was washed several times with 1 mL of distilled water, each mL being collected into a separate fraction that was used for FAMES analysis and zeta potential measurements. The fraction “1” represented in Figure 3.18 corresponds to the first fraction of cells that were washed out of the flow cell with the first mL used to clean the micro-reactor whilst fractions “3” and “4” were the last cells to be washed out. Cells adhesion to the surface of the cells in “1” should therefore be less strong than those in fraction “4”.

When ethanol was used as carbon source, the cells were mainly composed of SSFAs and MUFAs (Figure 3.18). In the fractions recovered from the flow cell, the bacterial cells also presented 10MBFAs. The degree of saturation of the cells increased with the number of fractions recovered. Since the first fraction contained the cells less attached to the flow cell and the last fraction recovered contained the cells more strongly attached the surface, this means that a membrane containing more SSFA (more saturated) should help the adhesion of the cells.

![Figure 3.18](image)

**Figure 3.18** – Lipid composition of membrane cells removed from the Flow Cell operating system. Left: PDMS/Glass flow cell after 17.7 h of operation. Right: PDMS/PDMS flow cells after 5.8 h of operation. At the second axis is presented the degree of saturation (Dsat) of the cell membrane. SSFAs – saturated straight chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFA – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.

Concomitantly to the increase of the degree of saturation and the percentage of SSFA in the membrane phospholipids of the cells, the surface charge of the cells attached also
increased with the order of flushing from the flow cell. So, the cells attached more strongly to the surface (flushed in the last fraction) were less negative than the cells less attached (Figure 3.19). In both PDMS-PDMS and PDMS-Glass flow cells, an increase of the surface charge of the cells could be observed, although the increase in the cells from the PDMS-PDMS flow cell was more abrupt when compared with that of PDMS-Glass flow cell. Moreover, the net values presented by the cells were less negative than that of cells attached to the PDMS-Glass flow cell.

![Figure 3.19 – Net Surface charge of planktonic cells at culture flask and biofilm attached in the two types of flow cells with 0.25 % v/v of ethanol as carbon source.](image)

As mentioned previously, net surface charge presented in Figure 3.19 is the average of the population, but in reality the samples recovered can present distinct subpopulations of cells. In the PDMS-Glass flow reactor, the majority of cells in the recirculating culture presented a surface charge of -37 mV, although 10% of the population had a charge of -9.09 mV (Figure 3.20). The cells from the first and second fractions flushed from the flow cell presented a more negatively surface charge, corresponding apparently to the most negatively charge subpopulation of the recirculating culture. Clearly, two populations were observed in the 3rd and 4th fractions. The 3rd fraction presented a subpopulation with -8.74 mV (43.9%) and the last fraction presented a subpopulation with a surface charge of -3.72 (55%). Apparently these cells are related to the 10% of the recirculating culture presenting less negative charge. The results thus suggest that an increase in the surface charge of the cells and in the degree of saturation (and consequently of the SSFAs) leads to more strongly adherent cells. The substratum characteristics of PDMS-Glass (hydrophilic) versus PDMS-PDMS (hydrophobic) may have influenced the difference on charge surface observed between the cells from the two flow cells.
With the PDMS-PDMS Flow cell (hydrophobic) an increase in the surface charge of the cells was also observed as in the PDMS-Glass micro-reactor. As expected, the cells in the recirculating culture presented a similar surface charge (-38.6 mV; Figure 3.21) when compared to those in the PDMS-Glass flow cell (-37 mV; Figure 3.20). Nevertheless, in this case a decrease of the cell charge in the first fraction flushed from the flow cell, as observed in the PDMS-Glass flow cell (Figure 3.19), was not observed (Figure 3.21). The first fraction of cells flushed from the PDMS-PDMS flow cell presented a surface charge (-36.6 mV) similar to cells in culture (-38.6 mV). The second fraction clearly presented two distinct subpopulations of cells: the major part (58.9%) having a surface charge of -22.7 mV and a second population (41.1 %) a positive charge of 2.46 mV (Figure 3.21). In the last fraction flushed, most of the cells (97.1%) presented a surface charge of -8.90 mV. This subpopulation appears between the two populations of the second fraction and seems to be an “intermediate” subpopulation in relation to the other two subpopulations flushed in the second fraction.

Figure 3.20 - Zeta potential distribution of biofilm cells on the PDMS-Glass flow cell with ethanol as carbon source (0.25% v/v). "Culture" represents the planktonic cells at the culture flask and replicates are indicated with numbers one and two.
The PDMS is a hydrophobic surface (contact angle of 109° [29]) and Glass is a hydrophilic surface (contact angle of 38.6º). Comparing the last fraction of *R. erythropolis* cells recovered from each flow cell (Figure 3.22) it is possible to observe that cells from the PDMS-Glass flow cell presented two distinguished subpopulation with surface charges of -35.3 mV (45%) and -1.73 mV (55%). This suggests that different surface materials on the flow cell may influenced the surface charge of the cells, resulting in different subpopulations. The PDMS-PDMS flow cell presented mainly a population with a zeta potential of -8.47 mV (97.1%), indicating that the subpopulation presenting the highest negative surface charge in the PDMS-Glass flow cell may be adhered to the glass. However, further studies will be necessary to elucidate these results.

![Figure 3.21 – Zeta potential distribution of biofilm cells from the PDMS-PDMS flow cell with ethanol as carbon source (0.25% v/v). "Culture" represents the planktonic cells in the culture flask and replicates are indicated with numbers one and two.](image)

![Figure 3.22 – Comparison of the zeta potential distribution of the cells from fraction 3 from the PDMS-PDMS flow cell and fraction 4 from the PDMS-Glass flow cell, with ethanol as carbon source (0.25% v/v).](image)
Compared to cells grown on ethanol, cells grown on hexadecane as carbon source presented a more complex lipid composition with significant variations in the composition of the cells from the several flushed fractions (Figure 3.23). The first fraction of *R. erythropolis* cells from the PDMS-Glass flow cell, had a lower percentage of SSFAs (17%) compared to the cells in the recirculating culture (49%), resulting in a low degree of saturation. In the following flushed fractions, the percentage of SSFAs increased as well as the degree of saturation of the cells. The same situation was observed in the flow cell using ethanol as carbon source (Figure 3.18). When grown on hexadecane, cells in the first two fractions exhibited dimethylacetal fatty acids (DMAFAs) and HSFAs which were not present in the cells from the last two fractions (Figure 3.23) whilst the percentage of BCFAs varied. The BCFAs may be more related to the carbon source being hexadecane than with the process of attachment, because BCFAs were not present in significant amount in the ethanol grown cells.

![Figure 3.23 – Lipid composition of membrane cells removed from PDMS-Glass flow cell operating system using hexadecane as carbon source. On the second axis the degree of saturation (Dsat) of the cell membrane is presented. SSFAs – saturated straight-chain fatty acids, MUFAs – monounsaturated fatty acids, BCFAs – branched-chain fatty acids, SCBFAs – saturated cyclopropyl-branched fatty acids, 10MBFAs – 10-methyl branched fatty acids, DMAFAs – dimethylacetal fatty acids, PUFAs – polyunsaturated fatty acids, HSFA – hydroxyl-substituted fatty acids.](image)

According to Curtis *et al.* [30] the incorporation of MUFAs of chain length lower than 18 carbon atoms (MUFAs<18C) on the cell membrane decreases the adhesion of cells. On the other hand, the incorporation of SSFAs containing more than 18 carbon atoms (SSFAs>18C) leads to an increase in cell adhesion. Under the three conditions tested in the present study, an increase of SSFAs>18C and a decrease in the MUFAs<18C was observed with the order of the fraction flushed from the flow cells (Figure 3.24). This suggests, as expected, that the “force” of cellular adhesion increases with the order of
the fraction recovered. In other words, since the cells recovered from the last fraction had a larger percentage of SSFAs >18C and a lower percentage of MUFAs <18C, these cells should present an increasing adhesion to surfaces when compared to cells recovered from the first fraction. This is in agreement with the logical assumption that cells recovered from the last fraction required more "force" to be washed out from the flow cell. Moreover, cells grown on hexadecane presented a percentage of SSFAs higher than the cells grown on ethanol, suggesting that cells in this carbon source are more strongly adherent to the flow cell surface.

Cell surface properties, including the surface charge, are important for cell adhesion. Curtis et al. [31] studied the association of adhesion and cell surface charge density and suggested that low surface charge densities are associated with high adhesion and vice-versa. Moreover, the incorporation of certain FAs may change the surface charge density and consequently influence cell adhesion. Using the electrophoretic mobility measurements of cells flushed from the flow cells using ethanol as carbon source, the surface charge density was calculated using the equation described by Krisinsky et al. [32]. In both flow cells, the negative charges at the cell surface decreased per area with the fraction recovered (Figure 3.25). The decrease in surface charge density verified in PDMS-PDMS cells was lower than that observed in cells recovered from the PDMS-Glass flow cell, which presented an abrupt decrease in surface charge density when analysing the cells from fractions 1 to 2. These results thus suggest that a decrease in cells surface charge density are correlated to high cell adhesion, which is in agreement with Curtis et al. [31], as previously hypothesised.

![Figure 3.24](image-url) – Fatty acid composition of the cells flushed from the flow cell systems. SSFAs >18C, Saturated straight-chain fatty acids with more than 18 carbon atoms. MUFAs <18C, monounsaturated fatty acids with less than 18 carbon atoms.
To assess the percentage of cells that were weakly or strongly attached to the surface of the flow cells, the percentage of cells recovered after each flushed fraction was calculated (Figure 3.26). When the cells were using ethanol as carbon source, the large majority (at least 75%) of the cells were recovered in the first fraction. However, in the system with hexadecane as carbon source, only 37.7% of cells were recovered in first fraction and 43.7% in the second fraction. The cells removed in the last fractions represented a small percentage of the total adherent biomass. The results thus indicate that, in a PDMS-Glass flow cell, the adhesion of *R. erythropolis* cells to the surfaces is stronger when using hexadecane as carbon source than when the cells are using ethanol. These results thus confirm what had been suggested by FAMEs and zeta potential analysis. Besides, the method of studying the percentage of cell in fractions obtained from flushing liquid through the flow cells, is sufficiently accurate as demonstrate by analysing the influence of time. The PDMS-PDMS flow cells were operated for only 5.75 h and, since the cells had not enough time to adhere to the surfaces and to form a proper biofilm, only three fractions containing cells were recovered and nearly 90% of the cells were recovered in the first fraction. 

**Figure 3.25** – Surface charge density ($\delta$) of cells flushed from the two flow cells (fractions 1 to 4) and culture (0) when grown on ethanol as carbon source.
The architecture of the biofilm formed in the flow cell systems was accessed by microscopy images acquired during the time of operation (Figure 3.27). The biofilms formed by cells grown on ethanol presented similar structure, with the cells embedded in a cloud type of exopolymeric lipids, regardless of the flow cell materials. On the other hand, the biofilm grown on hexadecane presented a distinct architecture from the biofilms grown on ethanol. Besides, during the operation of the flow cells on the two carbon sources differences were also noted in the biofilm behaviour. Biofilms grown during ethanol consumption were characterized by the agglomeration of cells in a structure like a “cloud” and by some movement inside the flow cell (data not shown). Contrarily, the biofilm developed in the hexadecane-containing flow cell was nearly “filamentous” like, and both cells and exopolymeric lipids adhered immediately at the entrance of the flow cell chamber. The attachment during a short period of time (1h) was noticeable as shown in the supplementary Figure S5 of chapter 5. In the flow cell systems tested, the deposition and attachment of cells occurred first at the entrance of the flow cell chamber and propagated from there to the rest of the chamber, probably as a result of a rapid decrease in pressure observed between the channel and chamber of the flow cell.

From the microscope observations it was perceptible that cells grown in hexadecane were “stickier” and more prone to adhesion and attachment than ethanol grown cells. These observations are in agreement with the analyses discussed before, e.g. regarding...
the increasing percentage of SSFAs with more than 18 carbon atoms in the cellular membranes (Figure 3.24) which is associated with higher cell adhesion ability.

5. The Role of Lipids

As discussed before in this work, the lipids in cellular membranes and the changes that *R. erythropolis* promote in their composition are essential to cell adhesion on different types of surfaces. The data related to the FAs composition of adherent cells on different surfaces (from the 6-well plates assay) and to the recirculating tube system with cells grown in mineral medium with ethanol as carbon source, were studied using principal components analysis (PCA). The data used is presented in Table V. Only the FA composition of the cellular membrane phospholipids of cells with similar age and from the same growth phase was used.
### Table V – Fatty acid composition of adhered cells in different surfaces used for the PCA.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Fatty Acids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Straight</td>
</tr>
<tr>
<td>Polystyrene (PS)</td>
<td>41.60</td>
</tr>
<tr>
<td>Al</td>
<td>37.80</td>
</tr>
<tr>
<td>Fe</td>
<td>36.16</td>
</tr>
<tr>
<td>Zn</td>
<td>38.16</td>
</tr>
<tr>
<td>Cu</td>
<td>38.59</td>
</tr>
<tr>
<td>Pb</td>
<td>40.31</td>
</tr>
<tr>
<td>Butyl rubber</td>
<td>45.77</td>
</tr>
<tr>
<td>Polietrafluoretileno (PTFE)</td>
<td>45.29</td>
</tr>
<tr>
<td>Polyethylene (PE)</td>
<td>45.02</td>
</tr>
<tr>
<td>Polyethylene terephthalate (PET)</td>
<td>44.88</td>
</tr>
<tr>
<td>Glass</td>
<td>43.08</td>
</tr>
<tr>
<td>Glass mosaic</td>
<td>39.68</td>
</tr>
<tr>
<td>Gas tubing</td>
<td>51.10</td>
</tr>
<tr>
<td>Marprene</td>
<td>30.45</td>
</tr>
<tr>
<td>Soft silicone</td>
<td>29.24</td>
</tr>
<tr>
<td>Silicone</td>
<td>27.30</td>
</tr>
<tr>
<td>Teflon</td>
<td>39.93</td>
</tr>
</tbody>
</table>

According to PCA, 87.2% of the variability of the data are explained by 2 components (PCA 1 and PCA 2). By scoring the experimental data on a plot having PC1 and PC2 as axes, it’s possible to observe the clustering of the data according to the FA composition and hydrophobicity character of the material (Figure 3.28). The data is separated along PC1 by the composition in FA: the main group of materials clustered at the right-side (Figure 28 – A) is separated from the left side by their composition in SSFAs (straight), 10MBFAs (10-methyl) and MUFA (Figure 3.28 – B). In this group, the metallic surfaces (Al, Fe, Cu, Zn and Pb) are very close to each other, being separated from some plastics (PET,PE), PTFE, glass and butyl rubber by the degree of saturation (i.e. percentage of monounsaturated and saturated straight fatty acids along PC2). Similarly the non-metallic surfaces silicone, soft silicone and marprene were separated from the other non-metallic surfaces by the percentage of saturated FA. According to this analysis, the gas tubing was the surface where cells presented a response that was most different from all others.
The relation between the FA composition of the cellular membrane phospholipids and the volume of biofilm of the cells formed on different surfaces was analysed by partial least squares (PLS). The statistical method tries to find the multidimensional direction in the FA composition that explains the maximum multidimensional variance in the biofilm data. The percentages of each FA type were therefore used as predictors (Table V), whilst the response was the biofilm volume (which was normalised to vary between 0 and 1; Table VI).

Figure 3.28 – Principal component analysis using the fatty acid composition of cells forming biofilms on different surfaces. A - Score plot with data represented as function of the surface to which the cells adhered; B – Loading plot.
Table VI – Normalized volume of the biofilm formed on each surface, used as response vector for the PLS analysis.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Biofilm, normalized (0-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene (PS)</td>
<td>0.736</td>
</tr>
<tr>
<td>Al</td>
<td>0.435</td>
</tr>
<tr>
<td>Fe</td>
<td>0.165</td>
</tr>
<tr>
<td>Zn</td>
<td>1.000</td>
</tr>
<tr>
<td>Cu</td>
<td>0.201</td>
</tr>
<tr>
<td>Pb</td>
<td>0.378</td>
</tr>
<tr>
<td>Butyl rubber</td>
<td>0.071</td>
</tr>
<tr>
<td>Polytetrafluoretileno (PTFE)</td>
<td>0.434</td>
</tr>
<tr>
<td>Polyethylene (PE)</td>
<td>0.240</td>
</tr>
<tr>
<td>Polyethylene terephthalate (PET)</td>
<td>0.157</td>
</tr>
<tr>
<td>Glass</td>
<td>0.562</td>
</tr>
<tr>
<td>Glass mosaic</td>
<td>0.036</td>
</tr>
<tr>
<td>Gas tubing</td>
<td>0.000</td>
</tr>
<tr>
<td>Marprene</td>
<td>0.004</td>
</tr>
<tr>
<td>Soft silicone</td>
<td>0.014</td>
</tr>
<tr>
<td>Silicone</td>
<td>0.012</td>
</tr>
<tr>
<td>Teflon</td>
<td>0.002</td>
</tr>
</tbody>
</table>

The results of the PLS analysis indicated that 2 components can predict 83.6% of the variance of data regarding the FA composition of the phospholipids of cells during adhesion to different surfaces and the amount of biofilm formed. According to the PLS coefficients, the 10MBFAs (10-methyl) are positively correlated with the formation of biofilm (Figure 3.29 – A). This is in concordance to/confirms the results obtained in section 1 (Figure 3.5). Comparing the PLS loading (Figure 3.20 - B) and score (Figure 3.29 –C) plots, it is possible to observe that the percentage of biofilm on MM increased with increasing percentage of 10MBFAs in the phospholipids of the cells which adhered to metallic and non-metallc surfaces clustered at the right side of Figure 3.29 – C. Outside this cluster, the surfaces silicone, soft silicone and marprene are separated because of the content in PUFAAs and BCFAs (branched). Once again, the cells changed the FA composition of the phospholipids during adhesion to the gas tubing in a different way from to all other surfaces.

The amount of biofilm increased in the direction from glass mosaic to zinc surface, because of the increase in the content of 10MBFAs (10-methyl) in the cellular membrane of *R. erythropolis*. On other hand, the biofilm size decreased in the direction of silicone to marprene.
After analysing the relation between the FA composition of the phospholipids of *R. erythropolis* adherent to different surfaces and the amount of biofilm produced, it was possible to conclude that cells, grown in mineral medium and using ethanol as carbon source, form and develop biofilms depending on the percentage of 10-methyl branched fatty acids in the cellular membrane.

![Figure 3.29](image)

**Figure 3.29** – PLS analysis of the relation between the FA composition of cellular phospholipids and the amount of biofilm formed. A - PLS coefficients, B - Loading plot, C - Score plot with data represented as function of the surface to which the cells adhered.
6. References


Chapter 4

Conclusion and Future Work

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2. Future work .......................................................................................................................... 87
1. Conclusion

The study of physicochemical properties of the surface and membrane composition of
*R. erythropolis* cells attached to surfaces with different properties led to some insights
on the cell adaptation mechanisms that determine the attachment of previously
planktonic cells and the identity of the lipids produced by the cells during the formation
of the biofilm.

In this study, it was possible to observe the changes in the fatty acid composition of the
cells after adhesion to different surfaces. The two media used also influenced the lipid
composition of *R. erythropolis* cells. These changes are in concordance to the
microscopic observations: the size of cell aggregates and biofilm development are
influenced by the surface material and the medium used. Using a recirculating biofilm
reactor it was observed that cells decreased their hydrophobicity with increasing contact
angles in hydrophilic surfaces, whilst in hydrophobic surfaces cells increased the
percentage of FAs with more than 16 carbons in their membrane with increasing contact
angle. Moreover, cells in early exponential phase had more capacity of adherence and
biofilm formation than cells in late exponential phase. But cell composition could be
influenced by adding limonene leading to less adhered cells. Nevertheless, the results
obtained suggest that no simple, linear correlation between the increase of contact angle
and the amount of adhered cells exists.

It was also observed that planktonic and biofilm cells increased the net surface charge
during the time of operation of a biofilm reactor. The cells grown on a short chain alkanol
(ethanol) presented surface charges lower than those grown on a long chain alkane
(hexadecane).

The construction of a flow cell system allowed the visualization of biofilm formation *in situ*
and in real time. The cells attached more strongly to the surface presented a net surface
charge less negative and a higher degree of saturation of the membrane phospholipids
compared to weakly adhered cells. Moreover, a larger percentage of saturated straight-
chain fatty acids with more than 18 carbon atoms and a lower percentage of
monounsaturated fatty acids with less than 18 carbon atoms in cell membranes of
*R. erythropolis* increased the “force” of cell adhesion to surfaces. Using statistic methods
was possible to conclude that *R. erythropolis* cells adherent to different surfaces, grown
in mineral medium and using ethanol as carbon source, formed and developed biofilms
depending on the percentage of 10-methyl branched fatty acids in the cellular
membrane.
The results obtained in the present work suggest that a very complex array of variables have to be taken into consideration, such as, composition of the substratum, carbon source and presence of toxic compounds to predict cell behaviour. The changes on membrane cell influence the production, the composition and thus the physical properties of the EPS and consequently, the formation of biofilms on solid surfaces. Such achievements may contribute to the knowledge and advance of biofilm development processes and lead to new biotechnological applications in the areas of bioremediation and biocatalysis or, on the contrary, to biofilm eradication.

1. Future work

The present study demonstrates that lipids play an important role in several steps of biofilm formation. Further studies are required for a better elucidation of the influence of lipids in biofilm formation and bacterial adaptation. Also, the identification of new drug targets such as the synthesis of specific lipids required for biofilm formation could be of extremely importance to understand the biofilm development. Thus, the following suggestions are presented:

- to study the influence of the composition of the cell wall (e.g. in mycolic acids) in cell adhesion;

- to determine the identity of the lipids involved in the cell anchorage and biofilm matrix;

- to study the defence and adaptation mechanisms of bacterial cells to antimicrobial compounds and extreme conditions (e.g. desiccation, osmotic shock, presence of solvents) at the lipid level;

- to evaluate which changes in the lipid composition of the mature biofilm lead to the release and dispersion of cells for the colonization of new surfaces.
Chapter 5
Supplementary Information
Figure S 2 - Images acquired with an optical microscope with fluorescent light (magnification of 30X) after 48 h of biofilm formation on metal surfaces in Mueller-Hinton (left) and mineral (right) media. Lipids are stained with Nile red (red). The right image of each set represent the respective calculated surface-plot.

Figure S 1 – Frequency of the size of the aggregates of cells of the images presented in Figure S1. MH – Mueller-Hinton medium. MM – mineral medium
<table>
<thead>
<tr>
<th></th>
<th>MH</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td><img src="image1" alt="Blank Image" /></td>
<td><img src="image2" alt="Blank Image" /></td>
</tr>
<tr>
<td>Butyl</td>
<td><img src="image3" alt="Butyl Rubber Image" /></td>
<td><img src="image4" alt="Butyl Rubber Image" /></td>
</tr>
<tr>
<td>Teflon</td>
<td><img src="image5" alt="Teflon Image" /></td>
<td><img src="image6" alt="Teflon Image" /></td>
</tr>
<tr>
<td>PE</td>
<td><img src="image7" alt="PE Image" /></td>
<td><img src="image8" alt="PE Image" /></td>
</tr>
<tr>
<td>PET</td>
<td><img src="image9" alt="PET Image" /></td>
<td><img src="image10" alt="PET Image" /></td>
</tr>
<tr>
<td>Glass</td>
<td><img src="image11" alt="Glass Image" /></td>
<td><img src="image12" alt="Glass Image" /></td>
</tr>
<tr>
<td>Glass Mosaic</td>
<td><img src="image13" alt="Glass Mosaic Image" /></td>
<td><img src="image14" alt="Glass Mosaic Image" /></td>
</tr>
</tbody>
</table>

**Figure S 3** – Images taken with an optical microscope with fluorescent light (magnification of 30X) during biofilm formation in non-metal surfaces in Mueller-Hinton (left) and mineral (right) media. Lipids are stained with Nile red (red). Right image of each set represent the respective calculated surface-plot.

**Figure S 4** – Frequency of the size of the aggregates of cells of the images presented in Figure S3. MH – Mueller-Hinton medium. MM – mineral medium Mueller
**Figure S 5** – Images taken during operation of PDMS-Glass flow cell when hexadecane was used as carbon source at 0.25% v/v. Images order by time of acquisition from A to F with a lapsed time of 1 hour. Cells were stained with Nile red. Amplification of 30x.