Biotechnology in Agriculture, Industry and Medicine

BIOFILMS

Formation, Development and Properties

William C. Bailey
Editor

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BIOTECHNOLOGY IN AGRICULTURE, INDUSTRY AND MEDICINE

BIOFILMS: FORMATION, DEVELOPMENT AND PROPERTIES

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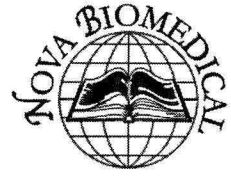
EDITOR



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PREFACE

A biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilms may form on living or non-living surfaces, and represent a prevalent mode of microbial life in natural, industrial and hospital settings. This new book presents research in the study of biofilms including bacteriophages and biofilms, dynamic models for in vitro biofilm formation, symbiotic biofilms and brain neurochemistry and mineralization of sedimentary biofilms. Also discussed herein are biofilms in urology, virulence properties in biofilms, biofilms and the upper respiratory system and microbial biofilms in the food industry.

Chapter 1 - Bacteriophages (phages) are the viruses of bacteria and biofilms represent a frequent niche for bacteria, one that potentially modifies phage-bacterial interactions away from those seen with planktonic bacteria. Bacteria in biofilms, especially, are structured into complex microcolonies and embedded in extensive extracellular polymer (EPS). Towards gaining a better understanding of the biology of phage-biofilm interactions, here I provide an overview of the subject, divided into four areas: (i) The many facets of phage-biofilm interactive biology including consideration of virus trapping, phage hydrolytic enzymessuch as EPS depolymerases, infection of biofilm bacteria, phage prevalence within natural biofilms, prophage-biofilm interactions including in terms of prophage modification of biofilm structure or function, and the potential for biofilms to resist phage attack. (ii) A critical review of the literature concerning phage use as biofilm prevention or eradication agents, that is,phage therapy or phage-mediated biocontrol of biofilms. (iii) Discussion of phage-plaque developmentas it occurs in the laboratory as a model for phage-biofilm interactions, since plaque formation is both related to and better understood than lytic phage infection of biofilms. And (iv) exploration of issues pertaining to phage penetration into bacterial microcolonies. I stress that key to understanding the dynamics of phage-bacterial interactions within biofilms is a combination of addressing how phages move toward as well asaway from target bacteria, including in terms of the phage potential to burrow into bacterial microcolonies. I argue that it may not be necessaryfor phages, even if they specialize on biofilm bacteria, to extensively destroy naturally occurring biofilms in order to prosper.

Chapter 2 - Chronic wounds, by definition, are those that remain in a chronic inflammatory state and therefore fail to follow normal patterns of the healing process. The chronic wounds present a challenge to physicians and patients alike because are very difficult to heal, inflict a huge cost to society and impair the quality of life for millions of people.

There are many factors that contribute to the development of chronic wounds. One of the most clinically significant impediments to wound healing is infections.

The growth of large aggregates of cells on a surface encased within a matrix of extracellular polymers produced by the sessile bacteria is known as a biofilm. In man, one of the surfaces that are available for attachment is ulcer beds. Bacteria biofilm formation in wounds is the best unifying explanation for the failure of chronic wounds to heal. It has been estimated that biofilms are associated with 65 percent of nosocomial infections. Biofilm bacteria are protected from host defenses, antibiotics and antiseptics.

Using good bacteria to obstruct bad ones—a strategy known as bacterial interference—is one application of so-called probiotics, a field with growing medical promise. Beyond using probiotics to replenish missing bacteria on digestive tract and vagina, we and other authors are working to interfere to pathogenic bacteria in infected wounds. Furthermore, it has been demonstrated in vitro that cells and/or the metabolic by-products of lactobacilli have antagonistic effects on pathogens; these results have also been found in vivo during trials with urinary and genital infections in humans and mice and in wounds infected with S. aureus.

We demonstrated that *L. plantarum* interferes with the pathogenic capacity of *P. aeruginosa* (quorum sensing, biofilm, virulence factors and growth).

Topical treatment with *L. plantarum* cultures is currently being carried out by our medical team with infected burns and chronic venous ulcers in humans with encouraging results. The cells from ulcer beds collected after treatment with *L. plantarum* showed a decrease in the percentage of polymorphonuclear, apoptotic and necrotic cells and a modulation of IL-8 production. The mode of action of *L. plantarum* on infection and wound healing seems to be related to acidity, presence of lactic acid, interference with *P. aeruginosa* quorum-sensing signals, perhaps inter-species communication through type 2 autoinducer and modification of tissue repair process.

Our findings will be useful for the formulation of effective and inexpensive products to resolve infected chronic wounds with biofilm-producing bacteria.

Chapter 3 - Since biofilms are resistant both to host defense mechanisms and to antimicrobial agents, they represent an ongoing source of infection.Investigations of natural biofilms are restricted because of problems with access and sampling, in addition to complications due to ethical aspects.In vitro biofilm models are important tools in experimental medical science to better understand the development and behavior of such microbial communities.Laboratory models that intend to mimic natural biofilms may be more controllable than in vivo protocols and therefore more useful to explain and predict their behavior.In vitro systems range from static mono-cultures to the development of diverse mixed cultures growing under dynamic conditions.Given that in vivo conditions are almost exclusively dynamic, studies evaluating biofilm formation under static conditions might be somewhat misleading.One of the most used dynamic models to study biofilm mode of growth is the flow cell system, which consists of a transparent chamber of fixed depth through which the growth medium passes.In conjunction with a microscope and camera, this method can be used to observe the early events of biofilm formation (microbial adhesion) and the interactions between microorganisms and substrata in real time.Chemostats can also be used to study the dynamic growth of microorganism populations on experimental substrates submerged within the chemostat.One of the most important features of chemostats is that microorganisms can be grown at a constant rate.Moreover, during biofilm formation, culture parameters such temperature and pH remain constant.Analogous to the operation of the

chemostat, there is another category of reactors in which biofilms are formed on thin filter membranes in a physiological steady state.These systems permitanalyses of the growth rate dependence and cell-cycle specificity of antibacterial agents by collecting the eluate passed thought the filter, which contains a cohort of freshly divided cells.Finally, there are constant depth reactors in which surface growth is periodically removed to maintain a constant geometry of biofilms.In these reactors, microorganisms can be grown in a physiological steady state with all culture parameters constant.The system can generate large numbers of biofilms with comparable and reproducible data from experiment to experiment and has been used extensively to investigate factors that may influence the growth of microorganism communities.Considering the information presented above, this chapter will explore the range of technologies and dynamic systems available for in vitro biofilm formation, development, and maturation.

Chapter 4 - An overwhelming majority of known species of microorganisms form biofilms, i .e., spatially and metabolically structured communities embedded in the extracellular biopolymer matrix. Biofilm development is a complex multi-stage process involving reversible and, at a later stage, irreversible attachment of microbial cells to the substrate surface, matrix formation, three-dimensional structuring of the whole community including the formation of mushroom- or pillar-shaped structures and, finally, the degradation of the biofilm and the dispersion of the cells involved. These processes are considered in the example of microorganisms that interact with the animal or human organism, playing the roles of symbionts or pathogens. In particular, the microorganisms of the gastro-intestinal (GI) tract interconvert between two different lifestyles: they can exist as planktonic cells in the intestinal lumen or form part of a biofilm attached to the mucous membrane of the GI tract. The GI microflora, including the biofilm-forming cells, is subject to regulation by the metabolites and chemical signals produced by the human/animal host.

The data published in the literature and our own findings suggest an important role of host-produced neuromediators, such as amines, peptides, and nitric oxide, which regulate biofilm formation by influencing microbial growth rate, the aggregation of microbial cells, the formation of microcolonies, and matrix synthesis. Our results were obtained using high efficiency liquid chromatography and revealed that cells of various symbiotic and pathogenic bacterial species contain serotonin, norepinephrine, and dopamine, as well as their precursors and oxidative deamination products. It follows from our studies with *Escherichia coli* that the culture of this symbiotic, biofilm-forming bacterium releases amine neuromediators and their precursors/products into the culture fluid in concentrations of 10--100 nM, which are sufficiently high to cause the host's physiological response. These facts and other relevant data are considered in the article in terms (i) of the autoregulatory role of neuromediators in the biofilm-forming microbial population and (ii) the microbially-produced neuromediator amines' impact on the human organism. Of particular interest in this respect are the data that the culture fluid of *E. coli* contains over 1 μM DOPA. DOPA, the catecholamine precursor, crosses the gut-blood and blood-brain barriers. In the brain, DOPA is converted to dopamine and thereupon to norepinephrine that regulate brain processes involved in locomotion, affection, sociable and dominant behavior, as well as aggression.

Chapter 5 - In most ecosystems, microorganisms mainly exist as surface-attached communities called biofilms. During the sequential process of adhesion and biofilm formation, bacteria undergo major adaptive changes leading to morphological and physiological changes in the cell. The switch from a planktonic state into the biofilm state is

regulated by a variety of environmenal and physiological cues, and during recent years, several of the underlying regulatory mechanisms have been revealed and dissected on a molecular level: Upon cell-surface contact, multiple environmental conditions give rise to the upregulation of various stress responses and two-component systems resulting in an altered expression of outer membrane proteins, as well as cell surface appendages and extracellular matrix. Notably, the transcription factors FhlCD and CsgD play central roles in the regulation of biofilm formation, and the secondary messenger c-di-GMP has emerged as an important and ubiquitous signal molecule mediating the transition in biofilm-specific gene expression. In this chapter, recent advances in the understanding of surface-induced signalling will be presented with special emphasis on concepts that have emerged during the last few years.

Chapter 6 - Microbial mats are sedimentary biofilms found in a variety of natural environments. Like other biofilms, they are composed of bacteria and archaea living in a hydrated polymeric matrix. They may also contain varying amounts of accreted sediment and harbor surface populations of sessile diatoms (Cohen et al., 1984; Paterson et al., 2008; Riding and Awramik, 2000; Underwood et al., 1995; Walter, 1976). Some of these sedimentary biofilms precipitate minerals (notably carbonates) while most do not. This intriguing variability makes modern systems ideal candidates for studies of mineral precipitation mechanisms. Precipitation requires favorable geochemical conditions and a suitable nucleation site (e.g. (Morse et al., 2007)), both of which can be microbially influenced (Dupraz et al., 2009). While environmental conditions posit important boundary effects, much recent emphasis has focused on the role of community composition, spatial distribution, bacterial metabolism, and the relative contributions of the bacteria and their matrix.

Chapter 7 - The epipelic biofilm is a biologic complex of autotrophs (algae) and heterotrophs (fungi, bacteria, microinvertebrates) embedded in a polysaccharide matrix that develops on the fine sediments (silt and clay) of many aquatic ecosystem worldwide of lowland fluvial streambeds. Biofilms play a key role in the energetic balance of the fluvial systems, contributing to the recycling of organic matter and, therefore, to their self-depuration. The structure and function of a biofilm are affected by a variety of factors, both natural and anthropogenic, that, in turn, determine the physical and chemical conditions of the water. The characteristics of biofilms on episammic and epilithic substrates have been widely described in the literature, but the features of epipelic biofilms have been only scarcely documented. In this chapter, we present a review of the use of the structural and functional parameters of epipelic biofilms in order to assess changes in water and habitat quality as a result of human impact. Methodologies for the sampling and analysis of biofilms are described; and selected study cases are discussed in order to provide information about specific composition, density, biomass, biological indices, primary production, respiration, and enzymatic activities of the epipelon in relation to different uses of the surrounding land. The structural and functional parameters of biofilms should be made an integral component in the routine assessment of stream health as well as in the establishment of baseline values for both disturbed and undisturbed systems to be incorporated into monitoring and compliance guidelines.

Chapter 8 - Naked amoebae are a fascinating group of single celled eukaryotic protists. Until recently the study of naked amoebae was largely beyond the scope of most ecological protozoologist. This was due to their small size (often near 10 microns), fragile cell structure, transparent cytoplasm, and tendency to adhere to substrates like soil aggregates and

suspended floc making them indistinguishable. The result was a dearth of research, and a widely held assumption that naked amoebae densities and ecological roles, were probably of lesser ecological significance than other more widely studied protozoa like ciliates and flagellates. However, recent advances in reliably enumerating naked amoebae have generated a great deal of renewed interest and understandings of the roles naked amoebae play in ecosystems. It is now known that not only are naked amoebae ubiquitously distributed in virtually every terrestrial and aquatic habitat, but there densities are very high, and in many cases even surpass those of ciliates and flagellates. Although our knowledge of naked amoebae ecology in native habitats has emerged rapidly, little is known about their capacity to fit into and exploit new surfaces and niches like biofilms that form when an invasive species becomes a dominant member of a previous stable ecological community.

In North America, zebra mussels (*Dreissena polymorpha*) are an invasive species notoriously known for altering the physical, chemical and biological attributes of aquatic ecological communities. Zebra mussel densities have been reported as high as 700,000/m^2 creating an enormous surface area for biofilm formation. In this study, the first investigating naked amoebae in zebra mussel biofilms, the abundance of naked amoebae documented in the biofilms of zebra mussels was compared to abundances on rock biofilms at approximately monthly intervals for one year. Data analyses showed no significant difference ($F = 1.44$; $p \leq .270$) in total naked amoebae abundances between the zebra mussel shells and rock surface biofilms. The combined naked amoebae abundance data from zebra mussels and rock biofilms showed a decline in mean numbers from winter to early spring, when naked amoebae were undetectable in some samples. This was followed by high abundance peaks in May (9604/g), and September (10,362/g). The similarities in naked amoebae abundances in biofilms established on the shells of living mollusks that are very new invaders of an aquatic ecosystem, and the surfaces of non-living rocks, indicates that naked amoebae seasonal patterns are independent of the substrate on which they form. In this study, the seasonally changing structure of biofilm communities, and not the substrate on which they form, is the most likely regulator of naked amoebae densities.

Chapter 9 - *Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia* and *Xanthomonas maltophilia*) is a widespread environmental bacterium that has become a nosocomial pathogen of increasing importance. In fact, it is the third most common nosocomial non-fermenting bacteria. Infection occurs principally in immunocompromised subjects, and in patients exposed to invasive devices and/or broad spectrum antibiotics. It has been isolated with increasing frequency from cystic fibrosis patients. *S. maltophilia* causes pneumonia associated with mechanical ventilation, catheter-related bacteraemia, haemodialysis-related infections and urinary tract infections.

S. maltophilia isolates are intrinsically resistant to β-lactams, aminoglycosides, macrolides, and many older quinolones. The full genome sequence of an environmental isolate, *S. maltophilia* R551-3, and a clinical isolate, *S. maltophilia* K279a, put in evidence several resistance-nodulation-division (RND)-type putative antimicrobial efflux systems.

The virulence factors of *S. maltophilia* include proteases and elastase as well as the ability to adhere to living and different abiotic surfaces, such as medical devices. Gene products implicated in the formation of intermediates of lipopolysaccharides and exopolysaccharides, as well as fimbriae 1 (SMF-1) are involved in biofilm formation. *S. maltophilia* uses a cell-cell signaling system mediated by a diffusible signal factor (DSF),

which controls the production of an extracellular protease, aggregative and biofilm behavior, and virulence in a nematode model.

We have studied *S. maltophilia* isolates recovered from patients with device-associated nosocomial infections at a university hospital in Argentina, between 2004 and 2008. The local isolates exhibited different abilitiesof biofilm formation on hydrophilic and hydrophobic surfaces. All isolates formed strong biofilms on polystyrene microplates, while strong, moderate or weak biofilms were formed on borosilicate or polypropylene tubes. The microscopic analysis of biofilms formed on glass coverslips revealed the presence of a matrix of exopolysaccharides and microcolonies typical of biofilms architecture. According to our results, twitching, a quorum sensing regulated motility, correlated well with attachment to the three abiotic surfaces tested, while swimming only showed a slight correlation with biofilm formation on polypropylene.

Due to its multidrug-resistance phenotype and its ability to attach, the selection of agents for use in the management of *S. maltophilia* infections presents a challenge. We have compared the in vitro effects of levofloxacin and ciprofloxacin on pre-formed biofilms and planktonic populations. Levofloxacin was more active than ciprofloxacin against local isolates according to MIC evaluation. However, *S. maltophilia* isolates sensitive to fluoroquinolones according to the MICs, were highly resistant when biofilm susceptibility was evaluated by the minimum regrowth concentration (MRC) assay. Thus, currently used concentrations of fluoroquinolones cannot be used in monotherapy for eradication of a biofilm. Nevertheless, these agents could be used for the lock technique.

Chapter 10 - Current biological processes for the treatment of effluents have undergone considerable evolution and one of the major contributions was the development of processes which use biofilms supported on particulate materials. Studies by several authors have highlighted some advantages of processes using adhered biomass in relation to conventional processes, including: the high concentration of biomass attained, resulting in a small space being required for the installation; capacity to tolerate toxic and recalcitrant compounds; application of a greater organic load; easy operation; and high pollutant removal efficiency. Reactors with biofilms have been employed in aerobic, anaerobic and denitrifying processes, in many different configurations. This chapter will describe some research studies related to the formation of the biofilm, the supports employed and the reactor configurations used in the treatment of liquid industrial effluents. Some cases of the application of reactors with biofilms in the removal of contaminants from liquid effluents will be presented, these being: the aerobic biodegradation of benzene, toluene and o-xylene (BTX) compounds in a batch reactor and in a continuous fixed-bed reactor with biomass adhered on activated carbon; the aerobic biodegradation of effluents from a textile plant in a fluidized-bed reactor using PVC as the support for the immobilization of the biomass; the biodegradation of a textile dye in a continuous fixed-bed anaerobic reactor with activated carbon as the support and in an aerated reactor with PVC as the support. The strategy for the immobilization of microorganisms on the support as well as their adaptation to the effluent under study, the way in which the biomass present in the biofilm is quantified and its efficiency in the removal of the compounds of interest will be addressed. This subject is of extreme importance to the industrial sector, since the application of biotechnological processes in the treatment of industrial effluents does not require the use of other chemical compounds, which generally increase the toxicity of the effluents.

Chapter 11 - Fungi are ubiquitous in nature and exist in soil, water, plants, and in animals and humans. Similar to bacteria, fungi also form confluent biofilms either singly (mono-species) or with other microbial species (mixed-species). Fungal biofilms are known to be highly resistant to the adverse environmental conditions including antimicrobials and biocide compared to its planktonic (free-floating) counterparts. Although bacterial biofilms have been studied in detail, relatively little is known of fungal biofilms, its properties and their role in infections.

Fungal biofilm infections, particular caused by *Candida* species have dramatically increased in the past decade due to increased numbers of compromised populations such as HIV/AIDS patients, organ transplant recipients and patients on chemotherapy. *Candida* biofilm associated infections are frequently refractory to antifungal agents owing to the properties that are unique to the biofilm phenotype.

Although various hypotheses have been proposed for the higher antifungal resistance of *Candida* biofilms, the exact mechanism is still elusive. Therefore, in this chapter, we review the current knowledge on the drug resistance mechanisms of fungal biofilms, focusing on that of *Candida* biofilms. We will also outline in brief, the technological platforms available to investigate such properties in fungal biofilms. Lastly we summarize areas that warrant further research in the field of fungal biofilms.

Chapter 12 - Urinary devices such as catheters, stents and implants are commonly used in urology. The urinary tract presents a unique environment for these biomaterials, considering the ability of host factors to coat the device surface and provide additional bacterial attachment points. Infection and encrustation related to biofilm formation are common problems and in some cases, limit the long-term use of these devices. Patients with recurrent urinary tract infections have also been shown to harbour persistent infections due to the intracellularization of bacteria and development of biofilms within host cells, a phenomenon that has only recently been described. The pathogens capable of forming such intracellular biofilms will be discussed, along with the mechanisms utilized for their survival and recurring infections.

Chapter 13 - Biofilms can be generally defined as a complex and dynamic ecosystem, constituted by a community of microorganisms adherent to a substrate and often embedded within a self-produced extracellular polymeric matrix. Biofilm formation is a well-recognized phenomenon, especially in medical and food industries and often leads to undesirable effects (nosocomial infections, energy losses, accelerated corrosion, food spoilage, and spread of foodborne diseases...). In this framework, surface engineering for preventing biofilm formation is a challenging question, which has fuelled an explosion of research in surface science for the development of antimicrobial and/or anti-adhesive materials by physical or chemical modifications. The surface treatment can prevent biofilm formation by limiting the initial microbial adhesion and/or by killing microorganisms as they come in close contact with the solid surface. Among the different approaches considered, a growing interest is focused on thin silver coatings, like silver-based composite materials, due to their extended time-release properties. In the present work, plasma-mediated thin films (~170 nm), containing silver nanoparticles embedded in an organosilicon matrix, were deposited onto stainless steel. The process originality relies on a dual strategy, associating silver sputtering and simultaneous Plasma Enhanced Chemical Vapour Deposition, in an argon-hexamethyldisiloxane plasma, using an asymmetrical radiofrequency discharge at 13.56 MHz.

SEM demonstrated the nanoparticle-based morphology of the deposited layer. X-ray photoelectron spectroscopy confirmed the presence of metallic silver nanoparticles embedded in the organosilicon matrix. The film anti-adhesive potentialities were evaluated *in vitro* towards the model yeast*Saccharomyces cerevisiae* by performing shear-flow induced detachment experiments, under well-controlled hydrodynamic and physico-chemical conditions. The maximal effect was achieved for the organosilicon matrix alone. When silver nanoparticles were incorporated, yeast detachment was less pronounced, probably due to the strong affinity of embedded silver for biological groups of the cell wall surface. The presence of methyl groups in the matrix network could also promote enhanced hydrophobic cell/coating interactions. An antifungal action of released silver (Ag^+ ions and/or nanoparticles) at the immediate vicinity of the coating surface occurred, since a 1.4 log reduction in viable counts was observed, compared to control conditions with bare stainless steel. TEM observations of the yeast ultrastructure demonstrated morphological and structural damages. The presence of electron-dense silver clusters was also detected not only on the cell surface but also within the cell. In parallel, the coating antimicrobial properties against bacteria were assessed (reduction in viable counts of 1.5 and 2.4 log for *Escherichia coli and Staphylococcus aureus*, respectively).

Chapter 14 - Difficulties in eradicating foreign-body infections are primarily related to the presence of bacterial biofilms. An orthopaedic prosthesis can often facilitate such infections, which may be usually caused by non-aggressive microorganisms with the ability to form biofilms even at low inoculum size. After bacterial adherence to the foreign body, the biofilm is quickly established and can reach maturity in just seven days.

The biofilm is responsible for several phenotypic changes in the bacteria including their antimicrobial susceptibilities. These changes affect all antibiotics to different degrees resulting in bacterial expression of high minimal bactericidal concentrations (tolerance to antibiotics). Thus, antimicrobial therapy needs to be carefully designed, and the use of antibiotics that are highly active against biofilms and non-growing bacteria (i.e. rifampicin or fluroquinolones) must be desirable. Other factors have also been related to difficulties in the treatment of orthopaedic device-related infections. Functional abnormalities in the activity of phagocytic cells in contact with the foreign body can lead to the presence of intracellular bacteria; this issue makes the microorganism more resistant to antibiotics and add complementary mechanisms of bacterial virulence. Moreover, the existence of small colony variants or other nutritionally defective bacteria associated with biofilms can make the management, evolution and resolution of orthopaedic prosthetic infections more complex.

Finally, in order to ensure complete eradication of orthopaedic device-related infections the antibiotic therapy must be combined with appropriate surgical treatment.

Chapter 15 - Adapted sessile microbial communities, so-called biofilms, warrant many advantages for cell consortia as cooperation on nutrient obtaining and increase in resistance against xenobiotics, among others. As medical relevant biofilms develop under unfavorable conditions (nutrient restriction, immune response, etc), microbes tend to adapt themselves to challenges or try to change the microenvironment where they grow. Invariably, both paths require the triggering of mechanisms that ensure microbes' survival. Unlike planktonic cells that may evade from adversity by flagella-mediated swimming, biofilms must endure environmental harsh conditions expressing self-protective attributes as release of insoluble extracellular polysaccharides, toxins, inactivating enzymes, etc. By other side, such communities have a dynamic physiology, which is supported by the release of a consistent

range of depolymerases as proteases, lipases, nucleases, and hemolysins. These substances are active in the dragging of nutrients and may be effective in the impairment of local immune system. Other strategies for survival/persistence on host's tissues involve invasion, dispersion, and seeding of cells for adjacent surfaces. In this review, some aspects regarding to the manifestation of virulence attributes of microorganisms living in biofilms are discussed.

Chapter 16 - Biofilms result as a consequence from free-floating bacteria, which when adhere to surfaces form very complex communities with unique characteristics. Due to the special characteristics of biofilms, they have been widely studied and have become one of the main etiologies for chronic and recurrent infections. The upper respiratory system is in constant interaction with free-floating bacteria in the environment. At the same time, bacteria have easy access to the upper respiratory system making this system one of the easy targets for bacteria to form biofilms and thus cause a significant amount of morbidity and disease to the human race. In this chapter, we will discuss the formation, development, properties and also the latest treatment options for biofilms in the upper respiratory tract.

Chapter 17 - Biofilm formation is a critical process in the treatment of wastewaters. A highly diverse number of microorganisms constitute these biofilms. The ability of microorganisms to generate specific microenvironments in these biofilms allows their development under adequate conditions ensuring the efficient processing of wastes. In spite of the importance of microorganisms and biofilms in wastewater treatments, scarce information is still available on their role in sewage processing. Better understanding of microorganisms and biofilm formation in these highly complex systems would allow introducing modifications in order to achieve desirable behavior of specific microorganisms and their metabolism. An example is the enhancement of microbial oxidation of sulfide during wastewater treatments aiming to obtain significant decreases in sulfide production and consequently a reduction of problems related with odors, toxicity and corrosion. Engineering microbial behavior within extremely complex systems such as wastewater treatment plants is a difficult objective which requires an understanding of the microorganisms involved, their metabolism, and their capacity to form and develop in microenvironments, specifically, the production of biofilms with the required metabolic capacities.

Chapter 18 - Most of the phototrophic microbial communities found in extreme acidic environments are distributed in extensive biofilms. As in other habitats, monospecies biofilms are relatively rare and thus most biofilms are composed of mixtures of microorganisms. The macroscopic shape and species composition of the biofilms vary greatly throughout these ecosystems. Some of them adopt filamentous morphologies in flowing water while others form thick colourful patches firmly attached to the mineral substrates. These biofilms are organized multicellular systems with a structural and functional architecture which influences metabolic processes, response to nutrients, predation and other factors of the ecosystem. Consequently, structural studies of microbial biofilms and their formation play a critical role in understanding the ecophysiological processes in natural habitats. Moreover, it is important to study how biofilms in highly acidic conditions affect geochemical processes as metal immobilisation and influences the ecophysiological rates of the microorganisms when compared to microorganisms growing in a planktonic form. This is even more important in extreme environments where forming a structured biofilm might protect the organisms from external stress conditions and allow them to resist more extreme conditions. All these issues will be discussed.

Chapter 19 - Although starch is considered one of the most promising natural polymers for packaging application, it also presents poor mechanical properties and high moisture sensitivity. To overcome such disadvantages, additives have been successfully incorporated into starch polymers. The isolated and combined effect of additives (sucrose, inverted sugar and sodium phosphate) on cassava starch biobased films protection performance was evaluated using response surface methodology. The additives reduced cassava starch films tensile strength and increased elongation percentage. No effect was observed on water vapor permeability properties. If lower oxygen permeability is requested, samples added with 0.7% sucrose, 1.4% inverted sugar and 0.3% sodium phosphate presented the best alternative and a good balance between tensile strength (>60 kgf/cm^2) and elongation percentage (>60%). Two glass transition temperatures were observed for all experimental samples, indicating phase separation related to the additives. Interaction of the film network with phosphate diminished the total mass loss after an endothermic peak. The exothermic peak was not observed for the control and is probably a crystallization phenomenon related to the additives.

Chapter 20 - Previous studies had indicated that additives such as sucrose, inverted sugar and sodium phosphate have resulted in significant increase on cassava starch films materials elongation. However, crystallization after storage has been reported. In order to evaluate the effect of such additives (sucrose, inverted sugar and sodium phosphate) on cassava starch films microstructure, color, water activity and solubility, a response surface methodology design experiment has been performed. None of the studied additives affected cassava starch water activity, solubility and color ("L", "a", "b" and haze) ($p<0.05$). However, when comparing the experimental samples to the control (cassava starch film with no additive), an increase on water activity, solubility and total color difference was observed. X-ray diffractograms and, SEM and light microscopy, have indicated that inverted sugar can prevent sucrose crystallization if utilized in the combination of a maximum of 0.8% sucrose and any concentration of inverted sugar, or the maximum of 1.2% sucrose with 0.4% inverted sugar. Increasing inverted sugar and phosphate concentration leads samples to a semi-crystalline behavior. Inverted sugar addition probably have resulted in cassava starch crystallites re-organization. Phosphate addition (> 0.48%) affected the gelatinization temperature of the starch presented in the film forming suspension, resulting in a totally amorphous structure.

Chapter 21 - Microorganisms are able to modify the electrochemical conditions in the metal/solution interface with biofilm formation. They cause the induction, acceleration or inhibition of corrosion. Within the biofilm adhered to the metal substrate, complex interactions are developed with the corrosion products. The mechanisms associated with corrosion and inhibitions influenced by microorganisms, are rarely generated by a single species of bacteria. In the case of corrosion inhibition, this may be due to neutralizing the action of corrosive substances present in the environment, or the formation of protective films before the development of biofilm. The corrosive action depends on the type of bacteria, the bacterial consortium, their development, and environmental conditions.

Chapter 22 - Contamination of food by spoilage and pathogenic microorganisms costs the food industry millions of dollars annually. Much of this contamination may be attributed to the presence of biofilms in the processing plants. Several studies have led to the discovery that bacterial biofilm formation on the surfaces of equipment used for food handling or processing has serious negative implications, being one potential source of contamination. In fact, it is generally recognized that the occurrence of biofilms in food processing environments can cause post-processing contamination leading to lowered shelf-life of

products and transmission of diseases. It is also demonstrated that biofilms not only present a considerable hygiene risk, but also cause economical losses by technical failures in water systems, cooling towers, heat exchangers, etc.

Until now, the major emphasis on biofilm research has been about the negativeimplications of biofilm formation in food industry, but not all biofilms cause problems and there are some successful examples of their positive use, even if this aspect remains little studied. This paper proposes a review of some potential positive uses of biofilms summarizing some recent approaches suggested in literature. For example, it is demonstrated that biofilm bacteria present in many natural environments are able to maintain the water quality biodegrading toxic compounds and minimizing the building of pollutants, thus acting as pollutant monitors.Biofilm microorganisms could also be successfully employed in bioreactors to improve the productivity and stability of the fermentation processes. More recently, a potential use of biofilms to guarantee food safety was suggested, such as a useful application of biofilms formed by Lactic Acid Bacteria against foodborne pathogens. Some studies about this innovative idea suggest that good results could be obtained directly employing biofilms in the field of food packaging by means of the active packing planning: ensuring the maintenance of a continuous metabolism, microbial biofilms could successfully act against dirty pathogens and spoiling food microflora to prolong food shelf life.

Chapter 23 - The Gram-negative bacteria*Escherichia coli* is one of the most widely used organisms in biotechnology. Despite this fact studies using *E. coli* as a model organism on bacterial biofilm formation are rare compared to the wealth of information concerning other bacteria like for instance the *Pseudomonas* genus. *E. coli* plasmids are used as vectors for recombinant protein production and in order to ensure plasmid stability on the cell a resistance marker for an antibiotic is often included thus preventing the growth of cells that have lost the plasmid. Cells harbouring plasmids face two different challenges that impact on their metabolism: they have to deal with the metabolic burden of maintaining the plasmids at a certain copy number and they have to produce an agent that will confer resistance to the antibiotic. Plasmid maintenance requires the replication and proofreading of large segments of DNA which is consuming both in terms of the energy drain involved but also on the expenditure of metabolic precursors such as nucleotides. Regarding the antibiotic, the resistance agent is often an enzyme that will act upon the antibiotic reducing or eliminating its activity. In this case protein synthesis is involved and precursors such as amino acids and energy are consumed. On this work we have assessed the impact of using *E. coli* cells that have been transformed with a plasmid conferring resistance to kanamycin and grown under selective pressure with the behaviour of non-transformed cells growing on a glucose containing medium in the absence of selective pressure. We have evaluated the cell growth on the planktonic state, the glucose consumption as an indicator of the energy drain and we have characterised the formed biofilms regarding their weight and thickness.

By analyzing the optical density of the planktonic cultures we observe that higher cell densities are obtained when the cells were not transformed with the plasmid. Although this may be an indication of the metabolic burden caused by plasmid maintenance and/or gene expression, we have also observed that the glucose consumption profiles are similar on both situations with higher glucose consumption for the transformed cells. By analysing the obtained biofilms we observe that thicker and heavier biofilms are obtained. We thus postulate that the metabolic load involved in maintaining the plasmid and/or the burden to

express a resistance gene are major contributors to physiological stress that favours biofilms formation.

Chapter 24 - Over the past few decades, it has become recognized that bacteria do not exist as planktonic cells, but exist predominantly in multi-cellular surface attached communities known as biofilms. A biofilm develops when the attached cells excrete a slimy, glue-like substance that facilitate adhesion, matrix formation, and alteration of the organism's phenotype with respect to growth rate and gene transcription. More than 99 percent of all bacteria live in biofilm communities. Biofilms are ubiquitous in natural, industrial, and medical settings where bacteria exist. The process of biofilm formation is believed to occur as a sequential, developmental process including diffusion, reaction and growth. In this context, it is assumed that each step needs to be finished before the onset of the next one, implying substantial, regulated modifications in gene expression. The biofilm community has a number of distinct properties including the production of extracellular polymeric substances, the formation of chemical and pH gradients and the development of high level resistance to a wide variety of antimicrobial agents. The adaptive and genetic changes of the micro-organisms during their transition from planktonic state to biofilm mode make them resistant to all known biocides. Biofilm architecture is heterogeneous both in space and time, containing microcolonies of bacterial cells, separated from each other by interstitial voids (water channels). Liquid flow occurs through these voids, allowing diffusion of oxygen, nutrients, and even antimicrobial agents. Formation of biofilms can have profound negative and positive impact in these environments, and, thus can have serious impact on natural and industrial systems, as well as human health. Many chronic human infections including infectious kidney stones, periodontal disease, otitis media, osteomyelitis, bacterial endocarditis, and cystic fibrosis lung infections that seem impossible to remove are caused by biofilms. In contrast, their unusual properties, such as their unusual strength and ability to withstand conditions in which planktonic cells would die, make biofilms ideal for many applications. For example, biofilms offer huge potential for bioremediating hazardous waste sites, biofiltering municipal and industrial water and wastewater, and forming biobarriers to protect soil and groundwater from contamination. Perhaps in the future we may harbor bacterial biofilms to be more efficient in humans' benefit. Who knows? Understanding bacteria in biofilms is one step in preparing for the future. At this point in time, this review discusses the biofilm form of life, the characterization of their morphology, the mechanisms of their development, physical and chemical properties.

Chapter 25 - *Pseudomonas aeruginosa*, an important human opportunistic pathogen responsible for lethal nosocomial infections, as emerged as a relevant animal pathogen. Treatment options are dramatically declining worldwide, due to massive antibiotic use and the microorganism large versatile genome. Low cell wall permeability may account for intrinsic antimicrobial resistance, besides the ability of *P. aeruginosa* to express acquired resistance mechanisms. Virulence can be further enhanced by other characteristics, such as production of β-lactamases and carbapenemases and biofilm expression, which facilitates bacterial persistence in the host, evading the immunological defences and surviving at high antibiotic concentrations.

The present work studied *P. aeruginosa* isolates of veterinary origin (n=34), including house pets, farm and zoo animals with clinical signs of infection, to investigate the relation between biofilm-forming ability and antimicrobial resistance. Susceptibility to amikacin (AK), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), chloranfenicol (C), carbenicillin