BIOFILM CONTROL WITH ANTIMICROBIAL AGENTS: THE ROLE OF THE EXOPOLYMERIC MATRIX

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"We live in an island surrounded by a sea of ignorance.

As our island of knowledge grows, so does the shore of our ignorance."

John Archibald Wheeler (1992)

*"Ser poeta é ser mais alto, é ser maior
Do que os homens! Morder como quem beija!
É ser mendigo e dar como quem seja
Rei do Reino de Aquém e de Além Dor!
É ter de mil desejos o splendor
E não saber sequer que se deseja!
É ter cá dentro um astro que flameja,
É ter garras e asas de condor!
É ter fome, é ter sede de Infinito!
Por elmo, as manhãs de oiro e de cetim...
É condensar o mundo num só grito!
E é amar-te, assim perdidamente...
É seres alma, e sangue, e vida em mim
E dizê-lo cantando a toda a gente!"*

Florbela Espanca (1923)

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This is the place reserved to make a retrospective on the last 3 years of my career, (life), and PhD dissertation. This has been a journey where I gained knowledge from different people who made a difference that mattered.

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ABSTRACT

Biofilms, accumulated microorganisms and extracellular compounds on a surface, are able to thrive in all environments. Biofilm presence in the food industry can cause negative effects, being associated to lower industrial operational efficiencies, as well as microbial contamination of the final product. There are many strategies that attempt to control biofilm proliferation, however, no control strategy is completely effective. Thus, the development of new and more effective treatments and improving of the conventional strategies is in demand. In an effort to overcome biofilm resistance new compounds must be discovered and their antimicrobial properties assessed. Additionally, the association between different chemical agents could potentiate their singular antimicrobial efficacy.

The main objective of this study was to develop biofilm control strategies and to understand the biofilm behavior to these conditions. Therefore a selection of factors associated with biofilm resistance were studied. *Bacillus cereus* and *Pseudomonas fluorescens* are common contaminants in the food industry and were selected as microbial models. Several antimicrobial agents were screened using a colony biofilm test. These consisted as biofilms developed in as colonies in the top of polycarbonate membranes. The efficacy of selected agents with putative antimicrobial quenching substances was studied using respirometry. The killing and removal efficacy of treatments with antimicrobial agents was assessed using 96-well microtiter plates. To mimic close-to-practice conditions, biofilms were developed in a flow cell system and characterized. Control strategies potentiating current antimicrobial agents, and new agents were performed using biofilms developed in the referred bioreactors.

The diffusion of ethanol, isopropanol, sodium hypochlorite, chlorine dioxide, hydrogen peroxide, bezalkonium chloride (BAC), benzyldimethyldodecylammonium chloride (BDMDAC), cetyltrimethylammonium bromide (CTAB), ciprofloxacin, erythromycin, streptomycin and tetracycline was assessed on colony biofilms. Ciprofloxacin, streptomycin, BAC and CTAB were selected to assess their biofilm control efficacy. These products had distinct abilities to diffuse through the biofilms (high diffusion − BAC and ciprofloxacin; low diffusion − CTAB and streptomycin). It was concluded that the diffusion ability of antimicrobial agents is not directly correlated with biofilm killing and removal efficacy. BAC and CTAB were selected for the following studies due use in industrial cleaning and disinfection practices.

Known constituents of the extracellular polymeric matrix of biofilms (alginate and humic acids), and selected disinfection-interfering agents from the European Standard EN − 1276 (bovine serum albumin and yeast extract) were used to challenge the antimicrobial efficacy of the selected quaternary ammonium compounds as soiling

agents. The minimum bactericidal concentration of the chemicals was assessed. The interfering agents simulated "clean" soiling conditions. Within the range of concentrations tested the interfering substances mildly reduced the action of the antimicrobial agents. Humic acids were able not only of reducing the antimicrobials efficacy, but also to increase *P. fluorescens* respiratory activity. It was shown that humic acids should be considered as a potential interfering agent when developing cleaning and disinfection solutions, due to its strong interaction with the quaternary ammonium compounds tested.

The biofilms grown in the flow cell system at varying linear flow velocities (applied in food industry) showed different characteristics. The biofilms developed at the lowest linear flow velocity ($u = 0.1$ m.s^{-1}) differed by being thicker and more hydrated than the biofilms developed at the two higher linear flow velocities ($u = 0.4$ m.s^{-1} and $u = 0.8$ m.s^{-1}). These biofilms were more compact, with higher bacterial cell numbers and more exopolymeric substances (proteins and polysaccharides). In spite of these differences, the dry biofilm mass per area was similar, as well as the expression of the major outer membrane proteins from the biofilm cells. The biofilms developed at higher linear flow velocities were selected for further studies of control strategies, due to higher resistance characteristics (cells and exopolymeric substances).

Halogen-based products are recognized for their relevant antimicrobial properties. Thus, selected halogen-based products (CTAB, 3-bromopropionyl chloride -BrCl, 3-bromopropionic acid -BrOH and sodium hypochlorite -SH) were used in order to understand their antimicrobial activity against both planktonic and biofilm cells of *P. fluorescens*. The mode of action of these products is cell membrane disruption, causing leakage of essential cellular constituents. The results demonstrate comparable effects of BrCl and BrOH to those of sodium hypochlorite that makes them a potential alternative to sodium hypochlorite. However, CTAB was the most efficient agent.

The addition of enzymes as an aid to biofilm control treatments, applied alone or in combination with BAC and CTAB, had the ability to kill and remove the biofilms developed in microtiter plates and in the flow cell system. The combination enzyme-biocides was synergistic on biofilm control. The treatments allowed both long term effects (additional biofilm removal and colony forming units reduction were observed in the hours following the treatments), as well as biofilm regrowth.

The presented studies in this thesis clearly underline the importance to study biofilm control strategies under representative conditions for practice, being stress conditions determinants of different biofilm responses. Biofilm control should be a multifactorial approach due to the many features that biofilms have that provides them an increased protection. It is, therefore, necessary to incessantly find new control strategies because microorganisms will adapt and find new ways to overcome the biofilm control treatments.

SUMÁRIO

Os biofilmes, acumulação de microrganismos e de substâncias exopoliméricas numa superfície, são capazes de prosperar em todos os ambientes. A sua presença na indústria alimentar pode causar efeitos negativos, devido à redução de eficiência de processos industriais, assim como a contaminação do produto final. Há muitas estratégias para controlar a proliferação de biofilmes, no entanto, nenhuma é totalmente eficaz. Deste modo, é necessário otimizar os tratamentos convencionais e também descobrir tratamentos novos e eficazes. Novos compostos devem ser procurados e estudados, para que superem a resistência dos biofilmes. Adicionalmente, a associação entre diferentes agentes químicos pode também potenciar a sua eficácia antimicrobiana.

O objetivo principal deste estudo foi desenvolver e otimizar estratégias para o controlo de biofilmes. Para tal, fatores relacionados com a resistência dos biofilmes foram estudados. *Bacillus cereus* e *Pseudomonas fluorescens* são duas bactérias responsáveis por contaminações na indústria alimentar, e por isso foram selecionadas como modelos microbianos representativos. Vários agentes antimicrobianos foram rastreados com um teste com biofilmes em colónia. A eficácia dos agentes selecionados com possíveis substâncias interferentes foi estudada usando respirometria. A morte e a remoção dos biofilmes foi avaliada recorrendo a placas de microtitulação de 96 poços. Para simular condições reais, foram desenvolvidos e caracterizados biofilmes em células de fluxo. Este sistema serviu para testar estratégias de controlo de biofilmes como a potenciação de agentes antimicrobianos, assim como o desenvolvimento de novos agentes.

A difusão de etanol, isopropanol, hipoclorito de sódio, dióxido de cloro, peróxido de hidrogénio, cloreto de benzalcónio (BAC), cloreto benzilldimetildodecilamónio (BDMDAC), brometo de cetiltrimetilamónio (CTAB), ciprofloxacina, eritromicina, tetraciclina e estreptomicina foi avaliada nos biofilmes em colónia. Destes agentes, foram selecionados a ciprofloxacina, a estreptomicina, o BAC e o CTAB para avaliar a sua eficácia na morte e remoção de biofilmes. Estes produtos difundiam de forma distinta através dos biofilmes (alta difusão - BAC e ciprofloxacina, baixa de difusão - CTAB e estreptomicina). Concluiu-se que a capacidade de difusão de agentes antibacterianos não está diretamente correlacionada com a sua capacidade para matar ou remover. No entanto, BAC e CTAB foram selecionados para estudos adicionais, devido ao seu uso corrente em práticas de limpeza e desinfeção.

Componentes da matriz extracelular dos biofilmes (alginato e ácidos húmicos) e agentes interferentes da desinfeção selecionados da Norma Europeia EN-1276 (1997) albumina de soro bovino e extrato de levedura) foram usadas, em condições "limpas", para desafiar a eficácia antimicrobiana dos compostos quaternários de amónio selecionados. A

concentração mínima bactericida dos compostos foi aferida neste estudo. Dentro da gama de concentrações testada, as substâncias interferentes reduziram levemente a ação dos agentes antimicrobianos. Os ácidos húmicos foram capazes não só de reduzir a eficácia dos agentes antimicrobianos, mas também de aumentar a atividade respiratória de *P. fluorescens*. Foi mostrado que estes devem ser considerados como um agente potencialmente interferente aquando do desenvolvimento de soluções de limpeza e desinfeção, devido à sua forte interação com os compostos testados.

Os biofilmes desenvolvidos nas células de fluxo apresentaram características diferentes de acordo com a velocidade do fluxo (semelhantes às aplicadas na industria alimentar) à qual foram desenvolvidos. Os biofilmes formados a uma velocidade de fluxo mais baixa ($u = 0.1$ m.s^{-1}) apresentaram-se mais espessos e hidratados do que os biofilmes desenvolvidos às duas velocidades de fluxo mais elevadas ($u = 0.4$ m.s^{-1} e $u = 0.8$ m.s^{-1}). Estes eram mais compactos, com mais células e substâncias exopoliméricas (proteínas e polissacarídeos). Apesar destas diferenças, a massa de biofilme seco por unidade de área foi semelhante, bem como a expressão das proteínas principais da membrana externa das células. Os biofilmes desenvolvidos às velocidades de fluxo mais elevadas apresentaram maiores densidades celulares e substancias exopoliméricas, razão pela qual foram selecionados para estudos de estratégias de controlo subsequentes.

Produtos à base de halogénio são reconhecidos pelas suas propriedades antimicrobianas. Portanto, os produtos à base de halogéneo selecionados (CTAB, cloreto de 3-bromopropionilo – BrOH, ácido 3-bromopropiónico – BrCl, e hipoclorito de sódio) foram utilizados com o intuito de compreender a sua atividade tanto contra células planctónicas como em biofilmes de *P. fluorescens*. O modo de ação destes produtos caracterizou-se essencialmente pelo rompimento das membranas celulares e a libertação de constituintes celulares essenciais. Os resultados demonstram que os efeitos de BrCl e BrOH são comparáveis aos do hipoclorito de sódio, o que lhes confere potencial como substitutos do último. Mas, CTAB foi o agente mais eficaz.

A adição de enzimas é, quando aplicada isoladamente e combinadas com BAC e CTAB, capaz de matar e remover os biofilmes formados nas placas de poliestireno e nas células de fluxo. Após os tratamentos foi possível verificar efeitos de longo prazo na redução da massa do biofilme e das células formadoras de colónias, no entanto, foi também observada uma eventual recuperação destes mesmos parâmetros.

Os estudos apresentados nesta tese enfatizam que o estudo de estratégias para o controlo de biofilmes deve ser feitos em condições representativas da prática, sendo que o comportamento dos biofilmes é determinado pelas condições de stress a que é sujeito. O controlo dos biofilmes deve ser realizado através de uma abordagem multifatorial, devido aos muitos recursos que estes dispõem para a sua proteção. É necessário desenvolver incessantemente novas estratégias de controlo, pois os microrganismos vão sempre encontrar novos métodos para superar os tratamentos.

THESIS OUTPUTS

The results presented in this thesis are published and/or submitted as following:

PEER REVIEWED JOURNALS

Araújo PA, Lemos M, Mergulhão F, Melo L, Simões M. 2013. The influence of interfering substances on the antimicrobial activity of selected quaternary ammonium compounds. *International Journal of Food Science*; 2013:9.

Araújo PA, Mergulhão F, Melo L, Simões M. 2014. The ability of an antimicrobial agent to penetrate a biofilm is not correlated with its killing or removal efficiency. *Biofouling* 1-9.

Araújo PA, Malheiro J, Mergulhão F, Melo L, Simões M. The influence of flow velocity on the characteristics of *Pseudomonas fluorescens* biofilms. *Submitted*

Araújo PA, Machado I, Mergulhão F, Melo L, Simões M. Evaluation of the synergistic potential of enzymes with quaternary ammonium compounds for biofilm control. *Submitted*

Araújo PA, Machado I, Mergulhão F, Melo L, Simões M. Decreased efficacy of enzymes on the control of flow generated biofilms. *Submitted*

Malheiro J, Araújo PA, Machado I, Mergulhão F, Melo L, Simões M. The effects of selected halogen-containing chemicals on *Pseudomonas fluorescens* planktonic cells and flow-generated biofilms. *Submitted*

BOOK CHAPTERS

Araújo PA, Lemos M, Mergulhão F, Melo L, Simões M. 2011. Antimicrobial resistance in biofilms to disinfectants. In: Science against microbial pathogens: communicating current research and technological advances. Badajoz, Spain: Formatex. p. 826-834.

Araújo PA, Lemos M, Simões M. 2012. Controlo químico de biofilmes industriais. In: Biofilmes – Na saúde, no ambiente, na indústria Porto, Portugal. Publindustria Lda.

Araújo PA, Mergulhão F, Melo L, Simões M. 2013. Diffusivity of antimicrobial agents through biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*. *In*: Worldwide Research Efforts in the Fighting against Microbial Pathogens: From Basic Research to Technological Developments. Brown Walker Press. p. 129-133.

Gomes LC, Araújo PA, Teodósio JS, Simões M, FJ Mergulhão. 2014. The effect of plasmids and other biomolecules on the effectiveness of antibiofilm agents. *In*: Antibiofilm agents: from diagnosis to treatment and prevention. Eds: Ahmad I, Rumbaugh K. Springer.

CONFERENCE PROCEEDINGS

Araújo PA, Lemos M, Mergulhão FJ, Melo LF, Simões M. Influence of interfering substances in disinfection. Poster presentation in Microbiotec'11 in the panel Industrial and Food Microbiology and Biotechnology PS1:120. Braga, Portugal, 1-3 December 2011.

Araújo PA, Mergulhão FJ, Melo LF, Simões M. Diffusivity of antimicrobial agents through biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*. Oral presentation in ICAR in the panel Antimicrobial surfaces - Biofilms - Quorum sensing. Lisbon, Portugal 22 November 2012.

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CHAPTER 1

THESIS OUTLOOK

1.1   RELEVANCE AND MOTIVATION

It is a natural tendency of microorganisms to attach to surfaces, multiply and produce extracellular polymeric substances (EPS), originating biofilms. Biofilm existence can represent a beneficial or a detrimental factor, affecting many areas, from the biomedical to the industrial [1, 2].

In food industry, process conditions are ideal for biofilm proliferation. Biofilm formation is very common in industrial settings, even when manufacturers apply comprehensive contingency plans. The EPS produced by bacteria in biofilms protects microorganisms from control strategies by hindering diffusion of antimicrobial agents and promoting antimicrobial quenching effects due to chemical reactions with the antimicrobial agents [3].

Flemming [4] described EPS as very complex and dynamic. Its exact functions of EPS are not yet clear e.g. because of the extreme heterogeneity. The EPS matrix is an intricate network that provides sufficient mechanical stability to maintain spatial arrangement for embedded-bacteria. It consists of various organic substances such as polysaccharides, proteins, nucleic acids and lipids [5]. This composition is affected by the environmental conditions under which biofilms are formed, and its arrangement is affected by the hydrodynamic stress [6, 7].

Disinfection procedures are commonly designed based on experiments carried out with planktonic bacterial cell cultures [8]. But, such tests do not mimic the biofilm and environmental conditions on surfaces in industrial processes. Actually, the European Standard EN-1276 (1997) [9], used as reference for the development of disinfection strategies for food, industrial, domestic and institutional areas, only provides a short list of potential interfering chemical substances to be considered when optimizing/ developing a disinfection process. Nevertheless, the conventional explanations for biofilm resistance and recalcitrance against current control strategies are based on the effects of the presence of a heterogeneous EPS matrix on transport limitations and chemical interactions with antimicrobial agents [3, 10, 11].

Knowledge of EPS is needed to develop effective biofilm control strategies. This knowledge can help overcome biofilm resistance. The treatment of biofilms with enzymes to weaken the EPS structure may enhance the effectiveness of other antimicrobial agents. Enzymes can degrade the EPS barrier and therefore increase the diffusivity of the chemical agents [12]. Moreover, due to the recognized antimicrobial

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resistance problem it is of importance to search and identify new and more effective antimicrobial agents and develop new control strategies [13].

Despite the definite importance of biofilms in microbial life style and their effects on human beings, the present knowledge about their structure, composition and behavior is still limited. Therefore there is a need to better understand biofilm resistance, by the identification of parameters linked with it, so that control strategies can be developed and optimized. Bioresist is a project financed by national funds through the Portuguese Foundation for Science and Technology (FCT) and MCTES (PIDDAC) and co-financed by the European fund of Regional Development (FEDER) through COMPETE – Operational Programme for Competitiveness Factors (POFC), with the reference PTDC/EBB-EBI/105085/2008. This project studied the influence of biofilm phenotype on its resilience and resistance. This PhD thesis was developed within the scope of this project.

1.2   MAIN OBJECTIVES

There are still no biofilm control strategies providing sustainable results in terms of inactivation, removal and prevention of biofilm regrowth events [14]. Development of approaches to control unwanted biofilms requires detailed knowledge about the biofilms [15]. It is necessary to develop strategies to control biofilms native of food industry, and simultaneously identify the resistance mechanisms associated with control strategies. Thus, the main objective of this study is to provide a contribution for the development of biofilm control strategies. Moreover, the outcomes of this thesis provide insight into how biofilms are affected by the food industry process hydrodynamics and how the biofilm phenotype is linked with its resistance.

1.3   THESIS ORGANIZATION

This thesis is essentially divided in eight chapters:

Chapter 1 describes the relevance and motivation, the objectives, and the work structure presented throughout this thesis are exposed.

Chapter 2 provides a review of the aspects of microbial resistance, and control strategies currently/recently applied, with particular emphasis on biofilms. It is the state of the art of major aspects related with the topic of this thesis.

Chapter 3 presents a study of the diffusivity of twelve biocides and antibiotics (ethanol, isopropanol, sodium hypochlorite, chlorine dioxide, hydrogen peroxide, BAC, BDMDAC, CTAB, ciprofloxacin, erythromycin, streptomycin and tetracycline) through *Bacillus cereus* and *Pseudomonas fluorescens* biofilms. BAC and CTAB were selected to be used in further experiments, taking into account their ability to diffuse through the biofilms and to inactivate the embedded cells.

In chapter 4, the influence of alginic acid, bovine serum albumin, yeast extract, and humic acids as interfering substances on the antimicrobial action of selected antimicrobial agents was assessed on planktonic *P. fluorescens and B. cereus*.

Chapter 5 presents the characteristics of *P. fluorescens* biofilms developed in a flow cell system. Three distinct linear flow velocities were used (u = 0.1, 0.4 and 0.8 m.s^{-1}). The biofilms demonstrating the highest complexity (cell numbers and EPS content) were selected for further studies.

In chapter 6 halogen-based chemicals, BrCl, BrOH, sodium hypochlorite and CTAB were tested on their potential to control *P. fluorescens* planktonic cells and biofilms. The effects caused by the exposure to the chemicals were studied in order to understand different aspects of the antimicrobial mode of action of these chemicals.

Chapter 7 presents a biofilm control strategy using enzymes (β-glucanase, protease, lipase, and α-amylase) and biocides (BAC and CTAB). Different types of treatment were tested, as an environmentally friendly method. The action of these treatments against planktonic cells was also assessed in order to understand the antimicrobial action of the enzymes and the interaction with the selected biocides.

In chapter 8, the main achievements of this thesis are exposed. Regards about follow up research are provided as well.

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This thesis is structured as a paper dissertation, consisting of a number of scientific articles. The chapters on the experimental work are presented in the way they have been submitted and/or published upon acceptance. Some repetitions are consequently unavoidable amongst individual chapters.

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CHAPTER 2

INTRODUCTION
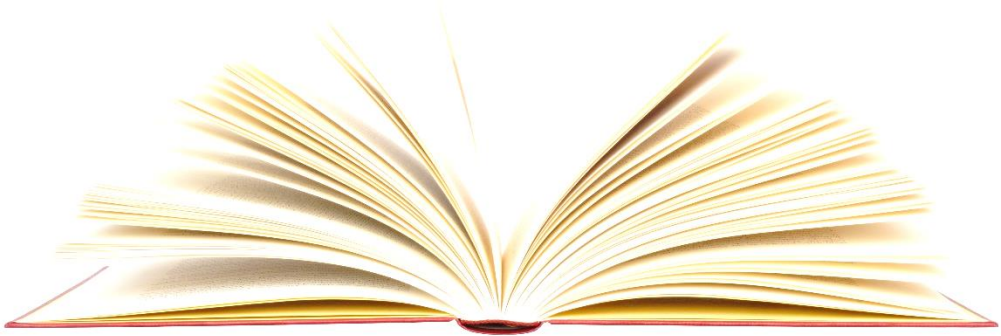
This chapter was based on:

Araújo PA, Lemos M, Mergulhão F, Melo L, Simões M. 2011. Antimicrobial resistance in biofilms to disinfectants. *In*: Science against microbial pathogens: communicating current research and technological advances. Badajoz, Spain: Formatex. p. 826-834.

Araújo PA, Lemos M, Simões M. 2012. Controlo químico de biofilmes industriais. *In*: Biofilmes – Na saúde, no ambiente, na indústria Porto, Portugal. Publindustria Lda.

2.1   BIOFILMS

Biofilm formation was reported early in fossil records [1]. The first recorded life forms on earth are biofilms, dated approximately 3.5 billion years ago. They have faced fluctuating and harsh conditions of primitive earth, such as extreme temperatures and ultraviolet light exposure [1]. Nowadays, biofilms can be found in the widest range of environments, on extremes of cold and hot temperatures, high pressures, high alkalinity or acidity, and even radioactivity [2]. There are also reports where biofilms were found in improbable environments such as a disinfectant solution [2, 3].

In 1684, Leeuwenhoek described in a report to the Royal Society of London "animalcules" that were found in plaque scraped from his teeth [4]. Later in 1940 Heukelekian and Heller stated in the Journal of Bacteriology that bacteria develop with bacterial slime or as colonies attached to surfaces [4]. In 1943, Zobell observed and described fundamental characteristics of attached microbial communities [4]. In 1975, the word "aufwuchs" (in German), meaning growth was the first conceptual term used to describe biofilms, however, it was later discarded by implying to be situated "around plants". Studies associated with biofilms started to have more attention in 1978, were the description of sessile communities was first described and termed by the group of Bill Costerton. The group described them as microorganisms with the ability to adhere to wet surfaces in ecosystems of fresh water [5]. Characklis and Marshall described biofilms as a community of microorganisms, either single or multi species, being anchored to a surface and entrapped in organic polymers excreted by them [6]. It is now commonly agreed that bacteria have a natural capability to attach irreversibly to surfaces, to multiply, and to embed themselves in a slimy matrix, establishing biofilms. The biofilm population is enclosed in a matrix adhered to each other, to a substratum or to an interface [7]. Although the population could be constituted by other organisms besides bacteria [8], single bacterial biofilms are often found in industry and in medicine [9]. Later on, Stoodley et al. distinguished some characteristics denominating these structures as we currently know them, they include the association with a surface, a high population density, and the presence of exopolymeric substances (EPS), which is the "glue" that holds biofilms together [8]. Yet, it is not uncommon to find biofilms lacking one of these characteristics due to the environmental characteristics to which the bacteria are exposed [10-13].

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BIOFILM FORMATION

Biofilm formation is a complex process that involves several stages [8]. Planktonic cells passing along a "conditioned" surface are deposited. The persistent microorganisms that remain on the surface bind irreversibly, and start to grow, multiplying and producing signaling molecules, as well as EPS [8, 14]. As the biofilm matures, an equilibrium between accumulation and erosion takes place. The biofilm could erode by dispersing (cells) or by sloughing (biofilm pieces) phenomena. After this stage the planktonic cells return to the beginning of their cycle on different locations [15-18].

The properties of the adhesion surface, and the surface of the bacteria, as well as their stage of growth, are determining factors for biofilm formation [19]. A suitable nutrient concentration, an optimum pH, and an appropriate hydrodynamic force exerted on the cells, provide favorable characteristics for attracting microorganisms to be adsorbed to the surfaces [20]. The genetic information of cells in biofilms is fairly different from their planktonic counterparts, this change is thought to be triggered when cells adhere to surfaces [21]. In some cases, the development of appendages such as flagella, fimbriae and pili help in biofilm formation [22].

The hydrodynamic conditions and adjacent environment contribute to biofilm formation [19, 23]. They affect the matrix structure, quantity and composition [13, 24]. The way how biofilms develop, the transfer of mass, the biofilm density and the conversion of substrate are all dependent on these parameters [25]. Biofilms developed under laminar regimes are different from the ones generated in a turbulent regime, as the access to deeper layers is made by an open structure to ease mass transfer [26, 27]. The shear stress exerted on the biofilms by the passing fluid determines their shape due to the erosion it causes. As new layers are formed, the force of the passing fluid dislodges the top layers. In contrast, when the biofilms attain a certain growth size, some of them are able to secrete surfactants with the ability to alter their internal properties [24].

The adhesion of microorganisms to surfaces, forming biofilms, represents an ecological advantage and is a prevalent form of survival in hostile environments. In fact, it is estimated that 99% of bacteria live in biofilms [4].

2.2   THE IMPACT OF BIOFILM FORMATION

Biofilms are able to thrive everywhere. The ability of biofilms to develop in nature or in engineered porous media could be used as an advantage on man-made processes [4]. When biofilms are used this way, they are called beneficial biofilms. For example, when planktonic cells are used in a reactor, their residence time is the same as the fluid flow time, however, if cells are in a biofilm, their residence time is as long as the time the biofilm is attached to a surface within the reactor [23]. Biofilms have been be used for environmental applications that include the degradation of organic substances, denitrification of waste or removal of phosphate and heavy metals [28, 29]. They have been employed as bio-control agents in the rhizosphere of plants, particularly against infections caused by fungi or bacteria. Biofilms formed by some microorganisms are able to produce antifungal and antibacterial substances, which provide protection to plants susceptible to phytopathogenic microorganisms. This has been a field of science with plenty of interest, since it enables the exploitation of new physiologically active products [30].

Conversely, biofilms could cause serious operation and management costs depending on where they appear. When biofilms appear in food industry they are highly unappreciated because they may contain pathogens and spoilage microorganisms, which constitute a risk to humans when contaminated and spoiled products are consumed. Typically, the emergence of biofilms is a result of an ineffective cleaning plan, increasing production costs due to production downtime. In other areas, such as the clinical area, biofilms are able to develop in medical devices, implants, venous or urinary catheters, resulting in an increased risk of infection [31]. In clinical settings biofilms have a higher importance due to their risk of causing infections, which could turn chronic [32]. Notwithstanding, environmental biofilms could contain pathogens as well. Foodborne diseases affect 48 million people in the United States of America each year. In this group, 2612 people did not survive infections related with microbial development, being estimated that 65% of all microbial diseases are a consequence of biofilm development [33-35]. Food poisoning has associated costs, according to Brooks and Flint these are difficult to estimate, however it was possible to make an estimative of approximately $90 million for New Zeeland, which is a country with only 4.5 million people [36]. It was also estimated that 25% of the total food produced is lost

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due to microbial activity, in spite of the diverse methods employed for food preservation, good manufacturing, quality control and hygienic measures [37].

Earlier in this chapter it was said that the characteristics of biofilms differ according to the environmental conditions under which they were formed, i.e. temperature, pH, type of nutrients available, and type of bacteria. It is also known that the type of microorganisms that forms biofilms is different according to the location where the biofilms were found. Dairy industries commonly have biofilms composed by *Pseudomonas fluorescens, Escherichia coli, Shigella* spp*., Staphylococcus aureus* and *Bacillus cereus*. Shrimp factories normally have *P. fluorescens* and *P. putida* as biofilm colonizers. In fish factories it is common to find biofilms composed by *Enterobacteriaceae* and *Serratia liquefaciens*. In caviar plants, biofilms of *Neisseriaceae* spp*., Pseudomonas* spp*., Vibrio* spp. and *Listeria* spp. were reported [38, 39]. *Pseudomonas* spp., *Klebsiella* spp., *Legionella* spp., *Helicobacter* spp., *Campylobacter* spp. and *Escherichia coli* were found in drinking water networks [2]. *Pseudomonas* spp. are ubiquitous in food industry environments and have been reported to be found in drains, and produce such as vegetables, meat and dairy products [40]. *Bacillus* spp. are found throughout dairy processing plants, accumulating on joints and pipelines of the equipment [40]. Both are able to form biofilms.

When these bacteria accumulate, they can cause other consequences besides product spoilage or infections. Biofilms are able to cause detrimental effects on many systems. Consequences are material corrosion and biodegradation, causing contamination of the raw or processed products in food processing plants. In cooling water towers and heat exchangers they cause energy loss due to increased fluid friction and resistance to heat transfer. In drinking water distribution systems, an increase in suspended solids and coliform contamination has been observed, in addition to pipe corrosion and pressure drop [2]. In paper manufacturing, the quality of the product is reduced. In ship hulls biofouling development increases drag and consequently energy loss as in reverse osmosis membranes, where the reduced permeability and material degradation are felt [41-43].

Industrial settings, particularly food processing plants, provide favorable environmental conditions, i.e. hydrodynamics and nutrients abundance, for biofilm proliferation (Figure 2.1). Biofilm contaminations are dangerous due to their mode of life which includes partial sloughing or detachment. Once on the fluid stream these could proliferate into other locations of the production line, restarting the process all

over again [8]. This ease of proliferation results in both economic and public health consequences, therefore, efforts have been directed for efficient industrial equipment design and the development of effective disinfectants [45, 46].
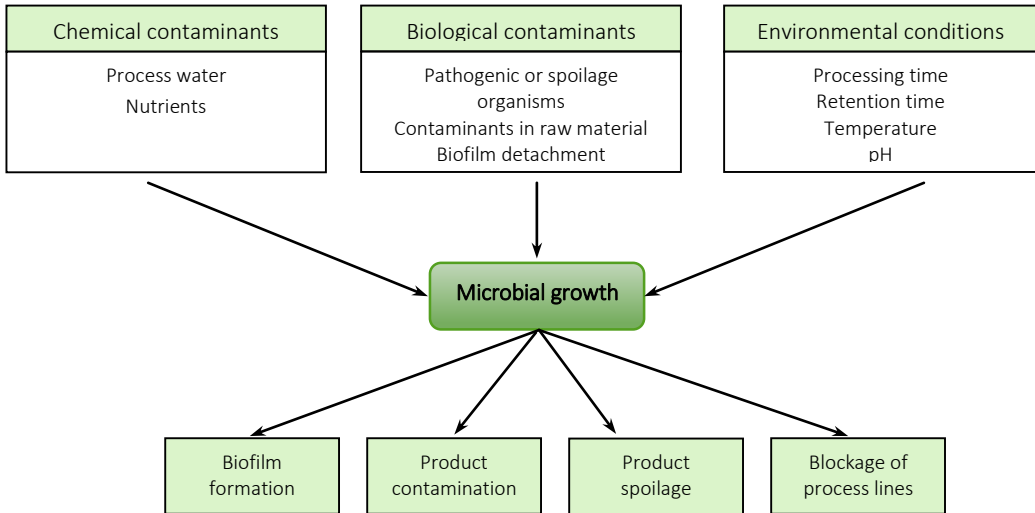
Figure 2.1 Microbial contaminations in food industry (adapted from [44]).

2.3   THE EXOPOLYMERIC MATRIX

The most recognized characteristic of biofilms is the EPS matrix, which provides favorable conditions to its inhabitants to thrive in the most diversified surroundings. Figure 2.2 is a micrograph of *P. fluorescens* biofilm developed in a flow cell system, the substance coating the cells is a dehydrated EPS matrix, as suggested in the work of Flemming et al. [2].

Biofilm characteristics such as porosity, tortuosity, density, water content, charge, sorption properties, hydrophobicity and mechanical stability are determined by environmental conditions [2]. Biofilm structure and spatial heterogeneity are essential to various biofilm processes such as convective and diffusional transport of oxygen and nutrients into the biofilms [47]. Biofilm heterogeneity is defined as a non-uniform structural, chemical and physical distribution within the biofilm [47].

The functions of the exopolymeric matrix are diverse, however, not all functions are fully understood [48]. Some of these are described in Table 2.1. One of the functions of EPS is to contribute to the mechanical stability of the biofilms, enabling them to

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withstand shear forces, dehydration or chemical attacks [49, 50]. EPS protects the embedded cells from UV light, radiation, pH changes, osmotic shock, or drying [51]. Furthermore, the matrix reinforces biofilm attachment to the substratum and stabilizes it, thereby reducing its susceptibility to sloughing by hydrodynamic shear stress [52, 53].
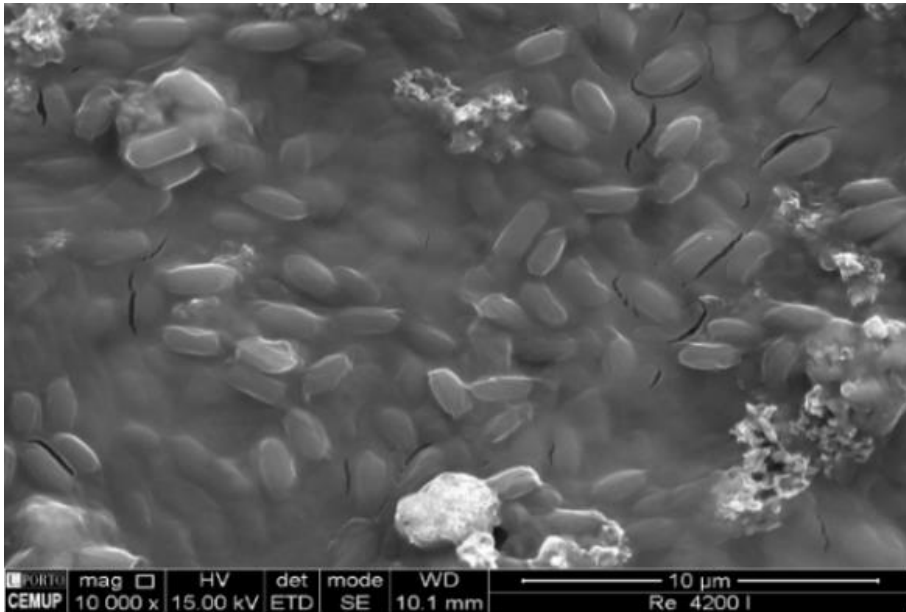
Figure 2.2 *P. fluorescens* biofilms developed for 7 days at a *Re* of 4000. Air dehydrated in a desiccator for two days, the thin layer covering the cells is believed to be EPS.

EPS are an intricate network formed essentially by polysaccharides and proteins [54]. The matrix differs according the microbial producer. In addition, between genus the matrix is likely to differ either in chemical composition or in terms of physical characteristics [51]. The composition of the matrix may also contain glycoproteins, lipoproteins, phospholipids, teichoic acids, nucleic acids and a variety of humic substances [22, 24, 55]. Any particles passing by the biofilm may be incorporated into it [56], therefore it is also possible to find mineral crystals, silt particles, milk residues as calcium phosphate and, sometimes, blood components or dirt [57]. EPS is able to retain water, the reason why biofilms are highly hydrated [2]. In fact, biofilms are composed essentially by water, as up to 97% of biofilm volume and mass is water [13, 58]. EPS composition is determined by the environmental conditions to which the

biofilm microorganisms are exposed [19, 55]. EPS are excreted by the cells, but also derive from natural cell lysis or hydrolytic activities [59]. Life in biofilms facilitates gene transfer and the retention of extracellular enzymes, that are useful to degrade biodegradable matter (lysed cells), that serve as nutrients for the living bacteria [60].

Table 2.1 Functions of EPS in bacterial biofilms. (Adapted from [2, 5, 51, 60, 61].)

Component function	EPS components involved	Relevance for biofilm organism
Aggregation of bacterial cells, formation of flocks and biofilms	Polysaccharides, proteins, DNA	Bridging between cells, immobilization of bacterial populations, basis for development of high cell densities; cell communication; biofouling and corrosion
Cell–cell recognition	Polysaccharides, proteins, DNA	Symbiotic relation with animals and plants; possible pathogenic processes
Retention of water	Hydrophilic polysaccharides/proteins	Maintenance of highly hydrated microenvironment organisms, desiccation tolerance in water-deficient environments
Protective barrier	Polysaccharides, proteins	Resistance to nonspecific and specific host defenses during infection, tolerance to various antimicrobial agents (e.g., disinfectants, antibiotics); protection against some grazers
Sorption of organic compounds	Charged or hydrophobic polysaccharides and proteins	Accumulation of nutrients from the environment; sorption of endogenous compounds
Sorption of inorganic ions	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate	Promotion of polysaccharide gel formation; ion exchange; mineral formation; accumulation of toxic metal ions (detoxification)
Enzymatic activity	Proteins	Digestion of exogenous macromolecules for nutrient acquisition; degradation of structural EPS allowing release of cells
		Accumulation, stabilization and retention of secreted enzymes on polysaccharides
Nutrient source	Potentially all EPS components	Source of C, N and P compounds for utilization by biofilm community
Genetic information	DNA	Horizontal gene transfer between biofilm cells

The resistance mechanism provided by the EPS is further reviewed in the next subsection.

2.4   RESISTANCE

The survival of the fittest is a biological principle applicable to all living beings, and although different organisms have developed their own survival mechanisms, all have one common factor that relates survival with the ability to adapt to constant changes in the environment. Microorganisms are particularly adaptable to environmental changes because of their high reproduction rates, which allows them to transfer survival characteristics to future generations in short periods of time [62]. When exposed to a harmful and/or stressful environment, bacteria will do all within their power to survive [63]. External stresses, such as environmental conditions, have different effects on different organisms, leading to natural responses like inhibition and/or inactivation of the cells. For instance, a deviation in the environmental conditions could result in reduced growth rates [64]. When bacteria are exposed to sub-lethal levels of biocides, and only minor cell damage is caused, a more resistant population could derive, with consequences that may include changes in the global phenotype of the community [63].

Resistance mechanisms are the means that living organisms have to respond to continuously changing environment in order to survive [65]. Resistance is the description of the relative insusceptibility, viability or multiplication of a microorganism to a certain chemical treatment under certain conditions. It may be temporary or permanent and relates either to the first generation of organisms or to the next [66]. Thus, there are three documented types of resistance: (1) inherent resistance, also termed natural or intrinsic to the microorganism, (2) adaptive resistance, due to the occurrence of a mutation, by continuous exposure to certain environments, and finally (3) acquired resistance which occurs through the acquisition of mobile genetic elements (plasmids) [67-69]. An example of intrinsic resistance is the difference between Gram positive and Gram negative cells. The main differences are in the outer cell layers. Gram positive cells present a large peptidoglycan layer after the phospholipidic membrane, where proteins and porins are located, while Gram negative have, from the inside to the outside, a smaller peptidoglycan layer followed by periplasm, an outer phospholipidic membrane and lipopolysaccharides, which gives the cells a hydrophobic character. Gram positive outer membrane works as a permeability barrier [70]. This cell wall is composed essentially by peptidoglycans and teichoic acids. Gram negative outer hydrophobic membrane limits the entry of the most diverse chemicals by working as an exclusion barrier [71]. Gram negative bacteria embedded in biofilms are known to have

a higher ratio of unsaturated to saturated fatty acids, a typical profile of resistant bacteria [72]. Their morphology limits the concentration of biocide to the corresponding targets [67]. The adapted resistance could be due to the continuous use of disinfectants, to which the embedded bacteria gain resistance as a consequence of the repetitive use of these chemical agents [54]. Similarly, microorganisms may acquire resistance to some antimicrobial agents through exposure to other agents of the same type, which is called cross-resistance [73]. A documented case of acquired resistance, provided by plasmids, is the horizontal transference of resistance to antibiotics from *Lactobacillus plantarum* to *Enterococcus faecalis* [74].

Changes at the phenotypic level, i.e. by forming biofilms as a response to the environmental conditions, is also a form of resistance [75]. As mentioned on the previous section, the proliferation of biofilms in industrial settings, especially in food industry, can result in serious operation and maintenance costs [36]. Their eradication is proved to be difficult as biofilm cells are known to be highly resistant to antimicrobial agents. Defense mechanisms against antimicrobial agents are frequently reported in literature [76-89]. The study of the resistance mechanisms to antimicrobial agents gradually unravels the mysteries of the biofilm tenacious nature and recalcitrance to control [90]. A deeper understanding of biofilm resistance mechanisms is required in order to develop new and more effective biofilm control strategies. Some resistance mechanisms are described in the following sections.

BIOFILM RESISTANCE

There are several characteristics that underlie the increased resistance of bacterial films, though some resistance mechanisms are shared with their planktonic counterparts. Nonetheless, adhered cells have a phenotype that confers them an increased resistance to antibiotics and biocides, when compared with suspended cells [67, 72, 91, 92].

Figure 2.3 exhibits several biofilm defence mechanisms differentiating them from their planktonic counterparts, such as specific resistance genes, restricted growth rates, the existence of persister cells, quorum sensing communication, stress response regulons, and the impervious EPS [93]. Cells in biofilms can be 10-1000 times more resistant to antimicrobial agents than their planktonic counterparts [94]. For instance,

control of biofilm cells of *S. aureus* requires 600 times more sodium hypochlorite than their planktonic equivalents [17].
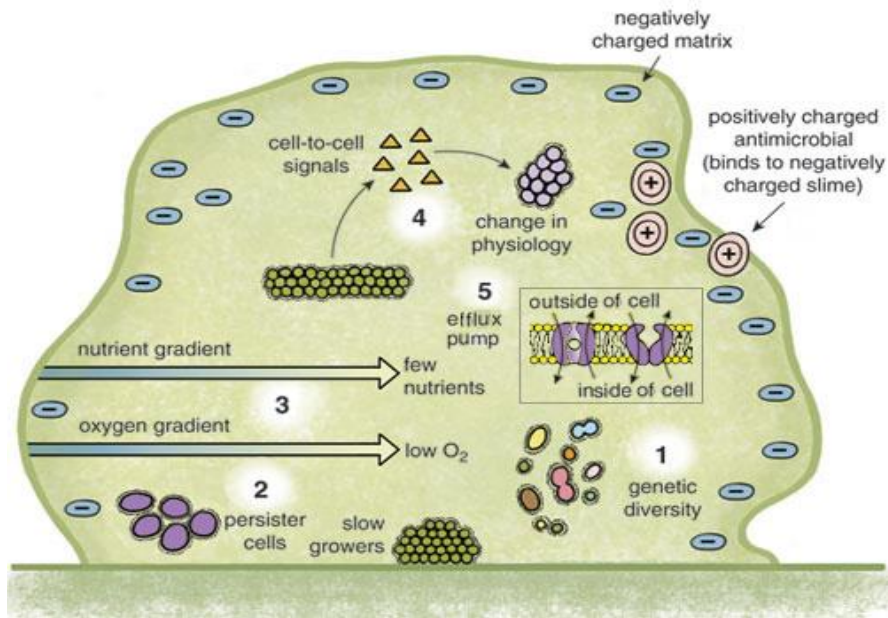
Figure 2.3 Biofilm resistance diversity to antimicrobial agents: (1) genetic expression of certain resistance genes, (2) restricted growth rates; level of metabolic activity within the biofilm; the existence of persisters, (3) mass transfer limitations, (4) quorum sensing and (5) multidrug efflux pumps. (Adapted from [95].)

CELL HETEROGENEITY

Cell heterogeneity is frequent within biofilms. It is common to find cells at different physiological states. In a given population, genetic and phenotypic diversity is triggered by the surrounding conditions [96]. Parameters such as space, nutrients, and age could contribute to the increased resistance of biofilms.

The unavailability of space can be a factor for resistance. The exopolymeric matrix can be hindering cell division in mature biofilms. The cells prefer to produce EPS than new cells, aging the population and increasing mass transfer limitations [97]. Bacteria have different degrees of resistance according to their state. Resistance increased as both *Burkholderia cepacia* planktonic and biofilm cultures approached the stationary phase [98]. Additionally, *P. fluorescens* cells are known to produce an exopolysaccharide lyase, which is triggered by the stress of feeling constrained. This enzyme degrades the

matrix with the purpose of obtaining nutrients from it and, also to free cells from the biofilm so that they can colonize other favorable locations [99].

In a high cell density population such inside biofilms [100], mass transfer might be hindered by cell packing [81]. Higher cell numbers promote horizontal gene transfer of resistance features [101]. The selection of resistant mutants by exposure to sub-lethal concentrations of antimicrobial agents is higher in highly populated biofilms [102]. The different microenvironments and depletion of nutrients and oxygen in the interior of biofilms can alter the metabolic activity of the cells [94, 103]. The activity is high at the surface and stratified going to to the interior, reaching its lowest levels in deeper layers. As cells are buried in the biofilms and the supply of nutrients and oxygen starts to be scarce, the cells go into a slow growing or dormant state [95, 104]. The antimicrobial agents that target the disruption of microbial processes are more effective, against rapidly growing cells [105]. When acting on slow growers or dormants, the activity of these antimicrobials is antagonized. In a biofilm, only pockets of this type of microorganism are found, however, some of these cells can be found with at least some degree of cellular activity [1]. Nonetheless, this mechanism is usually observed by an increase of resistance [106].

MASS TRANFER LIMITATIONS

The resistance mechanism is more evident in biofilms due to the presence of EPS. Both structure and composition dictate its susceptibility to antimicrobial agents. The biofilm constituents may act as an adsorbent or reactant, thus chemically interacting and impairing diffusion and, its structure (porosity and tortuosity) may physically reduce mass transport [30, 107-110].

The way how biofilms develop has influence on its degree of resistance: in a biofilm, which environmental conditions make it to be very porous, with large channels, the transport of substrates/nutrients would be easier than in a more compact one, with tight pores [111]. The carbon and nitrogen availability, pH and temperature, all influence EPS composition and, therefore, the resistance of biofilms can vary, by developing zones with different densities and consequentially different mass transfer parameters [28, 112]. The EPS matrix has sorption capacity for heavy metals, organic substances, and particles, including nanoparticles, all of which can be caught and accumulated within the biofilms [60].

The polyanionic nature of the bacterial EPS may be responsible for binding the antimicrobial agents before they have the opportunity to reach the cells [94, 113, 114]. Similarly, cells in biofilms are able to interact with the antimicrobial agents, wasting them, so that less quantities are available to reach the under layers of the biofilm [115]. This way, it is not only the matrix, but also other biofilm components that are able to hinder transport. Many examples are found to this reaction – diffusion phenomenon. Davison et al. observed retardation of a QAC by the exopolymeric matrix [116]. The work of Ciofu et al. demonstrated the increased resistance of mucoid biofilms. *P. aeruginosa* that overproduces EPS showed to be up to 1000 times more resistant to tobramycin than the normal EPS producers, in spite of similar planktonic MICs [117]. However, the selectivity of the biofilms might be related with the nature of the drugs. A study by Anderl et al. showed that the chlorine ions and the antibiotic ciprofloxacin were able to penetrate *Klebsiella pneumonia* biofilms, however ampicillin was retarded by the biofilm components [114]. Furthermore, biofilm composition may be a factor, as another group of investigators found that active chlorine ions were reacting with organic matter at the surface of *P. aeruginosa* biofilms faster than they could diffuse into deeper layers [118]. As a resistance mechanism, EPS works as barrier, hindering penetration, however, the bacterial matrix could not fully inhibit penetration of the antimicrobial agents, but instead it provides sufficient time for the induction of resistive mechanisms to respond to the attack [119].

SPECIFIC RESISTANCE GENES

The adaptation of planktonic cells to different conditions may promote changes at their genomic level [16]. These changes are the grounds for their specific adaptive response, i.e. to change into biofilms [120]. Biofilm formation is regulated by many genes, including some that are exclusive to biofilm growers [121, 122]. Mutations occur with more frequency in biofilm embedded cells than in the planktonic state, as seen on *P. aeruginosa* cells where mutations occur 105 times more when cell are in biofilms [123]. In an effort to control *P. fluorescens* cells with ethylenediamine tetraacetic acid (EDTA) and a QAC, Langsrud and Sundheim found that the cells developed resistance to these chemicals [124].

The contact with sub-lethal concentrations of antibiotics could be the cause for the adaptation of the outer structure of bacteria [115]. It was found that *P. stutzeri* had

resistance to QACs, triclosan and antibiotics, after developing resistance to chlorhexidine, probably through the alteration of the cell envelope [125]. The resistance to several agents might be encoded into the cell genome, stress activates these genes, deploying an active response [115].

In addition, plasmids were proven to be transferred inside biofilms. Kanamycin resistance genes were transferred from a donor population to other cells within the same biofilm, when this was exposed to sub-lethal concentrations of this antibiotic [126].

QUORUM SENSING

Quorum sensing (QS) is the term used to describe cell-to-cell signaling or intracellular signaling in bacteria. Small hormone-like signaling molecules termed auto-inducers are produced, released and detected by the bacterial populations. High bacterial populations inside the biofilms facilitate communication by signaling molecules [2, 60, 97]. The detection of a threshold concentration of these molecules triggers the expression or repression of genes, enabling bacteria in the biofilm to act as a multicellular organism [24, 95].

Many cell regulatory systems are governed by QS, i.e. several physiological processes such as formation, aggregation and dispersal of biofilms [127]. Deterioration of food by enzymatic activity is also regulated by QS [128]. Cellular repair and defense is also mediated by QS. The production of the enzymes catalase and superoxide dismutase is regulated by QS [95]. These enzymes play a role in defense by neutralizing toxic compounds [115]. Catalase breaks down hydrogen peroxide into water and molecular oxygen, and superoxide dismutase promotes the degradation of superoxide radicals [95]. *P. aeruginosa* biofilms presented higher catalase expression when exposed to sub-lethal concentrations of hydrogen peroxide to biofilm cells, however lethal to planktonic [129]. The exposure of biofilms to antimicrobials may trigger EPS production as a defense mechanism to that stressful situation [130].

MULTIDRUG EFFLUX PUMPS

Porins are proteins forming channels that allow the transport of specific molecules across the bacterial membrane [131]. These membranes could suffer structural

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alterations to act as a resistance mechanism to some antimicrobial agents [132]. This system is ingenious enough to select what goes out the cell, leaving the agents outside where the low permeability of the cells does not allow reentry, thus reducing the accumulation inside the cell and increasing the concentration needed to inhibit the bacteria [132]. The overproduction of efflux pumps has been reported as a biofilm resistance mechanism [133]. QS may be involved in the protection against antimicrobial agents, by increasing the production of these molecular pumps, with the intend to expel drugs from the cells [95]. This is a well-known resistance mechanism [95]. Multidrug efflux systems are usual on Gram-negative bacteria [134, 135].

PERSISTER CELLS

The indication of persister cells is the latest biofilm insusceptibility explanation [136]. Nonetheless, the first time persisters were mentioned was in 1944 by Bigger [137]. The surviving cells from the incomplete inactivation of *Staphylococcus* spp. with penicillin were able to regrow into a population with apparent penicillin susceptibility [137]. Recurring infection occurrences revived the interest into persister cells [138]. Small subpopulations of bacteria within the biofilms may differentiate into persisters [4, 136]. These cells, present in all bacterial cultures [48], are essentially invulnerable to lethal concentrations of antimicrobial agents [96, 139]. When exposed to antimicrobial agents, persisters neither grow nor die [140]. Nonetheless, when the drug is removed these bacteria give rise to a normal bacterial colony [95]. Typically, bacterial cells are persisters against antibiotics, however, there is a study where the disinfection of *P. fluorescens* biofilm embedded cells, treated with a multi-target biocide (*ortho*-phthalaldehyde), resulted in ineffective killing due to the differentiation of some of these cells into persisters [141]. The way how cells differentiate into persisters is not well known [142]. Persisters are not believed to be mutants [143], but instead they produce a toxin, RelE, that ceases bacterial activities, inducing an inactive state [95]. The formation of these cells could be considered as a method of adaptation to respond to environmental alterations, and could follow two ways: in the first, the population continues to grow, exhausting substrata risking extinction, and in the second, they simply suppress their functions waiting for favorable conditions [30].

The resistance mechanisms mentioned before, might explain biofilm persistence and resistance to control [8]. The resistance of biofilms is defined as a multifactorial mechanism and it is not universal, varying between different microorganisms [24].

2.5   BIOFILM CONTROL

Biofilms are a frequent source of infections and industrial process problems [105]. Many studies have been performed in order to control biofilms in food industry. Some strategies are already common practice in industry [144].

PREVENTION

Biofilms are difficult to eradicate, thus prevention of its occurrence is commonly implemented in food industry. Biofilms are considered heterogeneous in time and space, therefore research moved towards the study of biofilm mechanisms, covering many fields of science in order to characterize the processes governing biofilms [96].

Contamination is normally caused by biofilm development due to ineffective or complete lack of cleaning. Organic molecules are able to deposit in all types of surfaces, including the water used for manufacturing, conditioning the surfaces and providing favorable conditions for microbial growth [144]. The physical characteristics of the substratum has influence on initial attachment. To prevent bacterial attachment to surfaces, surface active substances are used. Surfactants provide uniform wetting of surfaces, reducing the surface tension of water by adsorbing at the liquid-gas interface and reducing the interfacial tension between the layers [24]. Additionally, the intricate process lines of industrial plants have critical points where built up of fouling is expected. These include gaskets, dead ends, joints, valves, corners, cracks, or crevices [57, 145]. Rational equipment design helps reduce the risk of microbial development by minimizing laminar product flow, reducing static product and facilitating cleaning and disinfection processes, with the aim of reducing attachment [22]. However, a specific design could be impractical or simply not implemented [40].

To prevent contamination, the materials that constitute the plant should be carefully selected [57]. Good hygienic properties must be attained, either by the material properties or by material modification to render them antimicrobial or to reduce attachment [146, 147]. However, the application of coatings on industrial

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surfaces could be restricted by toxicity or infeasibility by increased costs [22]. The most common materials used in food plants are stainless steel, grades 304 and 316, for their chemical and mechanical stability at the diversified food processing temperatures, ease of cleaning and resistance to corrosion [148]. The AISI 316 stainless steel shares the same characteristics with the AISI 304 grade with the addition of a higher tolerance to corrosion, given by the inclusion of molybdenum. Usually, corrosion could be caused by food, detergents or disinfectants [22]. Polyvinylchloride is a material that is not commonly used in food industry due to its increased risk of contamination, resultant from its deterioration over time [44]. Stainless steel may be a better option because it is more resistant to mechanical stresses like grinding, brushing, and electrolytic or mechanical cleaning. For that reason, in a cleaning and disinfection plan, it is of major importance to gather the maximum information about the system, together with flow diagrams (information about volume, residence time, cycle time, half-life time, etc.) to satisfy the sanitation regulations [57].

The risk of biofilm formation is increased in events such as intermittent operation, unattended risk areas (i.e. filters), inconsistent raw water composition, lack of cleaning after failures, and poor access to surfaces existing in the plant [149]. The risk can be lessened by the exclusion of light, use of short piping systems, inert and smooth materials, good air circulation, working at low temperatures, in fairly dry conditions, and general quality control [149].

The early detection of biofilms is also used as a prompt response to outbreaks [150]. There are currently many methods in the market. The conventional methods, such as the count of total viable cells, microscopy and spectroscopy techniques, impedance measurement and ATP analysis are broadly used [149, 151]. Pereira et al. reviews the principles behind each method and the importance of monitoring in the beverage and food industry [152].

Another strategy commonly used in food industry is the preconditioning of surfaces to reduce/inhibit bacterial attachment [93]. For instance, biosurfactants are known to have properties that prevent microbial attachment [153]. On the other hand, functionalized materials, or compounds that could be blended into the material surfaces have been reported in literature, the repulsion between the surfaces and the bacteria also have been reported to be effective in biofilm prevention [93]. Araújo et al. studied coated spacers used in reverse osmosis membranes with different antimicrobial coatings, as copper, silver, gold and Polydopamine, plus a spacer infused with Triclosan.

The objective was to prevent biofilm development, but this was instead only delayed in time. Only the first layers of biofilms were killed, leaving a conditioning film that was probably used for recolonization [154].

The frequency of cleaning is another strategy. The more often the food contact surfaces are cleaned, the lesser the risk of microbial attachment [22]. The process of irreversible attachment occurs so swiftly, that carefully determining the suitable cleaning frequency becomes extremely important to avoid accumulation of microbial and organic residues, influencing hygienic conditions as well as nutrient availability [22]. A thorough cleaning and disinfection process should occur several times a day on the surfaces that contact directly with the product, in regular intervals varying from 4 to 24 hours [148, 155].

CLEANING AND DISINFECTION

In all industries, particularly in the food industry, the proliferation of microorganisms is very common, even when manufacturers take all the "by-the-book" contingency plans. Therefore, biofilms occurrence is common, leading to a need for their control using cleaning and disinfection techniques. The aim of microbial control is the elimination or reduction of microorganisms and their activity to acceptable levels, as well as the prevention and control of the formation of biological deposits on process equipment [44]. Therefore, programs are established to control microbial proliferation: two examples are the Good Manufacturing Practice (GMP) and the Hazard Analysis and Critical Control Points (HACCP) plans [39]. HACCP is presented in Codex Alimentarius (CAC/RCP1- 1969- revision 2003), that was the basis for the Food Hygene directive by the European Eunion [156]. An example of this is to include all surfaces and vectors, such as the air or personnel that may disperse contaminations from areas such as the floor or walls. They too should be cleaned and disinfected [93].

Biofilm control in food plants normally includes a process called Clean-in-Place (CIP), which consists in cleaning of the plant without dismantling or opening the equipment. During CIP, alternated cycles of detergent and disinfectant solutions run throughout the plant with water rinses, with increased hydrodynamics (high turbulence and flow velocities) [145]. This method typically uses caustic acids, surfactants, biocides and, sometimes, includes enzymes [19, 145, 155, 157].

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The selection of a good cleaning and disinfection regime should be based in some principles to be efficient. It is of utmost importance that the nature and age of biofouling is known [158], as well as characteristics like location, type of microorganisms (bacteria, spores, yeasts, molds or protozoa) or biological entities (prions, viruses) [159]. The characteristics of the surface to be cleaned are also relevant, because of side-effects of cleaning on equipment materials (i.e. some cleaning agents can be corrosive) [57]. The total number of target cells should be taken into account since high initial numbers of bacteria may result in some persistent cells, which could result in biofilm regrowth [63]. In cases where the concentration of the disinfectant is limited, the bactericidal effect may be reduced in the presence of high numbers of bacteria. Also, the growth phase of bacteria will influence their susceptibility to disinfectants. It is known that bacteria in the exponential phase of growth are more sensitive to disinfectants than in the stationary phase [160]. Biofilm cells are more resistant to biocides as a result of their physiological heterogeneity and the presence of EPS, which hinders the diffusion of biocides into the cells [161]. Cleaning is important because, in general, disinfectants have poor diffusion and are not able to kill all the embedded cells [93]. To clean biofilms, methods such as the use of alkali-based and acid cleaning, scrubbing and brushing are used. However, when bases or acids are used, the environmental conditions have to be thoroughly defined, otherwise its efficacy is reduced [22]. Although biofilm removal can occur naturally by intrinsic processes, mechanical removal by human action is a common strategy in food industry, though, very expensive because of the need to open the process machinery [162]. Breaking up and removing the deposits on surfaces is of major importance in food industry [148]. The incomplete removal could lead to reattachment and consequent biofilm regrowth [155]. The right choice of disinfectants, single or in combination, point for biocide injection, concentration, temperature, exposure time and hydrodynamics should be carefully optimized for each system. The disinfectant should be kept at a concentration equal or superior to the minimum inhibitory concentration for the period of time defined as ideal for disinfection [44, 163]. All these parameters must be taken into account when designing a disinfection plan.

Additionally, in order to achieve long lasting stable results, follow up actions are required, such as monitoring the presence of microorganisms and the formation of deposits on surfaces [42].

BIOCIDES

The European Standard of 24 April 1998 (CE/8/98), defines biocidal products as active substances, or preparations containing one or more active substances, presented to the user in their final form, whose function is to destroy, stop the growth, render harmless, avoid or control, by any means, the action of pathogenic organisms, by biological or chemical processes. The use of biocides in biofilm control is well accepted and very common [14]. Although biocides are used for the reduction of the number of microorganisms, their simple use does not necessarily reduce the biofilm formation rate. It is essential to use biocides correctly, because their incorrect application is expensive and could lead to unwanted results [164].

The major groups of disinfectants used in the food industry are divided according to their mode of action, (1) oxidizing agents e.g. chlorine-based disinfectants, ozone, and hydrogen peroxide, (2) iodophores (iodine based disinfectants), (3) surface active compounds like QACs and (4) weak acids [22, 40]. However, current methods of disinfection include the application of other chemical compounds like alcohols, aldehydes, anilides, biguanides, bis-phenols, diamidines, halophenols, and heavy metal derivatives [165].

Most biocides kill bacteria by targeting the cytoplasmic membrane, resulting in membrane damage such as disruption, dissipation of the proton motive force and inhibition of membrane associated enzyme activity [70].

Each bacterial strain reacts differently to each chemical compound, either by its phenotypic characteristics (e.g. properties of the cell wall) or due to resistance mechanisms (coded by its genotype or induced). Thus, it is fundamental that upon biocide selection, an evaluation of the efficacy against the dominant microorganisms on the system is performed. Only after having information about the nature of the microbial population to treat it is possible to determine the relation between the minimum inhibitory concentration and the contact period of a biocide to a given contaminant [167].

Table 2.2 provides information on the mechanisms of action, typical targets, resulting effects and examples of biocides.

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Table 2.2 Mechanisms of interaction of several biocides according to their cellular targets and antimicrobial actions (adapted from [163]).

Cellular targets	Antimicrobial action	Interaction Mechanisms	Examples
Chemical reactions			
Thiol containing cytoplasmic and membrane bound enzymes e.g. dehydrogenases	Metabolic inhibition	Oxidation of thiol groups (predominantly)	Isothiazolinone Organomercury Salts of heavy metals Hypochlorite
Biomolecules (e.g. proteins, RNA, DNA) with amino, imino, amide, carboxyl and thiol groups (nucleophilic)	Inhibition of cellular metabolism and replication. Possible cell wall damage	General alkylation reactions	Glutaraldehyde Formaldehyde Chloroacetamide
Amino groups in proteins	Metabolic inhibition; lysis	Halogenation	Hypochlorite Chlorine-releasing agents
Enzyme and protein thiol groups	Metabolic inhibition	Free radical oxidation (e.g. hydroxyl radicals)	Hydrogen peroxide Peracetic acid
Divalent cation-mediated outer membrane integrity; principal target region Gram negative cell wall; metal ion-requiring enzyme processes	Release of cellular contents; high susceptibility to stress; metabolic inhibition.	Chelation of metal ions	EDTA Oxine
Intercalation between DNA base pairs	Damage in replication	Intercalation	Aminoacridines
Ionic interactions			
Cytoplasmic membrane integrity; membrane-bound enzyme environment and function	Leakage; respiratory inhibition; intracellular coagulation	Electrostatic interaction with phospholipids	QACs Clorhexidine Polyhexamethilene Biguanides
Physical interactions			
Transmembrane pH gradient; membrane integrity	Leakage; disruption of transport, respiratory and energy coupling processes	Penetration/partition into phospholipid bilayer; possible displacement of phospholipid molecules; intra membrane molecular cycling	Phenols Weak acids Parabens Tetrachlorosalisylanilide Phenoxyethanol 2-phenylethanol
Membrane integrity	Leakage	Solution of phospholipids	Aliphatic alcohols
Cytoplasmic membrane integrity; membrane-bound enzyme environment and function	Leakage, uncoupling of energy processes; lysis	Membrane-protein solubilization	Anionic surfactants

MECHANISMS OF ANTIMICROBIAL ACTION

A classical approach which is used to determine the mechanism of action of a biocide establishes a correlation between the minimum inhibitory concentration and the resulting biochemical and physiological changes in the organism [163]. An antimicrobial effect can be defined as an interaction between an active substance and specific targets in the microbial cell. In target approach, the active ingredients contact with a variety of cellular structures (cell wall, cytoplasmic membrane, membrane enzymes, cytoplasm, and genetic material). Experiments conducted to compare different strains revealed that Gram negative bacteria, which have the supplementary protection of the cell wall, are more resistant to the bactericidal effects than Gram positive bacteria [168-170]. The antimicrobial agents cross the cell wall through pores. This penetration, according to Paulus, is dependent on the size, charge and lipophilic properties of molecules [71]. If a substance is soluble in water and its molecular weight is around 600 Da, there is a greater probability of passing through the channel formed by the porin. It is also possible that the antimicrobial agent penetrates the cell wall after causing its destabilization and disintegration. Finally, the biocide reaches the cytoplasmic membrane as the primary site of action. Depending on the action spectrum, these substances could be designated as biostatics (if they only inhibit the microorganism growth or multiplication) or as biocides (if they are able to kill the microorganisms) [71].

The process of transporting the biocide to the cell surface, adsorption, diffusion, penetration and interaction with the target cell component is not instantaneous and the duration can be different according to the biocide. The differences depend on the action mode, including the chemical composition and physicochemical properties of the biocidal agent [163]. Biocidal compounds belong to different chemical classes.

Biocides could cause a series of self-destructive events in microorganisms, resulting from sub-lethal/lethal damage to cell death. Typical damage caused by biocidal compounds involves the disruption of the transmembrane proton motive force, leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane inhibition of respiration or catabolic/anabolic reactions; disruption of replication; loss of membrane integrity resulting in leakage of essential intracellular constituents such as potassium cation, inorganic phosphate, pentoses, nucleotides and nucleosides, and proteins; lysis and coagulation of intracellular material

[163]. Figure 2.4 shows the antimicrobial mode of action of biocide on diverse types of microorganisms.
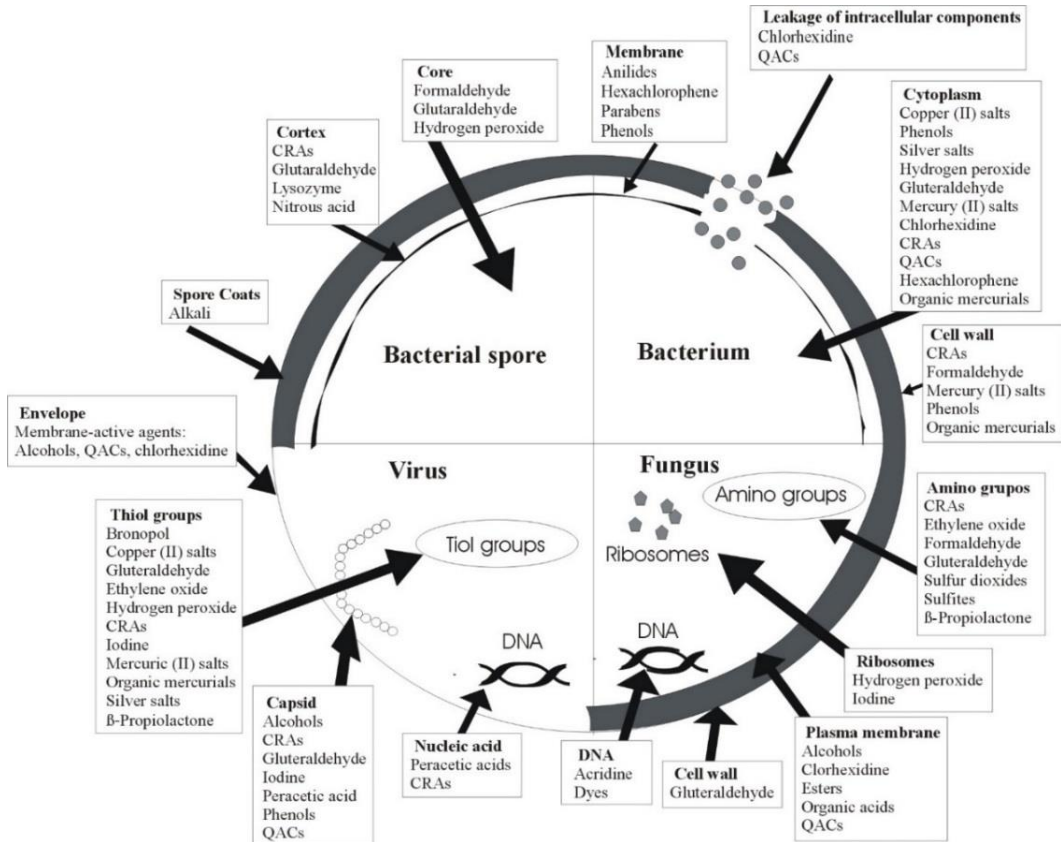
Figure 2.4 Antimicrobial mode of action of biocides (adapted from [164]). CRAs – chlorine removal agents; QACs – quaternary ammonium compounds.

FACTORS AFFECTING BIOCIDE ACTION

Cleaning is often inefficient in the removal of biofilms. Bactericidal activity is influenced by the surrounding media, but a correct cleaning plan is also very important. The main environmental factors that could influence the activity of a biocide are pH, water hardness, presence of additives and temperature [164]. Biocide concentration, exposure time, presence of organic compounds and type of microorganisms are key factors of the antimicrobial action as well. Many biocides have an optimum pH range of activity. For example, glutaraldehyde and cationic biocides such as chlorhexidine and

QACs are most active at alkaline pH, whereas hypochlorites and phenolics are more potent at acid pH. Additives, as corrosion inhibitors or conditioning agents may also influence and even reduce or inactivate activity. The activity of biocides against Gram negative organisms may be enhanced by permeabilizers that increase cell permeability. Russell reported that EDTA chelates divalent cations from the outer membrane, especially on *P. aeruginosa* [63]. Activity can also be increased by a combination of biocides. In general, the efficacy of disinfectants increases with temperature [66]. When disinfection occurs at low temperatures, the use of higher concentrations of biocides or prolongation of the contact time may increase effectiveness [160]. The antibacterial activity of biocides is determined by their chemical reactivity to certain organic groups. Biocides do not react independently with fixed groups or groups of the cell surface. Oxidizing biocides react with any oxidizing organic group, not only with living cells. In food industries, deficient cleaning may not eliminate contaminating substances, such as carbohydrates, fat, proteins, calcium phosphate, blood residues or dirt [57]. These contaminants may have a high impact on the cleaning and disinfection steps. This happens because the antimicrobial activity of chemical compounds may be reduced in the presence of organic material, through reaction/neutralization [94, 171].

The effect of disinfectants is concentration dependent. Generally, a user-concentration is given by the manufacturer based on simple laboratory tests that typically measure the efficacy in suspension and without additives, which may not be efficient to kill attached microorganisms [171]. In a practical disinfecting setting, the disinfectant may be diluted due to residual water left after the cleaning process. In order to avoid dilution, the equipment design should prevent, and thus facilitate, running of water off the surfaces instead of its accumulation. Furthermore, surfaces should be allowed to get reasonably dry before disinfection [160]. Biocides such as phenolics or alcohols typically lose their potency with dilution, whereas QACs, chlorhexidine, glutaraldehyde, ortho-pthalaldehyde retain much of their potency [63]. Nonetheless, the conventional protocols used for CIP have been unsatisfactory for biofilm control [172].

Resistance is a survival mechanism that will continuously morph, ensuring prevalence of the species. It will be necessary to find new antimicrobials and new ways to employ them to overcome bacterial resistance [173].

2.6   INNOVATIVE STRATEGIES FOR BIOFILM CONTROL

Many new developments have been made in order to overcome resistance, some of these have already been put into practice in food industry.

In an effort to replace chemical disinfectants, alternative physical treatments have been studied. Lately, the use of plasma radiation has been a theme in vogue. Ionizing radiation consists in atmospheric plasma, that is generated using high voltage discharges, to produce reactive oxygen species that kill microorganisms [22]. Another method combining the action of a photosensitizer, a non-toxic dye, with visible light and oxygen, is already used in food industry as a decontamination method. This procedure causes DNA damage and the destruction of cellular membranes and organelles, resulting in the leakage of cellular content [120]. In a paper by Buchovec et al. this method was able to remove up to 3 logs *L. monocytogenes* biofilms [174]. Ultrasonication is another technique used for control. It has been used in various food industry processes such as freezing, cutting, drying, softening, bleaching, sterilization, and extraction [144]. This process is able to generate shock waves with the ability to dislodge biofilms [175]. Besides the agitation, the ultrasounds are able to create small vacuum bubbles that generate high temperatures when collapsed [176]. It is used as a biofilm control strategy, already proven effective in cleaning the water in cooling towers [177] and drinking water, without the generation of disinfection by-products [178].

Ultraviolet radiation (UV) has been employed in many systems, including the treatment of municipal waters. Ultraviolet light is thought to be absorbed by the cells, disrupting some processes such as replication [179]. Contrary to what would be expected, the disinfection using ultraviolet to control biofilms present in water distribution systems resulted in no significant biofilm reduction after the treatment, due to the presence of other interfering substances in those systems [180, 181]. Nonetheless, UV radiation was told to be very effective eliminating planktonic and sessile bacteria on another paper [182].

Electric fields cause a bioelectric effect that is reflected on the increase of cell permeability [183] and, it is typically used to increase the intake of a drug into bacterial cells [93]. Racyte et al. studied the effect of electric fields in combination with activated carbon for disinfection of different types of bacteria in a fluidized bed electrode system [184]. They found this system more effective against Gram positive than Gram negative bacteria.

Nanoparticles were employed as a suitable biofilm control strategy. Biofilm formation may be inhibited in the presence of nanoparticles. In a study by Kalishwaralal et al., biofilm formation of *P aeruginosa* and *S. epidermidis* was repressed up to 95% using silver nanoparticles [185]. Moreover, nanoparticles have been used to carry disinfection agents [186]. They are a promising antimicrobial strategy, because of their high surface area to volume ratio they are thought to increase efficiency [187]. Ferreira et al. immobilized a QAC in nanoparticles. In a 1 hour disinfection process utilizing these reusable nanoparticles, approximately 90% of a *P. fluorescens* biofilm was killed.

The biofilm matrix is mainly composed of polysaccharides and proteins. The latest studies show the potential of matrix degrading enzymes on biofilm control by the disruption of the matrix components [120]. Enzymes hydrolyze the exopolimeric matrix in which the bacteria are embedded. Formulations that contain enzymes are optimized so that there is compatibility with low temperatures. They are efficient time-wise, reducing the cleaning and disinfection time [24]. Moreover, they can work in mild pH, temperature and high ionic strength without affecting, for instance, the membranes used for water filtration, which are easily damaged by many chemical classes. Enzymes were already used to control biofilms and were found to be enhancers of the action of antimicrobial agents [10, 161, 188]. Nevertheless, enzymes are substrate specific [24], and the efficacy of the method is dependent on the right use and right combination of enzymes, being often suggested the characterization of EPS before the enzymatic treatments [59].

The evolution of resistance [189], the possible failure of antimicrobial agents [79], and the formation of harmful byproducts [190] translate into the need for new antimicrobial agents [191]. These should be effective against the bacterial contamination [192]. However, as legislation restricts the use of toxic biocides, eco-friendly strategies represent a new approach for biofilm control [193, 194]. For instance, chlorine could react with organic matter, resulting in cancer-forming compounds that might enter the food chain [190]. Consequently, the exploitation of "green" biocides, from plant sources has been on course [192]. Valeriano et al. identified the antimicrobial properties of peppermint and lemongrass essential oils against biofilm formation of *S. enterica* [195]. *Rhodiola crenulata* (arctic root), *Epimedium brevicornum* (rowdy lamb herb), and *Polygonum cuspidatum* (Japanese knotweed) extracts also showed anti-biofilm properties against *Propionibacterium*

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acnes, reducing the biofilms 64.8%, 98.5%, and 99.2%, respectively [196]. Also *Melia dubia* (bead tree) bark extracts reduced *E. coli* formation and swarming by 84% and 75% [197]. Ferulic, gallic and salicylic acids, considered to be phenolic compounds, were tested against different bacterial biofilms with favorable control results [191, 198]. Chitosan, a polysaccharide, exhibited anti-biofilm properties against *S. mutans*. This compound was able to reduce biofilms by approximately 95% [199]. It was also tested againts *L. monocytogenes, B. cereus, S. aureus, S. enterica,* and *P. fluorescens*. The biofilms developed by these bacteria were reduced from a maximum of 6 logs (*L. monocytogens*) to a minimum of 1 log (*S. aureus*) [200].

QS interference is an alternative approach to biofilm control by targeting the signaling molecules that control various cell processes, including biofilm formation [127]. QS is a biochemical approach to a direct control of the rate and extent of biofilm development, as opposed to cleaning and disinfection techniques [201]. QS inhibitors impair the communication signals between cells in the biofilms [93]. One of the most potent QS quenchers are the halogenated compounds secreted by the red algae *Delisea pulchra* [202]. Other QS inhibitor substances are brominated furanones. Although the mode of action of these drugs is not yet fully understood, it is thought that it inhibits QS [203]. A biofilm treated with these chemicals is thought to have a higher susceptibility to disinfectants [24]. The strategy is to take advantage of the quorum signals used for biofilm regulatory mechanism [204]. In general, the study of which molecules regulate QS in food industry, to find corresponding inhibitors, could increase food safety and product shelf life [128]. The QS signaling molecules could be detected using biosensors [128]. Then, providing the inhibiting signals, or manipulating their mechanisms convincing bacteria not to form biofilms or triggering dispersal, biofilms could be controlled. For instance, *P. aeruginosa* produces rhamnolipid biosurfactants to detach from surfaces [205]. Inducing this bacteria to produce higher amounts of this chemical could result in effective control [204].

Phages are very simple, and the most abundant organisms on earth. They are viruses that infect bacteria, and like viruses they are only able to replicate inside their host [206]. Phages have known to be applied, initially in the early 20th century, to treat bacterial infections in Eastern Europe [144]. There are phages with extreme specificity and others which specificity is broader [206]. Treatments with phages showed potential to inhibit biofilm formation. As they are not chemical-based, their use eliminates the risk of surface corrosion, and due to their high specificity and non-toxicity they are good

candidates as therapy for biofilm infections in living hosts [144]. The co-existence of phages and bacteria is known in biofilms, which is one of the reasons why the combination of phages with disinfectants and polysaccharide depolymerases was suggested to be a novel control strategy [207]. Moreover, phages can be engineered to express biofilm degrading enzymes [208]. The use of phages as biofilm control resulted in a removal of 99.997% of bacteria [208]. The only drawback of the usage of phages is that their use for biofilm control might select resistant bacteria [36].

New strategies have been boosted by environmental restrictions. The combination of two or more strategies could control biofilms synergistically, approaching the problem in multiple fronts could be another alternative to overcome the persistence of biofilms [144, 209]. This technique is referred to as hurdle technology and it is widely used across industries. Any combination is valid as long as it is effective and abides to the current law. The right combination of hurdles should prevent, reduce or completely eliminate biofilms [144]. Therefore by combining different chemicals should broaden their antimicrobial spectrum, if they are able to work synergistically [210]. For example, treatments with ultrasounds in combination with enzymes and ozone were effective against established biofilms [22]. To prevent clinical infections in the operating block, surgical blades have been coated with a mixture of silver nanoparticles and lysozyme, effectively reducing infections by many clinical pathogens. [211]. Oulahal-Lagsir et al. reported the combination of an ultrasonic technique with a chelating agents (EDTA) to be effective in the removal of *E. coli* biofilms [212].

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CHAPTER 3

DIFFUSION OF ANTIMICROBIAL AGENTS THROUGH BIOFILMS
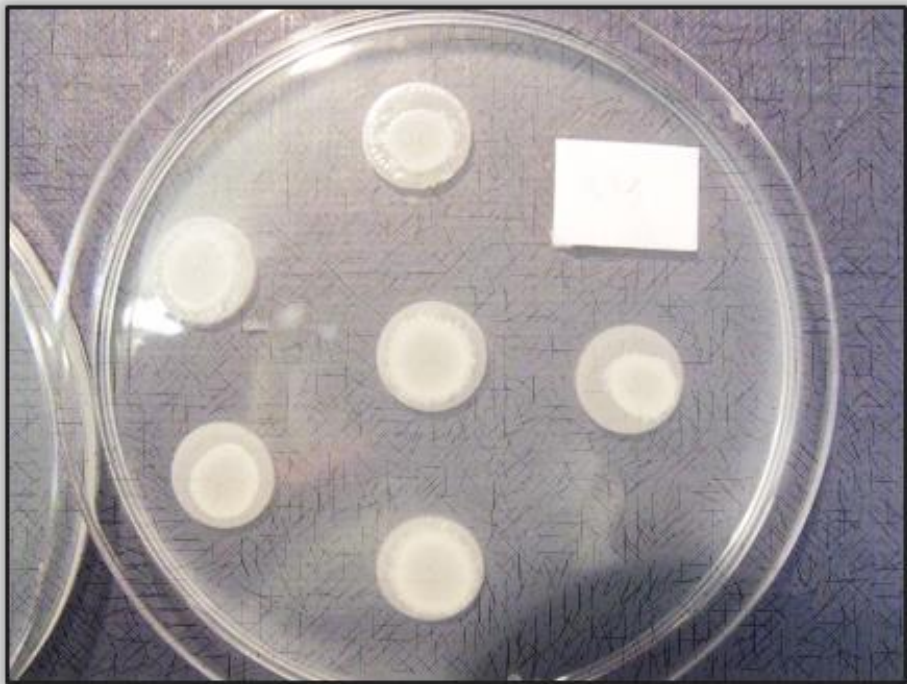
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ABSTRACT

The penetration ability of twelve antimicrobial agents was determined against biofilms of *B. cereus* and *P. fluorescens* using a colony biofilm assay. These antimicrobial agents included antibiotics and biocides. The surfactants benzalkonium chloride (BAC) and cetyltrimethyl ammonium bromide (CTAB), and the antibiotics ciprofloxacin and streptomycin raised interest due to their distinct activities. Erythromycin and CTAB were retarded by the presence of biofilms, conversely to ciprofloxacin and BAC (no retardation due to presence of biofilms). The removal and killing efficacies of these four selected agents was additionally evaluated against biofilms formed in microtiter plates. The most efficient biocide was CTAB, which enabled a higher killing of both bacterial biofilms. Ciprofloxacin was the best antibiotic although none of the selected antimicrobial agents promoted total biofilm removal and/or killing. Comparative analysis of the results obtained with colony biofilms and microtiter plate biofilms show that although extracellular polymeric substances and the biofilm structure are considered a determining factor in biofilm resistance, the ability of an antimicrobial agent to penetrate a biofilm is not correlated with its killing or removal efficiency. Also, the results reinforce the role of an appropriate antimicrobial selection as a key step in the design of disinfection processes for biofilm control.

3.1   INTRODUCTION

A biofilm is commonly defined as a microbial community with cells irreversibly attached to a substratum or attached to each other, and embedded in a matrix of extracellular polymeric substances (EPS) [1]. EPS protects bacteria from environmental adversities [2]. In all industries, especially in the food industry, the proliferation of microorganisms is very common even when manufacturers diligently follow all contingency plans [3]. The main objective of microbial control is to eliminate or reduce the numbers of microorganisms to acceptable levels, as well as to prevent and control the formation of biological deposits attached to the process equipment surfaces [4]. Currently, there is no control strategy capable of entirely eradicating biofilms [5]. At the same time, there is a need to continuously find new strategies to manage antimicrobial resistance [6, 7].

Resistance is the ability that microorganisms have to withstand antimicrobial treatments. Russell [8] and Chapman [9] documented three types of resistance: intrinsic resistance, e.g. Gram negative lipopolysaccharide layer [10]; acquired resistance, e.g. manipulated resistance mediated via plasmids; and adaptive resistance, e.g. exposure to sub-lethal concentrations of an antimicrobial agent that selects for mutation, conferring resistance to that agent or others of the same type (cross-resistance). The way how microorganisms develop resistance is not well understood. Biofilm formation, a case of adaptive resistance is considered a microbial survival strategy, enabling them to be 10-1000 fold more resistant to antimicrobial agents than their free-floating equivalents [11-13]. Antimicrobial resistance is multi-factorial and usually does not depend only on one specific mechanism [14, 15]. When biofilms are exposed to antimicrobial agents, they present specific survival strategies. In comparison with their planktonic counterparts, biofilm cells are physiologically distinct by having specific resistance genes that express protective factors such as multi-drug efflux pumps, stress response regulons and different cell physiognomies [16]. Moreover, they often present decreased respiration and growth/replication rates, despite having higher cell densities. Embedded cells are capable to communicate through quorum sensing, and the existence of persister cells enables them to survive [17]. Biofilm cells are protected by the EPS they produce. The functions of EPS are enabling the biofilm to withstand shear forces, dehydration and chemical attacks [18]. EPS enhances robustness and survival of the biofilm microorganisms on a substratum by serving as a chemically reactive diffusional transport barrier slowing down the penetration of antimicrobial agents.

Furthermore, this matrix reinforces the biofilm attachment to the substratum and promotes its mechanical stability [19, 20]. Moreover, it is where the convective and diffusional transport to the biofilm of oxygen, nutrients, and other substances takes place [21]. EPS composition and architecture has influence on how oxygen, nutrients and cell excreted products are transported [22]. The biofilm constituents may act as an adsorbent or reactant, thus chemically impairing diffusion, and its structure (porosity and tortuosity) may physically reduce transport [23-28].

In order to plan a disinfection procedure it is important to select a suitable antimicrobial agent with an appropriate effectiveness against the contaminants [5, 29]. The objective of this study was to understand the role of biofilms on the effectiveness of antimicrobial agents, with a specific focus on the selection of suitable chemical compounds capable of passing the EPS barrier, killing and removing the biofilm embedded cells of *B. cereus* and *P. fluorescens*. These bacteria are ubiquitous in industrial systems causing numerous process and end product quality problems [30, 31]. The production of extracellular enzymes by these bacteria results in food spoilage [30, 32, 33]. Moreover, they can represent a significant proportion of the contaminant biofilm microflora of dairy plants [34-37].

3.2   MATERIALS AND METHODS

MICROORGANISMS AND CULTURE CONDITIONS

The bacteria used in this work were *P. fluorescens* ATCC 13525^T and a *B. cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing [31].

Bacterial growth conditions were 30 ± 3 ºC and pH 7, with glucose as the main carbon source. Culture medium consisted of 5 g L^{-1} glucose, 2.5 g L^{-1} peptone and 1.25 g L^{-1} yeast extract, in phosphate buffer (pH 7, 25 mM) [38]. All the culture medium products were purchased to Merck (VWR, Carnaxide, Portugal). Bacterial suspensions were prepared by gently removing a small portion of bacteria from solid medium, and diluting it in a 1 L flask (Duran, VWR, Carnaxide, Portugal) containing 250 mL of sterile culture medium. This bacterial suspension was incubated overnight (16 h) at the given temperature, with agitation (120 rpm). After the growth period, the suspension was washed with phosphate buffer in two consecutive steps of centrifugation (3999 *g*, 10 min) in an Eppendorf centrifuge 5810R (Göttingen, Germany), and resuspended in

phosphate buffer (20 mM), in order to obtain a final bacterial concentration of 1×10^9 cells mL^{-1}.

ANTIMICROBIALS

The twelve antimicrobials used throughout the experiments (Table 3.2) were cetyltrimethyl ammonium bromide (CTAB), benzalkonium chloride (BAC), sodium hypochlorite, ethanol, hydrogen peroxide, streptomycin, and tetracycline that were obtained from Sigma-Aldrich (Sintra, Portugal). Benzyl dimethyl dodecyl ammonium chloride (BDMDAC) was obtained from Merck (VWR, Carnaxide, Portugal). Ciprofloxacin was acquired from Fluka (Sintra, Portugal). Chlorine dioxide was obtained from TwinOxide® (Salmon & Cia. Lda, Lisbon, Portugal) and, isopropanol and erythromycin were purchased from AppliChem (VWR, Carnaxide, Portugal). When possible, the compounds were used as they are commonly sold (♥). Some amounts of antimicrobial were previously optimized to obtain a detectable inhibition halo (♣), and others were used at the reported minimum inhibitory concentrations (MIC) (♦) [39, 40].

COLONY BIOFILM FORMATION AND PENETRATION TESTS

Colony biofilms were developed according to the method of Anderl et al. [41] and Singh et al. [42]. Biofilms were grown in sterile Mueller-Hinton agar plates (24 h, 30 ± 3 °C). A volume of 40 μL of cell suspension of *B. cereus* or *P. fluorescens* was placed on a 13 mm polycarbonate membrane, pore size 0.2 μm (Merk, Millipore, Carnaxide, Portugal) originating colony biofilms. Afterwards, the membranes with biofilms were transferred to a fresh plate containing the same growth medium, seeded with *Staphylococcus aureus* CECT 976 at a McFarland standard of 0.5 [41, 42]. Another polycarbonate membrane was placed on the top of the biofilm so that the sterile discs (Biochemica, VWR, Carnaxide, Portugal) were not in direct contact with the biofilms (Figure 3.1). The antimicrobial discs were impregnated with a 15 μL drop containing the different antimicrobials used, providing the amount per disc described in Table 3.2. The negative controls contained a 15 μL drop of sterile distilled water and the positive controls were obtained in the absence of biofilm.
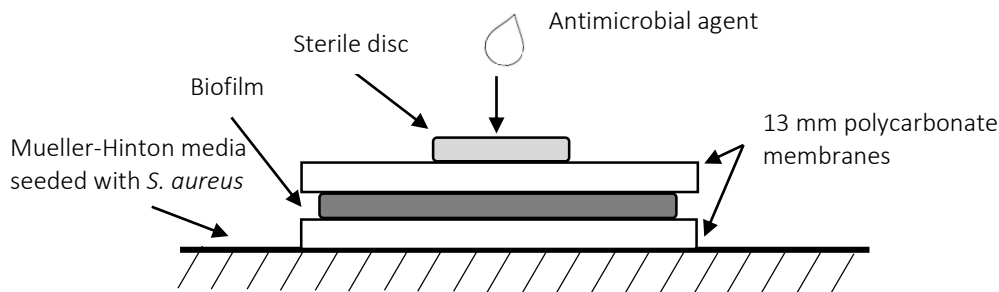
Figure 3.1 Array of polycarbonate membranes and biofilms for the study of the diffusion of antimicrobial agents through biofilms (adapted from Anderl et al. [41] and Singh et al.[42]).

The plates were incubated for 24 h at 30 ± 3 °C before the assessment of the inhibition halos. The positive controls were taken as 100% penetration and used to calculate the penetration rates when biofilms were present.

BIOFILM FORMATION IN MICROTITER PLATES

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. [43]. For each bacterium, at least 16 wells of a sterile 96-wells flat-bottomed polystyrene tissue culture plate with a lid (Orange Scientific, Braine-l'Alleud, Belgium) were filled with 200 µL of bacterial suspension at a density of 1×10^9 cells mL^{-1}. The negative controls were wells containing culture medium without bacterial cells. The plates were incubated for 24 h at 30 ± 3 ºC without agitation.

BIOFILM CHARACTERIZATION

Biofilms of *B. cereus* and *P. fluorescens* grown were removed from the polycarbonate membranes or from the microtiter plates using a stainless steel scraper and, afterwards resuspended in 10 mL of buffer solution (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7) and homogenized by vortexing (Heidolph, model Reax top, Schwabach, Germany) for 30 s with 100% power input, according to the method described by [31]. The homogenized biofilm suspensions were then characterized in terms of cell density, total and extracellular proteins and polysaccharides. Thickness was measured for the colony biofilms using a digital micrometer (VS-30H, Mitsubishi Kasei Corporation, Nagoya, Japan). Cell densities were assessed in terms of colony forming units (CFU) on

Plate Count Agar (PCA) (Merck, VWR, Carnaxide, Portugal), according to Simões et al. [44]. The biofilm suspensions were diluted to the adequate cellular concentration in buffer solution. A volume of 30 μL of the diluted suspension was transferred onto PCA plates. Colony enumeration was carried out after 48 h at 27 ºC.

To assess the total and extracellular proteins and polysaccharides, the method described by Simões et al. [45] was used. Biofilm extracellular proteins and polysaccharides were extracted using Dowex resin [46]. Dowex® resin Marathon® C sodium form, 20-50 mesh (Sigma, Sintra, Portugal) was added to the biofilm suspensions. The extraction took place at 400 rpm and 4 ºC for 4 h. The extracellular components (present in the supernatant) were separated from the cells *via* centrifugation (3777*g*, 5 min). The total (before extraction) and extracellular biofilm proteins were determined using the Lowry et al. modified kit (Sigma, Sintra, Portugal), with bovine serum albumin as standard. The procedure is essentially the Lowry method [47] as modified by Peterson [48]. The total and extracellular polysaccharides were quantified through the phenol-sulphuric acid method of Dubois et al. [49], using glucose as standard.

BIOFILM CONTROL IN MICROTITER PLATES

To ascertain the adequacy of antimicrobial penetration results to develop biofilm control strategies, 24 h aged biofilms formed in 96-well microtiter plates were exposed to selected antimicrobial agents. Biofilms were exposed for 1 h at 30 ± 3 ºC, without agitation, similarly to the colony biofilms. After antimicrobial exposure, the biofilms were analysed in terms of biomass and viability and the results are presented as percentage of biofilm reduction and killing.

BIOMASS AND VIABILITY QUANTIFICATION

The biomass was quantified using crystal violet (Merck VWR, Carnaxide, Portugal) staining, according to Simões et al. [50]. The bacterial biofilms in the 96-wells plates were fixed with 250 μL of 98% methanol (Vaz Pereira, Porto, Portugal) *per* well for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 μL of crystal violet *per* well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound

to the adherent cells was resolubilized with 200 µL of 33% (v/v) glacial acetic acid (Merck, VWR, Carnaxide, Portugal) *per* well. The absorbance was measured at 570 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc., Sunnyvale, USA). All tests were performed in three independent experiments with triplicates.

Biofilm removal was given by equation 1:

$$\%\,BR = \frac{OD_C - OD_W}{OD_C} \times 100 \qquad \text{(eq. 3.1)}$$

where %BR is the percentage of biofilm removal and OD is the optical density, OD_C is the OD_{570nm} value for biofilms not exposed to antimicrobial agents and OD_W is the OD_{570nm} value for biofilm exposed to the selected chemicals.

The modified alamar blue (Sigma-Aldrich, Sintra, Portugal) microtiter plate assay was applied to determine the bacterial viability of the cells as reported by Borges et al. [51]. For the staining procedure, fresh culture medium (190 µL) was added to the plates. To each well 10 µL of alamar blue (400 mM) indicator solution were added. Plates were incubated for 20 min in darkness at room temperature. Fluorescence was measured at the wave lengths $\lambda_{excitation}$ = 570 nm and $\lambda_{emission}$ = 590 nm with the same microplate reader. The percentage of biofilm killing was given by equation 2:

$$\%\,BI = \frac{FI_C - FI_W}{FI_C} \times 100 \qquad \text{(eq. 3.2)}$$

where %BI is the percentage of biofilm killing, FI_C is the fluorescence intensity of biofilms not exposed to antimicrobial agents and FI_W is the fluorescence intensity value for biofilms exposed to the selected chemicals.

STATISTICAL ANALYSIS

For each parameter tested, the average and the standard deviation were calculated. The statistical significance of the results was evaluated using the *t*-test (confidence level of 95%) with the statistical program IBM SPSS Statistics software (Armonk, NY, USA), version 20.0, to determine whether the differences between the controls and the antimicrobial tests could be considered significant.

3.3   RESULTS AND DISCUSSION

In order to ascertain possible factors involved in biofilm resistance/susceptibility to the selected antimicrobials, *B. cereus* and *P. fluorescens* biofilms were characterized in terms of their biovolume, CFU, total and matrix proteins and polysaccharides (Table 3.1).

Table 3.1 Characterization of *B. cereus* and *P. fluorescens* grown as colonies and as microtiter plate biofilms.

| | B. cereus | | | | | | P. fluorescens | | | | | |
	Colony			Microtiter plate			Colony			Microtiter plate		
Biovolume /(cm³)	0.019	±	0.002				0.018	±	0.001			
Log CFU/cm²	7.41	±	0.52	7.20	±	0.69	8.11	±	0.13	8.89	±	0.34
Matrix proteins/ (µg/cm²)	13.8	±	1.5	16.7	±	0.15	20.7	±	0.78	15.2	±	0.37
Total proteins/ (µg/cm²)	49.6	±	2.3	26.3	±	0.04	32.2	±	2.7	27.1	±	0.02
Matrix polysaccharides/ (µg/cm²)	20.4	±	2.6	20.4	±	0.09	7.17	±	0.56	17.4	±	3.0
Total polysaccharides/ (µg/cm²)	29.8	±	3.1	26.2	±	0.06	11.3	±	0.19	23.6	±	2.0

Colony biofilms of *B. cereus* covered approximately 5.5 ± 0.69 mm of the membrane whilst those of *P. fluorescens* covered 6.3 ± 0.44 mm. *B. cereus* biofilms were thicker than those of *P. fluorescens* ($P < 0.05$). The cell density of *P. fluorescens* biofilms (8.11 ± 0.13 CFU cm^{-2}) was significantly higher than for *B. cereus* (7.20 ± 0.69 CFU cm^{-2}) ($P < 0.05$). *B. cereus* biofilms had higher amounts of extracellular polysaccharides and lower extracellular proteins content in comparison to those found in the *P. fluorescens* biofilm matrix ($P < 0.05$). The resulting biofilms presented larger diameters, similar thickness values, and lower cell numbers than those used in the studies of Singh et al. [42] with *S. epidermis*. However, different growth conditions were used, particularly the growth period. Singh et al. [42] used 48 h old biofilms while the biofilms used in this study were 24 h old.

DIFFUSION OF ANTIMICROBIAL AGENTS THROUGH BIOFILMS

When antimicrobial agents were applied to the biofilms, inhibition halos were produced in the *S. aureus* culture underneath. The size of the halos was indicative of the ability of antimicrobial agents to penetrate the biofilms. The same characteristic is related to the antimicrobial potency of each antimicrobial agent against the *S. aureus* culture, i.e. a larger inhibition halo was indicative of a more powerful antimicrobial agent, in terms of penetration (Table 3.3).

In the diffusion test apparatus, ciprofloxacin and tetracycline were the antimicrobial agents that produced the largest halos (about 22 mm) after passing through the biofilms of *B. cereus* and *P. fluorescens*. This behavior was closely followed by BAC and BDMDAC (19 mm halos) for both types of biofilms. Erythromycin and ethanol were able to penetrate both biofilms (halos of about 13 mm were obtained). Isopropanol, sodium hypochlorite, chlorine dioxide and streptomycin produced inhibition halos of 5 mm. Hydrogen peroxide and CTAB caused insignificant inhibition halos ($P > 0.05$). In terms of antimicrobial retardation, values comprised between 5% and 20% were observed for ethanol, BDMDAC and tetracycline for both biofilms, and erythromycin for *B. cereus* biofilms. Erythromycin was retarded approximately 30% by *P. fluorescens* biofilms. The same percentage was only obtained with *B. cereus* biofilms treated with chlorine dioxide. *P. fluorescens* biofilms retarded streptomycin diffusion by 40% and isopropanol and chlorine dioxide by 50%. Isopropanol was retarded more than 70% by *B. cereus* biofilms. Total antimicrobial retardation (100%) was achieved with hydrogen peroxide and CTAB by both biofilms (for CTAB see Figure 3.2), and streptomycin by *B. cereus* biofilms. The statistical analyses showed that the retardation of hydrogen peroxide, BDMDAC, CTAB, streptomycin and tetracycline was significant for both biofilms ($P < 0.05$). *B. cereus* biofilms with isopropanol and erythromycin, and *P. fluorescens* biofilms with ethanol and chlorine dioxide also had significant effects on chemical retardation ($P < 0.05$). These results show that the presence of a biofilm markedly affected the diffusion of some antimicrobial agents. Biofilms have intrinsic resistance to antimicrobial agents. Amongst those resistance mechanisms, mass transfer limitations through biofilms is of utmost importance [52]. For the effective inactivation of bacteria in the deeper layers of the biofilms it is essential that the antimicrobial agent diffuses through the biofilm. In some cases, when biofilms are thick, cells can be in a dormant/low metabolic active state in the deeper layer. Those cells can show a

remarkable resistance to antimicrobials [16, 21]. Moreover, EPS protects the cells against an antimicrobial attack by hindering diffusion through the biofilms. The biofilm matrix is known to have the ability to bind to antimicrobial agents [53]. Anderl et al. [41] suggested that the diffusion of antimicrobial agents might be delayed because the biofilm has the ability to chemically react with them, resulting in their inactivation. Thus, less antimicrobial molecules are left to interact with the deeper layers of the biofilms.

Table 3.2 Antimicrobial agents and respective mass used for the biofilm colony tested. Inhibition halos (mm) of *S. aureus* due to antimicrobials in the presence of *B. cereus* and *P. fluorescens* biofilms. Percentage retardation caused by the presence of *B. cereus* and *P. fluorescens* biofilms. The average ± SD is presented

Antimicrobials	*B. cereus*			*P. fluorescens*	
	Mass/ (µg)	Inhibition halos/ (mm)	Retardation/ (%)	Inhibition halos/ (mm)	Retardation/ (%)
Alcohols					
Ethanol♥	8242	12 ± 1.3	12 ± 7.0	13 ± 0.96	9.3 ± 2.5
Isopropanol♥	11700	2.3 ± 0.47	70 ± 8.8	4.3 ± 2.1	52 ± 22
Oxidising					
Sodium hypochlorite♣	543 (Cl)	4.6 ± 0.50	5.0 ± 0.45	4.7 ± 0.10	1.9 ± 3.1
Chlorine dioxide♥	74 (Cl)	5.3 ± 0.84	32 ± 12	3.0 ± 0.71	47 ± 13
Hydrogen peroxide♥	500	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0	100 ± 0.0
Surfactants					
BAC♣	350	18 ± 1.3	0.11 ± 0.07	18 ± 0.25	0.0 ± 0.0
BDMDAC♣	350	19 ± 0.25	15 ± 1.1	19 ± 0.10	13 ± 0.54
CTAB♣	350	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0	99 ± 1.6
Antibiotics					
Ciprofloxacin♦	5	20 ± 1.0	0.0 ± 0.0	24 ± 0.50	0.0 ± 0.0
Erythromycin♦	15	14 ± 0.05	14 ± 0.32	12 ± 0.50	28 ± 3.1
Streptomycin♦	10	0.0 ± 0.0	100 ± 0.0	3.4 ± 0.05	40 ± 0.89
Tetracycline♦	30	22 ± 0.94	6.9 ± 3.9	24 ± 0.47	12 ± 1.8

♥ Commonly available/standard concentration; ♣ optimized concentration; ♦ MIC; Cl means chlorine
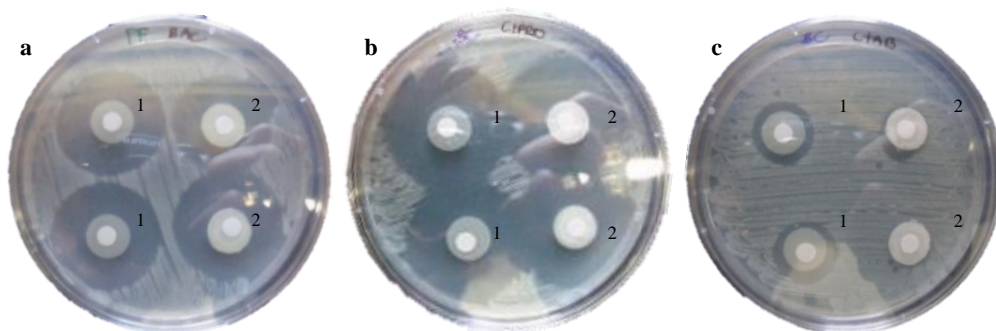
Figure 3.2 Inhibition halos on *S. aureus* using three antimicrobial agents. Condition 1 corresponds to the control where no biofilm is present and condition 2 represents the tests with biofilms. Both condition are duplicated in the same plate. The conditions tested were: (a) BAC test in the presence of a *P. fluorescens* biofilm, showing that this compound is not retarded; (b) ciprofloxacin in the presence of a *B. cereus* biofilm, showing that this compound is not retarded, also that the inhibition halos are large taking into consideration the small amount used (5 µg); and (c) CTAB in the presence of a *B. cereus* biofilm, showing that there is antimicrobial activity in 1, however, the compound was totally retarded by the presence of the biofilm (no halos were observed).

Christensen et al. [54] reported that the presence of alginate, a common EPS, caused mass transport limitations. Singh et al. [42] refers that the biofilm phenotype provides antimicrobial resistance. These authors indicated the existence of spatial heterogeneity in the biofilm structure as a possible explanation for the poor diffusion of antimicrobial agents into biofilms. Diffusion in biofilms may be affected by charge interactions between the matrix and the antimicrobial agents, by increasing the distance between the antimicrobial and the bacteria, by size exclusion, and by the viscosity of the matrix [55]. It has also been suggested that it is not the quantity of matrix that exclusively causes resistance, but its polyanionic nature that hinders the antimicrobial agents [55]. For instance, the polysaccharides can hinder antimicrobial action due to their charge and hydrophobic properties [21, 56]. In fact, the penetration of positively charged hydrophilic drugs is known to be delayed by the EPS matrix [56].

In this study, retardation percentages often differed between the types of biofilm (Table 3.2). Isopropanol, sodium hypochlorite and streptomycin diffused differently through the biofilms of both species. The highest retardation rates, over 70%, occurred for *B. cereus* biofilms. In fact, the distinct retardation rates are probably due to the distinct biofilm characteristics, particularly the type of EPS produced by each bacterium [57]. In addition, the amount of polysaccharides and proteins produced by both types of

bacteria is different. The high retardation rates observed for *B. cereus* biofilms might be related to the high proteins content present (Table 3.2). As many antimicrobial agents target protein-like structures [10], these might be adsorbed before penetrating the biofilm.

The function of antimicrobial agents is to extinguish or to discontinue the growth of an organism by biological or chemical processes [3]. The mode of action of antimicrobial agents may be another important factor contributing to mass transfer limitations through biofilms. Ethanol and isopropanol are membrane disruptors. These chemicals act by penetrating into the cells through the hydrocarbon part of the phospholipid bilayer, causing rapid release of intracellular components [15]. Even though a higher mass of isopropanol than ethanol was used, isopropanol retardation was higher, because it is slightly more reactive than ethanol against bacteria [10]. Chlorine based agents are the most broadly used disinfectants [10]. These chemicals are highly active oxidizing agents destroying the cellular activity of proteins. Sodium hypochlorite was slightly hindered (less than 5%) by the presence of a biofilm. In fact, oxidizing agents react strongly with cell constituents such as amino, carboxyl, sulfhydryl and hydroxyl groups in bacterial proteins as well as nucleic acids [10]. Hydrogen peroxide damages ribosomes which are responsible for the translation of RNA into a peptide chain, being also able to react with other cell constituents [15]. This compound has oxidative potential, producing hydroxyl free radicals that target lipids, proteins and DNA [10]. Peroxides are more active against Gram-positive bacteria than Gram-negative bacteria [10]. However, the ability of both bacteria to produce catalase or other peroxidases may increase tolerance to this compound [58, 59]. Quaternary ammonium compounds (QACs) are classified according to the ionic physiognomies of their hydrophilic group as anionic, cationic, non-ionic and zwitterionic [60]. The mechanism of action of cationic surfactants (BAC, BDMDAC and CTAB) is the same as the general mechanism of QACs. The hydrophilic headgroup of QACs is adsorbed to the cell wall and reacts with the cytoplasmic membrane, allowing the release of intracellular constituents [61-63]. The strong affinity of CTAB for proteins and lipid components of the membrane suggests that this QAC is spent before it reaches the under-layers of the biofilm [64]. Cationic surfactants are also known to bind to DNA and DNA-protein mixtures [65]. Fluoroquinolones such as ciprofloxacin are generally not hindered by the EPS of the matrix [14, 55]. This was also found in the present study. The penetration of aminoglycosides (streptomycin) is known to be delayed by *P. aeruginosa* biofilms [15].

Streptomycin was, in this case, 40% retarded by *P. fluorescens* biofilms and 100% when applied to *B. cereus* biofilms. This antibiotic acts by binding to prokaryotic ribosomes and has shown affinity for other nucleic acid targets [66]. Erythromycin, a macrolide, has the same mechanism of action as streptomycin. The antimicrobial mode of action of tetracycline is by binding to ribosomes [67]. In this study, lower retardation rates were expected, since a higher mass of this compound was used (Table 3.2). However, it seems that this antibiotic is strongly affected by the biofilms.

BIOFILM ACTIVITY SCREENING

The presence of inhibition halos on the *S. aureus* culture is indicative of the penetration efficacy of the antimicrobial agents through the biofilms. In fact, this assay does not allow the distinction between biofilm penetration and antimicrobial potency. Taking into account the results obtained with the antimicrobial retardation tests, selected antimicrobials agents were used in order to ascertain the reliability of the results obtained with the colony biofilm system. Therefore, biofilms of *B. cereus* and *P. fluorescens* were formed in microtiter plates. The effects of BAC, CTAB, ciprofloxacin and streptomycin were assessed on biofilm removal and killing. These antibiotics and biocides were those with the highest and lowest retardation values. The tests were performed using 96-well microtiter plates. This bioreactor permits the assessment of the biofilm killing and removal rates by the selected antimicrobial agents using a large number of replicates [68, 69]. The biofilms developed in the 96-well microtiter plates were characterized in terms of their cell density, total and matrix polysaccharides and protein content (Table 3.2). The cell densities of *B. cereus* and *P. fluorescens* biofilms formed in the microtiter plates were similar to those of colony biofilms ($P > 0.05$). Colony biofilms had a higher amount of total proteins in comparison to those formed in microtiter plates, for both biofilms ($P < 0.05$). *B. cereus* biofilms had similar polysaccharide content in either biofilms formed as colony and microtiter plates ($P > 0.05$), while the polysaccharides in *P. fluorescens* biofilms were lower in the colony system ($P > 0.05$)

   Table 3.3 depicts the killing and removal percentages with the selected antimicrobial agents for the biofilms formed in the microtiter plates. For *B. cereus* biofilms, removal was similar with ciprofloxacin and streptomycin (12-14%). Their killing efficiency was statistically similar ($P > 0.05$), even if streptomycin was the most efficient

antibiotic (40 *vs* 36%). The killing percentages of *B. cereus* biofilms caused by both QACs were approximately 50% and its removal was also similar (around 15%). *P. fluorescens* biofilms were equally removed (17-23%) and killed (about 15%) by both antibiotics. The same biofilm was easier to be killed by CTAB (26%) rather than by BAC (15%). The removal of *P. fluorescens* was similar to what was verified for *B. cereus* biofilms (about 15%). The removal and killing was significantly different between antibiotics and biocides ($P < 0.05$), which suggests that QACs are more efficient in biofilm killing than antibiotics. In general, biocides are known to perform better in the killing of biofilms, apparently due to their multi-target mode of action [70]. Between *B. cereus* and *P. fluorescens* biofilms, the removal was statistically similar in all cases ($P > 0.05$). *B. cereus* killing was higher for both QACs and antibiotics when compared to *P. fluorescens* ($P < 0.05$). This bacterium, as a Gram negative, is known to have higher tolerance to biocides [71], which is commonly explained by hindrances in penetration due to the presence of the outer membrane [15]. Between BAC and CTAB the killing percentages were not significant ($P > 0.05$).

Table 3.3 Percentage killing and removal of *B. cereus* and *P. fluorescens* biofilms. The average ± SD is presented.

	B cereus		P. fluorescens	
	Killing / (%)	Removal / (%)	Killing / (%)	Removal / (%)
BAC	46.6 ± 13	15.3 ± 2.7	15.5 ± 7.5	13.8 ± 5.4
CTAB	51.8 ± 10	15.8 ± 1.3	26.5 ± 6.9	16.0 ± 2.4
Ciprofloxacin	36.2 ± 8.3	11.8 ± 3.7	13.5 ± 2.7	22.7 ± 8.5
Streptomycin	40.0 ± 5.9	14.3 ± 3.5	15.3 ± 7.0	16.6 ± 1.3

The resistance of a biofilm is a very complex phenomenon. EPS plays an important role on antimicrobial interaction and mass transfer limitations; albeit, other phenomena can contribute to biofilm resistance [17, 72-74]. An antimicrobial agent that efficiently penetrates a biofilm does not necessarily kill the embedded cells. This means that the high penetration ability of some antimicrobial agents is not directly related with their efficiency, as proposed by the comparison between the results obtained with colony biofilms and those formed in the microtiter plates.

3.4   CONCLUSIONS

This study uses two simple biofilm formation systems (biofilm colonies and microtiter plates) to provide insights into the role played by a biofilm in the interaction with antimicrobial agents. The systems used formed biofilms with similar characteristics in terms of CFU, proteins and polysaccharides. The overall results demonstrate that the selection of a suitable antimicrobial agent, able to penetrate a biofilm and kill the bacteria, is of utmost importance when developing disinfection plans. At the same time, a diffusion test by itself does not provide enough information on the biofilm control efficiency of an antimicrobial agent. This reinforces the fact that antimicrobial resistance in biofilms is a multifactorial problem and transport limitations, although part of the problem, should not be implicated alone. Moreover, the assessment of biofilm killing and removal is important for the selection of an appropriate control strategy. Biofilm killing and removal are distinct phenomena.

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CHAPTER 4

INFLUENCE OF INTERFERING SUBSTANCES ON DISINFECTION

ABSTRACT

Standard cleaning processes may not remove all the soiling typically found in food industry, such as carbohydrates, fats, or proteins. Contaminants have a high impact in disinfection as their presence may reduce the activity of disinfectants. The influence of alginic acid, bovine serum albumin, yeast extract, and humic acids was assessed on the antimicrobial activities of benzalkonium chloride and cetyltrimethyl ammonium bromide against *Bacillus cereus* vegetative cells and *Pseudomonas fluorescens*. The bacteria (single and consortium) were exposed to surfactants (single and combined) in the absence and presence of potential disinfection interfering substances. The antimicrobial effects of the surfactants were assessed based on the bacterial respiratory activity measured by oxygen uptake rate due to glucose oxidation. The tested surfactants were efficient against both bacteria (single and consortium) with minimum bactericidal concentrations ranging from 3 to 35 mg.L^{-1}. The strongest effect was caused by humic acids that severely quenched antimicrobial action, increasing the minimum bactericidal concentration of the surfactants on *P. fluorescens* and the consortium. The inclusion of the other interfering substances resulted in mild interferences in the antibacterial activity. This study clearly demonstrates that humic acids should be considered as an antimicrobial interfering substance in the development of disinfection strategies.

4.1   INTRODUCTION

In order to prevent and control microbial proliferation in industrial settings, cleaning and disinfection plans are applied on a regular basis [1, 2]. In food processing plants, the control of microbial contamination generally involves clean-in-place (CIP) procedures which consist of running alternated cycles of detergent and disinfectant solutions with water rinses in high turbulence regimes through the plant and pipeline circuits without dismantling or opening the equipment [2-5].

Biocides are currently used in industrial processes as the most significant countermeasure to control microbial growth and proliferation [6]. Industry moved progressively towards the use of surfactants that are less toxic and more biodegradable [7]. Surfactants are classified according to the ionic physiognomies of their hydrophilic group as anionic, cationic, nonionic, and zwitterionic [6, 8]. Quaternary ammonium compounds (QACs) are cationic surfactants that are commonly used because of their hard-surface cleaning, odor removal and antimicrobial properties [9]. Besides killing bacteria, the chemical nature of QACs can cause modifications on the properties of abiotic surfaces, decreasing their tension and therefore preventing attachment of microorganisms [7]. The antimicrobial mode of action of cationic surfactants is proposed by some authors as a sequence of events: attraction by the negatively charged cell surface; adsorption to the cell wall through the hydrophobic headgroup; reaction with the lipids and proteins that compose the cytoplasmic membrane; and cell penetration and interaction with intracellular constituents [10, 11]. Thus, QACs damage the outer layers of bacteria [9], thereby promoting the release of intracellular constituents [12].

Antimicrobial efficacy tests require planning of an adequate strategy and should include all the parameters found in real settings [13]. Aspects such as the proper contact time under known water hardness and conditions of high or low soil content should be considered [14]. For an effective cleaning and disinfection plan, the choice of the disinfectant must follow specific criteria such as compatibility with the surfaces to be disinfected, economic constraints, safety in the workplace, toxicological safety, and biological degradability [15]. It should, most of all, target the type of bacteria and the type of soiling [16]. In fact, disinfectants can be seriously affected by the presence of organic matter [17].

Interfering substances have been studied in the last years and included in cleaning and disinfection plans regulated by the authorities such as the European Standard EN-1276 (1997) [18]. There are already some reports on the effects of interfering substances in disinfection. However, most of these studies only address the effects of bovine serum albumin (BSA) and water hardness [9, 14, 15, 19-21]. Aal et al. [15] evaluated the bactericidal activity of disinfectants referred in the German Veterinary Society guidelines as references for testing disinfectants used in dairy and food industries. In order to simulate the conditions found in practice, they used low fat milk as an organic load and reported the significance in choosing an appropriate disinfectant since the inclusion of a challenging substance (organic material) is important to assess the proper bactericidal activity. Bessems [14] demonstrated that a QAC tested on three microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*) had a similar killing rate in the absence of interfering substances and after the inclusion of 17 dH water hardness, a strong reduction of the killing activity was found for the Gram-negative bacteria. However, the same behavior was not verified for the other two microorganisms. Jonõ et al. [19] assessed the effect of dried yeast and human serum on the activity of benzalkonium chloride and concluded that the bactericidal activity of the QAC was inhibited by solutions of both interfering substances. The inhibition by yeast extract was more pronounced than the inhibition by human serum.

This work provides information on the influence of potential interfering substances (bovine serum albumin - BSA, alginate - ALG, yeast extract - YE, and humic acids - HA) on the antimicrobial activity of two QACs (benzalkonium chloride and cetyltrimethyl ammonium bromide) against *Bacillus cereus* and *Pseudomonas fluorescens*, as they are two major contaminants in the food industry, particularly the dairy industry, and are a known cause of produce spoilage and foodborne illnesses [2, 22-26]. Some of the interfering substances used throughout the experiments are proposed in the European Standard EN-1276 (1997) [18] as potential interfering agents in disinfection while the others are extracellular polymeric substances (EPS) from the biofilm matrix that have an important role in antimicrobial resistance [27].

4.2   MATERIALS AND METHODS

MICROORGANISMS AND CULTURE CONDITIONS

The bacteria used in this work were *Pseudomonas fluorescens* ATCC 13525T and a *Bacillus cereus* strain, isolated from a disinfectant solution and identified by 16S rRNA gene sequencing [28].

Bacterial strains were grown at a temperature of 30 ± 3 °C and pH 7, with glucose as the main carbon source. Culture medium consisted of 5 $g.L^{-1}$ glucose, 2.5 $g.L^{-1}$ peptone, and 1.25 $g.L^{-1}$ yeast extract in phosphate buffer (PB) (pH 7, 0.025 M) [29]. A bacterial suspension was prepared by inoculation of a single colony grown on solid medium into a 1 L flask containing 250 mL of sterile nutrient medium. This bacterial suspension was incubated overnight at the given temperature with agitation (120 rpm).

QACS AND INTERFERING AGENTS

The QACs used throughout the experiments were benzalkonium chloride (BAC) and cetyltrimethyl ammonium bromide (CTAB) (Sigma, Portugal) (Figure 4.1). Preliminary studies with a concentration range between 0 and 5000 $mg.L^{-1}$ were initially made. In order to ascertain the behaviour of bacteria to the QAC, the selected concentrations for further studies were 3, 5, 10, 20, and 35 $mg.L^{-1}$. The QACs were used individually and in combination (both chemicals were combined in equal volumes and concentrations).

The interfering substances used throughout the experiments were alginic acid sodium salt -ALG (Sigma, Portugal), bovine serum albumin - BSA (Sigma, Portugal), humic acids -HA (Acros organics, Fisher Chemical, Portugal), and yeast extract - YE (Merck, Portugal).
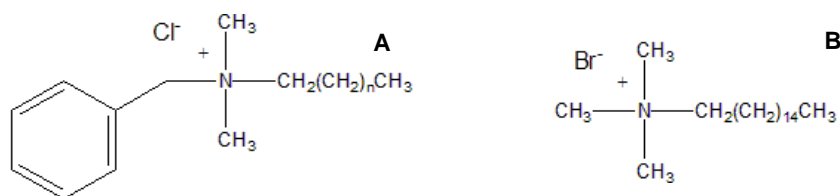
Figure 4.1 Chemical structures of benzalkonium chloride (A) and cetyltrimethyl ammonium bromide (B).

DISINFECTION PROCEDURE

After the growth period, the suspensions were centrifuged (3999 g, 5 minutes), washed two times, and resuspended in PB to a final cell density of approximately 1×10^9 cells. mL^{-1}. In the case of the consortium, both bacterial suspensions were washed two times resuspended in PB to a final cell density of approximately 1×10^9 cells.mL^{-1}, and combined in equal volumes to obtain the same cell concentrations of the single species tests. Afterwards, all bacterial suspensions were exposed to several concentrations of QAC for a period of 30 minutes [30]. The effects of the chemicals were evaluated by the assessment of the oxygen uptake rate due to glucose oxidation, according to Simões et al. [30].

To investigate the influence of interfering substances on the antimicrobial efficacy, the same procedure was followed with the addition of 300 mg.L^{-1} of BSA, ALG, YE, or HA to the bacterial suspension, simulating low concentrations of interfering substances according to the European Standard EN-1276 (1997) [18]. Three independent experiments, each with duplicate samples, were performed for each condition tested.

QACS NEUTRALIZATION

A neutralization process was performed after the disinfection procedure. The methodology was performed according to Johnston et al. [31] for a period of 10 minutes. BAC and CTAB were chemically neutralized by a sterile solution of (w/v) 0.1% peptone, 0.5% Tween 80, 0.1% sodium thiosulphate, and 0.07% lecithin dissolved in PB. All the chemicals were obtained from Sigma (Portugal). Control experiments were performed to ascertain the effects of the 10-minute exposure to the neutralization solution, and no effects were detected on the respiratory activity of *B. cereus* and *P. fluorescens*. After the neutralization step, the bacterial suspensions were centrifuged (3999 g, 5 min) and resuspended in the same volume of PB.

RESPIRATORY ACTIVITY ASSESSMENT

The respiratory activity was ascertained by measuring oxygen uptake rates in a biological oxygen monitor (Yellow Springs Instruments 5300A). Simões et al. [30] demonstrated that this procedure is more adequate and rapid than the assessment of

colony forming units to characterize the antimicrobial activity of biocides against heterotrophic aerobic bacteria [21]. Samples were placed in the temperature-controlled vessel of the biological oxygen monitor (T= 25 ± 1 °C) each containing a dissolved oxygen probe connected to a dissolved oxygen meter. Before measuring, the samples were aerated for 10 minutes to ensure oxygen saturation ([O_2] = 8.6 mg.L^{-1}). The vessel was closed, and the decrease of oxygen concentration was monitored over time. The initial linear decrease corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 12.5 µL of a 5 g.L^{-1} glucose solution was added to each vessel. The slope of the initial linear decrease in dissolved oxygen, after glucose injection, corresponds to the total respiration rate. The difference between these two rates is the oxygen uptake rate due to glucose oxidation [9].

The inactivation was calculated using metabolic activities according to the following equation:

$$\% \, Inactivation = \frac{(m_c - m_t)}{m_c} \times 100 \qquad \text{(eq. 4.1)}$$

where m_c is the metabolic activity of the control experiments (without antimicrobial exposure) and m_t is the metabolic activity of the bacterial solutions exposed to the antimicrobial. If % inactivation > 0 there was inactivation of the microorganisms whereas if % inactivation < 0 there was metabolic potentiation. The MBC for each situation was determined as the lowest concentration of QAC or QAC combination where no respiratory activity was detected [31].

STATISTICAL ANALYSIS

For each parameter tested the average and the standard deviation were calculated. The statistical significance of the results was evaluated using the Wilcoxon test (confidence level ≥ 95%), and for the MBC the independent t-test was used to investigate whether the differences between the resulting experimental values could be considered significant.

4.3   RESULTS

The antibacterial activity of BAC, CTAB, and their combination was investigated in the absence and in the presence of four selected interfering substances.

In the absence of interfering substances BAC caused the inactivation of *B. cereus* at 10 mg.L^{-1}, *P. fluorescens* at 35 mg.L^{-1}, and the consortium at 20 mg.L^{-1}. CTAB at 20 mg.L^{-1} completely inactivated *B. cereus* and at 35 mg.L^{-1} inactivated the total population of *P. fluorescens* and the consortium. The combination of both QACs was synergistic in the inactivation of *B. cereus* (total inactivation with 3 mg.L^{-1}) and indifferent for *P. fluorescens* (35 mg.L^{-1}) and the bacterial consortium (35 mg.L^{-1}). The inclusion of the selected interfering substances influenced the antimicrobial activity of the QACs to some extent (Figures 4.2-4.4). The inactivation of *B. cereus* (Figure 4.1) was not affected by the presence of any interfering substances ($P > 0.05$), except with HA. This interfering substance decreased the antimicrobial efficacy of BAC and the combination of QACs. The antimicrobial action of the QACs against *P. fluorescens* (Figure 4.3) was not significantly influenced by the presence of most potential interfering substances (P > 0.05), except for HA where interference was observed ($P < 0.05$). The antimicrobial activity of the QACs against the bacterial consortium (Figure 4.3) was affected by the presence of interfering substances. ALG and HA reduced significantly the activity of BAC ($P < 0.05$). HA reduced significantly the activity of CTAB at higher concentrations ($P < 0.05$). BSA and YE resulted in a significant reduction of the activity of the combination of QACs ($P < 0.05$).

Linear correlations were determined to assess the relationship between QAC concentrations and the inactivation data. The effect of increasing QAC concentration on bacterial inactivation shows that there are strong linear correlations (R > 0.850) for the control assays, with the exception of *B. cereus* (this bacterium was inactivated with low QAC concentrations). When interfering substances were added, the correlations decreased. The most extreme cases are the treatments with CTAB to *P. fluorescens* with ALG as an interfering substance (R = 0.771) and the bacterial consortium in the presence of YE (R = 0.738). Likewise, this decrease of linear correlation factors was found for *P. fluorescens* and for the consortium exposed to HA where the lowest correlation factor was 0.153, which was obtained for *P. fluorescens* treated with CTAB.
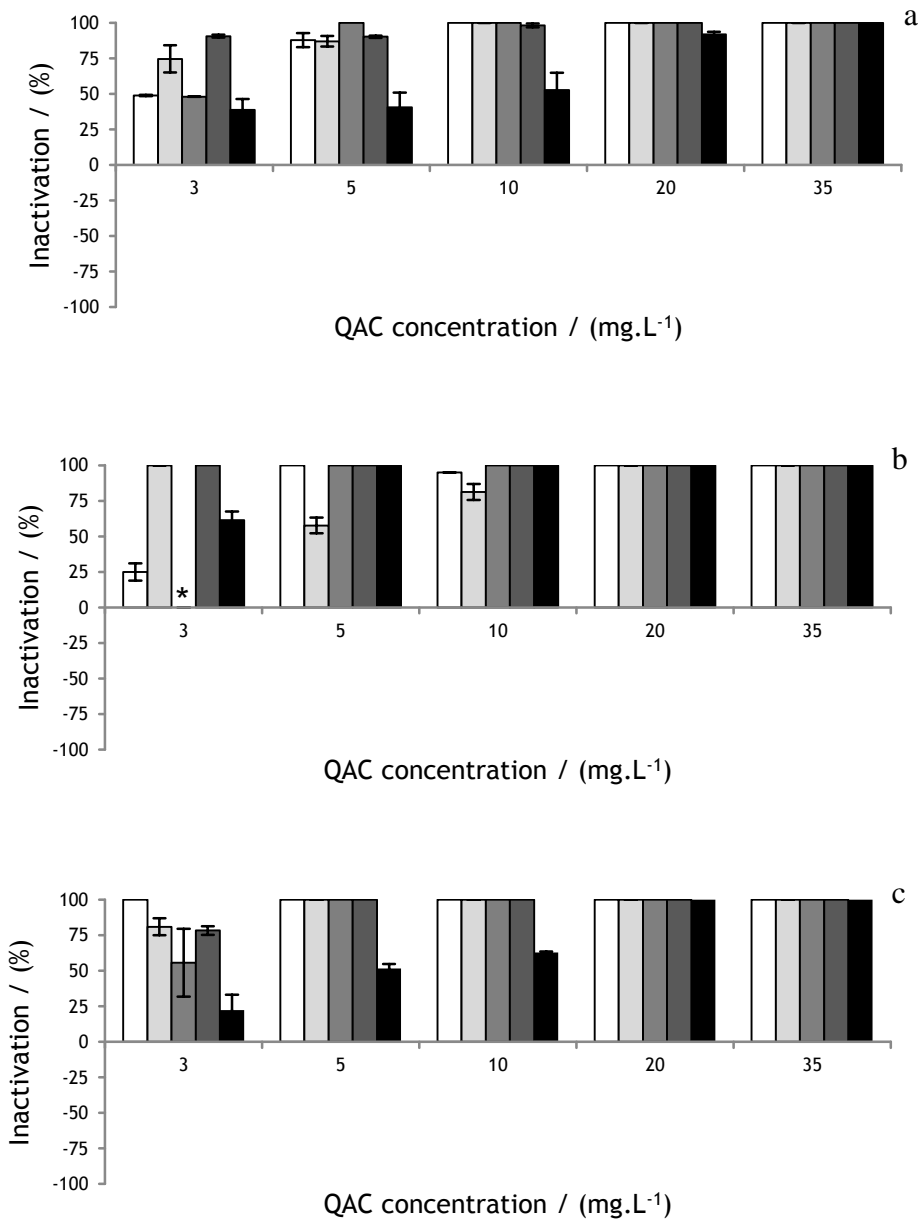
Figure 4.2 Inactivation of *B. cereus* by BAC (a), CTAB (b), and QAC combination (c), where solid white box is the control (no interfering substances), light grey box corresponds to BSA, grey box, is ALG dark grey box YE, and black box HA. ∗ means no inactivation. Average values ± standard deviation for at least three replicates are illustrated.
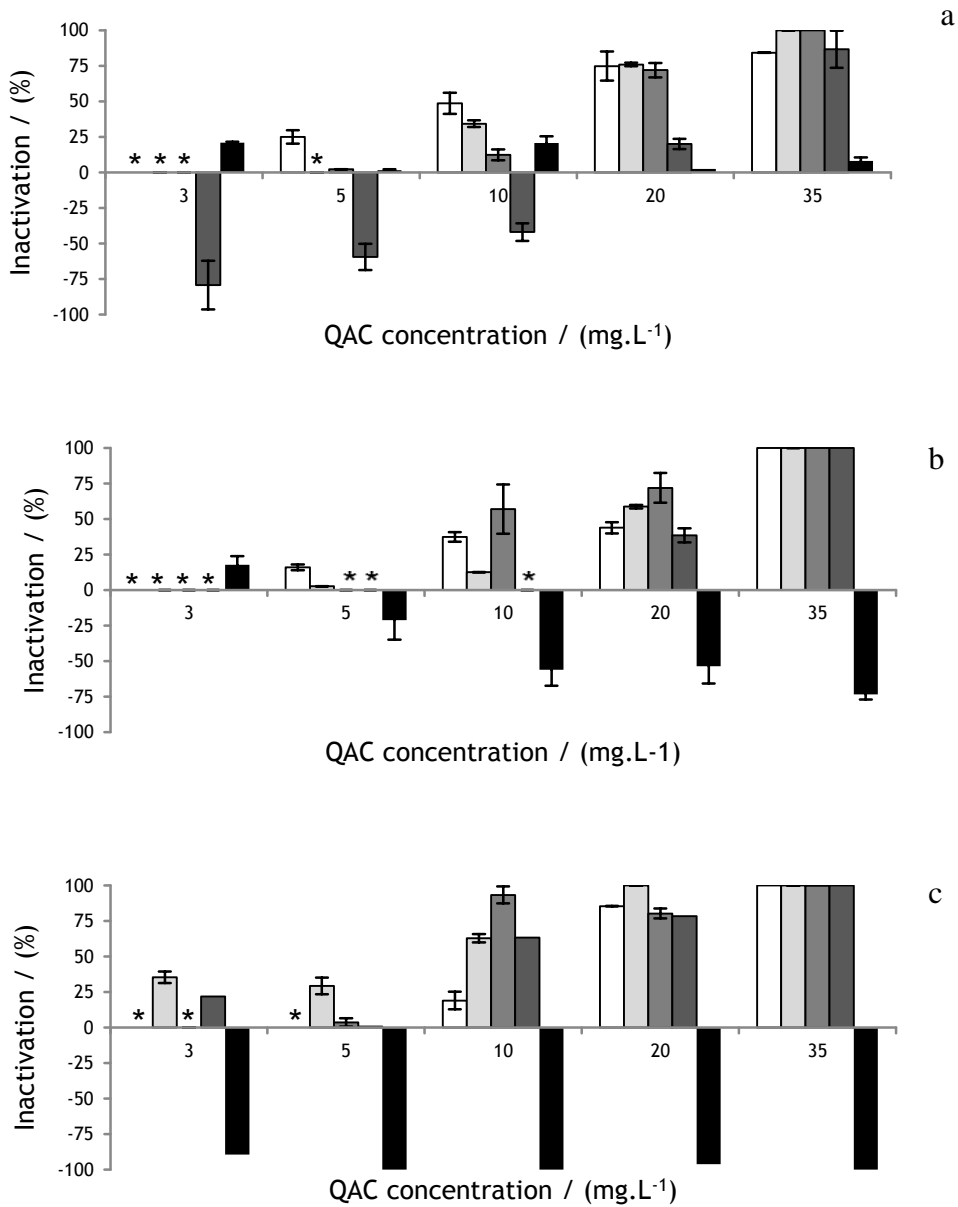
Figure 4.3 Inactivation *of P. fluorescens* by BAC (a), CTAB (b), and QAC combination (c), where solid white box is the control (no interfering substances), light grey box corresponds to BSA, grey box is ALG, dark grey box is YE, and black box is HA. * means no inactivation. Values below zero are indication that the metabolic activity increased in comparison with the control experiment. Average values ± standard deviation for at least three replicates are illustrated.
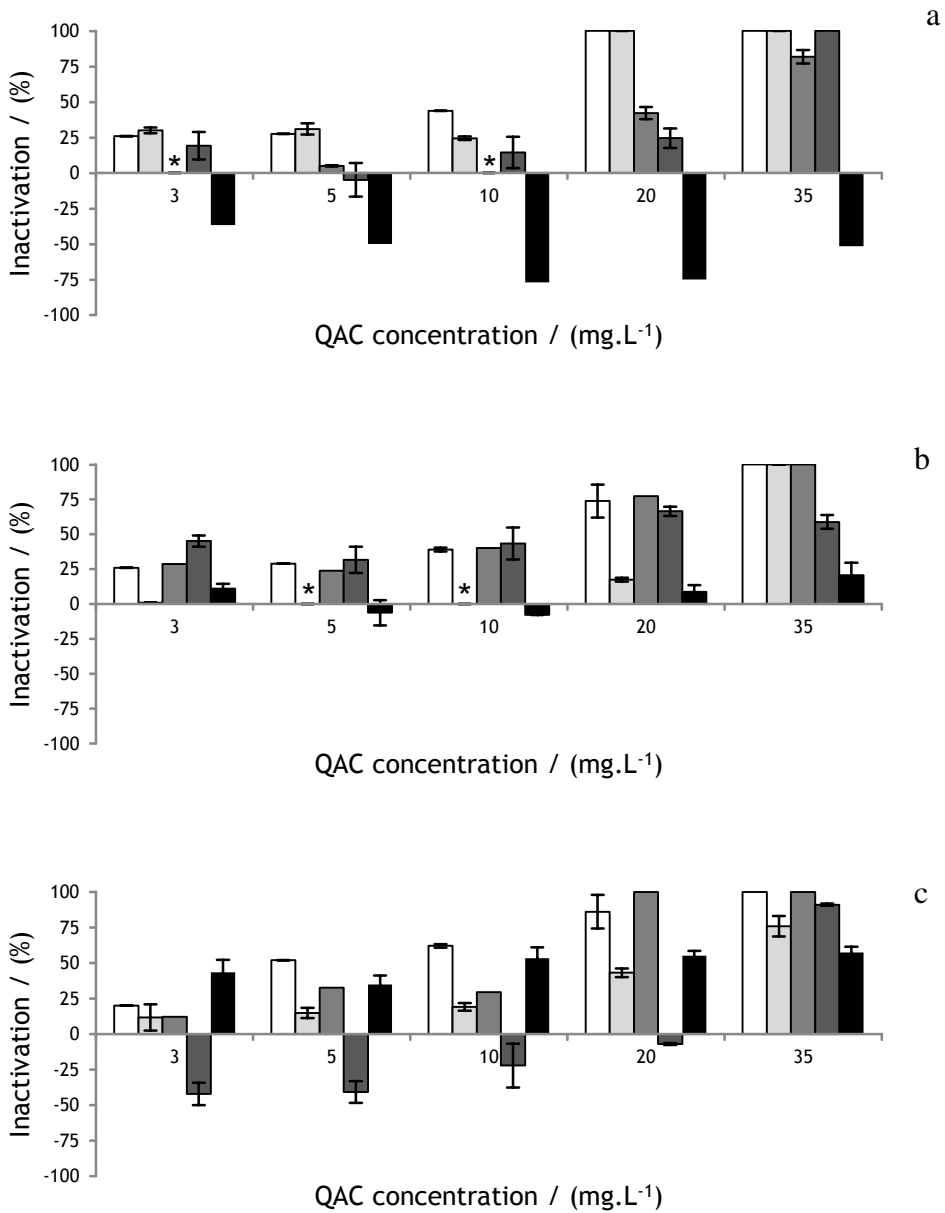
Figure 4.4 Inactivation of the bacterial consortium by BAC (a), CTAB (b), and QAC combination (c), where solid white box is the control (no interfering substances), light grey box corresponds to BSA, grey box is ALG, dark grey box is YE, and black box is HA. ∗ means no inactivation. Values below zero are indication that the metabolic activity increased in comparison with the control experiment. Average values ± standard deviation for at least three replicates are illustrated.

The results also demonstrate the occurrence of metabolic potentiation (inactivation below 0 %). This phenomenon only happened when the QACs were used on *P. fluorescens* and the bacterial consortium in the presence of YE and HA. The most significant cases of oxygen uptake rate increase were verified for *P. fluorescens* exposed to BAC (5 to 35 mg.L^{-1}) and CTAB (3 to 35 mg.L^{-1}) in the presence of HA and combination of QACs (3 to 10 mg.L^{-1}) in the presence of YE. A similar metabolic behavior was found for the bacterial consortium exposed to BAC (3 to 35 mg.L^{-1}) and CTAB (5 and 10 mg.L^{-1}) for HA and QAC combination (3 to 20 mg.L^{-1}) with YE.

The MBC values for the different conditions tested (single and combined QACs, in the absence and presence of potential disinfection interfering substances) are shown in Table 4.1.

Table 4.1 Minimum bactericidal concentration for *P. fluorescens, B. cereus* and the consortium with and without interfering substances.

		MBC (mg.L^{-1})		
		BAC	CTAB	QAC combination
Control	*B. cereus*	10	20	3
	P. fluorescens	35	35	35
	Consortium	20	35	35
BSA	*B. cereus*	10	20	5
	P. fluorescens	35	20	35
	Consortium	20	35	>35
ALG	*B. cereus*	5	5	5
	P. fluorescens	35	35	35
	Consortium	>35	35	20
YE	*B. cereus*	20	3	5
	P. fluorescens	35	35	>35
	Consortium	35	>35	>35
HA	*B. cereus*	35	5	20
	P. fluorescens	>35	>35	>35
	Consortium	>35	>35	>35

The presence of BSA increased the MBC of the combination of QACs for *B. cereus* (3 to 5 mg.L^{-1}) and the consortium. ALG increased the MBC of BAC for the consortium (20 to over 35 mg.L^{-1}) and QACs combination (3 to 5 mg.L^{-1}) for *B. cereus*. YE increased the MBC of BAC for *B. cereus* (10 to 20 mg.L^{-1}) and QAC combination (3 to 5 mg.L^{-1}). *P. fluorescens* MBC increased with the inclusion of YE with the combination of QACs. The MBC values for the consortium of cells increased in the presence of YE (BAC - 20 to 35 mg.L^{-1}, CTAB - 35 to over 35 mg.L^{-1}, and QAC combination - 35 to over 35 mg.L^{-1}). HA increased the MBC for all the scenarios, except of CTAB when applied to *B. cereus* (in this situation the MBC was reduced). The MBC was reduced in other situations such as, for *B. cereus*, in the presence of ALG when using BAC and CTAB (10 to 5 mg.L^{-1} and 20 to 5 mg.L^{-1}, respectively) and in the presence of YE when using CTAB (20 to 3 mg.L^{-1}). *P. fluorescens* inactivation by CTAB was reduced by BSA (35 to 20 mg.L^{-1}). ALG also reduced the antimicrobial activity of the combination of QACs against the bacterial consortium (35 to 20 mg.L^{-1}).

4.4   DISCUSSION

In disinfection practices, the environmental characteristics can influence the antimicrobial activity of biocides [32]. It is assumed that the organic material can potentially interfere with the antimicrobial agents by chemical and/or ionic interactions [15, 33]. Therefore, it is necessary to know the role of each potential interfering substance in the antimicrobial activity in order to develop effective disinfection strategies. The interfering substances tested are commonly found as residuals in the food industry (from food products and from microbial contaminants, biofilms) [18, 27].

In this study, higher inactivation rates were verified for *B. cereus* in comparison to *P. fluorescens* at the same QAC concentration. The inactivation profiles of the cell consortium are similar to *P. fluorescens*. In fact, when *B. cereus* and *P. fluorescens* are combined in a 1:1 bacterial suspension, it is expected that the first is more affected than the second. *B. cereus* is more susceptible due to the fact that it is a Gram positive bacterium that lacks an outer membrane, which typically provides increased protection to Gram negative bacteria. This fact is corroborated by previous reports which stated that Gram positive bacteria are more susceptible to cationic surfactants than Gram negative bacteria [34, 35].

BSA was already studied as an interfering substance in disinfection practices [9, 14, 19-21, 36]. The negative effect of BSA on the action of biocides against *P. fluorescens* was demonstrated by Simões et al. [9, 21]. *P. fluorescens* treatment with CTAB with the addition of 3 g.L^{-1} of BSA resulted in a 10-fold increase on the MBC of this QAC [9, 21]. In the present study, low BSA concentrations decreased the antimicrobial activity of the QACs. The efficacy of the combination of QACs against *B. cereus* and the cell consortium was also reduced. This effect of BSA as an antimicrobial quencher is apparently due to the strong ability of QACs to react with proteins [21]. Proteins can precipitate in the form of their anions. In this way, the negative-charged protein ions will cling to the positively charged molecules of the cationic compounds [37]. CTAB is a biocide that targets the membrane and has a strong affinity for proteins [21]. BAC is composed of a positively charged hydrophobic headgroup which clings to opposite charged surfaces [8, 37]. Jonõ et al. [19] studied the effect of the alkyl chain of BAC binding to BSA and dried yeast. Their conclusions were that BAC is often inactivated by organic matter, either by adsorption to the bacterial surface or by adsorption to the organic matter in general. These authors also suggested that the reduction in the activity of BAC was probably related to more than one physical property of the compounds like the chain length (longer chains result in more adsorption to the bacterial surface).

ALG is a common constituent of the extracellular polymeric substances of the biofilm matrix [38-40]. A function frequently attributed to EPS is their general protective effect on biofilm microorganisms against adverse conditions. The EPS matrix delays or prevents antimicrobials from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides [32, 41]. In this study, ALG either potentiated or hindered the antimicrobial activity of the selected QACs. The presence of this interfering substance was not obvious on the inactivation of *P. fluorescens*. On the other hand, the inactivation of *B. cereus* by BAC and CTAB and the consortium by the combination of QACs was easier in the presence of this interfering substance. The bacterial consortium treatments with BAC and *B. cereus* with the combination of QACs were hampered by the presence of ALG. Davies et al. [42, 43] found that the production of ALG was triggered by membrane perturbation induced by ethanol stress, nitrogen limitation, attachment to surfaces, or even high oxygen tension. This substance is suggested as one of the main biofilm resistance vectors either by reacting with the antimicrobials or by

hindering antimicrobials diffusion to the cells [44].The antimicrobial interference caused by ALG is apparently due to electrostatic interactions between the anionic ALG and the cationic-selected QACs [45].

The presence of YE as interfering substance resulted in three different outcomes on the antimicrobial activity of the QACs: (1) no effect/indifference, (2) the respiratory activity reduced, and (3) the respiratory activity potentiated. This interfering substance worked mainly as a hinderer of the antimicrobial activity by increasing the MBC of *B. cereus* in all cases except for CTAB, of *P. fluorescens* with the combination of QACs, and of the consortium of cells with CTAB and the combination of QACs. These results are in accordance with the available studies. YE is listed in the European Standard EN-1276 (1997) as an interfering substance native to the brewery industry [18]. The constituents of YE are very similar to the components of the bacterial cells, thus, it is expected that the antimicrobial agents that target the bacterial cells are also drawn to YE. In a similar study by Jonõ et al. [19] it was shown that the presence of dried yeast decreased the biocidal effectiveness of BAC.

Humic substances are found ubiquitously in the environment and can be found in the biofilm matrix [2, 46]. HA reduced the antimicrobial activity of the QACs in most of the cases, although in some cases it promoted the respiratory activity (potentiation). The presence of these compounds had the strongest effect compared to the remaining interfering substances. Like ALG, HA are known to be a part of the EPS composition [47]. Atay et al. [8] studied the sorption mechanisms of anionic and cationic surfactants to natural soils concluding that the dominant sorption mechanism of surfactants to clay is cation exchange. Ishiguro et al. [48] reported that cationic surfactants bind strongly to humic substances. Koopal et al. [49] also verified the formation of complexes HA-cationic surfactant. These observations are consistent with the present results.

Respiratory activity potentiation was verified with the addition of HA to *P. fluorescens,* and YE to the bacterial consortium. It is known that HA participates in cellular metabolism processes such as growth, respiration, photosynthesis, and nitrogen fixation [50]. On the other hand, HA were proposed to replace synthetic surfactants such as SDS, Tween 80, and Triton X-100 in industrial applications such as textile dying or washing [51]. It is therefore possible that the inclusion of humic substances in a solution of QACs may interfere with the chemical characteristics of the solution. The resultant mixture, with an apparent reduced antimicrobial efficacy, seems to potentiate the respiratory activity of the bacteria, particularly of *P. fluorescens*. As

QACs are membrane active agents, their use at sub-lethal concentrations could improve membrane permeability and consequently the nutrient influx, without compromising the bacterial viability. Also, there is the hypothesis that the potentially interfering agents could be used as nutrients. In fact, it was found that the growth rates of anaerobic and aerobic microorganisms increased when humic substances were added, which stimulated enzyme activity [52, 53]. In a similar way, YE is a nitrogen source widely used as a component of growth media [54]. HA are likely to be used for growth in the same way as YE, these might be broken down to smaller molecules that can be used by cells as a carbon [55] or nitrogen sources [51].

The antimicrobial activity of the tested QACs was enhanced in some cases, where the interfering substances were present. This is an unexpected result due to the recognized and observed potential of ALG, BSA, HA, and YE to interfere with disinfection. This effect is probably due to the low concentration of interfering substances tested that caused both respiratory activity reduction and potentiation. Cases of antimicrobial enhancement are widely known. Ethylenediamine tetraacetate (EDTA) was reported as early as 1965 to increase the biocidal effects of BAC and chlorhexidine diacetate on *Pseudomonas aeruginosa* [56]. Sagoo et al. [57] reported that chitosan (a polysaccharide) potentiated the antimicrobial action of sodium benzoate on spoilage yeasts. In dairy plants, disinfection is potentiated by prewashes with alkali or enzyme-based cleaning agents [58]. The antimicrobial potentiation of the QACs occurred in some cases. Most of these cases were observed for *B. cereus* (four occurrences), one was observed for *P. fluorescens*, and another one was observed for the consortium of cells. The MBC was improved by more than 50% in the cases of *B. cereus* and less than 30% for *P. fluorescens* and the consortium of cells. To our knowledge there are no reported cases of antimicrobial agents potentiation by BSA, YE, or ALG. Concerning the effects of HA, these molecules are reported to have detergent properties [51]. Although the exact chemical structure of HA has not yet been determined, HA could be chemically similar to the tested QACs, presenting a positive hydrophilic head and a hydrophobic tail. With this structure HA could act as detergents in conditions such as those observed in the treatment of *B. cereus* with CTAB [51].

The present work shows that increasing QACs concentrations lead to an increase in antimicrobial effectiveness. This is valid mainly when the QACs were applied in the absence of interfering substances. This means that disinfection was concentration dependent, as found for most of the antimicrobial chemicals [59]. However, the linear

dependency of inactivation *versus* concentration is not verified for most of the tests where interfering substances were added. This result evidences that the mathematical modelling of disinfection strategies requires a case-to-case analysis when interfering substances are present.

4.5   CONCLUSIONS

The overall results demonstrate that a disinfection process in the presence of the selected interfering substances can reduce the effectiveness of BAC, CTAB, and their combination. The bacteria were inactivated equally by all QACs, although in the absence of interfering substances CTAB was the most efficient solution. *P. fluorescens* was the bacterium with the highest resistance to inactivation, followed by the bacterial consortium. The tested interfering substances, referred in the European Standard EN1276 (BSA and YE), and known EPS constituents related with biofilm resistance (ALG) resulted in mild interferences on the activity of the QACs. HA were the interfering substance that resulted in the most severe effect by reducing the activity of QACs, causing, in some circumstances, significant respiratory activity potentiation. This interfering substance should, therefore, be considered when developing disinfection protocols.

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CHAPTER 5

HYDRODYNAMIC CONDITIONS AND BIOFILM DEVELOPMENT

This chapter was submitted as:

ABSTRACT

The characteristics of *Pseudomonas fluorescens* biofilms formed under three different linear flow velocities (u = 0.1, 0.4 and 0.8 m.s^{-1}) were studied. A flow cell reactor system was used to form biofilms. These biofilms were characterized in terms of thickness, morphological structure, mass, cell density, outer membrane proteins expression, and matrix and total proteins and polysaccharide content. The external mass transfer coefficients were also calculated.

Biofilms developed at the higher velocities (u = 0.4 and 0.8 m.s^{-1}) had similar characteristics, but different from those developed at the lower flow rate. High flow velocities formed thinner biofilms with higher cell densities, and higher contents of matrix proteins and polysaccharides. The external mass transfer coefficients suggest mass transfer limitations from the bulk fluid for the lowest velocity. Scanning electron microscopy images show cell-surface and cell-cell attachment structures appearing more frequently in biofilms formed at the two higher velocities. No major differences were found in the outer membrane proteins expression of biofilm cells, regardless of the linear flow velocity under which they were formed. The overall results show the effect of the hydrodynamic conditions under which biofilms were formed on selected macromolecular characteristics, demonstrating that higher flow velocities originate more complex and dense biofilms. However, cellular aspects as the outer membrane proteins expression are not affected by the flow velocity.

Understanding biofilm formation and corresponding characteristics allows the manipulation of hydrodynamic conditions as a control parameter in the improvement of biofilm control strategies in many engineered systems.

5.1   INTRODUCTION

Bacterial attachment to surfaces and the consequent biofilm formation is a well-recognized phenomenon in diverse areas such as the food and biomedical fields [1-3]. Especially in food industry, bacterial spoilage is a major concern with both economic and public health consequences. Therefore, efforts must be directed for efficient industrial equipment design and the development of effective cleaning and disinfection strategies [1, 4, 5].

Biofilms can be described as dense microbial communities associated to surfaces, which are highly hydrated clusters of bacterial cells surrounded by a matrix of extracellular polymeric substances (EPS) [6-8]. EPS, a result of bacterial secretion, cell lysis and hydrolysis, are constituted by biopolymers such as polysaccharides, proteins, extracellular DNA and lipids. These substances are responsible for the protection of bacteria from environmental stress, dehydration and chemical exposure and mediate bacterial adhesion to surfaces [6, 9-11]. In addition, EPS are essential for biofilm stability and architecture since their composition, structure and properties influences oxygen penetration and substrate absorption and transport [9]. Moreover, the biofilm structure depends on the microbial constituents and environmental factors like composition, pH and temperature of the contact fluid, surface properties and hydrodynamic conditions [12-14].

The hydrodynamic effects can induce a detachment force as a consequence of gas or liquid flow and particle-particle collision [10, 15]. Therefore, shear force has been considered a pivotal factor in biofilm formation, since it leads to equilibrium between biofilm thickness and density resulting in a steady state structure. Several authors found that higher shear force caused thinner and denser biofilms [10, 14, 16, 17]. Considering that the biofilm structure is influenced by the existing hydrodynamic conditions, the latter also influences the efficacy of substrate diffusion and the ecological selection within the biofilm [10, 14, 16, 18]. In fact, the three-dimensional biofilm structure has a physical impact on internal mass diffusivity, since it is dependent on the biofilm density [19] and tortuosity [20]. On one hand, substrate diffusion through biofilms could be enhanced by high turbulence which tends to produce thinner biofilms, but on the other hand it could be reduced by a shear-compacted biofilm structure [10]. A consequence of the hydrodynamic conditions is also the overproduction of EPS under high shear stress [10]. Thus, the diffusivity of a substance into the biofilm would be a result of

internal and external mass transfer effects [10]. In addition, the overproduction of EPS, especially polysaccharides, is useful in initial cell adhesion [10, 21]. Understanding the relationship between biofilm structure and function and also the factors that physically shape biofilms is important to the use and control of biofilms in industrial and biomedical fields [14, 22]. This study provides insights on macromolecular aspects of biofilms formed under three different linear flow velocities.

5.2   MATERIALS AND METHODS

MICROORGANISM AND CULTURE CONDITIONS

The bacterium used in this work was *Pseudomonas fluorescens* ATCC 13525^T. This bacterium is ubiquitous in industrial settings and has a high ability to form biofilms [23]. This strain was grown at 30 ± 3 °C, pH 7, with glucose as the main carbon source. Culture media consisted in 5 g.L^{-1} glucose, 2.5 g.L^{-1} peptone and 1.25 g.L^{-1} yeast extract, in phosphate buffer (PB), pH 7, 25 mM [24]. Bacterial suspensions were prepared by gently removing a small portion of bacteria from solid medium (agar at 10%), and diluting it in a 1 L flask containing 250 mL of sterile nutrient medium. This bacterial suspension was incubated overnight (16 h) with agitation (120 rpm). All medium components were purchased from Merck (VWR, Portugal).

BIOFILM FORMATION IN A FLOW CELL SYSTEM

The flow cell system used consisted of a 3.5 L recirculating bioreactor, two vertical perspex flow cells operating in parallel, one 0.5 L bioreactor, one peristaltic and two centrifuge pumps (Figure 5.1). The cross-section of the flow cells is semi-circular with diameter (d) of 2 cm. *P. fluorescens* was used to inoculate the smaller bioreactor (Bioreactor I), containing the culture medium defined previously, that operated continuously, dripping into the larger bioreactor (Bioreactor II) at a flow rate of 10 mL.h^{-1}. This larger bioreactor was fed with a medium that consisted of 0.05 g.L^{-1} glucose, 0.025 g.L^{-1} peptone, and 0.0125 g.L^{-1} yeast extract in PB (pH 7, 25 mM), at a flow rate of 0.833 L.h^{-1}. The dilution rate applied ensured that biofilm formation predominated over planktonic growth [25]. The flow cells were designed so that stainless steel coupons (1 × 2 cm) could be glued into structures to be inserted in specific slots of the flow cells. The coupons were fitted flush with the rest of the surface.

In this way, biofilm sampling was facilitated. The bacterial suspension from the larger bioreactor was allowed to recirculate in the flow cells, in order to form biofilms on the stainless steel (AISI 316) coupons at linear flow velocities (u) of 0.1, 0.4 and 0.8 m.s^{-1}.
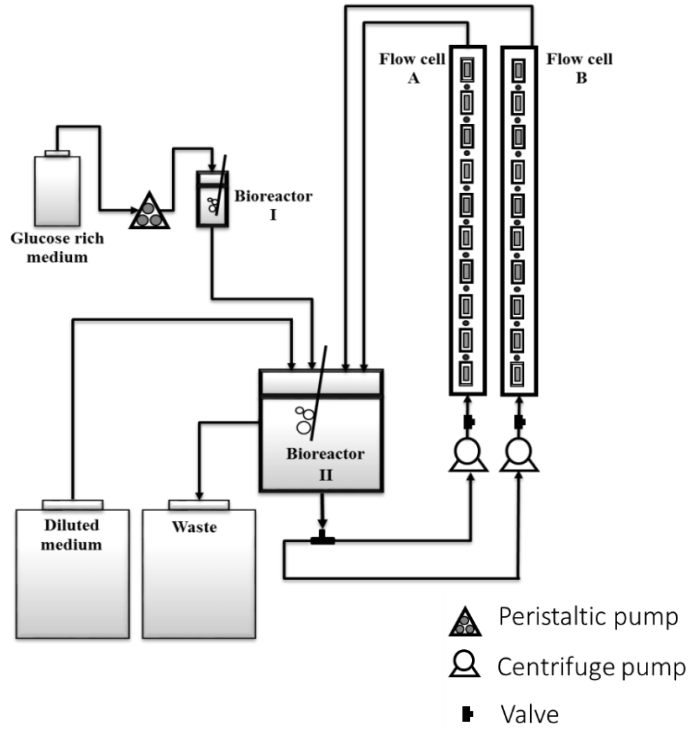
Figure 5.1 Depiction of the flow cell system used to develop biofilms at different linear flow velocities.

The linear velocity was calculated as a function of the duct design, using the hydraulic equivalent diameter (D_h) and the flow rate (Q):

$$D_h = 4 \times \frac{flow\ area}{wet\ perimeter} \quad \text{(eq. 5.1)}$$

For the semicircular duct [26]:

$$D_h = 4 \times \frac{\frac{\pi \times d^2}{8}}{\frac{\pi d}{2} + d} \quad \text{(eq. 5.2)}$$

where d is the semicircular duct diameter. The linear flow velocity was calculated as:

$$u = \frac{4 \times Q}{\pi \times (D_h)^2} \quad \text{(eq. 5.3)}$$

where u is the linear flow velocity (m.s^{-1}), Q is the flow rate (m^3.s^{-1}) and D_h is the hydraulic equivalent diameter (m). Biofilms were allowed to grow for 7 days to ensure

steady-state cell density and mass [27]. Two parallel similar flow cells were used simultaneously. Reynolds numbers were 1000 (u = 0.1 m.s^{-1}), 4000 (u = 0.4 m.s^{-1}) and 8000 (u = 0.8 m.s^{-1}).

BIOFILM SAMPLING AND ANALYSIS

The biofilms of *P. fluorescens* were characterized in terms of mass, thickness, cell density, total and extracellular proteins and polysaccharides.

The coupons were removed from the flow cell reactor and their thickness was immediately assessed, using a needle connected to a digital micrometer (VS-30H, Mitsubishi Kasei Corporation), as decribed by Teodósio et al. [28].

Afterwards, the biofilms that covered the coupons were completely scraped off, using a sterile scalpel and resuspended in extraction buffer (EB) (2 mM Na$_3$PO$_4$.12H$_2$O, 2 mM Na$_2$HPO$_4$.H$_2$O, 9 mM NaCl and 1 mM KCl) to assess biofilm mass, cell density, total and extracellular proteins and polysaccharides.

The dry biofilm mass accumulated on the slides was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions according to the Standard Methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF]), method number 2540 A–D [29]. According to this method the TVS assessed at 550 ± 5 °C in a furnace (Lenton thermal designs) for 2 h are equivalent to the amount of biological mass (cells and EPS). The dry biofilm mass accumulated was expressed in terms of biofilm mass *per* slide surface area (mg.cm^{-2}).

The biofilm number of cultivable cells was assessed in terms of colony forming units (CFU) in Plate Count Agar (Merck, Portugal), according to Ferreira et al. [30].

Biofilm extracellular proteins and polysaccharides were extracted from the cells suspension in EB using a Dowex Marathon® resin, C sodium form, 20-50 mesh (Sigma, Portugal), using the method described by Frølund et al. [31]. The extraction of proteins and polysaccharides took place at 4°C for 4 h, at 400 rpm. The extracellular components (present in the supernatant) were separated from the cells *via* centrifugation (3999 *g*, 5 min). The total (biofilm suspension before EPS extraction) and extracellular biofilm proteins were determined using the Lowry modified method (Sigma), with bovine serum albumin as standard. The procedure is essentially the Lowry et al. [32] method as modified by Peterson [33]. The total (biofilm suspension before EPS extraction) and

extracellular polysaccharides were quantified through the phenol-sulphuric acid method of Dubois et al. [34], using glucose as standard.

OUTER MEMBRANE PROTEINS EXTRACTION

The outer membrane proteins (OMP) were isolated according to the method described by Winder et al. [35]. Sessile cells were harvested by centrifugation (3999 g, 5 min, 4 °C). The pellet was suspended in 25 mM Tris and 1 mM $MgCl_2$ buffer (pH 7.4). The bacterial suspension was sonicated for 2 min, 50% power (Bandelin generator with a Microtip MS 72 probe) on ice to promote cell lysis. After sonication, the solution was centrifuged (7000 g, 10 min, 4°C) in order to remove non-lysed cells. The supernatant was collected and sarcosine (Sigma) was added to a final concentration of 2% (w/v), in order to solubilize the OMP. This solution was left on ice for 20 min. The solution was then centrifuged (13000 g, 1 h, 4 °C) to recover the OMP. The pellet containing the OMP was resuspended in 25 mM Tris buffer (pH 7.4) and stored at -20 °C until needed.

SDS-PAGE

The biofilm OMP were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as reported by Laemmli [36], using a 12% (w/v) acrylamide gel. The proteins content of each sample was standardized to 240 ± 10 µg.ml^{-1} for each sample. Electrophoresis was performed at a constant current of 170 mV. After electrophoresis, the proteins were stained with Coomassie blue for protein profile detection [37].

SCANNING ELECTRON MICROSCOPY

Twelve stainless steel slides covered with biofilms (four for each linear flow velocity) were observed by scanning electron microscopy (SEM). Prior to SEM observations, biofilm samples were fixed with 3% (w/v) glutaraldehyde in cacodylate buffer pH 7.2 [38] for 10 min and exposed to an ethanol dehydration series of 50, 60, 70, 80, 90 and twice 100% (v/v) ethanol, followed by a chemical dehydration series of 100% ethanol + hexamethyldisilazane (HMDS, Ted Pella, USA) at 50, 60, 70, 80, 90 and 2 × 100% (v/v) HMDS [39], 5 min for each concentration. The coupons were then air-dried for 1 day in a desiccator. Each coupon was sputter-coated with a palladium-gold thin film [23] using

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the SPI Module Sputter Coater equipment for, 90 s at 15 mA. The biofilms were analysed using a SEM/EDS (FEI Quanta 400FEG ESEM/EDAX Genesis X4M) under high-vacuum mode, at 10 kV. SEM observations were documented through the acquisition of, at least, 20 representative microphotographs.

DETERMINATION OF NUTRIENT AND CELL LOAD

The cell and nutrient loads were calculated as the number of cells, or the glucose mass *per* cross section area in a time unit. The cell number was calculated for Bioreactor II, which contained planktonic cells. The nutrient load was considered as the glucose content of the medium fed to bioreactor II.

DETERMINATION OF THE SHEAR STRESS

The shear stress was calculated using the dimensionless Darcy friction factor (f) obtained from the work of Teodósio et al. [40] applied to the following equation:

$$f = \frac{4 \cdot \tau_w}{\rho \cdot u^2 / 2} \quad \text{(eq. 5.6)}$$

where τ_w is the wall shear stress (Pa), ρ is the density of water at 25 °C (Kg.m^{-3}) and u is the fluid velocity (m.s^{-1}).

DETERMINATION OF THE MASS TRANSFER COEFFICIENTS

The external mass transfer coefficient, k_m (m.s^{-1}) was obtained using the mathematical model described by Moreira et al. [41]. The correlation uses the Sherwood (Sh) number which is a function of the Reynolds (Re) and Schmidt (Sc) numbers (for $2100 < Re < 3500$ and $0.6 < Sc < 3000$). For tubes with a fully developed concentration profile in laminar flow, $Sh = 3.66$:

$$k_m = \frac{Sh \cdot D}{D_h} \quad \text{(eq. 5.7)}$$

where D is the molecular diffusivity of glucose (m^2.s^{-1}), the growth-limiting nutrient in the medium and D_h is the hydraulic diameter of the flow channel (m) [42]. For turbulent flow in tubes:

$$Sh = 0.023 \cdot Re^{0.83} \cdot Sc^{1/3} \quad \text{(eq. 5.8)}$$

$$Sc = \frac{\mu}{\rho \cdot D} \quad \text{(eq. 5.9)}$$

$$Re = \frac{D_h u \rho}{\mu} \text{ (eq. 5.10)}$$

where µ is the viscosity of the water at 25 °C (kg.m^{-1}.s^{-1}).

STATISTICAL ANALYSIS

The data were analyzed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). The mean and standard deviation (SD) within samples were calculated in all cases. The experiments were replicated at least 3 times. The statistical significance of the results was evaluated using the *t*-test. Statistical calculations were based on confidence level equal to or higher than 95% ($P \leq 0.05$ was considered statistically significant).

5.3   RESULTS

The flow cell reactor was operated at three different flow velocities u = 0.1, 0.4 and 0.8 m.s^{-1}. The biofilms developed under each different condition were characterized in terms of thickness, dry and wet mass, cell density, matrix and total proteins and polysaccharides (Table 5.1). The influence of flow conditions, on biofilm superficial structure and morphology was assessed by SEM (Figure 5.3). Furthermore, the OMP expression was analyzed by SDS-PAGE electrophoresis (Figure 5.4). The hydrodynamic and external mass transfer coefficients were also calculated (Table 5.2).
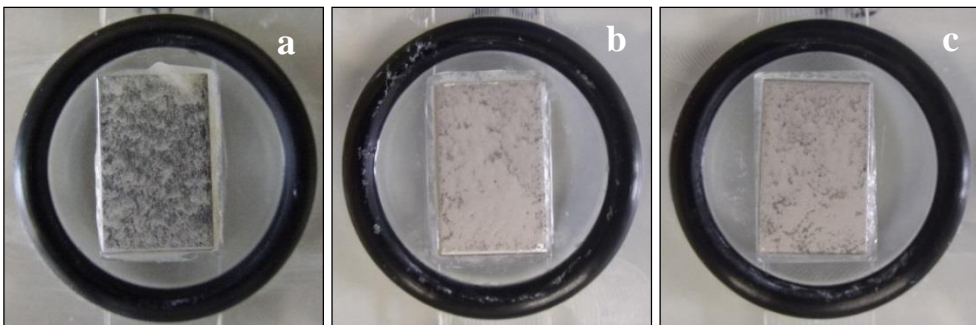
Figure 5.2 Photographs of the stainless steel coupons with 7 days old biofilms grown at (a) u = 0.1 m.s^{-1}, (b) u = 0.4 m.s^{-1} and (c) u = 0.8 m.s^{-1}.

Figure 5.2 demonstrates the morphological differences of biofilms developed on the coupons induced by different flow conditions. Biofilms developed at the lowest linear velocity (u = 0.1 m.s^{-1}) showed an apparent partial coverage of the stainless steel

surface (Figure 5.2a). Biofilms formed at u = 0.4 and 0.8 m.s^{-1} (Figures 5.2b and 5.2c) also showed a patchy appearance, but they appeared more homogeneous than those formed under low hydrodynamic stress.

Table 5.1 Characterization of *P. fluorescens* biofilms grown at different linear flow velocities.

Linear flow velocity (u) (m.s^{-1})		0.1	0.4	0.8
Thickness (mm)		0.213 ± 0.05	0.207 ± 0.06	0.178 ± 0.02
Biofilm mass (mg.cm^{-2})	Dry	0.297 ± 0.00	0.262 ± 0.04	0.269 ± 0.07
	Wet	33.6 ± 6.00	30.0 ± 4.56	30.6 ± 3.94
Log cell density (CFU.g$_{biofilm}^{-1}$)		8.11 ± 0.68	12.2 ± 0.63	11.5 ± 0.12
Matrix (mg.g$_{biofilm}^{-1}$)	Proteins	104 ± 15.2	125 ± 4.23	211 ± 11.5
	Polysaccharides	88.0 ± 20.1	135 ± 6.14	265 ± 9.39
Total (mg.g$_{biofilm}^{-1}$)	Proteins	294 ± 39.6	532 ± 42.8	471 ± 39.9
	Polysaccharides	198 ± 18.6	512 ± 30.7	621 ± 15.8

The thickness of the biofilms decreased with an increase of the linear flow velocity (Table 5.1). The thickness of the biofilms generated at the lowest velocity was different when compared with the thickness of those formed at u = 0.4 and 0.8 m.s^{-1} ($P < 0.05$). The biofilms wet and dry mass also differed with the flow velocity under which they were formed. The biofilm mass values were not statistically distinct ($P > 0.05$). Concerning the cell density of the biofilms, the biofilms formed at u = 0.4 m.s^{-1} had the highest cell density, being followed by those formed at u = 0.8 m.s^{-1}. Biofilms generated at the highest flow velocities had similar cell density values ($P > 0.05$), and different from those formed at 0.1 m.s^{-1} ($P < 0.05$). At u = 0.1 m.s^{-1} the amounts of extracellular proteins and polysaccharides in the biofilms were the lowest. In fact, the productivity of extracellular products increased with the flow velocity. Also, for most of the cases the total protein and polysaccharide content increased with increasing flow velocity, even if there was no statistical difference between the total proteins and polysaccharides content for the biofilms formed under the highest flow velocities ($P > 0.05$).

SEM micrographs highlight the morphological aspects of the biofilms developed under different flow regimes (Figure 5.3). These images show the presence of extracellular structures that apparently connect the cells to each other and to the

stainless steel surface. These structures are more frequent when the flow velocity increases. The selected SEM micrographs are intended to provide the representative inspection of the evidences of the existence of extracellular appendages connecting cells to each other and to the surface and are not representative of the numbers of cells in each biofilm.
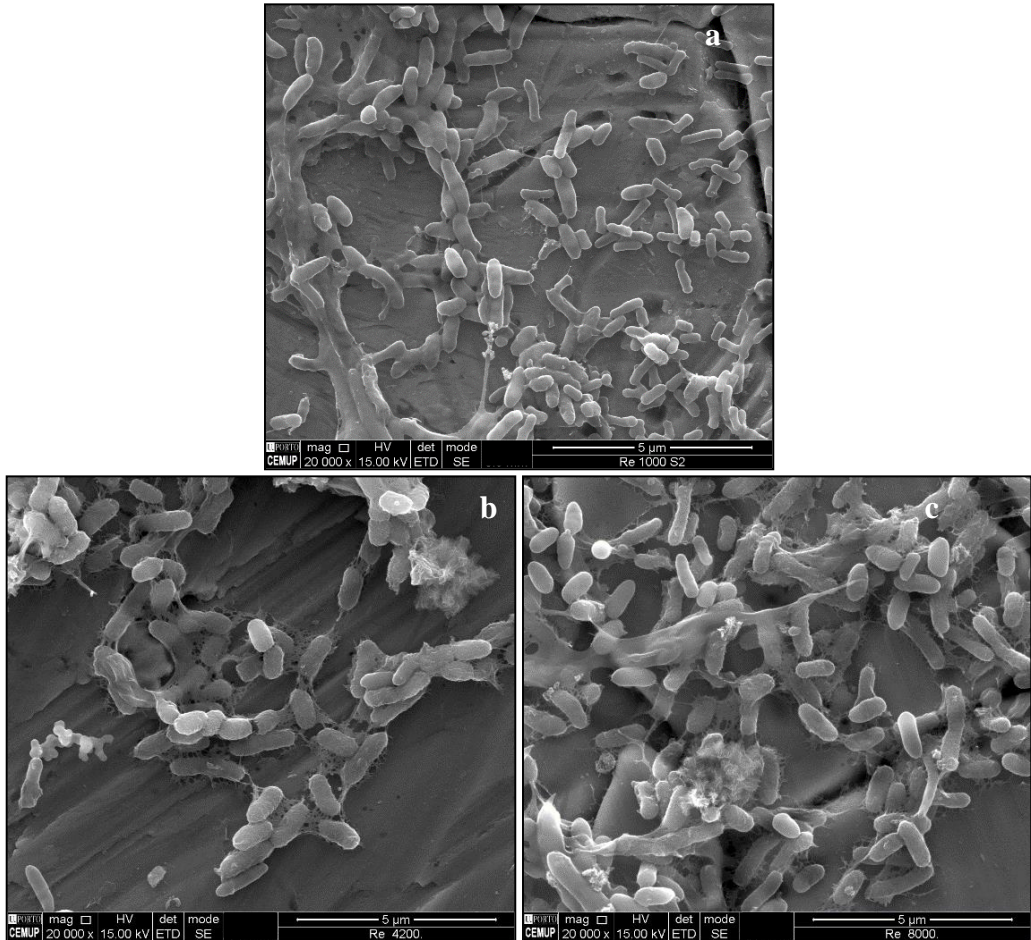
Figure 5.3 SEM micrographs of *P. fluorescens* biofilms developed on stainless steel surfaces at different flow conditions: (a) u = 0.1 m.s^{-1}, (b) u = 0.4 m.s^{-1} and (c) u = 0.8 m.s^{-1}. × 15000 magnification; bar = 5 µm.

Biofilm formation under different linear flow velocities had no apparent effects on the type of the OMP expressed (Figure 5.4). The major OMPs expressed by the biofilm bacteria had apparent molecular weights of 32, 36, 80 and 250 (±2) kDa. However, the results suggest that the low flow velocities induced the formation of lower quantities of

the major OMP. In fact, the protein with the apparent weight of 80 kDa appears to not be present in the gel, for the lowest velocity.
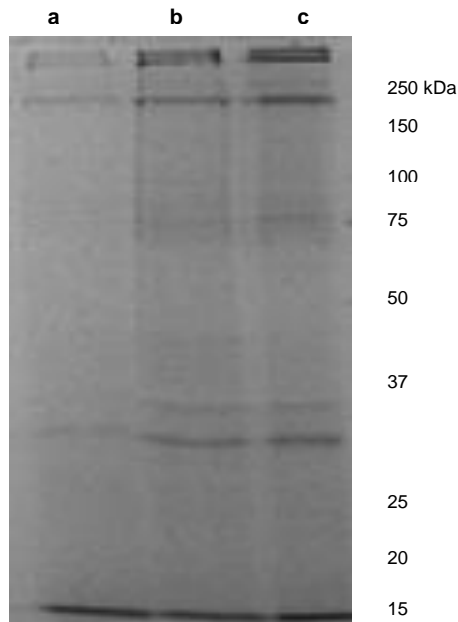
Figure 5.4 OMP profiles of *P. fluorescens* bacteria developed in different modes of growth. Biofilms formed at (a) u = 0.1 m.s^{-1}, (b) u = 0.4 m.s^{-1} and (c) u = 0.8 m.s^{-1}.

Some hydrodynamic and mass transfer coefficients, such as the feed flow, nutrient and cell loads, friction factor, shear stress, of the biofilms developed in the flow cell system are presented in Table 5.2.

Table 5.2 Hydrodynamic and external mass transfer coefficients of 7 days-old *P. fluorescens* biofilms grown at different flow velocities.

u / m.s^{-1}		0.1	0.4	0.8
Re		1000	4000	8000
Feed flow	L.h^{-1}	41	174	331
Nutrient load	g $_{glucose}$.m^{-2}.s^{-1}	3.65	15.4	29.2
Cell load	Log cells.m^{-2}.s^{-1}	13.3	13.9	14.2
Friction factor		0.063	0.037	0.033
Shear stress	Pa	0.042	0.44	1.43
Sc		n/a	1295	1295
Sh		3.66	255	435
k_m	m.s^{-1}	2.07×10^{-7}	1.44×10^{-5}	2.46×10^{-5}

The shear stress variation inside the flow cells next to the biofilm surface is shown in Table 5.2. The shear stress increase is more significant between the u = 0.1 m.s^{-1} (10.5 times lower) and 0.4 m.s^{-1}, than between 0.4 and 0.8 m.s^{-1} (3.25 times higher). The friction factor is higher in the low flow velocity biofilms (0.063), and similar (P < 0.05) for those formed under u = 0.4 m.s^{-1} (0.037) and u = 0.8 m.s^{-1} (0.033). The external mass transfer coefficient of biofilms developed at the lower flow velocity were 100 times lower than those calculated for the biofilms developed at the two highest flow velocities (P < 0.05). For these the k_m values were statistically distinct (P < 0.05).

5.4   DISCUSSION

The objective of this work was to determine how the hydrodynamic conditions under which biofilms were formed could influence their resistance characteristics. The biofilms were developed in a flow cell system at three different flow velocities, 0.1, 0.4 and 0.8 m.s^{-1}. In general, a linear velocity increase promoted a reduction of the biofilm thickness, however, the biofilm mass was kept constant despite the flow velocity. Therefore, a direct relationship between the increase of fluid flow velocity and the formation of more compact and denser biofilms was observed. This is in accordance with a previous study with *Escherichia coli* biofilms where the thickness of biofilms developed in a similar flow cell system was higher at lower flow velocities [41]. Biofilms grown at lower velocities are subjected to lower shear forces, growing faster and forming more open structures [43]. However, low flow-stressed biofilms are also known to have low mechanical strength, being more prone to sloughing events than those formed under higher flow rates [40]. Other authors also stated that the flow regime has a high impact on biofilm morphology; at lower flow rates the biofilms formed tend to be fluffy and thicker and, in opposition, higher flow rates yield compact, dense and smooth biofilms [44]. Verran [45] proposed that these structures with low mechanical resistance are critical on cleaning and disinfection practices. When biofilm erosion or sloughing occurs, bacteria are released to the bulk phase. These cells can attach to surfaces downstream and reseed a biofilm.

The cell load obtained from the bulk fluid containing planktonic cells, and the biofilm cell density increased with the flow velocity under which the biofilms were formed. The results showed that more cells were available to colonize the stainless steel for the higher flow velocities. The highest flow velocities resulted in biofilms with higher

cell densities. This fact is exacerbated by the shear stress, imposed by higher flow regimes, in the microorganisms, resulting in higher adhesion, and ultimately in biofilms with a higher cell density. Studies on electron transport systems provided evidence that the catabolic activity of biofilms can be stimulated by high shear forces, which could lead to higher cell numbers within the biofilms [46]. Simões et al. [23] studied the effects of hydrodynamic conditions on *P. fluorescens* biofilms. These authors showed that biofilm development under turbulent conditions gave origin to biofilms with more cells *per* unit area than those generated at laminar flow. That study also demonstrated an decreased bacterial metabolic activity of the biofilms developed under higher hydrodynamic stress conditions. They also proposed that higher flow velocities increase the availability of nutrients in the bulk fluid, stimulating bacterial metabolism. In this way, higher cell replication or EPS production was affordable.

The amount of matrix proteins and polysaccharides apparently increased with a feed flow increase. Chmielewski and Frank [47] stated that the biofilm structure and content are influenced by the flow regime, associating high turbulence with increased EPS production. Shear stress is the predominant force acting on biofilms [15] and an increase in linear velocity, reflected by an increase of shear stress, may influence biofilm accumulation [44]. Vrouwenvelder et al. [44] also reported that biofilms developed under high shear stresses are very stable against mechanical disturbances. In the present study, three distinct flow velocities were tested, corresponding to three different shear stress values. These hydrodynamic conditions allowed the formation of biofilms with different thicknesses. This result is in agreement with a previous study where higher shear stresses originated compact biofilms, characterized by low thicknesses values and high cell densities [44].

SEM micrographs showed structures that the biofilm-embedded cells use, apparently, to attach to each other and to the surface. Winn et al. [48] used a similar dehydration process as that used in this study and also observed microtubular-like structures used for microbial attachment and relevant for biofilm mechanical stability.

The hydrodynamic conditions used to form the biofilms had no significant effects on the OMP expression of biofilm cells. The OMP of 32 and 36 kDa is similar for the three biofilms. The protein with the apparent weight of 36 kDa could be correspondent to the one described by Kragelund et al. [49] as being the OprF, an outer membrane porin [50] known to be implicated in biofilm formation [51].

The external mass transfer coefficients increased with the flow velocity. A direct consequence of increasing the flow velocity is that the transport rate of nutrients to the biofilm surface was higher at the highest velocities [52]. The higher external mass transfer effects observed in the biofilms developed at the two higher flow velocities were a higher amount of cells and EPS. These results are in accordance with the findings of Simões et al. [23]. The higher flow velocities are correlated with higher shear stress imposed to the biofilm that is related by thinner biofilms. In spite of having more cells and EPS, they also have less limitations to mass transfer by being thinner, than the thicker biofilm (u = 0.1 m.s^{-1}) that have adapted its structure to be fluffier in order to facilitate the access to nutrients [13, 23]. Due to the higher shear stress they rather produce EPS, as seen on the results (u = 0.8 m.s^{-1}), than new cells to increase its cohesion and withstand the forces of the passing fluid [41].

5.5   CONCLUSIONS

In this study several characteristics of *P. fluorescens* biofilms were studied when formed at three distinct linear flow velocities. The biofilms developed at the two highest linear velocities were thinner, and both had approximately the same mass as that formed at the lowest velocity. The cell density and EPS content was also superior for the biofilms generated at the highest velocities. These features make these biofilms denser. The external mass transfer coefficient increased with the flow velocity. In general, biofilms formed under higher flow velocities were more complex, including the presence of attributes that can contribute to their antimicrobial resistance (higher cell density and EPS content) in a higher extent than those formed under lower flow velocities.

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CHAPTER 6

HALOGEN-BASED COMPOUNDS IN BIOFILM CONTROL

This chapter was submitted as:

ABSTRACT

Microbial biofilms are ubiquitous in nature and inherently resistant to an increasing range of antimicrobial agents. One of the most widely used disinfectants is sodium hypochlorite (SH), however, despite its proven efficacy, SH is surface corrosive, presents several health concerns, and resistance phenomena is already emerging. Therefore, the development of innovative biofilm control strategies are needed. Little is known about the usefulness of brominated products as food industry disinfectants. In this study, the control of *Pseudomonas fluorescens* biofilms was assessed with the halogen-based chemicals: cetyltrimethylammonium bromide (CTAB), sodium hypochlorite (SH), 3-bromopropionyl chloride (BrCl) and 3-bromopropionic acid (BrOH). The influence of these chemicals was assessed on several physiological aspects of planktonic cells, particularly their antimicrobial action, influence on cell surface properties and potassium release. While CTAB had the highest antimicrobial activity, BrOH had the lowest. All the chemicals promoted cellular disruption, with apparent pore formation in the cell membranes and consequent leakage of essential intracellular constituents. Only CTAB, BrCl and BrOH led to irreversible changes in membrane properties (charge and physicochemical properties) through hydrophobicity changes and decrease of negative surface charge. When these chemicals were applied to biofilms, no significant killing or removal was achieved (maximum killing of 1 log and 15% removal). Moreover, the chemicals allowed the biofilms to regrow after exposure. In fact, the overall results demonstrated similar effects with all selected chemicals. The overall data demonstrate that both BrCl and CTAB are advantageous alternatives to the currently used disinfectant, SH, since they present comparable efficiency with potentially less health and surface equipment damage concerns.

6.1. INTRODUCTION

The World Health Organization (WHO) refers to food safety as one of the top priorities and challenges of the century [1]. Nowadays, foodborne diseases are a prime public health concern in developing and developed countries. WHO reported 1.8 million mortality cases of diarrheal diseases worldwide. In the United States of America it is estimated that every year, about 48 million people suffer from foodborne diseases [1-3]. The Centers for Disease Control and Prevention (CDC) and the US National Health Institute (NIH), documented that biofilms are involved in over 65% of all microbial diseases. In addition, foodborne pathogens can form biofilms in produce, which makes them resistant to commonly used disinfectants [1, 4]. Consequently, the formation of biofilms has severe implications in several areas, from industrial processes to health-related fields, with huge economic losses [5, 6].

The sanitizers and biocides used in industry do not control microorganisms in biofilms as they are typically 10-1000 times more resistant than their planktonic counterparts [7, 8]. The efficacy of a disinfectant depends on several factors, such as the type of target microorganism and its susceptibility, the adhesion surface, temperature, exposure time, concentration and pH [9]. Also, antimicrobial resistance occurs as a multifactorial aspect that includes slow or incomplete penetration of the biocide into the biofilm, physiological alterations of the biofilm cells, expression of stress response with adaptive molecules, or even, differentiation as persister cells [10-12]. Biofilm prevention and control is, therefore, a priority in food industry, prompting a need to search for new biocides and/or sanitizers and to understand their potential to prevent and control biofilms.

Food contact surfaces are normally disinfected and cleaned with agents containing peroxides, chloramines or hypochlorite [5]. The free chlorine obtained by the use of hypochlorite can be very aggressive to stainless steel, interfering with its surface, and might facilitate further bacterial adhesion and biofilm formation [5, 13]. Furthermore, chlorine is the most widely used disinfectant in industry, however, there is a possibility that during disinfection it reacts with natural organic matter or contaminants in surface waters, and it can also produce a complex mixture of disinfection by-products which already demonstrated carcinogenic, mutagenic and teratogenic (abnormalities of physiological development) activity in animal studies [1, 14].

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Quaternary ammonium compounds (QACs), cationic compounds with a basic structure (NH_4^+) and a strong antimicrobial potential, are frequently used for disinfection and sanitation in a wide range of fields, such as hospitals and food manufacturing [15, 16]. Cetyltrimethylammonium bromide (CTAB) is a relatively safe and inexpensive product [17]. Nevertheless, it has been shown that its increasing use in a wide range of applications contributed to the emergence of resistant bacteria and, occasionally, multidrug resistance [17, 18]. Comparatively to chlorine, QACs are more expensive, however, they are an attractive alternative as they are less affected by the presence of organic matter, are not corrosive at low concentrations, are more stable, and could be stored for longer periods of time without compromising their antimicrobial activity [19].

The formation of biofilms is a microbial community behavior coordinated through cell-to-cell communication mediated by small, diffusible signals, a phenomenon called quorum sensing. Several phenotypes regulated by cell-to-cell communication are implicated in bacterial colonization and virulence [20]. Therefore, eukaryotes have developed a defense mechanism based on chemicals, including secondary metabolites that inhibit these phenotypes [20-22]. For example, furanones produced by the marine algae *Delisea pulchra* [23], oxidize halogen compounds produced by *Laminaria digitata* and, haloperoxidases produced by seaweeds are responsible for the production of the microbicidal compounds hypobromous acid (BrOH) and hypochlorous acid (ClOH) [22]. The natural furanones are halogenated at several positions by bromine, iodide or chloride and, as observed in field experiments, the concentration of furanones is inversely correlated with the degree of bacterial colonization [20]. Stabilized halogen antimicrobials are extensively used to control biofouling in industry and they have been shown to be more effective in penetrating and disinfecting biofilms than free halogen [22]. Considering this assumption, and the fact that 3-bromopropionic acid (BrOH) is used to synthetize several compounds with antimicrobial properties [24, 25], and that 3-bromopropionyl chloride (BrCl) has a comparable structure, these two halogenated compounds were selected for this study to be assessed on their antimicrobial properties against planktonic cells and biofilms of *Pseudomonas fluorescens* (Figure 6.1). Little is known about the utility of bromine as a disinfectant for food industry. Previous studies demonstrated that dibromodimethyl hydrantoin was as effective as chlorine against *Streptococcus faecalis* [26], however, less effective against *Bacillus cereus* spores [27]. Similarly to free chlorine, there are safety concerns about the production of brominated

organic compounds and their impact on human and environmental safety [28]. Because chlorine is widely used in industry as a disinfectant and sanitizer, it was used for comparison purposes.

The goal of this study was to assess the antimicrobial action of selected halogen-based chemicals against planktonic cells and biofilms of *Pseudomonas fluorescens*. This bacterium is a major contaminant in food industry, causing produce spoilage and foodborne illnesses [29, 30].

6.2. MATERIALS AND METHODS

ANTIMICROBIAL AGENTS

Sodium hypochlorite solution (SH) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma (Portugal), 3-bromopropionic acid (BrOH) was purchased from Merck (VWR, Portugal), 3-bromopropionyl chloride (BrCl) was purchased from Alfa Aesar (VWR, Portugal). All dilutions were performed using sterile distilled water.
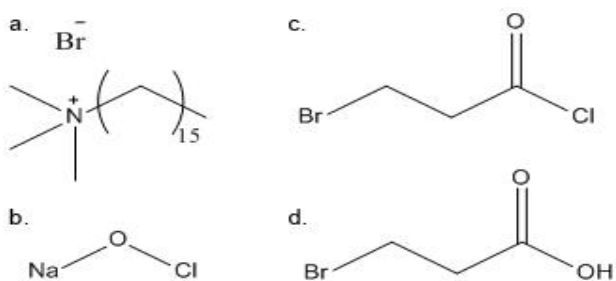
Figure 6.1 Chemical structures of the chemicals used: (a) cetyltrimethylammonium bromide (CTAB), (b) sodium hypochlorite (SH), (c) 3-bromopropionyl chloride (BrCl) and (d) 3-bromopropionic acid (BrOH).

MICROORGANISMS AND CULTURE CONDITIONS

The bacterium used in this study was *Pseudomonas fluorescens* ATCC 13525. Bacterial growth was obtained from overnight cultures (16 h) in culture medium (5 g.L^{-1} glucose, 2.5 g.L^{-1} peptone and 1.25 g.L^{-1} yeast extract in 0.025 M phosphate buffer, pH 7) and incubated at 30 ± 3°C, and 150 rpm of agitation [31].

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ANTIBACTERIAL SUSCEPTIBILITY TESTS

The minimum inhibitory concentration (MIC) of each agent was determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [32] using 96-well microtiter plates. Bacteria at a density of 10^9 colony forming units (CFU) *per* ml were inoculated into fresh culture medium. A volume of 200 µl was inserted in each well, along with the different concentrations of the chemicals (10% v/v). The bacterial growth was determined at 600 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.). The MIC was determined as the lowest concentration at which microbial growth was inhibited [33]. The cell suspension was plated in Plate Count Agar (PCA, Merck, Germany) and incubated overnight at 30 ± 3°C, after a neutralization step to quench the chemicals antimicrobial activity, by dilution, to sub-inhibitory concentrations [34]. The minimum bactericidal concentration (MBC) was considered the lowest concentration of the antimicrobial agent where no growth was detected on the solid medium [33].

PHYSICOCHEMICAL CHARACTERIZATION OF BACTERIAL SURFACES

The physicochemical properties of *P. fluorescens* cell surface were assessed by the sessile drop contact angle measurement on bacteria lawns, performed as described by Busscher et al. [35]. Contact angles were determined using an OCA 15 Plus (DATAPHYSICS) video-based optical measuring instrument, allowing image acquisition and data analysis. The measurements (≥ 15 *per* liquid and chemical) were performed according to Simões et al. [36], after bacterium incubation (1 h) with the chemical at the MBC. The liquid surface tension components reference values were obtained from the literature [37]. Hydrophobicity was assessed after contact angle measurement, following the van Oss method [38-40], where the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w)−(ΔG_{sws} mJ.m^{-2}). The surface is considered hydrophobic if the interaction between two entities is stronger than the interaction of each with water $\Delta G_{sws} < 0$. Otherwise, if $\Delta G_{sws} > 0$, the material is considered hydrophilic. ΔG_{sws} can be calculated using the surface tension components of the interacting entities of equation 6.1:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \text{ (eq. 6.1)}$$

where γ^{LW}, represents the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and donor parameters, respectively, of the Lewis acid-based component (γ^{AB}), where $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. The surface tension components, of a solid material, can be obtained by measuring the contact angles of three liquids with different polarities and known surface tension components (1): α-bromonaphtalene (apolar), formamide (polar), and water (polar). Upon obtaining the data, three equations of the type below can be solved:

$$(1 + \cos\theta)\gamma_L^{Tot} = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right) \text{ (eq. 6.2)}$$

where θ is the contact angle. The total surface energy is calculated as $\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$.

BACTERIAL SURFACE CHARGE

The zeta potential of bacterial suspensions was determined in sterile water using a Nano Zetasizer (Malvern Instruments). This determination was performed before and after 1 h bacterial exposure to the chemicals at the corresponding MBC.

POTASSIUM (K^+) LEAKAGE

The quantification of K^+ in bacterial solutions, before and after 1 h exposure to the MBC of each biocide was determined by flame emission and atomic absorption spectroscopy. Samples were filtrated (Whatman, pore size 0.2 μm) and analyzed in a GBC AAS 932 plus device using GBC Avante 1.33 software.

OUTER MEMBRANE PROTEIN EXTRACTION AND ANALYSIS

Outer membrane proteins (OMP) were isolated based on the method described by Winder et al. [41]. Briefly, an overnight inoculum of *P. fluorescens* was washed with 8.5% NaCl solution, diluted to approximately 10^9 CFU.ml^{-1} and incubated with each chemical at the MBC, for 1 h at 30 ± 3°C and 150 rpm of agitation. All suspensions were then harvested by centrifugation (3202 *g*, 25 min) and ressuspended twice with Tris-HCl 25 mM, pH 7.4 with 1 mM MgCl$_2$. Then, the suspension was sonicated for 2 min, 50% power (Bandelin generator with a Microtip MS 72 probe) on ice, to promote cell

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lysis. Next, the solution was centrifuged (7000 *g*, 10 min, 4°C) to discard cell debris. Sarcosine (Sigma, final concentration of 2%) was added to the supernatant and incubated for 20 min at 4 °C, to solubilize the OMPs. The solution was centrifuged (13000 *g*, 4 °C, 1h), to recover the OMP that were ressuspended in Tris-HCl pH 7.4 [42]. The concentration of proteins was determined by Bicinchoninic Acid Protein Assay Kit (BCA) (BCA - PIERCE Cat. No. 23225) and standardized to 240 ± 10 µg.ml^{-1} in each sample and applied to a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% bisacrylamide [43, 44]. The proteins were stained with Coomassie blue [45]. Electrophoresis was accomplished at constant 170 V. All electrophoresis components were purchased from BioRad (Portugal).

ASSESSMENT OF QUORUM SENSING INHIBITION

Quorum sensing inhibition was determined by the disc diffusion assay. The inoculum of *Chromobacterium violaceum* (ATCC 12472) was grown overnight (approximately 16 h) in Luria-Bertani broth (LB), Liofilchem, Italy (30 ± 3°C, 150 rpm). The MIC and MBC for all chemicals were determined with the antibacterial susceptibility tests as described before, with minor modifications. LB broth was used and *C. violaceum* growth was determined at 620 nm. Standard disc diffusion assay was performed for all chemicals at the MBC. Briefly, the bacterial suspension (approximately 10^8 CFU.ml^{-1}), was seeded on LB agar plates, using a sterilized swab. Next, sterile paper discs (6 mm diameter) were placed over the LB agar plates and 15 µl of each biocide was added. Antimicrobial and quorum sensing inhibition (halo of colorless but viable cells) halos were measured after 24 h of incubation at 30 ± 3°C [46, 47].

COLONY BIOFILM FORMATION AND PENETRATION TESTS

These tests were performed as explained in chapter 3, in the corresponding sub-section of material and methods.

BIOFILM FORMATION IN A FLOW CELL SYSTEM

Biofilm formation in the flow cell system was executed as explained in chapter 5, in the corresponding sub-section of material and methods.

BIOFILM CONTROL USING DIFFERENT CHEMICALS

The flow-generated biofilms of *P. fluorescens* were submitted to a disinfection process with the selected chemicals (CTAB, BrCl, SH) at their MBC. The flow cell was carefully emptied and the disinfection of the biofilms was made by recirculation of the chemical at the MBC, with a flow rate of 3.4 L.h^{-1}, for 1 h. After that period, the initial conditions were restored in the flow cell system. Control experiments with phosphate buffer were also performed. Four coupons, two from each flow cell were removed at different time periods: before chemical exposure, immediately after the antimicrobial exposure and 2, 12 and 24 h post-antimicrobial treatment. After biofilm chemical exposure, a neutralization step by diluting to sub-inhibitory concentrations was performed, according to Johnston et al. [34].

BIOFILM ANALYSIS

The *P. fluorescens* biofilms were characterized in terms of organic mass and cell density. The stainless steel coupons were removed from the flow cell and the biofilms that covered the coupons surface were completely scraped using a sterile scalpel and resuspended in 10 mL of phosphate buffer. The suspensions were vortexed (IKA TTS2) for 30 s at 100% input. The biofilm mass was determined according to the standard methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF]) [52]. The biofilm cell densities were assessed in terms of CFUs in Plate Count Agar (Merck, Portugal) [53].

STATISTICAL ANALYSIS

Data were analyzed applying the parametric paired *t*-test using the statistical program SPSS version 22.0 (Statistical Package for the Social Sciences). The average and standard deviation (SD) within samples were calculated for all cases. At least three independent experiments were performed for each condition tested. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant.

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6.3. RESULTS

The MIC and MBC values obtained for the tested chemicals against *P. fluorescens* are presented in Table 6.1. Overall, the MBC was higher than the MIC, with the exception of SH which a MIC and MBC were 500 µg.ml^{-1}. CTAB was the most efficient antimicrobial with a MBC 10 to 20 times lower than the other chemicals.

Table 6.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of each chemical tested.

	MIC / µg.mL^{-1}	MBC / µg.mL^{-1}
BrCl	650	700
BrOH	850	900
CTAB	20	50
SH	500	500

The parameters of the bacterial surface tension before and after the treatment with each chemical were determined to ascertain the effects of the selected chemical on the bacterial surface properties (Table 6.2).

Table 6.2 Surface tension parameters, hydrophobicity (ΔG_{sws}), apolar (γ_s^{LW}) and polar (γ_s^{AB}), of untreated *P. fluorescens* (control) and after 1 h treatment with the chemicals (BrCl, BrOH, CTAB or SH). The average ± SD is presented.

	Surface tension parameters / mJ.m^{-2}				ΔG_{sws} / mJ.m^{-2}
	γ_s^{LW}	γ_s^{AB}	γ_s^+	γ_s^-	
Control	22.8 ± 4.43	30.3 ± 4.79	4.14 ± 1.25	57.0 ± 4.71	30.7 ± 6.30
BrCl	19.4 ± 0.43	31.4 ± 2.92	4.40 ± 1.07	57.0 ± 3.96	29.8 ± 5.50
BrOH	20.3 ± 0.80	34.1 ± 3.61	6.10 ± 1.25	53.0 ± 3.51	23.4 ± 4.90
CTAB	12.0 ± 1.35	47.0 ± 7.10	10.4 ± 2.98	54.0 ± 0.81	14.0 ± 5.00
SH	29.4 ± 4.68	13.3 ± 1.83	0.89 ± 0.33	51.0 ± 6.00	33.5 ± 8.70

P. fluorescens is naturally hydrophilic ($\Delta G_{sws} > 0$ mJ.m^{-2}), however, this property was less pronounced when the cells were in contact with BrOH and CTAB ($P < 0.05$). Regarding the apolar parameter (γ_s^{LW}), only CTAB promoted a small decrease of the apolar component compared to the untreated cells. The polar parameter (γ_s^{AB}) of the bacterium increased with the application of CTAB and decreased in the presence of SH

($P < 0.05$). Moreover, when the capacity to accept (γ_s^+) or donate (γ_s^-) electrons was analyzed, it was possible to observe that the treatment with SH significantly decreased the surface capacity of the cell to accept or donate electrons ($P < 0.05$), while BrOH and CTAB increased the electron acceptor component of *P. fluorescens* surface ($P < 0.05$).

P. fluorescens untreated cells had a negative surface charge of -13.53 mV with a conductivity of 0.05 mS.cm^{-1} (Table 6.3). The exposure to CTAB, BrCl or BrOH modified *P. fluorescens* surface charge to less negative and increased its conductivity ($P < 0.05$), with the exception of CTAB that had no effects on the cell surface conductivity ($P > 0.05$). Conversely, SH enhanced conductivity ($P < 0.05$) without interfering with the cell surface charge ($P > 0.05$).

Table 6.3 Zeta potential and conductivity of *P. fluorescens* before and after 1 h treatment with different chemicals. The average ± SD is presented.

	Zeta Potential / mV	Conductivity / mS.cm^{-1}
Control	-13.5 ± 2.32	0.05 ± 0.02
BrCl	-2.88 ± 0.66	2.25 ± 0.06
BrOH	-4.96 ± 0.95	0.46 ± 0.09
CTAB	-8.14 ± 0.42	0.05 ± 0.01
SH	-13.0 ± 1.41	31.1 ± 0.14

To ascertain the effects of the chemicals in the cell integrity the intracellular K^+ release was assessed. Table 6.4 shows the K^+ concentration with and without exposure to the chemicals. All chemicals tested promoted an alteration in the cytoplasmic membrane permeability, causing K^+ release, regardless the chemical used ($P < 0.05$).

Table 6.4 Concentration of K^+ in solution of the untreated and after 1 h incubation of *P. fluorescens* with each chemical. The average ± SD is presented.

	Concentration of K^+ in solution / µg.ml^{-1}
Control	1.21 ± 0.08
BrCl	1.99 ± 0.21
BrOH	1.96 ± 0.25
CTAB	2.09 ± 0.28
SH	2.07 ± 0.26

The OMP expression, using 1-D SDS-PAGE, was assessed before and after biocide exposure for 1 h (Figure 6.2). No significant differences were found in the expression of

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the major OMP of *P. fluorescens* with and without the exposure to the selected chemicals, with the exception that CTAB and SH reduced significantly the amount of OMP expressed (Figure 6.2).

Figure 6.2 OMP profile of *P. fluorescens* cells when exposed to the MBC of different chemicals. The molecular weight marker (a) was used to extrapolate the molecular weight of some lanes of the OMP profile obtained from incubation in the (b) absence or in the presence of (c) BrCl, (d) BrOH, (e) CTAB and (f) SH.

The percentage of retardation gives an estimate on the efficacy of chemical products to cross the biofilm (Table 6.5). In this study, the penetration of BrCl was the most efficient followed closely by SH, with 0 and 1.90% retardation respectively. The biofilm penetration was retarded by 15% for BrOH and 100 % for CTAB ($P < 0.05$).

Table 6.5 Retardation caused by *P. fluorescens* biofilms, for each chemical used. Data is presented as average ± SD of the percentage of diameter measurements for halo readings compared with controls (no biofilm).

	Retardation / %
BrCl	0.00 ± 0.00
BrOH	15.7 ± 4.40
CTAB	100 ± 0.00
SH	1.90 ± 3.20

The disc diffusion assay for the detection of quorum sensing inhibition is depicted in Figure 6.3. Quorum sensing was not affected by the chemicals tested.
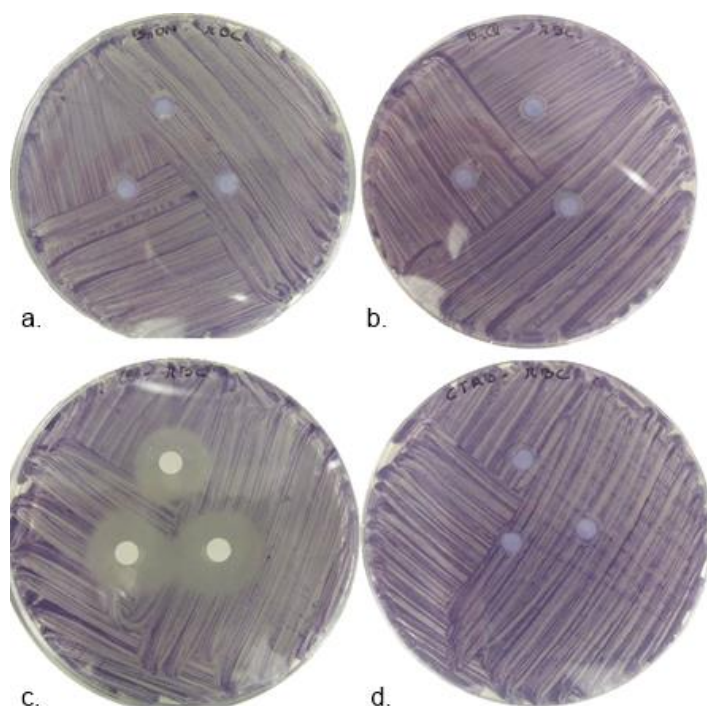
Figure 6.3 Disc diffusion assays for the detection of quorum sensing inhibition of *C. violaceum* by (a) BrOH, (b) BrCl, (c) SH and (d) CTAB.

BrCl was selected over BrOH for the biofilm assay, due to the lower MIC and MBC, higher capacity to change the surface properties of *P. fluorescens* and ability to penetrate the biofilm without being retarded. Therefore, BrCl, CTAB and SH were tested against 7-day old flow-generated biofilms, formed under conditions mimicking those found in industry, chapter 5. The effectiveness of the chemicals was assessed in terms of number of biofilm CFU (Figure 6.4a) and mass (Figure 6.4b).The results obtained for the number of biofilm CFU revealed a reduction after 1 h exposure to the MBC of CTAB and SH (Figure 6.4a). However, this effect was more pronounced for SH with 1-log reduction ($P > 0.05$). For CTAB a CFU log reduction of 0.4 was achieved and for BrCl the difference was almost negligible. In order to ascertain the role of the chemicals tested on biofilm regrowth, the CFU were determined during the 2, 12 and 24 h after chemical exposure. Two hours after the treatment the number of CFU increased for all chemicals tested, attaining similar values to those before the treatment ($P > 0.05$, Figure 6.4a).

The number of biofilm CFU, remained constant overtime for all the conditions tested, except for the 24 h BrCl-treated biofilms. In this case the number of CFU increased significantly ($P < 0.05$, Figure 6.4a) when compared to the control values (untreated biofilms). In terms of biofilm mass, the three chemicals promoted similar biomass removal (16%, Figure 6.4b). These values remained unchanged 2 h after the treatment ($P > 0.05$). When analyzing the biofilm, 12 h after the treatment, no significant biomass changes were found for the biofilms treated with CTAB and BrCl, in comparison to the biofilms immediately after exposure. The SH treated biofilms recovered significantly in terms of biomass ($P < 0.05$). However, 24 h after the treatment, the values obtained for the biomass, were as low as the values achieved with the SH treatment after the same time. During the recovery period, the biomass of CTAB-treated biofilms was similar to the value immediately after the treatment, while significant biomass regrowth of the BrCl-treated biofilms was found ($P > 0.05$).
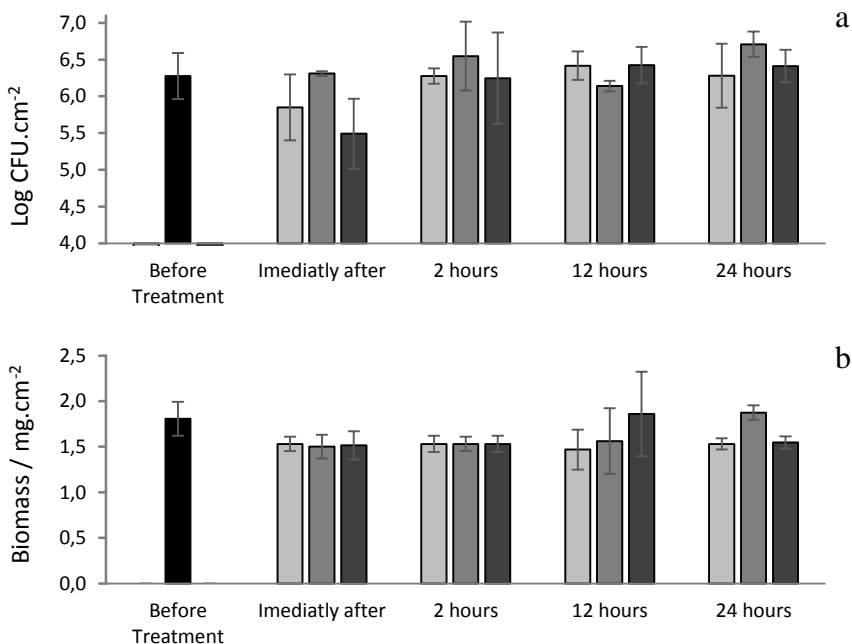
Figure 6.4 *P. fluorescens* biofilm log CFU.cm^{-2} (a) and mass (b) before and after treatment with CTAB (▢), BrCl (▨) and SH (■). Samples were collected before treatment (■), immediately after 1 h treatment and after 2, 12 and 24 h after chemical removal. Values are average ± SD.

6.4.   DISCUSSION

Antimicrobial resistance to conventional antimicrobial agents such as SH has been documented [54]. Also, the use of SH can result in the production of harmful disinfection by-products through their reaction with organic matter [55]. The best known and characterized products are the trihalomethanes, which include chloroform, bromoform, bromodichloromethane and chlorodibromomethane [56]. Drinking water quality regulation was specified to limit trihalomethane levels to 100 µg.L^{-1} [57].

In food industry, outbreaks of foodborne pathogens have been increasing in the last decades due to the high food demand to match population needs [58]. Therefore, the susceptibility to microbial contamination and biofilm formation requires major investments for produce decontamination and sanitation of the facilities [59]. However, resistance to disinfectants has been increasing, urging the necessity for the development of new formulations [10-12, 17, 18]. It is necessary to find alternative compounds capable of removing and/or killing undesired resistant microorganisms [60]. Brominated compounds might be a suitable replacement to the conventional chlorinated antimicrobials. In this work, the antimicrobial activity and capacity for biofilm control of chlorine (as sodium hypochlorite) and three bromine based chemicals (CTAB, BrCl and BrOH), against planktonic and biofilm embedded *P. fluorescens,* was studied. Additionally, several aspects of their interaction with the bacteria were assessed. The three brominated chemicals were selected based on their structure, particularly the presence of bromine [61, 62], chlorine [62, 63] and carboxyl group [64] known for their antimicrobial properties. *P. fluorescens* was chosen as a well-studied Gram negative bacterium, and ubiquitous in the natural, medical and industrial environments, that can cause serious problems in either its planktonic or biofilm states [33, 65]. In addition, this bacterium is known to form biofilms resistant to disinfectants [66]. Several bacterium physiological characteristics were assessed such as the MIC, MBC, hydrophobicity, potassium (K^+) release, and surface charge.

The MIC and MBC values of CTAB against *P. fluorescens* were 20 and 50 µg.ml^{-1}, respectively (Table 6.1). Previous use of CTAB, revealed MIC values of 4 µg.mL^{-1} against the Gram negative *Salmonella typhimurium* and *P. aeruginosa* and 18 µg.mL^{-1} for the yeast *Candida albicans* [67]. The MIC and MBC of SH were 500 µg.mL^{-1} (Table 6.1). A range of SH concentrations from 50 to 5000 µg.mL^{-1} has been determined by several authors, for a variety of conditions, and bacteria [68-71]. Differences between the

values obtained within the previous studies can be explained by the use of different methods and bacteria to determine these parameters. Moreover, it is widely assumed that no strain can characterize the behavior of a species [72]. To our knowledge, this is the first study reporting the antimicrobial properties of BrOH and BrCl. It was found that both chemicals have antimicrobial activity, with a MIC and MBC against *P. fluorescens* of 650 and 700 µg.mL^{-1} for BrCl and 850 and 900 µg.mL^{-1} for BrOH. The lower MIC and MBC of BrCl is possibly due to the presence of chlorine, known for its antimicrobial properties [63, 73].

In order to understand the action of the selected chemicals on *P. fluorescens*, several aspects of the interaction between the chemicals and the bacterial cells were assessed, particularly the surface physicochemical properties, charge and K^+ release. CTAB is a compound that binds to the negative cell surface of bacteria due to electrostatic attraction by chemisorption [74-76]. Azeredo et al. [75] revealed that when a concentration of CTAB higher than the MBC is used, hydrophobicity and surface charge properties can be enhanced and bacteria becomes hydrophilic and positively charged. Upon interaction with the surface, CTAB promotes cell membrane disorganization [77] or even disruption [78]. In this work, the effect of cell disruption was verified measuring by the amount of K^+ released.

Conversely to the effect caused by CTAB, *P. fluorescens* exposure to SH decreased the bacterial polar (γ_s^{AB}) character and, consequently the capacity to accept (γ_s^+) or donate (γ_s^-) electrons. According to Gottardi et al. [63], the action of active chlorine (hypochlorous acid - HOCl) in bacteria can be divided in two effects, non-lethal and lethal. The first implies reversible chlorination of the bacterial surface and the second is based on penetration into the bacteria combined with irreversible alterations. SH can also promote aggregation of essential proteins [73]. The present study corroborates the findings of Winter et al. [73] on the membrane destabilization effects. In fact, K^+ release is a consequence of membrane leakage. The interaction of active chlorine does not interfere with the cell surface charge, suggesting covalent links between the biocide and the bacterial membrane [63]. Moreover, SH dissociation in ions can originate salt formation that can help explain the increase in conductivity. BrOH decreased bacterial hydrophilic characteristics, and improved electron acceptance (γ_s^+). These results, together with reduction in negative surface charge suggest that, electrostatic interactions of bromine based chemicals with the membrane occur after Br$^-$ dissociation

from the structure, as it was described for BrOH [79]. The membrane interaction of the chemical may also promote destabilization and consequently potassium release into the solution. The effects of BrCl were less noticeable on the hydrophobicity values, but this was the chemical that most affected the bacterial cells charge. The difference in antimicrobial activity and mode of action of BrOH and BrCl can be due to the presence/absence of the chemical entities OH$^-$ or Cl$^-$. It seems that the presence of Cl$^-$ improves the antimicrobial activity of the molecules, causing a significant decrease in the cell surface charge and the leakage of intracellular K^+. Even if significant effects on the cell surface properties and charge were promoted by the biocides, no changes were induced on OMP expression. This may indicate that these compounds may not potentiate antimicrobial resistance. This hypothesis is based on the knowledge of the OMP importance in bacterial resistance to biocides and antibiotics [41, 80-83].

The penetration of CTAB through *P. fluorescens* biofilms was completely retarded due to the presence of biofilms. In fact, bacteria in biofilms exhibit less susceptibility to antimicrobials due to their spatial heterogeneity, which consequently originates nutrient depletion within the biofilm, reduced access of the chemicals to the bacteria inside the biofilm, or biocide interaction with extracellular polymeric substances, and the existence of degradative enzymes and neutralizing chemicals [65, 84, 85]. Simões et al. [86] supports the accessibility hypothesis as it was verified that less dense biofilms were more susceptible than denser biofilms. The retardation of SH was negligible. A previous study showed that chlorine effectively diffuses through biofilms with a diffusion coefficient in water estimated to be 0.84 cm^2.s^{-1} [87]. BrOH was moderately retarded by the biofilm, while BrCl was able to penetrate the biofilm without retardation. Again, this result supports the observations reported previously in this study, that the presence of ions Br$^-$, Cl$^-$ or OH$^-$ may define the activity of the molecule.

Biofilms are organized cell aggregates in a self-produced extracellular matrix and, can form on living or inert surfaces, which can create serious problems in several fields if disinfection protocols fail [65]. As biofilms are a major problem in industry, it is important to understand the chemical mechanism of disinfection and its efficacy on pre-established biofilms. Flow generated *P. fluorescens* biofilms were exposed, for 1 h, to the chemicals at their MBC values. Only modest reductions in the log CFU.cm^{-2} were obtained. This fact reinforces the higher resistance of biofilm cells compared to their planktonic counterparts [7, 88]. In terms of mass removal, the use of BrCl, CTAB or SH promoted low removal of the total biofilm mass (15%). It can be hypothesized that CTAB

acts eroding the biofilm, which may consist on eradication of the superficial bacteria, and disruption of the matrix. This assumption is based on the fact that CTAB is the most efficient chemical despite lacking the capacity to cross the biofilm layers. The possibility of BrCl and SH to pass through the biofilm and the lack of efficacy may be a consequence of an intrinsic or acquired resistance of biofilms [73, 89]. Furthermore, these chemicals did not interfere with quorum sensing. A screening protocol developed by McLean et al. [46] was used for the detection of quorum signal inhibition (targeting acylated homoserine lactones dependent signaling), no effects were found other than antimicrobial action.

To ascertain the ability of the biofilms to regrow after 1 h exposure to the antimicrobial agents, the initial conditions were reestablished, so that a disinfection practice in industry was mimicked. Biofilm regrowth was found on SH treated biofilms after 12h h, and on BrCl treated biofilms after 24 h. It was verified that CFU recovered to its initial values 2 h after exposure to all chemicals. This result can be explained by the possible presence of starved or injured cells or potentially viable but not culturable cells [10, 90, 91]. Also, Pereira et al. [92] found that 7-days old *P. fluorescens* biofilms formed in a flow cell system are in the stationary or stabilization phase. This means that the loss of biomass due to physical stresses is balanced by the growth of new cells at the edge of the biofilm [93]. The results obtained show low to moderate effects of the selected biocides on biofilm removal and killing, and rapid regrowth to the stabilization phase. These results suggest that the selected biocides, at the concentrations tested, had no significant effects on the dynamic behavior of the biofilms. It is apparent that the promising results obtained with the tests on planktonic cells did not provide relevant insights on their application in biofilm control, even if the same strain and antimicrobial concentrations were used in both tests. Moreover, in this study, it seems that the chemical nature of the biocide was not relevant on biofilm control, and the bromine-based products had no clear antimicrobial advantage on biofilm control over SH. It is possible that the combination of bromine-based products and chlorine might potentiate their antimicrobial action. Pioneer studies [94, 95], demonstrated synergistic antimicrobial relationship when bromine was added to chlorine solutions. However, as with free chlorine, there are safety concerns about the production of brominated organic compounds and their impact on human and environmental safety, even if bromide ion has a low degree of toxicity [28].

The effect of the halogenated compounds tested was similar between each other. Despite that BrOH and BrCl require higher MIC and the MBC than CTAB, they are both similar to SH. Also, the results propose that the chemicals tested share a similar mode of antimicrobial action against *P. fluorescens*. The overall results propose that the selected bromine-based products can be a potential alternative to chlorine-based products.

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CHAPTER 7

ENZYMATIC TREATMENTS FOR BIOFILM CONTROL

ABSTRACT

Current biofilm control strategies are inefficient in removing biofilms from equipment surfaces. The use of enzymes is considered a new and environmentally friendly approach for biofilm control. This work investigates the effects of a β-glucanase, a protease, a lipase, and α-amylase, alone and in combination with benzalkonium chloride (BAC) and cetyltrimethyl ammonium bromide (CTAB) in the control of biofilms formed by *Bacillus cereus* and *Pseudomonas fluorescens*.

Synergistic effects of the combination of the biocides with the enzymes were found against the biofilms developed using the microtiter tests. This biofilm control strategy was also applied against *P. fluorescens* biofilms formed in a flow cell system. In this case, the enzymes, when applied alone, resulted in low to moderate biofilm removal and CFU reduction. Following the enzymatic or CTAB-enzyme treatments, it was found biofilm regrowth and long term control events. The effect of the enzymes against planktonic cells was also evaluated by respirometry. Most enzymes showed to have the ability to reduce the respiratory activity of planktonic cells, except α-amylase that, instead, increased the activity of *P. fluorescens*. Moreover, protease, lipase and α-amylase hindered the antimicrobial activity of the biocides, apparently due to chemical neutralization.

The overall results show the synergistic potential of selected enzymes with BAC and CTAB in the control of biofilms and demonstrate that a careful selection and application of enzymes must be considered since these molecules can quench the activity of antimicrobial agents.

7.1   INTRODUCTION

Biofilms can be defined as microbial sessile communities, characterized by cells embedded in self-produced extracellular polymeric substances (EPS) [1]. The type and amount of EPS constituents can be strain dependent and vary with the environmental conditions under which biofilms are formed. However, there is unanimity in considering polysaccharides, proteins and DNA as its main constituents. EPS contribute to the mechanical stability of biofilms, enabling them to withstand shear forces, dehydration and chemical attacks [2]. EPS also enhance robustness and survival of the embedded-microorganisms on a substratum, by acting as a chemically reactive diffusional transport barrier, slowing the penetration of antimicrobial agents. Furthermore, the exopolymeric matrix increases biofilm attachment to the substratum and stabilizes it, thereby reducing its susceptibility to sloughing by hydrodynamic stress [3].

The use of water for diverse processes of food industry increases the chance for microbial contamination and biofilm formation. Typical consequences of biofouling are reduced operational efficacy in heat exchangers, increased operational pressure, blockage of tubes, increased energy consumption, accelerated metal surfaces corrosion, final product contamination and consequential potential health problems [4, 5]. Biofouling deposits can contain pathogenic and spoilage bacteria, increasing the risks for human consumption. Consequently, contingency plans to control microbial contaminations need to be applied. The main objective of microbial control is to eliminate and/or reduce the number of microorganisms and their activity, as well as to prevent and control the formation of biological deposits on process equipment [6]. As a result, programs such as Good Manufacturing Practice (GMP) and Hazard Analysis and Critical Control Points (HACCP) [7] are currently employed to control microbial proliferation. Biofilm control in food industry is usually performed without dismantling or opening the equipment in a Clean-in-Place (CIP) process. CIP consists on running alternated cycles of detergent and disinfectant solutions with water rinses with increased turbulence [8]. This method typically uses caustic acids, surfactants, biocides and, occasionally, enzymes [4, 8-10]. As the continued use of biocides can induce microbial resistance, new control strategies must be developed. Also, as regulation restricts the use of toxic biocides, eco-friendly strategies represent a new approach for the control of biofilms of food industry [11, 12].

Enzymes are already used in a wide range of applications, including the production of food and beverages, detergents, clothing, paper products, pharmaceuticals, fuel and monitoring devices [13]. For instance, glucanases are used to reduce the viscosity in barley and oats used in animal feed, enhancing their digestibility. Proteases are the most used enzymes in dairy industry, and in cleaning detergents. They are also used for protein hydrolysis, milk clotting, low-allergenic infant-food formulation, flavor improvement in milk and cheese, meat tenderization and prevention of chill haze formation in brewing. Lipases are used for flavoring cheese, *in-situ* emulsification for dough conditioning, support for lipid digestion in young animals, and synthesis of aromatic molecules. Amylases are used for starch liquefaction and sacharification, and also increase shelf life and, by retaining moist, they improve product quality. The elasticity and softness of bread is provided by amylases. In addition, flour adjustment, and low calorie beer are also processes that use amylases. Amylases are the second most widely used group of enzymes, along with cellulases [13, 14], in the formulation of enzyme detergents, mainly to remove food residues of starch-based foods. Enzymes have already been tested in biofilm control, and are proven to potentiate the action of some antimicrobial agents [15-18].

This work evaluates the effectiveness of an enzymatic treatment in the control of *Bacillus cereus* and *Pseudomonas fluorescens* biofilms. The combined action of enzymes and quaternary ammonium compounds (QACs) was assessed against biofilms formed in microtiter plates and in a flow cell system. Additionally, tests with planktonic bacteria were performed in order to ascertain the supposed action of enzymes as quenchers of antimicrobial agents.

7.2   MATERIALS AND METHODS

BACTERIA AND CULTURE CONDITIONS

P. fluorescens ATCC 13525[T] and a *B. cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing [19] were used in this study. Bacteria were grown at 30 $\pm$ 3 $^{\circ}$C, in the medium composed by 5 g.L^{-1} glucose, 2.5 g.L^{-1} peptone, 1.25 g.L^{-1} yeast extract, in phosphate buffer (PB), pH 7, 25 mM, that uses glucose (Merk) as the main carbon source [20]. Bacterial suspensions were prepared by inoculation of a single colony grown on solid medium (above medium supplemented with 10% agar)

into a 1 L flask containing 250 mL of sterile nutrient medium. This bacterial suspension was incubated overnight at 30 ± 3 °C in an orbital shaker (120 rpm).

ANTIMICROBIAL AGENTS AND ENZYMES

Two QACs were used: benzalkonium chloride (BAC) and cetyltrimethyl ammonium bromide (CTAB) (Sigma, Portugal). In order to ascertain the effect of the enzymes on the bacteria, QACs were used at their minimum bactericidal concentrations (MBC), previously assessed by Araújo et al. [21], chapter 4. The MBC of BAC was 10 mg.L^{-1} for *B. cereus,* and 35 mg.L^{-1} for *P. fluorescens*; CTAB was used at 20 mg.L^{-1} on the first and at 35 mg.L^{-1} on the second bacterium. QACs were prepared as concentrated aqueous solutions.

The enzymes tested were provided by Novozymes (Denmark). Their commercial names are Ultraflo® (3.2.1.6, β-glucanase), Alcalase® (3.4.21.62, protease-subtisilin), Lecitase® (3.1.1.3, lipase), and Fungamyl® (3.2.1.1, α-amylase), supplied as aqueous solutions containing 5-30% active protein. The enzymes were used diluted (1:100) in PB [17]. The solutions combining enzymes with biocides started as enzymatic solutions to which a concentrated solution of biocide was added to achieve the desired concentration.

BIOFILM FORMATION IN MICROTITER PLATES

After the growth period, bacterial suspensions were harvested by centrifugation, using an Eppendorf 5810R centrifuge with the A-4-62 rotor (Göttinger, Germany) (3999 *g*, 10 minutes) and resuspended in fresh culture medium to a final density of 1×10^9 cells.mL^{-1}. The methodology used to grow biofilms was based on the modified microtiter plate test, as proposed by Stepanović et al. [22]. For each bacterium, 200 µL of the bacterial suspension, 100-fold diluted, were transferred to the wells of sterile 96-wells flat-bottomed polystyrene tissue culture plates (Orange Scientific, Portugal). Plates were incubated in an orbital shaker (120 rpm) for 24 h at 30 ± 3 °C. Negative controls consisted of culture medium with no bacterial cells.

BIOFILM CONTROL USING ENZYMES

The effect of each enzyme and QAC, as well as different combinations of enzymes with antimicrobial agents on biofilms formed in microtiter plates was screened using the methods presented by Simões et al. [23] and Lequette et al. [17]. Briefly, the medium was removed and the wells were gently washed twice with PB to remove reversibly adhered bacteria. The remaining attached bacteria were submitted to a process that consisted in biofilm exposure to: (1) an enzymatic solution for 1 h, (2) a solution of enzyme and biocide for 1 h, and (3) an enzymatic solution for 30 minutes, followed by a gentle washing step with PB, and then, the biocide for 30 minutes (30 + 30 min). These tests were performed in an orbital shaker at 30 ± 3 °C, 120 rpm. Controls consisted of PB or biocide solutions, in the absence of enzymes. QACs were used at their MBC values [21]. This concentration will affect the biofilms only to a certain extent, not killing all the embedded bacteria, so that any effect caused by the selected enzymes, other than killing, could be recognized.

BIOFILM MASS AND VIABILITY ASSESSMENT

The biofilm mass was quantified using crystal violet staining (Merck, Portugal), and the modified Alamar blue (Sigma-Aldrich, Portugal) microtiter plate assay was applied to determine the bacterial viability of the biofilm-cells. Both methods were described by Araújo et al. [24], see chapter 3.

BIOFILM CONTROL ACTIVITY CLASSIFICATION

The effects of the enzymes, biocides, and combination of enzymes with biocides on biofilms were classified based on a ranking proposed by Lemos et al. [25]. Values of killing and removal percentage, or CFU reduction inferior to 10% represent *insignificant* efficacy of the product tested, 10% to 30% *low* efficacy, 30% to 60% *moderate* efficacy, 60% to 85% *high* efficacy and values superior to 85% *very high* efficacy.

The effect of the combination of enzymes with biocides was classified as described by Saavedra et al. [26], to elucidate the effects of the interaction. The combination of enzyme with biocide is considered *antagonistic* if [effect of combination enzyme and biocide − (effect biocide + effect enzyme)/2] < 0; *indifferent* if the 0 ≤ (effect of

combination enzyme and biocide) – (effect biocide + effect enzyme)/2 < effect enzyme or effect biocide; *additive* if (effect biocide) < [(effect of combination enzyme and biocide) – (effect biocide + effect enzyme)/2] < 2× (enzyme effect or biocide effect); and *synergistic* if (effect of combination enzyme and biocide) > 2× (effect enzyme or effect biocide).

BIOFILM FORMATION IN A FLOW CELL SYSTEM

To test these strategies using biofilms similar to those found in industrial environments, biofilms were developed in a flow cell system, see chapter 5 (Figure 5.1). The biofilms were left to develop for 7 days at a Reynolds number (*Re*) of 4000, corresponding to a flow velocity of 0.4 m.s^{-1}. Following this period, two coupons were analyzed as control (without any treatment) for each experiment; subsequently the biofilms were submitted to a similar treatment as the one used for microtiter plate biofilms. The flow cell was carefully emptied, and then the solutions of CTAB, enzymes, and solutions combining CTAB with enzymes ran through the flow cells at a flow velocity of 0.006 m.s^{-1}, for 1 h. After that period, the initial conditions of the system were restored. Control experiments with PB were also performed. Four coupons, two from each flow cell were removed at different time periods: before chemical exposure (control), immediately after the exposure, and then 2, 12 and 24 h post treatment, in order to observe putative long-term effects or regrowth events following the treatment.

FLOW GENERATED BIOFILM CHARACTERIZATION

Biofilm mass and cell density were assessed as indicators of control. The biofilms covering the coupons were completely scraped using a sterile scalpel, resuspended in 10 mL of PB, and vortexed for 30 s. The organic mass was determined according to the standard methods - American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF] [27]. The cell density was assessed in terms of colony forming units (CFUs) in Plate Count Agar (Merck, Portugal), according to Simões et al. [28]. The effectiveness of the control strategy was classified according to the rank based on the classification proposed by Lemos et al. [25].

TESTS WITH PLANKTONIC CELLS

To assess the antimicrobial potential of the selected enzymes in planktonic cells, the respiratory activity of *B. cereus* and *P. fluorescens* cell suspensions was ascertained by measuring oxygen uptake rates in a biological oxygen monitor (Yellow Springs Instruments 5300A) after the exposure to the QACs and the enzymes for 1 h. This method was described previously by Araújo et al. [21], see chapter 4. The concentration of O_2 used by the bacteria for the oxidation of glucose corresponds to the exogenous respiration rate, which is obtained by the difference between the total and endogenous rates [28]. The respiratory activity, in $mgO_2 . mg_{organic\ mass}^{-1} . min^{-1}$, was calculated according to the following equation:

$$Respiratory\ activity = \frac{exogenous\ rate \times O_{2\ solubility}}{bacterial\ mass} \text{ (eq. 7.1)}$$

where the metabolic rate was measured as the percentage of O_2 consumed by the cells in time, the O_2 solubility was considered 8.6 $mg.L^{-1}$ [29], and the bacterial mass obtained by the determination of total volatile solids (TSV) according to standard methods (APHA, AWWA, and WPCF, [27], expressed as $mg_{organic\ mass}.L^{-1}$. If respiratory activity < control, there was killing of the microorganisms, whereas if respiratory activity > control, there was metabolic potentiation.

STATISTICAL ANALYSIS

The experimental data was analyzed using the statistical program SPSS - Statistical Package for the Social Sciences, Version 22.0 (Armonk, NY, USA). The average and standard deviation were calculated for all cases, from at least three independent experiments performed for each condition tested. Normality of data distribution was assessed by the Kolmogorov-Smirnov method. The statistical significance of the average values obtained for biofilm biomass, biofilm activity and cell number were evaluated using the *t*-test. Statistical calculations were based on confidence level equal to or higher than 95% ($P \leq 0.05$ was considered statistically significant).

7.3   RESULTS

Several tests were performed in order to control *B. cereus* and *P. fluorescens* biofilms using selected enzymes: β-glucanase, protease, lipase and α-amylase, alone and combined with two QACs. An initial screening was performed using biofilms grown in 96-wells polystyrene microtiter plates (Figures 7.1 and 7.2). Afterwards, the enzymes were tested against biofilms developed in a flow-cell system (Figure 7.3). The effects of the enzymatic treatments were also assessed against planktonic cells (Figure 7.4).

THE EFFECT OF AN ENZYMATIC TREATMENT ON BIOFILM CONTROL

The 24 h-old biofilms formed in the microtiter plates were subjected to three different types of treatments (1) 1 h exposure to an enzymatic solution, (2) 1 h exposure to an enzymatic solution containing a QAC, and (3) exposure to an enzymatic solution for 30 min followed by 30 min exposure to a QAC (30 + 30). Control tests were performed with PB as negative controls, and the QAC solution as the positive. The control treatments were made to assess if the enzymes were acting in synergy with the QACs.

In Figure 7.1, biofilm killing and removal after the treatments with the duration of 1 h are depicted. The treatment with lipase resulted in no killing of *B. cereus* biofilms, and the treatments with β-glucanase, protease, and α-amylase promoted low killing ($P > 0.05$). The removal of *B. cereus* with the enzymatic solutions was low, with the exception of α-amylase that was insignificant ($P > 0.05$). *P. fluorescens* biofilms killing was insignificant with β-glucanase, protease and lipase ($P > 0.05$). The killing percentage was low with α-amylase ($P > 0.05$). The enzymatic solutions of β-glucanase, protease and α-amylase produced moderate removal ($P < 0.05$), while lipase caused low biofilm removal ($P > 0.05$) (Figure 7.1).

Both biofilms of *B. cereus* and *P. fluorescens* showed susceptibility to BAC and CTAB, when the biocides were applied alone. *B. cereus* killing was moderate with both biocides ($P < 0.05$), and removal was low with BAC ($P > 0.05$) and insignificant with CTAB ($P > 0.05$). *P. fluorescens* was insignificantly killed by both biocides ($P > 0.05$), and its removal was low with BAC and insignificant with CTAB ($P > 0.05$).

The combination of the selected biocides with the enzymes increased, in most cases, *B. cereus* and *P. fluorescens* killing percentage. The combined treatment BAC-enzymes improved *B. cereus* killing from low to moderate ($P < 0.05$), however, with

β-glucanase killing remained low ($P > 0.05$). With the addition of CTAB, the killing of *B. cereus* was insignificant with α-amylase, and moderate with the other enzymes. *P. fluorescens* biofilms killing with BAC-α-amylase was insignificant ($P > 0.05$), with BAC-protease and BAC-lipase remained insignificant ($P > 0.05$), and with BAC-β-glucanase increased to moderate ($P < 0.05$). Killing improved in all cases with CTAB: with protease, lipase and α-amylase increased to low, and with β-glucanase increased to moderate ($P < 0.05$). The removal of *B. cereus* with the combinations of BAC with all enzymes remained low despite an increase, in average of 5% ($P > 0.05$). *B. cereus* removal with CTAB improved slightly when combined with all enzymes ($P > 0.05$). The removal of *P. fluorescens* with BAC was low when combined with lipase and α-amylase, and moderate with β-glucanase and protease ($P < 0.05$). The removal of *P. fluorescens* with the combination CTAB-enzymes was similar to the case when only enzymes were used ($P > 0.05$).

Figure 7.1 Killing and removal percentages of *B. cereus* and *P. fluorescens* biofilms using the selected enzymes with and without the selected QACs. Where □ corresponds to β-glucanase, ▨ protease, ▨ lipase, ▨ α-amylase and ■ QAC. The enzymatic and QAC (biocide) solutions were applied for 1 h. *means no killing. Average values ± standard deviation for at least three replicates are illustrated.

The effect of the combinations of enzymes with biocides is *antagonistic*, when biofilm killing was reduced in comparison with the tests with the enzymes or the biocide

alone. This happened for *B. cereus* with the combinations of α-amylase with CTAB, and β-glucanase with BAC. The effect is indifferent as happened for *B. cereus* biofilms control using CTAB with β-glucanase, protease, and lipase, and for *P. fluorescens* using BAC with protease. The protease was an additive to killing when was used along with BAC on *B. cereus*.

The combination of enzymes with biocides was synergistic against *B. cereus* biofilms when control was improved. This effect happened with all enzymes when combined with BAC, except β-glucanase. *P. fluorescens* killing improved with the synergistic combinations of BAC with β-glucanase and lipase, and of CTAB with all enzymes tested. *B. cereus* removal with BAC and CTAB was indifferent with all enzymes. *P. fluorescens* removal was indifferent with BAC, but the enzymes worked as additives to CTAB.

The procedure that consisted in 30 minutes exposure to an enzymatic solution, followed by the same period of exposure (30 + 30 min) to the QACs resulted in higher killing and removal percentages (Figure 7.2).
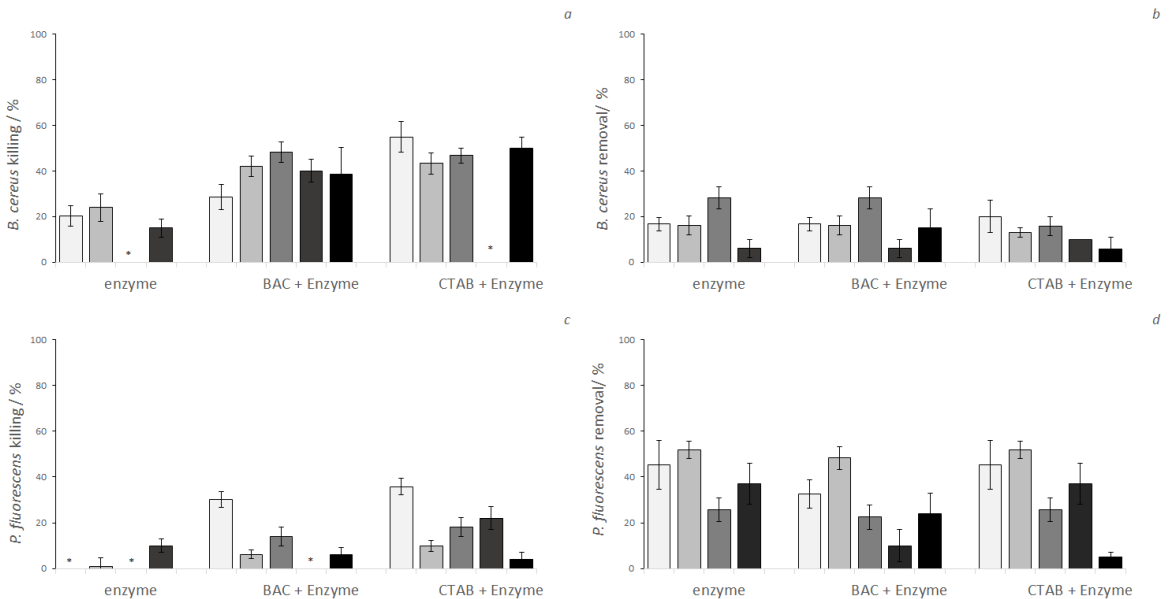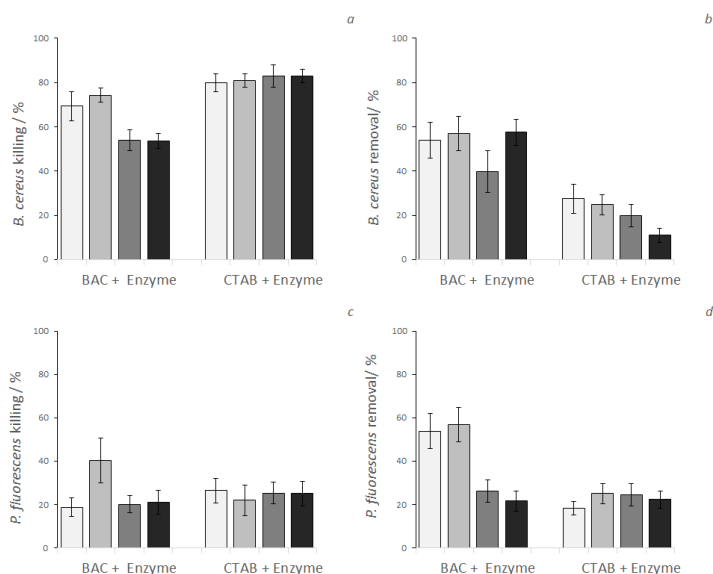

Figure 7.2 Killing and removal percentages for *B. cereus* and *P. fluorescens* biofilms using the selected enzymes. Where □ corresponds to β-glucanase, ▨ protease, ▨ lipase and ▪ α-amylase. The enzymatic solutions were applied for 30 min then removed and the biocide was applied for 30 min (30 + 30). Average values ± standard deviation for at least three replicates are illustrated.

High killing percentages of *B. cereus* were observed with the pre-treatment of β-glucanase and protease followed by BAC ($P < 0.05$), and for CTAB with all enzymes tested ($P < 0.05$). The pre-treatments of lipase and amylase followed by BAC were classified as moderate ($P > 0.05$). For *P. fluorescens,* biofilms killing with BAC combined with all enzymes was low, except with protease which was moderate ($P < 0.05$). With CTAB the killing efficacy was still low for all cases ($P > 0.05$). The removal increased in comparison with the approach, when the treatments were applied for 1 h, for *B. cereus* in all situations, and for *P. fluorescens* with BAC (all increased to moderate). Contrary to when the treatments were applied for 1 h, biofilm removal was, on average, more significant with BAC than with CTAB (*B. cereus* 41% *vs.* 20%; *P. fluorescens* 31% *vs.* 21%).

CONTROL OF BIOFILMS DEVELOPED IN THE FLOW CELL SYSTEM

P. fluorescens formed biofilms with the highest resistance to killing by QACs and enzymes. This bacterium was selected to develop biofilms in a flow cell system in order to understand the killing and removal efficacies of QACs and enzymatic treatments against biofilms with characteristics mimicking those found in industrial systems. The results before (control), immediately after 1 h exposure to the enzymatic and biocidal solutions, and up to 24 h post treatment were compared in terms of CFU and biofilm mass. In Figure 7.3 the biofilm mass reduction percentage and log CFU reduction are represented for the different treatments.

The treatments with the enzymes resulted, in most cases, in biofilm removal. β-glucanase, protease and α-amylase caused moderate biofilm removal, while lipase resulted in insignificant biofilm removal, immediately after exposure. CTAB caused low removal, 15% of total biofilm mass. When CTAB was applied in combination with the enzymes, the removal was insignificant for protease and lipase, and moderate with β-glucanase (57%) and α-amylase (36%), $P < 0.05$.

The application of enzymes caused log CFU reductions from 1 to 1.7. CTAB caused a log CFU reduction of 1.3, immediately after treatment. When CTAB was combined with the selected enzymes the efficacy of the treatment increased, except for the combination with lipase which reduced the efficacy of the treatment ($P < 0.05$). The CTAB-β-glucanase and CTAB-protease combinations caused the highest efficacy increase ($P < 0.05$). The log CFU reduction between these treatments is statistically similar ($P > 0.05$), and different from the treatment with the biocide ($P < 0.05$). The log

CFU reduction was slightly higher for the combination CTAB-lipase than the reduction caused by CTAB alone.
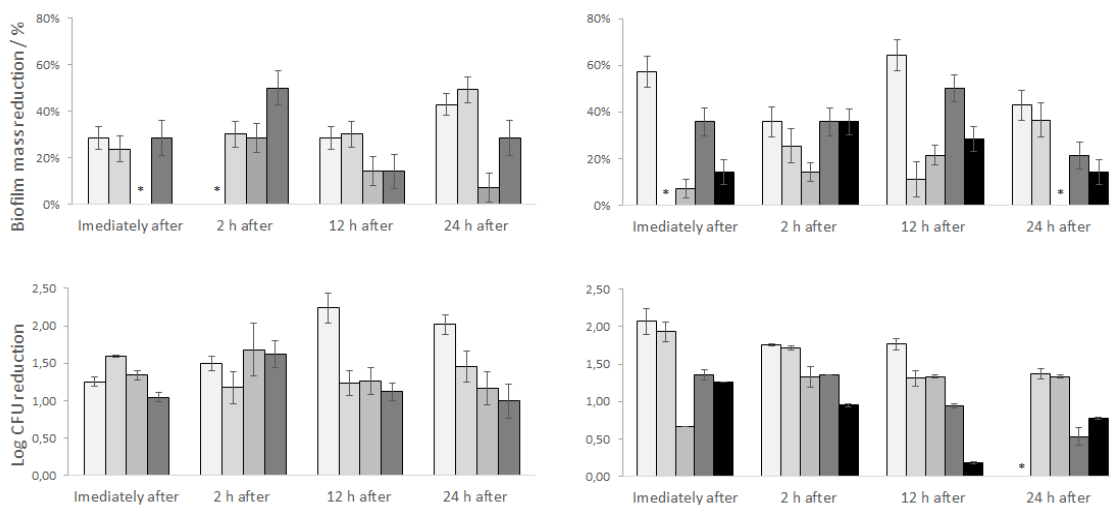
Figure 7.3 Mass and log CFU reduction of *P. fluorescens* biofilms overtime after the treatments with an enzymatic solution (left hand) and an enzymatic solution combined with CTAB (right hand). Where □ corresponds to β-glucanase, ▣ protease, ▢ lipase, ▨ α-amylase and ■ CTAB. *means no reduction. Average values ± standard deviation are depicted.

The biofilm behavior, in terms of biofilm mass and CFU numbers, was further analyzed 2, 12 and 24 h following the treatment, after the initial biofilm growth conditions were reestablished (Figure 7.3). Significant ($P < 0.05$) biofilm regrowth (biofilm mass reduction percentage was lower than immediately after the treatment) following the enzymatic treatment was found for α-amylase, 12 h after treatment. A long-term biofilm removal effect (biofilm mass reduction percentage was higher than immediately after the treatment) was found with lipase, 2 and 12 h after treatment, α-amylase, 2 h after treatment and with β-glucanase and protease, 24 h after treatment ($P < 0.05$). For the treatments with CTAB and CTAB-enzyme combinations, significant ($P < 0.05$) biofilm mass regrowth was found on the biofilms treated with β-glucanase, 2 and 24 h after treatment, and CTAB-lipase and CTAB-α-amylase, 24 h after treatment. Significant long-term effects were also found for CTAB, 2 and 12 h after treatment, for CTAB-lipase and CTAB-α-amylase, 12 h after treatment.

In terms of CFU regrowth, following the enzymatic treatment, no significant ($P > 0.05$) CFU increase was found. A long-term effect ($P < 0.05$) on CFU reduction was found 2, 12 and 24 h after β-glucanase, and 2 h after α-amylase treatments. For the

treatment of CTAB, alone, and combined with enzymes, regrowth was found 2 h after the application of CTAB alone and CTAB-β-glucanase and CTAB-protease. This regrowth behavior persisted for the 12 h (CTAB alone and in combination with protease) and 24 h (CTAB-β-glucanase) following treatment. Long-term effects ($P < 0.05$) in CFU reduction were found for CTAB-lipase, 2, 12 and 24 h after treatment.

PLANKTONIC TESTS WITH ENZYMES

Using respirometry, the respiratory activity of *B. cereus* and *P. fluorescens* was studied after the esposure to solutions of (1) enzymes, (2) biocides and (3) the combination of both (Figure 7.4).
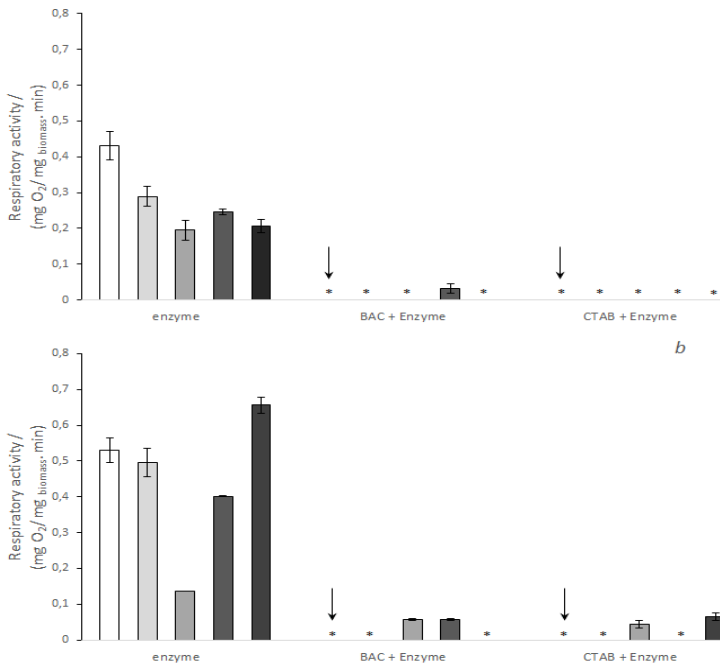
Figure 7.4 Effect of chemical treatment for 1 h of *B. cereus* (a) and *P. fluorescens* (b) planktonic cultures. Control □ (no treatment). The different enzymes □ β-glucanase, ▢ protease, ▢ lipase, and ■ α-amylase, were used alone and in combination with the two QACs. The results obtained with BAC and CTAB alone solutions are represented by arrows. Total inactivation of respiratory activity is indicated with an asterisk (*). Average values ± standard deviation for at least three replicates are depicted.

The use of enzymatic solutions, for 1 h, on *B. cereus* suspensions resulted in a decrease of the respiratory activity with all enzymes relatively to the control (no treatment). The respiratory activity was reduced by approximately 30% with

β-glucanase, 40% with lipase and 50% with both protease and α-amylase solutions. The presence of enzymes did not affect the antibacterial activity of BAC and CTAB ($P > 0.05$), with the exception of lipase ($P < 0.05$). In the presence of lipase, BAC at its MBC reduced the respiratory activity to 92% ($P < 0.05$).

For *P. fluorescens* there were reductions of the respiratory activity with the enzymatic solutions of protease (75%, $P < 0.05$) and lipase (30%, $P < 0.05$). No changes were observed in the respiration of the cells treated with β-glucanase ($P > 0.05$). Albeit, the exposure to α-amylase promoted cell activation (24%, $P < 0.05$) as the cells exposed to this enzyme were more active than the cells with no treatment. No total reduction of respiratory activity was observed with the combined solutions of BAC with protease and lipase ($P < 0.05$). Moreover, the effects of CTAB on the bacterial respiratory activity decreased when protease and α-amylase were present ($P < 0.05$).

7.4   DISCUSSION

The removal of *B. cereus* increased with the same enzymes. In this case, lipase was the best for biofilm removal. *P. fluorescens* biofilm removal was best with the enzymatic solution of protease, followed by β-glucanase, lipase and α-amylase solutions (Figure 7.1). Oulahal-Lagsir et al. [30] found that protease, α-amylase, and β-glucanase were effective in cleaning a simulated industrial biofilm formed during paper pulp manufacture. In another study [31], a lipase was unsuccessful when tested on the control of biofilm formed by a *Pseudoalteromonas* strain. In a study by Marcato-Romain et al. [18] two lipases were inefficient, or only slightly efficient for microbial multi-species biofilm removal. These results show that the efficiency of enzymatic treatments is strongly dependent on the biofilm type, particularly the species colonizers and the EPS they produce. Enzyme specificity is a fundamental stepping stone in designing an enzyme-based control strategy. The specific mode of action of enzymes makes the search for the correct enzymes for control challenging, because of the complex diversity of biofilm constituents [49] that differs between biofilms [50].

The preliminary studies with microtiter plates suggest that the enzymes worked synergistically with the biocides, even if biofilm killing and removal was modest. The biofilms of *B. cereus* were more effectively removed by BAC than the biofilms formed by *P. fluorescens* that were more effectively removed by CTAB. Simões et al. [32]

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showed that a *P. fluorescens* biofilms exhibited reduced susceptibility after a treatment with BAC.

A previous study by Walker et al. [33] proposed that treating biofilms with only one type of enzyme would only loosen the cells. These authors also suggested that enzymatic soaking should be used as a pre-treatment prior to the use of biocides. This corroborates the results obtained with the 30+30 min test performed in the present study (the tests providing the highest biofilm removal results).

Jacquelin et al. [34] reported the use of enzymes with surfactants to improve disinfection efficacy. However, the inclusion of enzymes is not certain to increase efficacy, because there are several factors contributing to the success of such treatment. Kim et al. [35] demonstrated that an enzymatic treatment with proteinase K and acylase I was not suitable against *P. aeruginosa* biofilms, as these were actually increasing the amount of proteins present. Marcato-Romain [18] tested the effect of concentration and contact time to discover that proteases were the best enzymes to remove biofilms, and that glycosidases and lipases only slightly removed biofilms from paper industry. It is known that exopolysaccharides and glycoproteins contribute to the adhesion of bacteria to a surface and bacterial accumulation in the biofilm [30]. In *P. fluorescens* biofilms α- and β- polysaccharides contribute to its cohesiveness [36]. Christensen et al. [36] studied the effect of alginate degrading enzymes to control pure culture biofilms. These authors concluded that bacterial alginate does not contribute to the cohesiveness of the biofilms tested. However, Johansen et al. [37] managed to successfully remove *Pseudomonas* spp. biofilms attached to stainless steel using polysaccharide hydrolysases. Lequette et al. [17] studied the effect of polysaccharidases and proteolitic enzymes on a CIP procedure, using bacteria commonly found in food industry. These authors found that proteolitic enzymes and polysaccharidases removed *P. fluorescens* biofilms attached to stainless steel. These enzymes were tested in several scenarios that differed from the current work, in parameters such as the pH and temperature.

In the current work, the selected enzymatic treatments were able to reduce low to moderately the flow-generated biofilms; however, this reduction is apparently lower than the removal observed for the biofilms developed in the microtiter plates (Figures 7.1 *vs.* 7.3 immediately after the treatment). The biofilms generated in the flow cell system are older, thicker and with resistance characteristics (high EPS content and cell density), see chapter 5. Flow-generated biofilms are known to be firmly attached to

the surfaces enabling them to withstand the shear stress caused by the passing fluid [38]. This result reinforces that the biofilm characteristics, particularly its age (7 days old biofilms formed in the flow cell system *vs.* 1 day old biofilms formed in the microtiter plates) are relevant in the control process. This comparison proposes that an adequate biofilm formation system, with the ability to simulate the conditions found in practice should be used in the development of control strategies.

The analysis of the flow generated biofilms following enzymatic and CTAB-enzyme treatments was followed up to 24 h after treatment. The results demonstrated some long-term effects on biofilm removal and CFU reductions. It is possible that the enzymes or CTAB-enzyme caused sustained effects in biofilm control. Even if the solutions are removed from the system, residual concentrations remain acting on the biofilms. Also, regrowth was found following some treatments. However, the long-term and regrowth effects found in this study were not specific for any particular enzyme and/or CTAB-enzyme combination. Also, none of the strategies was effective in completely killing and/or removing the flow generated biofilms. Parkar et al. [39] proposed that in cleaning industrial plants, a large decrease of cells (removal and killing) is not indicative of a successful treatment because the treatments leave cell debris that act as an organic conditioning film able to assist microorganism attachment and regrowth.

The function of the biocide is to kill bacteria, while the enzymes can cause EPS disruption. A part of biofilm resistance relies on internal mass transfer limitations caused by the intricate nature of biofilm architecture. The polyanionic nature of the bacterial EPS may be responsible for binding the antimicrobial agents before they have the opportunity to reach the cells, hindering their diffusion [40-42]. The antimicrobial agent can also react and be neutralized by components of the biofilms [43]. Augustin et al. [44] points out the possibility that inside biofilms there are altered chemical microenvironments able to inactivate enzymes. These authors also proposed that EPS may be impairing the diffusion of enzymes through biofilms, similarly to how oxygen does not fully penetrate biofilms [44]. The amount of enzymes available could be spent before these reach the under-layers of the biofilms [44, 45]. In order to overcome this issue, Pechaud et al. [46] allowed contact times of 20 hours to ensure total enzyme penetration inside the biofilms, which is a long downtime for cleaning and disinfection practices. Despite the long contact time, they observed that the enzymatic treatments were not efficiently removing biofilms.

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The use of enzymes depends on several factors such as the type of biofilm to treat, the surface to which it is attached, the contact time, the pH and the temperature [33]. The action of some agents could be antagonized by local accumulation of acidic waste products that might lead to pH dissimilarities. Differences greater than 1, between the bulk fluid and the biofilm interior, were previously found [44]. In this case, the neutral pH was selected because it is the pH commonly used to grow the selected bacteria [47]. In fact, the effects of the enzymes could only be assessed if the parameters tested were only the presence/absence of enzymes. Lequette et al. [17] found that alkaline buffers, promoted biofilm removal.

The results obtained with respirometry, demonstrated some inhibition of respiratory activity in the treatments where only enzymes were applied, except for *P. fluorescens* treated with α-amylase (which potentiated modestly the respiratory activity). However, when protease, lipase and α-amylase were applied with the QACs, the respiratory activity values of *B. cereus* and *P. fluorescens* indicated that the bacteria were in a viable state. In fact, the respiratory activity was higher than with the use of BAC and CTAB alone. Therefore, no advantage on microbial inactivation was found for these cases compared to the use of BAC and CTAB alone. These QACs were used at their MBC values and caused total bacterial inactivation. The antimicrobial activity of enzymes was already described [48]. In the last years many studies have been performed on the antimicrobial properties of bacterial cell wall hydrolases [49]. The hydrolysis of a sufficient number of specific bonds in the peptidoglycan layer results in weakening, or serious cell damage that ultimately results in bacteriolysis [50]. For instance, lysozyme is a bacteriolytic enzyme that hydrolyses polysaccharides which compose the cell wall [30]. The hydrolases used in this study could be acting in the same way. The exposure to protease resulted in high respiratory reduction of *P. fluorescens* cells. The other enzymes were unable to produce the same effect, with the exception of lipase that reduced the bacterial respiration in a low extent. This result is probably due to the presence of the outer membrane in *P. fluorescens* (Gram negative) compared with *B. cereus* (Gram positive).

It is currently known that environmental characteristics can influence the activity of antimicrobial agents [51]. These hindrances can be caused by organic material that potentially interferes with antimicrobial agents by chemical and/or ionic interactions [9, 52]. The results from the present study, the combination of lipase with BAC resulted in lower inactivation effects on *B. cereus* and that protease reduced the effects of BAC

on *P. fluorescens*. The antimicrobial effects of CTAB on *P. fluorescens* were reduced with protease and α-amylase. The work of Araújo et al. [21] (chapter 4) elucidates how the antimicrobial function of BAC and CTAB antimicrobial is affected by low concentrations of organic material such as bovine serum albumin (BSA), alginate, yeast extract and humic acids. The antimicrobial mechanism of QAC involves the disruption and denaturation of structural proteins and enzymes [53] that could lead to the impairment of the enzyme function and activity. A similar phenomenon could have happened in the present study. In the case of bacterial metabolism activation the enzymes might have been taken as nutrients, since these could be carbon and nitrogen sources. Bacterial activation was observed by Kim et al. [35] when using enzymatic treatments for biofilm control. In this case, the release of fatty acids from bacterial EPS was promoted by the treatments which in its turn were utilized by bacteria.

7.5   CONCLUSIONS

The preliminary studies with microtiter plates suggest that enzymes work synergistically with the QACs, even if biofilm killing and removal was modest. Similar effects were found on the treatments of *P. fluorescens* biofilms developed in the flow cell system. Long term effects were observed apparently due the interaction between enzymes and the biofilm components. On the other hand, for some treatments, the biofilms recovered some of their characteristics over the course of 24 h after the treatments. The enzymes were proven to work as antimicrobial agents against planktonic cells; however, when combined with biocides, some enzymes acted as interfering agents decreasing the activity of the QACs. The increase of concentration and contact time could be a solution for the low efficacy rates; however, higher concentrations of enzymes would be overly expensive, or the optimized contact time could be infeasible for industrial applications.

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CHAPTER 8

CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH

8.1   FINAL CONCLUSIONS

The results obtained in this thesis contribute to a better understanding of the phenomena associated with biofilm resistance to cleaning and disinfection. These studies also add information to develop cleaning and disinfection strategies to be applied in industry. Biofilm control was shown to be extremely complex, because embedded bacteria have dynamic resistance, enabling adaptation to varying environmental and engineered conditions.

When resilient contaminations occur, they are often related to biofilms. Furthermore, the embedded bacterial cells may gain additional resistance to conventional control treatments. From the work presented in this thesis some major conclusions can be drawn:

Antimicrobial agent penetration hindrances may be caused by the interaction of the agents with the biofilm components, or by diffusional limitations caused by the three-dimensional structure of the biofilm.

The ability of an antimicrobial agent to penetrate through a biofilm is not correlated with biofilm killing or removal efficacy.

Selected QAC (BAC and CTAB) showed reduced antimicrobial efficacy in the presence of BSA, YE and ALG. In the disinfection process of *P. fluorescens*, CTAB was severely hindered by low concentrations of HA, that inclusively increased the metabolic activity of this bacterium. The inclusion of HA substantially reduced the antimicrobial efficacy of the QAC. This substance is proposed to be included as an antimicrobial interfering agent for the testing protocols to develop disinfecting strategies.

The flow monitor system used for the development of flow generated biofilms at different linear flow velocities, resulted in biofilms with clearly different characteristics from those formed in microtiter plates. Nonetheless, both bioreactor systems demonstrated to be suitable to perform biofilm control studies.

The flow regime influences biofilm development. The biofilms developed at the highest linear flow velocities (u = 0.4 and 0.8 m.s^{-1}) have similar characteristics, and are different from those developed at the lowest linear flow velocity (u = 0.1 m.s^{-1}). Specifically, characteristics such as a higher occurrence of micro-tubular structures that cells are thought to use to adhere to the stainless steel surface and to each other, and more bacterial cells and EPS that were condensed in a more compact biofilm structure.

The most complex biofilms, developed in the flow monitor system at the highest linear flow velocities, were selected as a worst case scenario for the ensuing experiments on biofilm control strategies.

BrCl and BrOH, CTAB and SH demonstrated the ability to change the cellular membrane properties, changing hydrophobicity, and decreasing surface charge. The action mechanism of BrCl and BrOH is thought to be cellular disruption, with pore formation, as leakage of the intracellular constituents was observed.

BrOH, BrCl, CTAB and SH showed antimicrobial efficacy against planktonic *P. fluorescens*. The MIC and MBC values of SH were similar to the values for BrCl and BrOH, but the MIC and MBC of CTAB was the lower. Using the flow cell system, biofilm control with CTAB, SH and BrCl was modest at the tested concentrations. Also, the biofilms were able to recover after the treatments. The overall efficacy of BrCl and BrOH in biofilm control was comparable to that of SH, proposing that these brominated-based chemicals can be alternatives to SH. CTAB was the best antimicrobial agent.

The enzymatic treatments were able to reduce low to moderately the biofilm quantity of both *B. cereus* and *P. fluorescens* developed in microtiter plates and in the flow cell system. The enzymes showed synergistic potential with both BAC and CTAB. The effects of the enzymatic treatments were observed for the biofilms developed in the flow cell system. In the subsequent hours, the treatments alone and combined with CTAB showed both long term effects and biofilm regrowth. These effects occurred with no particular specificity to an enzyme or enzyme-CTAB treatment. Nonetheless, the potential of the application of enzymes on biofilm control was found for the selected bacteria.

When the enzymatic solutions were used on bacterial cell suspensions, all enzymes showed antimicrobial activity against the bacteria tested, except β-glucanase and α-amylase on *P. fluorescens*. The metabolism of this bacterium was stimulated when α-amylase was applied. Nevertheless, when enzymes were combined with the selected QACs, their antimicrobial potency was reduced. This phenomenon occurred when BAC was combined with lipase for both bacteria, BAC combined with protease, and CTAB combined with protease and α-amylase for *P. fluorescens*. A careful application of enzymes must be considered since these molecules can quench the activity of the antimicrobial agents.

8.2   FUTURE WORK

Biofilms can be a problem in industries where water is involved in the manufacturing process. The chemical control of biofilms is an important issue because of the severe operation, management and public health impact.

Biofilm control is proven to be even more challenging than the approaches taken against planktonic bacteria, however, the tests for chemicals efficacy are, most of the times, performed on planktonic bacteria. The selection of a suitable antimicrobial agent is of utmost importance for the development of a disinfection strategy. The antimicrobial efficacy should be tested against particular contaminations, because the loss of efficacy depends on many factors. Thus, it should be tested in conditions as close to practice as possible, as in many cases, the influence of interfering agents could severely hinder the antimicrobial efficacy. Therefore, it is proposed to widen the list of disinfecting interfering substances, through the investigation of the mechanisms of action of the antimicrobial agents and their interactions with different cellular targets and soiling agents.

Industrial processes have a need for biocides able to retain their activity in soiled conditions, work in low volumes, have low costs, and reduced corrosion. Particularly in food industry, consumers and governmental agencies demand chemical agents that are less toxic, less susceptible to microbial resistance, and stable so that disinfection by-products do not enter natural systems. Therefore, anti-biofilm specific compounds should be sought as alternative drugs with the function to selectively blocking virulence, quorum sensing, and biofilm formation. Consequently, natural products such as phytochemicals, have already been introduced into the market, however, in general, their effects are limited compared to conventional disinfectants. In this work, the combination of enzymes showed biofilm control potential, the addition/ combination of phenolic or other new chemicals to potentiate the action of the conventional antimicrobials is suggested as follow up research. Both strategies (combinations with enzymes and new products) need optimization for complete control.

When the biofilms were scaled up to the flow cell system, it was stressed that the way how biofilms develop is strongly connected with its degree of resistance. The study of the process of biofilm formation is required from the early stages to maturation, by a combined perspective of their physical, chemical and biological phenomena. When

the correlation of the processing characteristics is made with contamination occurrences, some solutions can be found: (1) the study of the effects that the environmental parameters have on biofilm should be deepened and related with the food processing line characteristics. Plant performance should be optimized to find an equilibrium when production is maximized and the microbial contaminations are minimized, (2) design materials with ability to inhibit soil accumulation must be sought, and (3) the development of improved cleaning regimes, incorporated with conventional/new but efficient chemical agents is in demand. A cleaning and disinfection plan should be developed complying with certain principals: the nature of the equipment (material and design), nature of the soiling agent, selection of a suitable antimicrobial agent, and optimum operational conditions at which the agent has maximum efficacy (temperature, concentration, hydrodynamics and exposure time). These suggestions should be performed not only for the model bacteria used for this thesis, but others such as *Escherichia coli*, *Salmonella* spp. *or Listeria monocytogene*s, commonly found in food industry, and their combinations. Understanding the different constituents that could emerge in food industry, will lead to a faster and more efficient response, by tailoring treatments to specific situations.

New strategies are currently being researched and many more will appear as a response to new resistance mechanisms or technological advances.

NOMENCLATURE

ABBREVIATIONS

ALG	alginate	-
APHA	American Public Health Association	-
ATCC	American Type Culture Collection	-
AVG	average	-
AWWA	American Water Works Association	-
BAC	bezalkonium chloride	-
BDMDAC	benzyldimethyldodecylammonium chloride	-
BrCl	3-bromopropionyl chloride	-
BrOH	3-bromopropionic acid	-
BSA	bovine serum albumin	-
CDC	Centers for Disease Control and Prevention	-
CFU	colony forming units	$CFU.mL^{-1}$
CIP	clean-in-place	-
CLSI	Clinical and Laboratory Standards Institute	-
CTAB	cetyltrimethylammonium bromide	-
DNA	deoxyribonucleic acid	-
EB	extraction buffer	-
EDTA	ethylenediamine tetraacetate	-
EPS	exopolymeric substances	-
GMP	Good Manufacturing Practice	-
HA	humic acids	-
HACCP	Hazard Analysis and Critical Control Points	-
HDMS	hexamethyldisilazane	-
LB	Luiria Bertrani	-
MBC	minimum bactericidal concentration	$\mu g.mL^{-1}$
MIC	minimum inhibitory concentration	$\mu g.mL^{-1}$
NIH	National Health Institute	-
OD	optical density	nm
OMP	outer membrane proteins	-
PB	phosphate buffer	-
PCA	plate count agar	-
QAC	quaternary ammonium compound	-
QS	quorum sensing	-
rRNA	ribosomal ribonucleic acid	-
SD	standard deviation	-
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis	
SEM	scanning electron microscopy	-
SH	sodium hypochlorite	-
SPSS	Statistical Package for the Social Sciences	-
TVS	total volatile solids	$mg_{biomass}.L^{-1}$
USA	United States of America	-

UV	ultraviolet	-
WHO	World Health Organization	-
WPCF	Water Pollution Control Federation	-
YE	yeast extract	-

INDEXES

BI	biofilm inactivation	%
BR	biofilm removal	%
D	molecular diffusivity of glucose	$m^2.s^{-1}$
D_h	hydraulic equivalent diameter	m
FI_C	fluorescence intensity of biofilms not exposed to antimicrobial agents	-
FI_W	fluorescence intensity value for biofilms exposed to the antimicrobial agents	-
k_m	external mass transfer coefficient	$m.s^{-1}$
m_c	metabolic activity of the control experiments	$mg\ O_2.\ mg_{organic\ mass}^{-1}.\ min^{-1}$
m_t	metabolic activity of bacteria exposed to the antimicrobial	$mg\ O_2.\ mg_{organic\ mass}^{-1}.\ min^{-1}$
OD_C	OD_{570nm} value for biofilms not exposed to agents	-
OD_W	OD_{570nm} value for biofilm exposed to the selected chemicals	-
Q	flow rate	$m^3.s^{-1}$
Re	Reynolds number	-
Sc	Schmidt number	-
Sh	Sherwood number	-
u	linear flow velocity	$m.s^{-1}$

GREEK

ΔG_{sws}	free energy of interaction between two entities	$mJ.m^{-2}$
γ^-	electron donor parameter	$mJ.m^{-2}$
γ^+	electron acceptor parameter	$mJ.m^{-2}$
γ^{AB}	Lewis acid-based component	$mJ.m^{-2}$
γ^{LW}	Lifshitz-van der Waals component	$mJ.m^{-2}$
γ^{Tot}	total surface energy	$mJ.m^{-2}$
μ	water viscosity	$kg.m^{-1}.s^{-1}$
ε	porosity	-
f	Darcy friction factor	-
θ	contact angle	-
ρ	density of water	$Kg.m^{-3}$
ρ_d	true density of dry biomass	$Kg.m^{-3}$
ρ_{dw}	mass per unit of wet volume	$Kg.m^{-3}$
τ	tortuosity	-
τ_w	wall shear stress	Pa