Activity of biosurfactant AC7 against different strains of *Candida albicans*: inhibition of adhesion and biofilm growth on medical-grade silicone

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SUMMARY

The formation of a biofilm by pathogenic microorganisms can cause severe infections that are very difficult to eradicate. Especially in a hospital environment, biofilm formation on medical devices can imply serious complications. A well-known problem nowadays is the increasing resistance of pathogenic microorganisms against current antimicrobial therapy. Furthermore, the bacteria or fungi present in a biofilm are even more resistant to antimicrobial agents than planktonic cells of the same strain. Research for new compounds that can prevent the formation of biofilms or that could even eradicate biofilm-related infections, is therefore important. Lately, lots of interest has gone out to biosurfactants, which are surface-active compounds produced by bacteria.

AC7BS is a biosurfactant isolated from of the supernatant of Bacillus subtilis AC7 strain. Its critical micelle concentration (CMC) was 45.7 µg/mL, indicating that AC7BS is a quite efficient biosurfactant.

To mimic a central venous catheter material, silicone disks were used for this study. They were pre-coated with AC7BS to examine its anti-adhesive and anti-biofilm effect on different Candida albicans strains: C. albicans 40, C. albicans 42 and C. albicans IHEM 2894. AC7BS showed a significant inhibition of adhesion (around 54 %) after 90 minutes with respect to biomass and cell viability. Similar results (around 49 % inhibition) were obtained against C. albicans biofilm formation (after 24 hours). A biosurfactant concentration of 2 mg/ml was the most effective.

In addition, the antifungal activity of AC7BS against planktonic cells and pre-formed biofilms of the three C. albicans strains was evaluated, but no effect was found.

We conclude that AC7BS shows a promising activity against C. albicans adhesion and biofilm growth. It therefore has potential to be used as a coating agent for medical material, in order to prevent biofilm formation on its surface. Considering further research, it would be interesting to evaluate the effect of AC7BS against other fungi or bacterial species. In this way, the overall preventive effect of pre-coating with AC7BS against biofilm formation could be examined. Improvements should be made to the pre-coating methodology in order to covalently attach AC7BS to the surface of the silicone disks. Moreover, combining the biosurfactant with an antifungal drug could result in a synergistic effect. Finally, pre-coating other kinds of surfaces with AC7BS is another interesting field to investigate.
SAMENVATTING


Het biosurfactant AC7 werd geëxtraheerd uit supernatants van *Bacillus subtilis* AC7. Zijn kritische micellaire concentratie (CMC) bleek 45.7 µg/ml. Dit geeft aan dat AC7BS een relatief efficiënt biosurfactant is.

Siliconen plaatjes werden gebruikt om het oppervlak van een centraal veneuze katheter na te bootsen. Ze werden geprecoat met AC7BS. Zo kon het anti-adhesief en anti-biofilm effect tegen de volgende fungi onderzocht worden: *C. albicans* 40, *C. albicans* 42 en *C. albicans* IHEM 2894. AC7BS veroorzaakte een significante inhibitie van adhesie na 90 minuten (gemiddeld 54 %), zowel in verband met het aantal levende cellen als voor de biomassa van de aangehechte *C. albicans* cellen. Gelijkaardige resultaten (rond 49 % inhibitie) werden gevonden voor biofilmvorming na 24 uur. De meest efficiënte biosurfactant concentratie bleek 2 mg/ml te zijn.

Vervolgens werd de antimycotische activiteit van AC7BS tegen planktonische cellen en voorgevormde biofilms van *C. albicans* geëvalueerd. Er werd geen effect vastgesteld.

We concluderen dat AC7BS een veelbelovende werking tegen *C. albicans* adhesie en groei van de biofilm vertoond. Om die reden heeft het potentieel in coatings voor medisch materiaal, om biofilmvorming op hun oppervlak te voorkomen. Met het oog op verder onderzoek zou het interessant zijn om het effect van AC7BS tegen biofilmvorming van andere fungi of bacteriën te evalueren. Op die manier kan het algemeen preventief effect van AC7BS onderzocht worden. De precoating kan nog geoptimaliseerd door het biosurfactant covalent te binden aan de siliconen disks. Daarnaast zou een combinatie van het biosurfactant met een antimycoticum voor een synergistisch effect kunnen zorgen. Ook precoating van andere soorten oppervlakten met AC7BS is een interessant onderzoeksveld.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BS</td>
<td>Biosurfactant</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Crystal Violet</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>MEL</td>
<td>Mannosylerythritol lipid</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rate per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SAC</td>
<td>Surface active compounds</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud Dextrose Agar</td>
</tr>
<tr>
<td>YNBD</td>
<td>Yeast Nitrogen Base Dextrose</td>
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1. INTRODUCTION

1.1. BIOFILM

1.1.1. Definition and structure

A biofilm can be defined as: “a community of microorganisms attached to a surface, producing extracellular polymeric substances and exhibiting an alternate phenotype compared with corresponding planktonic cells” (Roberts, 2013).

In their natural environment, most microorganisms do not live separately as planktonic cells. They rather have a tendency to attach to surfaces, where they will form a biofilm. The cells that are part of a biofilm commonly have different phenotypic characteristics in comparison with planktonic cells of the same strain (Donlan & Costerton, 2002; Ramage et al., 2006). Biofilms can be formed on living as well as on non-living surfaces, including human tissues and almost all kinds of medical devices (Tournu & Van Dijck, 2012).

The bacteria or fungi in a biofilm are surrounded by a self-produced matrix. This matrix acts as a cement between the cells to stabilize the biofilm, and it protects the cells from disturbing influences of their surroundings (Nobile et al., 2009). This explains partly why biofilms are, in comparison to planktonic cells, more resistant to both host immune defenses and to antibiotics or antifungal drugs (Ramage et al., 2006). The extracellular matrix is a hydrated and locally charged gel-like structure. It contains primarily exopolysaccharides, but also proteins, fatty acids and nucleic acids. The compounds of the biofilm vary with different factors such as species present in the biofilm, age of the biofilm and environmental influences (Al-Fattani & Douglas, 2006; Donlan, 2002).

The structure of a biofilm is heterogeneous and complex. Approximately 85 % of the total volume is occupied by the extracellular matrix, while the other 15 % consists of the sessile cells. The cells are mostly arranged into tower- or mushroom-formed microcolonies, embedded in the matrix (figure 1) (Donlan & Costerton, 2002). Water channels are present in between the microcolonies. This complex structure makes it possible to circulate nutrients
through the biofilm or to remove waste products (Al-Fattani & Douglas, 2006; Ramage et al., 2006).

![Figure 1.1: Schematic structure of a biofilm. (Donlan & Costerton, 2002)](image)

**1.1.2. Biofilms on medical devices**

In many cases, hospital-acquired infections involve biofilms on medical devices (Kojic & Darouiche, 2004). Besides, microbial infections in general are often associated with biofilms (Ramage et al., 2006). Biofilm-forming microorganisms can account for a variety of diseases, such as native valve endocarditis, otitis media, chronic prostatitis, cystic fibrosis and periodontitis (Donlan, 2002).

Biofilms have been reported on different medical devices and biomaterials, such as voice prostheses, central and urinary catheters, intra-uterine devices, contact lenses, stents and shunts (Andes et al., 2004; Chatterjee et al., 2014; Imamura et al., 2008; Ramage et al., 2001; Rodrigues et al., 2005).

When a medical device comes in contact with body fluids such as blood, urine or saliva, a conditioning film will coat its surface. Due to host proteins, this film facilitates adherence of microorganisms on the device and thus the formation of a biofilm (Donlan & Costerton, 2002; Ramage et al., 2001; Tournu & Van Dijck, 2012).

Biofilms have an enhanced resistance against antimicrobial agents in comparison to planktonic cells. Host immune defenses mostly do not have a great effect against biofilms.
either (Ramage et al., 2005). This is why biofilm formation on medical devices can cause severe infections that are very difficult to eliminate. In many cases, the device has to be removed or replaced to eradicate the infection, often with bad consequences for the patients’ health and high additional costs (Martinez & Fries, 2010; Ramage et al., 2006).

Indwelling medical devices can get contaminated by commensals of the patient that are present nearby the implanted device, or contamination can also occur due to manipulation by medical staff before or during implantation (Ramage et al., 2006).

In order to prevent contamination of medical devices, hygienic measures should be taken. Regular disinfection of equipment and environment and correct hand washing by medical staff are important to prevent the adhesion of microorganisms. On the other hand, too frequent disinfection can result in less efficiency (Fracchia et al., 2012). For reusable medical devices such as surgical instruments or endoscopes, biofilms do not get the chance to develop if the devices are sterilized or disinfected, rinsed and dried directly after use. Only if the microorganisms are in contact for a longer time on the device, they can get irreversibly attached (Roberts, 2013).

1.2. CANDIDA ALBICANS

1.2.1. Characteristics

*C. albicans* is a fungus that commonly occurs as a commensal in the gastrointestinal and vaginal flora of many healthy individuals. It is also an opportunistic pathogen: this microorganism can cause severe infections in case of a deteriorated immune system of the patient (Tournu & Van Dijck, 2012). For instance, cancer patients, HIV-patients, elderly people, patients who take immunosuppressive drugs or antibiotics, have a higher risk of uncontrolled *Candida* proliferation (Ramage et al., 2001). *C. albicans* is also one of the most common microorganisms causing nosocomial infections (Chandra et al., 2001).

*Candida* overgrowth can be found on superficial tissue such as skin, throat, vaginal mucosa, etc. It can also cause systemic diseases. Systemic candidiasis is commonly related to indwelling medical devices, since they form an excellent substrate for *Candida* biofilms. This
infection is often very severe, with a high mortality rate despite intensive antifungal therapy (Chandra et al., 2001).

**1.2.2. Polymorphism**

*C. albicans* is a polymorphic fungus, which is an important aspect of the virulence of *C. albicans* (Łukaszewicz, 2012). As figure 1.2 indicates, *C. albicans* can grow as yeast cells, pseudohyphae (ovoid cells that are connected at the sides) and true hyphae (elongated tube-shaped cells). These three morphologies can switch from one to another, although switching between pseudohyphae and true hyphae is not common.

![Yeast cells, Pseudohyphae, True hyphae](http://www.usask.ca/biology/kaminskyj/collab.html)

**Figure 1.2**: *C. albicans* grows in different morphologies: yeast cells, pseudohyphae and true hyphae. ([http://www.usask.ca/biology/kaminskyj/collab.html](http://www.usask.ca/biology/kaminskyj/collab.html), 19/04/2014)

Both yeast and hyphal forms are found in biofilms, and they are both necessary for survival and building of a biofilm. The transition between yeast and hyphal growth is why *C. albicans* is often marked as a dimorphic fungus. On the one hand, hyphal forms are more invasive in host tissue. Yeast cells, on the other hand, can leave the biofilm to spread and to start a new biofilm elsewhere (Berman, 2006; Fox & Nobile, 2012). Additional morphologies of *C. albicans* are white and opaque cells, and chlamydospores (Mayer et al., 2013).

**1.2.3. Virulence factors of *C. albicans***

Next to dimorphism and biofilm formation, various other factors contribute to the pathogenicity of *C. albicans*. Recently, Mayer et al. (2013) reviewed the most important virulence factors and pathogenicity mechanisms of *C. albicans*. 


For instance, to encourage the adhesion of the cell to other microorganisms or surfaces, *C. albicans* owns special proteins called adhesins. Furthermore, *C. albicans* has the ability to sense contact with a surface. Touching a surface will encourage switching from yeast cells to hyphal forms. This will facilitate biofilm formation and invasion of tissues. If all hyphae grow in a particular direction, this phenomena is called thigmotropism (Mayer et al., 2013).

Next to this, *C. albicans* is capable of synthesizing hydrolases, such as proteases, phospholipases and lipases. Phospholipases can rupture the cell membrane of host cells, which will help the fungus to penetrate host tissues. Proteinases can damage important proteins of the host including collagen, keratin, mucin, and cytokines (Borst & Fluit, 2003; Łukaszewicz, 2012; Mayer et al., 2013).

An important virulence factor of *C. albicans* is its cell wall, because this is the place of contact between the cell and its environment. The cell wall contains antigens and protects the cell against host immune defenses. The most important components of this fungal cell wall are glucan, chitin and mannoproteins (Chaffin, 2008; Ruiz-Herrera et al., 2006). The glucans, mainly β-1,3-glucan and β-1,6-glucan, give the cell physical strength and determine its specific shape together with chitin. Because of the different morphologies of *C. albicans*, this shape can change dramatically. The mannoproteins are positioned at the outside of the cell wall. They are responsible for its immunomodulatory activity (Chaffin et al., 1998; Kapteyn et al., 2000).

*C. albicans* is capable of surviving in very diverse environments. It perfectly adapts to a broad pH range, especially to acidic pH’s. This is necessary to deal with the diverse pH values in the gastrointestinal tract and in the rest of the human body. *Candida* strains can even slightly adjust the pH themselves by actively alkalinizing the environment, for example in times of starvation. The higher pH value will induce switching to hyphae. *C. albicans* can also adapt to changes in nutrient availability because of its metabolic flexibility (Mayer et al., 2013).

### 1.2.4. *C. albicans* biofilm formation

In figure 1.3, the different steps in *C. albicans* biofilm formation are illustrated. First, round yeast cells attach to a surface. In the case of a medical device, a conditioning film with
organic material is formed when the device comes in contact with a body fluid, and this will facilitate the adhesion.

In the second step, the yeast cells proliferate by budding. A basal layer of *C. albicans* cells is formed on the surface. Next, switching occurs from yeast cells to pseudohyphae and hyphae. Also, the extracellular matrix is produced, which contains mainly polysaccharides, carbohydrate and proteins. The biofilm is now mature (Fox & Nobile, 2012; Kojic & Darouiche, 2004; Roberts, 2013). It is a very complex structure that can reach a thickness of 350 µm (Martinez & Fries, 2010).

![Fig 1.3: C. albicans biofilm development over time (Fox & Nobile, 2012)](image)

Finally, yeast cells are dispersed from the biofilm to colonize new surfaces. The development of the biofilm can easily be disturbed or altered by a lot of factors such as temperature, nutrient availability, nature of the surface, flow rate and inoculum density (Roberts, 2013).

**1.2.5. Antifungal resistance of the *C. albicans* biofilm**

The resistance of a *C. albicans* biofilm against antifungal agents is much higher in comparison to planktonic cells. For example, for the common antifungal drugs amphotericin, fluconazole,itraconazole and ketoconazole, resistance can be up to 2000 times higher (Martinez & Fries, 2010). The fact that biofilms are so difficult to eradicate, is a major
problem in a hospital environment. Bloodstream infections are nowadays frequently caused by *C. albicans* biofilms, mostly on a catheter (Al-Fattani & Douglas, 2006).

The mechanisms behind biofilm resistance are complex and multifactorial. The fact that antifungal agents can have difficulties penetrating the biofilm matrix, is one of the contributing factors to resistance. The effect of the antifungal drugs is also reduced because of the lower growth rate of sessile cells in comparison with planktonic cells. In addition, the cells in the biofilm can express resistance genes, coding for efflux pumps. This pump will actively transfer the antifungal compounds out of the cell before they can have any effect. Another major problem is the presence of ‘persister’ cells in the biofilms, which are resistant to antifungal compounds. Persister cells are genetically identical to the other cells in the biofilm, but they are dormant-like cells: they have a very low metabolic activity (Barraud et al., 2013; Ramage et al., 2006).

### 1.3. BIOSURFACTANTS

#### 1.3.1. Definition

Many bacterial genera are capable of producing surface-active compounds (SACs). Those molecules are amphipathic: their molecular structure contains both a hydrophobic and a hydrophilic part (Bodour et al., 2004a). Surface-active compounds produced by microorganisms can be divided into two classes: biosurfactants and bioemulsifiers. Although these terms are often mixed, scientists generally refer to biosurfactants as low-molecular-weight compounds, which can reduce tension between air/liquid interfaces or between immiscible liquids. Bioemulsifiers on the other hand, are high-molecular-weight polymers. They have the ability to stabilize oil-in-water emulsions, without considerably lowering the surface tension (Fracchia et al., 2010).

The molecular structure of biosurfactants varies a lot. Especially for the polar hydrophilic parts, a broad range can be found. On this basis, biosurfactants can be divided into different classes such as glycolipids, lipoproteins, phospholipids or polymeric compounds (Bodour et al., 2004).
1.3.2. Characteristics

SACs are able to alter the properties of interfaces. When added to a heterogeneous system, they will migrate to the phase boundaries and form a molecular layer at the interface. Their hydrophilic moieties will face the polar or hydrophilic phase, while the hydrophobic moieties will prefer to stay in the hydrophobic or apolar phase. In this way, biosurfactants can be useful in many applications: among other things, they are capable of stabilizing emulsions, lowering the surface tension, wetting surfaces and foaming (Rodrigues et al., 2006; reviewed by Fracchia et al., 2012).

Biosurfactants have the ability to lower the surface tension at interfaces. The surface tension can be defined as “the surface free energy per unit area required to bring a molecule from the bulk phase to the surface” (Soberon-Chavez & Maier, 2011). The surface tension of the system varies with the concentration of surfactant. At low concentrations, adding more surfactant results in a larger decrease of the surface tension, because more amphiphilic molecules will accumulate at the interface. When the biosurfactant concentration reaches the critical micelle concentration (CMC), the lowest surface tension is obtained. At concentrations higher than the CMC, no more decrease of the surface tension is observed.

At the CMC, biosurfactant molecules will start to form structured aggregates inside the solution, such as micelles, vesicles or continuous bilayers. If the environment is aqueous, the hydrophilic moieties face the outside of the aggregates, while the hydrophobic parts are packed together in the center. If the environment is apolar, the biosurfactant molecules will be organized the other way around (Pacwa-Płociniczak et al., 2011; Soberon-Chavez & Maier, 2011).

In general, a biosurfactant with a low CMC is more efficient. If the concentration at which micelles are formed is low, only a low amount of surfactant is needed to obtain a sufficient decrease of the surface tension (Pacwa-Płociniczak et al., 2011).
1.3.3. Classification and structure

1.3.3.1. Low molecular weight compounds

The two best known classes of low molecular weight biosurfactants are lipopeptides and glycolipids. For lipopeptides, bacteria of the *Bacillus* genus are the main producers. The hydrophilic moiety of lipopeptides contains a peptide chain, often cyclic. For example, the best known biosurfactant in this class, surfactin, is formed by a ring of seven amino acids connected to a \(\beta\)-hydroxyl-fatty acid chain (figure 1.4).

![Figure 1.4: Structure of surfactin (Zhao et al., 2012)](image)

Surfactin is mostly produced by *Bacillus subtilis*. It causes a large reduction of surface tension, and is therefore known as one of the most powerful biosurfactants discovered so far. Generally, surfactin is found as a mixture of different isoforms, due to small differences in the peptide sequence or the length of the fatty acid chain. The availability of amino acids in the surrounding of the bacteria affects the formation of these different isoforms, which will also possess diverse physiological properties (reviewed by Fracchia et al., 2012; Zhao et al., 2012). Other important examples of lipopeptides besides surfactin are lichenysin, pumilacidin, iturin and fengycin (Reviewed by Fracchia et al., 2012).

Glycolipids, on the other hand, are mostly mono- or disaccharides acylated with fatty acids. Some members of this class are rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs). Rhamnolipids are mainly synthesized by *Pseudomonas* and *Burkholderia* species, while sophorolipids and MELs are both produced by different fungi. In figure 1.5, an example of a dirhamnolipid is presented (reviewed by Banat et al., 2010 and by Letizia Fracchia et al., 2012).
1.3.3.2. High molecular weight compounds

Another class of surface active compounds has a much higher average molecular weight than the previously discussed biosurfactants, because of their polymeric structure. This high molecular weight compounds or bioemulsifiers are polymers of polysaccharides, lipopolysaccharides, proteins or lipoproteins.

They do not have the ability to lower the surface tension as much as low molecular weight compounds. On the other hand, they are very useful to stabilize emulsions. Bioemulsifiers can be produced by various bacterial species, for example *Acinetobacter* species. The best studied high molecular weight compound is emulsan (figure 1.6). This is a lipopolysaccharide polymer synthesized by *Acinetobacter calcoaceticus* (Banat et al., 2010; Smyth et al., 2010).
1.3.4. Natural roles of biosurfactants

Surface active compounds produced by microorganisms are sometimes seen as secondary metabolites and possibly waste products. However, biosurfactants are often useful, and they can even be essential for survival of the microorganisms which synthesize it. Because of the very diverse chemical structure of biosurfactants, various natural roles can be attributed to them (Rodrigues et al., 2006; Ron & Rosenberg, 2001).

Some biosurfactants can help the cell in the uptake of insoluble substrates. Others are useful for increasing the bioavailability of hydrophobic substrates, thus facilitating microbial growth on its surface. Besides, biosurfactants can play an important role in cell-to-cell communication. They facilitate biofilm adhesion and formation, and they may play a role in quorum sensing. They can also give an advantage to the surfactant-producing cells during competition between microorganisms, for example thanks to their antimicrobial properties. Some microorganisms even need a biosurfactant to facilitate their motility. It has also been proved that surface active compounds such as some rhamnolipids and high molecular weight compounds, can bind heavy metals, thus eliminating toxic effects of them (Fracchia et al., 2010; Rodrigues et al., 2006; Ron & Rosenberg, 2001; Vatsa et al., 2010).

1.3.5. Advantages of biosurfactants over surfactants of chemical origin

Surfactants are used for a wide variety of industrial and other applications. In the past, almost all surfactants were chemically produced from petrochemical sources (Desai & Banat, 1997). Currently, a great interest goes out to biosurfactants, since they present many advantages over chemical surfactants.

First of all, biosurfactants often have very specific effects and are highly selective: some can specifically work at extreme temperatures or pH values. Biosurfactants are also considered safer to use than chemical surfactants. Their toxicity is much lower, they are generally more biodegradable, and are usually better digestible. These properties are important in food and pharmaceutical industries. Biosurfactants are also more environmentally friendly than chemical ones: this can mean a big advantage in times of high standards for health care and strict regulation against pollution (Desai & Banat, 1997; Fracchia et al., 2012; Kosaric, 1992; Rodrigues et al., 2006).
1.3.6. Biomedical applications of biosurfactants

Thanks to their antibacterial, antiviral and antifungal effects, biosurfactants can be used in medical settings. They are also known to have an anti-adhesive effect: they can prevent cells to attach to a surface or infection site (Banat et al., 2010). There even are biosurfactants which have a immunomodulatory or antitumoral action (Soberon-Chavez & Maier, 2011).

1.3.6.1. Antimicrobial action

Some biosurfactants can be considered as effective and safe therapeutic agents which could successfully substitute for current antifungal, antiviral and antibacterial drugs (Banat et al., 2010; Rodrigues et al., 2006; Rufino et al., 2011). Biosurfactants have antimicrobial properties due to their ability to damage the physical structure of membranes. This can lead to increased membrane permeability and leakage of metabolites. The biosurfactant molecules can also disrupt protein conformations of the membrane, resulting in defective membrane functions. Finally, cell lysis occurs and the microorganism is destroyed (reviewed by Banat et al., 2010 and by Fracchia et al., 2012).

This membrane rupture occurs to Gram-positive as well as to Gram-negative bacteria, suggesting a nonspecific effect of biosurfactants (Lu et al., 2007). Lipopeptides are the best known class of biosurfactants with antibacterial effects. Particularly a SAC synthesized by Bacillus subtilis named surfactin, has been shown to have a strong activity.

Flocculosine is a glycolipid produced by the fungus Pseudozyma flocculosa. Among other characteristics, it has been proven in vitro to have an effect against a broad range of fungi, including Candida species and Saccharomyces cerevisiae (Mimee et al., 2009; Mimee et al., 2005). Next to flocculosine, a whole panel of other biosurfactants, for example glycolipids as well as lipopeptides or rhamnolipids, show an inhibiting activity against fungi (Debode et al., 2007; Grover et al., 2010; Kulakovskaya et al., 2009; Varnier et al., 2009).

The antiviral activity of biosurfactants is much more effective for enveloped viruses, such as Herpes viruses or HIV, than for non-enveloped viruses. This is proof for the suggestion that the antiviral effect of biosurfactants is mainly due to the interaction with the envelope of the virus, which will lead to loss of important viral proteins (Vollenbroich et al., 1997).
1.3.6.2. Anti-adhesive effects

Biosurfactants can inhibit the adhesion of microorganisms to solid interfaces and infection sites (Rodrigues & Teixeira, 2010). When biosurfactants adsorb to a surface, its characteristics will change. The surface becomes more hydrophobic, which can inhibit microbial adhesion (Rufino et al., 2011). The primary adhesion of microorganisms to a surface is an important target to prevent its colonization and biofilm formation. By precoating the medical devices with a biosurfactant solution of the right concentration, a strong inhibition of both adhesion of microorganisms and biofilm formation on the surface has been found (Rodrigues et al., 2006).

1.3.7. Biosurfactants produced by B. subtilis

*B. subtilis* is a Gram-positive, spore forming bacteria that is able to synthesize surface active compounds (Priya & Usharani, 2009). Surfactin is the most important one, but among many others, fengycin and iturin are also produced. The biosurfactant solution extracted from the supernatant of *B. subtilis* strains can therefore be useful for many biomedical applications (Cheng et al., 2013; Kim et al., 2009). Thanks to the great emulsification activity of these biosurfactants, they are also promising for use in oil fields for enhanced oil recovery (Al-Bahry et al., 2013; Pathak & Keharia, 2013).
2. OBJECTIVES

The formation of a biofilm by pathogenic microorganisms can cause severe infections that are very difficult to eradicate. Especially in a hospital environment, biofilm formation on medical devices can imply serious complications. A well-known problem nowadays is the increasing resistance of almost all microorganisms against current antimicrobial therapy. Besides, the bacteria or fungi present in a biofilm are even more resistant to antimicrobial agents than planktonic cells of the same strain.

Research for new compounds that can prevent the formation of biofilms or that could even eradicate biofilm-related infections, is therefore important. Lately, lots of interest has gone out to biosurfactants, which are surface-active compounds produced by bacteria. Because of their antimicrobial and/or anti-adhesive effect, some biosurfactants can be considered as valuable alternatives for current antibiotics or antifungals (Rodrigues et al., 2006).

AC7BS is a biosurfactant produced by Bacillus subtilis AC7 with great potential as an anti-adhesive and as an inhibitor of biofilm formation. The aim of this master thesis is to extract AC7BS from the bacterial supernatant, and to examine its effect against the fungus C. albicans. To mimic central venous catheter material, silicone disks are used. Each experiment is carried out with the following three strains: C. albicans 40, C. albicans 42 and C. albicans IHEM 2894.

- AC7BS is isolated from the supernatant of B. subtilis AC7 and its critical micelle concentration is determined.
- The effect of pre-coating silicone disks with AC7BS is tested after both the adhesion phase (90 minutes of incubation) and the growth phase (after 24 hours).
  - The anti-adhesive effect of AC7BS at four different concentrations (0.5, 1, 2, 3 mg/mL) is examined by measuring the biomass of the attached C. albicans cells after 90 minutes using crystal violet staining.
  - The anti-biofilm formation effect of AC7BS at four different concentrations (0.5, 1, 2, 3 mg/mL) is examined by measuring the biomass of the mature C. albicans biofilms (after 24 hours) using crystal violet staining.
The anti-adhesive effect of 2 mg/mL AC7BS is examined by measuring the viability of the attached *C. albicans* cells after 90 minutes using the plate count (pour plate) method.

The anti-biofilm formation effect of 2 mg/mL AC7BS is examined by measuring the viability of the mature *C. albicans* biofilms (after 24 hours) using the plate (pour plate) method.

- The antifungal activity of different concentrations of AC7BS (0.03 - 3 mg/mL) on planktonic *C. albicans* cells is tested by comparing the absorbance values of the suspensions which were exposed to AC7BS to the suspensions which were not.

- The antifungal activity of different concentrations of AC7BS (0.06 – 3 mg/mL) on a pre-formed *C. albicans* biofilm is studied using the XTT method.
3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Instruments

- Autoclave: PBI International, FEDEGARI AUTOCLAVI SPA
- Balance 1: KERN 440-35N (sensitivity: 0.01g), LAISS
- Balance 2: CP324S-OCE (sensitivity: 0.0001g), Sartorius
- LAF unit: type HS15 and type HS18, Kendro Laboratory products
- Magnetic stirrer: ARED heating magnetic stirrer high power VELP scientific
- Microscope: SMZ200 Type 104, Nikon
- pH-meter: pH 211 microprocessor pH-meter, HANNA Instruments
- Rotary evaporator: RV10 basic, IKA
- Sonicator: Elma S30H, Elmasonic
- Spectrophotometer: Genova Plus, Jenway
- Tensiometer: Sigma 703, KSV
- Vortex: TOPMIX FB 15024, Fisher Scientific

3.1.2. Materials

- 0.4 μm Filter: single use filter unit, non-pyrogenic, Minisart
- 0.22 μm Filter: single use filter unit, non-pyrogenic, Minisart
- Petri dish: Enrico Bruno s.r.l.
- 12-well plate: Greiner bio-one
- 24-well plate: Costar 3526, Corning Incorporated
-96-well plate: Enrico Bruno s.r.l.

-Silicone disk: 12-well (15 mm Ø, 1.5 mm thick) and 24-well (1 cm Ø, 1.5mm thick)

TECNOEXTR s.r.l.

-Tube 50 mL: Sarstedt

-Tube 10 mL: Sarstedt

3.1.3. Media

-LB (Luria Bertani broth): this is a nutritionally rich medium, used for the general cultivation of microorganisms. Twenty-five grams of LB powder (Fluka) are weighed and dissolved in 1 L distilled water using the magnetic stirrer to dissolve all the powder. LB agar is prepared by adding 10 g of LB powder (Fluka) and 6 g of agar (Fluka) in 400 mL distilled water. The pH is 7.5. The medium is sterilized with the autoclave at 121°C for 15 minutes.

-SDA (Sabouraud Dextrose Agar): this is a complex medium which promotes fungal growth. Mycoligical peptone provides nitrogenous compounds. Dextrose is used as an energy source. The acidic pH favors fungal growth and inhibits growth of bacteria. Agar is used as a solidification agent. SDA is prepared by dissolving 12 g of Sabouraud Dextrose Broth and 6 g of agar (Fluka) in 400 mL distilled water. The pH is 5.6. The medium is sterilized with the autoclave at 121°C for 15 minutes.

-RPMI 1640 2% glucose (Roswell Park Memorial Institute) (2X): this is prepared by dissolving 1.0 g RPMI 1640 (Sigma-Aldrich), 3.45 g MOPS (Sigma-Aldrich) and 1.8 g glucose (Biolife) in 50 mL of distilled water. This pH is adjusted to 7.0 by adding 1M NaOH solution. As autoclaving can degrade media constituents, it is sterilized through 0.22 µm filter.

-YNBD (Yeast Nitrogen Base Dextrose): this medium is prepared by dissolving 0.67 g of YNB powder (Fluka) and 0.9 g of dextrose (Biolife) in 100 mL milli-Q water using a rotating magnet. In order to mimic the growth of the yeast at physiological conditions, the pH is set to 6.8 with 0.1 M NaOH solution. As autoclaving can degrade media constituents it is sterilized through a 0.22 µm filter.
-YNBD (2X): this medium is prepared by dissolving 0.67 g of YNB powder (Fluka) and 0.9 g of dextrose (Biolife) in 50 mL milli-Q water using a rotating magnet. In order to mimic the growth of the yeast at physiological conditions the pH is set to 6.8 with 0.1 M NaOH solution. As autoclaving can degrade media constituents it is sterilized through 0.22 µm filter.

3.1.4. Solutions

- PBS (Phosphate Buffered Saline): 4 g NaCl (Sigma-Aldrich), 0.1 g KCl (Sigma-Aldrich), 0.72 g NaH2PO4 (Sigma-Aldrich) and 0.12 g KH2PO4 (Sigma-Aldrich) are added to 500 mL distilled water. The magnetic stirrer is used to dissolve this mixture. The pH is at 7.4. The solution is sterilized for 15 minutes at 121°C.

- CV (Crystal Violet) solution: stock solution (CV 2%) is obtained by mixing solution B (0.8 g of ammonium oxalate (Fluka) in 80 mL distilled water) with solution A (2.0 g crystal violet (Fluka) in 20 mL 95 % ethanol (SigmaAldrich)). This final solution can only be used after 24 hours to obtain a 0.2 % working solution in distilled water.

- FBS (Fetal Bovine Serum) (Biochrom AG): This is the blood serum obtained from a bovine fetus. It is used to form a conditioning film that promotes Candida albicans early adhesion on the surface of silicone disks. It is stored at -20 °C.

- XTT solution (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma- Aldrich): Stock solution (5 mg/mL) is obtained by dissolving 5 mg of XTT in 1 mL of prewarmed PBS. Subsequently it is diluted 1:5 (vol/vol) in prewarmed PBS to obtain a 1 mg/mL working solution before use. Stock solution of XTT can be stored for a week at -20 °C.

- Menadione (Sigma- Aldrich) solution: Stock solution (10 mM) is obtained by dissolving 0.017 g of menadione in 10 mL of acetone. Subsequently it is diluted 1:10 (vol/vol) in PBS to obtain a 1-mM working solution before use. Stock solution of menadione can be stored for a week at -20 °C.

3.1.5. Bacterial and fungal strains

- Bacillus subtilis AC7: This endophytic biosurfactant-producer strain was isolated from a Robinia pseudoacacia tree in Novara. The culture is stored at -80 °C in Luria Bertani broth with 25 % v/v glycerol in order to prevent the formation of water crystals in the cells.
- *Candida albicans* 40: this strain has been isolated from a central venous catheter and obtained from the Microbiology Laboratory of the hospital “Maggiore della Carità” in Novara. The strain is stored at -80 °C in Sabouraud Dextrose broth, supplemented with 25% v/v glycerol for the same reason described above.

- *Candida albicans* 42: this strain has been isolated from a urinary catheter and obtained from the Microbiology Laboratory of the hospital “Maggiore della Carità” in Novara. The strain is stored at -80 °C in Sabouraud Dextrose broth, supplemented with 25% v/v glycerol for the same reason described above.

- *Candida albicans* IHEM 2894: this strain, isolated from the human tongue, is provided by the Belgian Co-ordinated Collections of Microorganisms (BCCM). The strain is stored at -80 °C in Sabouraud Dextrose broth, supplemented with 25% v/v glycerol for the same reason described above.

### 3.2. METHODS

#### 3.2.1. Production of biosurfactant AC7BS solution

3.2.1.1. Production

A few colonies of *Bacillus subtilis* are streaked off an agar plate and suspended in 10 mL of LB medium. This suspension is incubated at 28 °C for 4 hours on a rotary shaker with a rpm of 200. After that, 2 mL of the inoculum is added to 500 mL of fresh LB and again incubated at 28 °C, but now for 24 hours at 120 rpm. Subsequently, the suspension is centrifuged for 20 minutes at 5000 rpm. After collecting the supernatant, in which the biosurfactant is present, it is acidified with a 6 M HCl solution. Due to the obtained pH of 2, the biosurfactant will precipitate. The supernatant is stored in the fridge overnight. The cold temperatures facilitate the biosurfactant precipitation.

3.2.1.2. Extraction

A liquid-liquid extraction of the biosurfactant is done in a separatory funnel. As an extraction solvent, methanol/ethyl acetate (1:4) is used. Methanol is responsible for capture
of the biosurfactant from the supernatant, while ethyl acetate serves as a good organic solvent to ensure phase separation.

600 mL of the supernatant and 200 mL of the extraction solvent are added to the separatory funnel. The funnel is then shaken in order to mix the phases: at this moment, the biosurfactant moves from the supernatant to the extraction solvent. From time to time, the tap of the funnel should be opened while it is held upside down so that the excess of gas can be released. When a clear separation of the phases can be observed, the two phases are removed separately from the funnel. The aqueous phase is subjected to two other extractions with new extraction solvent in order to move all of the biosurfactant into the organic phase.

After the extractions, the collected organic phase will be turbid: due to the hygroscopic activity of methanol, water has been drawn into the organic phase. Therefore, anhydrous sodium sulfate (Na$_2$SO$_4$) is added, which has the ability to capture water. Subsequently, the organic solvent is evaporated under vacuum conditions by a rotary evaporator. The raw biosurfactant remains. It can be recovered by adding some acetone, which will evaporate spontaneously afterwards. Out of 1 L of supernatant, approximately 0.6 g biosurfactant can be obtained.

### 3.2.1.3. Preparation of biosurfactant stock solution

The preparation of 50 mL 6 mg/mL solution is explained. 300 mg of biosurfactant AC7BS is weighed in a beaker and 30 mL of PBS is added. The beaker is placed on a magnetic stirrer and a few droplets of NaOH 1 M are added to dissolve the biosurfactant. Afterwards, the pH is brought back to 7 with a HCl solution. PBS is added until the correct volume of 50 mL is obtained. To sterilize the solution, it is passed through a 0.4 µm filter into a sterile flask. For the experiments, the stock solution is diluted in PBS.

### 3.2.2. Critical micelle concentration of AC7BS

In order to find the critical micelle concentration of the obtained biosurfactant, the reduction in surface tension is measured with a tensiometer (KSV Sigma 703 D). This instrument will indicate the force necessary to pull a ring out of the solution with constant velocity. Serial dilutions (1:2) of a 3 mg/mL AC7BS solution are made with alkaline
demineralized water (pH 8). First, the tensiometer is calibrated by measuring the surface tension of the alkaline water at pH 8. Next, the surface tension of the AC7BS solutions with different concentrations are measured in quadruplicate. These values are plotted in a graph. The obtained curve can be divided into two sections: a concentration-dependent and a concentration-independent part. The CMC is the concentration value at the intercept of the two extrapolated trend lines of the curve.

3.2.3. Washing of the silicone disks

The silicone disks have to be washed and cleaned before they can be used in the experiments. During the whole procedure, it is necessary to wear gloves in order not to contaminate the disks.

The disks are first washed in a solution of milli-Q water containing 1.4 % detergent (RBS 50 solution by Fluka analytical). By sonicating the beaker containing this solution and the disks for 5 minutes, minuscule particles such as fatty acids are removed from the disks. Subsequently, the disks are transferred one by one into 1 L of milli-Q water, while the surface of the disks is rubbed with the hands. In the same way, the disks are transferred to another beaker containing 1 L of milli-Q water. Afterwards, the disks are put in approximately 50 mL of methanol and sonicated for 5 minutes. The rinsing in 1 L of milli-Q water is repeated twice. Finally, the disks can be dried in petri plates on a paper tissue and sterilized in the autoclave.

3.2.4. C. albicans adhesion and biofilm growth on disks pre-coated with AC7BS: biomass assays

The whole procedure should be done in sterility in a LAF unit, in order to avoid contamination.

3.2.4.1. Pre-coating of the disks with AC7BS biosurfactant

The cleaned and sterilized silicone disks are placed into a 12-well plate and exposed to different concentrations of biosurfactant (0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL). A pure PBS solution serves as a control. For each concentration, 3 different silicone disks are
tested. Figure 3.1 gives an overview of the plates and the concentration of added biosurfactant.

![Figure 3.1: schematic overview of the 12-well plates. Grey = control, green = 0.5 mg/mL, blue = 1 mg/mL, orange = 2 mg/mL, red = 3 mg/mL biosurfactant solution](image)

After adding the biosurfactant solution, the plates are placed on a rotary shaker (140 rpm) at 37°C for 24 hours.

### 3.2.4.2. Preparation of the inoculum

A strain of frozen *C. albicans* is cultured on a SDA plate and incubated at 37 °C for 24 hours. Three different *C. albicans* strains are tested: *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894. The inoculum is prepared by striking some *C. albicans* colonies with a cotton stick from the SDA plate and suspending them in 40 mL of a solution of PBS + 10% fetal bovine serum (FBS). To verify the amount of *C. albicans* cells in the suspension, the optical density is measured with a spectrophotometer at a wavelength of 600 nm: the absorbance should be 1.

To be sure that the inoculum contains approximately the right amount of cells (1 * 10^7 CFU per mL), the exact amount of cells in the inoculum has to be counted. Therefore, a dilution in PBS is made. The 10^{-5}, 10^{-6} and 10^{-7} dilutions are plated on SDA with the pour plate technique. Hence, 1 mL of the dilution is transferred into a petri dish, and after adding a sufficient amount of SDA, the petri dish is swirled in order to mix and to divide the *C. albicans* cells equally on the plate. The SDA should have a temperature of approximately 45 °C when it is added. It must not be too hot, otherwise the yeast cells won’t survive since they cannot tolerate heat. When the agar has become solid, the petri dishes are placed upside
down into the incubator at 37 °C. After some incubation time, the colonies can be seen and counted with the microscope.

3.2.4.3. Adhesion

After 24 hours, the biosurfactant solution is carefully removed from the wells of the 12-well plate described in section 3.2.4.1. After drying, 2 mL of the inoculum of C. albicans is added to the wells. Subsequently, the plates are incubated for 90 minutes at 37 °C in static conditions. After this time, the adhesion phase has occurred: C. albicans cells have attached themselves to the disks. Each well is gently washed three times with PBS in order to remove non-attached C. albicans cells.

3.2.4.4. Biofilm growth

The method as described in 3.2.4.1 and 3.2.4.2 is repeated. After 90 minutes incubation, the disks are transferred carefully to a plate containing 2 mL of a solution of YNBD (yeast nitrogen base with dextrose) with 10 % FBS. This medium will encourage the growth of the biofilm. The plate is incubated for 24 hours at 37 °C at 80 rpm. Afterwards, the medium is removed and the disks are washed twice with PBS.

3.2.4.5. Quantification of the biomass with crystal violet

First, the disks have to dry in the incubator for at least half an hour. The crystal violet staining is then performed. Crystal violet is a dye that attaches to adherent cells and biofilms. Thus, by measuring the amount of attached crystal violet, the mass of adherent cells or biofilm can be determined and compared with each other. As a blank, a new disk is added to the plates. This disk has not been exposed to the biosurfactant nor to the inoculum of C. albicans. It will display the amount of crystal violet that the disks themselves adsorb. This blank value will be subtracted from the values for the other disks.

After exposing the disks to a 0.2 % crystal violet solution for 10 minutes, the disks have to be washed carefully with water in order to remove the excess of dye. The disks can be placed in the incubator to dry when no more dye is released after adding new water.

After this, the remaining crystal violet can be measured. To dissolve the dye, 2 mL of acetic acid (33%) is added to each well containing a disk. The plates are placed on a rotary
shaker (120 rpm) for half an hour. Afterwards, 200 μL from each well is transferred into a well of a 96 well plate. The absorbance of the solution is then measured at a wavelength of 570 nm.

The inhibitory effect of the different concentrations of AC7BS is calculated by comparing the absorbance values with the absorbance of the controls:

\[
\text{% inhibition} = \left(1 - \frac{A_c}{A_0}\right) \times 100
\]  

(3.1)

Where: \(A_0\) = mean absorbance of the control wells  
\(A_c\) = mean absorbance of the wells for a specific concentration of AC7BS

Note: The control blank values should be subtracted from the obtained values.

### 3.2.5. C. albicans adhesion and biofilm growth on disks pre-coated with AC7BS: viability assays

#### 3.2.5.1. C. albicans adhesion and biofilm formation on the pre-coated disks

In this case, the inhibitory effect of AC7BS biosurfactant is only tested in a concentration of 2 mg/mL. In the same way as described in 3.2.4.2, a \(C.\ albicans\) \(\text{OD}_{600} = 1\) suspension is prepared. The procedure for \(C.\ albicans\) adhesion and the formation of the biofilms is equal to the methods described in section 3.2.4.3 and 3.2.4.4. Figure 3.2 shows a schematic overview of the plate for this test.

![Figure 3.2: schematic overview of the 12-well plate. Grey = control; orange = 2 mg/mL biosurfactant](image)
3.2.5.2. Measurement of the viability with the plate count method

After the washing of the disks, they are transferred into separate tubes containing 10 mL of PBS. The sonicator and vortex are used to remove the cells from the disks: the tubes are alternately inserted in the sonicator for 30 seconds and vortexed for 30 seconds. This cycle is repeated 4 times.

After this, dilutions of all the suspensions are made. The $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions (for adhesion phase) and $10^{-4}$, $10^{-5}$ and $10^{-6}$ (for growth phase) are plated using the pour plate method. After 24 hours at 37 °C, the colonies can be counted with the microscope.

The number of CFU per silicone disk is calculated according to equations 3.2 and 3.3:

$$N = \text{colony forming units/mL in primary dilution} = \frac{\sum C}{V (n1+0.1n2)d} \quad (3.2)$$

Where:
- $N$ = colony forming units/mL in primary dilution
- $\sum C$ = sum of colonies on the considered plates
- $V$ = Volume of inoculum plated (in ml)
- $n1$ = number of plates considered for the first dilution
- $n2$ = number of plates considered for the second dilution
- $d$ = factor of dilution corresponding with the first dilution

$$CFU/disk = N \times V_2 \quad (3.3)$$

Where:
- $N$ = colony forming units/mL in primary dilution
- $V_2$ = Volume of the first dilution.

The obtained results are expressed as log_{10} values of CFU/disk. Next, the difference between the colony count for the biofilms that were exposed to the biosurfactant and the colony count for the biofilms that were not is calculated:

$$\% \text{ inhibition} = \left(1 - \frac{X_c}{X_0}\right) \times 100 \quad (3.4)$$

Where:
- $X_0$ = CFU/disk for control disks
- $X_c$ = CFU/disk for disks treated with 2 mg/ml AC7BS
3.2.6. Evaluation of AC7BS antifungal activity against planktonic *C. albicans* cells

The effect of different concentrations of AC7BS on an inoculum of planktonic *C. albicans* cells is tested in a 96-well plate. The following concentrations of biosurfactant solutions are examined: 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.062 mg/mL and 0.031 mg/mL. Figure 3.3 shows a schematic overview of the plate for this test.

![Figure 3.3: schematic overview of the AC7BS concentrations added to the 96-well plate.](image)

Grey = control; brown = 0.031 mg/mL; yellow = 0.062 mg/mL; purple = 0.125 mg/mL; pink = 0.250 mg/mL; green = 0.500 mg/mL; blue = 1 mg/mL; orange = 2 mg/mL; red = 3 mg/mL.

The initial suspension is prepared by adding some *C. albicans* colonies into 5 mL of double concentrated RPMI 2 % glucose. At a wavelength of 550 nm, the suspension should have an optical density between 0.12 and 0.15. This suspension is diluted 1 to 10 in order to arrive at the right concentration for the inoculum. The exact number of cells in the inoculum is counted by making 1:10 serial dilutions. The $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions are plated with SDA.

First, for each BS concentration, 100 µL of a double concentrated BS solution is transferred into the corresponding wells. As a control, pure PBS is used. For each BS concentration, 3 wells are used to measure the effect on planktonic *C. albicans* cells, while 2 other wells serve as blanks. Next, 100 µL of the inoculum is added. For the blanks, double concentrated RPMI is used.
The 96-well plate is incubated at 37 °C in static conditions for 24 hours. Subsequently, the absorbance is measured at 450 nm.

### 3.2.7. Evaluation of AC7BS antifungal activity against a formed *C. albicans* biofilm

The effect of different concentrations of AC7BS on a fully formed *C. albicans* biofilm is tested. Therefore, *C. albicans* biofilms are pre-formed on silicone disks following a similar protocol as in 3.2.4.2., 3.2.4.3 and 3.2.4.4. Also, smaller disks that fit in a 24-well plate are used.

Briefly, an inoculum of *C. albicans* cells in PBS + 10 % FBS is made. The OD_{600} should be 1. The exact number of cells in the inoculum is counted after diluting. The 10^{-5}, 10^{-6} and 10^{-7} dilutions are plated with SDA.

The washed and sterilized silicone disks are transferred into the wells of the 24-well plates. One mL of the inoculum is added. For the blanks, 1 mL of PBS + 10 % FBS is used. The plate is incubated at 37 °C in static conditions. After 90 minutes, the disks are carefully transferred to new plates containing 1 mL YNBD + 10 % FBS in each well. The plate is then incubated for 24 hours at 37 °C at 80 rpm. After this time, the biofilms will be fully formed. The disks are transferred into new plates containing different concentrations of AC7BS, or just YNBD + 10 % FBS for the controls and the blanks. The following biosurfactant concentrations are examined, each of them in triplicate: 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.062 mg/mL. Figure 3.4 shows a schematic overview of the plate for this test.

After 24 hours at 37 °C in static conditions, the disks are transferred into new plates, containing 1 mL of PBS with 12.5 µL of XTT solution (at a concentration of 1 mg/mL) and 1 µL of menadione 1 mM. The plates are incubated for 5 hours at 37 °C at 80 rpm. The viable *C. albicans* cells will convert XTT into colored formazan. Menadione catalyzes this reaction. The optical density is an indication for the metabolic activity of the cells. In the end, 200 µL of each well is transferred into a well of a 96-well plate. The absorbance of the solution can then be measured at 490 nm.
Figure 3.4: schematic overview of the AC7BS concentrations added to the 24-well plate.

Grey = control; red = 3 mg/mL; orange = 2 mg/mL; blue = 1000 µg/ml; green = 500 µg/ml; pink = 250 µg/ml; purple = 125 µg/ml; yellow = 62 µg/ml; brown = blank.

The inhibitory effect of the different concentrations of AC7BS is calculated by comparing the absorbance values with the absorbance of the controls:

\[
\% \text{ inhibition} = \left(1 - \frac{A_c}{A_0}\right) \times 100
\]  

(3.5)

Where: 
\( A_0 = \) mean absorbance of the control wells  
\( A_c = \) mean absorbance of the wells for a specific concentration of AC7BS

Note: The control blank values should be subtracted from the obtained values.

### 3.2.8. Statistical analysis

The statistical program R (R Development Core Team, [http://www.R-project.org](http://www.R-project.org)) is used for the statistical analysis of the results. For the experiments to determine the effect of pre-coating the disks with AC7BS after 90 minutes and 24 hours (section 3.2.4 and 3.2.5), the student t-test is carried out. In the case of the plate count method (section 3.2.5), the number of cells, expressed as CFU/disk, is converted to a log-value in order to obtain a correct Gaussian curve. For the antifungal tests against planktonic cells (section 3.2.6) and biofilms (section 3.2.7), the one way ANOVA test is used.

The standard deviations are calculated and visualized in the graphs with error bars. The level of statistical significance is indicated by the sign *:

* = p < 0.05; ** = p < 0.01; *** = p < 0.001
4. RESULTS

4.1. CRITICAL MICELLE CONCENTRATION OF AC7BS

The CMC of the extracted biosurfactant is determined by measuring the surface tension of solutions with different concentrations of AC7BS. The obtained values are plotted in a graph (figure 4.1).

![CMC Graph](image)

**Figure 4.1: CMC of AC7BS. Plot of the surface tension (mN/m) as a function of the concentration of AC7BS (mg/ml)**

The red part of the graph represents the concentration-dependent section: the surface tension decreases when the BS concentration increases. The second part of the graph is concentration-independent. The CMC value is found at the intercept of the extrapolated linear functions of both sections. In this case, the CMC of AC7BS is 45.7 µg/mL.

4.2. EFFECT OF PRE-COATING DISKS WITH AC7BS ON C. ALBICANS ADHESION AND BIOFILM FORMATION

Silicone disks are pre-coated with different concentrations of biosurfactant AC7. Subsequently, they are exposed to an inoculum of *C. albicans*. The effect on adhesion of *Candida* cells on the disks is tested after 90 minutes. After 24 hours, the biofilms are fully formed and the difference in biofilm formation is measured.

The biomass is determined using crystal violet staining, while the viability is tested with the plate count method. The experiments are repeated for three different *C. albicans* strains: *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894.
4.2.1. Crystal violet assay: effect of AC7BS on biofilm mass

4.2.1.1. Reduction of adhesion

After 90 minutes of incubation, the adhesion phase has occurred. The difference in adhesion of *C. albicans* cells between the disks pre-coated with different concentrations of AC7BS can be observed. To this end, crystal violet is used. Figure 4.2 gives an example of how the disks look after treatment with crystal violet. To quantify the crystal violet staining, the disks are submerged in acetic acid, and the absorbance of these solutions is measured. The data are analyzed using the student t-test.

![Fig 4.2. View of the silicone disks after adhesion phase and crystal violet staining](image)

The inoculum with *C. albicans* 40 contained 1.03 * 10^7 CFU/mL. The obtained net values of absorbance are 0.625 for the controls, 0.481 for a AC7BS concentration of 0.5 mg/mL, 0.408 for 1 mg/mL, 0.256 for 2 mg/mL and 0.333 for 3 mg/mL. This implies a significant reduction of adhesion of 23 % (p<0.01), 35 % (p<0.001), 59 % (p<0.001) and 47 % (p<0.001) respectively for the disks treated with biosurfactant solutions of 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL. In figure 4.3, these results are plotted in a graph.

![Figure 4.3: Reduction of *C. albicans* 40 adhesion after pre-coating disks with AC7BS. Error bars represent the standard deviation. **p<0.01, ***p<0.001](image)
For *C. albicans* 42, the initial suspension contained $1.18 \times 10^7$ CFU/mL. The following net values of absorbance are found: 0.626 for the controls, 0.509 for a AC7BS concentration of 0.5 mg/mL, 0.451 for 1 mg/mL, 0.308 for 2 mg/mL and 0.402 for 3 mg/mL. Therefore, the percentages of reduction of adhesion at AC7BS concentrations of 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL are 19 % (p<0.01), 28 % (p<0.001), 51 % (p<0.001) and 36 % (p<0.001) respectively. Figure 4.4 shows the results.

![Figure 4.4](image1.png)

**Figure 4.4: Reduction of *C. albicans* 42 adhesion after pre-coating disks with AC7BS. Error bars represent the standard deviation. **p<0.01, ***p<0.001**

For *C. albicans* IHEM 2894, the inoculum contained $1.20 \times 10^7$ CFU/mL. The absorbance net values are 0.627 for the controls, 0.506 for a AC7BS concentration of 0.5 mg/mL, 0.427 for 1 mg/mL, 0.287 for 2 mg/mL and 0.378 for 3 mg/mL. The reduction of adhesion on the disks pre-coated with BS solution of 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL are significant: 19 % (p<0.01), 32 % (p<0.01), 54 % (p<0.001) and 40 % (p<0.001) respectively. These results are displayed in figure 4.5.

![Figure 4.5](image2.png)

**Figure 4.5: Reduction of *C. albicans* IHEM 2894 adhesion after pre-coating disks with AC7BS. Error bars represent the standard deviation. **p<0.01, ***p<0.001**
4.2.1.2. Reduction of biofilm formation

After 24 hours of incubation, the biofilms are fully formed. The difference in biomass of the *C. albicans* biofilms between the disks pre-coated with different concentrations of AC7BS can be observed. To quantify this, crystal violet is used. Figure 4.6 gives an example of how the disks look after treatment with crystal violet. To quantify the crystal violet staining, the disks are submerged in acetic acid, and the absorbance of the solutions is measured. The data are analyzed using the student t-test.

![Fig 4.6. View of the silicone disks after biofilm formation and crystal violet staining](image)

The inoculum with *C. albicans* 40 contained $1.03 \times 10^7$ CFU/ml. The obtained net values of absorbance are 3.599 for the controls, 3.390 for a AC7BS concentration of 0.5 mg/mL, 2.718 for 1 mg/mL, 1.700 for 2 mg/mL and 2.440 for 3 mg/mL. Therefore, at BS concentrations of 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL, a significant reduction of biofilm biomass is observed of 6 % (p<0.01), 24 % (p<0.05), 53 % (p<0.001) and 32 % (p<0.05) respectively. In figure 4.7, these results are plotted in a graph.

![Figure 4.7: Reduction of *C. albicans* 40 biofilm formation after pre-coating disks with AC7BS. Error bars represent the standard deviation. *p<0.05, **p<0.01, ***p<0.001](image)

For *C. albicans* 42, the initial suspension contained $1.18 \times 10^7$ CFU/mL. The following absorbance values are found: 3.536 for the blanks, 3.350 for a AC7BS concentration of 0.5
mg/mL, 2.746 for 1 mg/mL, 1.928 for 2 mg/mL and 2.581 for 3 mg/mL. This implies a significant reduction of biofilm mass of 5 % (p<0.01), 22 % (p<0.05), 45 % (p<0.001) and 27 % (p<0.01) for BS concentrations of 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL respectively. Figure 4.8 shows the results.

For C. albicans IHEM 2894, the inoculum contained 1.20 * 10^7 CFU/mL. The absorbance net value is 3.379 for the controls, 3.197 for a AC7BS concentration of 0.5 mg/mL, 2.529 for 1 mg/mL, 1.727 for 2 mg/mL and 2.412 for 3 mg/mL. The significant reduction of biofilm mass for BS concentrations of respectively 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL, is therefore 5 % (p<0.05), 25 % (p<0.05), 49 % (p<0.01) and 29 % (p<0.01). These results are shown in figure 4.9.
4.2.2. Plate count method: effect of AC7BS on viability of the biofilm

4.2.2.1. Reduction of adhesion

After 90 minutes of incubation, the adhesion phase has occurred. The difference in adhesion of *C. albicans* cells between the disks pre-coated with AC7BS (2 mg/mL) and those that were not can be observed. To quantify the viability of the *C. albicans* cells, the plate count method is used. The data are analyzed using the student t-test.

The inoculum with *C. albicans* 40 contained 1.20 * 10^7 CFU/mL. The mean log_{10} values for CFU/disk are 6.85 for the control disks and 6.49 for the disks treated with AC7BS. This indicates a significant reduction of adhesion of 56 % (p<0.01). The mean log_{10} CFU/disk values are plotted in a graph as a function of the AC7BS concentration (figure 4.10).

Fig 4.10: Reduction of *C. albicans* 40 adhesion after pre-coating with 2 mg/mL AC7BS: plate counting. Error bars represent the standard deviation. **p<0.01

For *C. albicans* 42, the initial suspension contained 1.14 * 10^7 CFU/mL. The mean log_{10} CFU/disk is 6.74 for the control disks, and 6.41 for the biosurfactant-treated disks. The adhesion is significantly reduced with 53% (p<0.01). Figure 4.11 gives a view of this result.
Fig 4.11: Reduction of *C. albicans* 42 adhesion after pre-coating with 2 mg/mL AC7BS: plate counting. Error bars represent the standard deviation. **p<0.01

The inoculum for *C. albicans* IHEM 2894 contained $1.20 \times 10^7$ CFU/mL. The \( \log_{10} \) CFU/disk is 6.83 for the control disks and 6.48 for the AC7BS pre-coated disks. This implies a statistically significant reduction of adhesion of 55 % (p<0.001). These results are shown in figure 4.12.

Fig 4.12: Reduction of *C. albicans* IHEM 2894 adhesion after pre-coating with 2 mg/mL AC7BS: plate counting. Error bars represent the standard deviation. ***p<0.001

4.2.2.2. Reduction of biofilm formation

After 24 hours of incubation, the biofilms are fully formed. The difference in viability of the *C. albicans* biofilms between the AC7BS precoated disks and the control disks can be observed. This is quantified using the plate count method and analyzed with the student t-test.
The inoculum with *C. albicans* 40 contained $1.20 \times 10^7$ CFU/mL. The mean log$_{10}$ values for CFU/disk are 7.59 for the control disks and 7.32 for the disks treated with AC7BS. This indicates a significant reduction of biofilm viability of 56 % ($p<0.01$). The mean log$_{10}$ CFU/disk values are plotted in a graph as a function of the AC7BS concentration (figure 4.13).

![Graph showing reduction of C. albicans 40 biofilm formation](image)

**Fig 4.13: Reduction of *C. albicans* 40 biofilm formation after pre-coating with 2 mg/mL AC7BS: plate counting. Error bars represent the standard deviation. **$p<0.01$

For *C. albicans* 42, the initial suspension contained $1.14 \times 10^7$ CFU/mL. The mean log$_{10}$ CFU/disk is 7.59 for the control disks, and 7.32 for the biosurfactant-treated disks. A 45 % reduction of biofilm formation is found ($p<0.01$). Figure 4.14 gives a view of this result.

![Graph showing reduction of C. albicans 42 biofilm formation](image)

**Fig 4.14: Reduction of *C. albicans* 42 biofilm formation after pre-coating with 2 mg/ml AC7BS: plate counting. Error bars represent the standard deviation. **$p<0.01$

The inoculum of *C. albicans* IHEM 2894 contained $1.20 \times 10^7$ CFU/mL. The log$_{10}$ CFU/disk is 7.67 for the control disks and 7.38 for the AC7BS precoated disks. This implies a significant reduction of biofilm formation of 48 % ($p<0.01$). These results are shown in figure 4.15.
4.3. ANTIFUNGAL ACTIVITY OF AC7BS AGAINST PLANKTONIC C. ALBICANS CELLS

The effect of AC7BS on planktonic C. albicans 40, C. albicans 42 and C. albicans IHEM 2894 cells is tested. The following concentrations of biosurfactant solutions are examined: 0.031 mg/mL, 0.062 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL. The effect is evaluated by measuring the absorbance of the suspensions at 450 nm. The results for the three C. albicans strains are shown in figure 4.16.

Figure 4.16: Antifungal activity of AC7BS against planktonic C. albicans cells. The absorbance at 450 nm is plotted as a function of the concentration of AC7BS.
The inocula of *C. albicans* 40, 42 and IHEM 2894 were $1.18 \times 10^7$ CFU/mL, $1.20 \times 10^7$ CFU/mL and $1.16 \times 10^7$ CFU/mL respectively. The results are analyzed with one-way ANOVA. The mean absorbance values of the control wells do not differ significantly from the absorbance values of the wells with the biosurfactant solutions ($p>0.05$). We conclude that no significant antifungal activity of AC7BS against planktonic *C. albicans* cells of the tested strains is found.

### 4.4. Antifungal Activity of AC7BS against a Pre-Formed *C. Albicans* Biofilm

The effect of AC7BS on a pre-formed *C. albicans* biofilm is examined. The following concentrations of AC7BS are tested: 0.062 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL. To quantify the antifungal activity of AC7BS, XTT is used. The results for *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 are plotted in a graph (figure 4.17).

![Anti-fungal activity of AC7BS](image)

**Figure 4.17:** Antifungal activity of AC7BS against a pre-formed *C. albicans* biofilm. The absorbance at 450 nm is plotted in function of the concentration of AC7BS.

The inoculum contained $1.23 \times 10^7$ CFU/mL for *C. albicans* 40, $1.21 \times 10^7$ CFU/mL for *C. albicans* 42 and $1.25 \times 10^7$ CFU/mL for *C. albicans* IHEM 2894. The results are analyzed with one-way ANOVA. The mean absorbance values of the control wells do not differ significantly from the absorbance values of the wells containing the biosurfactant solutions ($p>0.05$). We conclude that no significant antifungal activity against *C. albicans* biofilms of any strain is found for AC7BS.
5. DISCUSSION

The goal of this master thesis was to evaluate the activity of the biosurfactant AC7 against *Candida albicans*. AC7BS was isolated from the supernatant of *Bacillus subtilis* AC7, and its CMC was determined. Three *C. albicans* strains were examined: *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894.

In one part of our work, the preventive effect of AC7BS on *C. albicans* adhesion and biofilm growth was tested. Since biofilm formation on medical devices can cause severe infections which are very difficult to eradicate, research for compounds that could prevent the adhesion of microorganisms and biofilm formation is very useful. In order to mimic central catheter material, medical-grade silicone disks were used. They were pre-coated with AC7BS solutions and then exposed to a suspension of *C. albicans* cells. The inhibition of adhesion and biofilm growth was quantified in two ways: by measuring the biomass of the adherent cells or the biofilm with crystal violet staining, and by measuring their viability with the plate count method.

Another aspect of this thesis, was to examine if AC7BS has an antifungal activity against *C. albicans*. The effect of different concentrations of AC7BS against planktonic cells was evaluated by comparing the absorbance values of the suspensions which were en which were not exposed to AC7BS. The antifungal activity of the BS against mature biofilms was determined with XTT. This compound measures the metabolic activity of the cells inside the biofilm.

In this thesis, three different assays were used to quantify the adhered cells or the biofilm: crystal violet, XTT and plate counting. Other methods have also been described for this purpose. For example, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium derivate similar to XTT. It is also converted into a colored formazan product by metabolic active cells. Thus, the intensity of the color indicates the enzymatic activity of the cells in a biofilm. In contrary to XTT, MTT is water-insoluble (Cady et al., 2012). Next to that, biomass determination can be done by measuring the dry weight of the biofilm. To this end, the biofilm needs to be scraped off the substrate surface and filtered through a membrane filter (Chandra et al., 2008).
5.1. CRITICAL MICELLE CONCENTRATION OF AC7BS

The surface tension of AC7BS solutions was measured as a function of their concentration. The obtained graph was typical for a surfactant. At low surfactant concentrations, the surface tension decreased rapidly when the biosurfactant concentration increased. At higher concentrations of AC7BS, the curve was concentration-independent: the surface tension remained approximately equal. The CMC of the biosurfactant solution extracted from the supernatant of B. subtilis was calculated to be 45.7 µg/mL. At the CMC, the surface tension was only 34.8 mN/m. The CMC is an important value to measure the efficiency of a biosurfactant: a low CMC indicates an efficient surfactant.

In 2006, Nitschke and Pastore isolated a similar biosurfactant solution produced by B. subtilis LB5a. They also found a similar CMC: 33 µg/mL (Nitschke & Pastore, 2006). A biosurfactant obtained from a B. subtilis PTCC 1696 strain had a lower CMC, only 10 µg/mL (Ghojavand, et al., 2008).

Chemical characterization conducted by Prof. G. Allegrone (Laboratory of Pharmaceutical Analytical Chemistry) has discovered the exact composition of the biosurfactant AC7BS solution. Only approximately 80 % of the compounds in the mixture are biosurfactant molecules. The biosurfactant compounds are lipopeptides: the majority are different isotypes of surfactin, but also fengycin is present (Prof Allegrone personal communication).

In the literature, CMC values for surfactin and fengycin, which are the main constituents of AC7BS, are reported to be 10 µg/mL and 11 µg/mL respectively (Bodour et al., 2004). This means that the isolated biosurfactant solution in this test is less effective than its individual biosurfactant compounds. This is probably due to the impurities in the biosurfactant solution. Still, AC7BS is a strong surfactant: it can have a strong effect even at low concentrations.
5.2. INHIBITION OF C. ALBICANS ADHESION AND BIOFILM FORMATION BY BIOSURFACANT AC7

The anti-adhesive effect of biosurfactants has been studied before. Pseudofactin II, a biosurfactant secreted by Pseudomonas fluorescens BD5, showed a remarkable inhibition of adhesion for a few microorganisms, including C. albicans (Janek et al., 2012). Very recently, the effect of a biosurfactant solution produced by another Bacillus strain (AR2) on C. albicans biofilms was examined. This biosurfactant was composed of a mixture of iturin and fengycin. It also showed an inhibition of C. albicans biofilm formation and even a fungicidal activity (Rautela et al., 2014).

In this thesis, C. albicans biofilm formation was carried out according to Chandra et al. (2008). At first, the biomass of the adherent cells (after 90 minutes) and of the mature biofilm (after 24 hours) was measured using crystal violet staining. For this test, pre-coating with AC7BS was evaluated at four different concentrations: 0.5 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL. For all three C. albicans strains, and both after 90 minutes and 24 hours, a concentration of 2 mg/mL AC7BS showed the best inhibition. At this concentration, the maximal inhibition of adhesion for C. albicans 40 was 59 %, and maximal inhibition of biofilm formation was 53 %. For C. albicans 42, 51 % inhibition was found for the adhesion phase and 45 % inhibition for the biofilm growth. C. albicans IHEM 2894 showed an inhibition of 54 % and 49 % for respectively the adhesion and the biofilm formation phases.

Since 2 mg/mL was proven to be the most active AC7BS concentration, the viability tests were only performed at this concentration. These tests showed similar results as for the biomass tests: an inhibition of adhesion of 56 %, 53 % and 55 % for C. albicans 40, C. albicans 42 and C. albicans IHEM 2894, respectively. The inhibition of biofilm growth was 56 %, 45 % and 48 % respectively.

In summary, the obtained results demonstrate that the attachment of C. albicans to silicone elastomeric disks can be reduced by using biosurfactant AC7. The anti-adhesion and anti-biofilm growth effects of AC7BS are related to the amphiphilic character of the biosurfactant. Adsorption of AC7BS to the silicone material alters the hydrophobic properties of the surface, and this interferes with the adhesion of microorganisms (Janek et al., 2012).
We conclude that AC7BS shows a promising effect against *C. albicans* adhesion and biofilm growth. Therefore, it has potential as a coating agent on medical material to prevent biofilm formation on its surface.

### 5.3. ANTIFUNGAL ACTIVITY OF AC7BS AGAINST C. ALBICANS

In order to study all the mechanisms by which AC7BS inhibits *C. albicans* adhesion and biofilm growth, its antifungal activity was tested. The effect of AC7BS against planktonic *C. albicans* cells and pre-formed biofilms was examined. No antifungal activity was found for any of the three strains (*C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894). This indicates that AC7BS has no capability to kill *C. albicans* cells in planktonic or biofilm form. The inhibitory effect of AC7BS against *C. albicans* adhesion and biofilm formation is thus not due to an antifungal activity of AC7BS.

Biosurfactants isolated from *B. subtilis* AC7 did show antifungal activity against phytopathogenic fungi such as *Botrytis cinerea*, *Cercospora beticola*, *Pyricularia orza*, *Helminthosporium teres* and *Microdochium nivale* (Prof. L. Fracchia, personal communication). Plaza et al. proved the antifungal activity of surfactin produced by *B. subtilis* against a few phytopathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Plaza et al., 2013). Also, an antifungal activity of a *B. subtilis* biosurfactant was found against *Penicillium italicum* and *Aspergillus niger* (Ghribi et al., 2012). Fengycin, which is next to surfactin another substituent of AC7BS, has an antifungal activity against filamentous fungi, but not against yeasts (Wei et al., 2010).

### 5.4. FUTURE PERSPECTIVES

The results obtained in this thesis showed an interesting inhibition of adhesion and biofilm growth by AC7BS. Since research for new molecules that can prevent biofilm formation on medical devices is essential, further evaluation of AC7BS is recommended. The effect of AC7BS against other fungi or bacterial species should be measured in order to evaluate its overall potential for prevention of biofilm formation. Moreover, improvements of the pre-coating methodology have to be carried out in order to covalently attach AC7BS to the
surface of the silicone disks. In this way, the biosurfactant coating could better resist to external mechanical forces.

AC7BS is a mixture of surfactin and fengycin, and it contains a lot of impurities next to the biosurfactant molecules. Surfactin or fengycin could be purified from the crude extract and their individual effects could be examined.

The effect of AC7BS on other surfaces (such as contact lenses material, latex urinary catheters, stents or titanium) is another interesting field to explore.

Since AC7BS does not show any antifungal activity against C. albicans, combining this biosurfactant in a coating with an antifungal drug could result in a synergistic effect. Different antifungal drugs or antibiotics could be considered, in order to minimize chances of biofilm formation on medical material.
6. CONCLUSION

For this thesis, biosurfactant AC7 was extracted out of the supernatant of Bacillus subtilis AC7. Its CMC was 45.7 µg/mL, indicating that AC7BS is a quite efficient biosurfactant. The biosurfactant compounds were mainly surfactin and fengycin.

The effect of pre-coating silicone disks with AC7BS on Candida albicans adhesion was evaluated. Besides, its consequences for C. albicans biofilm growth were determined. Three strains were examined: C. albicans 40, C. albicans 42 and C. albicans IHEM 2894. The inhibitory effect was quantified by measuring both the biomass and the viability of the adherent cells (after 90 minutes) or the biofilm (after 24 hours). AC7BS showed a significant inhibition of adhesion (around 54 %) after 90 minutes with respect to both biomass and number of living cells. Similar results (around 49 % inhibition) were obtained against C. albicans biofilm formation (after 24 hours). A biosurfactant concentration of 2 mg/ml was the most effective.

No antifungal activity of AC7BS was found against the three tested C. albicans strains. Different concentrations of the biosurfactant could not decrease the amount of planktonic C. albicans cells in a suspension. AC7BS did not have a significant effect against pre-formed biofilms either. Thus, the previous found strong effect of AC7BS against C. albicans adhesion and biofilm formation, is not due to an antifungal activity of AC7BS.

We conclude that AC7BS shows a promising activity against C. albicans adhesion and biofilm growth. It therefore has potential to be used as a coating agent for medical material, in order to prevent biofilm formation on its surface.

Considering further research, it would be interesting to evaluate the effect of AC7BS against other fungi or bacterial species. In this way, the overall preventive effect of pre-coating with AC7BS against biofilm formation could be examined. Improvements should be made to the pre-coating methodology in order to covalently attach AC7BS to the surface of the silicone disks. Moreover, combining the biosurfactant with an antifungal drug could result in a synergistic effect. Also, pre-coating other kinds of surfaces with AC7BS is an interesting field to investigate.
7. BIBLIOGRAPHY


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