Compressive strength of fungal and oral biofilms
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CHAPTER 3

HYPHAL CONTENT DETERMINES THE
COMPRESSIVE STRENGTH OF Candida
Albicans Biofilms

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INTRODUCTION

Biofilms are populations of microorganisms embedded in an extracellular polymeric substance (EPS) (3). The biofilm mode of growth is beneficial for microorganisms as it provides a higher degree of stability compared to a planktonic existence and organisms within a biofilm are more resistant to environmental challenges (6), such as low-nutrient availability, high fluid shear, antibiotic and antimicrobial agents (5, 6, 24, 28, 48). The formation of biofilms and the damage caused by them is a widespread problem, ranging from pipeline corrosion, biofouling of ship hulls and food-processing equipment to clinical infections, such as endocarditis and cystic fibrosis pneumonia (7, 13, 19, 40). Many of the clinical infections due to biofilms are implant-related and occur when microorganisms adhere to the surfaces of biomaterials used in, for example, prosthetic heart valves, voice prostheses, joint replacements, vascular grafts and urinary catheters (1, 2).

Biofilms can consist of bacterial or fungal species or a mixture of both. For instance, in case of vascular catheter-related infections or voice prosthetic biofilms, the most commonly isolated microbial species are Gram-positive <i>Staphylococcus epidermidis</i> and <i>S. aureus</i>, and the fungus <i>Candida albicans</i> (31), in addition to streptococci found on voice prostheses. Numerous studies have been carried out to investigate bacterial biofilms and their role in infection (22, 23), but fungal biofilms have been given much less attention, despite the fact that they rank number four in frequency among species causing bloodstream infections (25). <i>C. albicans</i> is the most frequently isolated fungal species in clinical infections. Other <i>Candida</i> species, such as <i>C. tropicalis</i>, <i>C. parapsilosis</i>, <i>C. dubliniensis</i>, which are comparatively less pathogenic than <i>C. albicans</i>, recently emerged as clinically important pathogens (42).
Most *Candida* species grow as unicellular yeast but they are also capable of producing multicellular filamentous forms of growth, pseudohyphae and pseudomycelium. Two species, *C. albicans* and *C. dubliniensis*, can form true hyphae (9). The morphological transition from the yeast to the hyphal growth form is an important factor in virulence (29) and biofilm formation (33). Temperatures above 35°C and pH of 6.5 to 7 are known to be favourable for germ tube formation, while also serum and Lees medium can induce hyphal growth (6). At a high cell density of $10^6$ cells ml$^{-1}$ or above, hyphal formation is suppressed. This cell-density dependent effect is known as the inoculum size effect (31) and resembles quorum sensing (QS) in bacteria. Farnesol is a signalling molecule involved in QS in *C. albicans* (23). A two-component histidine kinase, Chk1p, has been implicated in the farnesol mediated response (28).

It remains unknown what role hyphae play in maintaining fungal biofilm integrity. Studies on the role of morphogenesis in the development and pathogeneity of *C. albicans* biofilms demonstrated that hyphal-defective mutants colonized polyurethane catheters poorly (34) and were unable to inhabit plastic surfaces and form biofilms (26). In another study, biofilms of a yeast-negative mutant did not produce any yeast cells and were easily detached from catheter disks, while biofilms of hypha-negative mutant were thin and densely-packed with yeast cells (3). In our experience while working with *C. albicans* biofilms, we observed that mature biofilms of a strain lacking both copies of *CHK1* were more resistant to washing. This prompted a further investigation into the factors that influence mechanical properties of *C. albicans* biofilms, which so far have received no attention. Mechanical properties are important for selection of treatment or dispersal of biofilm organisms due to a bodily fluid flow. In general, the mechanical properties of a biofilm determine
the deformation of a biofilm due to an applied force, such as shear originating from blood or urinary flow. Strength is one of the parameters used to describe mechanical properties. It is defined as a material’s ability to resist applied forces. Increased fluid shear (18, 36), presence of divalent cations (Ca\(^{2+}\)) (24), increased EPS production (19), EPS composition (40) and quorum sensing (12) are known to affect the strength of bacterial biofilms. For fungal biofilms, the factors affecting biofilm strength still remain to be determined.

EPS production and composition is known to play important role in maintaining integrity of bacterial biofilms (22). EPS consists of polysaccharides, proteins and extracellular DNA (eDNA). Polysaccharides represent the most abundant and widely studied component of bacterial EPS. Proteins in EPS have been studied less and it has been suggested that they serve to hold bacteria in a biofilm together via aggregation. Very little is known about role of eDNA in fungal biofilms. Extracellular DNA can be released into EPS by cells or can occur as a result of cell lysis. Recently it has been shown that DNaseI can dissolve established bacterial biofilms (23). For fungal biofilms, the composition and role of EPS in maintaining biofilm integrity has been studied less compared to bacterial biofilms. Al-Fattani and Douglas (1) have shown that EPS of \textit{C. albicans} and \textit{C. tropicalis} consists mostly of polysaccharides (30-41%) with a small amount of protein (3-5%). Upon application of DNaseI, biofilms of \textit{C. albicans} can be only partially detached from surfaces. In a manner similar to bacterial biofilms, \textit{Candida} EPS is known to play an important role in drug resistance.

In this study, biofilms of mutant \textit{chk1/chk1} (Chk24), the gene-reconstructed \textit{chk1/CHK1} (Chk23) and wild type \textit{CHK1/CHK1} (Caf2-1) \textit{C. albicans} strains were subjected to compression forces in a uniaxial low load compression tester (LLCT) (21) along with two non-\textit{albicans Candida} species, \textit{C. tropicalis} and \textit{C. parapsilosis}
isolated from used voice prostheses. Effects of increasing the shear during growth were evaluated only for *C. albicans* Caf2-1. LLCT evaluates the resistance of biofilms to compression and allows for accurate determination of biofilm thicknesses. In addition, cell morphologies within the biofilm, cell surface hydrophobicities, and EPS composition, were determined.

**MATERIALS AND METHODS**

**Microbial strains, growth conditions and biofilm formation**

*C. albicans* Caf2-1 (*CHK1/CHK1*), Chk24 (*chk1/chk1*) and Chk23 (*chk1/CHK1*) (19), *C. tropicalis* GB 9/9 and *C. parapsilosis* GB 2/8 grown on Tryptone Soya Broth (TSB, OXOID, Basingstoke, UK) agar plates were used to inoculate 10 ml Yeast Nitrogen Base (YNB, DifcoTM without amino acids, Becton Dickinson, Sparks MD, USA) containing 50 mM glucose, prepared according to manufacturers instructions with pH set to 7.0 using KOH. Cultures were grown at 30°C for 16 h in ambient air while shaking at 120 rpm. Cells were harvested by centrifugation at 5,000 x g for 10 min, washed once and resuspended in sterile phosphate-buffered saline (PBS, NaCl 8.76 g l⁻¹, K2HPO4 0.87 g l⁻¹, KH2PO4 0.68 g l⁻¹, pH 7.0).

Biofilms were grown on 1.5 x 1.5 cm polymethylmethacrylate (PMMA) slides in 6-wells tissue culture plates. Prior to growing biofilms, the slides were sterilized with 70% ethanol, rinsed with sterile, demineralised water and coated with 50% fetal bovine serum (Sigma-Aldrich, USA) for at least 30 min to enhance adhesion (18), washed once with sterile PBS, and placed into the wells. Three ml of cell suspension with a density of 1 x 10⁷ cells ml⁻¹ in sterile PBS was added to each well and cells were allowed to adhere at 37°C for 90 min while shaking at 60 rpm. The suspension was removed by aspiration and the slides were rinsed with PBS. Biofilm growth was
propagated by adding 3 ml per well of YNB pH 7.0 and incubated at 37°C (or 30°C in case of Caf2-1 grown at 30°C) while shaking at 60 rpm for 72 h or 144 h. For increased shear-grown biofilms, biofilms of *C. albicans* Caf2-1 were incubated while shaking at 90 rpm for 72 h at 37°C. Afterwards, the medium was discarded and biofilms were washed once with PBS.

**Low Load Compression testing**

Biofilm resistance against compression was measured during uniaxial compression in a low load compression tester (LLCT) (18) The LLCT apparatus consists of a linear positioning stage (Intellistage M-511.5IM, Physik Instrumente, Karlsruhe, Germany) connected to a cylindrical, moving upper plate with a diameter of 2.5 mm (Fig. 1). A stationary bottom plate is fixed to an automatic force compensating balance (SW 50/300, Wipotec, Kaiserslautern, Germany). The positioning stage and the load cell are interfaced to a PC. LabVIEW™ 7.1 software is used to control the positioning.

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**Figure 1.** Low load compression tester (front view) showing main components of the system.
stage and record the induced load. For biofilm thickness measurements, the substratum with biofilm was placed on the bottom plate and the top plate was moved downwards until it touched the area of the substratum without biofilm. This position was recorded as the bottom of the biofilm. In the second step, the top plate was moved downwards until it touched the biofilm and this position was recorded as well. Subsequently, biofilm thickness was calculated from the difference between both positions. For compression experiments, the biofilms were deformed at a rate of 1 µm s⁻¹ and both load and strain, defined as the fractional change in biofilm thickness from the initial contact point to the point where the plunger position was detected, were recorded. The induced load was normalized over the cross-sectional area of the plunger area to calculate the stress exerted, i.e. the force exerted per unit area of a biofilm. Biofilms were compressed until 0.5 (50%) strain was reached, at which point the compression was stopped because further deformation would only lead to compaction of the biofilms. Three measurements were taken for each independently grown biofilm and the average of three measurements was calculated and used for further analysis. Values of strain were plotted against corresponding values of stress to obtain stress-strain diagrams. These diagram help to acquire data about a material’s strength without regarding for its physical size or shape (16). The upper stress limit of a linear relationship between stress and strain in a stress-strain diagram was defined as the elastic limit. This limit was estimated at the point of 0.2 strain (20% deformation). At this point, the modulus of elasticity (E) was calculated from Hooke’s law as follows:

\[ E = \frac{\sigma}{\varepsilon} \]  

(1)
Quantification of biofilm density

PMMA slides with *Candida* biofilms were weighted, after which biofilms were scraped off the slides and the weights of the clean slides were measured. The difference between the two measurements yielded the wet weight of the biofilm. Subsequently, the biofilm density was calculated by dividing the biofilm wet weights by their volume (estimated as the area of the slide multiplied by biofilm thickness as determined from LLCT).

Cell morphology counts

Biofilms scrapings in PBS were subsequently vortexed for 2 min to break up the matrix. The resulting suspension was sonicated on ice 5 times for 30 s at 30 W to further break up biofilm clumps. Cooling on ice was done to prevent overheating and lysis. A cell suspension was placed into a Bürker-Türk counting chamber and the number of yeast and hyphal cell morphologies were counted using phase contrast microscopy (40x objective lens, Olympus BH-2, Japan). Hyphae-to-yeast ratios were calculated with a minimum of 150 cells counted per experiment.

Hydrophobicity measurements

Hydrophobicities of biofilms were assessed by water contact angle measurements. Biofilms grown on PMMA slides were air dried at room temperature, while measuring water contact angles as a function of drying time (6). Water contact angles became independent of drying time after 110 min, following which stable water contact angles could be established for at least another minute. A drop of water was placed on the surface of a biofilm and images were taken from the side using an Olympus MXR 5010 mounted with a 160 mm 4x macro lens, from which contact angles were calculated using Contact Angle Measurement System Ver. 2.07 software (MT, RuG,
The Netherlands). Measurements were performed in triplicate with independently grown biofilms, with three water droplets measured on each biofilm.

**EPS analysis**

For determination of the proteins and eDNA content of EPS, EPS was isolated using a modification of a protocol, previously developed by Baillie and Douglas (1). Biofilms were scraped from PMMA slides into PBS. The solution was vigorously vortexed for 3 min to disrupt the biofilms. Cell suspension was centrifuged at $5,000 \times g$ for 10 min at $10^\circ C$ and supernatant was collected for further analysis. The Quant-iTTM Protein Assay and dsDNA HS Assay Kits (Molecular Probes™, Invitrogen™, the Netherlands) were used to quantify proteins and DNA amount according to the instructions. Measurements were performed in triplicate with independently grown biofilms, with two samples measured on each biofilm. Amounts of proteins and DNA measured were normalized per unit biofilm wet weight.

Total polysaccharides in the EPS were quantified using the phenol-sulphuric acid method of Dubois *et al.* (12). Biofilms were scraped from PMMA slides into 1 ml of PBS. The suspension was mixed with 0.5 ml of 1M NaOH, vortexed and incubated while shaking at room temperature for 15 min. Further, the suspension was centrifuged at $10,000 \times g$ for 5 min. Supernatant was collected and mixed with 100% ice-cold ethanol (0.5 ml supernatant to 1.5 ml of ethanol) and placed in the freezer for at least 30 min. After freezing, 1 ml of the sample was combined with 25 µl of aqueous 80% (v/v) phenol solution in a glass test tube to which 2.5 ml of concentrated sulphuric acid was rapidly added. The tubes were vortexed and the mixture was incubated at room temperature for 10 min followed by incubation in $30^\circ C$ water bath for 20 min. After cooling to room temperature for 5 min,
absorbances of the mixtures were measured at 490 nm (neutral polysaccharides). Measurements were performed in triplicate with independently grown biofilms, with two samples measured on each biofilm. Amounts of polysaccharides measured were normalized per unit biofilm wet weight.

**Statistical analysis**

Statistical analysis was performed using SPSS software (Statistical Package for Social Sciences, version 14.0.0, SPSS, Chicago, Illinois, USA). Before analysis, data was tested for distribution using Kolmogorov-Smirnov goodness of fit test. Post hoc multiple comparisons were performed to quantify differences between variables using Tukey test with a level of significance of p < 0.05.

**RESULTS**

Hyphal content of *C. albicans* biofilms ranged from 0 to 79%, depending on the growth conditions, and biofilms of *C. tropicalis* and *C. parapsilosis* were comprised of yeast cells only (Fig. 2). Hyphal content of *C. albicans* Caf2-1 biofilms slightly increased with higher shear and decreased in older biofilms. When the growth temperature of *C. albicans* Caf2-1 was reduced to 30°C, hyphal content dropped to zero. There was no change in hyphal content of *C. albicans* Chk24 biofilms over time.

Analysis of EPS content of the biofilms showed that polysaccharides content was significantly lower in six days old biofilm of *C. albicans* as compared to other biofilms studied (Fig. 3A). Proteins content of EPS was variable across the different fungal biofilms and highest for three days old *C. tropicalis* biofilms (Fig. 3B). The amount of eDNA was also variable across the biofilms and was higher in six days old
biofilms of *C. albicans* than in three days old biofilms. The eDNA content was highest for the *C. albicans* Caf2-1 grown at 30°C.

**Figure 2.** Hyphal content of *Candida* biofilms. Error bars represent standard deviations over 9 independently grown biofilms.

Stress-strain curves (Fig. 4) showed that biofilms containing hyphae were able to withstand higher compressive forces than biofilms without hyphae, while hyphae content was influenced by growth temperature, shear rate during growth and the specific strain involved.
Hyphal content determines the compressive strength of Candida albicans biofilms

![Graph A]

Amount (mg g⁻¹)

A. Amount (mg g⁻¹) for different strains and ages of Candida albicans biofilms.

![Graph B]

Amount (mg g⁻¹)

B. Amount (mg g⁻¹) for different strains and ages of Candida albicans biofilms.
Figure 3. Amounts of polysaccharides (A), proteins (B) and eDNA (C) in the EPS of Candida biofilms, normalized with respect to the biofilm’s wet weight. Error bars represent standard deviations over 9 independently grown biofilms.

All biofilms, with exception of C. tropicalis had similar thicknesses ranging from 150 to 340 µm. C. tropicalis and all C. albicans biofilms were hydrophobic with contact angles higher than 50 degrees. Biofilms of C. parapsilosis were hydrophilic (Fig. 5). Biofilms wet density was around 1 g cm⁻³, with exception of C. albicans Chk24 and C. parapsilosis, which had wet density of 1.25 g cm⁻³ (Fig. 5). However, none of these parameters showed a systematic variation with biofilm strength, as can also be seen from Fig. 5.
Hyphal content determines the compressive strength of Candida albicans biofilms

Figure 4. Averaged stress-strain curves for (A) biofilms of *C. albicans* containing hyphal cells and (B) biofilms of *Candida* strains without hyphal cells. Note the different scales. Error bars represent standard deviation over 9 independently grown biofilms.

**DISCUSSION**

Hyphal content was found to be a determining parameter for the strength of fungal biofilms (Fig. 6). Biofilms with a high hyphal content were more resistant to compression and more difficult to disturb by vortexing and sonification, than biofilms with a lower hyphal content. The weakest biofilms consisted of yeast cells only. It appears that there was a linear relationship (linear correlation coefficient $R^2 = 0.943$) between biofilms strength and their hyphal content in three days old *Candida* biofilms (Fig. 6A). Interestingly, biofilms without hyphae and with the highest eDNA content, *C. albicans* Caf2-1 grown at 30°C, possessed the lowest compressive strength.
Figure 5. Moduli of elasticity ($E$) of *Candida* biofilms with biofilm characteristics, found to be unrelated with biofilm strength. Error bars represent standard deviations over 9 independently grown biofilms.

In *C. albicans*, hyphal production can be suppressed not only by reducing the growth temperature (4) but also by the presence of a QS molecule, farnesol (14). Here we see that in the absence of *CHK1*, more hyphal cells are produced in the biofilms of Chk24 compared to biofilms of the wild type strain Caf2-1, resulting in stronger biofilms. Interestingly, 6 day old biofilms for Caf2-1 showed a significant reduction in hyphal content, which was not seen for the strain lacking Chk1p. This could therefore illustrate the effect of farnesol, inducing yeast cells growth in wild-type biofilms while mutant biofilms are unable to sense farnesol and maintain a high
Hyphal content determines the compressive strength of Candida albicans biofilms

Hyphal content. Growth of C. albicans Caf2-1 under higher shear conditions also induced more hyphal production, although not to the extent that they were significantly stronger than Caf2-1 biofilms grown under lower shear.

Hyphal and yeast cells do not differ qualitatively in cell wall composition. The main differences, however, are in the proportions of main components, such as chitin or β-glucans (6). The cell wall of C. albicans consists for 80 to 90% of carbohydrates, most important of which are β-glucans (β-1,3 and β-1,6 glucose polymers), accounting for 47 to 60% of cell wall weight; chitin, which constitutes 0.6 to 9% and mannan, polymers of mannose covalently bound to proteins. Even though chitin content in the cell wall is small, it is an important component of the septa, bud scars, and the ring around the constriction between mother cell and bud. Hyphal cells contain at least three times as much chitin as yeast cells (4, 5). Chitin is a hydrophobic material, also found in insects, crabs, shrimps, and lobsters, where it provides rigidity to outer skeleton. Chitin has been shown to increase mechanical and flexural strength of bone substitutes (6). Higher amount of chitin due to the presence of more hyphal cells in biofilms may be responsible for increased compressive strength of the biofilm.

Furthermore, eDNA content in EPS showed an adverse effect on biofilms strength of C. albicans Caf2-1 and Chk24. EPS amount and composition were previously shown by others to have a significant influence on structure and cohesiveness of bacterial biofilms (10, 13). Moreover, presence of eDNA was shown to affect biofilm formation by P. aeruginosa (12). In our study we see that normalized amounts of eDNA increased with biofilm age and shear (Fig. 3C) and contributed to the decrease in strength of C. albicans Caf2-1 and Chk24 biofilms with approximately similar hyphal content, as suggested in Fig. 6B. In 6 days old biofilms of Chk24,
Figure 6. Modulus of elasticity $E$ as a function of (A) hyphal content in 3 days old *Candida* biofilms and (B) eDNA amount in *C. albicans* Caf2-1 and Chk24 biofilms. Arrows in Fig. 6B indicate the decrease in strength upon increasing the growth time from 3 to 6 days of *C. albicans* Caf2-1 and Chk24 biofilms and increasing shear rate at growth for *C. albicans* Caf2-1, which is concurrent with increased amounts of eDNA, while for the strains presented the hyphal content remains approximately constant.
Hyphal content determines the compressive strength of Candida albicans biofilms

decreased by 33%, compared to 3 day old biofilms, while hyphal content remained unchanged. In 3 day old biofilms of Caf2-1, strength of biofilms grown at higher shear decreased slightly, compared to the biofilms grown at normal shear, while the amount of eDNA increased, while here too hyphal content remained similar. Six days old biofilms of Caf2-1 showed 23% decrease in hyphal content, concurrent with 60% increase in the amount of eDNA and a major decrease in biofilm strength. This suggests that eDNA probably does not play the same essential role in Candida biofilms as it does in bacterial biofilms. This is in line with recent observations, that bacterial biofilms can be fully removed by DNaseI treatment (Nemoto et al., 2003), while Candida biofilms were only removed by 30% (2).

In addition to changes in eDNA content, polysaccharide and protein contents in EPS varied with biofilm age. Polysaccharide content decreased in ageing biofilms, while protein content increased, most likely due to cell lysis. Both polysaccharides and proteins did not seem to contribute significantly to biofilms strength. Additionally, no correlation was found between biofilms density, thickness and hydrophobicity.

This study contributes to a thin body of knowledge of fungal biofilms mechanics and helps to gain knowledge into which parameters are important for fungal biofilms strength. We show that hyphal cells are the most sturdy components in fungal biofilms and their presence is determinant for the compression strength of C. albicans biofilms. However, fungal biofilms strength can be adversely affected by the presence of eDNA. Without hyphae, fungal biofilms are weaker and can be more easily removed. Understanding what factors are important for biofilm integrity may help in development of new fungal-specific drugs.
REFERENCES


Hyphal content determines the compressive strength of Candida albicans biofilms


