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# GLASS SURFACE AS POTENTIAL *IN VITRO* SUBSTRATUM FOR *CANDIDA FAMATA* BIOFILM

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**Abstract:** The biofilm of *Candida* spp. is a three-dimensional structure consisting of a dense network of yeasts, blastospores and/or filamentous elements (hyphae or pseudohyphae). All species of *Candida* are able to form biofilm. The aim of this paper is to present data concerning biofilm formation under static conditions by oropharyngeal isolates of *C. famata* on a glass surface using non-invasive confocal scanning laser microscopy (CSLM). The changes in five parameters calculated using the CSLM technique, i.e. areal porosity (%), fractal dimension (D), length of edge line (mm/mm$^2$), length of skeleton line (mm/mm$^2$), number of cell clusters/mm$^2$, describing the biofilm structure of *C. famata* isolates after 1 h incubation (the adhesion step), 24 h incubation (biofilm formation) and 72 h incubation (mature biofilm), indicate the morphological reorganization of biofilm during maturation. The thickness of biofilm *C. famata* isolates after 72 h incubation ranged from 35.2 to 81.2 µm.

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## INTRODUCTION

Microorganisms in their natural or artificial habitats are usually found in the form of biofilms, i.e. complex microbial communities attached to surfaces and encased in exopolymeric matrix, but not as unicellular, free-floating (planktonic) forms [9, 10, 20, 21, 22]. Microbial cells are able to colonize natural (e.g. host epithelial cells) or artificial surfaces (e.g. glass, polychloride vinyl, polystyrene, silicone) [7, 12, 15, 24]. Biofilm development involves a series of steps starting with physicochemical interaction between microbial cells and substratum, followed by cell adhesion, multiplication and differentitation, leading to the formation of the mature biofilm. Recently, biofilm formation has been intimately associated with the ability of microorganisms to cause infections, especially those connected with medical devices, an *in vivo* substrata for attachment of microbial cells, followed by biofilm development [16, 17, 18].

Yeasts belonging to *Candida* spp. are a part of the normal human microflora, colonizing various niches, e.g. mucous membranes of oropharynx. Under predisposing conditions, pathogenic yeasts can cause a number of localized or deep-seated candidiases, and also those associated with biofilm formation on medical devices [9, 19, 23]. *Candida famata* is usually found in some food, including dairy products. This yeast species is also present in the normal microbiota of the human body [5, 8]. Nowadays, a number of clinical reports indicate that *C. famata* can be regarded as an important opportunistic pathogen involved in several infections, including those associated with medical devices [5, 6, 13].

In this paper, we present data concerning the biofilm formation under static conditions by oropharyngeal isolates of *C. famata* on a glass surface using the non-invasive method of confocal scanning laser microscopy (CSLM). This technique allows quantitative and qualitative assessment of intact biofilm development.

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## MATERIALS AND METHODS

**Yeast strains and culture conditions.** *Candida famata* isolates were obtained from oropharynx of patients with lung cancer undergoing pulmonary resection. The yeast suspensions were stored at -20ºC in 50% glycerol and then cultured on Sabouraud dextrose agar at 30ºC for 48 h; before each experiment, the isolates were subcultured on Sabouraud glucose broth (further called Sabouraud medium) at 30ºC for 48 h.

**Confocal scaning laser microscopy (CSLM) analysis.** Standardized yeast suspensions (optical density 0.5 Mc Farland standard, i.e. $5 \times 10^6$ colony forming units/ml) in Sabouraud medium were prepared. In order to assay the adhesion process, 350 µl of inoculum was added to four of the eight-well polystyrene culture chambers and then incubated for 1 h at 35°C, followed by gentle washing of the wells with sterile phosphate-buffered saline (PBS) to remove nonadherent cells. Next, to each well 200 µl of solution containing 0.1 mg/ml of concanavalin A Alexa Fluor 488 conjugate (CAAF) was added, and incubation continued for 45 min at 35ºC. In order to assay the early stage of biofilm formation, 350 µl of inoculum was added to four of the eight-well culture chambers and then incubated for 24 h at 35ºC, followed by gentle washing of the wells with sterile PBS to remove nonadherent cells. Next, to each well 200 µl of solution containing CAAF was added, and incubation continued for 45 min at 35ºC. In order to assay the mature biofilm, 350 µl of inoculum was added to four of the eight-well culture chambers and then incubated for 24 h at 35ºC. Next, nonadherent cells were removed by careful well rinsing with sterile PBS and then fresh Sabouraud medium was added. Medium changing and the culture chambers washing procedures after overnight incubation at 35ºC were repeated twice (the total incubation period lasted 72 h). After this time, 200 µl of solution containing CAAF was added and incubation was continued for 45 min at 35ºC. All assays were carried out in four replicates. The pictures for planimetric analysis were collected by LSM 5 Pascal confocal microscope (ZEISS, Germany), using an argon laser with excication wavelenght = 488 nm. The pictures for planimetric measurements were carried out in two-dimensional scans at 50 × magnification. Planimetric analysis was perfomed using Image J. v. l. 36b, Wayne Rasband, National Institutes of Health, USA. Statistical analysis was performed by applying Shapiro-Wilk's and Levene's tests using Statistica 6.0. All tests were performed with a confidence level of 95%.

## RESULTS

Five parameters of the biofilm formation by oropharyngeal *C. famata* isolates were calculated using the CSLM technique: areal porosity (%), fractal dimension (D), the length of edge line (mm/mm²), length of skeleton line (mm/mm²), number of cell clusters/mm² at various phases of the biofilm development, i.e. after 1 h incubation (adhesion step), 24 h incubation (biofilm formation) and 72 h incubation (mature biofilm); the overall incubation period lasted 72 h (Tab. 1). Two parameters, i.e. the areal porosity and the fractal dimension did not change significantly during the incubation period. In the case of the first *C. famata* isolate the length of edge line and the length of skeleton line increased after 24 and 72 h, while the number of clusters, representing the number of microcolonies, decreased after 24 and 72 h incubation. In the case of the second *C. famata* isolate the length of edge line and the length of skeleton line increased significantly after 24 h, followed by a slight decrease after 72 h, while the number of clusters decreased after 24, but increased after 72 h.

**Table 1.** Parameters describing biofilm formation *in vitro* under static conditions by *Candida famata* oropharyngeal isolates on a glass surface.

| Parameter | Incubation time (h) | *Candida famata* CF1 | *Candida famata* CF2 |
|---|---|---|---|
| Areal porosity (%) | 1 | 74.0 ± 7.8 | 76.0 ± 13.4 |
| | 24 | 80.0 ± 4.8 | 47.0 ± 13.4 |
| | 72 | 71.0 ± 5.1 | 70.0 ± 6.6 |
| Fractal dimention (D) | 1 | 1.52 ± 0.1 | 1.48 ± 0.16 |
| | 24 | 1.55 ± 0.09 | 1.81 ± 0.05 |
| | 72 | 1.69 ± 0.05 | 1.55 ± 0.06 |
| Length of edge line (mm/mm²) | 1 | 128.86 ± 38.72 | 125.71 ± 66.37 |
| | 24 | 148.25 ± 37.83 | 269.28 ± 49.02 |
| | 72 | 225.97 ± 34.24 | 136.68 ± 27.60 |
| Length of skeleton line (mm/mm²) | 1 | 45.52 ± 18.76 | 54.04 ± 40.60 |
| | 24 | 76.68 ± 18.67 | 166.73 ± 44.07 |
| | 72 | 131.34 ± 24.17 | 66.65 ± 16.59 |
| Number of cells clusters/mm² | 1 | 992.62 ± 54.57 | 886.37 ± 100.25 |
| | 24 | 857.82 ± 45.63 | 260.18 ± 111.67 |
| | 72 | 636.58 ± 159.32 | 477.58 ± 168.23 |
| Thickness of biofilm (µm) | 1 | nd | nd |
| | 24 | 16.28 ± 1.47 | 15.91 ± 1.43 |
| | 72 | 81.2 ± 7.71 | 35.2 ± 3.35 |

nd – not determined

Also, the thickness of the yeast biofilm was estimated after 24 h and 72 h incubation. As shown in Table 1, the thickness of biofilm of the first *C. famata* isolate, consisting of filamentous forms and sparse blastospores after 24 h incubation (Fig. 1A) and mainly of blastospores after 72 h incubation (Fig. 1B), increased from 16.28 to 81.2 µm, respectively, while the thickness of biofilm of the second *C. famata* isolate, consisting mainly of blastospores both after 24 h (Fig. 1C) and 72 incubation (Fig. 1D), increased from 15.91 to 35.2 µm, respectively.

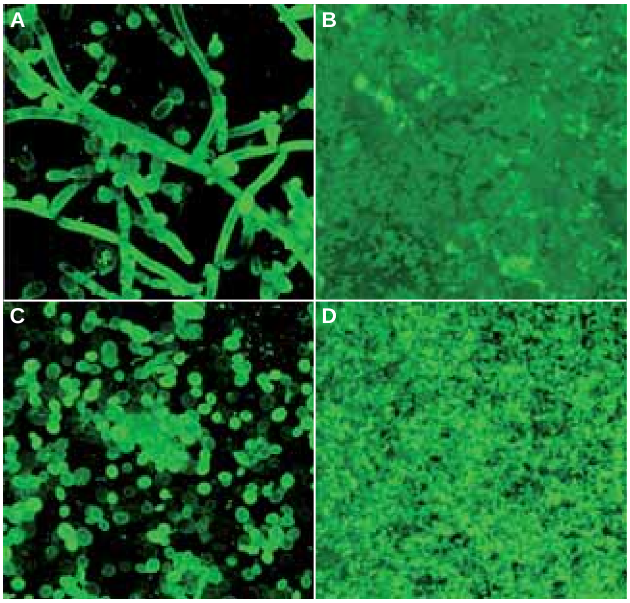


**Figure 1.** Morphology of biofilm formed *in vitro* under static conditions on a glass surface by *Candida famata* oropharyngeal isolates – CF1 after 24 h (A) or 72 h incubation (B), CF2 after 24 (C) and 72 h incubation (D).

## DISCUSSION

Most of the information on the structural characteristics of *Candida* spp., mainly *Candida albicans*, comes from *in vitro* experiments using a variety of the biofilm models on a number of abiotic surfaces, e.g. glass slides [15]. The overall organization of *Candida* spp. biofilm is similar; the mature biofilms consist of a dense network of yeasts and/or filamentous elements (hyphae or pseudohyphae) [2, 10, 11, 14]. However, there is scanty information on *C. famata* biofilm development [15]. Using the non-invasive CSLM technique, the structure of *C. famata* biofilms formed *in vitro* under static conditions on the glass surface was described in this paper; these biofilms consisted of mainly blastospores or blastospores, together with filamentous forms, depending on the strain and incubation time. Our data and those from the literature indicate [17] that dimorphism *per se* may not be an absolute prerequisite for biofilm formation by *Candida* spp., but filamentous elements may have an important role in the structural integrity and multilayered architecture of the mature biofilm. Indeed, according to our data, the thickness of *C. famata* biofilm consisting primarily of blastospores and filamentous elements, and then mainly of blastospores, was much higher (about 80 µm) than that consisting of mainly blastospores (about 35 µm).

The non-invasive CSLM technique allowed also the performance of quantitative analysis of *C. famata* biofilm development *in vitro* on the glass surface at various stages, i.e. during the adhesion and biofilm formation, leading to the mature biofilm. The calculated parameters describing the biofilm development, i.e. areal porosity, length of edge line, length of skeleton line, fractal dimension and the number of clusters indicate that during maturation the morphological reorganization within the biofilm occurred [3, 4, 25]. The yeasts within the biofilm continued to proliferate throughout 72 h, but the parameters of biofilm appeared to be similar to those after 24 h. Similar observations were described by Andes *et al.* [1]. In conclusion, a heterogeneity of the development and structure of the biofilm formed by *C. famata* oropharyngeal isolates has been found, suggesting that this characteristic appears to be common even within the yeast isolates belonging to the same species.

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