YEAST BIOFILMS

REAL-TIME MONITORING AND VISUALIZATION OF MONO- AND DUAL-SPECIES BIOFILMS

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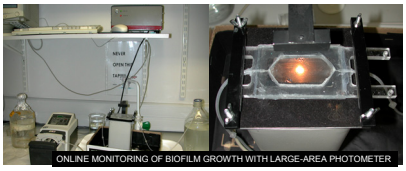
INTRODUCTION:

Since the recognition of biofilms as microbial phenomenon, most investigations have been focused on bacterial biofilms, with little reference to the involvement of yeasts in either single-species phenomena, or as part of multi-species complex biofilms. A better description of the extent and role of yeast in biofilms may be critical for therapeutic, control and exploitation strategies in clinical, industrial and natural environments. Increasing awareness of the impact of recombinant technology on the natural environment furthermore necessitates a broadened focus of microbial ecology to encompass microorganisms from various taxa and environmental niches, including yeasts – and specifically yeast biofilms. Yeast biofilms in medical devices may harbor pathogenic microbes resistant to conventional antimicrobial treatment, while pathogenic yeasts have been recognized to be harbored similarly in bacterial biofilms.

The dynamics of biofilm formation by pure cultures of *Cryptococcus laurentii*, *Saccharomyces cerevisiae*, and the recombinant yeasts *LKA1* and *LKA2*, was quantified using an optical large-area photometer, and compared. Dual-species *Cryptococcus-Pseudomonas* biofilms were similarly quantified, and compared to single-species biofilms of the same organisms. The effect of predation on yeast biofilm development and viability was subsequently monitored. Application of yeast (FUN-1™) and bacterial (*Bac*Light™) fluorescent viability probes, as well as Calcofluor™ White M2R cell wall stain, enabled visualization of mono- and dual-species biofilms with epifluorescence microscopy

METHODS:

Real-time monitoring: Overnight cultures (5 ml) of all yeast species (*Cryptococcus laurentii*, *Saccharomyces cerevisiae*, and recombinant yeasts *LKA1* and *LKA2* (*S. cerevisiae* strains expressing the raw starch-degrading genes encoding for α-amylases from *Lipomyces kononenkoae*)) grown in 10%YME, as well as the bacterial species (*Pseudomonas* CT03) grown in 3g.l^{-1}TSB (Merck), were used to inoculate the large area flowcell for online monitoring of biofilm growth. Ciliates isolated from sandy-loam soil by selective feeding with *Cryptococcus*, and addition of Streptomycin (0.2 mg.ml-1), were inoculated into flowcells after 3 days of biofilm growth, to evaluate the effect of predation on biofilm behavior. The optical large area photometer (OLAPH, German patent 19947651) was used in conjunction with a 10ml hexagonal Plexiglass flowcell covered on both sides with glass coverslips (75mm x 50mm). After inoculation and no flow for 3hrs, a peristaltic pump (Watson Marlow 205S) was used to irrigate developing biofilms with an oligotrophic growth medium consisting of 2.5 mg.ml^{1} sucrose and 6.7 mg.ml^{-1} Yeast Nitrogen Base (Difco). Photometric data was captured and analyzed using Labview-based software.

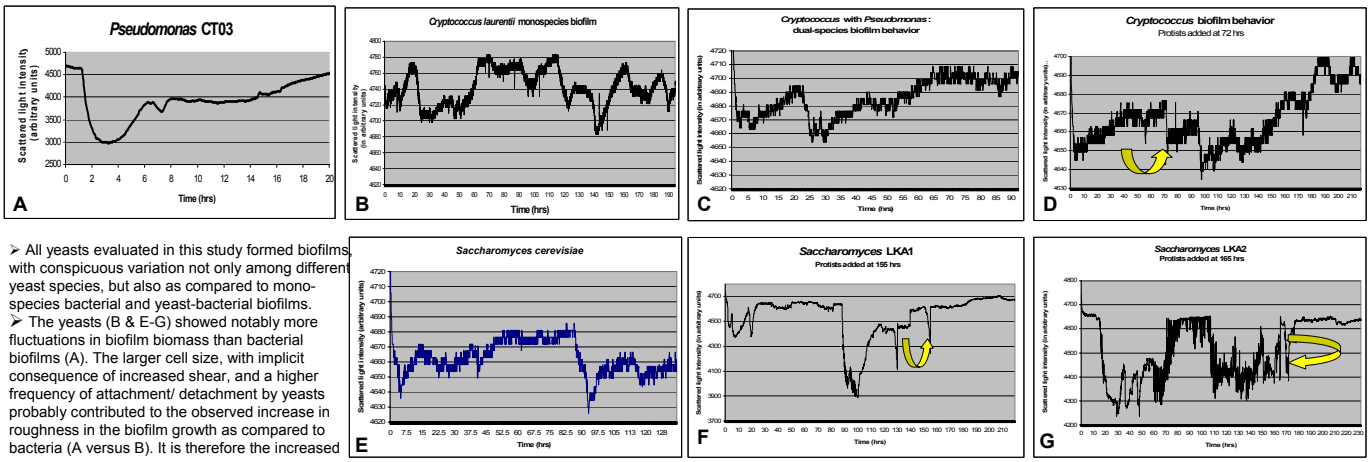


ONLINE MONITORING OF BIOFILM GROWTH WITH LARGE-AREA PHOTOMETER

Visualization: Multiple channel flowcells (each channel 30x4x2.2mm) with previously inoculated yeasts were subjected to staining with either FUN-1™ yeast viability probe, or Calcofluor™White M2R, (indicating 1-3 and 1-4 β D glucans), or both. In the case where the influence of predation on yeast biofilm viability was monitored, duplicate flowcell channels were stained with FUN-1, one and two weeks after inoculation with yeasts and protists. Live yeast cells containing typical red intravauolar structures indicating active metabolism of the viability probe, were statistically quantified in individual microscope fields at 400x magnification. Dual-species yeast-bacterial biofilms were stained with Calcofluor White and *Bac*Light bacterial viability probe. All fluorescent probes were obtained from Molecular Probes™(Invitrogen), with Calcofluor white staining cell walls fluorescent blue, *Bac*Light indicating live bacterial cells with green fluorescence and non-viable cells with red fluorescence, while FUN-1 indicated nonviable yeast cells with yellow-green fluorescence, and live cells with red intravacuolar structures. Microscopy was carried out with a Nikon Eclipse E400 epifluorescence microscope equipped with filter sets appropriate for viewing DAPI, as well as excitation/barrier filter sets of 465-495/515-555nm, and 540-580/600-660nm.

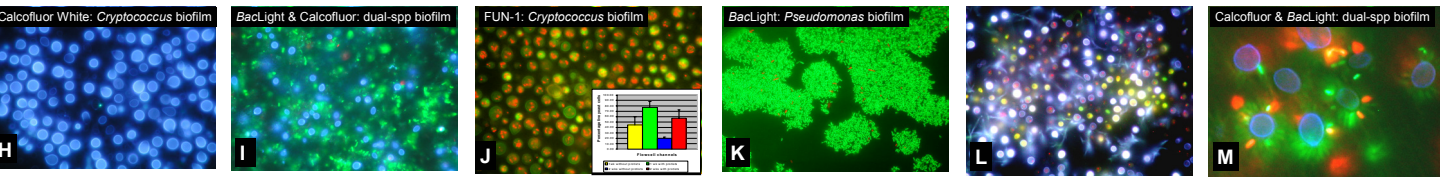
RESULTS:

REAL-TIME MONITORING:



- All yeasts evaluated in this study formed biofilms, with conspicuous variation not only among different yeast species, but also as compared to mono-species bacterial and yeast-bacterial biofilms.
- The yeasts (B & E-G) showed notably more fluctuations in biofilm biomass than bacterial biofilms (A). The larger cell size, with implicit consequence of increased shear, and a higher frequency of attachment/ detachment by yeasts probably contributed to the observed increase in roughness in the biofilm growth as compared to bacteria (A versus B). It is therefore the increased dynamics of the yeast biofilm, with a greater incidence of cell/cluster detachment and regrowth, which is reflected in the higher degree of variation in the yeast biofilm.
- The recombinant yeasts *LKA1&2* (F&G) revealed even more fluctuating biofilm behavior patterns than *Cryptococcus* (B) and the wild type *Saccharomyces* (E). Despite this, the biofilm biomass reached similar maxima in the wild and recombinant *Saccharomyces* strains, with *Cryptococcus* apparently having improved biofilm-forming potential.
- Dual-species *Cryptococcus-Pseudomonas* (C) biofilms showed less fluctuation than monospecies yeast biofilms, indicating a stabilizing, or 'glueing' effect of the co-inhabiting bacterial species on the biofilm structure. The profile of the dual-species biofilm resembles that of the monospecies bacterial biofilm (A). The EPS produced by the bacterial biofilm may be implied in this increased stability, with the frequency of detachment of yeast cells typical of the variable monospecies structure (B&D), consequently reduced in the dual-species yeast-bacterial structure..
- Inclusion of ciliate predators in yeast biofilms had the interesting consequence of increased biofilm biomass and metabolism (D&J-insert), as well as stabilization of the highly fluctuating structure (F&G). Yellow arrows indicate addition of protists in D, F&G.

VISUALIZATION



Selective staining of yeast, bacterial and yeast-bacterial biofilms with a range of fluorescent probes (Molecular Probes™) facilitated clear distinction of various microbes, revealed their relationships and abundance, and supplied information on viability (*Bac*Light) and metabolism (FUN-1). In the case of yeast biofilms the cell-wall stain Calcofluor White (H) enabled clear distinction of cells for quantification, while time-dependant staining with FUN-1 indicated viability in yeasts after metabolic conversion of the yellow fluorochrome into a red intravacuolar substance (J). Staining of a bacterial biofilm with *Bac*Light (K) indicated bacterial viability, while post-staining with Calcofluor White (I&M) furthermore allowed localization of yeast cells in a dual-species biofilm. Post-staining with Calcofluor White after staining with FUN-1 (L) allowed enhanced visualization of yeast-bacterial biofilms even in cases where the bacterial cell walls also stain blue with Calcofluor White. An interesting observation over time is the distinction of new biofilm growth after Calcofluor White staining, with newly-formed cells devoid of the blue stain, but still visible after incorporating the FUN-1 probe as fluorescent red intravacuolar structures (see L). As the toxicity of FUN-1and Calcofluor White has not been evaluated here, no quantification was carried out using this feature. Staining enabled quantification of image content, with selective staining and appropriate filters enhancing the comlexity of retrievable data.

CONCLUSIONS:

Bacterial biofilms have been extensively analyzed and described as occurring in discrete steps of surface conditioning, cellular adhesion and attachment, EPS production, biofilm growth and cellular detachment. The dynamics of yeast biofilms, however, received much less attention, with little reference to similar developmental stages. Various deviations from this hypothesized order of events lead to an inability to cultivate reproducible biofilms – a feature also illustrated in this study. Most notable is the increased variability of yeast biofilm structure as compared to bacterial biofilms – with other microbes impacting on this variation either by stabilizing the structure, or by stimulating increased growth. The demonstrated influence of various co-inhabiting species on biofilm structure cautions against the typical pure-culture planktonic approach generally relied on for empirical studies. These results exemplify the need to include multispecies biofilms for a realistic evaluation of microorganisms as environmental determinants. A holistic approach to biofilms as natural phenomenon is furthermore imperative in the translation of biofilm data into a meaningful account of this microbial lifestyle.