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Fabrication of micro-structured surface topologies for the promotion of marine bacteria biofilm

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MDPI

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Bacterial Ecophysiology and Biotechnology

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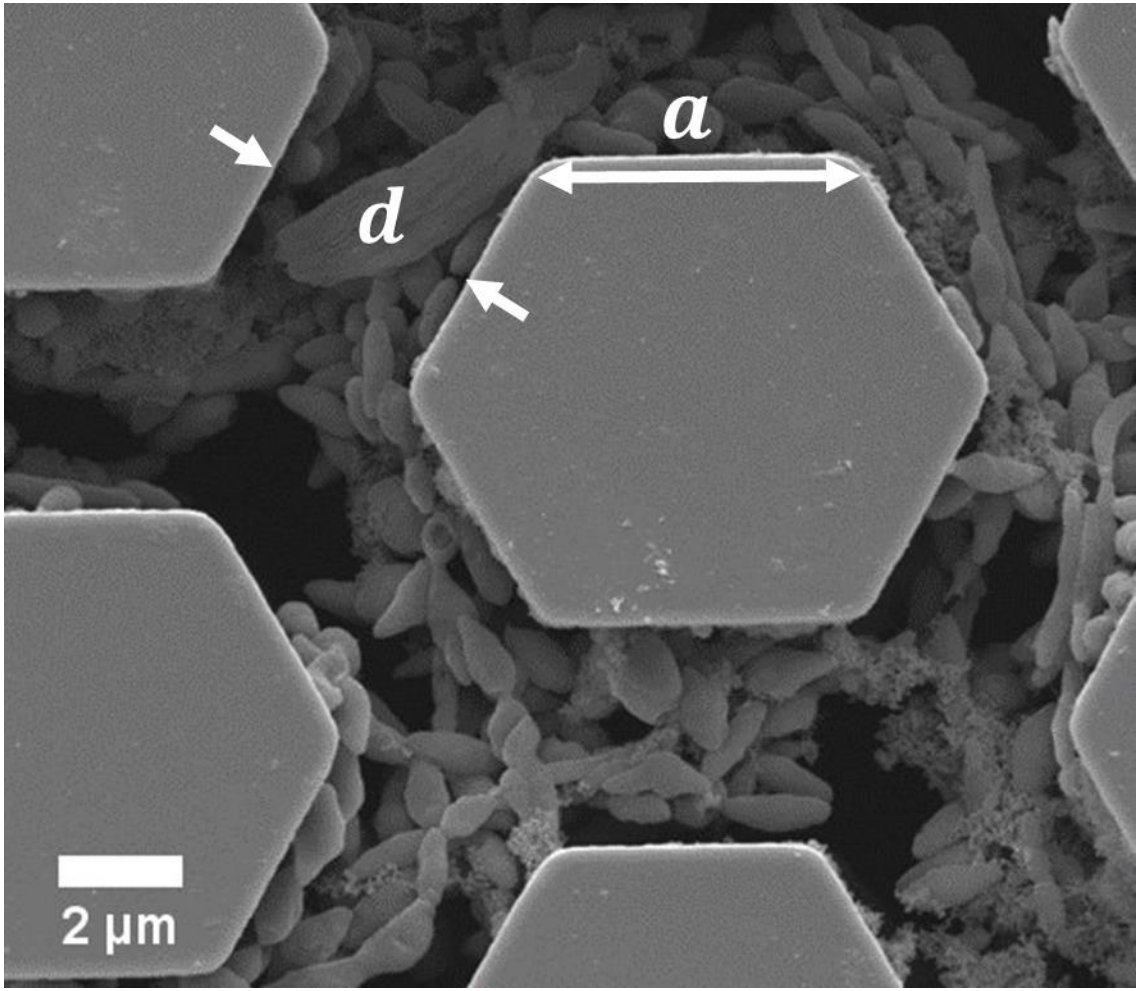


Fabrication of micro-structured surface topologies for the promotion of marine bacteria biofilm

Abstract:

Several marine bacteria of the *Roseobacter* group can inhibit other microorganisms and are especially antagonistic when growing in biofilms. This aptitude to naturally compete with other bacteria can reduce the need for antibiotics in large scale aquaculture units, providing that their culture can be promoted and controlled. Micro-patterned surfaces may facilitate and promote the biofilm formation of species from the *Roseobacter* group, due to the increased contact between the cells and the surface material. Our research goal is to fabricate biofilm optimal micro patterned surfaces and investigate relevant length scales for surface topographies as well as surface chemistry, which can promote growth and biofilm formation of the *Roseobacter* group bacteria.

In a preliminary study, silicon surfaces comprising arrays of pillars and pits with different periodicities, diameters and depths were produced by UV lithography and deep reactive ion etching (DRIE) on single-side polished silicon wafers. The resulting surface microscale topologies were characterized using optical profilometry and scanning electron microscopy (SEM).
Screening of the bacterial biofilm on the patterned surfaces was performed using green fluorescent staining (SYBR green I) and confocal laser scanning microscopy (CLSM). Different series of experiments were conducted by changing several parameters such as; growth time, shear stress corresponding to particular revolution per minute (rpm) and growth media. Preliminary results indicate that there is a correlation between the surface morphology, and the spatial organization of the bacterial biofilm.

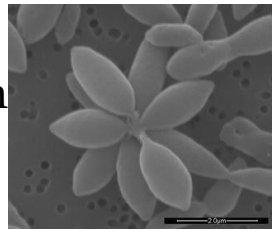
Our results indicate that further investigation leading to optimization of surface topology and surface chemistry will allow us to microfabricate polymer material surfaces where biofilm colonization is enhanced. Such surfaces will enable the introduction of beneficial bacteria in a variety of industrial processes including aquaculture.

Keywords: structured surfaces; silicon surfaces; microfabrication; bacterial biofilm; microbial adhesion

Motivation and Industrial Application

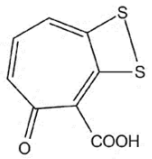
Industrial Application / Aquaculture systems
In fish production, transmission of pathogenic bacteria can be inhibited by introducing probiotic bacteria forming biofilms (Bruhn et al. 2005).
The focus is on marine bacteria and mainly the fish probiotic bacterium *Phaeobacter inhibens* (DSM 17395), which produces a **antimicrobial compound** on the biofilm mode of growth.

***Roseobacter* fish probiotic bacterium**
Antagonists of fish pathogens in marine environments includin live feed cultures.[1]



Scanning electron micrograph of *Roseobacter spp*. biofilm during growth on a planar surface. [2]

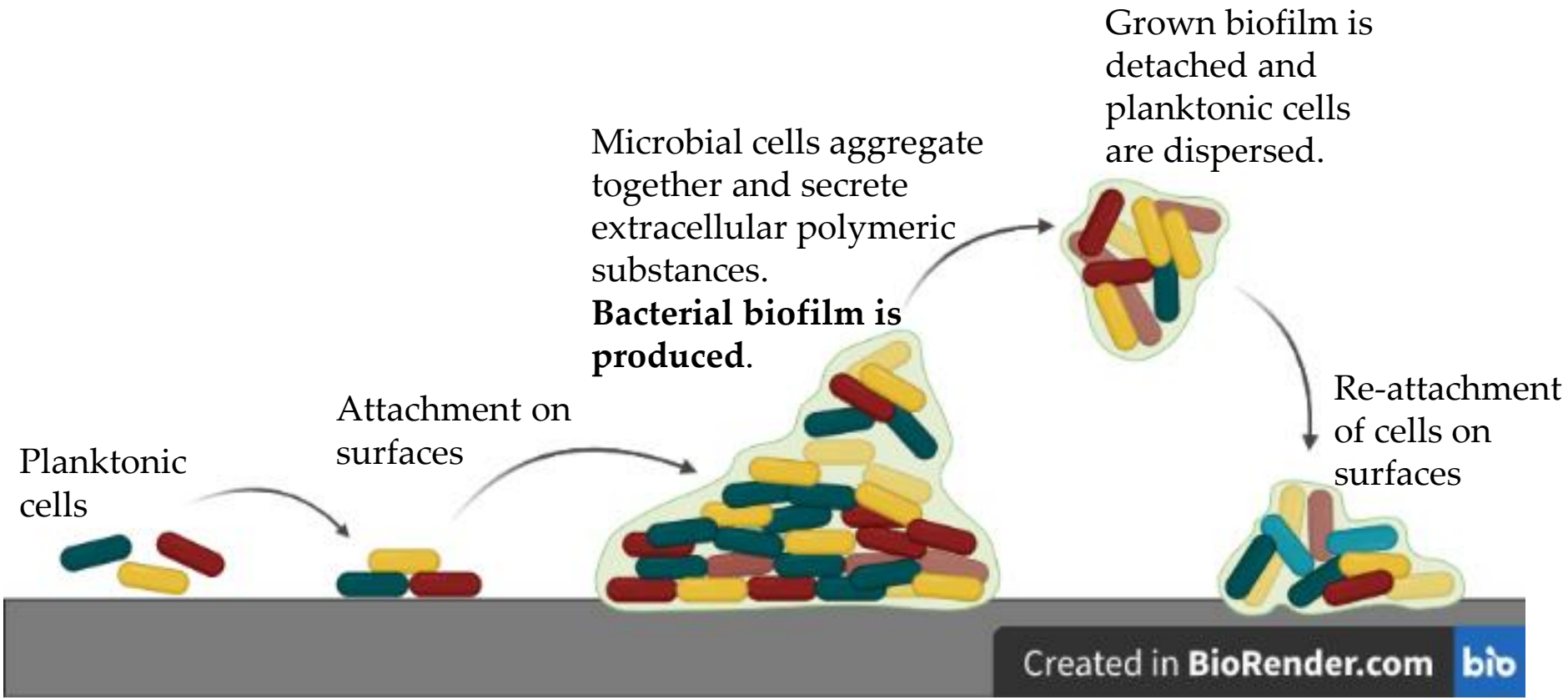
Beneficial effect in bacterial biofilm growth
Antimicrobial molecules on the membrane of bacteria (Tropodithietic acid)



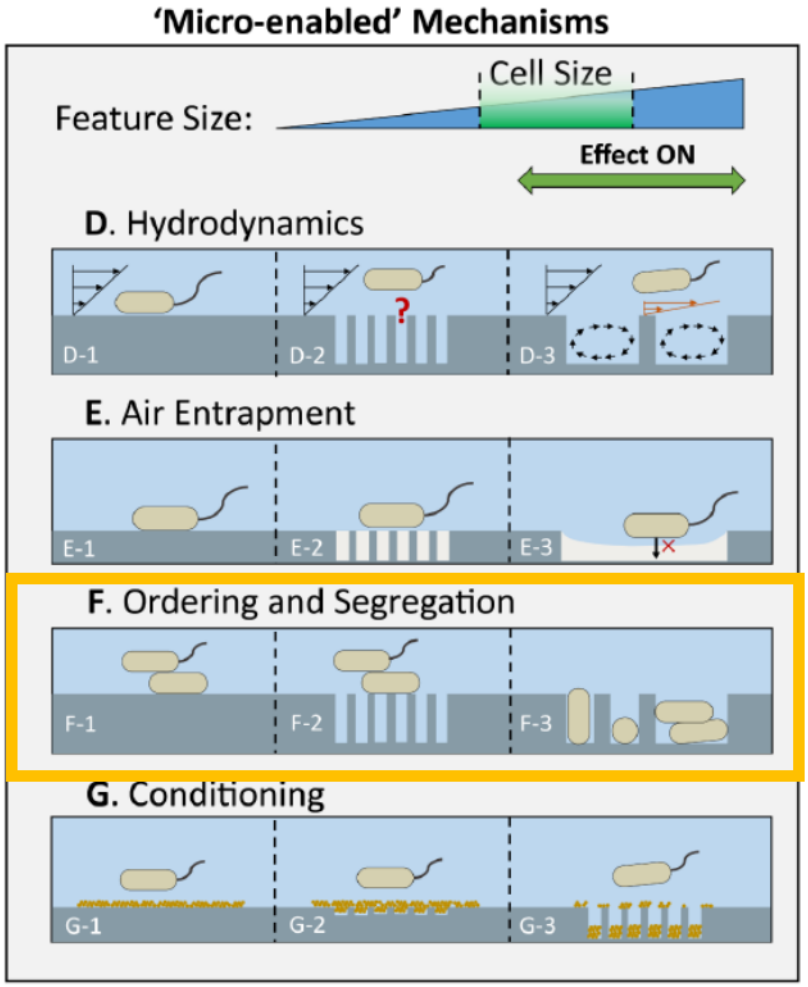
Tropodithietic acid (TDA)

1. D'Alvise, P., Lillebø, S., Prol García, M. J., Wergeland, H. I., Nielsen, K. F., Bergh, O., & Gram, L. (2012).Phaeobacter gallaeciensis Reduces Vibrio anguillarum in Cultures of Microalgae and Rotifers, and Prevents Vibriosis in Cod Larvae. PLoS One, 7(8), e43996. https://doi.org/10.1371/journal.pone.004399
2. Bruhn, J. B., Nielsen, K. F., Hjelm, M., Hansen, M., Bresciani, J., Schulz, S., & Gram, L. (2005). Ecology, Inhibitory Activity, and Morphogenesis of a Marine Antagonistic Bacterium Belonging to the Roseobacter Clade. Applied and Environmental Microbiology, 71(11), 7263–7270. https://doi.org/10.1128/AEM.71.11.7263-7270.2005

What is a biofilm?



Conditions of Biofilm growth



Scale-dependent effects of surface topography on various factors that influence initial bacterial attachment[3]

Various factors may influence bacterial attachment on surfaces

<u>CURRENT STUDY</u>

Focus on enhancement of bacterial attachment based on microscale topography

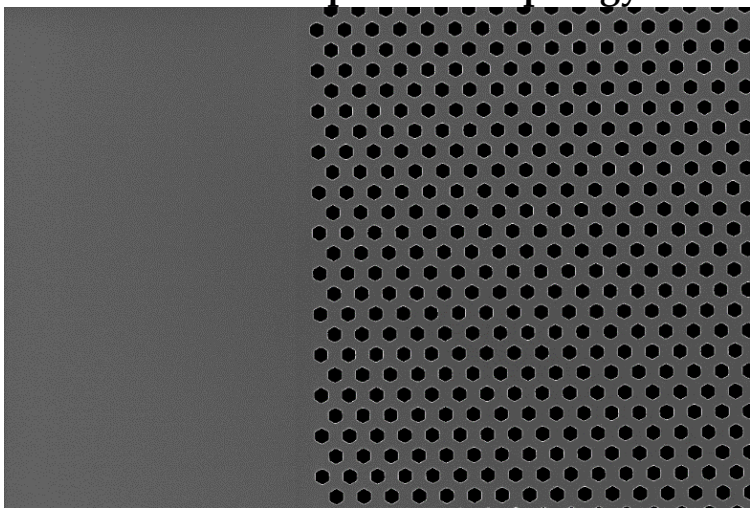
3. Cheng, Y., Feng, G., & Moraru, C. I. (2019). Micro-and nanotopography sensitive bacterial attachment mechanisms: A review. Frontiers in Microbiology, 10, 191. https://doi.org/10.3389/fmicb.2019.00191

Background

The biofilm formation by bacteria and their interaction with biotic and abiotic surfaces is necessary to develop appropriate materials and technologies to facilitate their biofilm formation.

For a better understanding on how a particular surface can facilitate the biofilm growth of *Phaeobacter inhibens*, micro-patterned surfaces with different length scales and surface topographies were fabricated to investigate if bacterial biofilm formation can be promoted.

Planar surface **Optimized topology**



Bacterial surface adhesion on surfaces[4]

Which surfaces facilitate biofilm formation?

4. Friedlander, R. S., Vlamakis, H., Kim, P., Khan, M., Kolter, R., & Aizenberg, J. (2013). Bacterial flagella explore microscale hummocks and hollows to increase adhesion. Proceedings of the National Academy of Sciences of the United States of America, 110(14), 5624–5629. https://doi.org/10.1073/pnas.1219662110

Objectives/ Aims

Aim:

Understand how a particular surface can facilitate the biofilm growth of marine bacteria in aquaculture units, such as the beneficial *Phaeobacter spp.*

Research Question:

How is the biofilm growth on the material surface (cell-surface interaction)?

Micro-topographic patterns may promote bacterial adhesion and biofilm formation.

Hypothesis:

By changing the size and space between pillars and pits (length scales and polarities), bacterial biofilm can be promoted.

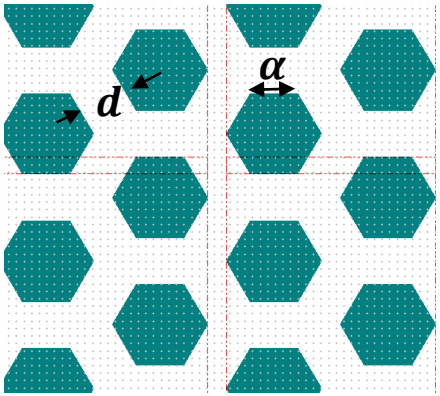
Material: Silicon substrate

Methods (1: Pattern Design)

Design of surfaces comprising arrays of pillars and pits with different periodicities, diameters and depths.

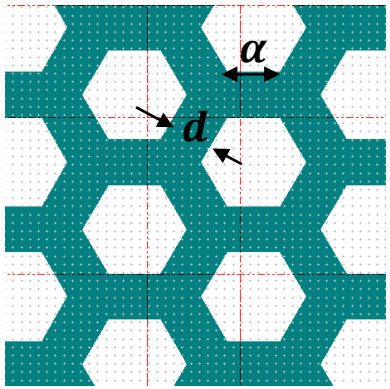
Surface Morphology
Honeycomb patterns: known shape in nature and used in literature
It allows to change the length scale and the filling

Different ***a*** (periodicity or side length) diameter ***d*** (trench distance) and depth
Different polarities: Pillars or pits (holes)



<u>Pillars</u>
Range of parameters

a = 2.5, 5, 10 µm
d=1, 2.5, 5, 10 µm

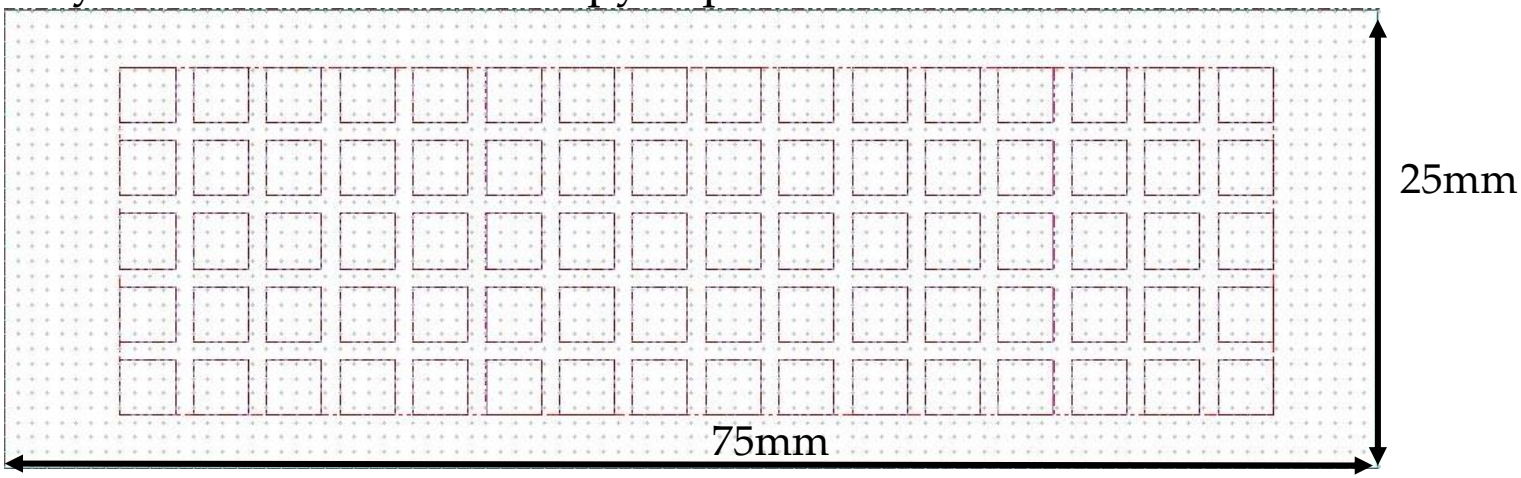


<u>Pits</u>
Range of parameters

a= 2.5, 5, 10 µm
d= 1, 2.5, 5 µm

Methods (2: Chip design)

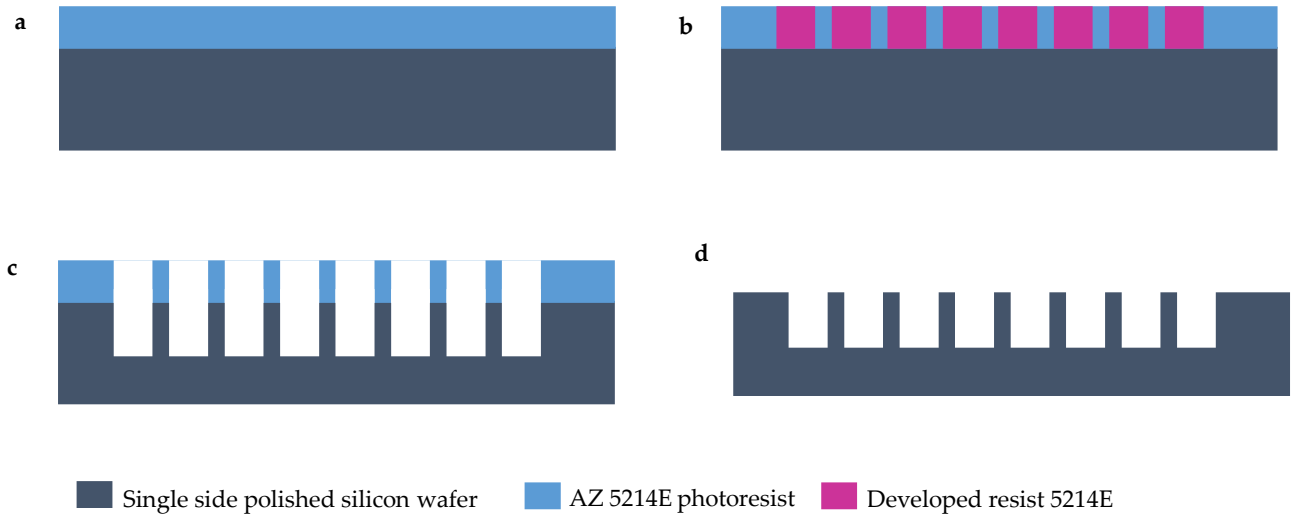
Design of microscopy slide as substrate (75mm x 25mm)
Easy to handle for microscopy experiments.



- **Honeycomb patterns**: different polarities
- Each chip one pattern
- 4 Replicates of each pattern in each slide – **randomized in different positions**
- **Control planar surfaces**

Methods (3: Fabrication)

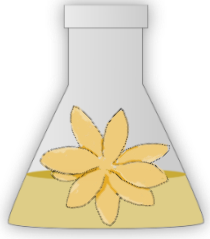
Fabrication by UV lithography and deep reactive ion etching (DRIE) on single-side polished silicon wafers.



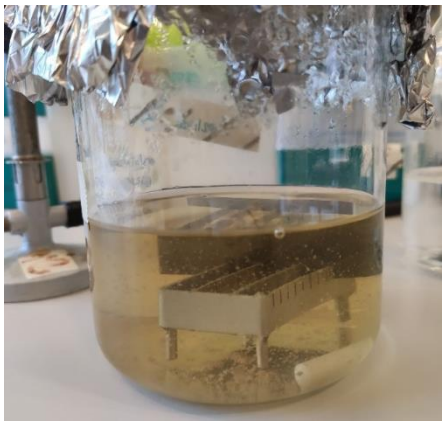
Fabrication steps for acquiring microscope slide.
Silicon master: Steps (a)-(d) are : (a) UV deposition of photoresist AZ5214E and UV exposure with MLA2, (b) Resist development, (c) Dry etching of silicon by Bosch process and (d) Plasma ashing of resist.

Methods (4: Bacterial culture)

Experimental setup: Closed system
Strain: *Phaeobacter inhibens* **DSM 17395**
Condition of shear stress: Low stirring (100 rpm) or Higher stirring (700 rpm)
Growth media: Marine Broth or Marine Minimal Media (inoculation at 25^{o}C)
Time: from 3 days up to 8 days



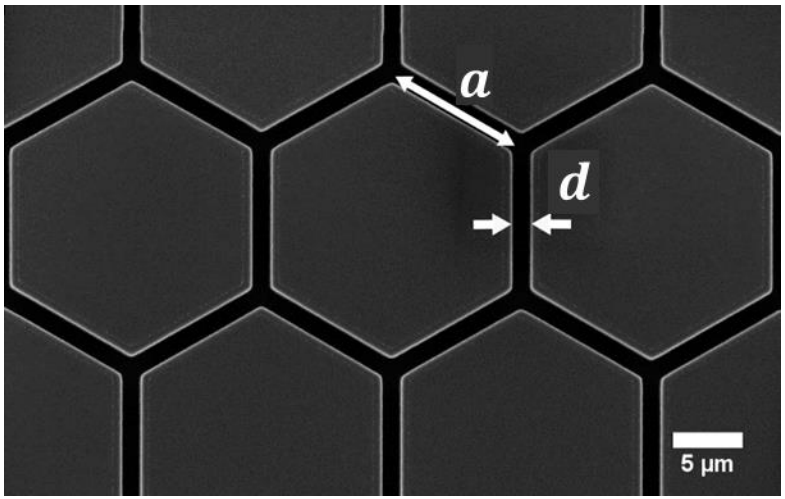
Before inoculation



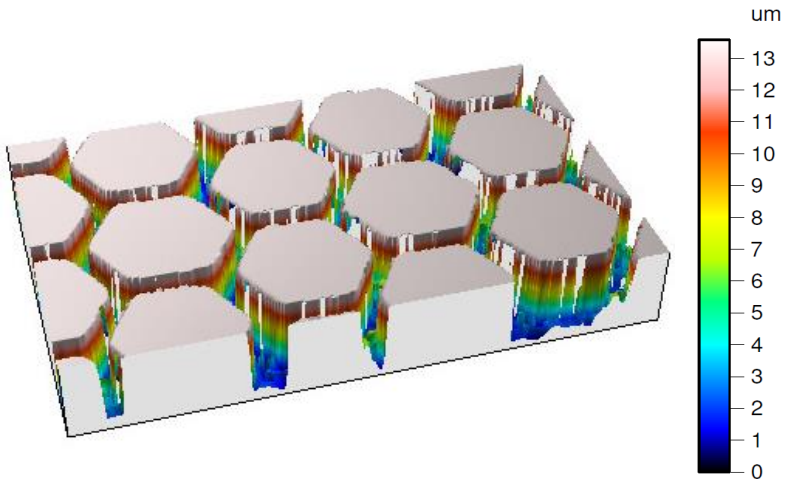
After inoculation

Results (1: Sample topology)

The resulting surface microscale topologies were characterized using optical profilometry and scanning electron microscopy (SEM).



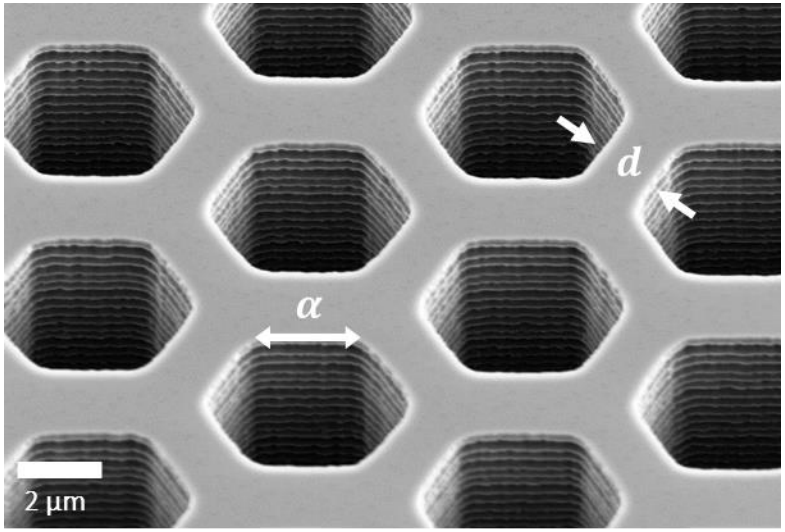
Silicon patterned microstructure of an array of honeycomb pillars with side length ***a*** ~ 10 µm and trench width ***d*** ~ 1µm.



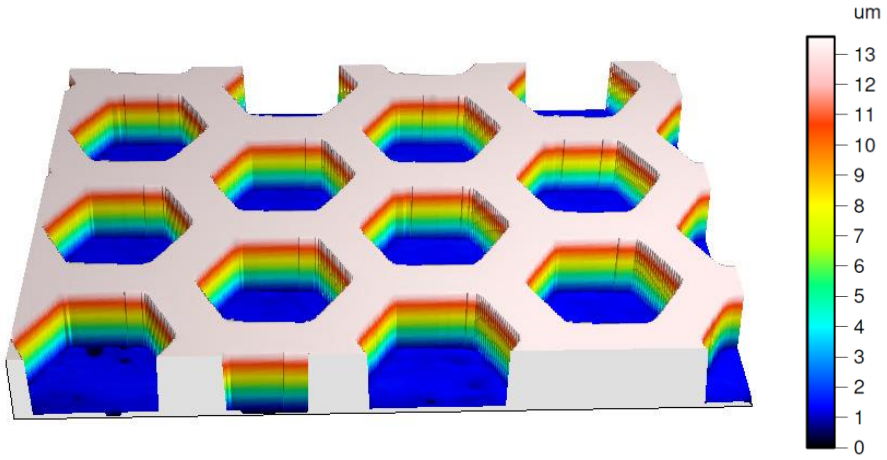
3-D profile taken with optical profiler (Sensofar PLu Neox 3D) ***d*** ~1 µm, ***a*** ~ 10 µm and height ~ 13 µm. The color code represents heights from blue (low) to white (high).

Results (2: Sample topology – continued)

The resulting surface microscale topologies were characterized using optical profilometry and scanning electron microscopy (SEM).



Silicon patterned microstructure of an array of honeycomb pits with side length $\boldsymbol{\alpha}$ ~ 5 µm and trench width $\boldsymbol{d}$ ~ 2.5 µm.

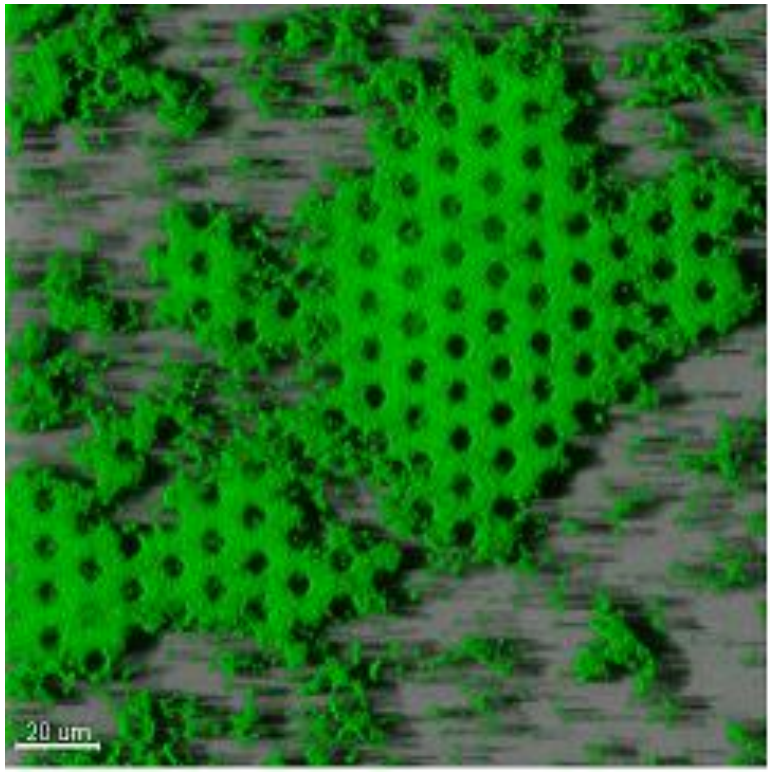


3-D profile of honeycomb pits with taken by optical profiler (Sensofar PLu Neox 3D) side length $\boldsymbol{a}$ ~ 5 µm and width $\boldsymbol{d}$ ~ 2.5 µm and depth ~12 µm. The color code represents heights from blue (low) to white (high).

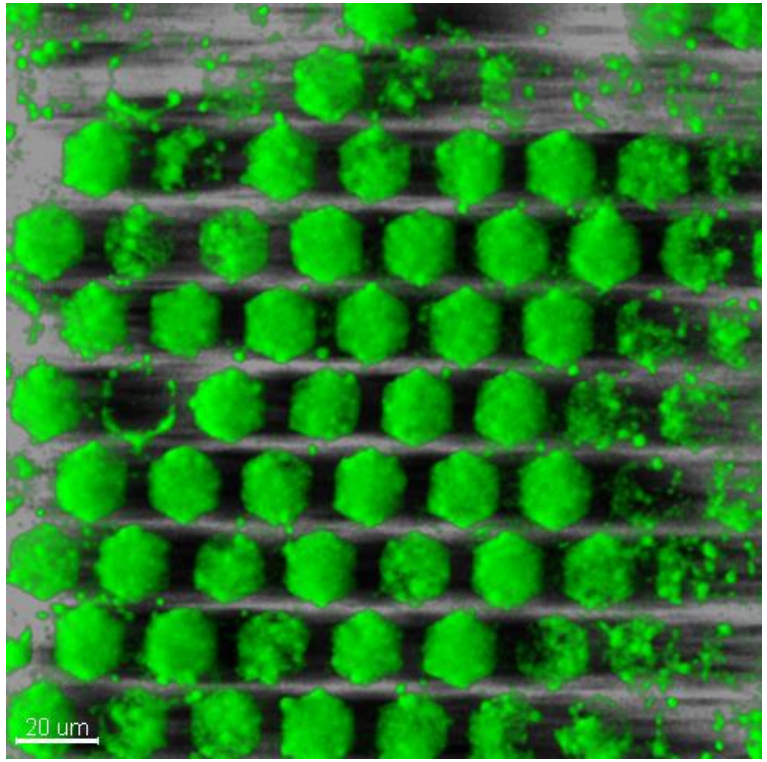
Results (3: Bacterial growth)

Screening of the bacterial biofilm on the patterned surfaces was performed using green fluorescent staining (SYBR green I) and inspection with confocal laser scanning microscopy (CLSM), using a Leica TCS SP8.

***Phaeobacter inhibens* biofilm stained with SYBR Green I. (Magnification 63X) Scale bar=20μm.**



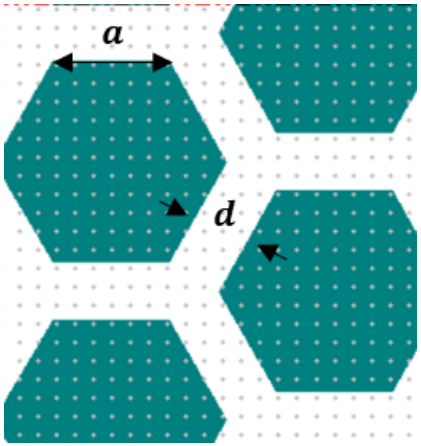
Biofilm grown on honeycomb pillars with side length ~ 2.5 μm, width (trench) ~ 10 μm and depth ~ 12 μm. (biofilm incubation with low stirring after 48 hours in 25 °C)



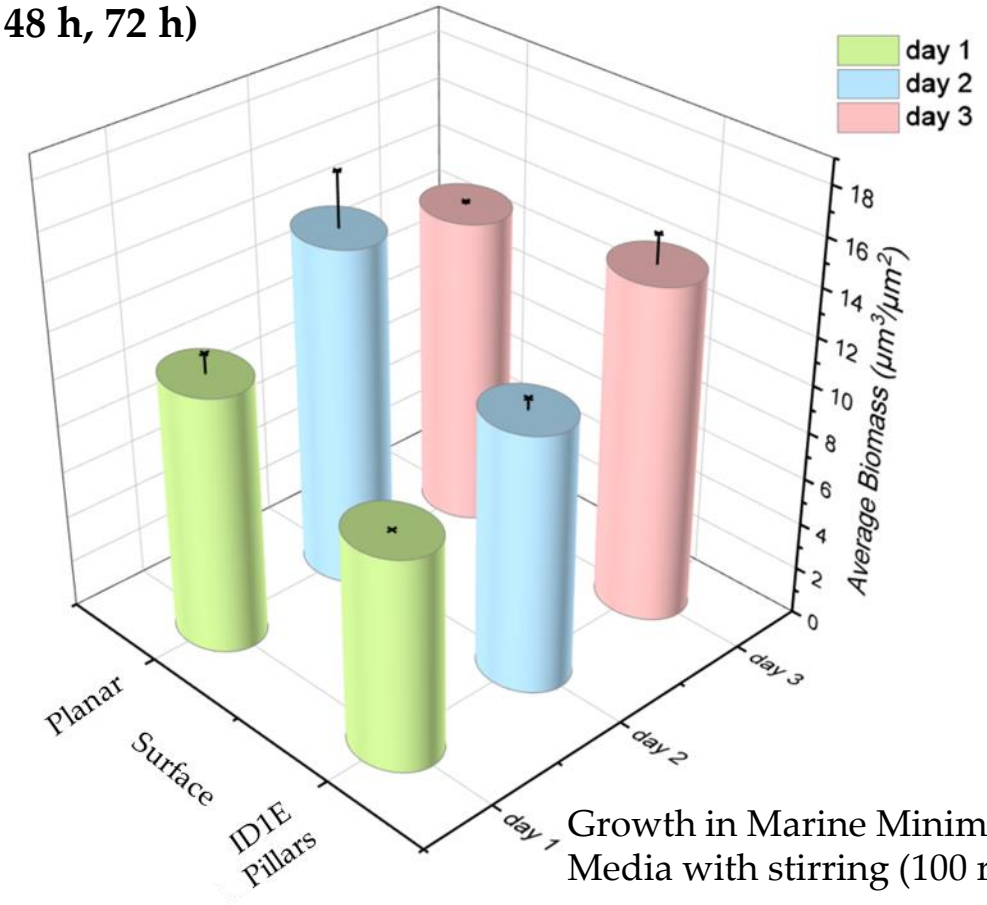
Biofilm grown on honeycomb pits with side length ~ 10 μm, width (trench) ~ 5 μm and depth ~ 12 μm. (biofilm incubation with low stirring after 48 hours in 25 °C)

Results (4: Biofilm quantification over time)

Time experiment (Incubation ≈ 20 h, 48 h, 72 h)



ID1E: Honeycomb pillars with side length $\boldsymbol{a}$ ~ 5 μm and width $\boldsymbol{d}$ ~ 2.5 μm



Growth in Marine Minimal Media with stirring (100 rpm)

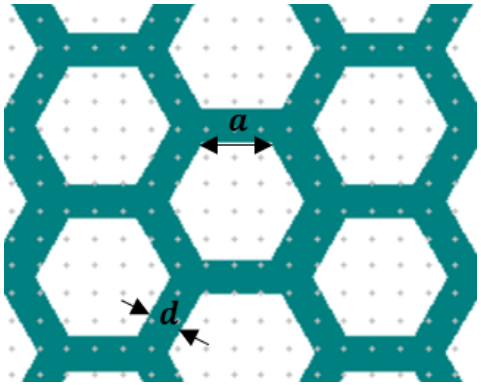
By quantifying the biomass with Comstat software results show that:

The average biomass (μm³/μm²) on planar surface (flat) is similar to biomass grown on patterned surfaces after 3 days of growing with low stirring.

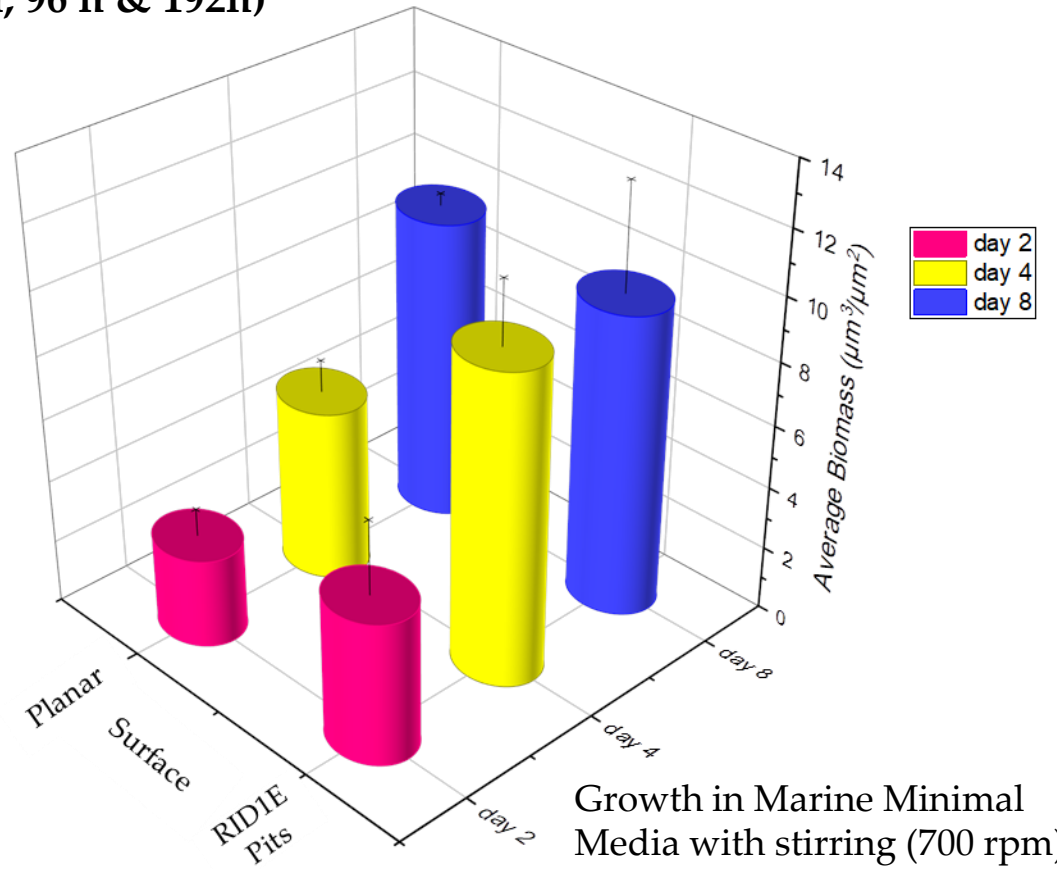
Comstat software:
(*Heydorn, A., et al., 2000)*
(Vorregaard, M., 2008)
www.comstat.dk

Results (5: Biofilm quantification under stress)

Stress experiment (Incubation ≈ 48 h, 96 h & 192h)



RID1E pits with side length ***a*** ~ 5 µm and width ***d*** ~ 2.5 µm



Growth in Marine Minimal Media with stirring (700 rpm)

Results indicate that:

The number of biomass ($\mu m^3/\mu m^2$) on planar surface(flat) is similar to biomass grown on patterned surfaces, however the biofilm is grown faster after 4 day on the patterned surface.

Discussion and Conclusion

Preliminary experiments on silicon substrate show that:

- ✓ There is a correlation between the surface morphology, and the spatial organization of the bacterial biofilm.
- ✓ The number of biomass ($\mu m^3/\mu m^2$) on planar surface(flat) is similar to biomass grown on patterned surfaces in silicon substrate, but further experiments are required to corroborate the results on patterned surfaces.

- ➢ Further optimization of surface topology and surface chemistry will be done by micro-fabrication of polymeric surfaces where biofilm colonization is enhanced.
- ➢ Polymeric surfaces will enable the introduction of beneficial bacteria in a variety of industrial processes including aquaculture.

Acknowledgments

DTU Nanolab
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POLYMIC
Polymer Micro & Nano Engineering

DTU Bioengineering
Department of Biotechnology and Biomedicine

Bacterial Ecophysiology and Biotechnology

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