MICROBIAL COLONIZATION OF A SMOOTH SUBSTRATUM: A KINETIC ANALYSIS USING IMAGE ANALYSIS

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INTRODUCTION

Microbial cells attach firmly to almost any surfaces submerged in an aquatic environment. The immobilized cells grow, reproduce, and produce extracellular polymers which extend free cells forming a matrix of molecular fibers that provide the structure to the sessile biofilm. Biofilms are sometimes distributed relatively evenly over the wetted surface and are other times quite "patchy" in appearance. In all cases, biofilms are the result of transport of bacteria to the substratum and consequent growth at the substratum. This secondary phase, the growth phase of biofilms, is well understood and can be described with predictive models of different complexity. Some simpler models are based on a single substrate limitation and a continuous detachment rate of cell back into the bulk liquid. Others include diffusion limitation of different nutrient or diffusivity and consumption of oxygen or any combination of it. But in all cases, they assume an initial homogeneous cell distribution at the substratum, which does not reflect the true initial colonization of a previously clean substratum.

RELEVANCE

The initial colonization of a clean substratum by cells is essential for the buildup of a biofilm; cells have first to be transported to the substratum, have to adhere to it, and grow. These few essential processes are influenced by a multitude of different parameters, biotic as well as abiotic, such as differential of surface charges between the cells and the substratum, shear stress of the liquid at the substratum, which can remove newly adsorbed cells from the substratum, or motility of the cells, which increases the probability that cells reach the substratum. Thus, these parameters can have an accelerating or inhibiting influence to the rate of microbial colonization of a clean substratum. This initial phase of biofilm development is a heterogeneous process which can be described with a stochastic model based on the probability of single events.

PROCESSES OF COLONIZATION

The rate of cellular colonization of a substratum is the net result of transport (e.g., adsorption, desorption) and growth processes. The initial events of biofilm accumulation (colonization) can be expressed in terms of two variables: colony forming units (CFU), and cells. This distinction is essential since cells can adsorb in groups or as single cells. Moreover, not all the cells accumulate at the substratum through transport alone. They might form at the substratum (growth and reproduction) changing the number of cells per colony with time, or any even glide away from their colony of origin to form a new colony. The following four processes in terms of CFU must be distinguished:

1. Diffusive or advective transport processes carry the CFU to a point adjacent to the substratum. In laminar flow, only diffusive transport is considered. In turbulent flow, advective transport generally dominates.

2. Adsorption in the linking of the CFU with the substratum. The cell is adsorbed to the substratum only if it has a linkage to it and, hence, becomes immobilized for a discrete, minimum time.
3. Desorption describes the breaking of the CPU-substratum linkage and its complete removal from the substratum. Desorption is the reverse of adsorption.

4. CPU-separation, although not related to adsorption or desorption, contributes to the accumulation of CPU at the substratum by changing the number of CPU adsorbed. A CPU with more than one cell can separate into two independent CPU as a result of fluid shear or even cell motility.

The four processes can be translated in terms of cells by determining the number of cells in each CPU. However, CPU-separation does not contribute to accumulation expressed in terms of cells. In addition to advection transport, adsorption, and desorption, two other processes are defined when cells is the variable:

1. Multiplication is related to cellular growth. Cells within a CPU multiply and the number of cells within this CPU increase. This does not change the accumulated number of CPU but does change the accumulated number of cells.

2. CPU-detachment recognizes that cells within a CPU can "detach" and, hence, reduce the cell number of the CPU. This process is the reverse of multiplication. Detachment is detachment of cells from a CPU which must be contrasted to desorption which is detachment of an entire CPU from the substratum.

**IMAGE ANALYSIS METHODS**

The individual processes contributing to the substratum colonization can be separated and measured independently with image analysis methods.

In the laminar flow experiments described in this chapter, microbial cells were grown in a chemostat and continuously flowed through a rectangular glass capillary. The chemostat operation minimized variations in cell physiological state and cell concentration throughout an experiment. The liquid wall of the rectangular capillary is the substratum and the processes occurring at the substratum were monitored continuously by a high-resolution television camera mounted on a microscope. The video signal was transmitted to an image analyzer which converted the grey image into a binary image. A single binary image of the specimen was stored on disk at constant time intervals (e.g., every five minutes) for later analysis.

**CONCEPTUAL MODEL OF SUBSTRATUM COLONIZATION: Laminar Flow**

The analysis of adsorption, desorption, and growth-related processes during the early colonization of a substratum cannot be considered in traditional terms of instantaneous mass balances. Rather, population balances must be used in which the single observed cells are individual members of the total population. Thus, the probability of an event within the total population is the key parameter.

**POPULATION BALANCE IN TERMS OF CPU**

The population balance will be made in terms of Colony Forming Units (CPU), a particle which consists of one or more cells. With an image analysis system, each CPU can be observed and treated individually and can be converted to cell numbers by determining the number of cells per CPU with the image analysis system. The stoichiometry of the system in terms of CPU can be described with the following equations:

\[
\begin{align*}
X_{Bu} & \quad \leftrightarrow \quad X_{re,\,rev} \quad \leftrightarrow \quad X_{re,\,irrev} \\
\text{suspended biomass} & \quad \text{reversible adsorbed biomass} & \quad \text{irreversible adsorbed biomass} \\
X_{re,\,rev} & \quad = \quad X_{re,\,irrev} \quad + \quad X_{re,\,irrev} \\
\text{Total adsorbed CPU} & \quad \text{total reversibly adsorbed CPU} & \quad \text{total irreversibly adsorbed CPU} 
\end{align*}
\]

CPU from the bulk liquid adsorb at the substratum first as reversible and then as irreversible CPU (Eq. 1.1). The population balances for the two forms are as follows:
Microbial colonization of a smooth substrate

**Reversible Adsorption (CFU):**

\[
\frac{d X_{rev,rev}}{dt} = \Gamma_{s,rev} - \Gamma_{d,rev} - \Gamma_{r,rev} \tag{9}
\]

Accumulation adsorption desorption transformation

where
- \( X_{rev,rev} \): CFU concentration at substrate [# CFU L\(^{-1}\)]
- \( \Gamma_{s,rev} \): adsorption rate for CFU [# CFU L\(^{-1}\) t\(^{-1}\)]
- \( \Gamma_{d,rev} \): desorption rate for CFU [# CFU L\(^{-1}\) t\(^{-1}\)]
- \( \Gamma_{r,rev} \): transformation from reversibly adsorbed CFU to irreversibly adsorbed CFU [# CFU L\(^{-1}\) t\(^{-1}\)]

**Irreversible Adsorption (CFU):**

\[
\frac{d X_{rev,irrev}}{dt} = \Gamma_{r,irrev} + \kappa_{s,irrev} \cdot X_{rev,irrev} \tag{4}
\]

Accumulation transformation CFU-separation

where
- \( \kappa_{s,irrev} \): probability of separation [t\(^{-1}\)]

**Total CFU:**

\[
\frac{d X_{tot}}{dt} = \frac{d X_{rev,rev}}{dt} + \frac{d X_{rev,irrev}}{dt} \tag{5}
\]

According to Equation 5, the total accumulation of CFU is the sum of the accumulation of reversibly and irreversibly adsorbed CFU. Thus, accumulation can be defined as:

\[
\frac{d X_{tot}}{dt} = (\Gamma_{s,rev} - \Gamma_{d,rev}) + (\kappa_{s,irrev} \cdot X_{rev,irrev}) \tag{6}
\]

Accumulation net adsorption "growth"

**Population Balance in Terms of Cells**

Similar to Equation 6, a population balance for cells can be defined with the following equation:

\[
\frac{d X_{tot}}{dt} = \Gamma_{s,cel} - \Gamma_{d,cel} + \kappa_{s,cel} \cdot X_{cel,cel} \cdot (\kappa_{d,cel} - \kappa_{e,cel}) \tag{7}
\]

Accumulation adsorption desorption multiplication erosion

where
- \( X_{cel,cel} \): Cell concentration at substrate [# cells L\(^{-1}\)]
- \( \Gamma_{s,cel} \): adsorption rate for cells [# cells L\(^{-1}\) t\(^{-1}\)]
- \( \Gamma_{d,cel} \): desorption rate for cells [# cells L\(^{-1}\) t\(^{-1}\)]
- \( \kappa_{s,cel} \): probability of cell attachment [t\(^{-1}\)]
- \( \kappa_{e,cel} \): probability of cell erosion [t\(^{-1}\)]

**Transport from Bulk Flow to Substrat**

Bowen et al. (1978) proposed an analysis with a first-order-reaction approximation for the substrate-particle capture rate, which leads to an expanded Griesz solution. This solution converges well for relatively large Péclet numbers and proved to be accurate for inert particles adsorbing to charged substrate. The resulting equation has the following form:
where

\[ \frac{r_{a,CFU}}{h} = \frac{\left( \frac{s_1}{s_2} \right)^{1/3}}{r\left( \frac{\xi}{\theta} \right) + \frac{1}{r\left( \frac{s_1}{s_2} \right)^{1/3}}} \]

According to Equation 8, the adsorption rate is directly proportional to the concentration and the diffusivity of the biomass in the bulk flow. This proportionality of adsorption rates to the bulk concentration is displayed in Figure 1 (Escher, 1987). Under laminar flow conditions, particles are transported to the substrate by diffusion perpendicular to the flow. Microorganisms with a size of 1 to 6 mm² have a very small Brownian motion and, hence, a small Brownian diffusivity. Therefore, motility is of considerable importance during the process of transport in laminar flow, but is often neglected in adsorption studies. Jang and Yen (1985) calculated the non-Brownian diffusivity (motility) for different microorganisms to be in the range of \( 3 \times 10^{-5} \) to \( 6 \times 10^{-5} \) m²/s, compared to the Brownian diffusivity of \( 0 \times 10^{-5} \) m²/s.

**Adorption Rates CFU, 0.5 N/sqm**

![Adorption Rates CFU, 0.5 N/sqm](image)

**Figure 1.** Adsorption rates at 0.5 N m⁻² shear stress in terms of CFU plotted against CFU concentration in the bulk flow.

**IMPORTANCE OF SUBSTRATE-PICTICLE FACTOR ε**

The dimensionless substrate-particle capture factor ε has a great importance for the analysis of experimental data. This factor, which is independent of bacterial transport rate to the substrate and geometry of the system, can be used to describe the interaction of the cells with the substrate during the process of adsorption. It is a function of different factors affecting the probability of adsorption of cells hitting the substrate. Thus, this factor can serve to compare different interactions due to different substrate charges, both of the cells and substrate, and different shear stresses in different experimental systems.

**RESULTS, PROGRESSION OF EXPERIMENTS**

The results are based on 15 experiments, 9 at 0.5 N m⁻² and two each at 1.0, and 1.25 N m⁻² CFU concentration in the bulk flow of the capillary tube ranging from 3.19×10⁻⁶ to 1.2×10⁻⁶ CFU ml⁻¹. Each experiment lasted 300 min for colonization, and images were taken at 5 min intervals. The progression of a typical substrate colonization is displayed in Figure 2a for adsorption, desorption, and accumulation of CFU, and in Figure 2b for area coverage. The same colonization data but in terms of cell is displayed in Figures 3a and 3b. This set of experimental data resulted from a shear stress of 1 N m⁻² and a bulk CFU...
concentration of 6.75 x 10^6 CFU m⁻³. The measured events of adsorption, desorption, multiplication and erosion are plotted cumulative to display their rates (slope) whereas accumulation is displayed as measured. These results indicate that growth related rates, i.e., CPU-separation and multiplication are a function of the surface accumulation, whereas the adsorption and desorption rates are independent of the surface accumulation. Thus, erosion related processes follow a zero order kinetic and growth related processes are first order rates in respect to accumulation at substratum.

Figure 2a. Typical progression of colonization in terms of CFU of a smooth glass substratum. Shear stress: 1.0 N m⁻², CFU bulk concentration: 6.75 CFU ml⁻¹. Adsorption, desorption, and CPU-separation are displayed cumulatively, whereas accumulation is shown as measured.

Figure 3a. Typical progression of colonization in terms of cells of a smooth glass substratum. Shear stress: 1.0 N m⁻², CFU bulk concentration: 6.75 CFU ml⁻¹. Adsorption and desorption are displayed cumulatively, whereas accumulation is shown as measured.

Figure 2b. Area coverage by biomass during surface colonization. Shear stress: 1.0 N m⁻², CPU bulk concentration: 6.75 CFU ml⁻¹.

Figure 3b. Multiplication and erosion are displayed cumulatively, whereas accumulation is shown as measured. Shear stress: 1.0 N m⁻², CFU bulk concentration: 6.75 CFU ml⁻¹.

RESULTS. SURFACE-PARTICLE CAPTURE FACTOR E

As stated before, the dimensionless surface-particle capture factor E describes the interaction between the particles and the substratum. Shear stress and CFU bulk concentration have been the only variables in the presented series of experiments. Therefore, a variation of the value of E can be attributed
SIMULATION WITH THE KINETIC RESULTS

The kinetic results can be used to simulate the accumulation of cells over an extended time (10 hours). The simulation uses the integrated equation 1 for a population balance in terms of cells. Figure 3 displays a simulation with constant shear stress (0.5 N m⁻²) and a variation of CFU concentration in the bulk flow. The simulations are displayed as lines. Accumulation of cells from an experimental series is marked with dots to compare the simulation with the actually measured values. The simulation with a constant CFU concentration of 5-10⁶ CFU ml⁻¹ has a cell concentration at the substrate in a similar range as the experiment. All the simulations show that the increased accumulation as the real experiments (see Figure 3a). The simulation suggests that accumulation under constant shear stress is proportional to the CFU concentration in the bulk flow. It appears that sorption related processes are important, but that after
about 100 minutes growth related processes start to contribute to the accumulation increasingly. The degree of contribution is, however, an indirect function of the CPA concentration, and, thus, of adsorption. This becomes evident in a simulation with a variation of shear stress and a constant CPA concentration in the bulk flow (Figure 4). At low shear, adsorption is greater than at high shear stress, resulting in a more rapid accumulation of cells at the substratum. This simulation suggests that, although growth related processes start to control accumulation after about 100 minutes, the influence of CPA concentration dictates the extent of accumulation with time.

CONCLUSION

The work presented uses a novel technique to measure early colonization of a smooth substratum. Several steps in the analysis of colonization processes have been developed for this work and have not been used before. Several conclusions with respect to the method and the experimental results have been derived:

1. The method of using an image analyzer allows direct measurement of essential independent processes contributing to colonization of substrates.

2. Behavioral characteristics of the organisms at the substratum, such as growth, rate and direction of motion, orientation, and more, can be measured in situ.

3. The method provides for a novel quantitative analysis of spatial distributions of organisms during adsorption.

4. Sorption related processes depend on shear stress and bulk CPA concentration, whereas growth related processes depend on the cell concentration at the substratum slime (under the experimental conditions).

5. Sorption related processes are zero order rates, whereas growth related processes are first order rates with respect to substratum concentration.

6. Although growth related processes are dominant within a short time, the extent of accumulation depends heavily on sorption processes.

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