Understanding and controlling detrimental bioreactor biofilms

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Unintentional biofilm formation within a bioreactor can have serious detrimental effects on bioreactor performance. These range from the obvious (reduced heat-transfer efficiency, increased frictional resistances, and fouling of internal diagnostic probes) to the more subtle (atypical econiches, species- or strain-specific adhesion, and inaccurate estimates of culture stoichiometric and kinetic parameters). Detection and control of unwanted biofilm formation in bioreactors are also considered.

Several years ago, a colleague inquired to what extent a minor amount of 'wall growth'[1] would affect the operation of a chemostat. The continuous-culture system (Fig. 1) was being used to determine the kinetic and stoichiometric growth parameters for a strain of *Hyphomicrobium* spp. My colleague's assumption was that any effect would be insignificant since:

> '. . . The system is at steady-state which allows me to determine yield from residual biomass and substrate concentrations . . . However, my estimate of the maximum dilution rate does seem exceptionally high.'

This attitude to adherent cell layers in bioreactors is common, with biofilm formation in a fermenter considered merely to be an aesthetic nuisance. Any detrimental effects are assumed significant only in small, lab reactors due to the high surface-area-to-volume ratio. Figure 2 illustrates an impeller assembly removed from an industrial-scale fermenter that was rendered completely inoperable due to massive biofilm growth[2]; a similar biofilm mass was also discovered completely covering the fermenter heat-exchanger coils.

As described by Blenkinsopp and Costerton[3], biofilms are collections of cells entrapped within a gelatinous matrix primarily comprising insoluble extracellular polymers that the cells secrete. Although the term is usually applied to bacterial cells and their exopolymers, any biologically active layer of cells (microbial, plant or mammalian) can be considered a biofilm[4]. Any surface in contact with a biological fluid is a potential target surface for the adhesion of suspended cells. Once cells are attached, subsequent growth and multiplication of surface-attached cells, cell exopolymer production, further recruitment of planktonic cells from the fluid phase, and various biofilm detachment processes comprise what is collectively known as biofilm formation and persistence[4–6].

Biofilms can play both beneficial or detrimental roles depending on whether their formation within a specific system is intentional or inadvertent. The beneficial aspects of biofilm reactors have been discussed in several excellent review articles[7–9]. This article focuses on both the obvious and less obvious detrimental effects that unintentional biofilm formation can inflict upon a bioreactor system. Research into the detection, control and prevention of biofilm formation within commercial bioreactors is also discussed.

Figure 1
Bacterial biofilm of *Hyphomicrobium* spp. within a two-litre laboratory bioreactor operated as chemostat.

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Detriments of biofilms in bioreactors

Biofilm formation and persistence can have diverse effects on the performance of a continuous-culture reactor system:

● *Effects on energy and momentum transfer*

Biofouling is the undesired biofilm formation that inevitably reduces the transfer efficiency of either momentum, energy or mass. Biofilm formation can lead to increases (of 200–300%, or more) in fluid-frictional and heat-transfer resistances[10]. Increases in fluid-frictional resistances translate to greater pressure drops (or reduction in volumetric flow in gravity-fed systems) as biofilms form within tubular reactors (e.g. within the lumen of hollow-fiber or ultrafiltration membrane reactors), decreased permeability in packed-bed reactors, and increased impedance across hollow-fiber membranes operated under 'starling flow'-convective-flow conditions. Within a conventional agitated fermentation vessel, biofilms on impeller surfaces will reduce the ease with which the impeller moves through the fluid, necessitating more torque to maintain the same rotational speed.

A biofilm can act as an ever-increasing layer of 'insulation' around heating coils and thermal detection/control probes in a fermenter, thus increasing the overall heat-transfer coefficient and the difference between the instrument surface and the bulk-liquid temperatures. As the biofilm develops in a temperature-controlled fermentation, the temperature-control system increases the electrical or steam input (i.e. heat flux) to maintain the pre-set culture temperature. These increases in mechanical (torque) or thermal (heat flux) energy are ultimately reflected in increased energy cost for the fermentation. Eventually, biofilms can become so extensive (Fig. 2) that the fermenter must be shut down and internal surfaces and exposed instruments cleaned or replaced.

● *Effects on mass transfer*

Biofilms can affect the mass transfer of a chemical species either actively or passively, depending on whether the bacteria and the diffusing component participate in a biochemical reaction.

In the passive sense, chemical species not reacting within the biofilm still encounter a stagnant region with a diffusivity that is different from that of the bulk liquid. Molecular-diffusion coefficients for various molecules diffusing within a biofilm have been determined to be ~30–100% of the values for the same solute diffusing in water[11,12]. Diffusion of charged molecules can also be affected by local electrostatic interactions[13]. Thus, as a biofilm develops, a linear concentration gradient can be established across the biofilm.

In the active sense, the biofilm constitutes a biologically reactive layer where a myriad of carbon/energy substrates, electron acceptors and donors, and essential nutrients are transported to and within the biofilm, where they are converted to products which, in turn, are utilized within or transported out of the biofilm to the bulk fluid. Transport within the biofilm is either by molecular diffusion[14] or by convective transport mechanisms[15]. As a biofilm develops, internal mass-transfer limitations to substrate conversion by attached cells create non-linear spatial gradients (1) in substrate concentration, (2) in local reaction rates, and, if the situation persists, (3) in bacterial species (see below).

Figure 2
Biofilm growth on an impeller from a 20-litre industrial fermenter (stirring speed, 500 rpm) after three-months operation. Reproduced, with permission, from Ref. 2.

In either passive or active situations, biofilm formation on *in situ* diagnostic probes (pH, Eh, dissolved oxygen, temperature, medium-level control, fluorescent NADH-based biomass probes, biochemical and immunological biosensors) will severely bias observation. Thus, conditions intended for the bulk liquid may not be realized due to erroneous sensor readings.

● *Atypical econiches*

Atypical niches can arise within a bioreactor due to (1) the preferential attachment of one strain or species at exposed surfaces and (2) the non-uniform growth conditions arising within established biofilms[16]. Unlike suspended cells which are subjected to the hydrodynamics of the fluid phase, immobilized cells are unaffected by residence-time constraints. Consequently, such organisms that would otherwise be selected out of a culture or physically 'washed out' when the dilution rate (D) exceeds the maximum growth rate (μ_{max}) have an advantage over suspended cells, remain attached to reactor surfaces, and increase as a proportion of the population. Due to biofilm-removal processes[17], detached cell progeny and eroded biofilm debris leave the biofilm surface and continually 're-inoculate' the

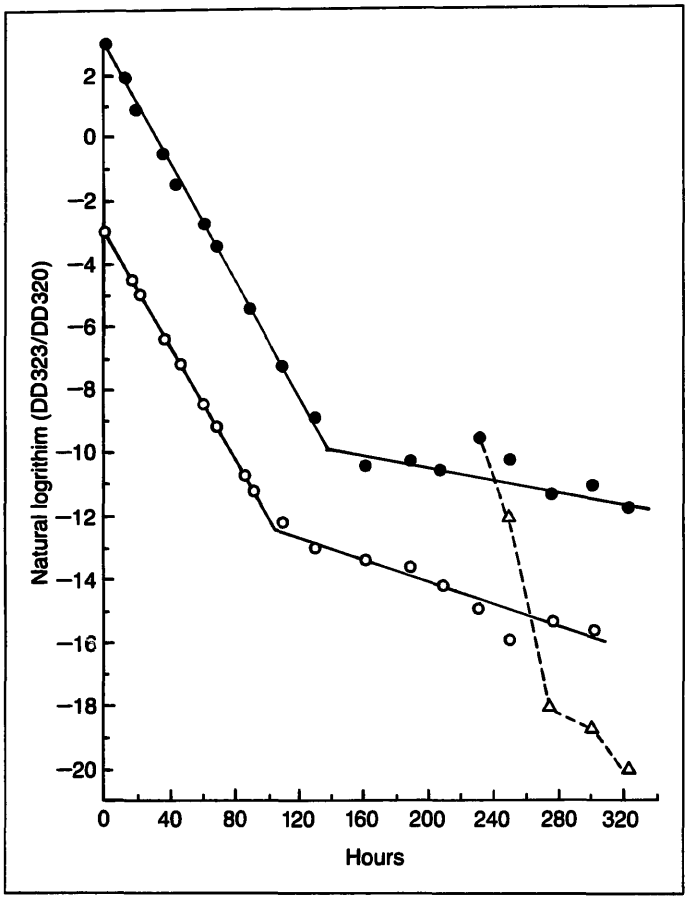

Figure 3

Selection against strain *E. coli* DD323 in maltose-limited chemostats (solid lines). Note the abrupt change in slope at 100–140 h. Transfer of culture to a fresh chemostat markedly increases the selection (dashed lines), indicating that the original decrease in slope is due to contamination of the liquid phase by a biofilm-bound population. Reproduced, with permission, from Ref. 18.

fluid phase. Thus, such 'unexpected floating organisms' (termed 'UFOs' within our lab group!) can persist in the culture fluid under otherwise negative selection pressures. Figure 3 illustrates[18] how the progeny of *E. coli* DD323 (*Str*^{r}*Lac*$^+$) detach from a growing biofilm and persist suspended in a medium that is selective for *E. coli* DD320 (*Str*^{s}*Lac*$^-$): the detached progeny enable DD323 to increase as a proportion of the population despite the selective medium, as manifested by the decrease in the rate at which the strain is lost by dilution from the chemostat.

Within biofilms containing multiple species, internal mass-transfer limitations and subsequent concentration gradients can, over time, lead to spatially distributed microcolonies[19]. For example, gradients in concentration of terminal electron acceptors within a facultative mixed population can give rise to a biofilm comprising aerobic, anoxic (denitrifying), and anaerobic (fermentative, sulfate-reducing, and/or methane-forming) layers or microcolonies. Irrespective of conditions maintained in the bulk liquid, a diverse ecology can arise spatially within a biofilm. Shear detachment of the biofilm's upper layers[20] or complete sloughing of sections of biofilm[21] could contaminate the bulk liquid with a variety of unexpected species or strains[20].

● *Effects on parameter estimation*

Chemostats provide an elegant way to determine the stoichiometric and kinetic parameters of a cell culture under substrate deficient growth conditions. Chemostat theory states that the yield coefficient (Y) can be determined from the concentrations of the residual biomass (X) and substrate (S) at any steady-state dilution rate D using Eqn 1:

$$Y = X/(S_{in}-S) \quad (1)$$

Unstructured models

Concentration of biomass, and limiting substrate, at a series of different dilution rates allows statistical regression estimates of saturation rate constants, half-saturation coefficient (K_s), and the maximum growth rate, μ_{max}. Such descriptions of a chemostat are known as unstructured models in that they treat growth as an increase in a single composite parameter, X (e.g. cell biomass dry weight, or cell number/volume). Topiwala and Hamer[1] presented an unstructured chemostat model that accounted for bacterial wall growth by assuming (1) a constant biofilm amount, (2) constant rate of biofilm detachment and entrainment, and (3) that kinetic and stoichiometric parameters were the same for both suspended and sessile bacteria. The Topiwala–Hamer model and subsequent refinements[21] predict that continual detachment from a biofilm extends the dilution rate at which suspended biomass will apparently remain in a chemostat, thus obscuring estimates of μ_{max} and K_s. In addition, Eqn 1 ignores substrate consumption by adherent bacterial populations, which will bias estimates of the culture yield coefficient.

Structured models

A structured model describes cell growth as the net result of changes in various subcellular components, each responding to changes in system conditions. Bakke *et al.*[22] present a structured model of biofilm formation in a chemostat that separates both suspended and attached biomass into two components: (1) viable cell carbon, and (2) extracellular polymer carbon. Separate, individual stoichiometric coefficients and growth-rate constants are assumed for each component in the attached and the suspended populations. Results of mathematical simulations and experiments indicate that attached cells grow at prevailing local substrate concentrations according to the same kinetic rate constants found for the suspended cells provided that viable cell concentration per area, rather than total biofilm mass/area, is employed as the biomass parameter. Yield coefficients for the conversion of substrate carbon to both cellular and extracellular polymer carbon were found to be different for suspended versus attached cells. Additionally, these stoichiometric coefficients were strongly influenced by the prevailing

substrate concentration. Erroneous estimates of culture growth parameters caused by ignoring biofilm populations in lab-scale reactors can adversely affect the scale-up design of industrial systems, severely overestimating reactor size and required heat-transfer areas.

Controlling biofilm formation in bioreactors

Biofilms that are intentionally cultivated form a subset of the class of heterogeneous reaction systems known as immobilized whole cells. To intensify process reactivity, a number of unique reactor geometries have evolved to increase surface-area-to-volume ratio and increase nutrient loading to levels that exceed the wash-out limits of suspended cell cultures. Examples of biofilm-reactor geometries include packed bed and fluidized beds of either solid biofilm supports (e.g. glass beads, plastic beads, quartz sand particles) or porous biofilm supports (e.g. stainless steel spherical webs, flexible polyester sponges, collagen beads), hollow-fiber and ceramic membranes, solid gel beads, and microgel capsules[6].

Unlike the biofouling of a conventional fermenter vessel which leads to the problems associated with uncontrolled biofilm accumulation, biofilm support systems were designed to confine the biofilm within known dimensions. Unfortunately, except in the gel-immobilized whole-cell systems (most of which are operated in a non-growth mode), these immobilized-cell systems can also develop excess, unwanted biofilm. In fluidized-bed bioreactors, prevailing turbulence and particle abrasion continually strip away any excess biofilm, maintaining essentially constant system reactivity. However, packed beds of solid or porous biofilm supports and hollow-fiber membrane reactors experience steady biomass accumulation until support cavities are filled, after which any excess biomass acts to 'plug' the void spaces between particles or membrane fibers. Irrespective of whether biofilms form unintentionally within conventional fermenter vessels or in heterogeneous, immobilized-whole-cell systems, the need for control of biofilm accumulation poses two problems, i.e. detection and remedial response.

Detecting biofilm formation

Control implies the ability to either promote, inhibit, or prevent biofilm formation. One major limitation to effective control of biofilm formation is the lack of detection methods within fermentation systems. Most fermenters are equipped with instrumentation to monitor and maintain liquid-phase conditions and provide aseptic sampling of resultant cell suspensions. While a number of elegant techniques for non-invasive biofilm diagnosis[3] have emerged in recent years (e.g. nuclear magnetic resonance [NMR] and attenuated total reflectance [ATR] waveguides coupled with Fourier transform infra-red [FT-IR] spectroscopy), these techniques are best suited for research purposes. Consequently, no commercially available fermenter system comes equipped to monitor biofilm formation. Techniques to measure biofilm development can detect either biofilm amount or the detrimental symptoms of extensive biofilm formation. In the latter case, one could measure the increase in torque required to maintain a constant stirring speed in the fermenter[9]. Alternatively, following a mild, rapid increase in heater surface temperature, one could determine the rate of heat dissipation to the liquid phase, which would decrease as biofilm accumulates[10]. Although methods measuring biofouling effects would forewarn against any unacceptable situations (e.g. Figs 1 and 2), such techniques (1) are not sensitive to early stages of biofilm development, and (2) since biofilm amount is not measured, estimates of true stoichiometric and kinetic rate parameters for the culture are not possible.

There are a number of very sensitive techniques to measure biofilm development directly by detecting the surface-bound cell numbers[20,22] and/or total biofilm quantity[6,10,17]. However, all direct methods require destructive sampling of an exposed reactor surface; a capability that normally does not exist in a fermentation system. Molin *et al.*[23] report the use of glass rods as removable sample surfaces; the rods pass through existing sample ports in the fermenter head plate and are submerged in the culture liquid. One promising new technique for on-line monitoring of biofilm formation is the development of an infra-red fiber-optic sensor. Biofilm thickness can be detected with a single cable, glass optic fiber 125/85 tapered to 10 μm, mounted within the reactor vessel wall by way of a sterile port[24]. The light source is a light-emitting diode (940 nm) with a square-wave frequency-modulated 500 Hz light; a standard high-responsivity photodetector is employed. Developers[24] indicate that two abrupt changes in detected light intensity are seen as the probe passes from the aqueous phase into the biofilm and again as the probe encounters the substratum surface. Our laboratory is refining a similar approach using newly available fiber-optic waveguides for infra-red light transmission coupled to a FT-IR spectrometer; the waveguide is covered except for a small 'window' situated flush to the target substratum. Due to the strong IR absorption of water, the IR wave can only analyse the organic events occurring within 1 μm of the substratum surface. As cells and extracellular polysaccharides accumulate at the surface, IR spectra detect increasing intensities in the various amide bands of adsorbed proteins and the C–O bond absorption peak (C–O stretch) of pyranose subunits in accumulating polysaccharides.

Preventing biofilm formation

Should a reliable biofilm-detection method be refined for bioreactor systems, what remedies are available to reduce or eliminate unwanted biofilm without affecting the liquid phase culture? Fermentation systems cannot use chemical agents (i.e. oxidants such as ozone or chlorine), biocides (employed in biofouling remediation of cooling water, and in oil recovery systems) or antibiotics, for obvious reasons. A developing biofilm could possibly be disrupted without influencing the suspended culture by periodically applying a pulse of EGTA (which specifically chelates the calcium

ions used to crosslink polysaccharides in the biofilm matrix)[25]. Past attempts at reducing or eliminating biofilms in lab-scale fermenters comprise: (1) bacteriostatic silicone (Protosil-28™; PCR Research Inc.) pre-treatment of reactor surfaces[26] to retard cell adhesion; (2) periodic use of a scraping device, retrofitted into the fermenter vessel[27]; and (3) suspending inert abrasive sand particles in the liquid media to continuously scour reactor surfaces[28]. For commercial-scale bioreactors, regular shut-down and manual cleaning of the reactor is the only current remedy to biofilm formation during operation.

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