biotechnology focus

Real-time monitoring of cellular biomass: methods and applications

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We review physical approaches to the problem of devising a real-time biomass probe. Direct measurement of the dielectric permittivity of cell suspensions at radio frequencies provides one possible solution to this problem.

The problem

'Without doubt, the single most vital yet problematical value sought during fermentation is biomass concentration.'[1] 'During a fermentation process the determination of the viable and total cell count is one of the most important.'[2]. 'The reliable on-line determination of the *number of cells* or the *weight of the cells* is still today the most important problem of measurement in biotechnology'[3]. 'Estimation of microbial biomass is by far the single most important parameter of any fermentation process'[4].

Given that, all else being equal, the instantaneous steady-state rate of production of a fermentation end-product is strictly proportional to the biomass present[5], quotations such as those above are not difficult to find in the fermentation literature. In the present brief review, then, we shall try to illustrate the ways in which one might seek to progress towards a solution of the problem of devising a real-time biomass probe, and the extent to which this has been achieved.

The desideratum

The first *desideratum*, and perhaps the most difficult of all strictly to satisfy, is to define what we actually *mean* by biomass! The more conventional definitions, which are dominated by the idea that 'biomass' must refer to cells which are capable of growth and division (usually under an arbitrary and somewhat restrictive set of environmental conditions), does not admit application in real-time systems (since perforce one must wait to see what fraction of the cells actually *have* divided). Even the other usual descriptors (dry weight, wet weight, cell number, 'viable' cell number) do not lend themselves easily to rapid and direct measurements. However, since the relative density of cellular biomass is in almost all cases essentially constant, and it is the possession of a cell membrane that makes a cell a cell, we have taken the view[6,7] that the *biovolume, i.e.* the proportion of the total volume that is enclosed by the cytoplasmic membranes of the cells in a suspension, constitutes the most suitable operational definition of biomass *sensu stricto* for use during fermentations. The biovolume may then be converted to any other realistic version of '*bio*'mass as the behest of the experimenter.

It might be argued that, since the macroscopic state of a fermentation does not change especially rapidly (seconds), off-line measurements, or those in sample loops, might be preferable to *in situ*, on-line measurements. In fact there are pros and cons to each approach, with a trend towards the generally more desirable on-line methods being discernible[8]. Thus we will confine our discussion to on-line methods, since these alone have the possibility of giving an 'instantaneous' reading of the sample *as it actually exists in the fermentor*. It might also be argued that since the cells in a culture at any instant are not identical, a knowledge of the distribution of, say, cell sizes might be of advantage. However, at the present state of knowledge of microbial physiology, it does not seem generally likely (filamentous or differentiating organisms are a possible exception) that such niceties as cell-size distributions would actually constitute useful and usable data in production fermentors in real time.

Other *desiderata* of fermentor probes in general, and of biomass probes in particular, are well known, but for convenience are given below.

- specific (or highly selective),
- continuous real-time assay,
- sensitive,
- probes biologically inert,
- non-destructive assay,

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- multiplexable,
- good lifetime,
- low cost,
- usable in turbid media,
- cleanable *in situ*,
- autoclavable *in situ*,
- linear to high biomass.

From this, we may conclude that the ideal approach would be reflected in a fermentor probe that was robust and which could in some way distinguish intact (viable) cells from dissolved medium constituents, gas bubbles and other flotsam. What kind of methods should we consider?

Physical, chemical, biological or mathematical approaches?

To date, most on-line methods for measuring biomass in large-scale fermentors have been indirect, and relate 'biomass' to the rate of O_2 uptake and/or CO_2 evolution, nowadays typically measured using a mass spectrometer. In general, any chemical that is produced or consumed by cells at an essentially constant rate during growth may be used to assess biomass, but, with equal generality, chemical methods suffer from the virtual impossibility of devising *in situ* or real-time assays, and of course are rarely specific for biomass over necromass. Under the 'biological' approaches we included classical and more modern forms of microscopy, and of course 'viable' counts obtained by petri plating; similarly, none can be called a 'real-time' method. Indeed, the 'mathematical' method[6], in which everything *except* the biomass is measured and the biomass obtained by difference, illustrates rather nicely the historical difficulty of obtaining a biomass probe. However, *physical* methods, in which one exploits one or more physical properties by which to assess the mass or number of cells and to distinguish this from everything else, are normally 'instantaneous' on a biologically significant timescale, and can thus provide a genuine hope of a real-time biomass probe[6,9].

Some physical properties of biological cells

For the present purpose, physical properties may broadly and conveniently be divided into optical, mechanical and electrical[10,11]; we consider each in turn.

The mechanical, *i.e.* acoustic, properties of biological materials are tolerably well understood, not least because of the emergence and success of ultrasound scanners[12]. However, attempts to apply similar approaches to fermentation broths[13] led to the (predictable) finding that the acoustic absorption of a typical cell suspension was due only in part to its biomass content, with gas bubbles, proton-transfer reactions and other factors affecting the most relevant overall property, *viz.* suspension compressibility. The dominance of acoustic properties by gas bubbles means that a sampling loop/degassing system must be used, and in these circumstances high-resolution acoustic resonance densitometry has proved capable of determining biomass concentration over a wide range in particle-free media, especially when used in a dual (suspension-*minus*-medium) configuration[14,15].

Optical methods

Due to the inconvenient similarity between the sizes of microbial cells and the wavelength range of optical radiation, such cells scatter light in a rather complicated fashion[6]. Both low-angle (forward) and large-angle scatter (as used conventionally if indirectly in 'optical density' measurements) may be used, and in favourable cases the extent of such scattering is proportional to biomass. However, optical measurements face two major problems if they are to be exploited in biomass measurements *in situ*: the failure of the Beer-Lambert law at only moderate concentrations of cells, and the presence in many fermentation media of non-cellular, scattering particulates. Although there has been some progress in the use of near-IR illumination, and commercially available optical biomass probes exist, only spectrofluorimetric monitoring has been widely used as a measure of fermentor biomass[15]. This method[16] relies upon the fluorescence of intracellular cofactors, predominantly reduced pyridine and flavin nucleotides, and as such is perhaps a better monitor of redox state than of biomass *per se*. Nonetheless, alone or in combination with other approaches, it can be a very useful indicator of the cellular metabolic state.

Needless to say, the most modern optical approaches exploit lasers as the source of illumination. As discussed by Clarke[15], the historically prohibitive costs of such instrumentation have been overcome by the introduction of laser diodes, avalanche photodiodes and fibre optics; further, with the use of a sampling system, flow cytometric approaches[17], based on such components, may be expected to provide a powerful, low(ish)-cost and convenient means for separating cellular from other signals. Limited population distribution analysis, including the presence of contaminants, may also be carried out in *non-flowing* streams containing small particles (<10 μm), via photon correlation spectroscopy[15]. It may be anticipated from the above that even more sophisticated optical methods, based on laser Raman or other infrared (and indeed microwave) photometry, will be applied to the problem before too long.

Electrical approaches

Both faradaic and non-faradaic electr(ochem)ical approaches may be used to detect microbial biomass. In the former case, one or more redox mediators, of suitable mid-point potential, (must be produced by the cells or) are added to exchange electrical current with an external anode, the mediators being reduced by the cells and the anodic current being therefore proportional both to the biomass and to its overall electron-transfer activity[6,18].

A dual-probe configuration (sample-*minus*-supernatant) has been described for use in fermentors[19].

The non-faradaic or 'passive' electrical properties of a system, such as a cell suspension, may be completely characterised by its macroscopic capacitance (in Farads) and conductance (in Siemens). These depend in part upon the size and geometry of the electrodes, and reflect respectively the system's intrinsic properties: permittivity (ability to store electrical energy) and conductivity (ability to dissipate it). Conductivity has the units Siemens/m, whilst permittivity is dimensionless. For plane-parallel electrodes of area A separated by a distance d, the relationship between the conductivity σ' and conductance G is

$$\sigma' = G\,(d/A)$$

where (d/A) is known as the cell constant and has units of reciprocal length. The capacitance C is similarly related to the permittivity ε' by

$$\varepsilon' = C\,(d/A\varepsilon_0)$$

where ε_0 is an experimental constant equal to $8.854 \cdot 10^{-12}$ F/m, such that a cubic electrochemical cell of unit dimensions containing water (which has a permittivity of 78.4 at 298 K) has a capacitance of some 6.94 pF. A variety of systems has been proposed by which to exploit these facts for the estimation of microbial biomass.

The commonest types[20] rely upon the biomass-dependent changes in bulk conductivity caused by the uptake and excretion of charged compounds, and/or biomass-dependent changes in the polarisation (*apparent* permittivity) of the electrode–electrolyte interfaces, to sense biomass, and do not attempt to distinguish the electrical properties of cell *per se*[6,21]. Conductivity does provide a generally useful (and, one might comment, greatly underused) sensor for particular kinds of metabolic activity at all stages of a fermentation, although these methods cannot be used accurately to estimate fermentor biomass *in situ* (since of course non-growing cells can also carry out these types of reactions). By contrast, *direct* measurement of the electrical properties of cellular suspensions, under appropriate conditions, can provide a direct and 'instantaneous' reading of biovolume.

Real-time measurement of biomass via dielectric measurements

The passive, non-faradaic electrical or 'dielectric' properties of cellular suspensions *per se* (as opposed to the suspending medium or the electrodes themselves) have been studied since the last century[21,22]. They are generally characterised by three major areas of frequency-dependence, known[22] (in order of increasing frequency) as the α-, β- and γ-dispersions (Fig. 1). The α-dispersion, centred in the audio-frequency range (kHz), is ascribed predominantly to the relaxation of ions tangential to the charged surfaces of cells or particles. It is much greater for Gram-positive than for Gram-negative bacteria[23], due to their substantially different envelope structures. The γ-dispersion occurs at ultra-high frequencies (GHz), and is mainly due to the rotation of dipoles such as water. Neither of these dispersions is thus selective for biomass.

However, the β-dispersion, centred in the radio-frequency region of the electromagnetic spectrum, is caused by the charging of the rather large membrane capacitance C_m displayed by all intact cells. This is typically of the order 1 μF cm^{-2}, and is due to the possession by cells (and by nothing else likely to be found in a fermentor) of non-micellar phospholipid membranes of molecular thickness. For spherical cells of radius r, present at a volume fraction P, the permittivity at low radio-frequencies exceeds that of the background by a value given to a close approximation by $9PrC_m/4\varepsilon_0$. For non-spherical cells the factor 9/4 is modified. *Thus, by measuring the dielectric permittivity of cell suspensions at low radio-frequencies, it is possible to design a biomass probe that*

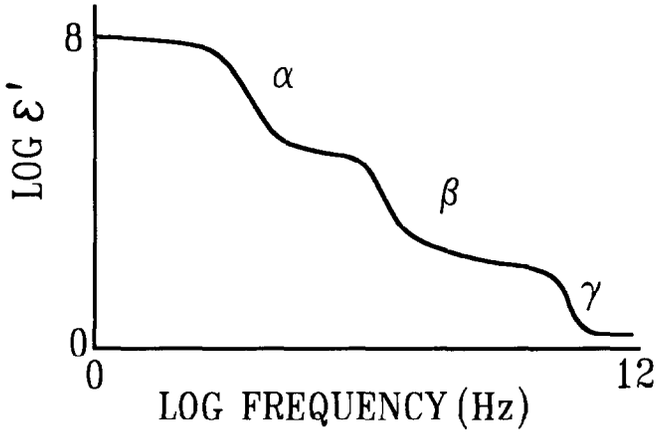

Fig. 1. Diagrammatic representation of the three major dielectric dispersions typically observable in tissue or cell suspensions. Note the logarithmic scale of the ordinate, indicating the substantial size of the β-dispersion.

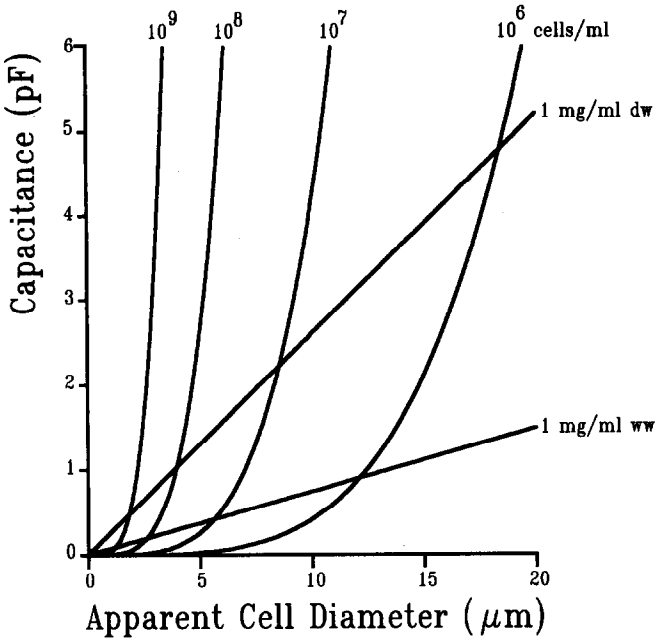

Fig. 2. The relationship between the electrical capacitance (relative to the background medium) of different cell masses and numbers as a function of cell size, assuming that the cells are spherical, have a relative density of 1, and are surrounded by a cytoplasmic membrane possessing a capacitance of 1 $\mu F\ cm^{-2}$, plotted according to the equation given in the text. dw = dry weight; ww = wet weight.

is specific for viable cells[7] (since necromass, particles, emulsions and gas bubbles do not have intact bilayer-type cell membranes). This dielectric increment (increase in capacitance over the background) for an electrochemical cell of cell constant 1 cm^{-1}, based on the above equation and assuming for the relevant traces a wet weight–dry weight ratio of 3:1, is given in Fig. 2.

Based on these principles, we have developed a biomass probe, the Bugmeter™, suitable for the real-time estimation of biomass in fermentors *in situ*. The (steam-sterilisable) probe itself consists of four gold electrodes in an insulating housing suitable for insertion in a standard 25-mm port. The outer two electrodes apply alternating current of a suitable frequency in the range 0.1–10 MHz and the inner two pick up the alternating voltage drop, an arrangement that more-or-less completely avoids measuring the properties of the electrodes themselves[21,24]. Fouling is obviated by the manual or automatic application of electrolytic cleaning pulses. The output may be chosen in terms of absolute capacitance, capacitance *minus* that upon inoculation, or mg/ml (via a previously determined calibration); an output of the conductance of the broth is provided as a bonus. This device is suitable for use in all kinds of fermentations, and the approach has been applied to a variety of prokaryotic and eukaryotic microbes[7], pitching control in breweries[25], plant cells[26], animal cells[27], immobilised cells[28], solid-substrate fermentations[29], and (because it measures *bio*mass) in assessing cytotoxicity[30]. Data from a typical fermentation run are shown in Fig. 3.

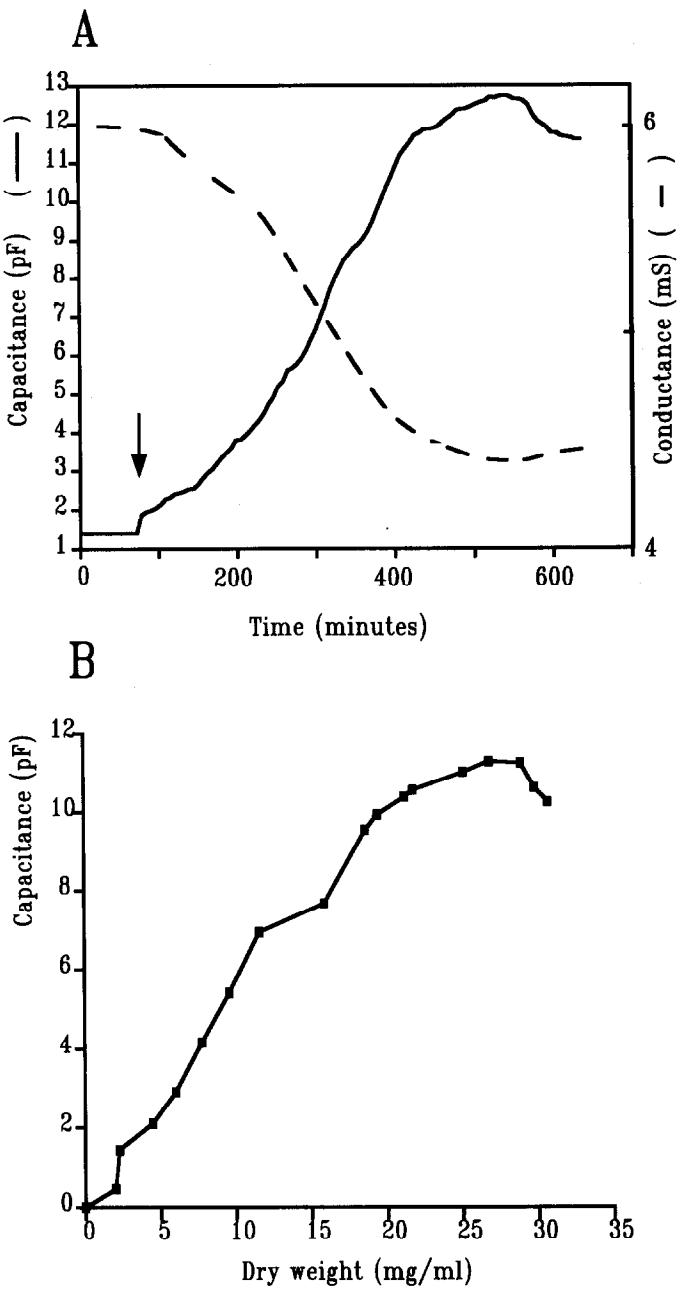

Fig. 3. Dielectric monitoring of a yeast fermentation. (A) Capacitance (——) and conductance (---) versus time. Cells were grown aerobically in a gas-lift fermentor (500 ml working volume) in a medium containing (per litre) 5 g yeast extract, 5 g balanced peptide, 1 g NH_4NO_3, 50 g glucose, initial pH 5 (with H_3PO_4). Capacitance and conductance were measured at 0.4 MHz, and S. cerevisiae cells obtained locally were inoculated at the arrow. (B) Capacitance versus the dry weight of samples taken from the fermentor. It may be observed (i) that the variables are closely correlated during the early stages of the fermentation, (ii) from the smoothness of the traces in A and B that most of the noise is due to the dry weight measurements, and (iii) that cell death as the cells enter stationary phase is monitored by the Bugmeter (on both conductance and capacitance channels) but not as a fall in dry weight.

A comparison of the *desiderata* (see above) with the power of this approach and its realisation indicates that dielectric spectroscopy may be regarded as providing an almost ideal solution to the problems of the real-time measurement of biomass as biovolume. As with any other physical approach, the signal determined depends partly on factors other than the absolute biomass, in this case notably the cell size and morphology. This means that individual fermentations need individual calibrations, and that morphological changes may be picked up in addition to a simple value for the total biomass.

The future

So far as the dielectric approach is concerned, we may expect the near future to bring the application of *rapid-scanning spectroscopic* analyses, the use of this device in feedback control loops for turbidostats, and the exploitation of chemometric techniques such as the Kalman filter to the on-line determination of growth rates (and contaminants), together with a greater integration of real-time biomass measurements into monitoring systems for the display of derived parameters such as *specific* oxygen uptake rate. From this point of view, and since the cost of instrumentation is negligible in large-scale fermentations, it is desirable also to exploit other suitable 'biomass probes' based on other physical principles, to maximise the information that is available to the fermentation technologist concerning this most important variable.

Summary

Since what distinguishes biological cells from other condensed matter is their possession of a molecularly thin, phospholipid cytoplasmic membrane, biovolume constitutes a convenient operational definition of biomass. Only physical approaches can offer the possibility of devising a real-time biovolume (biomass) probe for use in fermentors *in situ*. Optical, acoustical and electrical approaches are possible physical methods. Of these, direct measurement of the electrical capacitance at low radio frequencies permits the real-time estimation of biovolume.

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