Dielectric permittivity of microbial suspensions at radio frequencies: a novel method for the real-time estimation of microbial biomass

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The radiofrequency dielectric properties of microbial suspensions are a direct and monotonic function of the radius and volume fraction of the particles constituting the suspended phase. Measurement of these properties therefore permits the direct estimation of microbial biomass during fermentations, in situ *and in real time. The present approach to biomass estimation does not suffer significant interference from non-cellular particulate matter and retains its linearity at volume fractions two orders of magnitude greater than those at which the Beer–Lambert law fails.*

Keywords Biomass; dielectric spectroscopy; biosensors

Introduction

An accurate method for the real-time estimation of microbial biomass during laboratory and industrial fermentations remains an important goal which has yet to be attained. Carleysmith and Fox,[1] for instance, state; 'Without doubt, the single most vital yet most problematical value sought during fermentation is biomass concentration'.

An immediate difficulty in considering this problem is that no satisfactory definition of biomass exists[2,3] and in particular, if one defines biomass in terms of the amount of cellular material which is capable of growth and division, the time necessary to discern whether a cell actually has divided disallows, even in principle, the notion of a 'real-time biomass probe'. Nonetheless, not least to aid in the computerized control of fermentations, it would be extremely desirable to devise a probe which could respond in some way to the microbial content of a fermenter, in real time. For this reason, an operational definition of 'biomass' (which might in some cases also include 'necromass') is required. Since the density of bacterial protoplasm is thought in general to depend only rather weakly upon cultural conditions and since microbial viability is believed to depend rather strictly upon the possession of a relatively ion-impermeable cytoplasmic membrane (e.g. refs 4–6), an appropriate operational definition of microbial biomass is constituted by the biovolume, or volume fraction of the fermenter fluid which is enclosed within the boundary (cytoplasmic) membrane of the cells.[3,7]

As discussed in several recent reviews,[3,8–10] the only general types of method which may be used to give a real-time estimation of microbial biomass must exploit or rely upon some physical characteristic which distinguishes microorganisms from aqueous solutions, gas bubbles and particulate material. Thus the problem of designing a real-time biomass probe reduces to that of devising a probe which, by means of some physical principle, provides a rapid estimation of the volume fraction specifically enclosed within the cytoplasmic or plasma membrane of the cells of interest. The purpose of the present article is to point out that the radiofrequency dielectric properties of microbial (or other) cell suspensions are a direct and monotonic function of the volume fraction so defined (and differ significantly from those of particulate matter, gas bubbles and aqueous solutions), that their measurement may therefore be used to estimate microbial biomass, and to describe the theory and implementation of a device for the real-time estimation of microbial biomass which is based upon these principles.

Theoretical

The linear, passive electrical properties of condensed media are completely characterized by their (frequency dependent) electrical permittivity ε' and conductivity σ'. At frequencies <1 GHz or so, the passive electrical

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properties of ionic solutions are frequency independent, and may be assessed by measuring the capacitance C and conductance G of a sample held between two plane-parallel electrodes of area A separated by a distance d. Neglecting electrode polarization (see later) we than have:

$$G = \sigma'(A/d) \quad (1)$$

$$C = \varepsilon'\varepsilon_0(A/d) \quad (2)$$

where ε_0 is the permittivity of free space (8.854×10^{-14} F cm^{-1}) and (d/A) has the dimensions of reciprocal length and is known as the cell constant (e.g. ref. 11). The permittivity of water at 298 K is approximately 78.4, so that it may be calculated from equation (2) that a cell of unit dimensions containing water at this temperature possesses a capacitance of some 6.94 pF.

In contrast to those of simple ionic solutions, the passive electrical properties of biological cells generally, and microbial suspensions in particular, are strongly frequency dependent in the range DC–1 GHz, in the sense that their permittivity increases and their conductivity decreases as the frequency of measurement is lowered. When the passive electrical properties of such a system are frequency dependent, the phenomenon is referred to as dielectric dispersion and a voluminous literature indicates that biological cells including microorganisms generally possess three major dispersions in the range DC–1 GHz, known respectively as the α, β and γ dispersions (e.g. refs 3, 12–18). For the present purposes, we need discuss only the β-dispersion, which occurs in the radiofrequency range ($\approx$0.1–100 MHz).

Dielectric dispersions may generally be characterized: (a) by the values of permittivity (ε'_1, ε'_∞) at frequencies which are respectively very low and very high relative to the 'characteristic frequency' f_c (in Hz) at which the permittivity takes the value $\varepsilon'_\infty + (\varepsilon'_1 - \varepsilon'_\infty)/2$ where $\varepsilon'_1 - \varepsilon'_\infty = \Delta\varepsilon'$ is known as the dielectric increment; (b) by the relaxation time $\tau = 1/2\pi f_c$ and (c) by the distribution of relaxation times encompassed in an empirical value, the Cole–Cole α,[19] which may take the value $0 \leqslant \alpha < 1$. Similar relations exist for the frequency dependent conductivity, the conductivity increment $\Delta\sigma'$ being related for fundamental reasons (when the Cole–Cole α is small) to the dielectric increment by:

$$\tau = \Delta\varepsilon'\varepsilon_0/\Delta\sigma' \quad (3)$$

Figure 1 gives a normalized plot of the relation between the permittivity and the frequency for several values of α from 0–0.5; for $\alpha = 0$ we have a Debye dispersion with a single relaxation time. Thus (*Figure 1*), for reasonably small values of α, if the frequency of measurement f_m is about one decade lower than f_c, the permittivity measured will be within a few percent of the 'low-frequency' permittivity ε'_1.

Although other membrane-associated processes such as the translational and rotational motions of membrane lipids and proteins and the impeded motions of double-layer ions may make a contribution,[3, 16–18, 20] the RF dielectric properties of biological cells are thought to be dominated by the charging of the 'static' capacitance of the relatively ion-impermeable cell membrane by a Maxwell–Wagner type of mechanism.[3, 12–18, 20–26] For the case of spherical shell vesicles, at a volume fraction P, comprising a membrane of 'static' capacitance C_m farads per unit area separating internal and external solutions of conductivity σ'_i and σ'_o

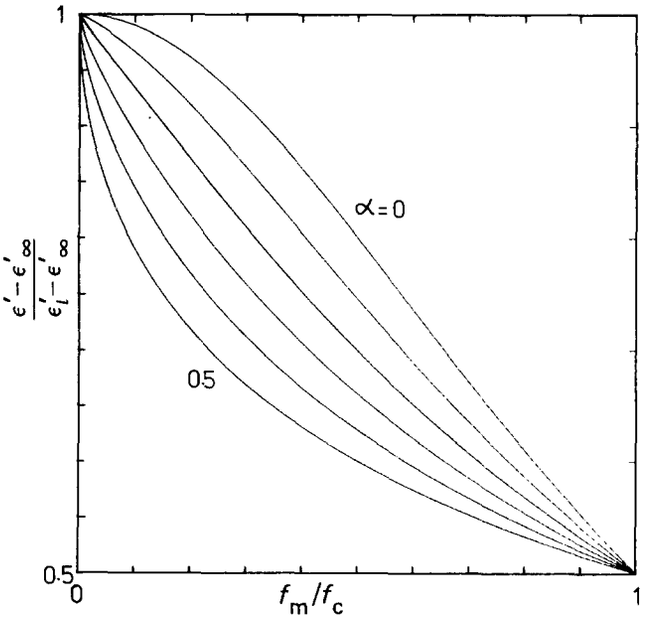

Figure 1 Normalized relation between the permittivity and frequency for a dielectric dispersion exhibiting Cole–Cole behaviour, using values for the Cole–Cole α of 0, 0.1, 0.2, 0.3, 0.4 and 0.5

respectively, the 'low-frequency' conductivity σ'_l is given by:

$$\sigma'_l = \sigma'_o(1 - P)/[1 + (P/2)] \quad (4)$$

This equation may be used under certain conditions to estimate P from measurements of σ'_l and σ'_o, but since their 'simultaneous' measurement requires (for the latter) removal of the suspended phase, it is most conveniently applied to immobilized-cell suspensions.[27]

The relaxation time for a Maxwell–Wagner dispersion of the present type is given by:

$$\tau = rC_m[(1/\sigma'_i) + (1/2\sigma'_o)] \quad (5)$$

where r is the cell radius, whilst for moderate values of the volume fraction ($P < 0.2$) and transmembrane conductance (<1 S cm^{-2}), the 'low-frequency' permittivity is given by:[12]

$$\varepsilon'_l = \varepsilon'_\infty + (9P\ r\ C_m/4\varepsilon_o) \quad (6)$$

For ellipsoidal cells of reasonably low axial ratio, the radius of the sphere of equivalent volume is appropriate.

For the β-dispersion, ε'_∞ differs little from the permittivity of the aqueous suspending medium, C_m is virtually a biological constant of 1 ± 0.5 μF cm^{-2}, (refs 18, 21), r is known and σ'_o may be measured. σ'_i may be determined from 'high-frequency' measurements of the β-dispersion and derived from the equation:[12]

$$\sigma'_\infty = \sigma'_o[(1 + 3P)(\sigma'_i + \sigma'_o)/(\sigma'_i + 2\sigma'_o)] \quad (7)$$

Alternatively, and particularly since τ is not in most cases an enormously sensitive function of σ'_i (see equation 5 and later), we may use values from the literature, the range known to us being from $\approx$2 mS cm^{-1} for Gram-negative bacteria and *Saccharomyces* (e.g. refs 28, 29) to $\approx$9 (ref. 30) or (uniquely) 15 mS cm^{-1}. (ref. 31) Thus, to derive P from measurements based upon equations (5) and (6) the following steps are appropriate:

(a) derive τ (and hence f_c) from equation (5) – a nomograph for this purpose has been given elsewhere;[32] (b)

choose a measuring frequency that is somewhat lower than f_c such that $\varepsilon' \approx \varepsilon'_1$; (c) derive P from equation (6).

One difficulty arises in that whilst σ'_i may be presumed to be relatively independent of the physiological state of a given culture, σ'_o may vary due to the production, uptake and excretion of charged metabolites, a principle which may itself be used (though not in real time) to estimate the microbial load in sparse samples (see refs 3, 33). Changes in σ'_o will thus, to a greater or lesser extent, affect τ which will in turn affect the measured permittivity if the condition $f_m \ll f_c$ does not hold. Thus we have plotted in *Figure 2* the effect of changes in σ'_o upon f_m/f_c for different values of f_m. It may be seen that for reasonable values of σ'_o, changes in σ'_o have an essentially negligible effect upon the ratio of the measuring to the characteristic frequency over a wide range of measuring frequency.

Finally, from a technical standpoint, it should be mentioned that unless a magneto-inductive[8–10] or other non-contacting system is used, the measurement of ε' and σ' may always be subject to artefacts due to the phenomenon of electrode polarization (see e.g. refs 11, 14, 26). A four-terminal arrangement may, in principle, be used to overcome this (e.g. refs 26, 34), and probe fouling may be obviated by exploiting electrolytic cleaning pulses.[35]

In what follows, we describe measurements that we have made in the light of the foregoing considerations. It is concluded that the measurement of the RF dielectric properties of microbial suspensions can provide an extremely convenient means for the direct estimation of microbial biomass, *in situ* and in real time.

Experimental

Measurement of RF dielectric properties

Dielectric properties were measured using a four-terminal impedance bridge (model BM 212, Dulas Engineering Ltd, Llwyngwern Quarry, Machynlleth, Powys, UK). A block diagram of the apparatus is shown in *Figure 3*, and the cell constant was obtained using solutions of known specific conductivity, as described.[22] Electrodes were of Pt plated onto the edges of shielded, insulated, Au-plated printed circuit boards.

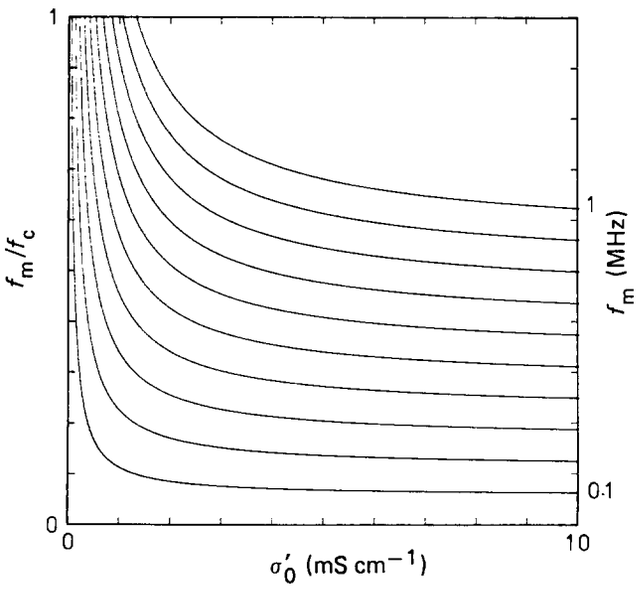

Figure 2 Effect of external conductivity on the ratio of the measuring to characteristic frequencies, at different values of the measuring frequency, for a system exhibiting a β-dielectric dispersion. Calculations are based on equation (5) and assume that the internal conductivity of the cells is 2 mS cm^{-1}, membrane capacitance 0.6 μF cm^{-2} and cell radius 3 μm. These values are typical of *Saccharomyces cerevisiae*[22, 28]

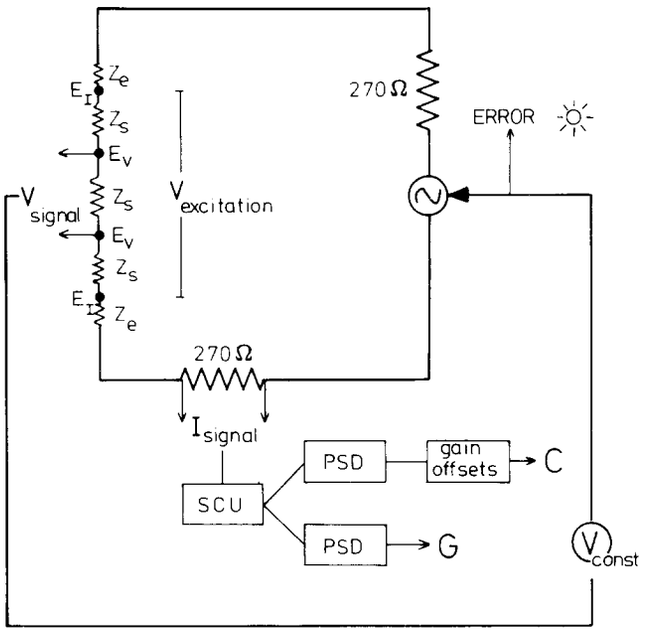

Figure 3 A block diagram of the four-terminal impedimetric device used in the present work. The principle of operation (cf. ref. 34) is as follows. The alternating voltage supply, whose frequency may be set manually at all frequencies in the range 0.2–10 MHz, is electronically adjusted until the voltage drop across the two inner electrodes (as measured with a voltmeter of high input impedance, such that negligible current flows and negligible electrode polarization is created) is equal to a set reference value (50 mV). When this condition is satisfied, the current flowing in the circuit (which is a function of the sample impedance) is measured (as the voltage drop across a precision resistor). This current is filtered, phase-shifted as appropriate and processed to give the conductance and capacitance of the sample. Under these conditions the measured impedance is in principle independent of contributions from the electrode impedance.[34] Symbols are: E_I, current (outer) electrodes; E_V, voltage (inner) electrodes; Z_e, impedance of current electrodes; Z_s, impedance of sample; $V_{excitation}$, voltage supplied across outer electrodes; V_{const}, reference voltage; V_{signal}, voltage drop across inner (voltage) electrodes; I_{signal}, current flowing when $V_{signal} = V_{const}$; SCU, signal conditioning unit; PSD, phase-sensitive detector; C, capacitance; G, conductance

Biological material

Saccharomyces cerevisiae was obtained from the Distiller's Company Ltd and stored, suspended and treated as previously described.[22] Reaction mixtures are given in the legends to the figures. For growth experiments, the cells were grown in a gas-lift fermenter described previously.[27] The medium contained 5% (w/v) malt extract and 0.5% (w/v) yeast extract. The initial pH was adjusted to 4.5 and the temperature was 30°C. The conductance of this medium was $\approx$1.57 mS cm.$^{-1}$ In this case, the electrodes were inserted into the base of the air-lift fermenter.

Chemicals and biochemicals

Chemicals and biochemicals were of analytical grade, or of the highest purity available and were obtained from the Sigma Chemical Company, Poole, Dorset, UK or from BDH Chemicals Ltd, Poole, Dorset, UK. Water was singly distilled in an all-glass apparatus.

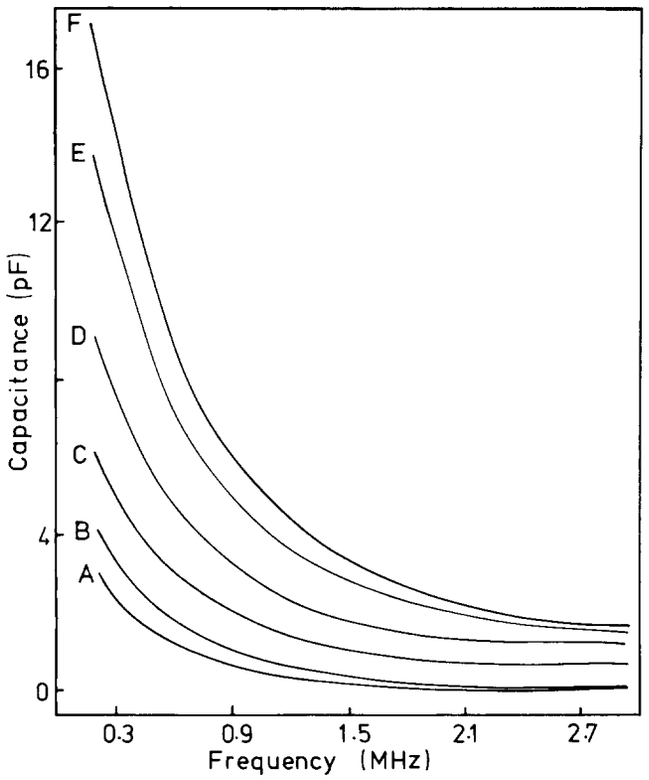

Figure 4 Radiofrequency capacitance of yeast cell suspensions. The reaction medium (initial volume 40 ml) was contained in a 100 ml beaker and consisted of 0.2 M sorbitol, 5 mM Tris–Cl pH 7.0 and the following final concentrations of yeast cells (mg ml^{-1}); A, 0; B, 1.7; C, 4.4; D, 7.1; E, 13.6; F, 18.9. The conductance was titrated with small aliquots of 3 M KCl to give a value of 0.86 mS. The cell constant was 0.58 cm^{-1} and experiments were carried out at 20°C

Results

Figure 4 shows the frequency dependent capacitance of a number of yeast cell suspensions in the range 0.2–3 MHz. It is evident that there is a sizeable, and cell concentration-dependent, dielectric dispersion over this frequency range, corresponding in magnitude and relaxation time to the β-dispersion measured previously using a two-terminal arrangement.[22,28] The impedance bridge used in the present work does not measure below $\approx$200 kHz; we therefore chose a measurement frequency of 300 kHz to assess the utility of the present approach in estimating microbial biomass in real time.

Figure 5a shows the time-dependent changes in conductance and capacitance at 300 kHz as aliquots of a stock suspension of yeast cells are added to a solution initially lacking cells. It is obvious not only that the probe responds instantly to the increased biomass present but that its response to the addition of a large aliquot of non-cellular, particulate suspended matter is negligible for practical purposes. In the present case, the observed probe response is limited by the time constant of the chart recorder, some 0.25 s. Given the cell constant (0.58 cm^{-1}), the change in permittivity at this frequency may be calculated to be $\approx$4 permittivity units mg dry $weight^{-1}$ ml^{-1}. It may also be calculated from equation (6) (given the parameters used in *Figure 2*) that the change in 'low-frequency' permittivity for the β-dispersion is approximately 7 permittivity units mg dry $weight^{-1}$ ml^{-1} (see also ref. 22). The linearity of the probe response is excellent (*Figure 5b*), and extends to at least 100 mg ml^{-1} (not shown, but see ref. 22). Since stirrer noise etc. is absent for practical purposes, and

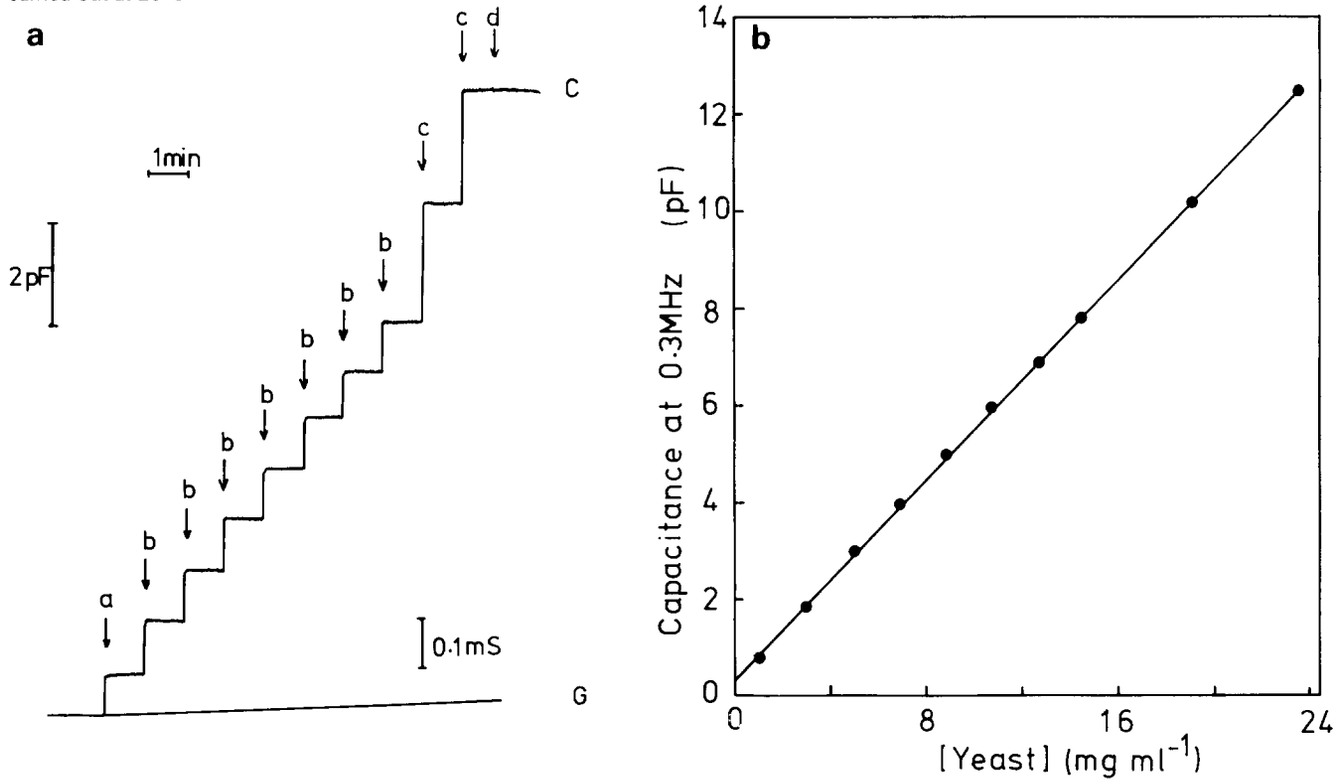

Figure 5 Capacitance and conductance of yeast cell suspensions at 300 kHz. The reaction medium was as described in the legend to *Figure 4*. (a) Time dependent behaviour. At the point marked a, yeast cells were added from a concentrated suspension to a final concentration of 1 mg ml^{-1}, whilst at the points marked b aliquots corresponding to a change in biomass concentration of 2 mg ml^{-1} were added. At the point marked c, incremental concentrations in biomass corresponding to 5 mg ml^{-1} were effected. At the point marked d, powdered calcium carbonate was added to a final concentration of 3% (w/v). The starting conductance was 2.86 mS and the temperature was 20°C. The initial capacitance was backed off to read approximately 0 pF. (b) Capacitance of yeast cell suspensions at 300 kHz. Data are those in *Figure 5a*

leaving aside for the moment any systematic errors, the minimum sensitivity is governed by the smallest capacitance change which may reliably be determined. In the present case this amounts at worst to ≈ 0.1 pF or some 0.2 mg dry weight ml^{-1}.

As discussed above, it may not always be possible to make estimations of the permittivity at a frequency at which the 'low-frequency' permittivity of the β-dispersion is manifested. Similarly, there may be a significant overlap between the α- and β-dispersion,[16, 36] so that one might then also wish to make measurements at a frequency nearer to the characteristic frequency. *Figure 6* therefore shows the effect of external conductance (and hence relaxation time) on the permittivity of a yeast suspension of constant biomass. Whilst this effect is fairly substantial (under the conditions selected, which were indeed chosen to illustrate this), it is easily taken into account by means of equations (5) and (6) and *Figure 1*.

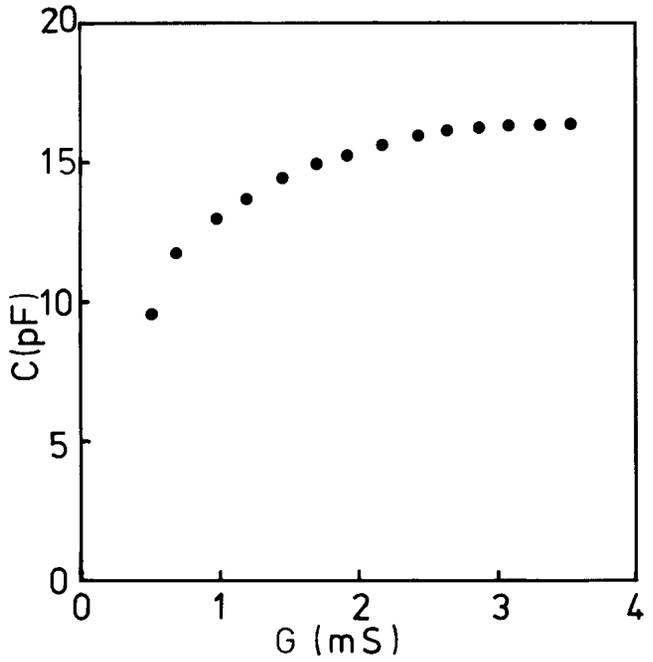

Figure 6 Effect of conductance on the capacitance of a suspension of yeast cells at 0.3 MHz. The reaction medium contained 0.2 M sorbitol, 5 mM Tris-Cl pH 7.0 and 30 mg ml^{-1} yeast cells. The conductance was increased by the addition of small aliquots of 3 M KCl. The cell constant was 0.58 cm^{-1} and the temperature 20°C

To assess the utility of the present approach to microbial biomass estimation in growing cultures, the experiment displayed in *Figure 7* was undertaken. It is evident (*Figure 7*) that the measurement of the RF permittivity provides an extremely convenient assessment of the biomass present, as judged by its linearity with the optical density of (appropriately diluted) samples taken from the culture.

Discussion

That the presence of cellular material affects the dielectric properties of ionic solutions has been known since the last century[37, 38] and it is of great historical significance that Fricke[39] was able, from measurements of the audio- and radiofrequency dielectric properties of erythrocytes, to calculate, using a model similar to that used herein, that biological membranes are of molecular thickness. In spite of this long history, the principle of estimating the volume fractions of biomembrane-enclosed matter by measuring the RF permittivity of cell suspensions does not seem to have been considered in the literature to date, although Clarke and his colleagues have described the general approach to the direct estimation of microbial biomass by measuring the magneto-inductive properties of bacterial cultures.[8–10]

The present work has shown that the RF permittivity of suspensions of yeast cells is indeed a linear and monotonic function of the biomass present (*Figure 5*) may be estimated with a rapidity exceeding the ability of a potentiometric chart recorder to respond (*Figure 5a*), and is for practical purposes independent of the presence of non-cellular material (*Figure 5a*). This, in contrast to say an absorbance measurement, is due to the fact that the Maxwell–Wagner mechanism of dielectric relaxation in the present cases depends on the possession by the suspended phase of an internal and external conducting aqueous phase. Thus, despite a statement to the contrary,[1] the present general approach could still be exploited in media containing oil droplets, the dielectric properties of which differ substantially from those of microbial cells.[40] Similarly, the

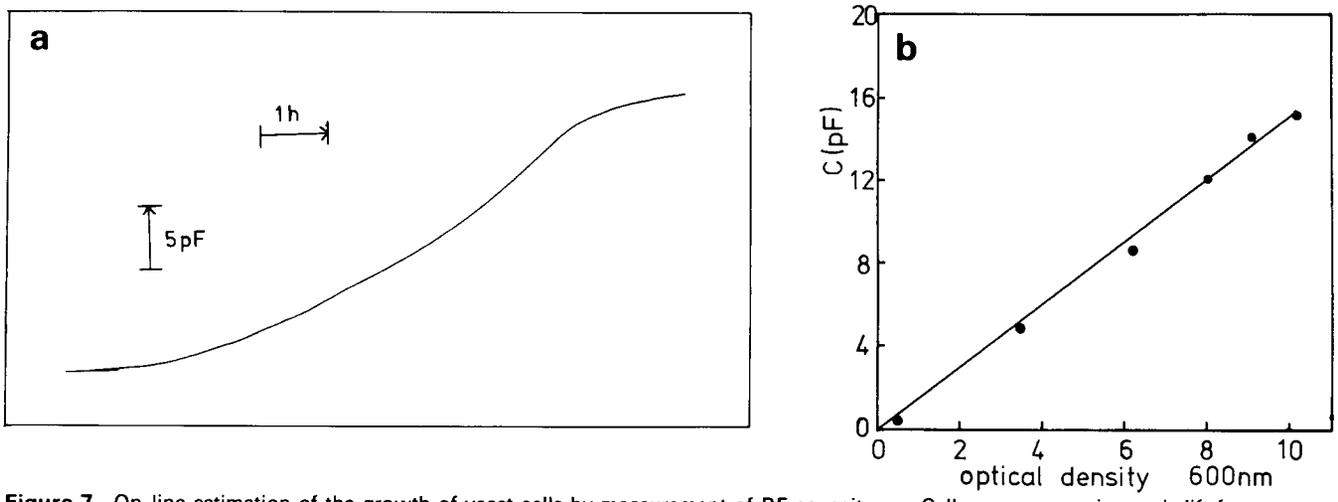

Figure 7 On-line estimation of the growth of yeast cells by measurement of RF capacitance. Cells were grown in an air-lift fermenter as described in Experimental. The initial probe reading was backed off to give a capacitance of 0 pF and the change in capacitance at 300 kHz is plotted versus time (a) or versus the optical density of a sample taken from the fermenter at different intervals and appropriately diluted (b). The cell constant was 1.58 cm^{-1}

Beer–Lambert law fails for yeast cells at concentrations of more than ≈ 1 mg ml^{-1} (data not shown), whilst the RF permittivity is linear with biomass to cell concentrations of at least 100 mg ml^{-1}.[22] The present approach therefore possesses clear advantages over optical methods.

Naturally, the conductivity and permittivity of gases are much lower than those of aqueous media, and the present probe would suffer interference if gas bubbles are actually present in the neighbourhood of the electrode surfaces. However this is easily avoided by appropriate probe design and placement. Alternatively, one might exploit the cross-correlation function between the conductivity and permittivity signals not only to assess any such interference but to gain information about the gas dynamics and bubble-size distribution.[26, 41, 42]

The choice of measuring frequency is under the control of the experimenter. However, the simple model used in the present work indicates that provided the (fixed) measuring frequency is somewhat lower than the characteristic frequency of the β-dispersion little error in biomass estimation will result from the effects of time-dependent conductance changes on the relaxation time, and can be accounted for in terms of the classical explanation of the β-dispersion. In any event, the growth dependent changes in the conductivity of the extracellular phase are in general rather modest, particularly in yeast cultures (*Figure 7*).[33]

We conclude that the measurement of RF permittivity provides an extremely powerful and convenient means for the assessment of microbial biomass, *in situ* and in real time.

Acknowledgements

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