On-Line, Real-Time Measurements of Cellular Biomass using Dielectric Spectroscopy.

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Introduction

All else being equal, the productivity of a biological process is determined by the quantity of biomass present. There is therefore a major requirement for the accurate measurement and control of the biomass within fermentors, at both laboratory and industrial scales. Presently the range of sensors available that can be used *in situ* and reliably for the monitoring and regulation of biotechnological processes in general is rather limited. These sensors normally rely upon physical (e.g. optical, mechanical and electrical) or chemical variables (e.g. pH and concentration) rather than biological ones *per se* (Sarra *et al.*, 1996; Pons, 1991). However only physical methods allow the on-line, real-time estimation of biomass (Harris and Kell, 1985). As well as physical methods, any easily determinable chemical that is produced or consumed by cells at an essentially constant rate during cell growth may also be used to assess biomass, e.g. carbon dioxide evolution and oxygen consumption. In these indirect methods biomass is then calculated based upon mass balances, stoichiometric relationships or empirical constants. However, this type of approach has the great disadvantage that it does not generally discriminate between biomass and necromass (Kell *et al.*, 1990).

Even if biomass was easily measurable there is still the question of what is biologically relevant information for fermentation control and how can one define and quantify it (e.g. metabolism, viability, vitality, morphology) (Kell *et al.*, 1987; Kell, 1987a;

Kell *et al.*, 1990). Such data are usually complex in their acquisition (mainly off-line), time-consuming to measure, prone to human error and usually subjective in their interpretation, e.g. microscopic cell counting and viability staining.

In this survey we will review the measurement of biomass based on the passive electrical (dielectric) properties of biological materials. As will be discussed later, this technique has the advantage that it only detects cells with intact plasma membranes and so gives values that do correlate with biomass rather than necromass. The physics that forms the basis of this technique has been studied since the last century and has been reviewed in great detail by a number of authors (Grant *et al.*, 1978; Pethig, 1979; Pethig and Kell, 1987; Takashima *et al.*, 1988; Foster and Schwan, 1989; Davey and Kell, 1995; Rigaud *et al.*, 1996) and how this theory relates to (dielectric) biomass measurements has also been extensively reviewed and researched (Irimajiri *et al.*, 1975; Harris and Kell, 1983; Harris *et al.*, 1987; Kell,1987b; Kell and Davey 1990; Markx *et al.*, 1991a,b,c,d; Mishima *et al.*, 1991a; Sonnleitner *et al.*, 1992; Davey, 1993a,b; Davey and Kell, 1994; Matanguihan *et al.,* 1994; Davey and Kell, 1995; Asami and Yonezawa, 1996; Siano, 1996; Asami *et al.*, 1998; Davey and Kell, 1998a,b; Asami *et al.*,1999; Krairak *et al.*, 1999 ; Markx and Davey, 1999).

For the purpose of this review we will concentrate on the Biomass Monitor (BM, formerly called the βugmeter, Aber Instruments Ltd, Science Park, Cefn Llan, Aberystwyth, SY23 3AH and see http://www.aber-instruments.co.uk) (Harris *et al.*, 1987; Davey *et al.*, 1999) as this has a large publication base and is the only commercial system available at the moment that can work in real fermentation environments, on-line and in real-time. The other more laboratory-based systems under development will be considered but unless otherwise specified the work being discussed will be studies using the Biomass Monitor (BM).

The theory and practice of dielectric biomass estimation

THE β-DISPERSION

For the purposes of this article a simplified approach to dielectric dispersions and the dielectric approach to the estimation of biomass will be employed. References with more exacting explanations were mentioned in the introduction. For modelling purposes a suspension of cells can be regarded as being composed of three separate parts. Within the (spherical) cells and surrounding them are conducting aqueous ionic media, the cell's cytoplasm and the suspension medium respectively. The cytoplasm is a highly complicated and structured mixture of salts, proteins, nucleic acids and smaller molecules (Clegg, 1984). In addition in eukaryotes various internal membrane bound structures are also present, which can affect the cell's dielectric properties (Foster and Schwan, 1989; Asami *et al.*, 1996). Surrounding the cell's conducting core is the plasma membrane which is essentially non-conducting (Takashima *et al.*, 1988). Thus electrically a cell suspension can be regarded as a suspension of spherical capacitors each containing a conducting matrix (cytoplasm) and all surrounded by a conducting suspension medium.

When an electric field is applied to a suspension of cells in an aqueous ionic solution, the ions in that solution are forced to move. The positively charged ions are pushed in the direction of the field whilst the negatively charged ones are pushed in the opposite direction (*Figure 1*). The ions both inside and outside the cells can only move so far before they encounter the plasma membranes which acts as an insulating physical barrier preventing further movement. This results in the development of a charge separation or polarisation at the poles of the cells (see *Figure 1*). The magnitude of the suspension's field induced separations is measured by its capacitance (C) in Farads (F), however as a Farad is a very large capacitance one normally sees its values expressed in pico-Farads (pF). Thus by measuring the capacitance of the suspension at one or more appropriate frequencies its biomass can be estimated (Harris *et al.*, 1987; Kell *et al.*, 1990; Davey *et al.*, 1993; Davey, 1993a,b; Kell and Todd, 1998) because, as the volume fraction of the cells increases there are more polarised membranes, which in-turn gives a higher measured capacitance. Dead cells

(operationally defined - Kell *et al.*, 1998; Barer *et al.*, 1998) and non-biomass solids do not possess intact plasma membranes and so do not polarise significantly, therefore they do not contribute significantly to the capacitance of the cell suspension (Harris *et al.*, 1987; Stoicheva *et al.*, 1989). Similarly, if oil droplets or gas bubbles are present in the medium they do not contribute directly to the measured capacitance as they are also not membrane-enclosed particles. However, if they are present at high concentration they will reduce the net suspension capacitance by virtue of the fact that they are reducing the cellular volume fraction. In some rare cases non-biomass solids do contribute a significant capacitance that interferes with biomass measurements; however these contributions can be removed using multivariate techniques (Nicholson *et al.*, 1996).

To this point we have only considered the electric field moving in one direction. If the electric field's direction is reversed, so is the polarity of the resulting charge separations (*Figure 2*). However the magnitude of the polarisations remains unchanged and so the capacitance of the suspension also remains unchanged. The rate at which the electric field changes direction can also be varied. The number of times the field changes direction per second is measured by its frequency in Hertz (Hz). The greater the rate of change the higher the frequency. Frequency has a marked effect upon the capacitance of a cell suspension as the ions moving up to and polarising the plasma membranes take a finite time to reach them and cause the polarisations (Pethig, 1979; Foster and Schwan, 1986; Pethig and Kell, 1987).

Figure 3 illustrates the polarisations induced across the cells within a suspension as the frequency of the electric field is increased. At low frequencies, below approximately 0.1 MHz ((A)), many ions have time to reach the cells' plasma membranes before the field is reversed driving the ions in the opposite direction. In this case the induced polarisations are large and hence the capacitance of the cell suspension is high. As the frequency is increased over 1 MHz ((B)) fewer ions have time to reach the plasma membranes before the field is reversed and, therefore, the extent of the transmembrane polarisation is less and hence the capacitance of the suspension is lower. At very high frequencies, typically 10MHz and above ((C)), even fewer ions have time to polarise the membranes and so the resulting membrane

polarisation is small, giving a negligible contribution to the overall measured capacitance. What remains is a background capacitance due largely to the dipoles of the water in the suspending medium.

From *Figure 3* one can see that as the frequency is increased the capacitance of the suspension falls from a high low-frequency capacitance plateau (maximal cell polarisation) to a low high-frequency plateau (minimal cell polarisation). This fall in capacitance due to the loss of induced charging of the cells' plasma membranes as frequency is increased is called the β-dispersion and for most cells it is centred between 0.5 and 3 MHz (Pethig, 1979; Foster and Schwan, 1986; Pethig and Kell, 1987; Davey and Kell, 1994).

The residual high-frequency capacitance due to the medium is termed C_∞ and the height of the low-frequency plateaux above this the ΔC or capacitance increment of the β-dispersion (see *Figure 3*). The frequency at which the fall in capacitance is half completed (i.e. at $C_\infty + (\Delta C/2)$) is termed the characteristic (or critical) frequency (f_c). The measure of the steepness of the capacitance fall during a dispersion that is usually used (which is not its slope) is called the Cole-Cole α (Cole and Cole, 1941; Cole 1972). The Cole-Cole α is a dimensionless quantity with values $>= 0$ but < 1 which nominally describes the distribution of relaxation times in the suspension (however see Markx *et al.*, 1991a for evidence that this cannot be the reason in the case of the β-dispersion of biological cells, and also for example Jonscher, 1983). Typical values for biological cells are up to 0.2 (Davey and Kell, 1995) with some yeasts and bacteria going as high as 0.4. The effect these non-zero α values has on biomass measurements will be discussed later.

With increasing biomass concentration neither the f_c or the C_∞ are significantly changed. However what does change is the magnitude of ΔC, which increases monotonically with biomass concentration. Thus in order to estimate the biomass content of a cell suspension, one simply measures the magnitude of the ΔC of the β-dispersion.

MATHEMATICAL MODELS OF THE β-DISPERSION: ΔC AND CELL SUSPENSION STRUCTURE.

To discuss how the ΔC of the β-dispersion relates to the structural features of the cells in the suspension we must consider both capacitance and conductance in more detail. Capacitance gives a measure of a material's ability to store electrical energy as charge. Conductance (in Siemens (S), which the BM also measures) gives a measure of the ability to conduct charge and dissipate the electric field's energy as heat. Conductance increases as the concentration, valency and mobility of the ions in the solution increases. Both capacitance and conductance depend not only on the material being measured but also on the geometry of the electrodes (and range setting of a BM: high or low) being used. Thus for theoretical work in particular it is convenient to normalise the values to a standard electrode geometry. Doing this converts capacitance (C, in Farads (F)) into relative permittivity (ε (dimensionless), as it is relative to a vacuum), and conductance (G, in Siemens (S)) into conductivity (σ, in $S.m^{-1}$)(Kell, 1987a; Pethig and Kell, 1987; Kell and Davey, 1990).

The electrode geometry (and BM range setting) is encapsulated in the cell constant (K in m^{-1}) of the system. To convert a measured conductance into conductivity one uses *Equation 1*:

$$\sigma = G\,K \qquad \text{....(1)}$$

As tables of the σ for aqueous KCl solutions at known temperatures are available one can measure the conductance of a reference KCl solution (at a known temperature) and then calculate the cell constant of the system. For example, a dielectric spectrometer (e.g. a BM) gives a conductance of 1.83×10^{-3}S for 10 mM KCl at 20°C which from the tables has a conductivity of 0.1278 $S.m^{-1}$. From *Equation 1* we can calculate the cell constant as 70 m^{-1}.

A measured capacitance can be converted to permittivity using:

$$\varepsilon = C\ (K/\varepsilon_0) \qquad \text{....(2)}$$

where ε_0 is a constant called the permittivity of free space and is equal to 8.854×10^{-12} F.m^{-1}. The relative permittivity is numerically equal to the capacitance of the standard electrode with the material in it, divided by the capacitance of the same electrode containing a vacuum.

An important point about both *Equations 1* and *2* is that the unnormalised values (G and C) are converted to their normalised counterparts (σ and ε) by being multiplied by a constant (K and (K/ε_0)) for the given measuring system. This means that the basic shape of the β-dispersion curve in *Figure 3* is unchanged. All that happens is that ΔC becomes $\Delta\varepsilon$ (dielectric increment) and C_∞ becomes ε_∞, whilst the f_c and Cole-Cole α are unchanged. If a dispersion had a ΔC of 10×10^{-12} F when measured with a spectrometer system with a K value of 70m^{-1} then by *Equation 2* the $\Delta\varepsilon$ is 79.

Of course as the ΔC of the β-dispersion is proportional to biomass content (up to moderate levels - see below) so is its $\Delta\varepsilon$, and the theoretical work of Schwan (1957) allows us to relate the latter directly to the physical make-up of the suspension. His equation, which has been extensively checked experimentally (e.g. Harris and Kell, 1983; Davey and Kell, 1995), is:

$$\Delta\varepsilon = (9\ P\ r\ C_m) / (4\ \varepsilon_0) \qquad \text{....(3)}$$

where P (dimensionless) is the volume fraction of cells with intact plasma membranes (i.e. biomass), r is the radius of the spherical cells (m) and C_m is the plasma membrane capacitance in F.m^{-2} (Ferris *et al.*, 1990). C_m gives a measure of the charge-storing ability of the membranes and has been found to be effectively a biological constant of 0.01 ± 0.005 F.m^{-2}. This is perhaps not surprising as it is governed by the thickness and permittivity of the hydrophobic core of membrane material (Hanai *et al.*, 1964; Hanai *et al.*, 1965; Everitt and Haydon, 1968; Coster and Smith, 1974; Laver *et al.*, 1984).

If we assume a C_m for yeast of 0.01 $F.m^{-2}$ then from *Equation 3* a suspension of yeast (radius, $r = 3x10^{-6}$ m) present at a volume fraction (P) of 0.01 will give a β-dispersion Δε of 76.2. A BM standard 25 mm diameter probe used with a BM set to its most sensitive setting (low range) has a cell constant (K) of 70 m^{-1}. From *Equation 2* we can calculate that this Δε corresponds to a measured ΔC on the BM of $9.6x10^{-12}$ F (9.6 pF). If we had the same volume fraction of coccoid bacteria ($r = 0.5x10^{-6}$m) under the same conditions then *Equation 3* would give the Δε as 12.7 and hence a capacitance on the BM of $1.6x10^{-12}$ F (1.6 pF). This makes the general point that dielectric biomass estimations are proportionately more sensitive to larger cells than smaller ones.

If one is using *cell concentration* as the measurement of biomass then *Equation 3* becomes:

$$\Delta\varepsilon = (3 \pi r^4 C_m N) / \varepsilon_0 \qquad ....(4)$$

where N is the cell concentration in cells.m^{-3}. *Figure 4* is a plot of Δε versus cell radius for different cell concentrations calculated using *Equation 4*. Once again one can calculate the capacitances equivalent to the Δε values using *Equation 2*.

For most fermentations r is constant which means that *Equations 3* and *4* predict that there is a linear relationship between the volume fraction or concentration of intact cells and the measured Δε (ΔC). In fact r can change slightly during a fermentation as cells often get slightly larger during exponential growth. To apply *Equations 3* and *4* crudely to non-spherical cells we can think in terms of an equivalent radius, thus if the cells change morphology over time this equivalent radius will also change. For most fermentations these changes do not introduce significant errors in biomass measurements. The other point made clear by *Equations 3* and *4* is that the working definition of biomass used in dielectric biomass estimations is that biomass means cells with intact plasma membranes. Non-culturable, dormant or biochemically inactive cells which retain intact or largely intact plasma

membranes are also measured as biomass. However, it has been found experimentally that this is not usually a source of major error under fermentation conditions (see the later literature review sections).

For cell suspensions with high volume fractions (P>0.15) it has been found experimentally that $\Delta\varepsilon$ (ΔC) no longer increases linearly with volume fraction as predicted by *Equation 3*, but begins to plateau off. This plateauing has been successfully modelled by multiplying the right-hand-side of Equation 3 by the additional term $1/(1 + (P/2))^2$ (Schwan and Morowitz, 1962; Schwan *et al.*, 1970; Harris and Kell, 1983; Davey *et al.*, 1992).

Schwan (1957) also gives an equation for the characteristic (critical) frequency (f_c) of the β-dispersion:

$$f_c = \frac{1}{2\pi r C_m \left(\left(\frac{1}{\sigma_i} \right) + \left(\frac{1}{(2\sigma_o)} \right) \right)} \qquad \text{....(5)}$$

where σ_i and σ_o are the conductivities of the cytoplasm and suspending medium respectively. Thus a yeast of radius (r) $3x10^{-6}$m and C_m of 0.01 $F.m^{-2}$ with an internal conductivity (σ_i) of 0.5 $S.m^{-1}$ suspended in a medium of conductivity (σ_o) 0.8 $S.m^{-1}$ has a β-dispersion f_c of $2.0x10^6$ Hz (2.0MHz) (For useful values to use in calculations such as these see Davey and Kell, 1995). Research has shown that the cytoplasmic conductivity of such cells does not change significantly as one varies the external conductivity of a suspension (Beving *et al.*, 1994). Thus during a fermentation it will largely be changes in the external medium conductivity (σ_o) that will cause the β-dispersion to move. Typically such changes will result from the cell's metabolic activity or from the acid and alkali used for pH control. The implications of movements in f_c for biomass measurements will be discussed later.

To model non-spherical cells more accurately alternative models to the ones above must be used (Asami *et al.*, 1980; Asami and Yonezawa, 1995). A detailed description of

these is beyond the scope of a review such as this as they typically involve the use of complex numbers and 3D geometry. *Figure 5* shows the effect of cell morphology on the β-dispersion (at a constant volume fraction) calculated using the equations in Asami *et al.* (1980). As the cells become more elongated (prolate) a secondary low-frequency component to the β-dispersion becomes increasingly prominent. For very prolate cells one can clearly see the two β-dispersions for the two different semi-axes of the cells. Also shown on the plot is the frequency range for the BM. It is clear that for filamentous fungi the limited frequency range of the BM results in only a fraction of the total signal being registered. It also explains why the dielectric spectra of tempe (a solid-state fermentation using the filamentous fungus *Rhizopus oligosporus*) that were measured using a BM were seen as a gentle slope rather than a defined step change (Davey *et al.*, 1991; Penaloza *et al.*, 1991; Penaloza *et al.*, 1992).

MEASUREMENT OF THE ΔC OF THE β-DISPERSION

To estimate the biomass concentration by measuring ΔC (Δε) we need convenient and practical ways of making these measurements. There are three means of estimating ΔC: the first two rely on measurements at spot-frequencies and the third uses frequency scanning and curve fitting. In all three cases one has first to perform off-line calibration measurements before actual biomass measurements can be carried out on-line. A dilution series of the cells of interest is made and the ΔC of each is measured along with the required biomass output e.g. the dry weight, cell concentration etc. The resulting straight line calibration graph of capacitance versus required biomass output can then be used to convert measured on-line ΔC values back to dry weights etc.

Figure 6 illustrates a β-dispersion showing what one might actually see because of the presence of electrode artefacts (electrode polarisation, see later) which can cause the capacitance to tip up at low frequencies. In single-frequency biomass measurements one selects a single frequency well onto the low-frequency plateau of the β-dispersion (f-low, typically 0.3 to 0.5 MHz) but above the frequency range in which the time-dependent

electrode polarisation effects cause significant interference. Prior to inoculation one backs the capacitance of the medium at this frequency to zero, in effect backing C_∞ to zero. During cell growth the ΔC of the β-dispersion increases as the biomass content does and so does the estimate of ΔC recorded at f-low.

Also shown on *Figure 6* is a spot-frequency (f-high) on the high-frequency plateau of the β-dispersion (typically at 10 MHz). The capacitance at f-high is approximately equal to C_∞ whilst that at f-low approximately equals ($C_\infty + \Delta C$). Thus if the capacitance is measured simultaneously at f-low and f-high and the capacitance at f-high (C_∞) is subtracted from that at f-low ($C_\infty + \Delta C$), one is left with ΔC and therefore an estimate of biomass. This is the method referred to as dual-frequency biomass measurement.

Both the single- and dual-frequency methods of biomass measurement using the Biomass Monitor have been used successfully with a variety of cells and fermentation configurations. The dual-frequency method offers greater stability to long term instrumental drift (Davey, 1993a) which can occur during long term installations.

Leaving aside electrode polarisation which will be discussed later, both these methods do have potential problems because of the need to be well onto the plateaux of the β-dispersion. *Figure 7* shows the effects of non-zero Cole-Cole α values on a β-dispersion when seen over the limited frequency range of a BM. As the α value increases the spot measuring frequencies are progressively off the plateaux so reducing the quality of the biomass measurements.

Using spot-frequencies off the plateaux makes the measurements prone to the second source of error, namely "f_c-offset" effects. *Figure 8* shows three dispersions (lines (A), (B) and (C)) where we have attempted to estimate ΔC (and hence biomass concentration) with f-low values on or off the low-frequency plateau. The three lines are exactly same dispersion, the only difference being that the f_c has moved due to gross changes in suspending medium conductivity (σ_o). *Equation 5* shows that a fall in σ_o will lower the f_c (line (B) on *Figure 8*), a rise will increase the f_c (line (C) on *Figure 8*). With an f-low well onto the low-frequency

plateau these movements have little effect on the estimation of ΔC. However, for an f-low off the plateau, movements in f_c have caused changes in the estimate of ΔC and hence biomass concentration, where no such changes have actually occurred. This effect is only a problem for cell suspensions where the medium conductivity is very low (compared to σ_i) and where large medium conductivity shifts occur during a fermentation. These conditions do not occur in normal industrial fermentations where nutrient levels are high.

The third method that has been used to estimate biomass is by scanning the frequencies over the β-dispersion region to generate a capacitance curve similar to that in *Figure 3*. One then fits this curve to the Cole-Cole equation, which models the shape of dispersion, to give best fit values of ΔC, f_c, Cole-Cole α and C_∞. This approach has been used for the non-contact electrode method from Hewlett-Packard (Asami *et al.*, 1996; Siano, 1996) and with the Biomass Monitor (Davey *et al.*, 1993; Davey and Kell, 1998b). On the BM one typically scans between 15 and 50 frequencies in random order under the control of an external computer. Curve fitting of the data is then undertaken on the computer with a program that uses the Levenberg/Marquardt algorithm for non-linear least-squares fitting (Marquardt, 1963; Bevington, 1969; Grant *et al.*, 1978; Press *et al.*, 1990; Davey *et al.*, 1993). For noisy data the influence of outlying points is minimised using "robust weighting" (Mosteller and Tukey, 1977; Leatherbarrow, 1992).

The main advantage of frequency scanning is that one can extrapolate to the plateaux of the β-dispersion if they are outside the frequency range of the instrument and so f_c-offset errors are eliminated as are the adverse effects of large Cole-Cole α values (see above). On the down-side scanning can take from several seconds to several minutes, which can cause problems in rapidly changing systems: e.g. ones where gas hold-up fluctuates rapidly or where there is poor mixing or homogeneity. The computational overheads in the curve fitting procedure and the need to check that the resulting fits are indeed plausible also need to be taken into consideration for real industrial applications.

ELECTRODE POLARISATION

The major limiting factor on the performance of dielectric biomass estimation for systems where the electrode metal is in direct contact with the growth media is electrode polarisation. The charged metal electrodes used to apply the electric field to the cell suspensions attract a counter layer of ions around themselves which act as a large capacitance in series with the suspension of interest (Schwan, 1963; Bockris and Reddy, 1970). This manifests itself in frequency scans as a sharp increase in capacitance in the frequency range where one wishes to measure biomass (i.e. below about 100-500 kHz)(see *Figure 6*). As this polarisation capacitance increases as the electrodes become fouled or as the medium conductivity increases, one can see that electrode polarisation could be a limiting factor in the present applications of capacitive biomass measurements (Cerckel *et al.*, 1993; Degouys *et al.*, 1993; Siano, 1996; Davey and Kell, 1998a,b).

A variety of methods have been used in order to remove the polarisation's contribution to biological spectra with varying degrees of practicality (Davey and Kell, 1998b). The Biomass Monitor uses a four-terminal electrode design using “non-polarisable” platinum electrode pins to reduce the innate polarisation of the electrodes (Schwan, 1963; Schwan, 1968; Schwan and Ferris, 1968; Ferris, 1974; Harris *et al.*, 1987; Kell, 1987a; Kell and Davey, 1990). This arrangement consists of two pairs of electrodes, the outer two apply an alternating current at a suitable frequency in the range of 0.2 MHz-10 MHz (from 0.1 MHz on older machines), while the inner two, which are connected across the terminals of a high impedance voltmeter, pick up the alternating potential difference (Kell and Woodward, 1991). With a high input impedance voltmeter, negligible current crosses the electrode interface, and hence polarisation is reduced. In practice this configuration works well (Kell and Davey, 1990; Davey *et al.*, 1997), but it does not remove all of the polarisation. This is particularly evident at high conductances combined with low biomass (Cerckel *et al.*, 1993).

In addition to its electrode design the BM uses electrolytic cleaning pulses to keep the electrodes clean and to reduce electrode polarisation. 10V pulses are applied to the electrodes which generate gas bubbles by electrolysis which lift off any adhering materials

and also exposes fresh uncontaminated metal. Both these factors serve to keep the polarisation of the electrodes low in the vast majority of fermentations. However, if the protein content of the medium is very high, as in some brewery worts or animal cell media containing albumin, the pulses can actually precipitate the protein onto the electrodes (Yardley *et al.*, 1999).

For situations where polarisation is still a problem on the BM two methods have been devised to reduce its influence: the polarisation control method and the 2f method. The polarisation control method (Schwan, 1963; Grant *et al.*, 1978; Davey *et al.*, 1990) is frequently used as an off-line method using the Biomass Monitor. A frequency scan of the cell suspension is performed and the conductance at the lowest frequency noted. A sample of the suspending medium is then taken and its conductance adjusted at the lowest frequency to that of the suspension using either distilled water or solid KCl. When this solution is scanned, we have an estimate of the polarisation as a function of frequency which can be subtracted from the cell suspension scan to provide data that is largely free from polarisation.

The 2-frequency (2f) method (Davey and Kell, 1998a,b) for reducing electrode polarisation on BM dielectric spectra has recently been developed. Electrode polarisation can be modelled by a power law and the parameters of this model can be simply modified to give reliable and intuitive measures of the magnitude ($^{L}C_{P}$) and the rate of fall of polarisation with increasing frequency (^{h}f). Although the magnitude of electrode polarisation can change it was found that the ^{h}f value remained constant. This latter fact enabled the capacitance of a suspension at two frequencies to be used to estimate the polarisation's contribution to the spectra and then eliminate it from the suspension data. This method is on-line and is easily capable of being put under full computer control. Where this is of particular use is where the β-dispersion curve has become embedded in a large polarisation curve due to low biomass concentration and a highly conductive growth medium.

A recent and highly significant electrode development involves inductively coupled electrodes based on the use of magnetic fields (Asami *et al.*, 1996; Siano, 1996). This removes the need for any direct electrode/suspension contact and so eliminates electrode

polarisation completely. The probe consists of two coaxial toroidal coils covered with epoxy resin. When an alternating voltage is applied to one toroidal coil, a current which depends on the sample impedance is induced in the other coil by electromagnetic induction. The sample impedance is determined from the relationship between the output current and the input voltage. These electrodes have been prototyped by Siano (1996) and have been marketed by Hewlett Packard as the Colloid Probe. Recent studies using this instrument include those of Asami and Yonezawa (1995) and Asami *et al.* (1996).

DIELECTRIC SPECTROMETER BASELINE ARTEFACTS

The baselines of the spectra obtained with dielectric spectrometers (e.g. a BM) are typically not flat as a function of frequency, by virtue of the fact that one is operating at radio frequencies where strays become increasingly important as one ventures above 1MHz. Thus most dielectric biomass measurement systems rely on calibration/compensation methods to generate reliable data (e.g. Siano 1996). For instance in the BM there are electronic compensations in the machine itself. This has recently been complemented by a full mathematical model of the baseline artefacts of the machine as functions of both frequency and growth medium conductance, which can be implemented in software (Yardley *et al.*,1999).

Biomass measurements on model systems using instruments other than the Biomass Monitor.

There are several other types of commercial, commercially based or experimental instruments for making capacitative biomass measurments apart from the BM. All these instruments involve using/augmenting the features of existing analysers such as those produced by Hewlett-Packard. However, none of these systems is as yet ready to be used as an "off-the-peg" biomass instrument in real industrial environments and all the published research has been on model laboratory systems.

Researchers from Kobe Steel Ltd have developed a capacitance probe based upon a Yokogawa Hewlett-Packard 4194A Impedance/Gain Phase Analyser controlled with a NEC PC9801 (Mishima *et al.*, 1991a Mishima *et al.*, 1991b). These researchers investigated both immersible and installed electrode arrangements (Junker *et al.*, 1994). The distance between the platinum blacked electrodes employed using the immersible system was approximately 30mm; however it was notably greater for the installed platinised platinum plate electrodes placed on opposite sides of a 10 litre fermentor. A wide variety of cell types were studied in order to examine the suitability of the combination of the measuring instrument and the electrodes for biomass determination. These cell types were *Saccaromyces cerevisae* (as a suspension or immobilised on calcium alginate beads) , *Escherichia* coli (suspension), *Aspergillus niger* (suspension), human leukemia (K562) cells (suspension), Madin-Darby bovine kidney (MDBK) cells (microcarrier suspension) and *Sesamum indicum* L. (plant cells in suspension). The effects of chemical (glucose concentration, salinity and pH) and physical parameters (aeration and agitation) upon the dielectric measurements where assessed. There was an excellent linear relationship between capacitance and cell concentration for all these cell types even when the physical and chemical parameters mentioned above were varied. Further it was shown that the capacitance signal reflected biomass as opposed to necromass.

Bragos *et al.* (1998) used the HP4192A impedance analyser to measure the biomass of *Saccharomyces cerevisiae* and *Candida rugosa* (measured on-line) and two bacteria, *Escherichia coli* and *Rhodobacter capsulata* (measured off line). *S cerevisiae* was also measured off-line. Liposome suspensions where measured in order to validate the method and to determine the relationship between particle size and the estimation sensitivity (Gamez, 1996). Two numerical models of biomass estimation were used, the first using two frequencies derived from the Resistor/Capacitor (RC) model of a generic cell suspension and the dependence of its parameters on the cell volume fraction (P) as described by Foster and Schwan (1989) and the second using the parameters of the Cole-Cole impedance model (Cole and Cole, 1941). In order to reduce artefacts (caused by electrode polarisation and cabling) the analyser was connected to the electrodes through a remote front end (Gersing, 1991). This

was used in conjunction with a triple reference calibration method adapted from the methodology of Bolk (1985). This work concluded that the off-line results showed a detection threshold, linearity and sensitivity.

Yelamos *et al.* (1998) have further developed the use of a front-end to reduce the effects of electrode polarisation when again used in conjunction with an HP4192A. They used a modified front-end from that of Bragos *et al.* (1996), using a common-mode feedback voltage and a high input impedance instrumentation amplifier. From this work it was found that the low-frequency systematic error due to the instability of the electrodes was cancelled out as well as the error induced as common mode by interference.

There is a recent, commercially-available instrument based upon electromagnetic induction. It consists of a Hewlett-Packard E5050A Colloid Dielectric Probe (described above), a 4285A precision LCR meter and a personal computer (H.P. Vectra), and is capable of measuring within the frequency range of 100kHz to 20MHz (Asami and Yonezawa, 1995; Asami, *et al.*,1996; Siano 1996).

Siano (1996) used the Colloid Dielectric Probe with data acquisition from 75KHz to 30MHz to measure the biomass of suspensions of aerated and agitated *Escherichia coli*, *Saccharomyces cerevisea*, hybridoma cells, Chinese Hamster Ovary and two un-named proprietary cell lines. Both fixed frequency and spectral permittivity data analysis were compared. For the cells measured the results indicated accurate and reliable biomass estimations, interference was negligible, there was good linearity and the detection limit was below the inoculation concentration. The cell constants used in this arrangement are some three orders of magnitude greater than those used in the BM, and so the capacitances measured tend to fall in the (femto) fF rather than the pF range.

Biomass measurements on model systems and real world industrial applications using the Biomass Monitor.

REQUIREMENTS FOR AN INDUSTRIAL BIOMASS MONITORING INSTRUMENT

As we have emphasised the distinction between instruments capable of measuring biomass in laboratory model systems and those capable of being used in industry it is worth exploring the requirements for true "off-the-peg" industrial biomass instruments. The major design consideration for an instrument capable of real industrial work is that industrial fermentation halls can be extremely hostile environments. This means that the whole system must be robust to things such as water and steam, personnel climbing on the equipment and large sudden ambient temperature changes. Under these conditions the machine must be able to operate in a stable manner for prolonged periods without the electrode being removed from the fermentor. As fermentation halls can be extremely large one must design the system so that the measuring electronics can be at some distance from the electrodes, and multiplexing to multiple fermentors should also be possible. The measuring system must be designed so that no modifications to the user's fermentors are required, thus its probes must fit standard fermentor ports. Any calibrations required must be infrequent and simple and the electrical output of the data has to be compatible with industrial fermentor monitoring systems.

The design of the electrodes is critical. The system should not be overly susceptible to electrode polarisation, and there should be an *in-situ* cleaning system to prevent growth on the electrodes themselves. The construction materials used must be inert and the electrode construction has to be robust. It must be able to withstand high pressures and repeated *in-situ* chemical/heat sterilisation and above all must not present a microbial contamination risk. The Biomass Monitor has evolved over the past decade to fulfil all of the above requirements (see *Figure 9*) (Davey *et al.*, 1999).

In the sections that follow various applications of the BM are described with particular emphasis on the more demanding systems and on real world industrial applications of the machine.

ASSESSMENT OF CYTOTOXICITY

The major site of cytotoxic action of organic solvents is the cytoplasmic membranes of cells (Tanford, 1980), due to the hydrophobicity or amphipathicity of such molecules and their

ability to partition into, and to dissolve, such membranes (Seeman, 1972). Therefore, a screen based on the assessment of membrane damage is indicated.

Stoicheva *et al.* (1989) noted the effects of octanol upon the β-dispersion of *S. cerevisiae*. As this partitioned into the plasma membranes it first caused an increase in capacitance due to the expansion of membrane area (Seeman, 1972) which was followed by a rapid decrease due to cell lysis. This effect held for a number of other substances tested. The work of Salter and Kell (1992) confirmed that cell membrane damage is the likeliest major mechanism of toxicity, and that it was readily assessable using the Biomass Monitor. Davey *et al.* (1993) noted a reduction in cell "viability" after solvent exposure, according to the methylene blue and ethidium bromide tests. This emphasises the fact that biomass rather than necromass is detected using dielectric measurements. From this work it was concluded that the dielectric approach was a novel and convenient means by which to screen solvents and indeed substrates for their biocompatability. These works and others are included in the recent review of solvent effects on microbial cells by Salter and Kell (1995).

BACTERIA AND BIOFILMS

The formation of biofilms can be measured using dielectric spectroscopy as an on-line method. Markx and Kell (1990) observed the formation of a biofilm caused by *Klebsiella rubiacearum*. The biofilm was grown in a plate system under a constant flow of medium with the tip of a BM probe flush to the plate wall. Dielectric measurements were recorded on the Biomass Monitor by registering capacitative changes of the culture in the frequency range 0.1MHz-10MHz using a 4-terminal gold electrode. It was shown that the biofilms could be removed from the probe by using the Biomass Monitor's electrolytic cleaning pulses. A number of biocides were assessed, including cetrimide, chlorine and glutaraldehyde. Chlorine both removed and inhibited further bioflim formation and it was also demonstrated that the automated addition of chlorine in response to changes in capacitance allowed for the control of biofilm formation on-line.

FILAMENTOUS BACTERIA AND FUNGI

Fehrenbach *et al.* (1992) decided that the Biomass Monitor had reached a stage of development where it could be installed in pharmaceutical production facilities working to cGLP/cGMP regulations. Their work was performed on three scales, with 20 litre, 1500 litre and 2000 litre total bioreactor volumes. They used *Saccaromyces cerevisiae*, *Pitchia pastoris* and *Streptomyces virginiae* for biomass estimations in suspension culture. It was concluded that the Biomass Monitor gave an on-line capacitance measurement that could be related directly to biomass concentration. The instrument was also particularly useful in following mycelial growth under industrial conditions, for which precise off-line measurements did not exist. Under these circumstances, the instrument gave data which were closer to physiological reality and could be interpreted more readily and easily than the traditional off-line methods.

Saccharopolyspora erythraea was grown in submerged culture at 2 agitation speeds by Sarra *et al.* (1996) on a soluble medium with glucose as the main carbon source. They concluded that the BM gave good agreement during the growth phase when compared with biomass concentrations as determined by dry weight methods, and that the Biomass Monitor was unaffected by mycelial fragmentation and a lowering of viscosity.

SOLID SUBSTRATE FERMENTATIONS OF FILAMENTOUS FUNGI

The direct measurement of microbial biomass on-line and in real time in liquid substrate fermentations has been problematical and in solid substrate fermentations virtually impossible. Davey *et al.* (1991) showed it was possible to exploit the dielectric properties of cells in order to overcome this problem using the accretion of tempe as a biological model (*Figure 10*).

Solid-substrate fermentation processes using moulds are traditionally exploited in the manufacture of a wide variety of oriental foods including tempe. Tempe is a typical example of a solid-substrate fermentation and is traditionally a soya bean product fermented by the filamentous fungus *Rhizopus oligosporus* Saito. The tempe was cultured at 31^0C in Petri dishes using soya beans, Andean bitter lupins (*Lupinus mutabilis* Sweet) and Quinoa seeds

(*Chenopodium quinoa* Willd) as substrates. The Biomass Monitor electrode was introduced centrally through the perforated lid of a petri dish into the culture, with the electrodes and probe body penetrating 3-7mm into the substrate. Fermentations were followed over a period of five days and samples where taken from replicate dishes in order to monitor the culture's pH, moisture content and biomass as hyphal length per gram dry weight. It was shown that capacitance and hyphal length during the growth phase were closely related, with the linear regression correlation coefficients being close to unity. In this case capacitance was proven to be a reliable, reproducible and on-line measurement of biomass in solid substrate fermentations.

Peñaloza *et al.* (1991) exploited dielectric spectroscopy in order to identify the effects of potassium on the growth of *Rhizopus oligosporus* in solid substrate fermentations. The sources of potassium were K_2CO_3, K_2HPO_4, and KCl, and were introduced to the culture medium at known concentrations. Mycelial potassium salt levels were measured using an Auto Analyser and capacitance readings recorded using a Biomass Monitor at a fixed single frequency of 0.3MHz. It was concluded that the on-line measurement of fungal biomass via capacitance was extremely useful in determining the effect of potassium ions on mycelial growth, and led to a significant improvement in both the medium composition and the speed of the fermentation.

Further to this work Peñaloza *et al.* (1992) used the Biomass Monitor at a single set frequency of 0.3MHz to optimise the solid-substrate tempe fermentation of *Chenopodium quinoa* Willd by *Rhizopus oligosporus* Saito. From the accurate determination of biomass via capacitance the optimum combination of strain and fermentation conditions were deduced for tempe production. This consisted of an initial moisture content of some $620g.kg^{-1}$ an initial pH of 6.4 and an inoculum of $3x10^4$ colony forming units of strain UCW-FF8001 per gram of substrate.

YEAST

Fermentations

The dielectric properties of yeast cell suspensions have been studied in great depth both on- and off-line by many authors (e.g. Asami and Yonezawa, 1996; Harris *et al.*, 1987; Kell, 1987b; Kell *et al.*, 1987; Davey *et al.*, 1992; Asami *et al.*, 1999). Infact yeast provides the standard models for studying the dielectric properties of cells in general and for dielectric biomass measurement studies in particular. Indeed the first Biomass Monitor publication (Harris *et al.*, 1987) was on studies of yeast growing in an air-lift fermentor (*Figure 11*). Leading on from this work further research has led to other yeast studies and applications.

Salter *et al.* (1990) described a novel method of yeast cell immobilisation in ceramic microspheres which allowed high cell densities to be achieved. A suspension of *S. cerevisiae* was passed through a column of microspheres into which a BM electrode had been built. The cells rapidly colonised the microspheres with an even distribution along the entire column length. Cell loading was determined off-line using a protein assay and optical density. From this it was possible to correlate the measured capacitance from the Biomass Monitor to the column loading. The conversion factors produced were 1mg dry wt.ml^{-1} = 39.6 x 10^{6} cells.ml^{-1} = 1.74(6) pF. Overall the Biomass Monitor proved to be very reliable irrespective of whether the cells were resting or growing.

Kronlof (1990) monitored immobilised yeast cells in a continuous brewery fermentation, which due to the insoluble nature of the carrier can cause problems for the more traditional approaches to viable biomass monitoring. The problems are associated with the complete inhabitation of the immobilisation system and how to ensure the complete removal of adhering cells. Further, when the cells had been removed it was unclear as to how to differentiate between viable and non-viable cells as this is impossible when using traditional protein estimation methods to calculate biomass. Several biomass determination methods were evaluated: gravimetric, haemocytometer, methylene blue, ATP determination and glycogen estimation. The results were compared to those obtained from the Biomass Monitor. It was found that the background effect due to non-cellular material could be eliminated and a wide range of biomass concentrations reliably monitored. The conclusion was that the

Biomass Monitor is equally suitable for viable biomass estimations in both suspended and immobilised systems.

The Biomass Monitor has also found a significant niche in fermentation process control (Kronlof, 1990; Kronlof, 1991; Markx *et al.*, 1991b; Austin *et al.*, 1994; Davey *et al.*, 1996). Markx *et al.* (1991b) grew bakers yeast in a novel type of turbidostat; or more correctly permittistat, in which a constant biomass level was continuously maintained by a feedback mechanism based upon the dielectric permittivity of the culture. Dielectric biomass estimations were made using the two frequency method at 0.4MHz and 9.5MHz. Other parameters where also compared to the permittivity data at each setpoint to validate the fermentor control. These included dry weight, fresh weight, the optical density at 600nm, percentage viability (from the methylene blue assay), bud count, ethanol concentration, glucose concentration, and the cell size distribution was measured using flow cytometry. Good linear relationships between setpoint permittivity and dry weight, wet weight and OD were obtained. It was concluded that any changes in the physiological properties of the yeast had a negligible effect on the ratios between permittivity set (and measured) and the steady-state dry weight or optical density of the cultures.

Davey *et al*. (1996) studied the fluctuations in growth rate of a permittistatically controlled yeast culture as estimated from the rate at which medium was pumped into the fermentor to maintain the permittivity setpoint (biomass concentration). They found that permittistatic control provided an excellent method of maintaining and monitoring a constant biomass level within a fermentor and were the first to show that cellular growth could exhibit deterministic chaos.

A study by Austin *et al.* (1994) utilised the Biomass Monitor in a control loop to maintain set-point levels in a cyclic reactor under perturbations. A linear relationship was found between capacitance measurements and cell counts of brewers yeast suspensions, and importantly a correlation was also demonstrated between capacitance and viable biomass concentration.

Brewery yeast management

Yeast management within breweries has received a considerable amount of attention in recent years. It is necessary to ensure that there is the correct amount of yeast in the wort at the start of a fermentation as this has a major influence on the final quality of the beer. A great deal of research has been undertaken to assess practical ways in which yeast concentration can be monitored prior to and during pitching (Carvell, 1994). Traditionally yeast pitching rate is calculated from either the direct weighing of yeast cake or more usually by metering a volume of yeast slurry with a predetermined solids content. Both methods have disadvantages and these may lead to errors in calculating the correct quantity of yeast in the pitch. The yeast cake method is prone to errors due to variable moisture levels and the spun solids content can be inaccurate at high concentrations. With the yeast slurry method it is necessary to correct the yeast content for trub (insoluble non-yeast material) and yeast viability by off-line methods. These problems make process automation difficult.

Viability is a measurement that has vexed brewers for many years. Viable and non-viable yeast cells can purportedly be discriminated using the methylene blue staining method (Fraser, 1920), which is still widely considered to be the standard. In addition to the question of membrane permeability, metabolically active cells reduce those molecules of methylene blue which do cross the cell membrane to a colourless form. The methylene blue method is a subjective test that tends to overestimate the number of viable (culturable) cells (since metabolic activity can remain long after culturability is lost; Davey and Kell, 1996; Barer *et al.*, 1998; Kell *et al.*, 1998). Even after viability staining the production brewer may well add approximately 10% extra yeast "in order to be on the safe side". This addition can lead to fermentation problems as over pitching can be the cause of poor yeast vitality, reduced hop utilisation, and variable consistency in terms of product flavour and process optimisation.

For the purposes of estimating on-line viability of yeast the 316B Yeast Monitor was developed from the BM specifically for the brewing industry. The Yeast Monitor is capable

of measuring the viable yeast count per ml directly on-line and is unaffected by trub, proteins or gas bubbles. The 316B was evaluated by a major British brewing company (Bass Brewery PLC, High Street, Burton-on Trent, Staffordshire, DE14 1JZ, U.K) and their results were presented at the 22nd European Brewing Convention (Boulton, 1989). The results in that paper showed a linear relationship between capacitance and yeast biomass over a range extending to at least 50% wet weight/volume which was equivalent to 100mg.ml^{-1} dry weight or 1x10^{9} cells.ml^{-1}. It was also noted that with the correct yeast pitch as determined by the Yeast Monitor a typical fermentation was completed in 55 hours as opposed to the periods of up to 74 hours occasioned by conventional pitching procedures. These correct pitching values in turn led to enhanced fermentor performance and therefore to an increased turnover. In this paper a schematic representation of a yeast pitching control system was also suggested (*Figure 12*), which was later implemented in the breweries of many companies worldwide. Leading on from this article the application of the Yeast Monitor to control yeast pitching rates received further attention: Boulton *et al.*, 1991; Lawrence, 1992; Boulton and Clutterbuck, 1993; Maca *et al.*, 1994; Kell and Todd, 1998.

In the study of Maca *et al.* (1994), Yeast Monitor readings were used to calculate the yeast slurry volumes required to pitch fermentations at the Miller Brewing Company, Milwaukee, USA. These volumes were then compared to a conventional spin down wet solids measurement method of calculating pitching rates. This method gives an estimate of the yeast volume to be pitched based upon the determination of the volumes of specific layers within a centrifuged sample of yeast. Of six fermentors pitched using the Yeast Monitor all were on target for viable cell count immediately after pitching. In comparison only one out of the five fermentors that was pitched using the traditional spin down method was on target. It was also concluded that the Yeast Monitor is not affected by the high and varying levels of trub which, in the Miller yeast, can interfere with the spin down method.

During 1993 Alfa Laval Brewery Systems, Sweden, launched the Dynapitch controlled yeast pitching system, which at its heart is controlled by a 316B Yeast Monitor. This instrument was evaluated by Dymond *et al.* (1994) and is a self-contained, skid-

mounted, computer-controlled module designed to minimise the requirements for on-site engineering and installation. The system is currently installed in a number of European breweries, allowing the full automation of yeast pitching.

The Yeast Monitor has recently undergone further metamorphoses. One of the new variants is the Yeast Monitor 320, which utilises probes positioned at different heights within a large production fermentor (Carvell, 1997). This instrument is multiplexed (up to 16 probes) and designed to monitor yeast profiles and mixing patterns within production fermentors, a process which is not well understood. Further development work by Aber Instruments has led to the 800 series Lab Yeast Analyser (Pateman, 1997), which has been designed as a bench-top brewery laboratory tool requiring little sample preparation.

A further application of the Yeast Monitor is the control of yeast feed rate to centrifugal separators in breweries. This process can be difficult to optimise leading to yeast slurries of varying concentration which can cause centrifuge blockages and hence process down time. The combined use of the Yeast Monitor coupled to a variable-speed centrifuge allows the brewer to recover beer from the yeast slurry more efficiently and reliably with lower running costs. Centrifugation is but one process in the recovery of yeast from the fermentation process (yeast cropping). Yeast cropping can also be automated using a Yeast Monitor so that among other things only viable yeast is stored ready for re-pitching (*Figure 13*) (Boutlon and Clutterbuck, 1993; Carvell, 1997; Siems, 1997).

ANIMAL CELLS

Biotechnological processes performed at a semi-pilot or industrial scale using mammalian cells lack appropriate probes to evaluate on-line, in real-time, non-invasively and reliably, the biomass content of a bioreactor (Kell *et al.*, 1990; Konstantinov *et al.*, 1994).

The application of the Biomass Monitor to animal cell culture monitoring has only occurred in earnest in the last few years (Cerkel *et al.*, 1993; Degouys *et al.*, 1993; Beving *et al.*, 1994; Davey *et al*., 1995; Noll, 1995; Noll *et al.*, 1996; Noll and Biselli, 1998; Davey *et al.*, 1997; Guan and Kemp, 1997; Guan *et al.*, 1998; Zeiser *et al.*, 1999). In these works the Biomass Monitor has been used to study the growth of a wide variety of animal cells whether in suspension or in an immobilised state.

Cerckel *et al.* (1993) investigated the dielectric properties of Chinese Hamster Ovary (CHO 320) cells and HeLa cells grown in suspension culture at a concentration of 0.5-3 x 10^6 cells.ml^{-1} and scanned at frequencies between 0.2 and 10MHz using a BM. Cell numbers were determined using a Coulter counter model Z_b and a linear relationship between capacitance and cell number was observed. Low-frequency dielectric spectra did however prove to be unreliable due to the high conductance of the growth medium and the corresponding increase in electrode polarisation. It was found that using 0.5 MHz as the measuring frequency gave the best compromise in terms of loss of sensitivity verses quality of biomass evaluation.

Zeiser *et al.* (1999) grew batch suspension cultures of *Spodoptera frugiperda* Sf-9 (insect cells) which were infected with a baculovirus expressing recombinant β-galactosidase. Permittivity measurements were made on-line using a Biomass Monitor set at a frequency of 0.6MHz. It was observed that during the growth phase there was an increase in the relative permittivity; this reflected an increase in viable cell numbers which remained broadly matched with permittivity during the time-course of the experiment. From these data the most appropriate point on the growth curve for the addition of the baculovirus could be determined. The virus initiates the arrest of cell division, and the infected cells increase in size eventually lysing and releasing the recombinant protein. The use of dielectric spectroscopy allowed for the optimisation of the time of infection and hence lead to the maximum yield of the recombinant protein.

Macroporous carriers are a useful means of increasing the numbers of cells in a culture which can be low particularly in batch cultures using CHO 320 cells (Guan and Kemp, 1997). One of the problems in using macroporous carriers to cultivate animal cells in

culture has been to assess cell viability on-line; this is because many of the cells inhabit the macroporous infrastructure of the bead. Guan and Kemp (1997) measured the cell concentration of CH0 320 cells grown on Cytopore 1 microcarrier beads (Pharmacia) using off-line protein estimations and compared them to dielectric measurements made using a BM. The results indicated that the dielectric estimations of biomass in the microcarriers was more accurate than the protein estimations and was also able to give a viable cell count.

Degouys *et al.* (1993) used the Biomass Monitor to evaluate the concentration of anchorage dependant HTC cells grown on Cytodex 3 (Pharmacia) in spinner vessels. Capacitance values measured at 0.8 MHz on the Biomass Monitor were compared to measurements from a Coulter counter Z_b. It was found that the cellular biomass estimations made from the Biomass Monitor were extremely accurate when seeded concentrations of Cytodex of 5g/L and higher were used. These microcarrier concentrations are those commonly used in the biotechnology industry for the mass production of recombinant anchorage-dependent cells.

Davey *et al.* (1997) used suspensions of immobilised Chinese Hamster Ovary (CHO 320) cells which had been genetically adapted to produce interferon-γ to evaluate the relationship between capacitance and the concentration of viable cells. Dielectric data were compared with data from a Coulter counter (Model D) and from flow cytometry, comparisons were also made with traditional microscope counts (haemocytometer) and to the fluorescein diacetate and ethidium bromide viability assay. An excellent relationship was again observed between capacitance and viable cell number. This is important as the conventional means of assessing biomass are not possible with immobilised cells.

Guan *et al.* (1998) combined on-line BM and microcalorimetric measurements to control a stirred aerobic batch culture of CHO 320 cells which had been genetically modified to produce human interferon-γ. This approach was chosen as cell growth is associated with an enthalpy change which is a direct reflection of metabolic rate. A specific heat flow measurement was acheived by dividing heat flow rate by the capacitance of the cell

suspension with detection limits of ca. $2.0x10^{-6}$ W.cm^{-3} and $1.4x10^{5}$ cells.cm^{-3} respectively. The results of this work have led to the patenting of a specific heat flow sensor as a means of metabolic control of mammalian cell cultures with the advantages of on-line reliability, robustness and with long term advantages in the way of little recurrent cost to the user.

Noll and Biselli (1998) evaluated the BM using immobilised hybridoma cells grown in continuous suspension in a fluidised bed bioreactor batch culture. Both capacitance and conductance were measured on-line at a frequency of 0.6MHz, while control measurements were made off-line to ascertain cell density. The capacitance data provided information that led to computer optimisation of on-line medium dosing as it was found that a constant ratio existed between glutamine consumption and capacitance. This allowed a closed loop control of the medium feed rate, which was directly linked to the capacitative signal produced by the Biomass Monitor during the entire course of a continuous fermentation.

PLANT CELLS

Markx *et al.* (1991c) measured the biomass of plant cell suspensions of *Festuca arundinacea* using the on-line measurement of the permittivity of the culture with a BM and also by measuring the conductivity of the suspending medium and the cell suspension as a whole using a "Bruggeman probe" connected to a bench conductivity meter. The Bruggeman method of biomass estimation (Bruggeman, 1935; Lovitt *et al.*, 1983) proved accurate and could be applied on-line and it also gave results which correlated with biomass concentrations as determined from measurements of the radio-frequency dielectric permittivity of the culture. However the Biomass Monitor's results based upon dielectric permittivity were more convenient to use on-line as no mechanical pumping was required. Although one has to say that the Bruggeman approach has the potential to form a very cheap biomass measuring system for systems where the cells/immobilised cells settle out very quickly or can be easily filtered.

Further work by Markx et al. (1991d) showed that dielectric spectroscopy using a BM could be used to measure the shear sensitivity of plant cells by measuring the permittivity fall

in a plant cell suspension culture under shear stress. This was demonstrated using suspension cultures of *Catharanthus roseus*, *Nicotiana tabacum*, *Cinchona robusta* and *Tabernaemontana divaricata*. All of the cultures showed an initial rapid decline in viable cell number followed by a slower decline as observed dielectrically. These results were compared with fresh weight, dry weight, packed cell volume and cell number. It was concluded that the sensitivity of the cells to shear stress depended strongly on the cell line but only slightly upon the cell's age.

Conclusions

It is clear from the many publications cited that capacitative (dielectric) biomass measurements are generally an accurate and reliable method of determining viable cellular biomass, both on- and off-line. However of the several instruments that have been used to make these measurements it is only the Biomass Monitor and its derivatives that can be used for "off-the- peg" applications, particularly within an industrial environment.

The Biomass/Yeast Monitor continues to be incorporated and exploited within the brewing and pharmaceutical industries, with its use being not purely for measurement but also as a control instrument, capable of controlling valves, centrifuges etc. Within the brewing industry the Yeast Monitor has been utilised in the control of yeast pitching, yeast reclamation, the monitoring of cell growth and feed rate control for beer recovery processes. The use of the Yeast Monitor has in some breweries wholly superseded traditional methods of yeast measurement and has led to greater process performance, with important capital cost savings.

Perhaps the major future advances in dielectric biomass estimation will come with fully-developed instruments that can operate below 100 kHz without significant electrode polarisation effecting the results. This would not only give more reliable biomass estimations but would allow the study of low-frequency dielectric phenomena related to cell surface charge effects (the α-dispersion). The extension of dielectric studies from the linear to the

nonlinear domain has already begun (Woodward and Kell 1990; Woodward *et al.*, 1996) and could lead to important new on-line methods of monitoring cell physiology.

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Legends

Figure 1. When an electric field is applied to a suspension of cells in an aqueous ionic solution, the positive ions are pushed in the direction of the field and the negative ions in the counter direction. The ions can only move so far until they encounter the cell's plasma membranes, which prevents further movement. This results in a charge separation or polarisation at the poles of the cells.

Figure 2. *Figure 1* showed the applied electric field pointing in one direction only. If the direction of the electric field is reversed then the only effect is to change the polarity (but not the magnitude) of the polarisations of the cells. This results in the measured capacitance of the cells remaining unchanged.

Figure 3. The capacitance of a cell suspension as a function of frequency shown along with the equivalent polarisations of the cells (the field is shown in one direction only). (A) is at approximately 0.1MHz and many ions have time to reach the cells' plasma membranes before the electric field is reversed. As the frequency is increased over 1 MHz ((B)), fewer ions reach the cells' plasma membranes resulting in reduced membrane polarisation and hence the suspension's capacitance is lower. At very high frequencies typically over 10MHz ((C)) fewer ions have time to polarise the cell membranes before the field is reversed, this results in fewer polarisations and hence to a negligible contribution to the measured capacitance. Also shown on the figure are the terms used to describe a dispersion curve. The residual high-frequency capacitance is called C_∞ and the height of the low-frequency plateau above this is the capacitance increment (ΔC). When the fall in capacitance is half completed we have the characteristic (critical) frequency (f_c) and the steepness of this fall in capacitance is characterised by the Cole-Cole α.

Figure 4. This shows a plot of permittivity increment ($\Delta\varepsilon$) verses cell numbers/ml (N) as calculated using *Equation 4*. The three cell radii (r) used were 2, 4 and 6 μm these correspond to the lines (A), (B) and (C) respectively (C_m was assumed to be 0.01 $F.m^{-2}$). From *Equation 4* one can see that the gradient of these lines depends on the fourth power of the radius of the cells.

Figure 5. From this simulation we can observe the effects of cell morphology on the β-dispersion (at a constant volume fraction of 0.05). The lowest of the lines corresponds to a spherical yeast cell with a radius of 3μm. In the subsequent traces the yeast cell has been drawn-out into a hypha of increasing length. The cross section of this hypha is a circle of radius 3μm and each line above that of the sphere corresponds to an increase in total length of a further 6μm up to the top trace of tip-to-tip length of 60μm. The cells are assumed to be a homogeneous population of randomly orientated rigid prolate spheroids of revolution lacking a cell wall and with a completely insulating plasma membrane. The other assumptions are: membrane thickness =4nm; membrane permittivity =4.5 (C_m is thus 0.01 $F.m^{-2}$); suspension medium ε =80; suspension medium σ =0.8 $S.m^{-1}$; cytoplasmic ε =60 and the cytoplasmic σ =0.5 $S.m^{-1}$. The BM's frequency range is denoted by the two vertical lines.

Figure 6. A hypothetical frequency scan of a cell suspension (solid line) showing the contribution of electrode polarisation at low frequencies. The dashed line indicates the true low-frequency plateau of the β-dispersion unadulterated by electrode polarisation. Also shown on the figure are the typical spot frequencies (f-low and f-high) at which single- and dual-frequency biomass estimations are made on the BM.

Figure 7. The effect of increasing the Cole-Cole α of the β-dispersion seen within the BM's frequency range. The plots are for a β-dispersion with ΔC = 20pF, f_c = 1.5MHz, C_∞ 5pF and

in ascending order are α values of 0.1, 0.2, 0.3, 0.4 and 0.5 respectively. It can be seen that as the α value increases the spot measuring frequencies are progressively off the plateaux. At very high Cole-Cole α values the β-dispersion can fail to reach either of its plateaux within the BM's available frequency range; this can therefore reduce the quality of biomass measurements.

Figure 8. The effect on biomass measurements resulting from the movement of the β-dispersion's f_c when the f-low frequency used to estimate ΔC is on and off the low-frequency plateau of the β-dispersion. Line (A) shows the capacitance verses frequency plot for a hypothetical cell suspension at a given f_c value. In line (B) the f_c has moved to a lower frequency and this has resulted in an artefactual decrease in the estimate of ΔC and hence biomass concentration estimated with the f-low off the plateau but not for the one on the plateau. Line (C) shows the effect of the f_c moving to a higher frequency than that of line (A). This time there is an artefactual increase in the biomass estimated using the f-low off the plateau. Once again using an f-low on the plateau prevents this artefact from occurring. These artefactual changes in the estimation of ΔC (and hence biomass concentration) due to changes in f_c are called "f_c-offset" errors.

Figure 9. A Biomass Monitor system contained in a water proof housing (background) suitable for an industrial installation. The system consists of a Biomass Monitor (bottom) linked to a Muliplexer Controller (middle) and a Multiplexer (top). For clarity only two probes and cabling are shown connected to the system (foreground). The probes fit into standard 25mm fermentor ports and are screwed into head amplifiers (small boxes) that do some signal conditioning prior to passing the measured signal to the main BM units.

Figure 10. The growth of the filamentous fungus *Rhizopus oligosporus* on soya beans during the solid substrate tempe fermentation. For details of the methods used see Davey *et al.*

(1991). This figure compares the variation of hyphal growth in km of hyphae per gram of dried tempe (open circles) with capacitance (pF) (closed circles) over the course of the fermentation. The capacitance data were recorded continuously, on-line and in real-time using a BM but only the data points corresponding to the off-line hyphal length measurements are shown. There is an excellent linear relationship between capacitance and hyphal length throughout the 48 hour growing period. During the lytic phase the body of the tempe collapses away from the beans as the cells lyse. The loss of intact membranes causes the capacitance to fall while hyphal length remains unchanged as this is based on the measurement of the unaffected cell walls

Figure 11. The growth of yeast in an air-lift fermentor (see Harris *et al.* 1987 for full details of the methods used). (a) The on-line real-time estimation of biomass using a BM. The capacitance of the suspension was measured at 0.3MHz using single-frequency biomass measurements. (b) The data from (a) plotted against the equivalent off-line optical densities (after appropriate dilution) measured at 600nm. An almost perfect linear relationship between the two methods of accessing biomass is demonstrated.

Figure 12. A schematic diagram of an automated pitching rate control system incorporating a Yeast Monitor to ensure an accurate amount of viable yeast slurry is delivered from a yeast storage vessel to the fermentor. The brewer sets the amount of yeast to be pitched and the timing of the pitch within the brew. The system then monitors the concentration of viable yeast passing the probe in the pitching main. The resulting concentration signal is then integrated with the output from the flow meter, giving a measure of the amount of viable yeast pitched into the fermentor. When the target is reached the controller will turn off the yeast pump. With the addition of a flow meter in the wort line the wort flow can be used to control the viable yeast rate and hence provide continuous pitching over the entire length of the brew (Carvell, 1997).

Figure 13. This photogragh shows a Yeast Monitor (background) installed in a working brewery environment (Bass PLC, U.K.). This system utilises the Yeast Monitor to automatically control yeast cropping, thereby ensuring that waste is minimised and the storage of viable yeast suitable for re-pitching, is maximised.

References

ASAMI, K., HANNA, T. AND KOIZUMA, N. (1980). Dielectric analysis of Eshcherichia coli suspensions in the light of the theory of interfacial polarisation. *Biophys. J.* **31**, 215-280.

ASAMI, K. AND YONEZAWA, T. (1995). Dielectric behaviour of non-spherical cells in culture. *Biochimica et Biophysica Acta* **1245**, 317-324.

ASAMI, K. AND YONEZAWA, T. (1996). Dielectric behavior of wild-type yeast and vacuole-deficient mutant over a frequency range of 10 kHz to 10 GHz. *Biophysical Journal* **71**, 2192-2200.

ASAMI, K., YONEZAWA, T., WAKAMATSU, H. AND KOYANAGI, N. (1996). Dielectric spectroscopy of biological cells. Bioelectrochemistry and Bioenergetics. **40**, 141-145.

ASAMI, K., GHEORGHIU, E. AND YONEZAWA, T. (1998). Dielectric behavior of budding yeast in cell separation. *Biochimica et Biophysica Acta* **1381**, 234-240.

ASAMI, K., GHEORGHIU, E., AND YONEZAWA, T. (1999). Real-time monitoring of yeast cell division by dielectric spectroscopy. *Biophysical Journal* **76**, 3345-3348.

AUSTIN, G.D., WATSON, R.W.J. AND D'AMORE, T. (1994). Studies of on-line viable yeast biomass with a capacitance biomass sensor. *Biotechnology and Bioengineering*. **43**, 337-341.

BARER, M. R., KAPRELYANTS, A. S., WEICHART, D. H., HARWOOD, C. R. AND KELL, D. B. (1998). Microbial stress and culturability: conceptual and operational domains. *Microbiology UK* **144**, 2009-2010

BEVING, H., ERICKSSON, L.E.G., DAVEY, C.L. AND KELL, D.B. (1994). Dielectric properties of human blood and erythrocytes at radio frequencies (0.2-10 MHz); dependence on cell volume fraction and medium composition. *Eur. Biophys. J.* **23**, 207-215.

BEVINGTON, P. R. (1969). *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

BOCKRIS, J. O'M. AND REDDY, A. (1970). *Modern Electrochemistry, Vols 1 and 2*. Plenum Press, New York

BOLK, W.T. (1985). A general digital linearising method for transducers. *J. Phys. E. Sci. Instrum*. **18**, 61-64.

BOULTON, C.A., MORGAN, P.S. AND LOVERIDGE, D. (1989). The application of a novel biomass sensor to the control of yeast pitching rate. *Proceedings of the 22nd European Brewing Convention*, Zurich. pp 653-661.

BOULTON, C.A., JONES, A.R. AND HINCHCLIFFE, E. (1991). Yeast physiological condition and fermentation performance. *Proceedings of the 24th European Brewing Convention*, Lisbon. pp 385-392.

BOULTON, C.A. AND CLUTTERBUCK, V.J. (1993) Application of a radio frequency permittivity biomass probe to the control of yeast cone cropping. *Proceedings of the 24th European Brewing Convention*, Oslo. pp 509-516.

BRAGOS, R., YANEZ, A., RIU, P.J., TRESANCHEZ, M., WARREN, M., CARRENO, A. AND CINCA, J. (1996). Espectro de la impedancia de miocardio porcino in situ durante ischemia. *Parte 1: sistema de medida, XIV. Congreso Annual de la Sociedad Espanola de Ingenieria Biomedica, Pamplona, Spain*. pp 97-99.

BRAGOS, R., GAMEZ, X., CAIRO, J., RIU, P.J. AND GODIA, F. (1998). Biomass monitoring using impedance spectroscopy. *Proceedings of the X. International Conference on Bio Electrical Bio-Impedance, Barcelona, Spain*. pp 337-340.

BRUGGEMAN, D.A.G. (1935). Berechnung verschiedener physikalischer Konstanten von heterogrnen Substanzen.1. Dielectrizitatskonstanten und Leitfahigkeiten der Mischkorper aus isotropen Substanzen. *Ann.Phys*. **24**, 636-679.

CARVELL, J.P. (1994). Automatic yeast pitching control using a yeast sensor. *The Brewer* **80**, 57-59.

CARVELL, J.P. (1997). Development in on-line monitoring of viable yeast in the brewing process. *Institute of Brewing (Asia Pacific Section), Regional Technical Symposia in April*. pp 261-265.

CERKEL, I., GARCIA, A., DEGOUYS, V., DUBOIS, D., FABRY, L. AND MILLER, A.O.A. (1993). Dielectric-spectroscopy of mammalian-cells:Evaluation of the

biomass of Hela-Cell and CHO-Cells in suspension by low-frequency dielectric spectroscopy. *Cytotechnology*. **13**, 185-193.

CLEGG, J. S. (1984). Properties and Metabolism of the Aqueous Cytoplasm and Its Boundaries. *American Journal of Physiology* **246**, R133-R151.

COLE, K.S. AND COLE, R.H. (1941). Dispersion and absorption in dielectrics. 1. Alternating current characteristics. *J. Chem. Phys*. **9**, 341-351.

COLE, K.S. (1972). *Membranes Ions and Impulses*. University of California Press, Berkley.

COSTER, H.G.L. AND SMITH, J.R. (1974). The molecular organisation of bimolecular lipid membranes. A study of the low-frequency Maxwell- Wagner impedance dispersion. *Biochim. Biophys. Acta* **373**, 151-164

DAVEY, C.L., MARKX, G.H. AND KELL, D.B. (1990). Substitution and spreadsheet methods for analysing dielectric spectra of biological systems. *Eur. Biophys. J.* **18**, 255-265.

DAVEY, C.L., PEÑALOZA, W., KELL, D.B. AND HEDGER, J.N. (1991). Real-time monitoring of the accretion of Rhizopus oligosporus biomass during the solid-substrate tempe fermentation. *World Journal of Microbiology and Biotechnology* **7**, 248-259.

DAVEY, C.L., DAVEY, H.M. AND KELL, D.B.(1992). On the dielectric properties of cell suspensions at high volume fractions. *Bioelectrochemistry and Bioenergetics* **28**, 319-340.

DAVEY, C.L., DAVEY, H.M., KELL, D.B. AND TODD, R.W. (1993). Introduction to the dielectric estimation of cellular biomass in real time, with special emphasis on measurements at high volume fractions. *Analytica Chimica Acta* **279**, 155-161.

DAVEY, C.L. (1993a). *The Biomass Monitor Source Book* (Aber Instruments, Aberystwyth, U.K.).

DAVEY, C.L. (1993b). *The theory of the β-dispersion and its use in the estimation of cellular biomass* (Aber Instruments, Aberystwyth, U.K.).

DAVEY, C.L., MARKX, G.H. AND KELL, D.B. (1993). On the dielectric method of measuring cellular viability. *Pure Appl. Chem.* **65**, 1921-1926.

DAVEY, C.L. AND KELL, D.B. (1994). *The low-frequency dielectric properties of biological cells and tissues* eds. D. Waltz, H. Berg and G. Milazzo (Birkhauser Verlag, Geneva). pp 189-207.

DAVEY, C.L. AND KELL, D.B. (1995). The low frequency dielectric properties of biological cells. In *Treatise of Biochemistry* ed H.Berg, Vol.VI.

DAVEY, C.L., GUAN, Y., KEMP, R.B., AND KELL, D.B. (1995). Real-time monitoring of the biomass content of animal cell cultures using dielectric spectroscopy. *Proceedings of the Symposium on Process Analysis of Animal Cell Culture (JAACT'95), Fukuoka, Japan*. 6-10 Nov.

DAVEY, H.M., DAVEY, C.L., WOODWARD, A.M., EDMONDS, A.M., LEE, A.W. AND KELL, D.B. (1996). Oscillatory, stochastic and chaotic growth rate fluctuations in permittistatically controlled yeast cultures. *BioSystems* **39**, 43-61.

DAVEY, C.L., GUAN, Y., KEMP, R.B. AND KELL, D.B. (1997). Real-time monitoring of the biomass content of animal cell cultures using dielectric spectroscopy. *Animal Cell Technology*: *Basic and Applied Aspects* **8**, 61-65.

DAVEY, C.L. AND KELL,D.B.(1998a). The influence of electrode polarisation on dielectric spectra, with special reference to capacitive biomass measurements 1. Quantifying the effects on electrode polarisation of factors likely to occur during fermentations. *Bioelectrochemistry and Bioenergenics* **46**, 91-103.

DAVEY,C.L. AND KELL,D.B.(1998b). The influence of electrode polarisation on dielectric spectra, with special reference to capacitive biomass measurements 2. Reduction in the contribution of electrode polarisation to dielectric spectra using a two frequency method. *Bioelectrochemistry and Bioenergenics* **46**, 105-114.

DAVEY, C.L., TODD, R. AND BARRETT, J. (1999). From concept to market in industrial impedance applications. In *Electrical Bioimpedance Methods* eds. P.J. Riu, J. Rosell, R. Bragos and O. Casas, pp 239-244. The New York Acadamy of Sciences, New York.

DAVEY, H.M., KAPRELYANTS, A.S., WEICHART, D.H. AND KELL, D.B. (1998). Estimation of microbial viability using flow cytometry. In: *Current Protocols in Cytometry: Volume 11 Microbial Cytometry*, ed. Robinson, J.P, pp.1-20. Wiley, New York.

DEGOUYS, V., CERKEL, I., GARCIA, A., HARFIELD, J., DUBOIS, D., FABRY, L. AND MILLER, A.O.A. (1993) Dielectric spectroscopy of mammalian cells: 2, Simultaneous in situ evaluation by aperture impedance pulse spectroscopy and low-

frequency dielectric spectroscopy of the biomass of HTC cells on Cytodex 3. *Cytotechnology* **13**, 195-202.

DYMOND, G., HOGAN, A. AND OWEN, R. (1994). Pitching the perfect amount of yeast. *Brewers Digest International*, May, 24-25.

EVERITT, C.T. AND HAYDON, D.A. (1968) Electrical capacitance of a lipid membrane separating two aqueous phases. *J. Theoret. biol*. **18**, 371-379.

FERENBACH, R., COMBERBACH, M. AND PETRE, J.O.(1992). On-line biomass monitoring by capacitance measurement. *Journal of Biotechnology* **23**, 303-314.

FERRIS, C.D.(1974) *Introduction to Bioelectrodes*. Plenum Press, New York.

FERRIS, L.E., DAVEY, C.L. AND KELL, D.B. (1990). Evidence from its temperature dependence that the β-dispersion of cell suspensions is not due soley to the charging of a static membrane capacitance. *Eur. Biophys. J.* **18**, 267-276.

FOSTER, K.R. AND SCHWAN, H.P. (1986). Dielectric properties of tissues In: *CRC Handbook of Biological Effects of Electromagnetic Fields* eds. C. Polk and E. Postow. CRC Press, Boca Raton, FL.

FOSTER, K.R. AND SCHWAN, H.P. (1989). Dielectric properties of tissues and biological materials: a critical review. *Critical Reviews in Biomedical Engineering* **17**, 25-104.

FRASER, C.G. (1920). The action of methylene blue and certain other dyes on living and dead yeast. *J. Phys. Chem*. **24**, 741-748.

GAMEZ, X., SABES, M., BRAGOS, R., RIU, P.J., CAIRO, J. AND GODIA, F. (1996). Biomass monitoring using multifrequency impedance measurements: relationship between particle size and electrical impedance. *Proc. of the 1st European Symposium on Biochem, Eng. Sci*., Dublin. pp94.

GERSING, E. (1991). Measurement of electrical impedance in organ-measuring equipment for research and clinical applications. *Biomedizinsche Technik* **36**, 6-11.

GRANT, E.H., SHEPPARD, R.J. AND SOUTH, G.P. (1978). *Dielectric Behaviour of Biological Molecules in Solution*. Clarendon Press, Oxford.

GUAN, Y. AND KEMP, R. (1997). The viable cell monitor: A dielectric spectroscope for growth and metabolic studies of animal cells on macroporous beads. *Proc. 15th E.S.A.C.T. meeting, Tours* ed. O. W Merton, Kluwer Publ., Dordrecht.

GUAN, Y., EVANS, P.M. AND KEMP, R.B. (1998). An on-line monitor and potential control variable of specific metabolic rate in animal cell culture that combines microcalorimetry with dielectric spectroscopy. *Biotechnology and Bioengineering*. **58**, 463-477.

HANAI, T., HAYDON, D.A. AND TAYLOR, J. (1964) An investigation by electrical methods of lecithin-in-hydrocarbon films in aqueous solutions. *Proc. R. Soc. Ser. A*. **281**, 377-391.

HANAI, T., HAYDON, D.A. AND TAYLOR, J. (1965) Polar group orientation and the electrical properties of lecithin bimolecular leaflets. *J. Theoret. Biol*. **9**, 278-296.

HARRIS, C.M. AND KELL, D.B. (1983). The radio-frequency dielectric properties of yeast cells measured with a rapid, automated, frequency-domain dielectric spectrometer. *Bioelectrochemistry and Bioenergetics* **11**, 15-18.

HARRIS, C. M. AND KELL, D. B. (1985). The Estimation of Microbial Biomass. *Biosensors* **1**, 17-84.

HARRIS, C.M., TODD, R.W., BUNGARD, S.H., LOVITT, R.W., MORRIS, J.G. AND KELL, D.B. (1987). The dielectric permittivity of microbial suspensions at radio frequencies: a novel method for the estimation of microbial biomass. *Enzyme Microbial Technol*. **9**, 181-186.

IRIMAJIRI, A., HANAI, T. AND INOUYE, A. (1975). Evaluation of a conductometric method to determine the volume fraction of the suspensions of biomembrane-bounded particles. *Experientia* **31**, 1373-1374.

JONSCHER, A. K. (1983). *Dielectric relaxation in solids*. Chelsea Dielectrics Press, London for purely physical systems.

JUNKER, B H., REDDY, J., GBEWONYO, K. AND GREASHAM, R. (1994).On-line and in-situ monitoring technology for cell density measurement in microbial and animal cell cultures. *Bioprocess Engineering* **10**, 195-207.

KELL, D.B., SAMWORTH, C.M., TODD, R., BUNGARD, S.J. AND MORRIS, G. (1987). Real-time estimation of microbial biomass during fermentations, using a dielectric probe. *Studia biopysica* **119**, 153-156.

KELL, D.B., (1987a). The principles and potential of electrical admittance spectroscopy:an introduction In: Biosensors: *Fundamentals and Applications* eds. A.P.F.Turner, I. Karube and G.S. Wilson, pp 427-468. Oxford University Press.

KELL, D.B.(1987b). Forces, fluxes and the control of microbial growth and metabolism. The twelfth Fleming lecture. *J. Gen. Microbiol*. **133**,1651-1665.

KELL, D. B., KAPRELYANTS, A. S., WEICHART, D. H., HARWOOD, C. L. AND BARER, M. R. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues,. *Antonie van Leeuwenhoek* **73**, 169-187.

KELL, D.B., MARKX, G.H., DAVEY, C.L. AND TODD, R.W. (1990).Real-time monitoring of cellular biomass: methods and applications. *Trends in Analytical Chemistry* **9**, 190-194.

KELL, D.B. AND DAVEY, C.L. (1990). Conductimetric and impedimetric devices In: *Biosensors: A practical Approach* ed. A.E.G. Cass, Oxford University Press, Oxford.

KELL, D.B. AND WOODWARD, A. W. (1991). Non-faradic electrochemical sensors: principles and practice. *Analytical Proceedings* **28**, 378-379.

KELL, D.B. AND TODD, R.W. (1998). Dielectric estimation of microbial biomass using the Aber Instruments biomass monitor. *Trends Biotechnol*. **16**, 149-150.

KELL, D. B., KAPREELYANTS, A. S., WEICHART, D. H., HARWOOD, C. L. AND BARER, M. R. (1998). Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* **73**, 169-187

KONSTANTINOV, K., CHUPPA, S., SAJA, E., TSAI, Y., YOON, S. AND GOLINI, F. (1994). Real-time biomass-concentration monitoring in animal-cell cultures. *Trends Biotechnol*. **12**, 324-333.

KRAIRAK, S., YAMAMURA, K., NAKAJIMA, M., SHIMIZU, H. AND SHIOYA, S. (1999). On-line monitoring of fungal cell concentration by dielectric spectroscopy. Journal *of Biotechnology* **69**, 115-123.

KRONLOF, J. (1990). Estimation of living biomass of brewers yeast on the basis of capacitance measurement.[in Finnish]. *Eripainos Mallas ja Olut*. **5**, 140-149.

KRONLOF, J. (1991). Evaluation of a capacitance probe for determination of viable yeast biomass. *Proc. EBC*. pp233-240.

LAVER, D.R., SMITH, J.R. AND COSTER, H.G.L. (1984). The thickness of the hydrophobic and polar regions of glycerol monooleate bilayers determined from the frequency-dependence of the bilayer capacitance. *Biochim. Biophys. Acta* **772**, 1-9.

LAWRENCE, D. (1992). The evolving science of brewing microbiology. *European Microbiology*. **1**, 34-39.

LEATHERBARROW, R. J. (1992). *GraFit Version 3.0*, Erithacus Software, Staines.

LOVITT, R. W., WALTER, R. P., MORRIS, J. G. AND KELL, D. B. (1983). Conductimetric assessment of the biomass content of immobilised (gel entrapped) microorganisms. *Applied Microbiology and Biotechnology* **23**, 168-173.

MACA, H.W., BARNEY, M., GOETZKE, G., DANIELS, D. AND RYDER, D. (1994). The use of radio frequency capacitance for the measurement of yeast viable biomass and its use in the automatic pitching of fermentations. *MBAA Technical Quarterly* **31**, 146-148.

MARKX, G.H. AND KELL, D.B. (1990). Dielectric spectroscopy as a tool for the measurement of the formation of biofilms and their removal by electrolytic cleaning pulses and biocides. *Biofouling* **2**, 211-227.

MARKX, G.H., DAVEY, C.L. AND KELL, D.B. (1991a). To what extent is the magnitude of the Cole-Cole α of the β-dispersion of cell suspensions explicable in terms of the cell size distribution? *Bioelectrochemistry and Bioenergetics* **25**, 195-211.

MARKX, G.H., DAVEY, C.L. AND KELL, D.B. (1991b). The permittistat: a novel type of turbidostat. *Journal of General Microbiology* **137**, 735-743.

MARKX, G.H., DAVEY, C.L., KELL, D.B. AND MORRIS, P. (1991c). The dielectric permittivity at radio frequencies and the Bruggeman probe: novel techniques for the on-line determination of biomass concentrations in plant cell cultures. *J. Biotechnol*. **20**, 279-290.

MARKX, G.H., ten HOOPEN, H.J.G., MEIJER, J.J. AND VINKE, K.L. (1991d). Dielectric spectroscopy as a novel and convenient tool for the study of the shear sensitivity of plant cells in suspension culture. *Biotechnology* **19**, 145-147.

MARKX, G. AND DAVEY, C.L. (1999). The dielectric properties of biological cells at radio frequencies: Applications in biotechnology. *Enz Microbial Tech*. (In press).

MARQUARDT, D.T. (1963). An algorithm for least squares estimation of non-linear parameters. *J. Soc. Ind. Appl. Math*. **11**, 431-441.

MATANGUIHAN, R.M., KONSTANTINOV, K.B. AND YOSHIDA, T. (1994). Dielectric measurement to monitor the growth and the physiological states of biological cells. *Bioprocess Engineering*. **11**, 213-222.

MISHIMA, K., MIMURA, A., TAKAHARA, Y., ASAMI, K. AND HANAI, T. (1991a) On-line monitoring of cell concentrations by dielectric measurements. *J. Ferment.Bioeng*. **72**, 291-295.

MISHIMA, K., MIMURA, A. AND TAKAHARA, Y. (1991b). On-line monitoring of cell concentrations during yeast cultivation by dielectric measurements. *J. Ferment.Bioeng*. **72**, 296-299.

MOSTELLER, F. AND TUKEY, J.W. (1977). *Data Analysis and Regression*, addison wesley, reading, ma.

NICHOLSON, D.J., KELL, D.B. AND DAVEY, C.L. (1996) Deconvolution of the dielectric spectra of microbial cell suspensions using multivariate calibration and artificial neural networks. *Bioelectrochemistry and Bioenergetics* **39**, 185-193.

NOLL, T. (1995). Dielectrical measurement of immobilised mammalian cells. *Presented on 11th October, at Biotechnia, Hannover.*

NOLL, T., BISELLI, M. AND WANDREY, C. (1996). On-line biomass monitoring of immobilised hybridoma cells by dielectrical measurements. [*poster abstract from,*

Symposium on Animal Cell Technology; from Vaccines to Genetic Medicine (14th meeting of E.S.A.C.T.), Algarve, Portugal.]. 20-24 May.

NOLL, T. AND BISELLI, M. (1998). Dielectric spectroscopy in the cultivation of suspended and immobilised hybridoma cells. *Journal of Biotechnology* **63**, 187-198.

PATEMAN, B.R. (1997). Improving yeast handling by in line monitoring of viability. *Brewing & Distilling International* **28**, 57-59.

PEÑALOZA, W., DAVEY, C.L., HEDGER, J.N. AND KELL, D.B. (1991). Stimulation by potassium ions of the growth of Rhizopus oligosporus during liquid- and solid-substrate fermentations. *World Journal of Microbiology and Biotechnology* **7**, 260-268.

PEÑALOZA, W., DAVEY, C.L., HEDGER, J.N. AND KELL, D.B. (1992). Physiological studies on the solid-state quinoa tempe fermentation, using on-line measurements of fungal biomass production. *J. Sci. Food. Agric*. **59**, 227-235.

PETHIG, R. (1979). *Dielectric and Electronic Properties of Biological Materials* .Wiley, Chichester.

PETHIG, R. AND KELL, D.B. (1987). The passive electrical properties of biological systems: their significance in physiology, biophysics and biotechnology, *Phys. Med. Biol* **32**, 933-970.

PONS, M. N. (1991). Bioprocess monitoring and control. Hanser, Munich.

PRESS, W .H., FLANNERY, B. P., TEUKOLSKY, S.A. AND VETTERLING, W.T. (1990). Numerical Recipies: *The Art of Scientific Computing (FORTRAN Version)*. Cambridge University Press, Cambridge.

RIGAUD, B., MORUCCI, J. P. AND CHAUVEAU, N. (1996). Bioelectrical impedance techniques in medicine .1. Bioimpedance measurement - Second section: Impedance spectrometry. *Critical Reviews in Biomedical Engineering* **24**, 257-351.

SALTER, G.J., KELL, D.B., ASH, L.A., ADAMS, J.M., BROWN, A.J. AND JAMES, A.J.(1990). Hydrodynamic deposition: a novel method of cell immobilisation. *Enzyme Microb. Technol*. **12**, 419-430.

SALTER, G.J. AND KELL, D.B. (1992). Rapid determination using dielectric spectroscopy, of the toxicity of organic solvents to intact cells. In: *Fundamentals of Biocatalysis in Non-conventional Media*, pp 291-297. Elsevier, Amsterdam..

SALTER, G.J. AND KELL, D.B. (1995). Solvent selection for whole cell biotransformations in organic media. *CRC Crit. Rev. Biotechnol*. **15**, 139-177.

SARRA, M., ISON, A. P. AND LILLY, M.D. (1996). The relationships between biomass concentrations, determined by a capacitance-based probe, rheology and morphology of Saccharopolyspora erythraea cultures. *J. Biotechnol*. **51**, 157-165.

SCHWAN, H.P.(1957). Electrical properties of tissue and cell suspensions. *Adv. Biol. Med. Phys*. **5**, 147-209.

SCHWAN, H. P. AND MOROWITZ, H.J. (1962). Electrical properties of the membranes of the pleuropneumonia-like organism A 5969. *Biophys. J.* **2**, 395-340.

SCHWAN, H.P.(1963) Determination of biological impedances. In: *Physical Techniques in Biological Research,* Vol. V1B. 323-407, Academic press, New York.

SCHWAN, H.P. (1968). Electrode polarisation impedance and measurements in biological materials. *Ann NY Acad Sci*. **148**, 191-290.

SCHWAN, H.P AND FERRIS, C.D. (1968). Four terminal null techniques for impedance measurement with high resolution. *Rev. Sci. Instrum*. **39**, 481-485.

SCHWAN, H.P., TAKASHIMA, S., MIYAMOTO, V .K. AND STOCKENIUS, W. (1970). Electrical properties of phospholipid vesicles. *Biophys. J*. **10**, 1102-1119.

SEEMAN, P. (1972). The membrane action of anaesthetics and tranquilisers. *Pharmicol. Rev.* **24**, 583-655.

SIANO, S.A. (1996). Biomass measurement by inductive permittivity. *Biotechnol. Bioeng*. **55**, 289-304.

SIEMS, G., (1997) First experience of using the yeast monitor. *Brauwelt International* **11**, 132-137.

SONNLEITNER, B., LOCHER, G. AND FIECHTER, A. (1992) Minireview: Biomass determination. *Journal of Biotechnology* **25**, 5-22.

STOICHEVA, N.G., DAVEY, C.L., MARKX, G.H. AND KELL D.B. (1989). Dielectric spectroscopy: a rapid method for the determination of solvent biocompatability during biotransformations. *Biocatalysis* **2**, 245-255.

TANFORD, C. (1980). *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*. New York : John Wiley.

TAKASHIMA, S., ASAMI, K. AND TAKHASHI, Y. (1988). Frequency domain studies of impedance characteristics of biological cells using micropippette technique. 1. Erythrocyte. *Biophys. J.* **54**, 995-1000.

WOODWARD, A.M. AND KELL, D.B. (1990). On the nonlinear dielectric properties of biological systems. Saccharomyces cerevisiae. *Bioelectrochem. Bioenerg*., **24**, 83-100.

WOODWARD, A. M., JONES, A., ZHANG, X., ROWLAND, J. AND KELL, D. B. (1996). Rapid and non-invasive quantification of metabolic substrates in biological cell suspensions using nonlinear dielectric spectroscopy with multivariate calibration and artificial neural networks. Principles and applications. *Bioelectrochem. Bioenerg*. **40**, 99-132.

YELAMOS, D., CASAS, O., BRAGOS, R. AND ROSELL, J. (1998). Improvement of a front-end for bioimpedance spectroscopy. *Proceedings of the X. International conference on bio electrical bio-impedance, Barcelona, Spain*. 383-386.

YARDLEY, J.E., TODD, R., NICHOLSON, D.J., BARRETT, J., KELL, D.B. AND DAVEY, C.L. (1999). Correction of the influence of baseline artefacts and electrode polarisation on dielectric spectra. (submitted).

ZEISER, A., BEDARD, C., VOYER, R., JARDIN, B., TOM, R., KARMEN, A.A. AND KARMEN, T. (1999). On-line monitoring of the progress of infection in Sf-9 insect cell cultures using relative permittivity measurements. *Biotech. Bioeng*. **63**, 122-126.