Appl Microbiol Biotechnol (1986) 23:168–173

Applied and Microbiology Biotechnology

Conductimetric assessment of the biomass content in suspensions of immobilised (gel-entrapped) microorganisms

Robert W. Lovitt, Robert P. Walter, J. Gareth Morris, and Douglas B. Kell

Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK

Summary. The problem of obtaining a rapid estimate of the microbial content of an immobilised cell suspension is addressed. The "low-frequency" conductivity of free-living cell suspensions of *Clostridium pasteurianum* is lower than that of the medium in which they are suspended, by an amount conforming to the Bruggeman relation. The conductivity of the cell wall makes a negligible contribution to the measured conductivity under the conditions used. Calcium alginate beads (lacking microbial cells) lower the conductivity of a solution with which they have been equilibrated by an extent which is a function of the concentration of alginate gel used in forming the beads. When this is taken into account, the ratio of the conductivity of a suspension of gel-immobilised cells to that of the suspending medium can be used to give a rapid and convenient assessment of the amount of microbial biomass present.

Introduction

Despite the well-known advantages of cell immobilisation, there is a great need, in particular, for methods which would allow one rapidly and reliably to assess the biomass content of immobilised cell suspensions.

Now, as recently discussed elsewhere (Blake-Coleman et al. 1984; Clarke et al. 1985; Harris and Kell 1985a), the only really appropriate methods with which one might hope to assess the biomass content of a fermentor or bioreactor in real time must exploit or rely upon some *physical* characteristic which adequately distinguishes microorganisms from aqueous solutions, gas bubbles and particulate material. Further, it is widely recognised that microbial viability depends, not least for bioenergetic reasons (Konings and Veldkamp 1980), upon the cell's possession of a relatively ion-impermeable cytoplasmic or plasma membrane. Thus, as has been known for many years from studies on inhomogeneous media generally (Landauer 1978), the (low-frequency) conductivity of a suspension of "viable" biomembrane-bounded vesicles is, neglecting the cell wall conductivity (see later), less than that of the medium in which the cells are suspended by an amount that is a monotonic function of the volume (fraction) of the suspended phase (e.g. Harris and Kell 1983; Clarke et al. 1985). Indeed, a version of this principle forms the basis of electronic particle counters and sizers such as the Coulter Counter™ (e.g., Grover et al. 1969, 1982), in which individual cells are forced hydrodynamically to pass through an orifice across which an electrical field is maintained. Under most circumstances, conditions are, or may be, so arranged that the measured change in electrical resistance of the orifice, that is occasioned by the passage of the particle of interest, is proportional to the particle volume (Kubitschek 1969), although the volume-dependence of the measured signal is not absolutely independent of the particle shape or orientation (Hurley 1970; Grover et al. 1982; Bator et al. 1984).

In bulk, isotropic suspensions, the (low-frequency) conductivity σ of a suspension (volume fraction $P<0.5$) of non-conducting spheroids suspended in a medium of conductivity σ_0 is given (Fricke 1924; Fricke and Morse 1925) by:

$$P = \frac{x(\sigma_0 - \sigma)}{\sigma + x\sigma_0}, \qquad (1)$$

Offprint requests to: D. B. Kell

where x is a so-called form (shape) factor. For spheres, $x=2$, and equation (1) in general reduces to:

$$\frac{\sigma}{\sigma_0}=\frac{1-P}{1+(P/x)}. \quad (2)$$

(Note that this equation was misprinted, although correctly plotted, in Harris and Kell 1983). For ellipsoidal particles, the form factor more closely approximates 1.5. A plot of the left hand side of equation (2), the ratio R of the low-frequency suspension conductivity to that of the suspending medium, versus the volume fraction P, gives a locus which (a) is only very weakly dependent upon the value of x, and (b) for values of P less than approx. 0.3 is effectively indistinguishable (Harris and Kell 1983) from a straight line and from that of the Bruggeman approximation (equation (3)) which, it has been claimed (Jrimajiri et al. 1975), is more accurate for non-spherical particles:

$$\frac{\sigma}{\sigma_0}=R=(1-P)^{3/2}. \quad (3)$$

However, before we might wish to exploit measurements of σ and σ_0 to obtain the values of P, and hence the biomass, in immobilised cell suspensions by means of equation (3), four possible problems need to be discussed. The first problem relates to the fact that cell membranes are not *completely* non-conducting. However, the available evidence indicates overwhelmingly (e.g., Grover et al. 1982) that only in the very rarest cases (Ginzburg et al. 1978; Zmiri et al. 1984) does the electrical size of a microorganism, as measured at low field strength (Zimmermann et al. 1973), appear artefactually low as a result of significant transcellular ion flow. The full equations, in this case, are given by Schwan and Foster (1980).

The second problem relates to the fact that the so-called suspension equations, as embodied, for example, in equations (1) to (3), strictly apply only to suspensions with volume fractions less than 0.2, and whilst this condition will normally hold for the majority of cases of gel-immobilised microorganisms considered in toto, it may not be true *within* the beads. However, since (a) no exact treatment exists for such cases, (b) the orientation and organisation of cell suspensions within gel beads remain matters of uncertainty both in the general case and in any specific case of interest, and (c) the potential errors introduced by the use of the simpler "low volume fraction" equation (3) are small (and see later), we feel justified for the present purposes, and for reasons of simplicity, in ignoring any second-order effects which might be manifested at very high volume fractions.

The last two potential problems with the use of conductimetry in assessing the biomass content of immobilised cell suspensions relate to the fact that both the microbial cell wall and the gel-forming material (a) may themselves make a significant contribution to the conductivity of the suspension, and (b) will serve to decrease the conductivity of the suspension by virtue of the fact that they exclude the bulk, conducting extracellular fluid. Investigations designed to assess the significance of these contributions are discussed in the body of this article.

Experimental

Organism. Clostridium pasteurianum ATCC 6013 was maintained in reinforced clostridial medium (Difco), and grown in batch culture at 37° C under a N_2 atmosphere in TYE medium containing 3% (w/v) glucose. TYE medium contains 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% KH_2PO_4 pH 6.9. Cell suspensions were harvested in mid-exponential phase, and washed and resuspended anaerobically in KCl solutions of the molarity indicated in the Figure legends.

Immobilisation of non-growing cells in alginate beads. Harvested cells in 0.1 M KCl were mixed with an equal volume of 5% (w/v) sodium alginate. This mixture was drawn (under vacuum), through an array of 23-gauge needles embedded in the bung of a Buchner flask, into a solution of 2% (w/v) $CaCl_2 \cdot 2H_2O$ which was contained therein. The beads so formed were allowed to cure for 1 h. The size distribution of freshly prepared beads was approximately normal; their diameter has a mean value and standard deviation of 2.7 and 0.26 mm respectively. Some shrinkage of beads was noted upon prolonged storage. Beads were also prepared with pure sodium alginate solutions (lacking cells) of various strengths using the above method. Bead suspensions were then equilibrated with the desired solutions.

Conductimetric measurements were carried out using a model ECC 251 conductivity meter (EDT Research, 14 Trading Estate Road, London NW10 7LU) together with a conductivity cell of conventional design incorporating parallel plate Pt black electrodes (cell constant 1.58 cm^{-1}). The operating frequency of this instrument, as measured with a Solarton 1200 Signal Processor, was 1423 Hz, and the applied signal had a value of 86 mV p-p. To ensure good temperature control, measurements were carried out in a constant temperature room (37° C).

Media for the fermentor. The media used in the fermentor experiment were prepared in 20 l carboys and contained (g/l): NH_4Cl 4; $MgCl_2$ 0.5; $CaCl_2 \cdot 2H_2O$ 3.35; MES 0.2 g. Vitamin solution 1 ml/l (Wolin et al. 1963) and trace element solution of George et al. 1983, 0.25 ml/l were also present. Glucose, 10 g (50 ml of 200 g/l) and KH_2PO_4 0.26 (in the glucose solu-

tion) per l were added separately after sterilisation. The starter batch cultures used as the bead inoculum were supplemented with 5 g $KHCO_3$ to provide additional buffering required for batch culture and the $CaCl_2$ was omitted. Phosphate was omitted for "low-phosphate" media.

The bead fermentor

The cells in the beads were then grown in a gas lift fermentor which was constructed in our laboratory. It consisted of a pyrex glass tube (internal diameter 43 mm, 2 mm walls, length 260 mm) fitted with rubber bungs at the base and top to seal the system. The top bung was fitted with a pH probe (Russell) which in turn was connected to a pH control apparatus (LH Engineering); the pH was maintained at 6 by the controlled addition of 2 M KOH via an 18 gauge needle through the top bung. The top bung also contained a sample port and gas outlet which was connected to the gravity weir (outlined further below). Medium was pumped from a reservoir using a peristaltic pump (Watson Marlow) through a grow-back trap (Evans et al. 1970) and entered the fermentation vessel via a 17-gauge syringe needle.

The base bung was fitted with a heater and temperature sensor (LH Engineering) and the temperature was maintained at 37° C. Gas (oxygen-free N_2) entered the system via a 17-gauge 3-inch needle which passed through the centre of the base bung (flow rate approximately 300 ml/min). The gas passed through the needle into a 1 cm ID glass tube of 180 mm length which was attached to the temperature probe and suspended 1 cm from the base. A circulating flow up the central tube and down the surrounding annular space was thus generated. Liquid left the system via a port in the base bung of the fermentor, which was fitted with a stainless steel mesh (1 mm) to exclude the passage of the beads. Liquid levels in the system were controlled by a gravity weir external to the main part of the fermentor, which was constructed using a plastic T-joint connected via butyl rubber tubing to the liquid outlet in the base and the gas outlet in the top of the fermentor. Liquid levels in the fermentor were then controlled by the movement of the T-joint relative to the fermentor. The total volume of the fermentor was 350 ml; initially the beads occupied a packed volume equivalent to approximately 30% of this (110 ml); with subsequent sampling this decreased to approximately 20% of the total volume (70 ml).

Preparation of beads for continuous fermentation

Beads for the fermentor were prepared from 5% (w/v) alginate, which was sterilised at 115° C for 10 min. After cooling, equal volumes of alginate solution and exponentially growing cultures (approximate $OD_{680} = 1.2$) were mixed. The beads were produced as described above, except that the $CaCl_2$ gelling agent contained 0.5% w/v cysteine and 0.02% resazurine. The beads were conditioned for 1 h and were then transferred to the anaerobic medium in the fermentor via a small funnel inserted into the sample port. Sampling was effected via the sampling port in the top of the fermentor. Beads (400–500) together with medium were drawn up a 5 mm tube into a sampling bottle. The beads were then used for the conductivity measurements, blot dried with tissue paper and several 1 g samples were analysed further for protein content.

Protein assays

Protein assays were performed by the method of Lowry et al. (1951). The protein content of the entrapped cells in alginate was estimated after the beads were dissolved by the addition of 2 ml of 0.5 M KH_2PO_4 and 2 ml NaOH. This treatment dissolves beads in 2–3 h, after which the alginate/cell mixture was diluted to 25 ml and the protein was then extracted from a 5 ml sample by boiling in 0.5 M NaOH, according to the method of Herbert et al. (1971).

Chemicals and biochemicals were from sources described (Clarke et al. 1982): sodium alginate (cat. no. A2158; 2% solution has a viscosity of 0.25 Pa s) was from the Sigma Chemical Company, Poole, Dorset. Water was singly distilled in an all-glass apparatus.

Results and discussion

Figure 1 shows the ratio of the conductivity of a number of suspensions of free-living *C. pasteurianum* to that of the medium in which they were suspended under two different conditions. In Figure 1 it is shown that the conductivity ratio *R* was monotonically (and linearly) related to the cell (dry weight) concentration, and that, at a given osmotic potential, it was independent of the bulk ionic conductivity. This finding shows that the conductivity of the cell wall and its double layer makes a negligible contribution to the total conductivity at this frequency, in confirmation of other studies of both Gram-positive and Gram-negative organisms (Harris et al. 1984; Harris and

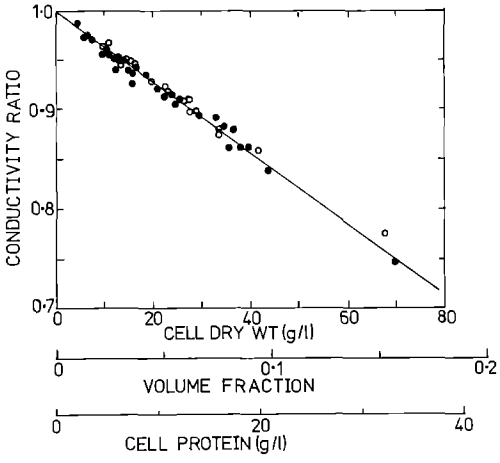
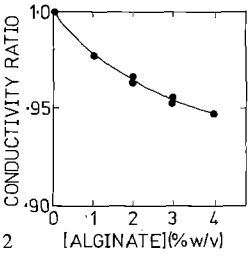


Fig. 1. The conductivity ratio of suspensions of free-living *C. pasteurianum*. The conductivity ratio R represents the conductivity (at 1.42 kHz) of the suspension divided by that of the supernatant obtained following centrifugation of the suspension. Cells were suspended either in 0.15 M KCl (○) or in 0.1 M KCl containing 0.1 M lactose (●). Lactose is not taken up by this organism. The conductivity ratio and protein content of the suspensions were measured as described in the experimental section. The volume fraction *P* was calculated by fitting the line to equation 3. The specific enclosed volume of the cells under these conditions was therefore 2.25 ml/g dry weight

Kell 1985b). We have given the biomass (Fig. 1) in terms of dry weight, protein and enclosed volume fraction. Since cell size and macromolecular composition can of course vary independently of cell number, viability and volume fraction, we regard the last expression, especially for the present purposes, as an appropriate description of the biomass content of a cell suspension (Harris and Kell 1985a).

Figure 2 shows the conductivity ratio of suspensions of calcium alginate beads (lacking microbial cells) made with different concentrations of sodium alginate in the gel-forming solution; the gel itself also had the effect of decreasing the conductivity of the bead suspension below that of the supernatant. Measurements of the complex permittivity of these beads, using the dielectric spectrometer described previously (Harris and Kell 1983), showed that they possess a measurable dispersion centred around 1 MHz or so, but that the conductivity was frequency-independent below 5 kHz (data not shown). This result indicates, as with the microbial cells above, that the *bound* ions and counterions in the gel make a negligible contribution to the DC or "low frequency" (1.4 kHz) conductivity of the system. Thus, knowing the percentage of gel in a gel-forming mixture containing immobilised microorganisms, one may, without significant loss of accuracy, merely subtract the contribution made by the gel to the conductivity ratio measured in an immobilised cell suspension.



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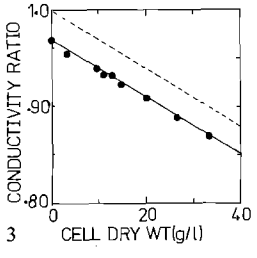

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Fig. 2. The effect of alginate concentration on the conductivity ratio of gel beads lacking cells. Gel beads were formed as described in the experimental section, and suspended and equilibrated in 0.1 M KCl. The conductivity ratio refers to the conductivity of a closely packed bead suspension relative to that of the supernatant

Fig. 3. The conductivity ratio of alginate beads containing known quantities of non-growing cell suspensions of *C. pasteurianum*. Cells were immobilised as described in the experimental section, and the beads resuspended and equilibrated in 0.1 M KCl. The final gel concentration was 2.5% w/v, and the dotted line gives the expected conductivity ratio when account is taken of the contribution of the gel (Fig. 2) to the conductivity ratio

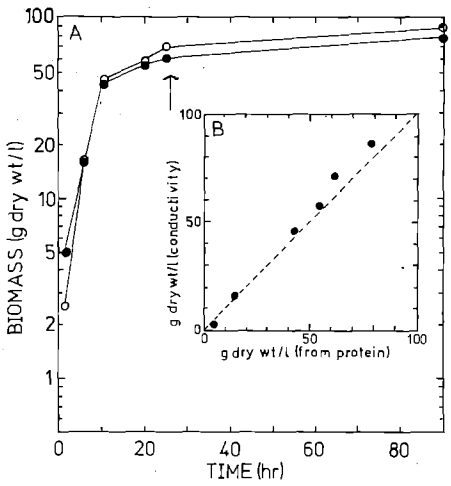

Fig. 4A, B. Conductimetric assessment of the growth of immobilised *C. pasteurianum* during a continuous fermentation. Cells were immobilised as described in the experimental section. The medium given therein was passed through the fermentor at a dilution rate of 2.07 h^{-1}. At the *arrow*, the medium was changed to the "low-phosphate" medium given in the experimental section, and the dilution rate changed to 0.69 h^{-1}. **A** Growth as assessed conductimetrically (○) or by a protein assay on a sample of beads (●). **B** (Inset) A comparison of cell dry weights, using the data in A, as estimated conductimetrically or from protein assays using the conversion factor (Fig. 1) 1 g dry weight cells = 480 mg protein

Figure 3 shows the conductivity ratio of Ca-alginate beads containing known quantities of immobilised *C. pasteurianum* cells. It is evident (Fig. 3) that, when account is taken of the contribution of the gel to the decrease of the conductivity ratio below 1, the conductivity ratio can provide an accurate assessment of the biomass present in the gel.

The above experiment was performed with non-growing cells, and it was of interest to use the method to assess the growth of cells during an immobilised-cell fermentation. Figure 4 shows the time-dependent increase in gel-immobilised biomass during a continuous fermentation as assessed (a) from the conductivity ratio and (b) as determined by a protein assay and assuming (Fig. 1) that the cells contain 48% of their dry weight as protein. The inset to Fig. 4 serves to compare the two methods, wherein it may be observed that the rapid and convenient conductimetric method gives a very fair estimation of the microbial biomass present.

Conclusion

We have here presented a novel method for the estimation of microbial (or other) biomass. Com-

pared with other methods, such as protein extraction and colorimetry (Freeman et al. 1982), the method is obviously rapid and convenient. Provided, of course, that beads and supernatant are measured at the same temperature, the only significant potential source of error in this method lies in mass transfer effects (Radovich 1985) leading to a significant lack of equilibration between the 'bulk' medium in the gels and the bulk supernatant.

The saccharolytic metabolism of *C. pasteurianum* produces copious quantities of acids (acetate and butyrate) and gas (H_2 and CO_2) (Thauer et al. 1977), so that a significant lack of equilibration of their activities in the gels and the bulk supernatant will lead respectively to an underestimation and an overestimation of the immobilised microbial biomass by means of the present conductimetric method. Whilst conductimetry using needle-type electrodes which pierce gas bubbles has been used to estimate the size and velocity distribution of such bubbles (Serizawa et al. 1975; Buchholz and Schügerl 1979; Sekoguchi et al. 1984; Kell 1986), the present arrangement does not seem excessively to be affected by mass-transfer considerations, even under rather stringent conditions (Fig. 4).

We conclude that the conductimetric method described herein provides a rapid and convenient means of assessing the biomass content of immobilised microbial cells. It might also find use in the measurement of the growth of filamentous organisms.

Acknowledgements. We thank the Biotechnology Directorate of the Science and Engineering Research Council, UK, for financial support, Anthony Pugh for photographic assistance and Jane Watts for typing the manuscript.

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Received June 14, 1985/Revised August 26, 1985