THE USE OF CAPACITANCE MEASUREMENT IN FERMENTATION MONITORING

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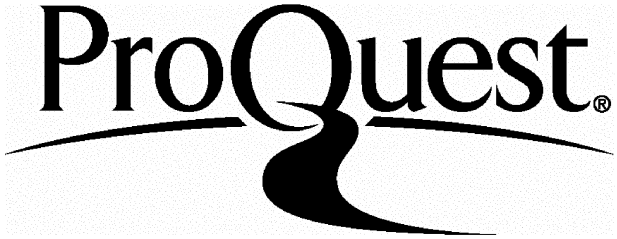

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ABSTRACT

On-line biomass measurement of fermentations has the potential to facilitate the use of real-time control strategies that can improve fermentation performance. This work investigates the utility of capacitance measurement as a microbial biomass measurement technique, used on-line in fermentation vessels and off-line. The capacitance of a cell suspension increases with the introduction and proliferation of microbial cells by an amount which is directly proportional to the volume bound within the cell membranes of the cells. This means that cell size and viability has a profound effect on the capacitance measurements.

Four representative organisms were used in the experimental work: *Saccharomyces cerevisiae; Pseudomonas putida; Penicillium chrysogenum* and *Streptomyces sp.* Experiments were carried out monitoring on-line changes in capacitance during a variety of fermentations of these organisms and correlating these with conventional biomass measurements. The variety of scales, modes of fermentation and media composition were used to examine the effects of these on capacitance and its correlations with other measurements. Other experiments were carried out off-line looking at changes in capacitance resulting from homogenisation of cell suspensions.

In all fermentations monitored on-line, capacitance was found to increase with increasing biomass. Correlations were found with most others measures of growth, particularly metabolic measures such as Carbon Dioxide Evolution rate. Correlations were also found in the off-line experiments where capacitance accurately detected decreases in cell viability due to homogenisation. Specific capacitance measurements were calculated from the data and found to be consistent for the experiments carried out in this work and very similar to those found by other researchers using similar organisms. In addition, the values found in this work were compared with theoretical values derived from the equations describing capacitance and its relationship to cell size. The values from this work were found to be comparable to the theoretical equivalents calculated. This work shows that on-line capacitance measurement is a good tool for the on-line measurement of biomass and for the off-line measurement of cell viability.

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1. INTRODUCTION

1.1 Fermentation processes

Fermentation processes are used in the production of a wide variety of materials. These processes range from the ancient arts of brewing and wine making to the modern production of antibiotics and recombinant therapeutic proteins. These fermentations vary considerably in complexity of the process, and thus also, in the level of control required. This then generates a need for accurate and timely monitoring of the relevant variables of the process to facilitate this control.

A fermentation can be regarded as a complex system of interactions between the micro-organisms being grown and their environment, the fermenter fluid. In order that the environment may be held in the state that best aids microbial growth and product formation, detailed knowledge of both the micro-organism and its environment is desirable.

The methods employed for the monitoring and control of fermentation processes are typically those which were originally developed for chemical processes. Fermentation fluid or broth is more complex than the solutions to be found in chemical processes as it typically comprises at least three phases: organic and inorganic media components dissolved in liquid; the gases present both in the liquid and in the headspace of the fermenter and the cells.

This introduction is divided into two sections. The first section concerns fermentation monitoring and control as broad concepts and will by necessity be rather limited in the examination of the detail of this large area of research. In order to obtain the knowledge required to assess the state of the fermentation broth, an extensive range of analytical methods need to be employed, and these will reviewed in this section.

The variable which imparts most of the added complexity of fermentation processes, the cells (or biomass), is the variable of interest in this work. The second section will deal with measuring techniques related to biomass monitoring in fermentation in general. The subsequent chapter will then concentrate specifically on the use of capacitance measurement to monitor fermentation biomass.

1.2 Fermentation monitoring

This section will concentrate on what can be termed the theory of fermentation monitoring and will deal specifically with the monitoring of the fermentation environment.

1.2.1 Off-line and on-line monitoring

The monitoring of a fermentation can be carried out both on-line and off-line. On-line means that the parameter of interest is monitored by a device which is either physically in the fermenter or is connected to the fermenter by a sample loop. These are normally hard-plumbed into the fermenter. While such loops solve some of the problems of on-line monitoring, they also have their own problems. These include representivity of the sample and maintenance of vessel sterility, especially when the sample loop returns the sampled fluid to the fermenter after testing.
Off-line means that the fermenter fluid is sampled and the removed sample is then tested. It is preferable to use an on-line method as off-line monitoring can be problematic for the following reasons:-

1. There are often difficulties in ensuring that any sample taken is representative of the state of the fermenter fluid, the statistics of sampling error are well documented but in fermentation problems such as incorrect placing of the sampling point, partial blocking of the sample port and errors inherent in sample processing methods also have a significant effect in reducing the accuracy of the results obtained.

2. Most fermenters are run under monoseptic conditions to ensure that only the organism of choice is growing in the fermenter fluid. Care must be taken when removing a sample that good aseptic technique is used to avoid contamination. While good design of the sampling system can minimise the possibility of contamination, it is an ever present risk due to sampling.

3. To maintain a sampling rate similar to that possible with most on-line devices would be impossible not only in terms of time but also in terms of loss of volume from the vessel. Even using quite moderate sampling rates, the possibility of removing too much volume from the vessel must always be borne in mind, even in fed-batch or continuous culture.

4. The time needed for sampling and sample processing can vary from minutes to hours. This can lead to problems, especially if the data from the sampling are to be used to attempt to control the fermentation as the values are consistently as old as the time taken to yield the result.

5. A final problem is one of manpower. There are very few automatic sampling systems available, which means that a person must be present to take the sample, and often process it as well. As most fermentations last upwards of 24 hours this means that either someone is present at night to take and process samples or no data is gathered for the period outside normal working hours. While it is possible to ensure overnight sampling, especially on production plants which are manned continuously, many research facilities operate without inspection and supervision overnight.

On-line monitoring of parameters is much more desirable. Harris and Kell (1985) set out criteria for an on-line biomass probe, but they can equally be applied to any type of on-line probe. These criteria are:-

1. Continuous, real-time measurement.
2. Sensitive to significant changes.
3. Probe must be biologically inert.
4. Non-destructive assay with no added reagent.
5. Compatibility, i.e. many probes using the same equipment.
6 Good lifetime.
7. Low cost.
8. Can be used in optically opaque and turbid solutions.
9. Cleanable *in situ.*
10. Sterilisable *in situ.*

Using these criteria, it can be seen that the demanding environment of the fermenter fluid, coupled with the requirement for process information from that environment has required the development of robust probes and sensors for on-line fermentation monitoring. In the next section, the parameters commonly monitored on-line and the means to do this are reviewed.

1.2.2 Parameters commonly monitored on-line

The parameters commonly monitored on-line can broadly be divided into physical and chemo-biological variables.

1.2.2.1 Physical variables

Temperature is easily monitored on-line by a variety of methods including: thermocouples; conventional mercury thermometers clad in stainless steel and the thermistor, a semi-conductor based device which relates changing resistance to temperature. The monitoring and control of temperature is critical in fermentation processes as all microorganisms have optimal temperatures for growth. The growth of micro-organisms can be considered as a series of chemical reactions and the rate at which these reactions proceed is a function of temperature.

In addition to being a requirement for optimal growth, fermenter fluid temperature can also be manipulated as part of the bioprocess. An example of this is the use of temperature inducible promoters to control protein expression in recombinant organisms (Helmuth *et al.*,1994).

Vessel pressure is commonly monitored on-line in fermenters. This not only to ensure that the vessel is operated safely, but also, the use of overpressure as an aid to sterility maintenance is common. Another common use of overpressure is to change the partial pressure of oxygen and CO_2 in the fermenter to improve gas transfer in fermentations which have a high oxygen demand. Pressure measurement devices commonly used are pressure transducers based on strain gauges and electronic manometers.

Two related variables, agitation speed and power input determination are often measured on-line in fermentation. Agitation speed is an important variable as it is

crucial in ensuring good mixing and maintaining dissolved oxygen at a level which is required by the organisms for growth. Indeed, it is very common for the agitation speed to be controlled as a function of dissolved oxygen concentration. The agitation speed also needs to monitored and controlled as high agitation rates can cause conditions of high shear in the fermenter fluid which can damage cells, especially the mycelia of filamentous organisms and mammalian cells. Power input determination is important at large scale for both economic considerations, and when looking at oxygen transfer, fluid viscosity and shear effects in large fermentation systems.

Another variable related to dissolved oxygen which is measured on-line is airflow into the vessel. This can be measured simply by a rotameter on the air supply. More accurate flow rates can be determined by mass flow meters to give the accuracy required for calculating fermentation rates such as carbon dioxide evolution rate (CER). Due to the problems of making measurements of a gas containing large amounts of water vapour, exit gas flow rates are normally determined by measuring the flow of an inert gas such as argon or nitrogen rather than measuring exit airflow directly.

For calculation of the volumetric fermentation rates of a fermentation it is often necessary to monitor the liquid level in the fermenter. This can be done using simple conductivity probes or by more sophisticated methods such as ultrasound. Conductivity probes are also often used as foam probes to control excessive foaming by triggering the addition of antifoams when the foam reaches the level of the probe inserted through the head plate of the vessel.

Another method of monitoring fermenter volume is to place the vessel on a load cell so the weight of the fermenter contents can be monitored. In addition to this, load cells can be used to monitor the weight of addition vessel such as those containing nutrient feeds or pH control solutions, and thus calculate (and control) the amount being added to the vessel.

1.2.2.2 Chemical and biological variables

pH is a crucial variable in fermentation processes. All micro-organisms have a relatively narrow pH optima for growth, and often cannot withstand changes in environmental pH of more than 2 pH units. Most fermentation media are designed

to have a good buffering capacity, usually in the form of phosphates or insoluble carbonates. pH is the negative log of the H^+ ion concentration and is typically measured in fermentation vessel by means of a combination probe, comprising of a measuring electrode and reference electrode, which operates as a voltage generating electrochemical cell.

pH probes are prone to fouling by protein in the fermenter medium therefore off-line measurement of the pH of samples is often necessary to check on the on-line probe performance. As pH is a variable which profoundly effects the metabolic state of the micro-organisms, it has also been used as means of inducing expression in recombinant protein production (Tolentino *et al.*,1992).

As mentioned above, dissolved oxygen concentration is another important variable to be measured on-line in fermentation vessels. The majority of biotechnological fermentation processes are aerobic and thus have a requirement for molecular oxygen to be present in the fermenter fluid. Aerobic growth of most industrial micro-organisms gives the most efficient utilisation of substrate. Conditions of low dissolved oxygen generally result in changes in metabolism, *e.g. E.coli* produce more acetate when oxygen limited. It is a matter of routine to monitor and control dissolved oxygen levels in fermentations which are designed to give high cell densities (Reisenberg 1991) and some of these processes are designed so that the level of dissolved oxygen is controlled by the nutrient feed and thus the growth rate of the microorganisms.(Mori *et al.,* 1979). Two types of probe are in frequent use: galvanic and polarographic. As with pH probes, fouling can easily occur and careful calibration is necessary to yield meaningful results.

From the examples given above, it can be seen that important environmental variables such as pH, temperature and dissolved oxygen can be measured on-line, thus giving a more accurate indication of the environment in the fermenter by avoiding any errors due to sampling. However, the problems of accurate on-line measurement caused by the demanding environment of the fermenter fluid mean that there are some key environmental variables which are not routinely monitored on-line.

Some of the main exceptions are nutrient concentration and product concentration which, due to the inherent differences from process to process cannot be measured by a ubiquitous device or system. It requires a separate measuring system to be developed for each component off-line, even before the possibility of an on-line

system can be addressed. Furthermore, most nutrient or product measuring methods are chemical in nature which makes it very difficult for them to be used on-line, although work has been carried out in this field, mainly looking at glucose probes (Phelps *et al.*, 1995, Rishpon *et al.*, 1990) or sample loop connections to analytical systems such as HPLC (Mathers *et al.*, 1986, Turner *et al.*, 1994).

1.3 Fermentation control and modelling

The data obtained from the fermentation monitoring methods described above is collected, collated and acted upon to maintain the fermentation environment in the desired state to ensure best or most efficient use of substrate by the organisms being grown. The need for precise monitoring of fermentation processes is due to their dynamic and inter-related nature, which also presents problems in ensuring adequate and accurate control of the process. The use of models of the processes has been combined with the process data to initially give a better understanding of the process and, ultimately, better control over the process (Ritzka *et al.*,1997, Olsson *et al.*, 1998).

In this section, the inter-related fields of fermentation control and modelling will be outlined and the major types of control and models utilised in fermentation processes will be described. Finally, the theoretical reasons for seeking an on-line biomass measurement will be reviewed, to lead in to a examination of some of the possibilities derivable from an on-line, real-time measure of biomass.

1.3.1 Control of single variables

Due to the extensive nature of bioprocess monitoring, it follows that the level of control exercised is considerable. Allied to this are the varying levels of complexity of control. In this section, the methods used to control single variables will be examined first, leading into the field of process modelling and control.

Open-loop control is the simplest level of control used in fermentation processes. The value of the variable controlled is not used for adjustment of the control parameters. The control of the parameters is pre-set, derived from a calibration carried out beforehand. The control of the rate of nutrient addition by a previously determined and set pump rate is an example of open-loop control.

When the measured value of the variable to be controlled is used to adjust the control parameters to improve accuracy, this changes the control to closed-loop control. From the example above, the installation of a flow metering device would then facilitate closed-loop control of the nutrient feed rate and be able to improve the accuracy of the control.

Within closed-loop control, there are further subdivisions of control loops which can be distinguished, these are feedback, feedforward, cascade and adaptive control. These are used both independently and in combination to improve the power and accuracy of the control system.

Feedback controllers compare the measured value of the parameter to be controlled with the required setpoint and if there is a deviation beyond acceptable limits, set in motion steps to reduce that deviation until it is acceptable. Two types of feedback control can be identified: on/off feedback control and modulated feedback control. In on/off control, the means available to remove the deviation from the setpoint is either off or on fully. This type of control is obviously rather crude and not suitable for use with systems where the allowable deviation between the measured value and the setpoint is small. The possibility of overshoot is inherent in the use of this type of control, especially in systems where the response time of the measured value to the actions of the modifier is relatively long. To try and minimise these problems, modulating feedback controllers were developed. The most common of these are proportial (P) controllers, integrating (I) controllers, differentiating (D) controllers and systems which use a combination of these control modes.

P controllers work by controlling the response of the modifier such that this response is proportional to the deviation away from the setpoint. The effect of this is to speed up the response and reduce the deviation in a crude fashion. I controllers use the integral of the deviation to control the response of the modifier and allow finer control towards the setpoint but have a tendency to overshoot and cause oscillatory patterns. D control action is also known as anticipatory control and is only ever used to in conjunction with P and I controllers. It anticipates the future behaviour of the deviating signal by considering its rate of change. It is used to decrease process response time as a sudden change in the deviation between the measured value and the setpoint will result in a sharp change in the amount of modification applied by the controller. The most common combinations of these

control types are PI and PID. In simplistic terms, P control stops the deviation increasing and thus minimises it; the addition of an I term eliminates the offset but tends to make the response overshoot; the addition of D control then reduces the oscillation and response time and allows the value to stabilise (Fordyce *et al.*, 1990).

Feedforward control uses the measured values of variables other than the variable to be controlled to carry out a control action. It is most often used in conjunction with feedback control and indeed can be regarded as a form of feedback control. In general feedforward control acts best in system with a slow response time. An example given by Dusseljee and Feijen (1990) is that of a feedback control loop controlling temperature. If a sensor measuring the pressure of the cooling water indicates a change in pressure, this will affect the cooling capacity of the water. As heat transfer is a relatively slow process, the change in flow will not immediately affect temperature. If a feedforward control loop detects the change in pressure and then increases the flow to compensate, the temperature will not be affected and a deviation was avoided by the implementation of the feed-forward control loop.

Cascade control is a means of improving response time in a closed-loop control system by linking two measurement and control systems, one subservient to the other. It is usually used to control dissolved oxygen (DO) in fermentation vessels. In cascade control, two variables are measured and controlled but the net result is still within the remit of single variable control. In the case of DO control, the DO variable is the primary variable and in one example of these systems the agitation speed is the secondary variable. In order to control the DO at a set-level, the DO controller measures the DO level, compares it to the setpoint and calculates an output to the agitation controller. This secondary controller compares the agitation rate to the rate requested by the DO controller and adjusts the agitation speed accordingly to give the required rise in DO level. (Stephanopoulos 1988).

Adaptive control is now used in fermentation control. (Jorgenson *et al.*, 1992) The dynamic nature of fermentation processes means that linear, time-invariant control models such as those described above in feedback control can run into problems if the process deviates sufficiently from the desired operating region. This can result in anything from poor controller performance to controller instability and thus process instability. A solution to this would be to adapt the controller to the present process conditions, not to an idealised version of the process control

conditions. This is what is meant by adaptive control. The input and output variables are retrospectively used to update the process model parameters. This is then followed by adjustment of controller setting based on the current process variables. This technique is potentially very powerful and may lead to the development of reliable self-tuning PID controllers (Astrom 1983).

1.3.1.2 Process modelling and control

In this section, the systems which model and control fermentation processes will be discussed.

The power of the single-variable control processes should be obvious. In many cases, the combination of these techniques is sufficient to control the fermentation environment to pre-set parameters to allow growth of the culture and product formation. The pre-set parameters are largely determined by a combination of informed guesswork and trial and error derived experience. The successful maintenance of the fermentation environment does not necessarily imply that the fermentation is being operated under optimal conditions.

In order to combine all the information and control from single variables and use this information to try to improve both the process and the process control, it is necessary to have a theoretical construct within which the inter-relationships of the variables within the processes can be defined, examined and manipulated. This is done by constructing a model of the fermentation process. Like the fermentation processes they represent, these models vary enormously in their complexity. Dhurjati and Leipold (1990), Montague *et al.,* (1989) and Lubbert and Simutis (1994) reviewed the use of modelling in fermentation control and the following summary is derived from these works.

Six distinct types of fermentation model can be identified. These are;

1 material balance models
2. unstructured models
3. structured models
4. segregated models
5. rule-based models
6. neural-networks

Material balance models are primarily used in control applications to calculate gas analysis variables such oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ). The control model is then derived to maintain one these variables at a set level or profile. This type of model was developed as a response to the lack of an on-line biomass measurement and uses these metabolic measures as a representative of the level of biomass in the fermentation process.

Unstructured models of fermentation processes are probably the most widely used form of model used for control. In these models, the fermentation is treated as a single, homogeneous, organism wherein the cell is a "black box" with respect to physiological and metabolic processes. The most widely used representation of cell growth is that of Monod (quoted in Agar, 1985) given here in Equation 1.1:

$$\mu (X,S) = \mu_m S / (K_m + S) \qquad \textbf{Equation1.1}$$

where:

μ is the specific growth rate

μm is the maximum specific growth rate

Km is the Monod constant for growth on substrate

X is the biomass concentration

S is the substrate concentration.

Although this model has shortcomings, and a number of alternatives have been suggested to it, variations of this model are the most widely used because it is simple and thus is mathematically easy to use. An example of the use of models, descended from the Monod equation, to control fermentation processes is the use of feeding a growth limiting substrate to control the specific growth rate in recombinant *E.coli* fermentations (Curless *et al.*, 1991, Korz *et al.*, 1995).

Structured models are designed to take into account some of the detail of the biological processes occurring in the cell. Partial and algebraic equations are used to describe the dynamic behaviour of the growth process. In most cases, an intracellular characteristic which describe metabolism and growth of the biomass and the cell are measured and input into the structured model (Fredrickson, 1976,

Peretti and Bailey, 1986). By their nature this makes them considerably more complex than the models discussed above. As a consequence of this and also due to the difficulties in obtaining accurate measurements of intracellular components, the use of structured models for fermentation processes has been limited, though some work has been carried out in using the greater availability of reliable measurement techniques to develop these models (Nielsen and Villadsen, 1992). Segregated models take into account the changing physiological states of cells. In these models, cells can be viewed as having either a continuously changing distribution of states or a finite number of identifiable states. This makes them difficult to use as process control models due their complex and changing nature.

All of the models described so far have been largely mathematical. In contrast to these, rule-based models are constructed more or less linguistically to aid interpretation of the relationships between numerical data values. The development of routinely available computing power has lead to the extensive use of computers in control functions. Rule-based models are used in expert systems, a computer intensive form of control. The rule-based model is formulated from the previous process data. These models often take the form of " if (condition) then (consequence)" rules. An advantage of these systems is that they can take of advantage of non-numerical data as well as aiding interpretation of numerical data. At present, these systems are used in advisory capacities to aid decision making in the control of the fermentation using the expert knowledge from previous fermentation runs and suggest a solution.

Another computer intensive modelling strategy are neural networks. This modelling technique also makes use of both numerical and non-numerical and process data. The models are formulated from available data and can represent unknown, non-linear relationships in complex multi-variable systems. Montague and Morris (1994) have applied this method to a SmithKline Beecham penicillin G production process to gain on-line, real-time estimates of biomass from gas analysis data and use the data to "tune" nutrient feed-rates to optimise biomass and penicillin production. As this is a complex media fermentation, conventional mass-balance models would be unable to estimate biomass, and would certainly be unable to control the fermentation.

1.4 On-line biomass measurement

All of the modelling techniques described above require accurate data about the process to ensure the accuracy of the model and the accuracy of the model-derived control. It is interesting, though not surprising, to note that all the models described above have a biomass component to them, and there were several whose very function was to derive a measure of biomass to input into a control model.

An accurate real-time, on-line measure of biomass would have numerous applications in both modelling and control and in real-time fermentation efficiency analysis and optimisation (Ritzka *et al.*, 1997, Olsen *et al.*, 1998).

In many modelling cases, a real-time measure of biomass would turn the model from an open-loop control system to a closed-loop control system and allow fine-tuning of the control while the process was running. An example of this would be in control of specific growth rate (μ) by substrate addition rate in *E.coli* fermentations controlled using an unstructured model. Korz *et al* (1995) proposed the following model as a means of controlling μ in this manner:

$$Ms(t) = \{(\mu set / Ysx) + m\} \, Vtf \, Xtf \, \varepsilon^{\mu set(t-tf)} \qquad \textbf{Equation 1.2}$$

where:

Ms(t) is the mass flow of substrate in g/h at time t

μset is the operator set specific growth rate

Ysx is the yield coefficient

m is the maintenance coefficient, set to 0.025 g/g/h (for *E.coli* growing on glucose)

tf is the time at which feeding commences

Vtf is the vessel volume at time f in L

Xtf is the biomass at time f in g/L

$\varepsilon^{\mu set(t-tf)}$ is 2.7183 to the power of the set growth rate multiplied by the length of time the feed has been on.

If an on-line measure of biomass (Xtf in this model) was available to be input to this model, the control model could be fine-tuned in real time and the mass flow of

substrate required to maintain the required growth rate could be directly controlled and manipulated in real time, thus improving the control of the fermentation.

This technique has been used successfully to control an *E.coli* fermentation. Yamane *et al* (1992) used an on-line optical density probe to provide control using an unstructured model similar to the Korz model. In both of these examples, the accuracy of the control is determined by the accuracy and frequency of the measurements.

The production efficiency of a fermentation is often of concern in industrial fermentations. The analysis of fermentation efficiency was originated by the work of Gaden (1959). Two bases for expressing fermentation rate were proposed:

1. The volumetric rate, the rate of change of concentration with time
2. The specific rate, which is the volumetric rate divided by the cell concentration.

For analysis of kinetic processes, the specific fermentation would yield more useful data as the production rates achieved are corrected for the amount of biomass made to produce the product. From this, the efficiency of the fermentation, in terms of relating energy input to biomass and product formation can be derived. At present it is possible to do this retrospectively. From this retrospective data, the relationship between these factors can be determined and modelled to allow predictive proposals to be made and then tested by further experimentation. With an on-line measure of biomass, the optimisation of the fermentation efficiency by the manner suggested above is possible while the fermentation is running. Some industrial fermentation lend themselves to this more readily than others. The long antibiotic fermentations where growth and product accumulation is relatively slow would appear to be better suited to this than short fermentations such as those typically seen with bacterial cultures.

In summary, the usefulness of an on-line, real-time measure of biomass in fermentation monitoring, control and analysis is clear and it is still one of the most sought after measurements in fermentation monitoring. The aim of this work was to investigate the methods currently available for on-line biomass measurement and assess the accuracy and the nature of the biomass signal yielded by the best available method In analysing on-line biomass measurement, a ubiquitous probe would also fulfil the following abilities, additional to those quoted above from

Harris and Kell (1985) for general on-line measurements, and these are the ability to:-

1. monitor the growth of both filamentous and unicellular organisms.
2. operate in media containing undissolved solids.
3. differentiate between biomass and necromass (dead cells).

This introduction will firstly look at the available methods for biomass measurement and then concentrate on the chosen method for this work, capacitance measurement.

1.4.1. Biomass Measurement

1.4.2 Definition of biomass

The most basic but also the most fundamental question which can be asked concerning a fermenters microbial population regards the amount present. This parameter, which is commonly called the biomass, would seem to be a simple issue. However, what is actually meant by the term biomass depends on what the area of interest is and what the method of measurement is. Biomass can variously mean:- the number of visible cells on a microscope slide (Becker *et al.*, 1990); the number of cells possessing an intact membrane (Kell and Davey, 1990) or simply the weight of cells present (Malette, 1969). For the purposes of this work, the general term, biomass, will be taken to mean the weight of cells present in grams [g] dry weight per unit volume of the growth medium in litres [L]. However, more specific biomass-related terms will be introduced during the progress of this work, when considering data obtained from various biomass measurement systems.

1.4.3 Methods of biomass measurement

There are a large range of methods and devices available for biomass determination (Ison and Matthew, 1997). These vary from the very simple methods such as wet weight analysis to complex devices such as near-infra red spectroscopy. In this section, a review of the application of these methods in monitoring biomass

accretion, both off-line and on-line, in fermentation will be carried out in order to establish the prior art in this field.

The methods available can broadly be divided into three categories:-

1. Chemical
2. Microscopic
3. Physical

1.4.3.1 Chemical methods

The chemical methods of biomass determination can be divided into 2 types. These are:-

1. Non-biomass specific elemental analyses such as total carbon (Hashimoto *et al.* 1982) or protein content (Cooney, 1982).

2. Biomass specific methods such as ATP measurement by bioluminescence (Lundin, 1989) or DNA content (Herbert, 1971).

All are indirect measurements. They work by relating the amount of a growth related chemical to the likely amount of biomass present. In fermentation, the fermenter fluid must first be aseptically sampled, the biomass is then separated from the growth medium before being allowed to react with the reagents to provide the result. This sampling and sample processing is time consuming and can lead to errors. They also mean that these methods are unsuitable for adaptation to on-line measurements. The biomass specific methods are primarily useful for detecting low levels of microbial activity, (bioluminescence can detect 10^4 cells/mL) and are used mainly by microbial ecologists. All of these methods have no theoretical upper limit of detection, though the non-specific methods can have large errors (up to 50%).

1.4.3.2 Microscopic methods

Microscopic methods again involve sampling before the biomass determination can be carried out.

1.4.3.2.1 Direct count microscopy

Direct counts by microscopic examination of the number of visible cells is the simplest and most common method used (Koch, 1981). This involves taking an aliquot of the sample, diluting and staining it, before placing into a haemocytometer for viewing. The haemocytometer is an enclosed counting grid of known volume, the grid pattern being used to define the sample area and also break down the viewed area into smaller sections to make counting easier. The differences in direct count microscopy occur in the type of stain used and consequently the type of microscopy used to view the cells. These methods have the advantage of being able to give a count of the number of intact cells present in the sample.

1.4.3.2.2 Epifluorescence microscopy

The most sensitive method for viable counts is epifluoresence where stains such as acridine orange cause intact DNA and RNA to fluoresce and allow viewing under an epifluoresence microscope (Hobbie, 1977). Both of these methods are only suitable for unicellular organisms. As the samples are diluted to bring the number of cells viewed under the microscope to an easily countable number, these methods can be used over the full range of cell concentrations. These methods have the problems of sampling and sample processing before any biomass determination can be made. Again, these problems seem to make these methods unsuitable for on-line use, although work by Suhr *et al.* (1995) viewing cells *in-situ* using an epifluoresence microscope inserted into port in the fermenter has shown that these problems are not insurmountable.

1.4.3.2.3 Image analysis

Both of the preceding techniques are only suitable for suspensions of dispersed, unicellular organisms. Image analysis shows promise as an off-line measurement of both unicellular and filamentous organisms and work is currently progressing to enable it to be used on-line. It is a computer based system which works by

digitising the picture obtained under the microscope so that the computer can differentiate between blank slide and biomass (Packer and Thomas, 1990; Brooks and Coleman, 1989). Obviously it still involves sampling but the use of the computer image analyser speeds up and automates biomass determination. Work has shown that it is possible to distinguish between undissolved medium solids, cells with intact cytoplasm and degenerated cells. Thus mycelial biomass concentrations may be measured in the presence of undissolved solids and the physiological state of the mycelia may also be determined. It has been shown that this method is able to measure biomass over a range of concentrations from 0.03 to 38 g/L (Packer *et al.*, 1992, Vanhoutte *et al.*, 1995)

1.4.3.3 Physical methods

The physical methods of biomass determination available are very varied. The simplest physical methods are ones which measure biomass by weight.

1.4.3.3.1 Wet weight and packed cell volume

Wet weight can be obtained by sampling the fermenter fluid and then separating out the biomass from the liquid before weighing it (Gerhardt, 1981). A similar method to this is packed cell volume analysis. The sample is centrifuged in graduated containers. During centrifugation all the cells will be sedimented at the bottom of the container so that the volume that the packed cell mass takes up can be quickly read off against the graduations. This yields a very rough but quick result. Both of these methods can be used over the full range of biomass concentrations. However, the lack of accuracy available would make it inadvisable to use these methods at low cell concentrations (Malette, 1971).

1.4.3.3.2 Dry weight

The method which can be regarded as the "gold standard" for biomass is dry weight. This involves the same procedures as wet weight analysis but includes a subsequent drying of the sample after separation. The drying can be carried out in a number of ways, the most common being dry heat (typically 80°C for 24 hours).

Drying using a microwave oven can speed up the process thus yielding the result faster. This method is as sensitive as the balance used to make the measurements and has a potentially unlimited range (Mallete, 1971). Once more, the errors of sampling and the time to yield the result are problems. Other problems with these methods are the possible presence of suspended and dissolved non-biomass solids which can lead to a false result and the presence of debris from dead cells also confusing the result. Careful washing of the sample should eliminate most non-biomass solids but the problem of cell debris is inescapable. Cooney (1982) estimates that dry weight analysis yields a result which is +/- 30% the actual biomass concentration in the vessel. The need for sample processing and the time needed to obtain a result again makes these methods unsuitable for on-line use.

1.4.3.3.3 Filtration probe

A device which relies on packed cell volume is the Filtration Probe (Nestaas and Wang, 1981). This was developed to monitor mycelial biomass in penicillin fermentation. It operates by taking a sample of the fermenter fluid, degassing it before applying a constant pressure across the top of the sample, forcing the filtrate out of the bottom of the unit. The filtrate volume is measured by a load cell and the filter cake by an optical sensor, this information is used by the computer system to calculate the mycelial portion of the sample and give an approximation of biomass. It is estimated that this system can operate in the range of 2 to 40 g/L dry weight. Typically this system is in a sample loop, hard plumbed into the fermenter although more recent *in-situ* versions have been tried (Thomas *et al.*, 1985). The system can operate at the rate of 1 sample every 30 minutes which is too slow to be considered as a "real" on-line monitor of biomass, and the degree of accuracy may also be a problem. However, it does yield useful results under ideal circumstances and any future development of the device would bear examination.

1.4.3.3.4 Optical density measurement

The measurement of the proportion of a light beam which passes through a microbial suspension is called turbidimetry or optical density measurement (OD). The OD of microbial suspensions is closely related to the concentration of cells in

suspension. The attenuation of the incident beam caused by the cells in the suspension can be described by an empirical relationship similar to the Beer-Lambert law for light absorption. The relationship between OD and cell concentration is proportional up to 0.3-0.5 Absorbance units (depending on cell size). The sensitivity of OD varies with the instrument and wavelength used and the type of cells being measured but is typically between 2.4 x 10^6 and $1.8x10^8$ cells/mL (Malette, 1971). For light in the 600-700 nm range, dilute bacterial suspensions have virtually the same absorbance per unit of dry weight concentration, regardless of cell size. Thus 1 absorbance unit corresponds to approximately 1.5 g/L dry cell weight. Therefore samples for OD measurement are beyond the linear range at between 0.2-0.33 g/L. Samples may be diluted with fresh media to bring them into the linear range. Otherwise calibration versus dry weight and non-linear regression analysis is necessary to convert absorbances higher than 0.5 to biomass concentration (Cooney, 1982; Phillips, 1990, Choo and Chang, 1995). This is not the only problem with OD as a biomass measuring method. The presence of non-biomass solids and gas bubbles in the sample greatly distorts the reading and while it may be possible to eliminate gas bubbles even in an on-line system (Coppella, 1990, Hatch and Veilleux, 1995) it is much more difficult to remove suspended solids. While the monitoring of bacterial or yeast growth is feasible, the diffuse nature of filamentous organisms makes it impossible to calculate their biomass with this method. Once again the problems of sampling and sample processing lead to difficulties in obtaining a fast accurate result.

There are available a number of commercial on-line OD probes, the more modern of which are interfaced with a micro-processor to cope with the problem of non-linearity and remove the need for sample processing (Cox, 1989). Some also have electronic circuitry which enables the monitoring system to recognise anomalies caused by gas bubbles and ignore them (Dekovitch *et al.* 1989). In a trial of various types of commercially available OD probes in mammalian cell fermentations, Wu *et al* (1995) found that the probes which work, by measuring backscattered radiation gave the most linear responses with cell density and the best resolution.

However, despite these advances the problem of suspended solids remains, and it seems unlikely that this will change. Despite this, the currently available OD probes are useful in monitoring bacterial or yeast fermentations in media without

suspended solids and some are used routinely for control purposes (Yamane *et al.*, 1992).

1.4.3.3.5 Fluorescence

Another technique available using light radiation is fluorescence. Zabriskie and Humphrey (1978) pioneered its use in monitoring fermenter biomass. They irradiated the culture with near ultra-violet radiation at 366 nanometers and detected the resultant fluorescence at 460 nm. This fluorescence is mainly caused by NADH (> 50%), the levels of which correlate to some extent with biomass. Other known fluorophores such as tryptophan, pyridoxine and riboflavin can also be used as biomass indicators using multiple excitation fluorometric systems (Li *et al.* 1991). However the dependence of success on the correct conditions and the possibility of the presence of unknown fluorophores makes this method generally unreliable as a stand-alone biomass probe (Liden, 1993). It has been used on-line in characterised systems (Greer *et al.* 1989) and it can also be used as an indicator of general metabolic activity (Walker and Dhurjati, 1987, Marose *et al.* 1998) rather than biomass *per se.*

1.4.3.3.6 Mass balance analysis

One different type of physical method which is common at present is fermenter exit gas analysis. The gases vented out from the fermenter are analysed by a mass spectrometer. The values obtained are used to calculate carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR). These parameters are combined with the information about the other environmental parameters and input to mass balance models of the process to yield an approximation of the biomass present. This means that the system is as sensitive as the available model and is able to work over the full range of biomass concentrations (Gbewonyo *et al.*, 1989; Wu *et al.*, 1989). This method can only estimate the biomass concentration and is naturally dependent on the accuracy of the available model of the process.

1.4.3.3.7 Acoustic Densitometry

The work carried out by Blake-Coleman *et al.* (1986) used a commercially available device which measures the attenuation of a sound wave by a sample much in the same way that OD measures the attenuation of a light wave. The measurement depends on the relationship between the density and resonant frequency of sample enclosed in a test chamber, electromagnetically excited to vibrate at its natural frequency. Biomass is determined by reference against a cell-free media sample. It measures mass per unit volume of the sample and therefore will include cell debris, non-biomass solids and gas bubbles in any measurement. It is also very temperature dependent. The range in which this device operates is not yet known but the lower limit is 10^6cells/mL. The version used in the initial experiments was used off-line but an on-line version has been developed and used to measure mammalian cell biomass with some degree of success (Kilburn *et al.* 1988). This system is one which should improve with further work.

1.4.3.3.8 Pieozoelectric membranes

This method is similar to acoustic densitometry, the difference being that this method measures sample compressibility rather than density. The method used was devised by Ishimori *et al.* (1981). Sound generated at one piezoelectric membrane is transmitted across the contained sample to cause vibration at the other piezoelectric membrane, which converts it to a proportional output voltage. Again, biomass must be calculated after comparison with a reading taken from cell free media. As the compressibility of a cell varies with cell size, area and volume, calibration of cell species compressibility characteristics with cell number is necessary for each species to be monitored. This gives this method the capability of identifying cell species; this may make it a useful tool in other areas of microbiology. However, it cannot differentiate between biomass and the common confounding factors of cell debris, non-biomass solids and gas bubbles. As yet this method has been shown to be accurate within the range of 10^6 to 10^8 cells/mL and it is also temperature dependent. Another problem in using this method as an on-line system is the comparative inability of the piezoelectric membranes to stand

up to heat sterilisation. This problem has been overcome by the development of a piezoelectric gum membrane which is much more robust, (Endo *et al.*, 1989) but the problem of bubble and solids interference have yet to be overcome. Zips and Faust (1989) have patented an off-line, impulse echo version of this system so perhaps the efforts of two groups working on solving the problems with the system may yield some encouraging results.

1.4.3.3.9 Coulter counting

Coulter Counters work by measuring the change in resistance when a saline suspension of micro-organisms passes through a 30-100 μm diameter aperture (Kubitschek, 1969). The increase in resistance is proportional to cell volume which means that these devices can both count the number and the range of sizes of cells in a given volume of saline. The device can count around 3 - 5 x 10^3 cells/second, thus it can operate over the full range of fermentation biomass concentrations. However, most bacteria are near to the minimum detectable size and this should always be borne in mind. This device is only suitable for unicellular organisms and great care must be taken to avoid clumping of the cells which can result in false measurements and can also block the measuring aperture. Once again, this method cannot differentiate between biomass, cell debris, non-biomass solids and gas bubbles. These problems, along with the need for sample processing, preclude it from being used on-line.

1.4.3.3.10 Calorimetry

When micro-organisms grow, they produce heat, known as the heat of enthalpy. This heat production can be related via enthalpic and elemental balances to give an estimation of biomass. Previous attempts to do this have failed because the technology available to monitor these temperature rises against the normal temperature fluctuations in a fermentation was not good enough. However, improvements have been made and microcalorimetric measurements both in a sample flow loop (Greer *et al.*, 1989) and on-line (Randolph *et al.*, 1990) have been attempted with some success. This method has the sensitivity and range of the measuring technology available and it deserves further work as it measures a

change in the fermenter fluid which can be directly related to biomass. It is also not hampered by the need for sampling or sample processing nor is it interfered with by the presence of cell debris, non-biomass solids or gas bubbles. It cannot, however, differentiate between growing cells and respiring but non-growing cells but it is still an area of measurement well worth pursuing.

1.4.3.3.11 Near-infra red spectroscopy

Near-infra red spectroscopy (NIR) is used for multi-component analysis in a number of industrial applications and has recently been applied to fermentation monitoring (Vaccari *et al.,* 1994, Ge *et al.,* 1994, Macaloney *et al.,* 1994). The operating principle is based on the absorption of NIR radiation (700-2500nm) related to the components present in the sample analysed. The sample is irradiated with NIR radiations of selected frequencies. A part of the radiation is absorbed by the sample, while the remainder is reflected and measured. The radiation absorbed is proportional to the number of molecules present which absorb radiation of that frequency. This technique is useful as it is fast (30s) enough to be used as a real-time measurement and the technique can be carried out non-invasively through a viewing port in the vessel, thus avoiding any problems associated with sampling and invasive probes. The technique does require significant computing power for deconvolution of the signals and identification of the sample components from the spectra of known materials. This also requires an extensive calibration and validation phase before the technique can be used to yield real time results. The instrumentation and the computing required to analyse the signals are also expensive, making this technique impractical for small-scale research laboratories. Despite these reservations, NIR spectroscopy of fermentation processes will continue to become more widespread and thus the technique will become more refined and cheaper.

1.4.4 Conclusions

The degree of usefulness of the devices and methods discussed varies considerably. The chemical methods and most of the microscopic methods are largely unsuitable for on-line use because of the need for sample processing.

Capacitance measurement, which is discussed in the next section, is the only method currently available which fulfils all the imposed criteria, although it does have its problems. All the other methods have difficulties, mainly with media containing undissolved solids or with detecting viable cells. All of these methods and devices are worth considering as long as their limitations are borne in mind. Some are still experimental and still need to be developed to their full potential, thus it is difficult to give an accurate assessment of their true usefulness. The possibility of using one or more of these methods in tandem should also be given serious consideration, with each method covering for the deficiencies of the other. Until the ideal, ubiquitous biomass probe is developed, these methods are the best available and if they are used to their fullest extent in the appropriate conditions they can yield useful biomass data.

2. DIELECTRIC SPECTROSCOPY

2.1 Capacitance and conductance

The field of dielectric spectroscopy is vast, and it is inappropriate for the purposes of this work to give more than an overview, so that its use in deriving and analysing biomass measurements can be understood. In this section, the behaviour of charged particles in electrical fields, the nature of capacitance and its dimensionless equivalent, permittivity, will be examined to illustrate the theory behind the biomass measurements. As conductance and its equivalent, conductivity, have a much smaller bearing on this technique as a biomass measurement, they will receive less attention.

An electrical field can be created by applying a voltage between two electrodes. This field can be regarded as a force which moves electrical charges, for instance ions in solution, in specific directions related to the polarity of the electrodes. It is dependent on the fields' direction and nature and on the charge of the ions. An electrical field acts on ions in solution to move positive ions towards the negative electrode and the negative ions towards the positive electrode. This charge separation or polarisation is what is measured by capacitance. The dielectric properties of a material are wholly described by its capacitance and conductance. Capacitance can be regarded as the propensity of the material to retain charge, while conductance is the ability to conduct this stored charge and thus dissipate the energy as heat. Thus each is the antithesis of the other. As capacitance is the source of the biomass measurement, it will be dealt with first.

2.2 Capacitance and β-dispersion

As discussed above, capacitance is defined as the extent of charge separations induced in a material by the application of an electrical field to that material (Pethig, 1979). In electrical terms, all biological cells in suspension can be regarded as a poorly conducting membrane separating a conducting cytoplasm from a conducting suspending solution. If cells are introduced into the theoretical

ionic suspension described above and the electrical field applied is of radio frequency (in this case between 0.1-10 MHz), then the measured capacitance of the solution increases by an amount which is directly proportional to the number or more accurately, volume of cells present. The electrical field is still causing the polarisation of the free ions in solution, but it also causes the cytoplasmic ions of the cells to become polarised. The cytoplasmic membrane of cells is selectively permeable and will not allow the egress of these polarised ions, thus the ions accumulate at the membrane and are retained there due to electrostatic attraction. This effect causes a charge separation across the cell due to the insulation of the electrically conductive cytoplasm from the similarly conductive suspending medium. This charge separation is measured as the capacitance due to the cells, which causes the rise in the overall capacitance of the solution. If the electrical field applied is alternating current, then the dielectric properties of the cell suspension are strongly frequency dependent.

Figure 2.1. shows an idealised plot of the change in capacitance (ΔC, measured in pF) versus the log of the frequency of the alternating current applied. The frequency of an alternating current is a measure of how often the direction of the electrical field changes per second and is measured in Hertz (Hz). A low frequency means that the electrical field is changing direction more slowly than at a high frequency. When an alternating current is applied to a cell suspension, the direction of the polarisation across the cell membrane changes every time the direction of the electrical field changes. This does not effect the extent of the charge separation or polarisation which is the same regardless of direction and only varies with frequency.

The range of frequencies shown in this plot represent the operating range of the instrument used in this work. The frequency related fall in capacitance illustrated in this figure was termed β-dispersion by Schwann (1957) and is primarily due to the following factors.

At the low frequency end of the range shown above, the intracellular ions go to the cytoplasmic membrane and cause the extra capacitance, as described above. As the frequency of the applied electrical field is increased , the time available for these ions to migrate to the poles of the cells (*i.e.* the cell membrane) and cause the charge separation decreases.

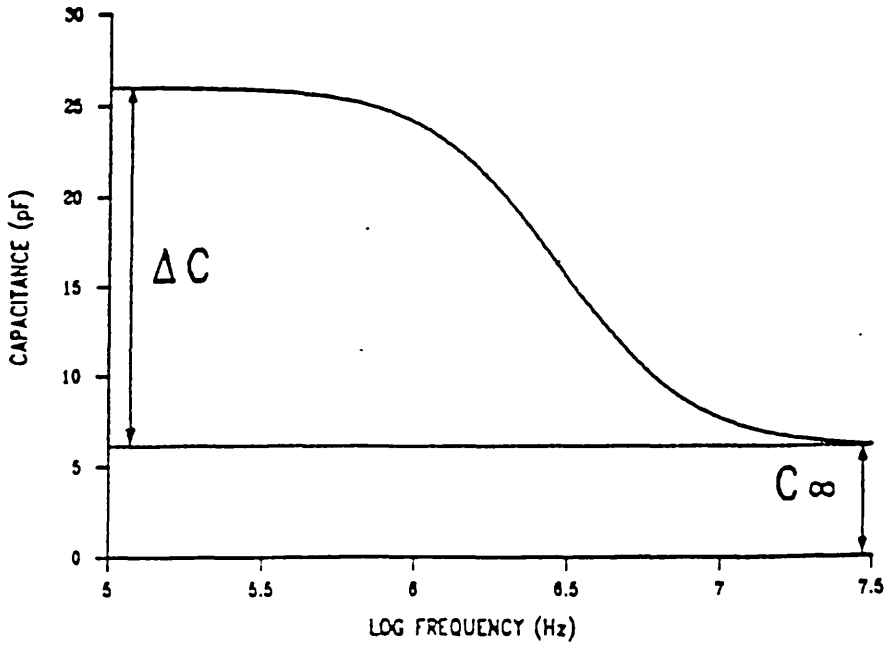


Figure 2.1: Idealised plot of capacitance (pF) versus log frequency (Hz) adapted from Davey (1991)

This available time continues to decrease with the increase in the frequency of the electrical field until at high frequencies, such as 9.5 MHz, the electrical field is switching direction too rapidly to allow the ions to reach the cell membrane and cause the polarisation and, therefore, the capacitance due to the presence of the cell is effectively removed. This capacitance measure, termed $C\infty$, primarily corresponds to the capacitance of the water in the suspension, caused by the small charge separations within the water molecule.

In using this phenomena to derive a biomass estimate, it is necessary to choose a measuring frequency which is on the low frequency plateau to give a maximal Δ capacitance signal. Typical measuring frequencies in this work were between 0.4-0.5 MHz. The term f_c is called the critical or characteristic frequency and it is the point in the frequency where the drop in Δcapacitance is half the original Δ capacitance value. The position of the f_c is dependent on, in descending order of importance: the conductance of the suspending medium; the conductance of the cell cytoplasm and the electrical properties of the cell membrane. The last two factors can be expected not to change significantly over the course of a fermentation, while it is likely that some changes in the conductance of the media will occur. A fall in conductance will cause the f_c to fall slightly while a rise will cause it to rise slightly. If the measuring frequency is correctly selected so that it is well on to the low frequency plateau, then these conductance derived changes in f_c will show little or no effect on the Δcapacitance and thus the biomass estimate.

The steepness of the fall in capacitance is measured by what is termed the Cole-Cole α value, a term reflecting the breadth of the dispersion, which is effected by the relative homogeneity of the cell populations' electrical properties (Pethig and Kell, 1987). An α value of 0 indicates a steep fall, as depicted in Fig 2.1 above, while a value of 1 would indicate a dispersion which had a shallow fall in capacitance. These parameters, f_c and the Cole-Cole α value are characteristic for a known cell suspension of known radius and cell number, in a suspending medium of known conductance.

From work carried out by Markx *et al.* (1991), it has been suggested that the determination of Cole-Cole α values may be useful in the rapid characterisation of morphological changes in microbial cultures. Work by Siano (1997) concluded that changes in cell shape in yeast suspensions had an effect on the Cole-Cole α

value in that deviations from a spherical cell shape resulted in an increase in the Cole-Cole α value.

2.2.1 Capacitance and permittivity

Capacitance is a dependent variable. It is related to the size and geometry of the electrode used to measure it. This means that capacitance measurements made using one measurement system should be converted to an independent variable before they can be compared with capacitance measurements made with another measuring system. The independent equivalent of capacitance is permittivity. Its relationship to capacitance is described by the following equation:

$$\varepsilon' = \frac{CK}{\varepsilon 0} \qquad \textbf{Equation 2.1}$$

ε' = relative permittivity (no units)

C = Capacitance as measured by Biomass Monitor in Farads

K = Cell constant in metres^{-1}

$\varepsilon 0$ = permitivitty of free space (a physical constant equal to 8.854 x 10^{-12} Farads per metre)

(Pethig 1979)

The term ε' is called relative permittivity as it the ratio of the capacitance of the standard electrode with the material to be measured in it, divided by the capacitance of the electrode with a vacuum in it. The standard electrode is an imaginary capacitance measuring system devised to allow the conversion of probe dependent capacitance measurements to independent permittivity measurements. It can be thought of consisting of a cube of the test material 1m^3 with two plate electrodes at opposite sides, which gives a cell constant of 1m^{-1}. As the parameter

K is constant for a given measuring system and the parameter ε_0 is also a constant, the capacitance and permittivity of a substance will show the same relative characteristics when measured over a frequency range.

2.2.2 Conductance and conductivity

In practice, it is simplest to use the relationship between conductance and conductivity to calculate the K value of a given measuring system. As described earlier, conductance is related to the number, mobility and charge of the ionic species present in solution and can be regarded as the opposite of capacitance. As with capacitance, conductance is a dependent variable related to the electrode used to measure it. Due to their interrelated nature, the formula for converting conductance to its dimensionless equivalent, conductivity, contains the same cell constant term as the capacitance equation. The equation for converting conductance to conductivity is:

$$\sigma' = G K \qquad \textbf{Equation 2.2}$$

σ' = conductivity in Siemens per metre

G = conductance in Siemens (as measured by Biomass monitor)

K = Cell constant of Biomass monitor electrode system in metres^{-1}

Conductance is strongly temperature dependent as the mobility of the charged ions in solution increases with temperature, thus increasing the conductance and altering the capacitance. A rise in temperature of 1°C will give a 1-2.5 increase in conductance (Davey 1991). For this reason, it is important that off-line and on-line measurements of cell suspensions are temperature controlled. As discussed, one of the most important effects of conductance is on the f_c of the β-dispersion. This can then effect the Δcapacitance measurement used to estimate biomass. In general terms, if the medium conductance is low then the f_c is low and is more prone to gross changes due to changes in medium conductance. If the conductance is high then the f_c is high. In extreme cases, especially with small cells, this can result in

the f_c being beyond the instruments measuring frequency range and this has the effect of reducing the measured Δcapacitance and the resultant biomass estimate.

2.3. Capacitance and biomass

If permittivity were plotted against log frequency, the plot would be identical to the curve shown in Figure 2.1. The Δpermittivity value (Δε, also known as the dielectric increment) is given by the following equation (Schwann 1957):

$$\Delta\varepsilon = 9PrC_m / 4\ \varepsilon_0 \qquad \textbf{Equation 2.3}$$

where:

Δε = the dielectric increment, change in permittivity from low to high frequency

P = the volume fraction of the biomass, i.e. the fraction of the total volume of the suspension which is enclosed by the plasma membranes of the cells.

r = the cell radius (m)

C_m = membrane capacitance of the cell per unit of membrane area (Fm^{-2})

ε_0 = permittivity of free space (a constant, $8.854 .10^{-12}$ F m^{-1})

As the capacitance measurements described in this work are directly related to the permittivities, this equation gives a useful means of estimating likely capacitance values for the organisms studied and allows a degree of interpretation of the results from the capacitance monitoring. It should be born in mind that this equation was derived to describe the dielectric increments of solutions of spherical cells. The ratio 9/4 changes for different cell morphologies (Kell *et al.,* 1990). Davey (1993) suggests ways of correcting for this effect with suspensions of ellipsoidal and rod-shaped cells but no method is proposed for filamentous organisms. For a given cell suspension, the cell radius, r, and the membrane capacitance Cm can be regarded as constants. As ε_0 is also a constant, this means that Δε is linearly proportional to P, the volume fraction of the biomass in suspension, which is the biomass. Equation 2.3 is true only for low values of P. At high volume fractions,

the electrical field effects on an individual cell become distorted by the cells around it, and a plot of $\Delta\varepsilon$ versus volume fraction (and thus biomass) begins to plateau out at values of $P > 0.15$, which corresponds to an approximate wet weight of 150 g/L, a dry weight of between 50-75 g/L. A modification to this equation was suggested by Schwann and Morowitz (1962) to allow for this non-linearity:

$$\Delta\varepsilon = 9PrC_m / 4\,\varepsilon_0 \cdot P/\,[1+(P/2)]^2 \qquad \textbf{Equation 2.4}$$

This is Equation 2.3, with an additional term $P/\,[1+(P/2)]^2$. This additional factor clearly only depends on the volume fraction of the cells present. Some recent work by Davey *et al.* (1992) has shown that this additional term does correct the equation for cell suspensions of high volume fractions. This allows the dielectric increment data to correlate with volume fraction and thus biomass measurements, even from high volume fraction cell suspensions by the application of a curve-fitting routine of non-linear least squares to the data from high-volume fraction cell suspensions.

One of the biggest advantages of using capacitance measurement to estimate biomass is that only viable cells will contribute to the capacitance signal. Viability is a relative term and means different things to different people. In this instance, viability is taken to mean the possession of an intact cell membrane, which is a reasonable definition of viability in general and the most appropriate definition of viability for this work. As discussed above, to contribute to the capacitance attributed to the cells, a cell must possess an intact cell membrane to allow a polarisation and charge separation to occur. This theory is acceptable for entirely viable cells which have an intact membrane and entirely non-viable cells which are fully disrupted and their cytoplasmic ions cannot be contained by their cell membranes. The cells which fall somewhere between these two extremes are still of interest. The contribution of partially lysed and leaky cells to the capacitance signal of a cell suspension has not previously been investigated. The normal turnover of a cell population and the sometimes harsh environment of a fermenter, with the stresses caused by shear forces, will result in a portion of the fermenter's cell population falling between these two extremes. This is especially true of filamentous organisms which are very shear sensitive, have a tendency to lyse and

also change morphology during typically long antibiotic fermentations such as the growth of *P. chrysogenum* to produce penicillin G. Mishima *et al.* (1991a) demonstrated that, with *Aspergillus niger*, homogenisation reduced the capacitance of the cell suspension by around 95%, indicating the viability component of capacitance.

The effect of non-intact cells on capacitance could be examined using variable, low-pressure homogenisation to disrupt a portion of the cells and look at the changes in capacitance, viability measured by conventional means and if possible cell morphology. This technique was employed in this work and will be discussed in detail in later sections.

Another advantage of the capacitance method of biomass measurement is that it should not be interfered with by non-biomass solids, oils, antifoams and gas bubbles in the suspending media. It is relatively common for fermentation media, especially industrial fermentation, to contain all of these potential interferents. These all give problems for on-line optical density probes. As they are not significantly polarised by the applied electrical field, they do not contribute to the capacitance of the cell suspension. If a large amount of non-polarisable species are present in the fermentation fluid, (*e.g.* the gas hold-up is high or there is a large solids or immiscible liquids content) then the capacitance will be reduced over the full frequency range as a finite volume of polarisable material (cells and water) has been replaced with this non-polarisable material. The effect of gas hold-up should be noted. A highly mixed and aerated fermentation vessel, especially a small (< 20L) one can show a significant increase in volume over the liquid volume due to the hold-up time of the air being input into the vessel. Again, this has the effect of reducing the overall capacitance of the fermenter fluid due to the effect described above. As this effect is more pronounced in small vessels of the type predominately used in this work, a method of correcting for this was determined (see Section 3.1.6.4). Where possible, the agitation speed and gas flow were kept constant in small fermenters to avoid exacerbating this problem.

Another potential problem is concerned with the use of non-biomass solids and immiscible oils as substrates in some fermentations. If the presence of the non-polarisable substrates in large concentrations was to reduce the capacitance of the fermentation fluid at the start of the fermentation, then the breakdown of the non-polarisable substrates by the organisms for growth would possibly give a falsely

high biomass estimate as the capacitance of the fermentation fluid in increasing due to the increase in cell number and the decrease in the presence of non-polarisable substrates.

2.4 The Biomass Monitor

The instrument used to measure capacitance in this work is the Biomass Monitor (BM, Aber Instruments, Aberystwyth UK). The *in-situ* sterilisable probe consists of four 24-carat solid gold electrode pins encased in a thermally stable inert resin. The two outer electrodes apply the alternating current of the required frequency in the range 0.1-10 MHz and the inner pins pick up the alternating voltage drop. The probe effectively "sees" the capacitance of an area of a few centimetres directly around it and as with any on-line probe should be placed in a position in the vessel that ensures a representative measurement can be obtained. The signals from the probe are boosted by a head amplifier which is located on the top of the probe, outside the vessel and sends the amplified signal to the instrument box to be filtered and then displayed. This probe geometry is optimised for use in biomass measurement (Kell and Todd, 1989) and is designed to minimise problems with the measuring electrodes (Harris *et al.* 1987, Kell and Davey, 1990)

2.4.1 Single and dual frequency capacitance measurement

In order to minimise the problems discussed above, and to take full advantage of the characteristics of the β dispersion curve as shown in Figure 2.1, it is possible to determine the dielectric increment of the cell suspension of interest every time a measurement is made. To do this, the instrument used to make the measurement must be capable of switching instantly from a low frequency to a high frequency. In the case of the Biomass Monitor, the high frequency is factory set to 9.5 MHz and the low frequency is set by the operator. Typically the low frequency is between 0.4 and 0.6 MHz as these values are generally well on the low frequency plateau of the β dispersion but they avoid the possible problems of electrode polarisation (discussed in the next section) seen at below 0.4 MHz. In using this method, the changes in background capacitance are corrected for on-line and in

real-time and the effects of gas-hold and noise on the signal due to bubble interference are minimised, making the final signal much smoother.

2.4.2 Electrode polarisation

Although the BM probe is designed to minimise artefactual measurements, they cannot be entirely avoided. Charged electrodes in an ionic solution will become surrounded by a layer of oppositely charged ions and a charge separation occurs at the electrode surface. As capacitance is a measure of the charge separation present, the instrument sees this polarisation of its own electrodes as capacitance. This electrode polarisation is frequency dependent and is most marked at very low frequencies, typically those below 0.4 MHz. It can be corrected for using the method given by Davey (1993), but choosing a measuring frequency of 0.4 MHz or above precludes the need for this.

2.4.3 Conductivity and crosstalk

The major limitation in the use of the BM is the conductance range within which it can accurately operate. The newest BM instruments have a maximum operating conductance of some 20mS. This is roughly equivalent to 200mM salts solution at 25°C (Aber Instruments Ltd, 1991). To place this in perspective, all of the conventional defined fermentation media used in this work (detailed in Section 3.2) had to be modified to allow the use of the BM in these fermentations. The industrial fermentations were exclusively complex media fermentations, did not have high concentrations of fine chemicals and were not outside the conductance operating range of the BM.

Another problem in the practical use of the BM is what is termed crosstalk. While not caused by conductance, it is strongly effected by changes in conductance and should be corrected for. Crosstalk can be regarded as an artefactual contribution to the capacitance measured by the BM caused by the electronics of the instrument itself. The magnitude of this phenomena is related to the conductance of the solution being measured and as such will change if the conductance changes significantly. This in turn will cause a change in the capacitance readings and adversely effect the biomass estimate based on capacitance. As the crosstalk is

caused by the electronics of the instrument, any calibration of this effect must be carried out using the correct instrument. Section 3.1.6.5 gives details of how to correct for this effect.

2.5. The use of capacitance measurement in biotechnology

The BM has been commercially available since 1989. It is the most used instrument for capacitance measurement for fermentation monitoring and similar applications. The initial versions of the instrument were little more than working prototypes and the problems with these early instruments has caused potential users to be sceptical of the usefulness of the instrument available now. However, the improvement in the reliability and ease of use of the BM has led to work being published on its use. Considerably more work than that discussed in the review below has been carried out, especially in the brewing industry but often this work is of a commercially sensitive nature and thus is not published. The next section of this introduction is a review of the work published in using capacitance measurement in biotechnology, primarily, but not exclusively using the BM . It is arranged according to species measured and to specialised applications.

2.5.1 Yeast

The majority of work using capacitance measurement in biotechnology has been carried out on yeast-based applications. This is primarily because yeast suspensions are ideally suited for monitoring with this device as the cells are large spherical, and thus have a large specific capacitance and generally give an easily detectable capacitance reading in culture. ,

Industrial fed-batch fermentations of the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* have been monitored using the BM (Fehrenbach *et al.,* 1992). The biomass was measured off-line in samples from these fermentations by OD, and plate counts to give colony forming units per mL (CFU). For both organisms, good agreement was noted between the rises in both capacitance and the other measures of biomass during the fermentations. The *S.cerevisiae* fermentation gave a peak rise of 35pF at 4.5 $x10^9$ CFU, while the *P.pastoris* gave a peak of 14.5pF at $2.75x10^{10}$. A specific capacitance of 0.6pF/g/L for *S.cerevisiae* and 0.17 of

P.pastoris was determined from these results. The difference in the specific capacitance values was attributed to the differences in cell size, *S.cerevisiae* having a mean diameter of around 4μm and *P.pastoris* of around 2μm. Plotted against fermentation time, the specific capacitances values for *S.cerevisiae* were constant during the majority of the fermentation, then declined, which was attributed to a fall in average cell size towards the end of the fermentation. The authors also noted oscillations of around 1-2pF in the signals from the *S.cerevisiae* fermentation at 20L scale due to changes in gas hold-up, which were caused by oscillations in agitation speed due to a poorly tuned PID controller. This was noticeable because the changes in gas hold-up were proportionally large in the 20L vessel and thus effected the conducting volume of the culture. The same paper also showed work carried out on filamentous organisms, which is discussed later.

Another device has been used to measure capacitance on-line in fermentations. (Mishima *et al.*, 1991a). The device was developed by the Kobe Steel company and will be referred to in this work as the Kobe Steel Capacitance Probe (KSCP). The device was used on-line in a *S.cerevisiae* batch fermentation at 10L scale and off-line, resuspending the yeast in NaCl solutions. Good correlations between capacitance and dry weight and viable cell concentration were found both during the growth phase of the fermentation and with the off-line measurements. Further work has been carried out with this device by Matanguihan *et al.* (1994) obtaining similar correlations monitoring *S.cerevisiae* on-line in a batch fermentation.

As discussed at the beginning of this review, the brewing industry has used this technique extensively but has published little. Three papers have been published on the evaluation of capacitance measurement in the brewing industry. Boulton *et al.* (1989) showed that capacitance measured by the BM was linear with dry weight, wet weight and cell number over a range from 0-100g/L dry weight for a selection of brewing strains of yeast suspended in KCl. It was noted that the specific capacitance (pF/g/L wet weight) differed by up to 50% depending on the strain used. The results from this work were sufficient to lead to a proposal that capacitance could be used as the criteria to determine yeast pitching rates in brewing and it is thought that such a scheme is now in regular use in at least one major brewery. Kronlof (1991) also found good correlations between capacitance measured for suspensions of brewing yeast strains in KCl and the other measures of biomass used; dry weight, wet weight, optical density and cell number, with wet

weight giving the best correlation. It was also noted that capacitance correlated well with viable biomass though not with methods to determine "vitality" (a brewers term reflecting metabolic activity) as measured by ATP and glycogen levels. Work was also carried out using immobilised yeast, which is discussed later. Austin *et al* (1994) used the BM on-line to monitor the growth of a brewing yeast strain in an agitated bioreactor. The fermentation was sampled throughout the run and the dry weight and percentage viability of the samples determined. Plots of capacitance against viable biomass concentration, calculated by multiplying the dry weight by the viability, gave excellent linear correlations for data throughout the run. Work was also carried out using the BM to control yeast levels in the fermenter, this is discussed below in the section concerning applications of the instrument.

2.5.2 Bacteria

There is very little published work on using the BM in bacterial cultures, although it is known that a number of studies have been carried out. It is presumably because of the sensitive nature of these fermentations that the data has not been published. However, batch and fed-batch fermentations of a recombinant *E.coli* have been monitored on-line using the KSCP (Mantaguihan *et al.,* 1994). Good correlations between capacitance and dry weight, OD and VCC were found during the growth phases of these fermentations. In the batch fermentation, a decline in capacitance and VCC was observed after the carbon source was exhausted, supporting the correlation between capacitance and viability.

2.5.3 Filamentous organisms

An industrial fermentation of a filamentous bacteria *Streptomyces virginiae* has been monitored on-line using the BM (Fehrenbach *et al.,* 1992). A good correlation was found between capacitance and packed mycelial volume (PMV) and dry weight during the growth phase of this fermentation, with a peak capacitance of 10.5 pF. Replotted, the data showed that the specific capacitance constant (Cs) declined over time during the fermentation, starting at around 0.5pF

and falling to 0.4 by the end of the fermentation, possibly indicating cell lysis and morphological changes.

Work by Sarra *et al.* (1996) showed that using the Biomass Monitor on-line in fermentations of *Saccharopolyspora erythraea* grown in submerged culture gave a good correlation between biomass, measured as dry weight, and capacitance during the growth phase of the cultures and a separate correlation between the two measures during the decline phase of the culture. The decline phase measurements also showed that at higher agitation speeds, the decline in capacitance was faster indicating the increased cell lysis commonly associated with high agitation speeds in filamentous fermentations.

Suspensions of the fungal organism, *Aspergillus niger*, were harvested and then, resuspended at varying biomass concentrations in their supernatant (Mishima *et al.*, 1991b). The capacitance of these suspensions were measured using the KSCP and found to be linear with biomass concentration. Similarly, working off-line using samples harvested from a fermentation vessel, Ciureanu *et al.* (1997) found linear correlations between capacitance and dry weight for a strain of Streptomyces. This work reported specific capacitance values, calculated against dry weight, of 0.9pF/g/L.

Capacitance has also been used to monitor biomass accretion in sold-substrate fermentations of filamentous organisms. The growth of the filamentous fungus *Rhizopus oligosporus* on a mixture of soya beans, lupins and quinoa to produce the fermented food product, tempe, was monitored on-line by capacitance measurement (Penazola *et al.*, 1991, Davey *et al.*, 1991). The hyphal length of the filamentous organisms was also measured by image analysis on samples from the tempe fermentation. Good correlations were found between capacitance and hyphal length during the growth phase of these fermentations. After inoculation, both the capacitance and the hyphal length increased rapidly to a peak after around 24 hours. The peak rise in capacitance was around 100pF for the fermentations monitored. After the growth peak, the capacitance declined and the rate of increase in hyphal length slowed markedly, associated with a rise in pH indicative of hyphal lysis. The problems in obtaining any biomass measurement in solid-substrate fermentation are obvious and the success of capacitance measurement in this system is very encouraging in that it shows that capacitance measurement works

both with filamentous organisms and on solid-substrates to give a biomass related signal.

2.5.4 Applications

The use of capacitance measurement not just to monitor but to control the biomass level in a fermentation is the most obvious ultimate goal of the use of the BM. Markx *et al.* (1991) devised a continuous culture system where the simple (on/off) control of the media feed pump (set at a fixed rate) was derived from the capacitance measurement (converted to its equivalent permittivity) from a probe in the fermentation vessel. The organism grown was baker's yeast and the fermentation was carried out anaerobically in a complex medium with a fixed agitation rate of 500rpm. The "permittistat" culture used the addition of fresh media to maintain the permittivity setpoint. It showed a good level of control, maintaining the permittivity level in the vessel within a hysteresis of between 0.1-0.2 pF of the required setpoints, which were varied throughout the fermentation. Correlations between permittivity and dry weight and OD were noted and a capacitance of 1.93 pF per g/L dry weight and 0.93 pF per OD unit calculated. This work shows that it is possible to use the BM as a control parameter, albeit under restricted conditions, and with yeast. Similar work carried out by Austin *et al.* (1994), in reaching and maintaining a capacitance set-point by using capacitance to control a pump-rate in a cyclic reactor vessel support these conclusions.

Another specialised application of capacitance measurement has been in examining solvent toxicity. The use of often toxic organic solvents in microbially catalysed biotransformations is now a common practice in this expanding field of biotechnology. The fact that capacitance gives a measure of the amount of viable biomass has been used to give on-line indications of the toxicity of the solvent being used to the cells being monitored. (Stoicheva *et al.*, 1989, Salter and Kell, 1992). Work by Markx and Kell (1995) has combined these two specialised applications of the BM to both assess solvent toxicity and then attempt a continuous culture (permittistat) selection process to select for more solvent tolerant yeast cells by stepwise addition of increasing concentrations of the solvent of interest, benzaldehyde.

The immobilisation of cells in or on porous material is now a common technique in biotechnology and gives the same problems as growth on a solid-substrate as regards biomass measurement. Work in monitoring biomass by capacitance measurement in immobilised yeast cultures has been successfully carried out using the BM by Salter *et al.* (1990) and Kronloff (1990) and by Mishima *et al.* (1991b) using the Kobe Steel Capacitance Probe (KSCP). Mishima *et al.* also measured biomass related capacitance in mammalian cell cultures immobilised on microcarrier beads.

The application of capacitance measurement to the estimation of biomass in mammalian cell fermentations has until recently been limited. This is primarily due to the high conductance of the media typically used to cultivate mammalian cells. Davey *et al.* (1988) have shown that the technique can be applied to mammalian cells after harvesting and resuspending in buffer to remove the media and found good correlations between the dielectric increment and both volume fraction and cell number. Mishima *et al.* (1991b) used the KSCP to monitor the growth of two types of mammalian cell off-line, one on microcarrier beads, and found good correlations between capacitance and cell number from haemocytometer cell counts.

Following on from these preliminary works, the increasing use of mammalian cell fermentations has resulted in more work being carried out on using capacitance measurement in mammalian cell fermentations. Cerckel *et al.* (1993) have shown that it is possible to obtain correlations between off-line capacitance and mammalian cell number for CHO and HeLa cells by constantly correcting for the changes in medium conductance and the resultant changes in capacitance, caused by crosstalk. Degouys *et al.* (1993) have shown that using similar techniques to Cerckel *et al.* (1993), the adhesion of HTC cells to microcarriers can be demonstrated. The difficulties in measuring capacitance on-line in mammalian cell fermentations have been overcome in the work of Noll and Biselli (1998). Through careful selection of the optimum measuring frequency, interesting results have been obtained monitoring hybridoma fermentations on-line in suspended and immobilised batch culture. In the suspension culture, the capacitance measurement gave a growth-related profile which slightly anticipated the VCC growth profile and showed a marked decline at the end of the fermentation, correlating with the fall in cell density and viability. The specific capacitance (calculated per 10^5 cells)

showed a good correlation with the intracellular nucleotide concentration, which is a good indicator of cell metabolic activity. This work also showed that, in common with the work of Degouys *et al.* (1993), capacitance can be used to monitor the adhesion of mammalian cells to microcarriers. In this case, the monitoring was done on-line and was extended to included the monitoring of the growth of the adherent cells in culture. Only an approximate correlation with VCC was obtained in this work. Similar to the previous experiment, measurements of metabolic activity, in this case glutamine consumption rate showed an excellent correlation with capacitance This correlation was used in subsequent experiments to allow optimised control of the glutamine feeding rate, based on the on-line capacitance measurement. This was used successfully and allowed closed loop control which gave a constant glutamine concentration of 0.48 mmol/L in the vessel. These results show the utility of capacitance measurement, and also indicate that the monitoring of capacitance could provide more information than biomass levels, under the correct conditions.

The latest extension to the use of the Biomass Monitor has been in the monitoring of insect cell fermentations. Zeiser *et al.* (1999) have reported using on-line capacitance monitoring in submerged cultures of Sf-9 cells and in Sf-9 infected with baculovirus expressing β-galactosidase. They found that capacitance, expressed as relative permittivity, had linear correlations with VCC during the growth phase of the culture and with measured biovolume throughout the culture. This was found to be consistent for both infected and non-infected cells. In the infected culture, changes in permittivity correlated with changes in biovolume associated with the progress of infection and allowed on-line tracking of the infection of the culture with baculovirus.

2.4.5 Conclusions

The work reviewed shows that the application of capacitance monitoring in biotechnology has been mostly of a preliminary nature. Despite this, some more specialised and interesting application are now being carried out and are starting to be reported.

The experimental work carried out in this project was begun before all but the very earliest of these publications appeared so some of this work is similar in content to

what is now published. This work has sought to examine the relationship between capacitance and biomass more closely to aid the further use of this technique in routine fermentation monitoring, and ultimately control.

2.4.6 Rationale behind the practical work

In order that the utility of the Biomass monitor be tested under the full range of conditions it may be required to work in, a number of different organisms and fermentations were chosen. Representative yeast, bacterial and fungal organisms were grown under a variety of conditions, media and scales to examine the effects of organism, media, scale and mode of fermentation on the accuracy and utility of the capacitance measurement obtained. To examine the accuracy of the viable biomass measuring capabilities of the Biomass Monitor, disruption of the organisms using homogenisers was used to simulate loss of culture viability.

As is outlined above, the most successful use of the Biomass Monitor has been in work with yeast. The yeast chosen for the experiments in this work was *Saccharomyces cerevisiae.* Packed yeast from the Distillers Yeast Co. Ltd. (UK) was used as an easily available test organism and *S.cerevisiae* GB4918 also from the Distillers Yeast Co. Ltd. (UK) was used for the yeast fermentations as it was the organism which was most closely related to the yeast in the packed yeast supply. To examine the effects of cell size and media solids, experiments were carried out using MC1, a yeast which is smaller (4μm) than the other yeast used, grown in a complex media containing undissolved solids

As this work was industrially sponsored, the choice of the other organisms in this work reflects the various interests of the companies sponsoring the work. *Pseudomonas putida* ML2 was chosen as the representative bacterium, as this was of interest to Zeneca. *P.putida* is a gram negative rod, approximately 0.5 by 1 μm. The small size of this organism allowed a test of the ability of the Biomass Monitor to detect smaller total cellular volumes shown by smaller organisms in fermenter culture.

SmithKline Beecham's interest in this work meant that *Penicillium chrysogenum* was the representative fungal culture examined. This was grown at small scale at UCL under batch fermentation conditions using a fully defined medium. The results of these experiments were then compared with pilot and production scale

fed-batch fermentations of an industrial strain of *Penicillium* grown on an industrial complex media.

As a further examination of the effect of cell size and morphology on capacitance, an industrial strain of the filamentous bacterium, *Streptomyces* sp. was grown in fed-batch mode on a complex media at pilot scale while working at SmithKline Beecham.

The next section gives details of the materials and methods used in this work. In particular, it describes in detail the methods developed to obtain reliable results from the BM both off-line and on-line.

3. MATERIALS AND METHODS

3.1 Biomass Monitor

The Biomass Monitor (Aber Instruments, Aberystwyth, U.K.) is a sensitive capacitance and conductance measuring system, developed primarily for use in fermentation vessels. It measures the capacitance and conductance of a suspension using a constant voltage, 4-terminal, phase-sensitive detector system. This consists of a radio-frequency probe (either 25mm Ingold type or 19mm long type designed for top entry) which is inserted into the vessel and is steam sterilisable. Attached to the non-vessel end of the probe is a head amplifier which boosts the signal obtained from the four gold terminals embedded in the end of the probe and sends this signal to the instrument.

The instrument has a number of features which control the measurements made and the signal received. It allows the measurement frequency to be set between 0.2 and 10 MHz. The instrument has two ranges, "hi" and "lo", which allow measurements to be made at either 0-10 mS or 0-20 mS. The available ranges are expressed as the conductance units mS as the ability of the instrument to operate in a given fermentation depends on the conductance of the fermentation medium being within this range The range setting also affects the capacitance signal, with the signal on the hi setting being approximately half that on the lo setting The output capacitance signal can be viewed on the LED display and recorded on a external device as: capacitance; Δcapacitance, using the facility which allows the background signal to be offset or g/L, if the system being monitored has been sufficiently well characterised. The mode of measurement can be either single or dual frequency. The latter means that the device measures both at the low frequency set by the operator and at 9.5 MHz, the factory set high frequency and that the Δcapacitance readings are the capacitance at the low frequency minus the capacitance at the high frequency. The capacitance display and output can be made subject to a response time filter to stabilise noisy signals. This can be set to either zero, 1 or 5 seconds. Typically, the 5 second filter would be used in a stirred and agitated fermentation, while 1 second or zero are used for off-line work. A bipolar cleaning pulse can be applied across the measuring pins to dislodge any adhered

material. It can be applied either manually or automatically using the built-in timer. One application of the cleaning pulse is usually sufficient to dislodge small particles and to decrease changes in probe performance due to electrostatic charge build-up on the measuring pins. In filamentous fermentations, if the pins become tangled with mycelia then the cleaning pulse is not capable of dislodging the adhered mycelia and the probe signal will be adversely affected. In practise, this has only happened on one occasion out of around twenty mycelial fermentations carried out in the course of this work.

3.1.1 Biomass Monitor off-line set-up

The probe is primarily meant for operation in fermentation vessels (on-line). However, a variety of tests and calibration procedures should be undertaken off-line. Figures 3.1a and 3.1b shows the typical off-line set-up for both 19 and 25mm probes.

It is important that the probe is immersed 6cm into the suspension and that the measuring terminals are at least 3cm away from the bottom and sides of the container. The suspension should be gently mixed, typically by a magnetic stirrer and flea.

The probe should be positioned so that the terminals are not in any vortices caused by the mixing. It is necessary to earth the probe to the magnetic stirrer by means of the earth wire attached to the head amplifier to avoid electrical interference. When the procedure being carried out involves removing the probe and then replacing it to repeat measurements, it is very important that the probe is replaced to exactly the same position.

The Duran bottle set-up adopted for use with the 25mm probe was found to be excellent in this respect as the tightening of the cap with the probe inserted through it ensured that the probe was located exactly every time allowing comparisons to be made with a greater degree of confidence that all probe variables are the same. The Duran bottle set-up is temperature controlled as capacitance and conductance are very temperature dependent and the time taken for measurement can sometimes allow temperature fluctuations to occur and interfere with the accuracy of the signal. The routine use of this procedure allowed accurate off-line measurements to be made.

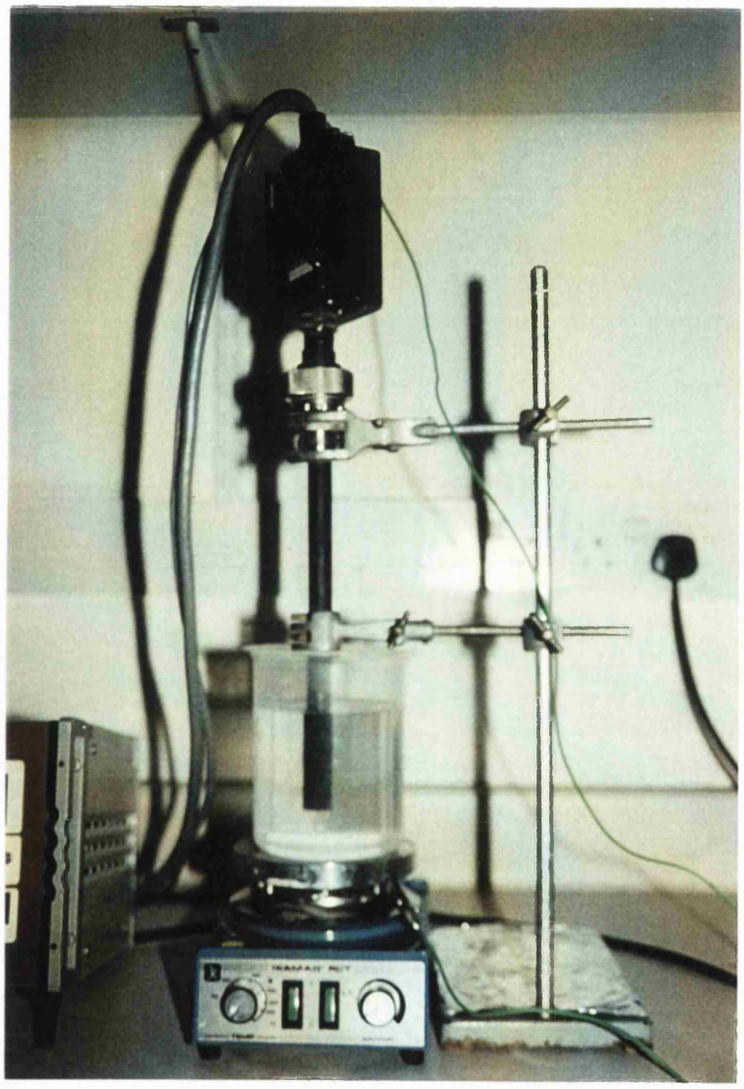
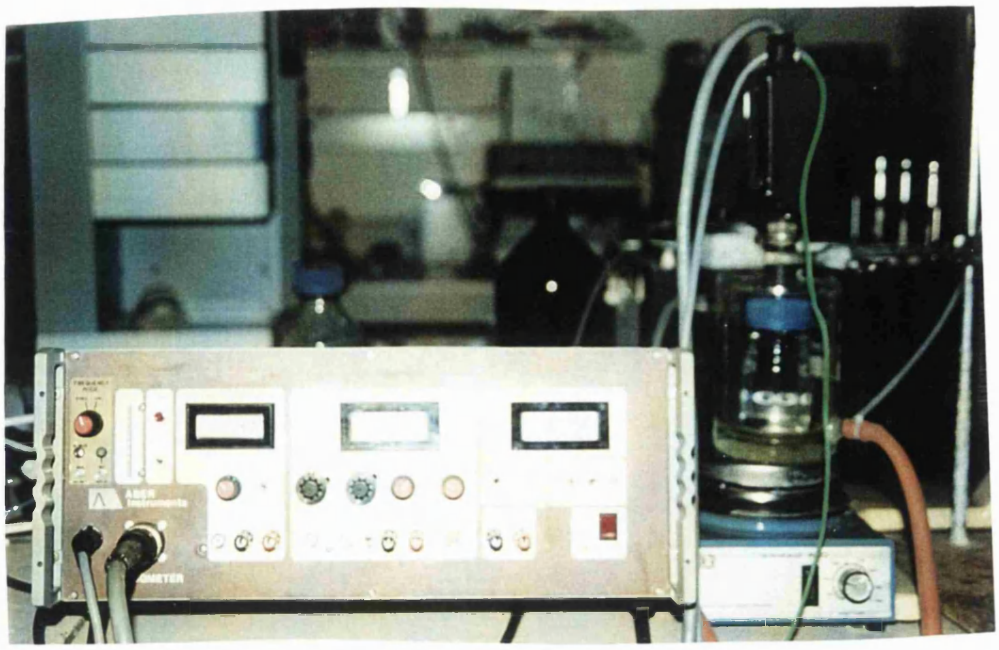


Figure 3.1a: Off-line set up of 19mm probe



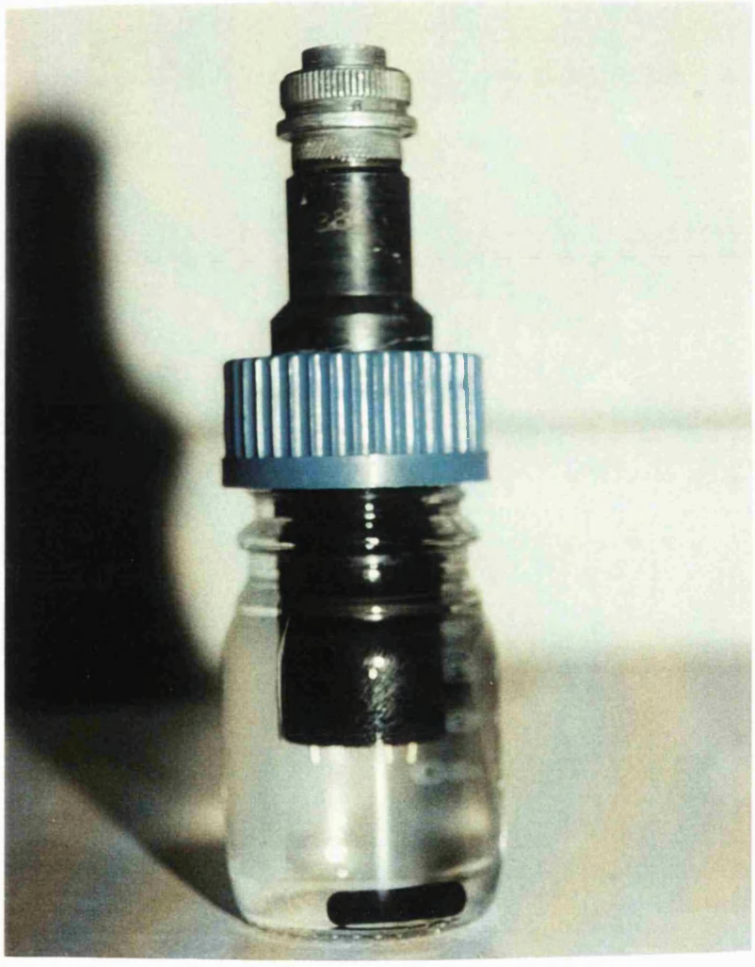


Figure 3.1b: Off-line set up of 25mm probe

3.1.2 Biomass Monitor cell constant determination

Capacitance and conductance as measured by the Biomass monitor are relative measurements which are dependent on the electrode configuration and geometry of the probe used. To allow comparisons to be made with measurements taken using other probes or equipment, capacitance and conductance must be converted to their independent variables, permittivity and conductivity. These are related to the capacitance and conductance as measured by the probe by a Cell Constant which is attributable to the measuring system used. Equations 2.2. and 2.1. show the relationship.

$$\sigma' = G K \qquad \textbf{Equation 2.2}$$

σ' = conductivity in Siemens per metre

G = conductance in Siemens (as measured by Biomass monitor)

K = Cell constant of Biomass monitor electrode system in metres^{-1}

$$\varepsilon' = \frac{CK}{\varepsilon_0} \qquad \textbf{Equation 2.1}$$

ε' = relative permittivity (no units)

C = capacitance as measured by Biomass monitor in Farads

K = Cell constant in metres^{-1}

ε_0 = permitivitty of free space (physical constant equal to 8.854 x 10 $^{-12}$ Farads per metre)

The reason that ε' has no units (and why it is called relative permittivity) is that it is the ratio of the capacitance of the standard electrode in the material of interest divided by the capacitance of the electrode in a vacuum.

To calculate the Cell Constant of an electrode set up, the conductance of a known ionic solution must be measured with the electrode set-up of interest and then this must be compared with standard conductivity measurements for that solution at the temperature used for measurement. Aber Instruments recommend using 0.1M KCl for this purpose and provide tables of known conductivities of this solution at a range of temperatures to enable the calculation of the Cell Constant of a Biomass monitor set-up.

3.1.2.1 Method used for cell constant determination

Using the set-up shown above in Figure 3.1, a 1M KCl was made up by dissolving 74.56g of Analar grade KCl (BDH) in 1L of reverse osmosis (RO) water. An appropriate dilution of this stock solution was made with RO water to give 1L of 0.1M KCl solution. This was placed in the beaker and the probe was immersed to a set depth and orientation in the solution, which was gently stirred. A thermometer was used to determine the temperature of the solution throughout the procedure. The instrument was set on the scale of interest (either hi or lo) and this was noted, the display outputs were set to capacitance and the conductance and capacitance at the frequencies of interest were measured. The temperature of the solution at the time of measurement was noted. Using the tables provided in the Biomass Monitor manual, the conductance measured can be converted to conductivity at the temperature used.

Thus if we rearrange Equation 3.1 to make the Cell Constant, K the subject,

K= conductivity/conductance measured.

The Cell Constant should be calculated for the frequency at which biomass measurement is to be carried out. As well as enabling comparisons to be made with measurements carried out using other electrode configurations, it is useful to check the Cell Constant on a regular basis as changes in the position of the

measuring terminals of a probe can change the Cell Constant of the Biomass Monitor set-up used, which in turn will change the capacitance and conductance apparently measured.

3.1.3 Frequency scans

Frequency scans entail measuring the capacitance and conductance across the full frequency range of the Biomass Monitor. They can be carried out in any situation where the Biomass Monitor is used. They yield more information than spot capacitance measurements and are of great use in understanding the dielectric nature of any suspensions of interest. They can be carried out manually or automatically.

3.1.3.1 Manual frequency scans

Manual frequency scans are done by setting the mode switch to single frequency, the display to capacitance and the time filter to off. The frequency is initially set to 0.2 MHz, the time filter is switched to 5s and the capacitance and conductance values noted. The time filter is then switched off and the frequency adjusted to 0.3MHz, the time filter is switched back on and the capacitance and conductance readings taken. This routine is repeated for 0.2-1MHz in 0.1 increments, then from 1-8 in 1MHz increments, then finally at 9.5 MHz, the factory-set high frequency for dual frequency scanning The capacitance and conductance values are then plotted against frequency (with frequency plotted on a logarithmic scale) to give the dielectric spectrum of the suspension.

3.1.3.2 Automatic frequency scans

Automatic frequency scans can be carried out by computer. The Biomass Monitor frequency control was switched to external and the instrument was connected to a Zenith Data Systems 286 personal computer by a bi-directional RS232 link. A program BMSCAN (donated by Dr C.L. Davey, University of Wales, Aberystwyth, U.K.) which has been developed for Aber Instruments was used. This program both controls the frequency at which the Biomass Monitor makes its

measurements and logs the frequency, capacitance and conductance values from the instrument's outputs. The program can increase the frequency sequentially, or set it randomly from a predetermined group of values designed to give an accurate spread across the logarithmic frequency values. The results can then be displayed either as the figures or as a graph of capacitance or conductance versus frequency, with frequency plotted on a logarithmic scale. The program can be controlled so that it scanning is initiated automatically using a timer or manually by the operator.

3.1.4 Biomass Monitor off-line calibration

This method can be used both as a classic calibration and as another check on the integrity of the signal from a given Biomass monitor set-up. Using the beaker set-up shown above in Figure 3.1, 1L 50mM (pH 6.7) phosphate buffer was added and allowed to equilibrate. The temperature of the solution was set to 26°C. The capacitance display and outputs were switched to Δcapacitance and the capacitance of the buffer was backed off to zero. The range and measuring frequency were then noted to allow comparison to be made with other experiments performed at a later date. Packed high activity baker's yeast (*S.cerevisiae,* Distillers Yeast Company Ltd, Menstrie UK) was added in 20g (wet weight) aliquots to a final concentration added of 200g/L (wet weight). Each aliquot of packed yeast was allowed approximately five minutes to disperse before the Δcapacitance, conductance and temperature readings were taken. Subsequent readings were taken after the next aliquot of yeast was added.

A plot of Δcapacitance versus yeast added should give a linear correlation and act as a calibration curve if the organism sequentially added in the above experiment is the one to be monitored on-line. If the above experiment was performed using the same instrument and set-up at a later date then the calibration curve obtained should be very similar. If this is not the case then a full check on the instrument and set-up should be carried out to determine the source of the error. As discussed above, any change in the electrode geometry can have significant effects on the Cell Constant and thus the capacitance as measured by the probe and this is the most likely source of error.

In this way this experiment can act as a check on the functional properties of the Biomass Monitor set-up being used. This method could be extended to become a

calibration with viable biomass by carrying out viable cell concentration measurements on the suspension after each capacitance measurement; however this is beyond the requirements of a routine performance check.

3.1.5 Biomass Monitor off-line media and organism tests

To determine the suitability of a growth medium and organism for use with the Biomass Monitor, it is advisable to test them in the off-line set-up described in Figure 3.1 above. Frequency scans were made as described above in Section 3.1.3.1.

Initially the scans were carried out on the uninoculated medium at the temperature and pH to be used during growth. KCl of the same conductance as the medium was then made up, and another frequency scan carried out A plot of capacitance against log frequency from these two experiments show whether the media had any capacitance above that of KCl in the low frequency region where biomass measurements are to be made. The most common problem with fermentation medium affecting the use of the Biomass Monitor is that the conductance of the medium is too high to allow the instrument to operate. Defined media containing large amounts of salts have a high ion concentration which gives them a high conductance. The Biomass Monitor signal is more prone to noise and crosstalk beyond 19mS and cannot operate in media with a conductance greater than 20mS.

If the medium was suitable, the frequency scans were then carried out on fermentation samples, preferably fresh, of both uninoculated media and media containing biomass of the organism of interest. Ideally these samples should be from a range of time throughout the fermentation. If this is not possible then the best sample to use is one from the point of maximum biomass of the fermentation. Plots of the capacitance of the cell suspensions and the uninoculated media against log frequency will indicate the best frequency to use for biomass monitoring.

3.1.6 Biomass Monitor fermenter set-up.

As mentioned above, the probes supplied for the Biomass Monitor come in two sizes. The 25mm probe is a standard Ingold type device, designed for entry through ports either at the side or on the bottom of the vessel, protruding

approximately 15cm into the suspension medium. The 19mm version is considerably longer (30cm) as it is designed to enter through ports on the top plate of the vessel and thus must pass through the vessel head-space in order to be fully immersed in the suspending medium. In operational aspects, the two types of probe are intended to be identical.

3.1.6.1 Probe installation

Prior to attempting to place the probe into a vessel, it is necessary to examine the internal and external arrangement of the vessel.

Internally, it is important that the probes' measuring terminals are at least 5 cm from the vessel wall, any baffles, sample tubes or other metal protuberances. It is also important that the probe does not sit in any vortices caused by the vessels impellers or in a zone of very high aeration caused by spargers. The positioning of the probe in any of these situations will result in interference in the signal, usually to the extent of it being meaningless for the purposes of biomass detection.

Externally, the problems to be aware of stem from the head amplifier, which connects with the top of the probe It is quite large (8 x 5 x 4 cm) which can cause problems when using small vessels in cramped conditions. It is also important to ensure that the connection is secure and that the head amplifier will not be disturbed during the routine operations of the fermentation. The head amplifier is an important part of the instrument and both is not waterproof and is sensitive to temperature fluctuations and draughts. Thus some thought should be given to its positioning to avoid potential problems and sources of error.

3.1.6.2 Probe sterilisation

3.1.6.2.1 *In-situ*

Once the probe is securely installed in the vessel and the connections at the top of the probe have been covered with cotton wool, aluminium foil or some similar waterproof cover, the vessel and the medium can be sterilised in the normal way.

3.1.6.2.2 Chemical

If the vessel is sterilised separately from the medium in an autoclave then it is necessary to install a blank plug in place of the probe and replace the separately sterilised probe aseptically after sterilisation. In this case the probe can be sterilised chemically using a suitable chemical sterilisation technique. Immersion in Kloroxide for 30 minutes, then rinsing with sterile water has been used successfully in the course of this work, although sterilisation *in-situ* is the preferred method.

3.1.6.3 Instrument set-up

When the vessel has cooled to the temperature to be used for growth, the head amplifier can be connected. The medium pH should then be adjusted to that to be used at the start of the fermentation. Under ideal conditions the probe should be allowed an hour to equilibrate before the set-up procedure is carried out. Ensure that the temperature and pH are correct before commencing. Turn off the aeration, wait for a period to allow the bubbles to clear and then turn off the agitation. If it is not possible to turn these off completely then turn them to their lowest possible settings. Once the medium has stabilised at the operational temperature and pH, if dual frequency mode is to be used switch the mode to dual frequency. Use a micro-screwdriver to set the "set medium control" so that the bi-colour L.E.D. is either colourless or flashing red/green. Set the measuring frequency to the value determined previously (see Section 3.1.5). Switch the display and output to Δ capacitance and adjust the offset control so that the display reads 0.0pF $\pm$ 0.2 pF. It may be necessary to set the time filter to 1s to make this easier. Switch the agitation and aeration back on and set to the level to be used in the fermentation and turn off the time filter to allow any changes to occur quickly. Allow around 30 minutes for the instrument to adjust to the aeration and agitation levels. Check the Δcapacitance display. If this has changed significantly from the value set previously, reset back to 0.0pF $\pm$0.2pF using the Gain Balance control. Switch off the aeration and agitation and check if the display is still 0.0pF, $\pm$0.2pF. If not, start again at the set medium stage until set-up is completed correctly.

3.1.6.4 Corrections for changes in agitation

As the primary method of controlling dissolved oxygen (dO_2) in fermentation is by manipulating the agitation speed, it is necessary to look at the effects of changes in agitation on capacitance. The Biomass Monitor and the fermentation were set-up as described above in a 5L fermentation vessel. The mode was set to single frequency and the capacitance displayed set to capacitance, with no offset used. The aeration was constant at 0.5 VVM. The agitation was set to 100rpm using the TCS controller of the fermenter. The capacitance was allowed to settle down for five minutes and then the capacitance and conductance values were noted. The agitation was then set to 200rpm and again the capacitance was allowed to change for five minutes before being noted. This routine was repeated for 100-1000 rpm at 100rpm intervals. Capacitance and conductance was plotted against agitation speed, and this was used as calibration curve to correct for changes in capacitance caused by changes in agitation for this set-up in this vessel under the fermentation conditions. Figure 3.2 shows an example of a calibration graph of agitation versus capacitance for a 19mm probe in a 5L working volume fermentation vessel.

3.1.6.5 Crosstalk corrections

The inter-related nature of capacitance and conductance means that changes in conductance can cause changes in capacitance during the course of monitoring a fermentation. This Crosstalk, as it is known, is only of major significance when the measured Δcapacitance values are small. It can also occur in situations where the instrument is operating at the extremes of its range, *i.e.* in high conductivity media.

To determine the extent of this crosstalk, the 5L fermenter of interest was set-up as described above with all parameters being identical to those to be used for microbial growth, including agitation and aeration. The capacitance and conductance measurements were set-up as if growth was to be monitored, as described above. The growth medium conductance was increased in increments of 0.5 mS to around 15mS by addition of KCl. The highest and lowest capacitance values at each point in the conductance scale were noted and averaged, and the averages plotted against conductance to give a calibration curve.

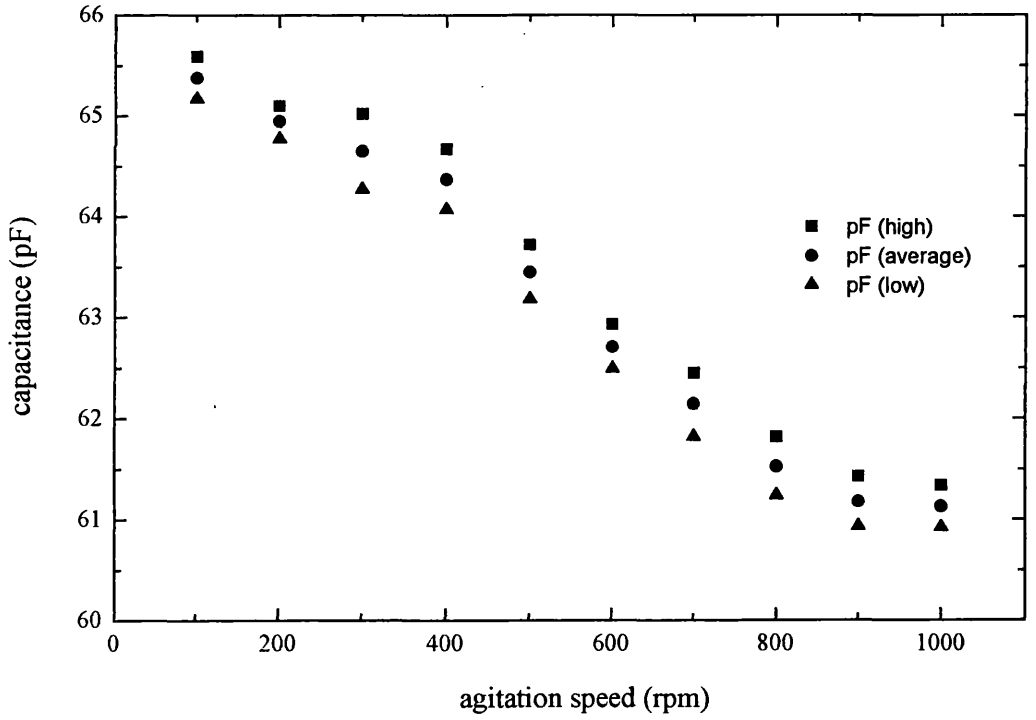


Figure 3.2: agitation speed versus capacitance (pF)
5L working volume fermenter
P. putida ML2 media
0.5 vvm airflow

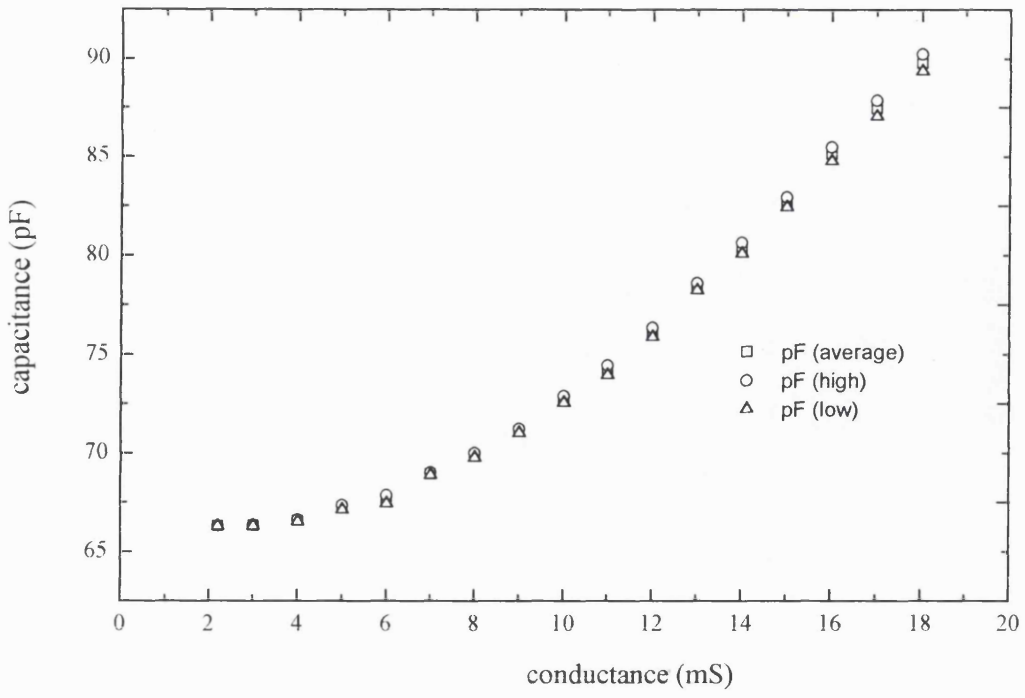


Figure 3.3: conductance (mS) versus capacitance (pF)
5L working volume fermenter
KCl solution
0.5 vvm airflow

A more generally applicable alternative of the above method is to do this experiment using RO water and simulate the full range of possible conductance values from 1mS to 20mS, noting the highest and lowest capacitance values at each conductance value. A plot of the capacitance changes resulting from the conductance changes allows the calculation of a general correction factor for use with this system. Figure 3.3 shows a typical conductance versus capacitance calibration curve for a 19mm probe in a 5L working volume fermentation vessel.

3.1.7 Biomass Monitor signal recording techniques

3.1.7.1 Manual spot readings

As the capacitance and conductance values displayed on the LEDs often have a high degree of fluctuation, especially when the instrument is in on-line use, a protocol was adopted to aid accurate readings. Any spot reading takes the form of the highest and lowest values displayed over a two minute period. The range of these readings depends on the inherent stability of the instrument, the time filter used and the noise generated by the agitation and aeration of the fermentation. This means that any value used is a mean of these two readings to improve accuracy.

3.1.7.2 Chart recording

For the majority of the time dependent experiments in this work, the capacitance and conductance output values were recorded using a chart recorder. The chart speed used for fermentation monitoring was 0.01 mm/s^{-1}, the full scale deflection of the instrument was 25pF and 20mS and manual checks were used to indicate sample points using the event marker. The values used for capacitance and conductance fermentation graphs were taken from the chart recorder data to ensure accuracy and were checked with the routine manual readings noted when samples were taken. The values taken were the average of the highest and lowest values read off the chart at suitable time intervals. Any corrections for gross changes in conductance or agitation necessary were calculated on the values from the chart and applied before the fermentation graphs were plotted.

3.1.7.3 Automatic recording

The output capacitance and conductance values for the *P.chrysogenum* fed-batch fermentations in this work were recorded using a 12-bit Squirrel Data Logger (Grant Instruments). Due to the length of these fermentations (180h), the Squirrel was setup to record the mean reading of the past 10 readings, taking a reading every minute. The data used for fermentation graphs of this work were the spot mean values every hour from inoculation.

The BMCSAN program discussed in Section 3.1.3.2 was used to monitor several fermentations. The automatic mode of the program was used. It was set to scan every ten minutes. The frequency scanned were 0.2-1.0 MHz in 0.1 MHz intervals, then 2, 4, 6, 8 and 9.5 MHz and the order of scanning was sequential. The Biomass Monitor set-up used for these experiments was as described above in Section 3.1.6 with a few changes. These were that the frequency control was external and that the time filter was switched off. The data from each of the frequency scans was stored as separate, time identified .DAT file on the hard-disk of the Zenith Data Systems 286 EQ14 computer BMSCAN contains a function which allows time series data files to be generated for a specific frequency. At the end of the fermentation, time series data files were generated for the measuring frequency (0.4MHz) and the high frequency used in dual frequency scanning (9.5MHz). These were copied on to a floppy disk to allow data processing. This allowed the production of both single and pseudo-dual frequency fermentation profiles for these fermentations.

3.2 Fermentation Protocols and Equipment

Unless specified, all fine chemicals used were obtained from BDH and SIGMA and were Analar grade or equivalent. All microbiological media were obtained from Oxoid.

3.2.1 *Saccharomyces cerevisiae*

3.2.1.1 Organism

The organism used was *S. cerevisiae* GB4918, obtained from The Distillers Yeast Company Ltd (Menstrie, UK). This organism was chosen as it is the strain used in the packed yeast experiments described earlier. The stock culture was maintained on YMB agar slopes at 5°C. When required for liquid culture, this was then subcultured on to YMB agar plates (see below) and grown at 30°C for 7 days.

Table 3.1 YMB agar

component	g/L
yeast extract	10
glucose	20
peptone	20
technical agar no.3	20
pH (pre sterilisation)	6

3.2.1.2 Inoculum preparation

The plate culture was then used to inoculate shake flasks containing the following sterile medium, adapted from Fiechter (1981):

Table 3.2

Media SC1

component	g/L
solution A	
$(NH_4)_2 SO_4$	8.9
$KH_2 PO_4$	2.85
NaCl	0.1
solution B	
glucose	30
$MgSO_4 . 7H_2O$	1.2
$CaCl_2.2H_2O$	0.15
EDTA	0.243
meso-Inositol	0.16
$FeCl_3.6H_2O$	0.1

All concentrations given above are final concentration in SC1.

Table 3.2.1

trace elements solution

component	mg/L
$ZnSO_4.7H_2O$	30
$MnSO_4.2H_2O$	32
$CuSO_4.5H_2O$	0.8
$NaMoO_4.2H_2O$	5
$CoCl_2.6H_2O$	5.6
H_3BO_4	15
KI	2

Table 3.2.2

Vitamin solution

component	mg/L
d-Biotin	0.1
Ca Panthothenate	2
Nicotinic acid	15
Thiamine-HCl	4
Pyridoxine-HCl	10

Solution A was sterilised in flasks, solution B in separate containers, both were autoclaved at 121°C for 30 minutes and added to A aseptically to give the final concentrations above. The trace elements solution was made up at x500 concentration, 10mL of 10mM citric acid was added to prevent precipitation before the solution was sterilised and stored at 4°C. Trace elements solution was added to the fermentation media aseptically to a concentration of 2mL/L. The vitamin solution was also made up at x500 stock concentration and stored at 4°C. This was added to the fermentation medium aseptically by sterile filtration through a 0.2μm pore size syringe filter to a final concentration of 2mL/L In shake flasks, the pH of solution A was adjusted to 5 with 2M H_2SO_4.

Using a sterile inoculation loop, two loopfuls of the YMB agar culture were inoculated into 125mL (final volume) of SC1 medium in a 1L flask and grown in a shaking incubator set at 200rpm and 30°C for 48 hours.

25mL of this culture was then subcultured into 2L flasks, with a final YMB volume of 250mL. Sufficient flasks were inoculated to provide a 10% (v/v) inoculum for the fermentation vessel. These were then grown for 24 hours in a shaking incubator at 200rpm and 30°C.

3.2.1.3 Fermentation

Solution A was sterilised in the vessel at 121°C and 1 bar overpressure. Aseptically, the appropriate sterile volumes of solution B, trace elements solution and vitamins solution were added once the vessel had cooled to the growth temperature of 30°C. The pH was adjusted to 5 in the vessel using 2M H_2SO_4 and maintained at this level throughout the fermentation by the addition of 2M NaOH. This and the other fermentation parameters of temperature, air flow rate, and agitation rate were controlled using the T.C.S. (Turnbull Control Systems, Worthing UK) control system of the fermenter. The Biomass Monitor was set up described in Section 2.1. The instrument was set on the "hi" range, measuring frequency used was 0.5MHz for dual frequency scanning and a 5 second time filter was used. The Δcapacitance and conductance outputs were recorded using a J.J.Lloyd Instruments CR600 chart recorder with a full scale deflection of 60 pF and 6 mS. After a suitable period to achieve a stable baseline for the Biomass Monitor, the fermenter was inoculated with the shake flask culture (10% of vessel volume). The air flow rate used was 0.5 VVM and the agitation rate was 700 rpm. The air exhaust gas composition was monitored using a VG mass spectrometer. The data from this and the other fermentation parameters such as pH and DOT were logged by a DEC PDP 11-73 computer running the fermentation monitoring software, Bio-i, from BCS (Biotechnology Computer Systems) and RTDAS (Real Time Data Acquisition Systems)

3.2.1.4 Recombinant *Saccharomyces cerevisae*

3.2.1.5 Organism

The organism used was *S. cerevisiae* MC1, kindly donated by E.H. Creaser, Department of Molecular Biology, Research School of Biological Sciences, Australian National University, Canberra, Australia. The organism was modified at the active site of the ADH-1 enzyme, which was changed by replacing Trp93 with Phe and Thr48 with Ser. This allowed the modified enzyme to work at higher alcohol levels (Murali and Creaser 1986).

The stock culture was maintained on agar slopes at 5°C. When required for liquid culture, this was then subcultured on to YMB agar plates (see above) and grown at 30°C for 7 days.

Batch complex media fermentations were carried out using this strain in the following complex medium:

Table 3.3

Complex media

component	g/L
yeast extract	10
glucose	30
mycological peptone	20
malt extract	20
PPG antifoam	0.1mL/L

3.2.1.6 Fermentations

The inoculum was prepared by autoclaving three 200mL in 2L shake flasks of the Complex media at 121°C for 30 minutes. These were then allowed to cool to growth temperature and the pH was adjusted to 5 using 2M H_2SO_4. The flasks were inoculated using 2 loopfuls of the YMB agar plates described above. These were grown at 30°C and 200rpm until an OD600 of 10 had been reached. The cultures were then pooled into a sterile inoculation flask and aseptically inoculated into the pre-sterilised 42 L fermenter.

Fermentations of this strain were carried out at 42L scale, sterilising the media *in-situ* at 121 °C and 1bar for 40 minutes. The vessel was cooled to the growth temperature of 30°C and the pH was adjusted to 5 in the vessel using 2M H_2SO_4 and maintained at this level throughout the fermentation by the addition of 2M NaOH. This and the other fermentation parameters of temperature, air flow rate, and agitation rate were controlled using the T.C.S. as described previously. The Biomass Monitor was set up described in Section 2.1. The instrument was set on the "hi" range, measuring frequency used was 0.5MHz for dual frequency scanning and a 5 second time filter was used. The Δcapacitance and conductance outputs were recorded using a J.J.Lloyd Instruments CR600 chart recorder with a

full scale deflection of 60 pF and 6 mS. After a suitable period to achieve a stable baseline for the Biomass Monitor, the fermenter was inoculated with the shake flask culture, prepared as described above. The air flow rate used was 0.5 VVM and the agitation rate was 700 rpm. The air exhaust gas composition was monitored using a VG mass spectrometer. The data from this and the other fermentation parameters such as pH and DOT were logged as described previously.

3.2.2 *Pseudomonas putida*

3.2.2.1 Organism

The organism used was *Pseudomonas putida* ML2, donated by Shell Research Ltd (Sittingbourne). This organism was used as it has the *Tol* plasmid which enables it to metabolise aromatic compounds as a carbon source. The stock culture was stored as freeze-dried stocks at -70°C. When required, the culture was revived by resuspension into Nutrient Broth (Oxoid) and used as an inoculum for the shake flask 1 fermentation described below. To screen for plasmid retention, this culture was then plated out onto Indole agar.

Table 3.4

Indole Agar

component	g/L
KH_2PO_4	1.33
K_2HPO_4	2.65
Yeast Extract	3.0
$MgSO_4.7H_2O$	0.4
Peptone	0.04
Indole	0.1
Technical agar No.3	15

Colonies retaining the plasmid were black and these were then sub-cultured onto nutrient agar plates and used as a working stock culture for 1 month.

3.2.2.2 Inoculum preparation

The working stock culture was used as an inoculum for the shake flask1 fermentation. The medium used for this comprised:

Table 3.4.1

Shake flask 1 media	
component	**g/L**
$(NH_4)_2SO_4$	1.0
$MgSO_4.7H_2O$	0.2
$FeCl_2$	0.0016
Na_2HPO_4	3.0
KH_2PO_4	3.0
$CaCl._2H_2O$	0.015
Trace Element solution	2.0mL
pH (pre sterilisation)	6.8

Table 3.4.2

trace element solution	
component	**g/L**
$CaCl_2.2H_2O$	0.66
$ZnSO_4.7H_2O$	0.18
$CuSO_4.5H_2O$	0.15
$MnSO_4.4H_2O$	0.15
$CoCl_2.6H_2O$	0.18
$H_3BO_3.H_2O$	0.1
$Na_2MoO_4.2H_2O$	0.3

100mL of SF1 media was sterilised *in-situ* in 500mL Quick-Fit flasks at 121°C and 1 bar in an autoclave. Each flask was inoculated with several colonies from the nutrient agar stock culture. 100µL of benzene was added to each flask as a carbon source and the flasks were immediately stoppered with sterile Suba-seals to prevent loss of the volatile compound.

The culture was then incubated at 28°C and 200rpm in a shaking incubator for 48 hours. After this period, the organism was then subcultured into the shake flask 2 media. The base media composition and sterilisation for this was identical to that shown above for shake flask 1 but the carbon source added (post sterilisation) was 5mL of 600 g/L fructose, to give a final concentration of fructose in the shake flask of 30g/L. The flasks were inoculated with 10mL of the shake flask 1 culture and incubated at 28°C and 200 rpm in a shaking incubator for 24 hours. The carbon source was changed to fructose to condition the inoculum for the fermentation, sufficient flasks were inoculated to ensure a 10%(v/v) inoculum for the final large scale fermentation

3.2.2.3 Fermentation protocol

The vessel used for these fermentations was a 7L glass walled fermenter (LH Fermentation, Reading U.K.) with a 5L working volume. The vessel was agitated at a fixed rate of 850 rpm using a top driven spindle carrying three 6.75 cm diameter Rushton type turbines and was sparged with air at a rate of 0.7 v.v.m. The medium used was comprised as follows:

Table 3.4.3

fermentation media

component	**g/L**
solution A:	
$(NH_4)_2SO_4$	5.0
K_2HPO_4	8.7
KH_2PO_4	6.8
polypropylene glycol	10mL
RO water	3.4L
solution B:- filter sterilise	
$MgSO_4.7H_2O$	4.6
$FeSO_4.7H_2O$	0.16
Trace element solution	7.7mL
RO water to	10mL

solution C: autoclaved

fructose	150g
RO water to	1L

The trace element solution used was identical to the one described previously. The 10mL of solution B was added into the vessel post-sterilisation through the inoculation port after being filter sterilised using a sterile 0.2μm Acrodisc syringe filter (Gelman Sciences). All solution A and B concentrations given above are final concentration in media, while the final concentration of fructose in the media was 30g/L. Solutions A and C were sterilised at 121° C for 30 minutes, A *in-situ* and B in an autoclave.

Once all the medium components were added, the pH was set to 6.8 and maintained at that level using 4M NH_4OH and 2.5M H_2SO_4.

Once the vessel was ready for inoculation, the Biomass Monitor was set up as described in Section 2.1. The Biomass Monitor set up used was a measuring frequency of 0.4MHz, single frequency mode and a time filter of 5 seconds. The Δcapacitance and conductance output were recorded using a J.J.Lloyd Instruments CR600 chart recorder with a full scale deflection of 25 pF and 6 mS. After a suitable period to achieve a stable baseline for the Biomass Monitor, the fermenter was inoculated with 10% (v/v) of shake flask culture.

The air exhaust gas composition was monitored using a VG mass spectrometer. The data from this and the other fermentation parameters such as pH and DO_2 were logged as described previously and samples were taken at regular intervals for dry weight, optical density and viable cell concentration measurements.

3.2.3 *Penicillium chrysogenum* defined media batch fermentation

3.2.3.1 Organism

Penicillium chrysogenum P1 (School of Biotechnology, University of Westminster) was used in these fermentations. The culture was originally developed by Panlabs Inc. The strain is capable of Penicillin-V production using phenoxyacetic acid (POA) as a precursor.

3.2.3.2 Spore production and storage

Table 3.5.1

spore production medium

component	g/L
glycerol	7.5
molasses	2.5
yeast extract	1.0
$MgSO_4.\ 7H_2O$	0.05
KH_2PO_4	0.06
bacteriological peptone	5
NaCl	10
$FeSO_4.\ 7H_2O$	0.003
$CuSO_4.\ 5H_2O$	0.001
Technical agar No.3	20

The medium components excluding agar were dissolved in RO water and the pH was adjusted to 6.7 with 2M NaOH. 100mL of the liquid medium was dispensed into 500mL mediflat bottles together with 2g of agar. The mixture was shaken vigorously and autoclaved at 121°C for 25 minutes. The bottles were lain flat to cool to allow a flat agar lawn to form. Each bottle was inoculated with 0.5mL of the stock culture spore suspension. The bottles were then incubated for 10 days at 26°C, after which the agar lawn was covered with green spores.

The spores were harvested with sterile glass beads and a 20 ml harvesting solution which comprised : sucrose, 20% (w/v) Tween-80, 0.1% (w/v) dissolved into phosphate buffer saline solution at pH 7.0. The buffer solution was made by dissolving one tablet of phosphate buffer saline (Oxoid Ltd) in 100mL of RO water. The spores suspension was harvested and dispensed into sterile Universal bottles containing 4 glass beads of 3mm in diameter. The working spore suspension was then stored at -20°C. When required, the spore suspension was thawed and mixed vigorously using a Vortex mixer. Spore counts were carried out on the suspension using a Haemocytometer (see Section 3.3.3.1 for method) and the number of spores per mL noted. All fermentations carried out used the

same generation of spores to avoid mutation problems. The master stock cultures were stored in a number of bottles at -70°C.

3.2.4.3 Inoculum preparation

The inoculum was grown in the medium adapted by Makagiansar (1992) from Hosler and Johnson (1953).

Table 3.5.2

inoculum media

component	g/L
sucrose	10
lactose	10
KH_2PO_4	3
Na_2SO_4	0.5
$FeSO_4.7H_2O$	0.1
$ZnSO_4.7H_2O$	0.02
$MnSO_4.7H_2O$	0.02
$CuSO_4.7H_2O$	0.005
$MgSO_4.7H_2O$	0.25
$CaCl_2$	0.05
EDTA	0.55
mycological peptone	5
$(NH_4)_2SO_4$	15

The sucrose was prepared in 10% of the final volume and sterilised separately at 121°C for 20 minutes as was the $(NH_4)_2SO_4$ and mycological peptone mixture. The pH of the solution containing the other media components was set to 6.7 with 2M NaOH prior to sterilisation in flasks at 121°C for 20 minutes. 200mL final media volume per 2L flask was used for fermenter inoculum production from a spore inoculum. Spores were inoculated into the flasks required at a final concentration of 0.8 x 10^6/mL and were grown in a shaking incubator set at 200rpm and 26°C for 39 hours. Sufficient shake flasks were inoculated to provide a 10% (v/v) inoculum for the fermentation.

3.2.3.4 Fermentation protocol

Table 3.5.3

fermentation medium

component	g/L
sucrose	10
lactose	50
KH_2PO_4	4
Na_2SO_4	1
$FeSO_4.7H_2O$	0.18
$ZnSO_4.7H_2O$	0.05
$MnSO_4.4H_2O$	0.05
$CuSO_4.5H_2O$	0.008
$MgSO_4.7H_20$	0.7
$CaCl_2$	0.05
EDTA	0.55
mycological peptone	5
$(NH_4)_2SO_4$	2
antifoam	1mL/L

As with the inoculum preparation medium, the sucrose and the mixture of peptone and ammonium sulphate were sterilised separately. The medium pH was set in the vessel after sterilisation and the addition of all the components at 6.7 with 5M NH_4OH and maintained using this and 2M H_2SO_4.

Once the vessel was ready for inoculation, the Biomass Monitor was set up as described in Section 2.1. The Biomass Monitor set up used was a measuring frequency of 0.4MHz, single frequency mode and a time filter of 5 seconds. The Δcapacitance and conductance output were recorded using a J.J.Lloyd Instruments CR600 chart recorder with a full scale deflection of 25 pF and 6 mS. After a suitable period to achieve a stable baseline for the Biomass Monitor, the fermenter was inoculated with 500mL of shake flask culture.

The air exhaust gas composition was monitored using a VG mass spectrometer. The data from this and the other fermentation parameters such as pH and DO_2 were logged as described previously. Samples were taken at regular intervals for dry weight, optical density and morphological measurements.

3.2.4 *Penicillium chrysogenum* fed-batch fermentations

3.2.4.1 Pilot scale

The fermentations were carried out at 3,000 litre scale in stirred fermenters at the SmithKline Beecham pilot plant in Worthing, U.K. The organism used was a commercial strain of *Penicillium chrysogenum*. The processes monitored were the standard SmithKline Beecham complex media, fed-batch fermentation with no allowances made for the use of the Biomass Monitor. The instrument used was BM25 and 25mm probe CT108, hired from Aber Instruments. The instrument was set up as described in section 2.1 of this work and Δcapacitance and conductance were monitored in two frequency mode using 0.4 MHz as the selected low frequency point. Periodic frequency scans were carried out to check on the reliability of the outputs. The probe was located in a Ingold style port situated in the lower third of the vessel and was sterilised *in-situ*. The Δ capacitance and conductance signals from the Biomass Monitor were logged using a 12 bit Squirrel data logger (Grant Instruments). The regime used was to read a value every 1 minute and then every 10 minutes, log an average of the readings obtained. On-line gas analysis was used to give exit CO_2 measurements and other fermentation parameters were logged as routine. Samples were taken at regular intervals for viscosity, dry weight, DNA content analysis and morphological measurement.

3.2.4.2 Laboratory scale

The fermentations were carried out in 14L glass walled, stirred fermenters. They were run with 10 litre working volume. The organism and process used were the standard SmithKline Beecham Pen G fed-batch, complex media process and was identical to the process monitored at pilot scale. The vessel was sterilised in an

autoclave, therefore it was necessary to chemically sterilise the probe in Kloroxide for 1 hour before inserting the probe into a port in the top plate of the vessel. Δ capacitance was monitored in two frequency mode using 0.4 MHz as the low frequency point. For the first fermentation, Δ capacitance and conductance were recorded using a J.J.Lloyd Instruments CR600 chart recorder with a full scale deflection of 60 pF and 6 mS. For the second fermentation, a 12-bit Squirrel datalogger (Grant Instruments) was used to log Δ capacitance and conductance using the regime described above for the pilot scale fermentations. On-line gas analysis was used to give exit CO_2 measurements and other fermentation parameters were logged as routine. Samples were taken at regular intervals for viscosity, dry weight, DNA content analysis and morphological measurement.

3.2.5 *Streptomyces sp.* pilot scale fed-batch fermentations

The fermentations were carried out at 30L scale in a stainless steel stirred fermenter. The processes used were standard production fed batch, complex media processes. The organism used was a commercial strain of *Streptomyces sp.* The Biomass Monitor used was BM25 and probe CT079. The probe was situated in an Ingold style port in the lower third of the vessel and was sterilised *in-situ*. Δ capacitance and conductance were monitored in two frequency mode. The low frequency point used was 0.5 MHz, which was chosen after off-line frequency scans on mid-run broth from a previous *Streptomyces* fermentation. Δcapacitance and conductance were recorded using a 12 bit Squirrel data logger as described previously. Exit CO_2 was also monitored on-line, as were the normal fermentation parameters. Samples were taken at regular intervals for viscosity and DNA content measurement. Dry weight measurement was attempted but was found to be very time-consuming and inaccurate.

3.3 Analytical techniques

3.3.1 Dry weight determinations

3.3.1.1 Eppendorf method

Three Eppendorf tubes were dried for 1 hour at 95°C. They were then weighed to four decimal places and the weights noted. 1mL of well-mixed sample was pipetted using a Finnpippette into each of the tubes and a batch was spun in a micro-centrifuge at 8-10,000 *g*. The supernatant was poured off and retained for biochemical tests. The pellet was resuspended in RO water and the centrifugation procedure was repeated. The supernatant was poured off and the tubes placed in racks and then dried in an oven at 95°C for 24 hours. After 24 hours, the tubes were re-weighed to four decimal places and the weight of cell mass per tube calculated. The average and range of the three values was calculated and multiplied up to give the final dry weight in g/L.

3.3.1.2 Glass tube method

This method is identical to the one described above except that the containers used were glass Pyrex 12mL centrifuge tubes, with 10mL of sample spun down at 3-4,000 *g* in a benchtop centrifuge.

3.3.1.3 Filtration method

This method was only used for *P.chrysogenum* samples.

Whatman GF/A glass microfibre filters (0.45μm pore size, 4.7cm diameter) were pre-dried at 95°C, and then weighed to four decimal places. The filter was placed in a Sartorius SM vacuum filter holder connected to a vacuum supply. 10mL of sample was pipetted onto the surface of the filter and the vacuum supply allowed to remove the bulk of the moisture from the mycelial sample. The mycelium was washed with distilled water and re-dried, twice if suspended solids were present in

the media. Where possible, depending on available sample volume, duplicates were carried out. The filter was then dried for 24 hours at 95°C and stored in a desiccator until the weight remained constant. The dry weight of cell mass on the filter was calculated. If sufficient sample volume was available to allow duplicates, an average dry weight was calculated and then multiplied up to give the dry weight in g/L.

3.3.2 Absorbance determination

The presence of bacterial or yeast cells in a liquid causes an increase in the visible spectrum light absorbance of that liquid by an amount which is directly related to the number cells present, within the linear range of the Beer-Lambert Law.
A variety of visible light spectrophotometers were used throughout this work for absorbance determination. Yeast cultures were measured at 600nm and bacterial cultures at 670nm. A 1mL aliquot of the sample was pippeted into 9mL of RO water and mixed using a Vortex mixer. 3mL of RO water was pipetted into a disposable plastic cuvette and used to set the zero of the instrument used and then as the blank for sample measurement. 3mL of the diluted sample was pipetted into a plastic cuvette and the absorbance at the appropriate wavelength was measured against the blank. As the linear range of the Beer-Lambert is operable only to around 0.3-0.5 absorbance units, any samples found to be outside this range were diluted again by 1:10 with RO water and measured again. The absorbance measurements shown in fermentation graphs in this work are corrected for dilution.

3.3.3 Viable cell concentration determinations

3.3.3.1 Direct microscopy counts

This method is suitable only for yeast samples. The sample was diluted into sterile Universal bottles containing 9mL of sterile water, by a factor known to be suitable from experience. In practice, this was found to be the same as the dilution required to reach a suitable concentration to enable absorbance measurement. The bottles were Vortex mixed before and after every step to ensure that the dilutions

were representative. A 2mL aliquot of the appropriate dilution was transferred to a bijou bottle and two drops of a 0.01% methylene blue aqueous solution were added and the solution was Vortex mixed. The methylene blue stain is added to test for the presence of dead or damaged cells. It is a auto-oxidisable dye which is taken up by all cells but is only decolourised by healthy cells, thus in this case the stained cells are classed as the dead cells.

The counts were made using a Petroff-Haas Haemocytometer kit, viewed using an Olympus microscope set to bright field illumination with a x40 objective lens and x10 eyepiece lens The coverslip was pressed onto the surface of the slide and the stained cell solution was pipetted into the counting chamber by capillary action until the area was filled. Care was taken to ensure that the volume of cell suspension in the counting chamber was correct. For speed of measurement, only the cells in five of the small measuring squares were counted. The squares counted were the four corner of the measuring chamber and the square in the centre. The number of stained and unstained cells were counted and noted. The coverslip was removed and the slide and coverslip were washed with ethanol and dried before a repeat measurement was carried out. To give good statistical accuracy, the number of cells in each square should be more than 5 or less than 200, if the measurements were not within these boundaries they were repeated using a more appropriate dilution. The total number of cells; the number of stained cells and unstained cells in the five squares for each of the repeats was measured, and the average values were calculated. These cell numbers were then converted into the number of cells per mL in the culture as follows:-

(Number of visible cells counted x $4x10^4$) x dilution of sample used

$4x10^4$ is the calculation factor due to volume of the counting chamber used. This calculation thus gives a measure of the total cell number of the culture, the total viable cell number of the culture and the total number of dead cells present in the culture.

3.3.3.2 Serial dilution plate counts

Eleven Universal bottles per determination were prepared with 9mL of 50mM Na_2HPO_4 buffer (pH 6.7) and sterilised by autoclaving. Care was taken to seal the

lids of the bottles to prevent liquid loss. The method used was a classical microbiology technique. The first (cooled) Universal was opened and the neck flamed in the Bunsen, the sample was vortex mixed and, using a Finnpipette with a sterile tip, 1mL was removed and added to the 9mL in the open Universal. The neck was flamed again and the cap firmly replaced. The sample bottle neck was then lightly flamed and the cap replaced. The Universal was then vortex mixed, and the cap then loosened, the next Universal was prepared by removing the cap and flaming the neck. Again, using the Finnpipette with a fresh tip, 1mL of the cell suspension was removed and added to the 9mL in the fresh Universal. This procedure was repeated until all eleven of the bottles had received a dilution of the cell suspension. This procedure produces a range of dilutions from 10^{-1} to 10^{-11}. The full dilution procedure was repeated for each sample to ensure accuracy

Three Nutrient Agar (Oxoid) plates per dilution of each determination were prepared earlier and dried for 30 minutes at 50°C prior to use. The dilutions appropriate to the estimated cell concentration were plated out on the Nutrient Agar, for early fermentation samples this would be from 10^{-4} to 10^{-11}. Later in the fermentation the minimum dilution required would rise to 10^{-6}. The Universal bottle containing the first dilution of interest was vortex mixed, the neck was heated in the Bunsen flame and 0.1mL removed using a Finnpipette and sterile tip. The aliquot of diluted cell suspension was pipetted on to the surface of the agar and spread across the surface of the plate using a sterile glass spreader. The spreader was sterilised before and after every use by immersion in ethanol and flaming in the bunsen which burnt off any remaining ethanol. This procedure was repeated three times to give three plates per dilution for statistical validity.

Once all the dilutions of interest had been plated out, the plates were marked with the relevant details and the time and date of inoculation before being placed in an incubator at 28°C. After 24 hours, the plates were removed and the number of colonies on each one counted. If a plate had more than 250 and less than 10 colonies then it was discarded. The appropriate dilution to use for determining cell number should be clear. Preference was given to the dilutions with higher cell concentrations as this gives greater statistical validity. The average cell number for the three plates of the appropriate dilution was calculated and this was then averaged with the average of the three plates from the same dilution of the repeat.

The average cell number yielded was then multiplied by the dilution to the number of viable cells per mL of the culture.

3.3.4 Morphological measurements by image analysis

3.3.4.1 Sample preparation and storage

Samples from *P.chrysogenum* fermentations were taken for morphological measurement of the mycelia. Immediately after removal from the vessel, they were mixed with an equal volume of fixative solution before being stored at 4°C until required for analysis. The fixative solution comprised of:
40% formaldehyde, 13mL
glacial acetic acid, 5mL
50% (w/v) ethanol, 200mL

The fixed samples were diluted with fixative solution prior to analysis. The dilution used was dependent on the cell concentration and the viscosity of the culture. The diluted sample was stained with Trypan Blue solution, which was made by dissolving 0.25g Trypan Blue into 100mL of Lactophenol solution. Wet mount slides were prepared by spreading 80μL of fixed and stained sample from a pipette onto a slide and covering with a glass cover slip. The edges of the coverslip were sealed with nail varnish to prevent distortion of the measurements due to sample evaporation

3.3.4.2 Morphological measurements

The measurements were carried out using a Magiscan MD Image analyser (Joyce Loebl Ltd, Gateshead, U.K.) using a semi-automatic method adapted from Packer *et al* (1991). The slide of the wet sample was placed on the stage of a Polyvar microscope set for brightfield illumination (Leica Ltd, Cambridge, U.K.) and focused under the x 40 objective manually, using the automatic light setting of the image analysis equipment. The image obtained through the objective lens was sent to the image analysis equipment by a Hitachi EQ414 video camera.

The image processing equipment was controlled by the Genias software package supplied by Joyce Loebl. Some modifications had been made to this by Packer and Thomas (1990) to allow morphological measurements to be made. The main steps involved in the Genias program making morphological measurements were image selection and focusing, thresholding, image cleaning, skeletonisation, and measurement within a predefined active measuring frame. The program was run in semi-automatic mode with manual field of view selection and focusing. The picture from the video camera was defined as an image. A greyness threshold was set to a level to differentiate the mycelia from the background. The image was cleaned of non-mycelial objects such as dust and media particles by excluding circular objects. Skeletonisation of the image clarifies the edges of the remaining objects. The processed binary image from the skeletonisation phase was then divided into measurable organisms and clumps. Mean main hyphal length, mean hyphal length, mean branch length, number of tips and percentage of clump area were determined. 100 or more measurements were taken for each of the morphological measurements.

3.3.5 *S. cerevisiae* cell number and volume determination

Samples were taken from the fermenter and diluted as required in KH_2PO_4 (0.1M pH 6.5) buffer, to a coincidence level of less than 1%. Cell number was determined using an Elzone 280PC instrument (Particle Data Inc, Elmhurst, IL, U.S.A.) running PDI software (version EW8005). The orifice (30μm) was calibrated using latex standards (1 and 5 μm, PDI) suspended in buffer (KH_2PO_4). A 20 μL manometric section was used to determine number concentration. Six counts were made for each sample and the values averaged. From the histogram plot of particle size versus number, the section in which the yeast cells were observed was selected by means of size markers and the average volume for the particles within this section was obtained from the program. The total cell volume for the fermentation was calculated from this average volume and the cell number counts, taking the dilution factors into account.

3.3.6 Total soluble protein assay

The method used was the Bradford type Coomassie Blue protein assay. (Bio-Rad Laboratories 1992). It is a dye-binding assay based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs. The Coomassie blue reagent was calibrated using Bovine Serum Albumin, fraction V (Sigma Chemical Co Ltd.) in the 0-1 mg/L range. Samples (0.1mL) were added to the binding reagent (3mL) and the absorbance recorded at 595nm after 5 minutes using Coomassie blue reagent as the blank. Samples were diluted using KH_2 PO_4 (0.1M) to keep the absorbance of the sample below 0.5 and thus in the linear range. The total soluble protein in the samples was then calculated from the absorbance with reference to the standards and the dilution factor.

3.3.7 DNA content assay

The assay is based on the principle that DNA is precipitated by cold perchloric acid and returns to solution in hot perchloric acid. As perchloric acid is hazardous, gloves and protective clothing were worn and all manipulations of perchloric acid were carried out in a fume cupboard. A DNA standard solution was made up using the DNA sodium salt from salmon testes (Sigma Chemical Company Ltd) to a final concentration of 1mg/mL. This was then diluted with RO water to give a range of concentrations from 0.01-1 mg/mL to use as a standard curve. 2mL of cold 0.4N perchloric acid was added to 2mL of standard in a 10mL centrifuge tube and gently mixed. The tube were kept on ice when not being handled to ensure the precipitation of the DNA. The mixture was then centrifuged for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 2mL of 0.2N perchloric acid. This mixture was then placed in a water bath set to 100°C for 20 minutes, with marbles used as stoppers to prevent excess evaporation of the solution. The samples were then decanted into Eppendorf tubes and spun for 10 minutes. The supernatant was then poured into a 1mL quartz cuvette and the absorbance at 280nm (with the deuterium lamp on) measured using RO water as the blank. A standard curve was plotted from these results. The above procedure was then repeated for the fermentation samples. These were mixed 1:1 with 0.4N

perchloric acid immediately after removal from the fermenter and stored at -4°C until required for assay. Where necessary, the fermentation samples were diluted with RO water to bring them within range of the standard curve. After the assay as described above was carried out on the fermentation samples, the DNA content was calculated from the standard curve with reference to any dilutions necessary.

3.3.8 High pressure homogenisation

The equipment used for the homogenisation studies in this work was the Gaulin Micron Lab 40 high efficiency homogeniser. (APV Gaulin GmbH). The equipment was turned on 30minutes prior to use to allow the glycol cooling loop to cool the homogeniser head to prevent excess protein denaturation due to heat. 40mL of the cell suspension to be homogenised was placed into the product cylinder with care to avoid the introduction of air bubbles or excess particulates. The product cylinder was then mounted onto the working piston of the valve assembly and the homogeniser head assembly was secured into position and clamped. The operating pressure was then selected in the range of 100-1600 bar. Homogenisation was achieved by the working piston forcing the cell suspension through a narrow aperture at the set pressure to collide with an impact ring and thus become disrupted. The product cylinder and valve assembly were rinsed with sterile water between passes to avoid carry over.

4 RESULTS

In this section the results of the experiments carried out using the Biomass Monitor (BM) to measure the capacitance of the organisms and fermentations discussed in section 1, both on-line and off-line are presented. The work is divided up by organism monitored.

As discussed in Section 2.2, capacitance is a dependent variable which can change with the system and instrument used to measure it. A variety of instruments and set-ups were used in the course of the practical work, the results of which are described in this section. In order that the values shown may be directly compared, they have all been corrected to the cell constant of the main instrument used, 0.93 cm^{-1}.

Alternatively, all values could have been converted to permittivity. However, the majority of published work, with which this work is to be compared, have expressed their measurements as capacitance (in pF), therefore, it is appropriate that this convention be maintained here.

The conversion of the capacitance measurements to permittivity would also lead to a greater discussion of the biophysics of these results than is appropriate for this program of work. The work described in this thesis was designed to evaluate the accuracy and reproducibility of the capacitance measurements, to compare the capacitance measurements made by the BM with other available measures of biomass, and therefore to determine the suitability of capacitance measurement as a measure of biomass for all the organisms and fermentation conditions described. The influence of conductivity changes, crosstalk and electrode polarisation on the measured capacitance have all been discussed in Section 2.4. Where required, the results presented here have been corrected for these effects by the methods described in section 3.1.

4.1 *Saccharomyces cerevisiae* Experiments

4.1.1 *Saccharomyces cerevisiae* initial off-line experiments

Initial experiments to examine the applicability of the Biomass Monitor were carried out using *S.cerevisiae* (Distillers Co. Ltd) as an easily available test

organism. Using the set-up described in Section 3.1.1, 20g aliquots of the packed yeast were added to 1L of 50mM, pH 6.8, phosphate buffer. The capacitance of the suspending buffer was backed off (as described in Section 3.1) prior to the addition of the packed yeast. Figure 4.1 shows the result from one of these experiments.

The correlation coefficients and Y-axis intercepts of the linear regressions applied to these data are R^2 =0.996, Y intercept = -1.53pF and, derived from the linear regression, giving a specific capacitance constant (QCdw) of 0.76pF/g/L for capacitance against dry weight of the yeast suspension. From these correlations it can be deduced that the capacitance measured by the Biomass Monitor does accurately reflect the level of biomass present in a yeast suspension under ideal test conditions.

4.1.2 *Saccharomyces cerevisiae* Fermentations

To investigate the ability of the Biomass Monitor to operate on-line in a fermentation vessel under normal conditions, a series of fermentations were carried out growing *S.cerevisiae* GB4918 under the conditions described in Section 3.2.1. The capacitance measurements shown are average values at hourly intervals taken from the real-time continuous measurements recorded by the chart recorder. The background capacitance of the fermentation media was removed by the offset facility of the Biomass Monitor. To investigate further the nature of the relationship between the capacitance measured and the biomass, the capacitance measurements were compared with other indicators of growth for each of these fermentations. Exit gas CO_2 concentration, expressed as its percentage amount present in the air exhaust from the vessel, was measured on-line and in real-time by mass spectrometry. In addition, the cumulative total of this percentage, calculated off-line, was also calculated and both these measures were used as indicators of growth and measures of metabolic activity. Cell number and the total volume of cells present in the vessel were calculated by both conventional microbiological methods and by using an Elzone particle sizing and counting instrument off-line from samples taken at regular intervals from the vessel, using the methods described in Section 3.3.5. In all cases where the Viable Cell Concentration (VCC)

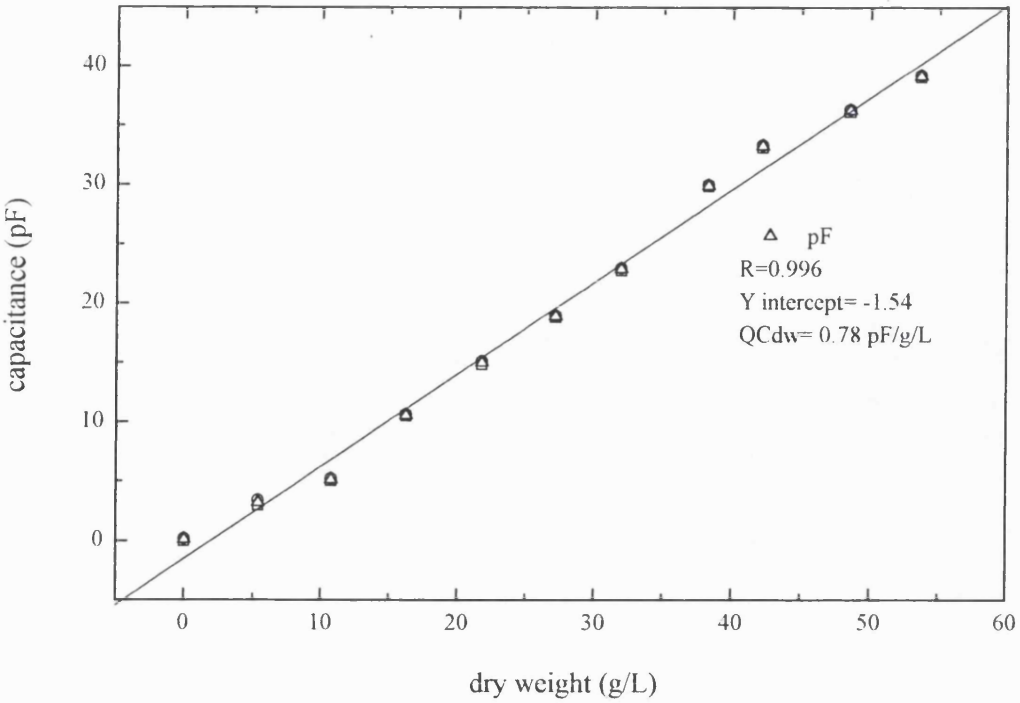


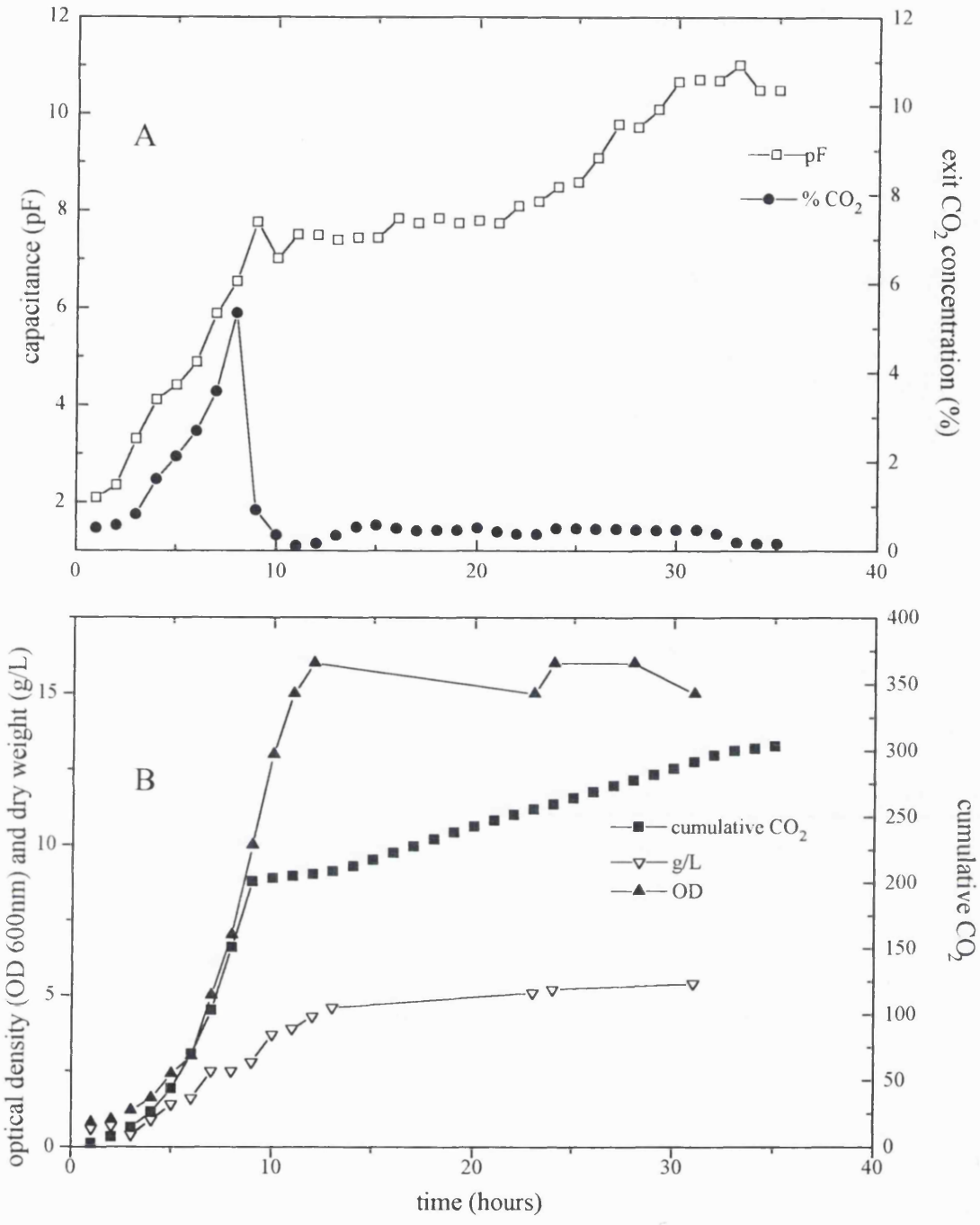
Figure 4.1: Dry weight versus capacitance (pF)
Saccharomyces cerevisiae

was measured on fermentation samples by methylene blue staining, the percentage viability of the samples was above 99%. The most commonly used measures of biomass, dry cell weight and optical density (OD) were also calculated off-line using these samples from the vessel.

4.1.2.1 *S.cerevisiae* GB4918 14L defined media fermentations

Figures 4.2a-c and 4.3a-c show the results from two separate fermentations carried out under the same conditions, in the same vessel, to examine the reproducibility of the system. The parameters measured are all plotted against time after inoculation. In Figures 4.2a and 4.3a, the initial growth peak, as determined by exit CO_2 occurred at approximately 9 hours and at a concentration of 5.5% CO_2 for both the fermentations, indicating that both fermentations were reproducible in terms of growth. In both cases, the rise in capacitance from the level post inoculation was 5.5pF at the peak point of growth, and showed good agreement with exit CO_2 during the exponential growth phase. Capacitance continued to rise at a much slower rate beyond the end of the exponential growth phase, while exit CO_2 concentration fell to a much lower level. This indicates that the culture is utilising the ethanol made during the initial growth on glucose The difference between capacitance and exit CO_2 is due to exit CO_2 being given as a spot value while capacitance is a cumulative value. Consequently, the plots of cumulative exit CO_2 show better agreement with capacitance throughout the time course of the fermentation than the spot values.

In Figures 4.2c and 4.3c, the cell number and total cell volume plots show similar growth profiles to those of capacitance. In 4.2c, the cell concentration, and consequently the total cell volume is slightly higher than the fermentation depicted in Figure 4.3c, but with no apparent difference in the capacitance value. In Figure 4.3c, the Viable Cell Concentration (VCC) is also shown. This is lower than the Elzone Cell Number (TCN), possibly reflecting that the mode of operation of the Elzone does not allow it to differentiate between viable and dead, but intact cells. In both fermentations, the TCN plots show better agreement with capacitance than does the VCC plot, as capacitance and TCN continue to rise slowly, indicating the growth on ethanol, after the initial (glucose) growth peak, while VCC levels off.



Figures 4.2a and b : capacitance (pF) and exit CO_2 concentration (%), cumulative exit CO_2 concentration, OD600 (OD) and dry weight (g/L) versus time

Saccharomyces cerevisiae 14L defined media fermentation SC1

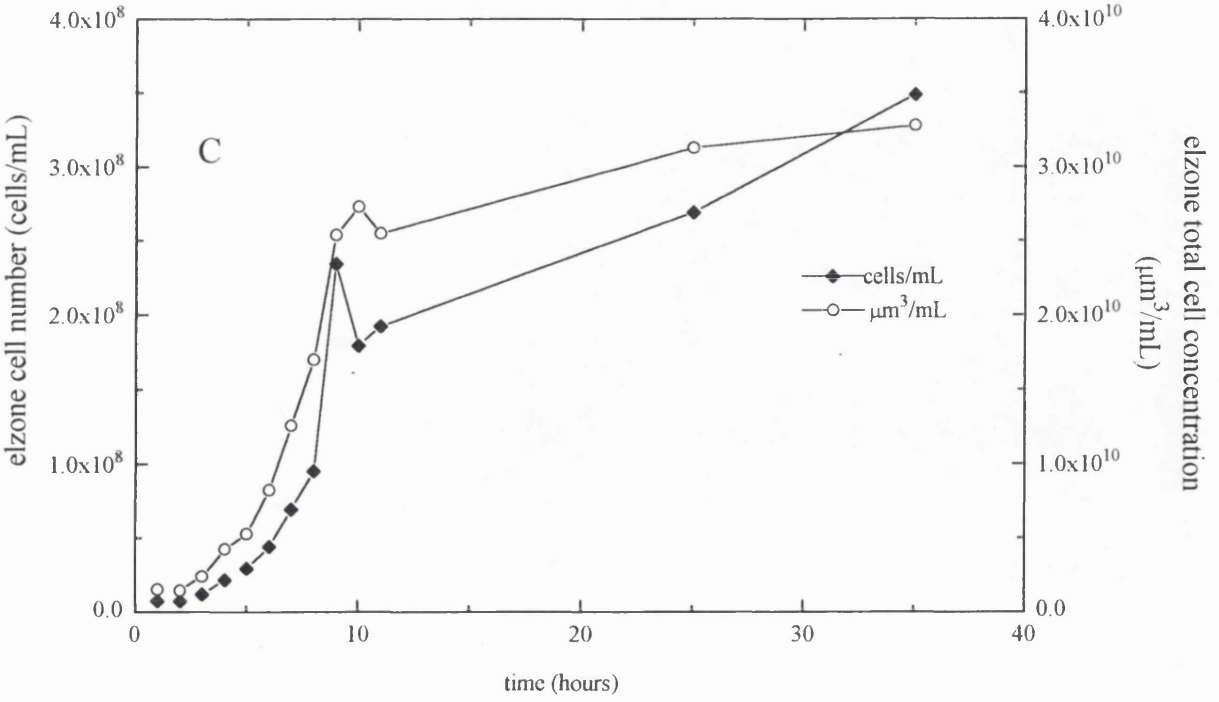


Figure 4.2c: elzone cell number (cells/mL) and elzone total cell volume (μm^3/mL) versus time
Saccharomyces cerevisae 14L defined media fermentation SC1

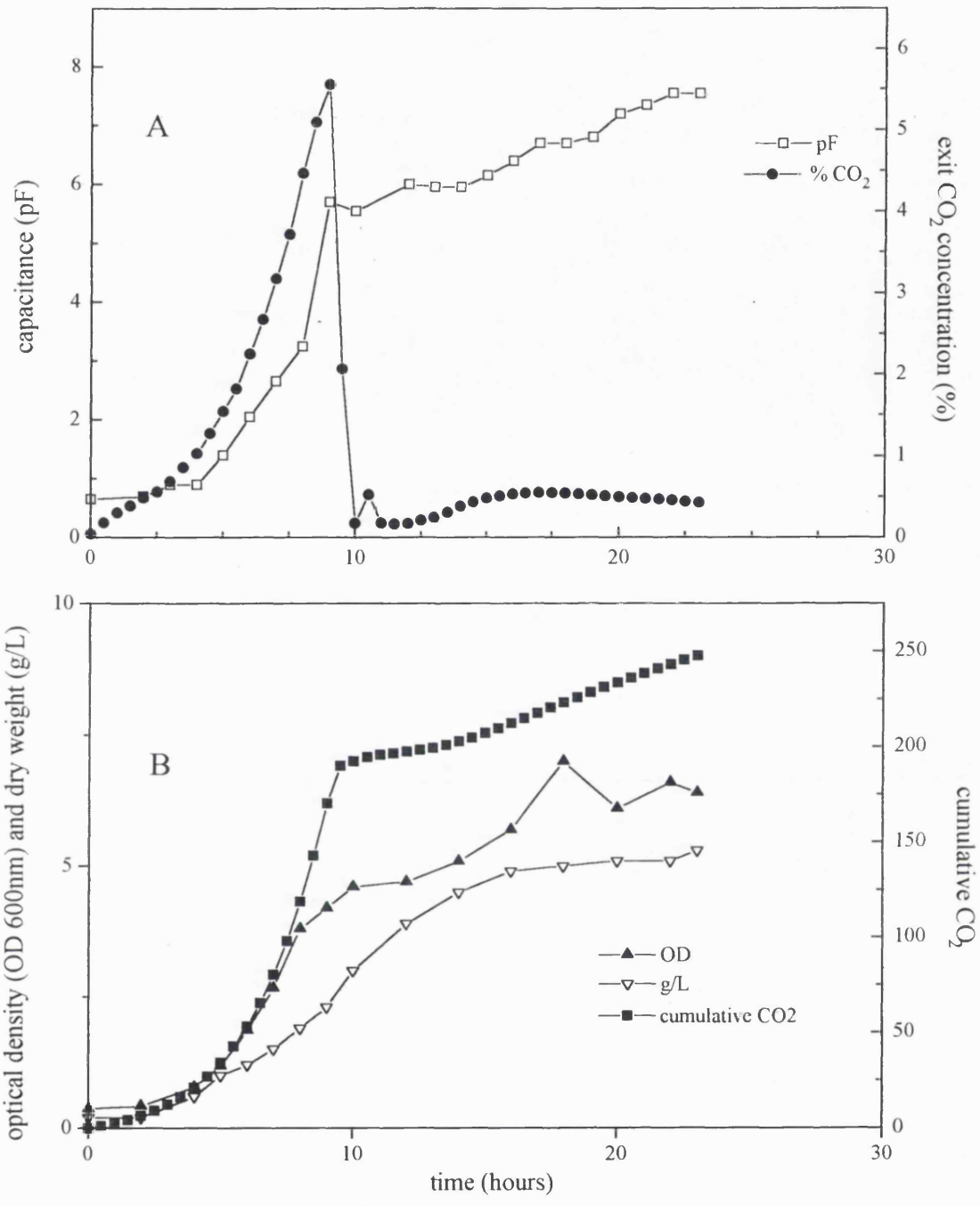


Figure 4.3a and b: capacitance (pF) and exit CO_2 concentration (%), cumulative exit CO_2 concentration, OD600 (OD) and dry weight (g/L) versus time

Saccharomyces cerevisiae 14L defined media fermentation SC2

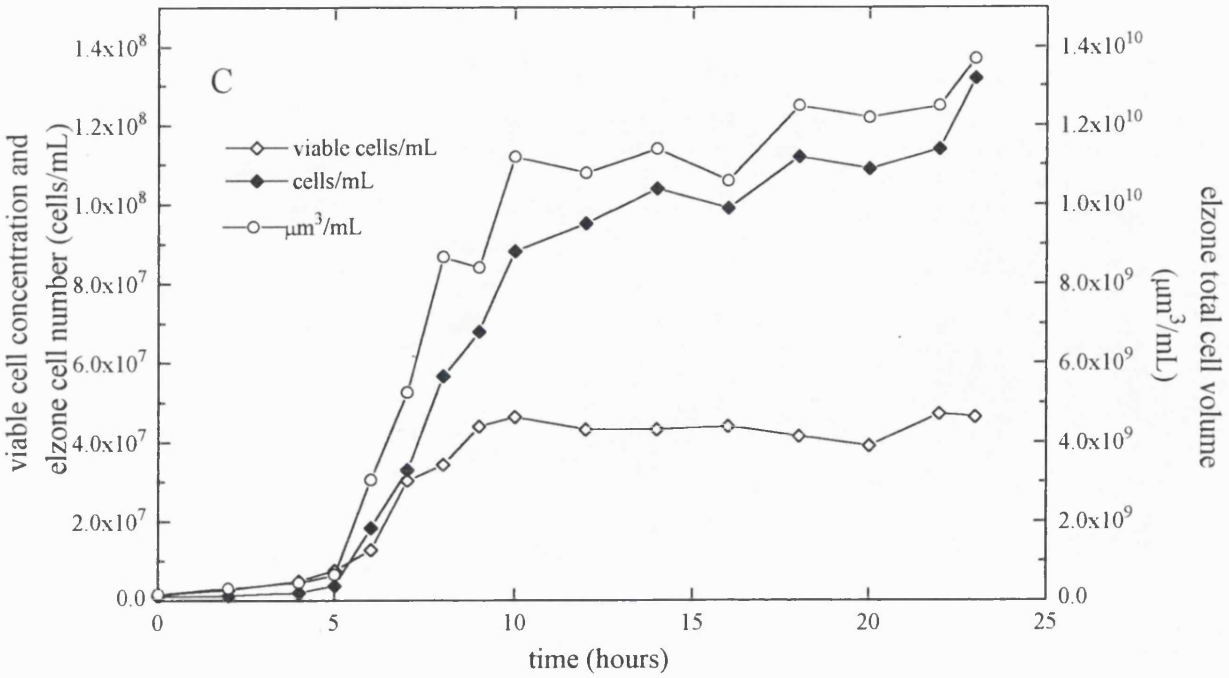


Figure 4.3c: capacitance (pF) and viable cell concentration (cells/mL) , elzone cell number (cells/mL) and elzone total cell volume (μm^3/mL) versus time
Saccharomyces cerevisiae 14L defined media fermentation SC2

This is contrary to what would be expected but is almost certainly due to the much greater statistical accuracy of the cell counting instrument over conventional microbiological techniques.

In Figures 4.2b and 4.3b, the dry weight and OD profiles were consistent with the other indicators of growth. In both fermentations, the peak dry weight was around 5 g/L, indicating a rise in capacitance of around 1pF per g/L. The differences in the levels of optical density of the two fermentations was due to using two different spectrophotometers to measure the values.

To examine the relationship between the capacitance measured on-line for these fermentations and the other indicators of growth measured, Table 4.1 shows data from linear regression plots of the data for these variables.

For these fermentations, the best correlation is seen between capacitance and cumulative exit CO_2 (R^2=0.971 and R^2=0.996) All the other parameters have correlation coefficients of 0.920 or greater except the spot percentage exit CO_2 measurements which show poor correlations with capacitance for the reasons outlined above.

The specific growth rates (μ) of the initial exponential growth phase, calculated from semi-logarithmic plots of the measured variables of these fermentations (SC1 and SC2) are given in Table 4.2.

The data from the μ calculations show that the fermentations are very similar in terms of metabolic activity as measured by exit gas analysis and this also reflected in the rates calculated from the capacitance measurements. The μ values obtained from the other measured parameters are varied, which reflects the relative accuracy of the other biomass measurements.

The data from these two fermentations show that the BM can be used effectively and reproducibly to obtain an on-line biomass measurement in yeast fermentations.

Table 4.1

Correlation coefficients (R^2) for capacitance against various biomass measurements for *S.cerevisiae* 14L defined media fermentations.

R^2 values versus capacitance

	%CO2	cCO2	DW	OD	VCC	TCN	TCV
SC1	0.30	0.97	0.94	0.92	N/A	0.95	0.96
SC2	0.21	0.99	0.96	0.98	0.94	0.98	0.97

Table 4.2

Specific growth rates for *S.cerevisiae* 14L defined media fermentations, calculated from various biomass measurements.

parameters used to calculate μ

	Cap.	CO2	cCO2	DW	OD	VCC	TCN	TCV
SC1	0.25	0.29	0.47	0.29	0.34	not done	0.21	0.20
SC2	0.26	0.31	0.42	0.22	0.33	0.45	0.50	0.56

4.1.2.2 *S.cerevisiae* GB4918 5L defined media fermentation

Data from an experiment growing the same organism under identical conditions (detailed in Section 2.2.1) in a smaller vessel are shown plotted against time after inoculation in Figures 4.4a-c.

In Figure 4.4a, the initial peak of growth after the exponential growth on glucose again occurred after around 9 hours but at a lower level of CO_2 in the exit gas, 3.5% as opposed to 5.5%. The initial capacitance peak was also around 9 hours but was lower than for the fermentations described earlier at around 3pF, as opposed to 5.5pF. As in the previous fermentations, cumulative exit CO_2 showed

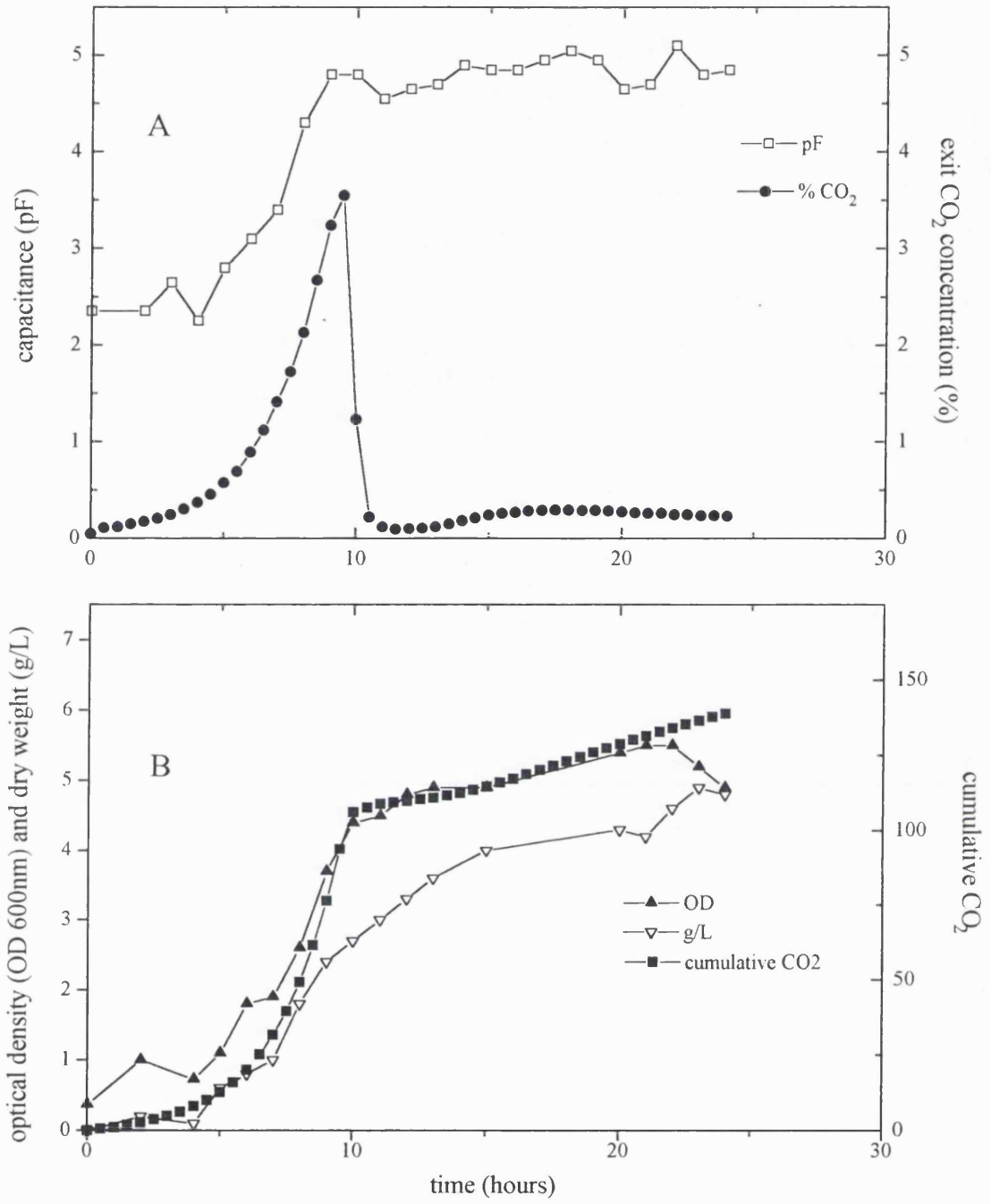


Figure 4.4a and b: capacitance (pF) and exit CO_2 concentration (%), cumulative exit CO_2 concentration, OD600 (OD) and dry weight (g/L) versus time

Saccharomyces cerevisiae 7L defined media fermentation SC3

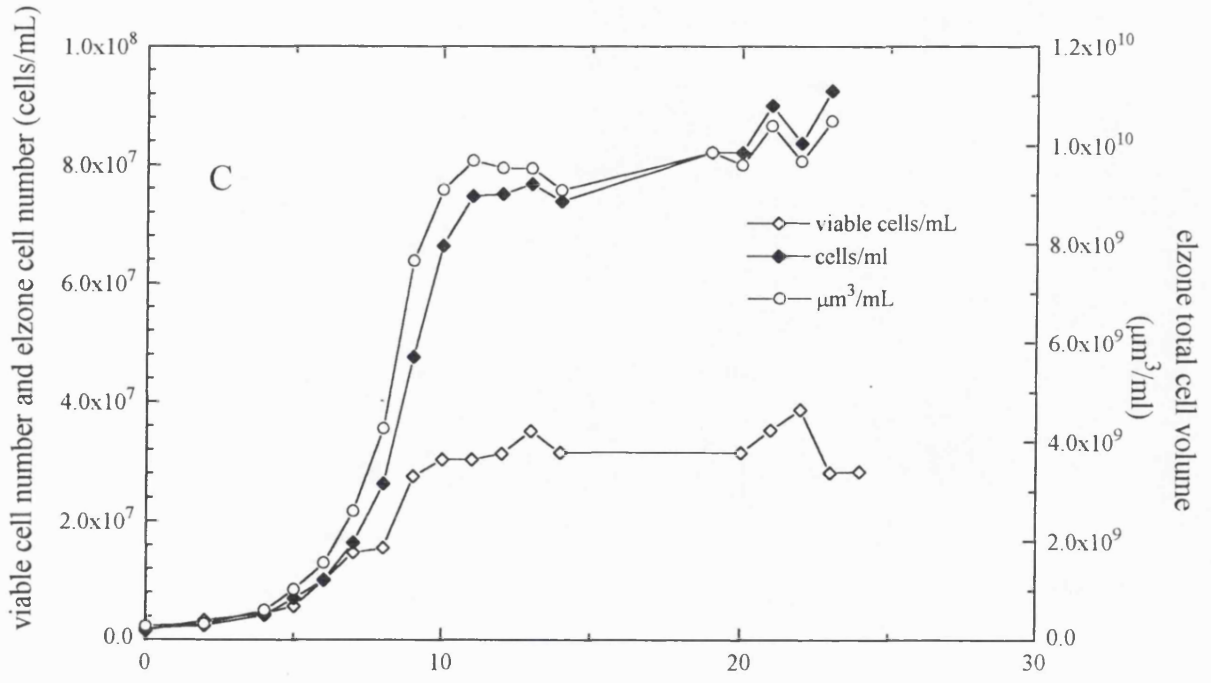


Figure 4.4c: Viable cell concentration (cells/mL) , Elzone cell number (cells/mL) and Elzone total cell volume (μm^3/ml) versus time
Saccharomyces cerevisiae 7L defined media fermentation SC3

good agreement with capacitance throughout the course of the fermentation. All these values indicate less growth in this fermentation In Figure 4.4c, the VCC, TCN and TCV plots showed similar profiles to those discussed previously, but the values were lower. This is in agreement with the capacitance and CO_2 values for this fermentation and indicates that the level of growth obtained in this fermentation is less than at the 14L scale. The relationship between VCC and TCN remains the same, with the viable cell concentration being lower than the cell count which does take viability into account. In Figure 4.4c, the dry weight and OD profiles are similar to those discussed previously, and the values are again slightly lower than those obtained from the fermentations at 14L.

To analyse the relationship between capacitance and the other measured variables, Table 4.3 shows correlation coefficients from linear plots of the data from this fermentation.

In this case, the VCC has the best correlation with capacitance (R^2=0.956). TCV is very close to this with an R^2 value of 0.955 and as in the larger scale fermentation, the exit CO_2 spot values show the worst correlation with capacitance. Although the fermentation at 5L scale was significantly different in metabolic terms from the 14L fermentations, the capacitance as measured by the BM again gave a good on-line biomass estimate.

4.1.2.3 *S.cerevisiae* MC1 42L complex media fermentation

To examine the effects of both the cell size and of the presence of undissolved media solids on the biomass measurement, a strain of *S.cerevisiae*, MC1, was grown at 42L scale in a complex media as described in Section 3.2.2. The theory of operation of the BM predicts that while the media components should have no effect on the capacitance signal yielded by a cell suspension, the cell size should have an effect.

Figures 4.5a-c show the results from this experiment plotted against time after inoculation. In Figure 4.5a, the exit CO_2 data is missing for the first 7 hours of the fermentation due to technical problems. From the available data, it can be inferred that the peak of exit CO_2 for this fermentation occurred at approximately 8 hours at 1.75% and then the CO_2 declined slowly over the remainder of the fermentation.

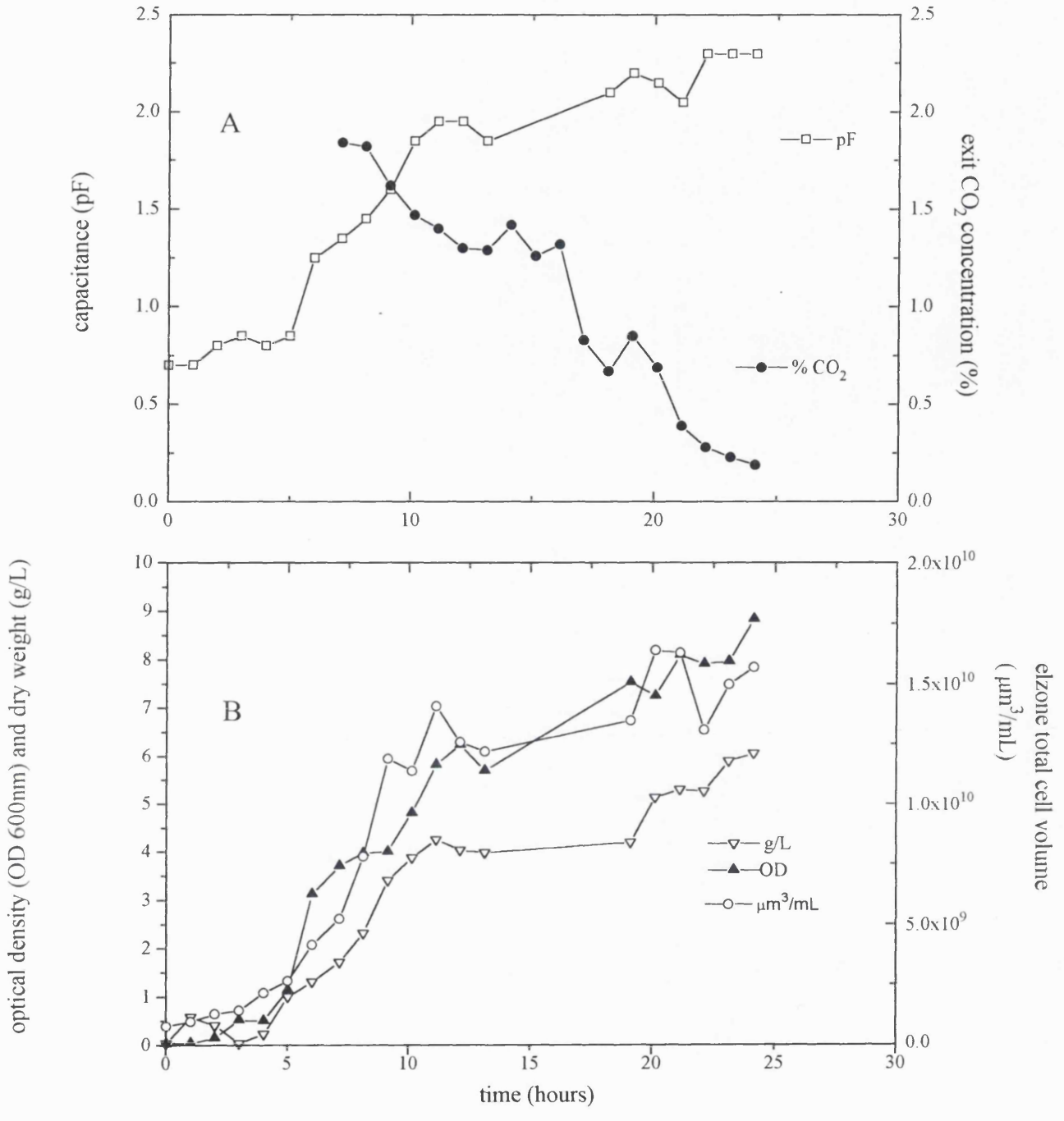


Figure 4.5a and b: capacitance (pF) and exit CO_2 concentration (%), OD600 (OD) elzone total cell volume (μm^3/mL) and dry weight (g/L) versus time
Saccharomyces cerevisiae 42L complex media fermentation SC4

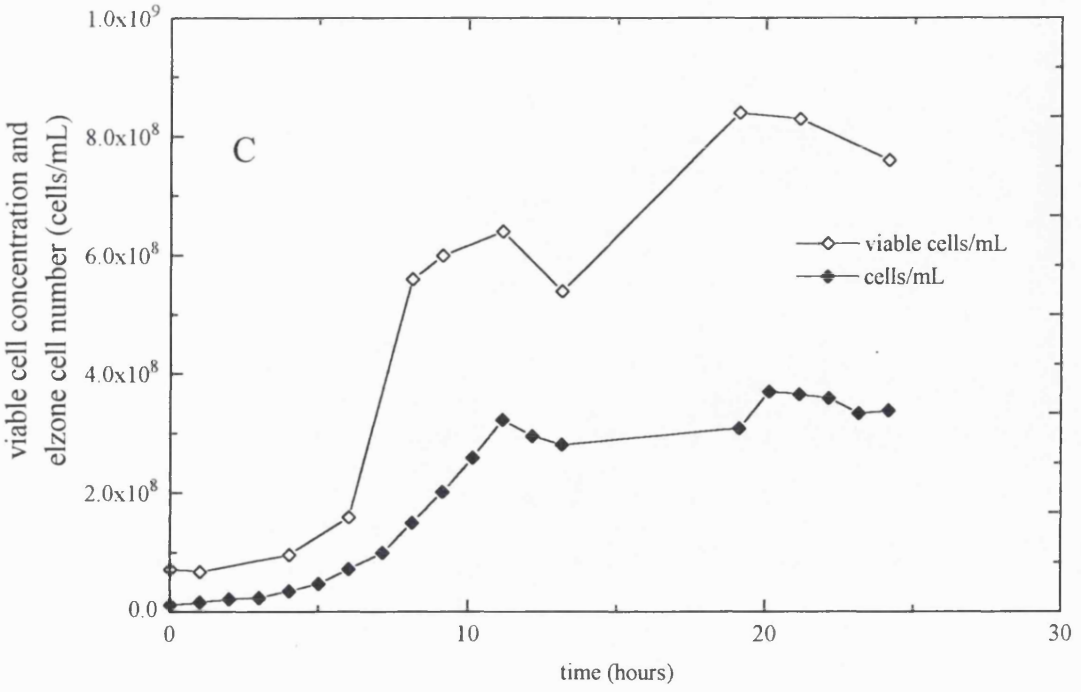


Figure 4.5c: viable cell concentration and elzone cell number (cells/mL) versus time
Saccharomyces cerevisiae 42L complex media fermentation SC4

The capacitance value does not show a growth peak which corresponds to this CO_2 peak. The capacitance rises sharply after a lag phase of around 5 hours, this rapid rise slows after 11 hours at around 2pF, before rising again at a slower rate till the end of the fermentation with a final value of 2.3 pF. The data for dry weight and OD show profiles which are in good agreement with capacitance and indicate a maximal dry weight of around 6 g/L , which is probably an overestimate due to the presence of complex media solids. The VCC, TCN and TCV fermentation profiles show good agreement with capacitance profile. In this case, the VCC values are higher than the TCN values, possibly due to experimental error in the VCC counts.
To examine the relationship between capacitance and the other measured variables for this fermentation, Table 4.4 shows the correlation coefficients calculated from linear plots of the data from this fermentation.
All of the measured biomass values correlate with capacitance, with OD having the best correlation (R^2=0.987) but all of the variables have a correlation of 0.95 or above.
Semi-logarithmic plots of the measured values show that the peak of the exponential growth phase was at around 10 hours for capacitance, dry weight, TCN and TCV, while OD and VCC peak at around 8-9 hours. The μ values for the initial growth phase of this fermentation and the 7L fermentation SC3, described above, are shown in Table 4.5.

The differences in the growth rates, both between the fermentations, and within the fermentations again shows the inherent variability in the biomass measurements. The μ values calculated from CO_2 for SC3 are higher than those for the larger scale fermentation, although the overall growth yield and peak are lower. This would seem to indicate that there were differences between SC3 and the larger scale fermentations which are not attributable to the change in scale.

Table 4.3

Correlation coefficients for capacitance against various biomass measurements for *S.cerevisiae* 5L defined media fermentation.

R^2 values versus capacitance

	%CO2	cCO2	DW	OD	VCC	TCN	TCV
SC3	0.05	0.95	0.92	0.95	0.96	0.92	0.95

Table 4.4

Correlation coefficients for capacitance against various biomass measurements for *S.cerevisiae* MC1 42L complex media fermentation.

R^2 values versus capacitance

	%CO2	cCO2	DW	OD	VCC	TCN	TCV
SC4	N/A	N/A	0.98	0.98	0.95	0.98	0.97

Table 4.5.

Specific growth rates for *S.cerevisiae* 7L defined media (SC3) and 42L complex media (SC4) fermentations, calculated from various biomass measurements.

parameters used to calculate μ

	Cap.	CO2	CCO2	DW	OD	VCC	TCN	TCV
SC3	0.37	0.43	0.50	0.36	0.31	0.36	0.24	0.49
SC4	0.30	N/A	N/A	0.30	0.31	0.37	0.38	0.37

4.1.3 *S.cerevisiae* packed yeast homogenisation experiments

The results shown above have indicated that the BM can give a biomass related capacitance signal in both an idealised off-line situation and on-line in fermentation vessels. The attempts to correlate capacitance with the other indicators of growth and biomass have had mixed results. In order to further examine the relationship between capacitance and biomass, and in particular viability, a series of experiments were carried out using a homogeniser to disrupt suspensions of packed yeast in buffer (as described in Section 3.3.8). The effects of homogenisation on capacitance and other indicators of viability and cell integrity were examined.

4.1.3.1 *S.cerevisiae* 1400 bar homogenisation

In order to determine the effects of homogenisation on the capacitance signal, a 200 g/L packed yeast in phosphate buffer suspension was homogenised by carrying out three passes through the homogeniser at 1400 bar. This pressure and method was chosen as it was known to give maximal protein release, indicating maximum homogenisation. The method used is outlined in Section 2.3.8.

The results shown in Figures 4.6 and 4.7 are from two identical experiments, each of which contains three repeat capacitance measurements pre and post homogenisation. The capacitance of the suspending buffer was also measured. As another measure of the background capacitance, the disrupted cell suspension was centrifuged to remove the cell debris and the capacitance of the supernatant was measured. The post homogenisation supernatant was used as the background capacitance for calculating the Δcapacitance values shown in Figure 4.7. The total protein release values shown are each the average of three separate measurements. In addition to these measurements, dry weight and VCC measurements were carried out on the cell suspensions pre and post homogenisation. Table 4.6 summarises these results.

Figures 4.6 and 4.7 indicate that the homogenisation at 1400 bar is sufficient to decrease the capacitance from around 37pF (Δcapacitance) to 1.5pF above that of the background measurements, a 96% decrease in capacitance.

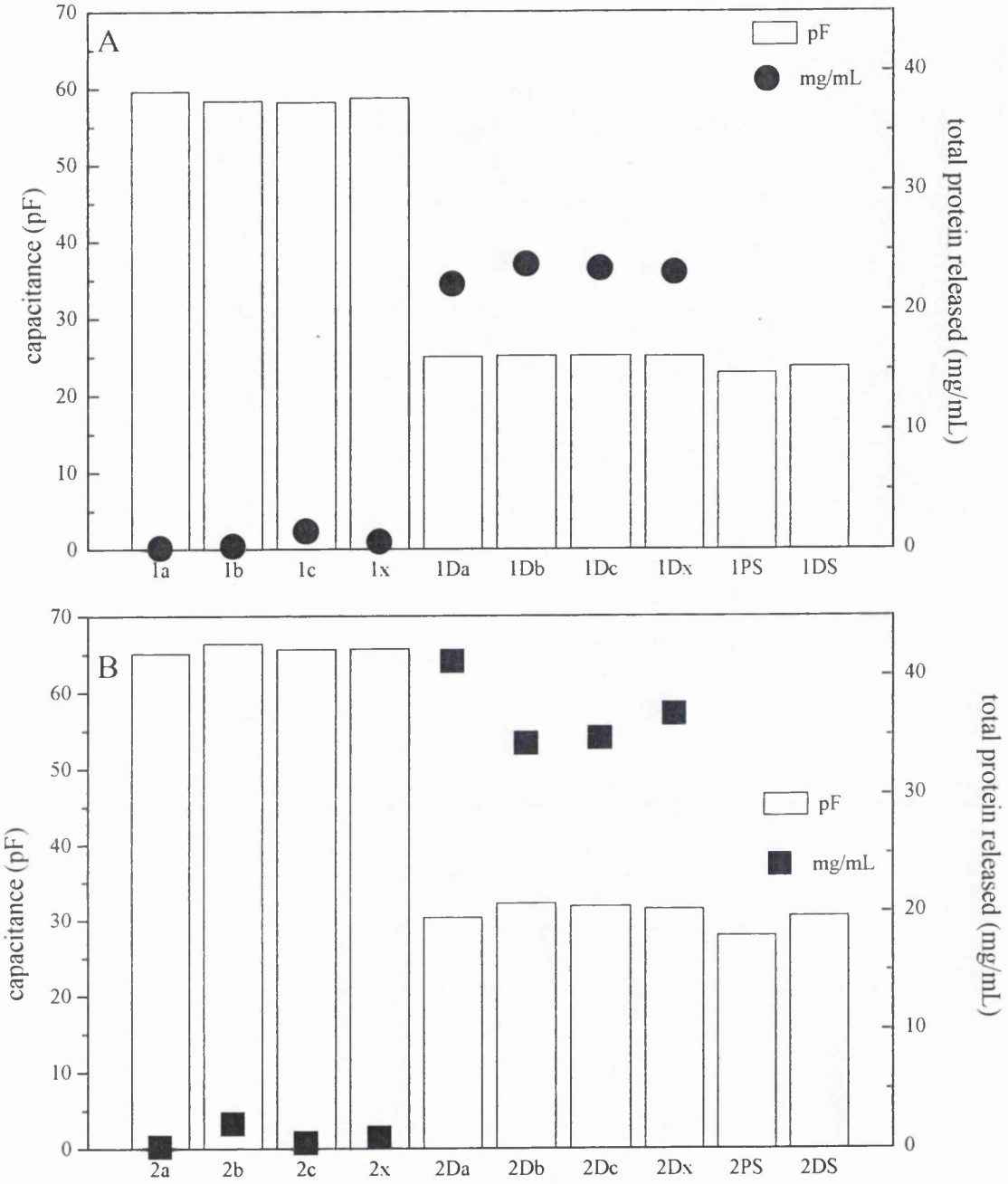


Figure 4.6: capacitance (pF) and total protein released (mg/mL) 1400 Bar Disruption (3 passes) of *Saccharomyces cerevisiae*

Figure 4.6A Experiment 1:

1a-c, 1x pre-disruption values and average

1Da-c, 1Dx post disruption values and average

1PS and 1DS pre and post disruption supernatent values

Figure 4.6B Experiment 2

1a-c, x pre-disruption values and average

1Da-c, x post-disruption values and average

1PS and 1DS pre and post disruption supernatent values

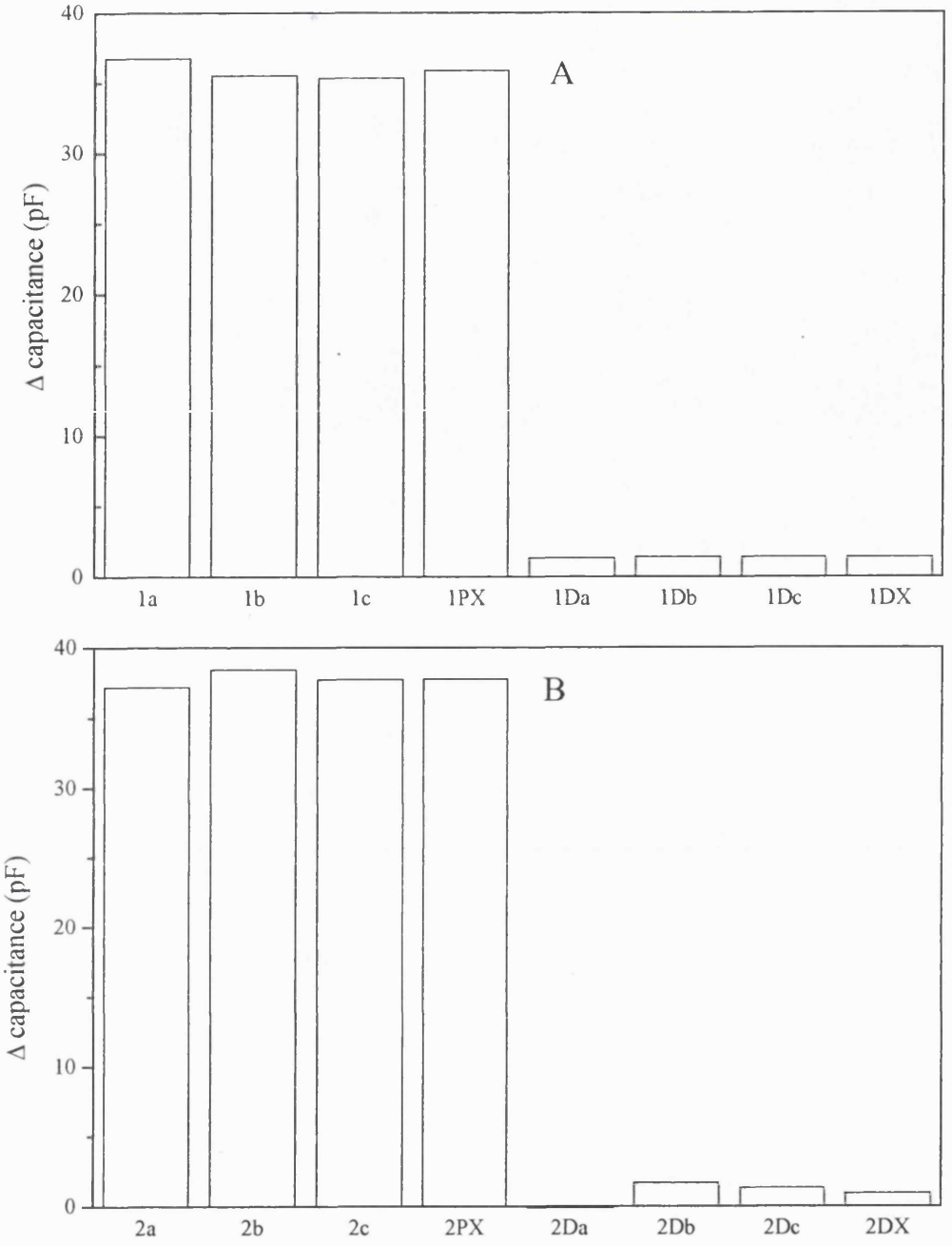


Figure 4.7: Δ capacitance (pF)
1400 Bar Disruption (3 passes) of *Saccharomyces cerevisiae*
Figure 4.6A Experiment 1:
1a-c, 1x pre-disruption values and average
1Da-c, 1Dx post disruption values and average
Figure 4.6B Experiment 2
1a-c, x pre-disruption values and average
1Da-c, x post-disruption values and average

The reproducibility of this effect is good, both within each experiment and between the two repeats of the experiment. The levels of protein release are maximal within each experiment, indicating complete homogenisation of the yeast suspension. The reproducibility of the protein release data is good within each experiment, but the levels differ between the two repeats, post homogenisation. The dry weight and VCC data are also very similar and show a large decline after homogenisation. The VCC figure, calculated from microscopic examination, post-homogenisation is still apparently quite large. However, serial dilution plate counts carried out on post homogenisation samples from both experiments gave no growth at any dilution.

Table 4.6

Summary of data obtained from 1400 bar homogenisation experiments.
DW is expressed in g/L and VCC is expressed in CFU/mL.
Results shown are pre and post homogenisation

	DW pre	DW post	VCC pre	VCC post
expt 1	54.4	4.8	2.4e 10^{10} (97.8% viable)	1.9e 10^{8}
expt 2	50.9	5.35	2.5e 10^{10} (98.3% viable)	3.6e 10^{8}

4.3.1.2 *S.cerevisiae* homogenisation at a range of pressures

A packed yeast suspension identical to the one used in the experiments described above in Section 4.3.1.1 was disrupted by a single pass at a range of pressures using the same homogeniser. The capacitance, total protein released, VCC and dead cell concentration (DCC, measured by counting the number of intact cells

which take up but do not decolourise methylene blue, see Section 3.3.4) were measured pre and post homogenisation. Three pre homogenisation capacitance measurements were taken at intervals throughout the experiment and averaged. The cell suspension was centrifuged pre homogenisation to remove the cells and the capacitance of the supernatant used as a measure of background capacitance. The post homogenisation measurements are single readings taken immediately after homogenisation. As before, the total protein release values shown are the average values from three separate measurements. The VCC and DCC counts are the average values of three repeats for each sample pre and post homogenisation. Dry weight and VCC determination on the pre-homogenisation cell suspension gave a value of 51.4 g/L and a percentage viability of 99%, respectively. Figure 4.8 show the results of this experiment.

In Figure 4.8, the decrease in capacitance with increasing homogenisation pressure was linear between 100 and 1000 bar and showed a drop of around 3.3pF per 100 bar pressure increase in this region. The decrease in capacitance above 1000 bar was much less, dropping by around 3pF from 1000 to 1600 bar. It is interesting to note that the background capacitance was lower than the value after homogenisation at 1600 bar by around 4pF, indicating that even after homogenisation at 1600 bar, there is still a Δcapacitance attributable to the presence of the cells. The increase in total protein was also linear with increasing homogenisation pressure, between 100 and 700 bar. Above 700 bar, the increase in protein release slowed until it peaked at 1400 bar and then declined slightly at 1600 bar.

In Figure 4.8b, the decline in VCC followed the decline in capacitance with increasing homogenisation pressure, though not as smoothly as capacitance. The pre homogenisation supernatant VCC measurement again has a lower value than that for the cell suspension post homogenisation at 1600 bar, which is agreement with the capacitance data for those points.

The DCC value rose for 100, 300, 500 and 700 bar , before falling sharply to a plateau after 1000 bar. As with the capacitance and VC values, the DCC value for the pre homogenisation supernatant was lower than that for the value post homogenisation at 1600 bar.

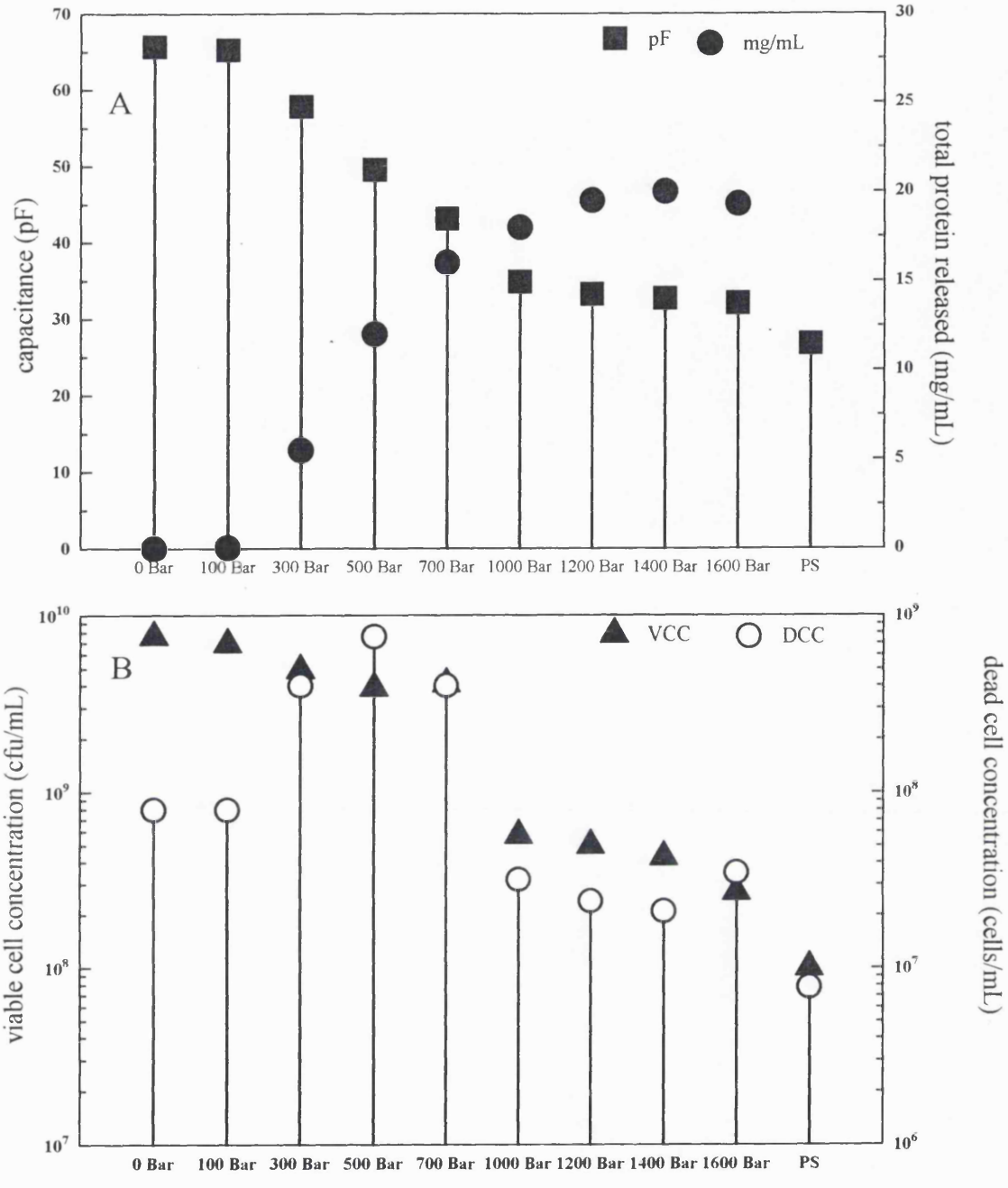


Figure 4.8:
capacitance (pF), total protein released (mg/mL), viable cell concentration (cfu/mL) and dead cell concentration (cells/mL) versus disruption pressure (single pass) of *Saccharomyces cerevisiae*
PS pre-disruption supernatent

4.3.1.3 The effect of repeated low pressure homogenisation on viability

The work described above shows the effect of homogenisation, both at high pressure for three passes and at a range of pressures for a single pass. An experiment was carried out to examine the effects of multiple pass, low pressure (500 bar) homogenisation on the viability of a *S.cerevisiae* packed yeast suspension, as determined by capacitance and VCC/DCC measurements. Total protein release and decline in dry weight were used as indicators of damage. In this experiment, a 250 g/L (wet weight) suspension of yeast in 50mM pH 6.7, phosphate buffer solution was used. The capacitance of the solution pre homogenisation was measured twice during the experiment and an average value determined. The cell suspension was centrifuged to remove the cells and the capacitance of the supernatant measured and used as a background value. Dry weight and VCC determinations on the pre-homogenisation cell suspension gave a dry weight of 75 g/L and an initial viability of 100%.

Figure 4.9 shows that at 500 bar, the majority of damage had occurred in the first two passes and the decrease in capacitance levelled out after 4 passes. Around 45% of the drop in capacitance occurred after the first pass and a further 30% after the second pass. The protein release data was not as clear cut but again the increase in protein release reached a plateau after 4 passes.

In Figure 4.9b, the VCC measurements declined slowly through the range of passes and showed only a small decline after the first pass and no plateau after 4 passes. The DCC rose after the first pass before declining steadily through the range of passes.

The data presented in this section of the results has shown that the BM can be used very successfully to give an accurate on-line estimate of biomass in yeast fermentations at a variety of scales and using defined and complex media. The nature of the relationship between capacitance and biomass has also been investigated, and it was found that, capacitance is biomass-related and is strongly affected by cell viability.

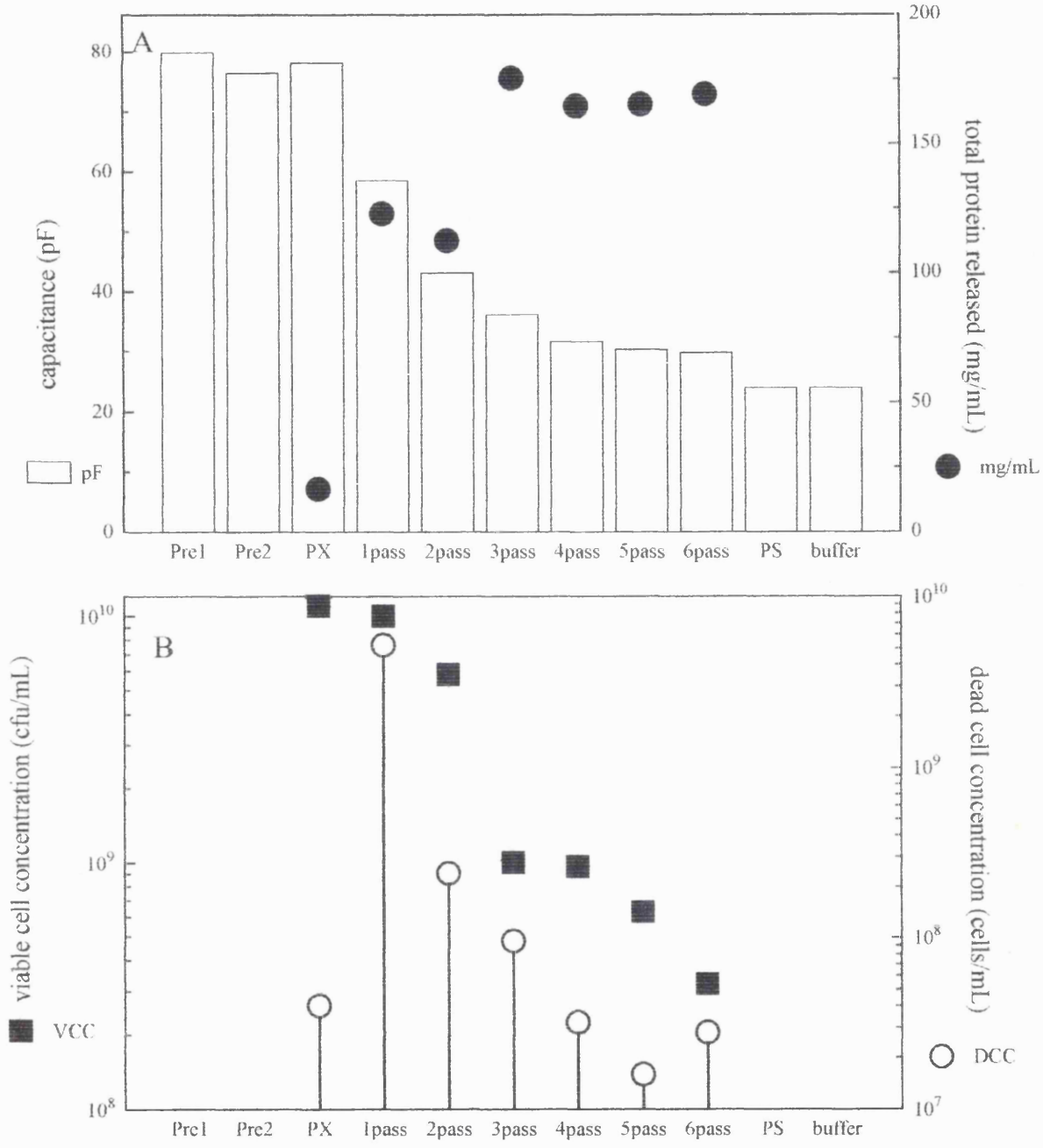


Figure 4.9:
capacitance (pF), total protein released (mg/mL), viable cell concentration (cfu/mL) and dead cell concetration (cells/mL) 500 Bar Disruption (multiple passes) of *Saccharomyces cerevisiae*
Pre, Pre 2, PX pre-disruption values and average
1-6 Pass number of cumulative passes at 500 Bar
PS pre-disruption supernatent
buffer pre-disruption buffer

4.2 *Pseudomonas putida* ML2 experiments

Experiments to examine the applicability of the Biomass Monitor for bacterial cells were carried out using *P.putida* ML2 as the test organism.

4.2.1 *P.putida* ML2 5L defined media fermentations

To investigate the ability of the Biomass Monitor to measure bacterial biomass on-line in a fermentation vessel under normal conditions, a series of fermentations were carried out growing *P.putida* ML2 under the conditions described in Section 3.2.2.

The capacitance measurements shown are average values taken from the real-time continuous measurements recorded by the chart recorder and have had the background capacitance of the fermentation media removed by the offset facility of the Biomass Monitor. To fully investigate the nature of the relationship between the capacitance measured and the biomass, the capacitance measurements were compared with other indicators of growth for each of these fermentations. Carbon Dioxide Evolution Rate (CER) was measured and calculated on-line and in real-time, and the cumulative CER, calculated off-line. These were used as indicators of growth and measures of metabolic activity. Viable Cell Concentrations (VCC) of the cultures were calculated using conventional microbiological methods off-line from samples taken at regular intervals from the vessel. The optical density (OD) at 670nm was also calculated off-line using the same samples from the vessel.

Figures 4.10 and 4.11 show the results from two fermentations carried out under the same conditions in the same vessel. The parameters measured are all plotted against time after inoculation.

In Figures 4.10, the growth peak, as determined by CER occurred around 31 hours when CER reached 72 mmol/L/h. The peak in capacitance was 3.2pF at the peak point of growth, and showed good agreement with CER during the exponential growth phase. Capacitance fell by 50% at the end of the exponential growth phase, while the CER fell to a much lower level, indicating the cessation of growth associated metabolic activity.

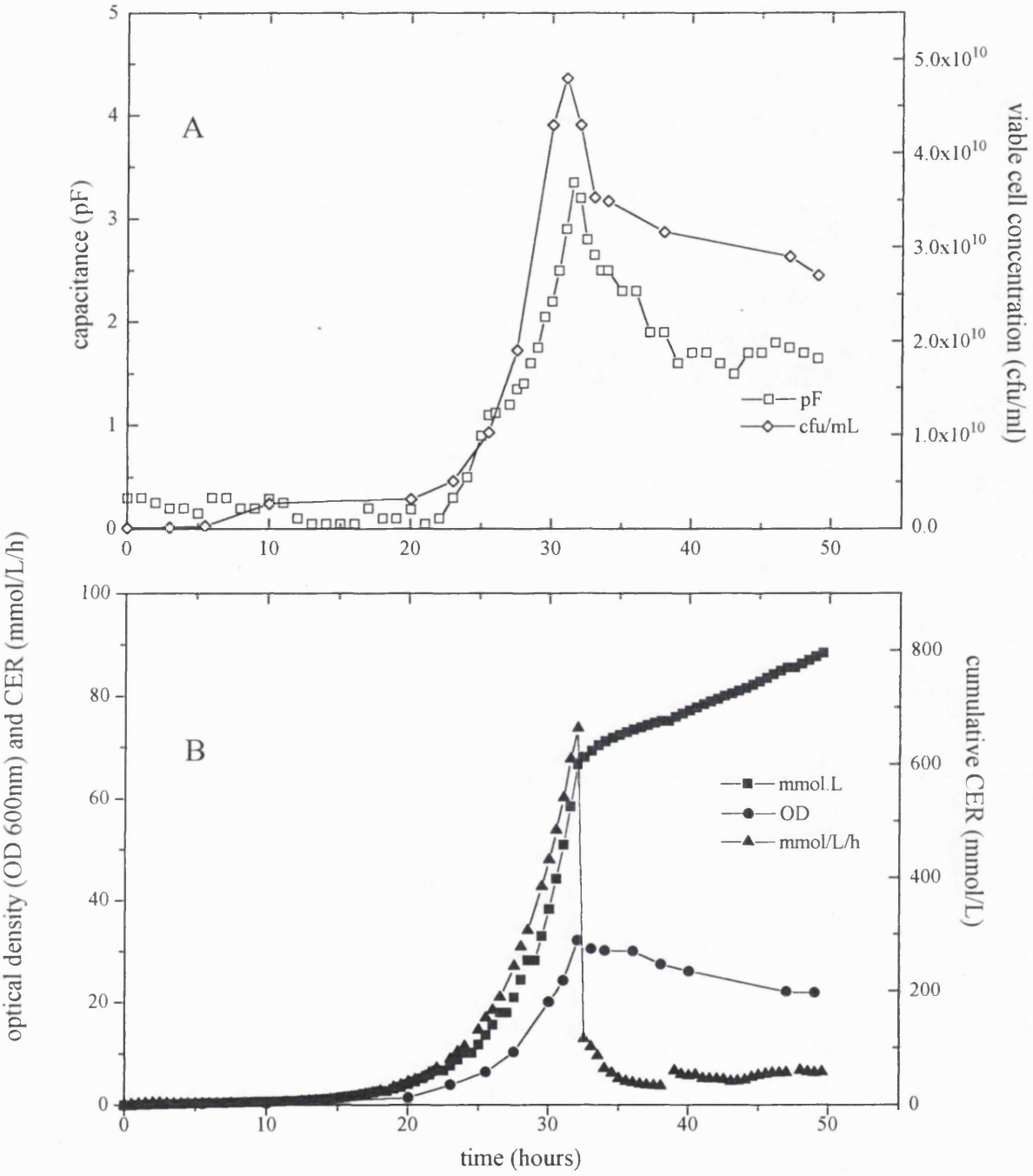


Figure 4.10: capacitance (pF) and viable cell concentration (cfu/mL), OD600 (OD), carbon dioxide evolution rate (CER, mmol/L/h) and cumulative CER (mmol/L) versus time *Pseudomonas putida* ML2 5L fermentation PP1

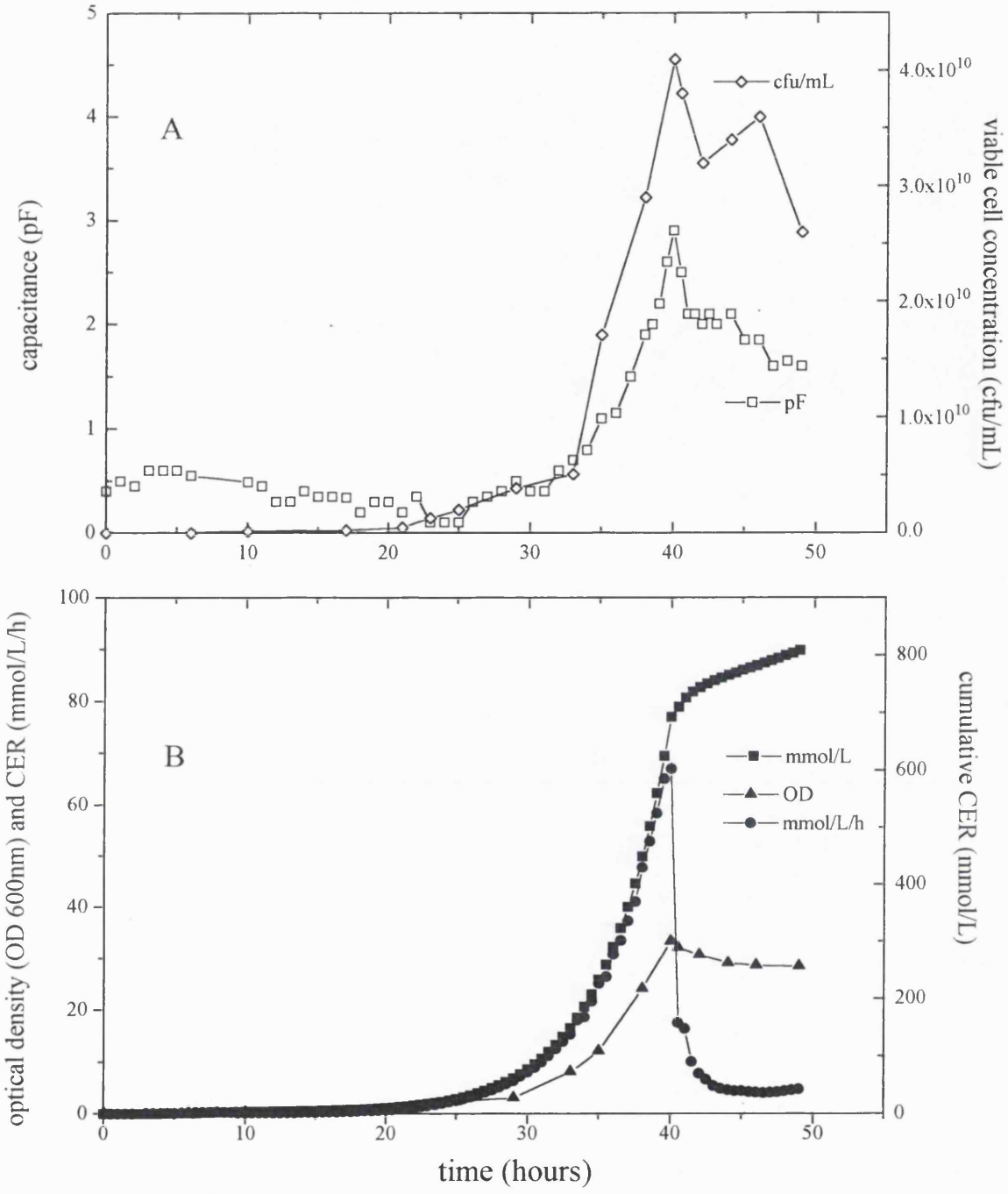


Figure 4.11: capacitance (pF) and viable cell concentration (cfu/mL), OD600 (OD), carbon dioxide evolution rate (CER, mmol/L/h) and cumulative CER (mmol/L) versus time.
Pseudomonas putida ML2 5L fermentation PP2

The difference between capacitance and CER is again due partly to CER being a spot value while capacitance is a cumulative value. However, the plots of cumulative CER continue to rise after the end of log growth, while the capacitance values decrease at the end of the fermentations.

The VCC plot showed a similar growth profile to that of capacitance. The cell concentration peaks after 31 hours at 4.8 x 10^{10} and declined during stationary phase. The OD profile was consistent with the other indicators of growth, and peaked after 31 hours at 32 absorbance units before declining by 30% in the stationary phase of the culture.

In Figure 4.11a-b, the growth peak, as determined by CER, occurred after 39 hours at 68 mmol/L/h. The capacitance peak also occurred after 39 hours at 3 pF. The metabolic measure, CER, fell to a resting level immediately after the end of exponential growth. The capacitance measurement declined in the stationary phase, falling by around 40%. The lag phase of this fermentation was eight hours longer than in Figure 4.10 and as a result, the carbon source was exhausted before the growth, as measured by CER and capacitance, reached the levels shown in Figure 4.10. This was as a result of the some of the available carbon in this carbon-limited fermentation being used for maintenance during the longer lag phase. However, the exponential growth phases have the same duration. The VCC measurements and the OD measurements in Figure 4.11 peaked after 39 hours at 4.2 x 10^{10} and 28. These are both slightly less than in Figure 4.10, illustrating that the growth is less in this fermentation due to the longer lag phase and the consequent greater utilisation of the substrate for maintenance prior to the exponential growth phase.

To examine the relationship between the capacitance measured on-line for these fermentations and the other indicators of growth measured, Table 4.7 shows the correlation coefficients from linear plots of the data for these variables.

Table 4.7

Correlation coefficients for capacitance against various biomass measurements for *P.putida* ML2 fermentations.

R^2 values versus capacitance

	CER	cCER	OD	VCC
PP1	0.66	0.82	0.94	0.97
PP2	0.65	0.93	0.96	0.98

For these fermentations, the variable which correlates best with capacitance throughout each of the fermentations is viable cell concentration (R^2=0.98 and R^2=0.97) All the other parameters have correlation coefficients of 0.8 or above except the CER measurements and have poor correlation's with capacitance for the reasons outlined previously.

The specific growth rates (μ) of the initial exponential growth phase, calculated from semi-logarithmic plots of the measured variables of these fermentations (F1 and F2) are given in Table 4.8.

Table 4.8

Specific growth rates for *P.putida* ML2 5L fermentations, calculated from various biomass measurements.

parameters used to calculate μ

	Cap.	CER	cCER	OD	VCC
PP1	0.21	0.22	0.22	0.27	0.22
PP2	0.25	0.24	0.24	0.23	0.30

The data from the μ calculations show that the fermentations were very similar, despite the longer lag phase shown in PP2. The correlation between the two

fermentations was closest in terms of metabolic activity as measured by exit gas analysis, which reflects the accuracy of this measurement. The values from capacitance are very similar to those for the metabolic activity, though the difference between the two fermentations is greater. The μ values for the other measured parameters were varied, which reflected the relative accuracy of the other biomass measurements.

4.2.2 *P. putida* ML2 off-line experiments

To look at the relationship between capacitance and biomass at high bacterial biomass levels, off-line experiments were carried out using the set-up described in Section 3.1.1. Cells were harvested from a *P.putida* ML2 fermentation at the peak point of growth and centrifuged to yield a firm pellet. The dry weight of the pellet was estimated from the wet weight using previous data and sufficient of the pellet was added to 50mM phosphate buffer, pH 6.7, to give a 60 g/L dry weight solution of bacterial cells. The capacitance of the suspending buffer was backed off prior to the addition of the bacterial cells. The addition of the harvested cells was repeated at varying concentrations to give a range of biomass levels. Figure 4.12 shows the results of these experiments.

The correlation coefficients and Y-axis intercepts of the linear regression applied to these data are R^2=0.99, Y intercept =0.05942 for capacitance against dry weight of the bacterial suspension. From these correlations it can be deduced that the capacitance measured by the Biomass Monitor shows good linearity to high biomass levels for *P. putida* . It is interesting to note that the best agreement with the linear regression is found at the higher biomass levels.

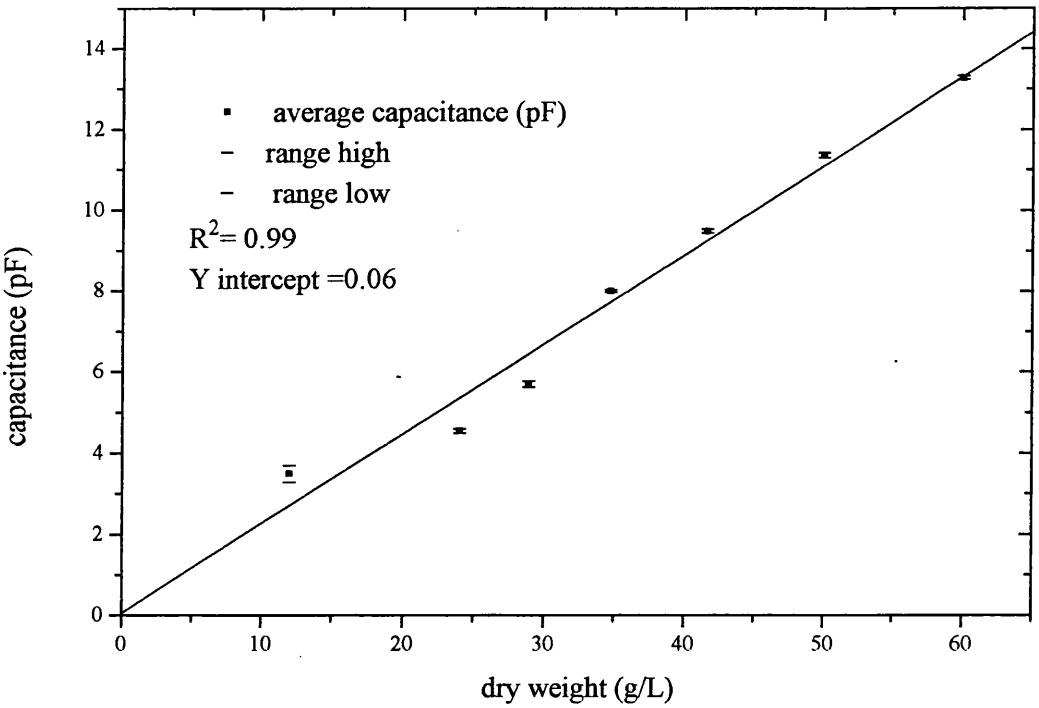


Figure 4.12: Off-line capacitance measurement of concentrated *P.putida* ML2 in 50mM, pH 6.7, phosphate buffer.

4.2.3 *P.putida ML2* homogenisation experiments

The results shown above have indicated that the Biomass Monitor can give a biomass related capacitance signal in both an idealised off-line situation and on-line in fermentation vessels. The attempts to correlate capacitance with the other indicators of growth and biomass have shown good results. In order to further examine this relationship between capacitance and biomass, and in particular viability, two experiments were carried out using a homogeniser to disrupt cell suspensions of *P.putida* ML2 (as described in Section 3.3.8).
The effects of this homogenisation on capacitance and other indicators of viability and cell integrity were examined.

4.2.3.1 *P.putida* ML2 1400 bar homogenisation

In order to determine the effects of homogenisation on the capacitance signal from the cell suspension, a 14 g/L dry weight *P.putida* ML2 suspension, harvested from a fermentation at peak point of growth, was disrupted by carrying out three passes through the homogeniser at 1400 bar. This pressure and method was chosen as it was known to give maximal protein release, indicating maximum homogenisation. The method used is outlined in Section 3.3.8.
The results shown in Table 4.9 are from two identical experiments (C1 and C2), each of which contains three repeat capacitance measurements pre and post homogenisation. The data shown is the change in capacitances measured off-line at 0.4 MHz before and after homogenisation. As measures of viability and cell homogenisation, VCC and total protein released were also measured. The VCC measurements were carried out as described previously. The total protein release values shown are each the average of three separate measurements on each of the three separate homogenisations. As a measure of the background capacitance, the undisrupted cell suspensions were centrifuged to remove the cells or cell debris and the capacitance of the supernatants were measured. The capacitance value given below is derived from subtracting the capacitance of the cell supernatant from that of the cell suspension.

Table 4.9

Δcapacitance (pF) total protein concentration (mg/mL) and viable cell concentration (cfu/mL) pre and post homogenisation (two experiments)

	ΔCap	protein pre	protein post	VCCpre	VCCpost
C1	-3.2	1.01	5.26	$4.1x10^{10}$	0
C2	-2.8	2.8	4.78	$3.8x10^{10}$	0

The drop in capacitance due to the homogenisation of the cells was similar, 3.2pF for C1 and 2.8pF for C2. The total protein release data were also in agreement, although the release after homogenisation in C1 was greater than that seen for C2. The higher initial protein level in C2 could explain this, and also could explain the smaller capacitance signal attributable to the cells in C2. As the background protein level was higher in C2, this could indicate some cell lysis in the suspension prior to homogenisation. The data for VCC for the two experiments shows that the loss of viability of the cells was total, and that the VCC of C2 was less, initially, than C1, which was in agreement with the capacitance data and also supports the data from the protein measurements. The capacitance values attributable to the cells are as would be predicted from the on-line fermentation data described above as are the VCC values.

The data from these experiments shows that the biomass monitor can be used to detect the presence of bacterial cells, both off-line and on-line. The correlations shown with VCC, both on-line and off-line support the theory that the device only measures intact, viable cells.

4.3 *Penicillium chrysogenum* P1 experiments

Experiments to examine the applicability of the Biomass Monitor for fungal cultures were carried out using *P. chrysogenum* P1 as the test organism.

4.3.1 *P. chrysogenum* P1 defined media batch fermentations.

To investigate the ability of the Biomass Monitor to measure fungal biomass on-line in a fermentation vessel under normal conditions, a series of fermentations were carried out growing *P. chrysogenum* P1 under the conditions described in Section 3.2.3. The fermentations were carried out using a defined medium in batch mode, with two carbon sources, sucrose for the initial growth phase and lactose for the "production" phase.

The results shown in this section attempt to illustrate the differences found in capacitance measurements yielded by different growth forms and morphologies of *P. chrysogenum* P1, when grown in submerged culture. The capacitance measurements shown are average values taken from the real-time continuous measurements recorded by the chart recorder and have had the background capacitance of the fermentation media removed by the offset facility of the Biomass Monitor. To investigate the nature of the relationship between the capacitance measured and the biomass, the capacitance measurements were compared with other indicators of growth for each of these fermentations. Carbon Dioxide Evolution Rate (CER), calculated from the exit gas CO_2 concentration, was measured on-line and in real-time, and the cumulative CER, calculated off-line, were used as indicators of growth and measures of metabolic activity. Dry weight of the fungal biomass in the vessel was calculated by conventional microbiological methods off-line from samples taken at regular intervals from the vessel. The mean main hyphal length, an indicator of fungal morphology was also measured off-line from these samples using the method described in Section 3.3.4.

Figure 4.13 shows the results from a fermentation in which the growth form of the fungal mycelia was as loose pellets. The parameters measured are all plotted against time after inoculation. In Figure 4.13, the growth as determined by CER, capacitance and dry weight did not show the diauxic profile usually associated with growth of *P. chrysogenum* on two carbon sources. The culture grew slowly, and reached a plateau at around 50 hours which lasted until around 80 hours, after which metabolic activity declined and capacitance and mean hyphal length fell.

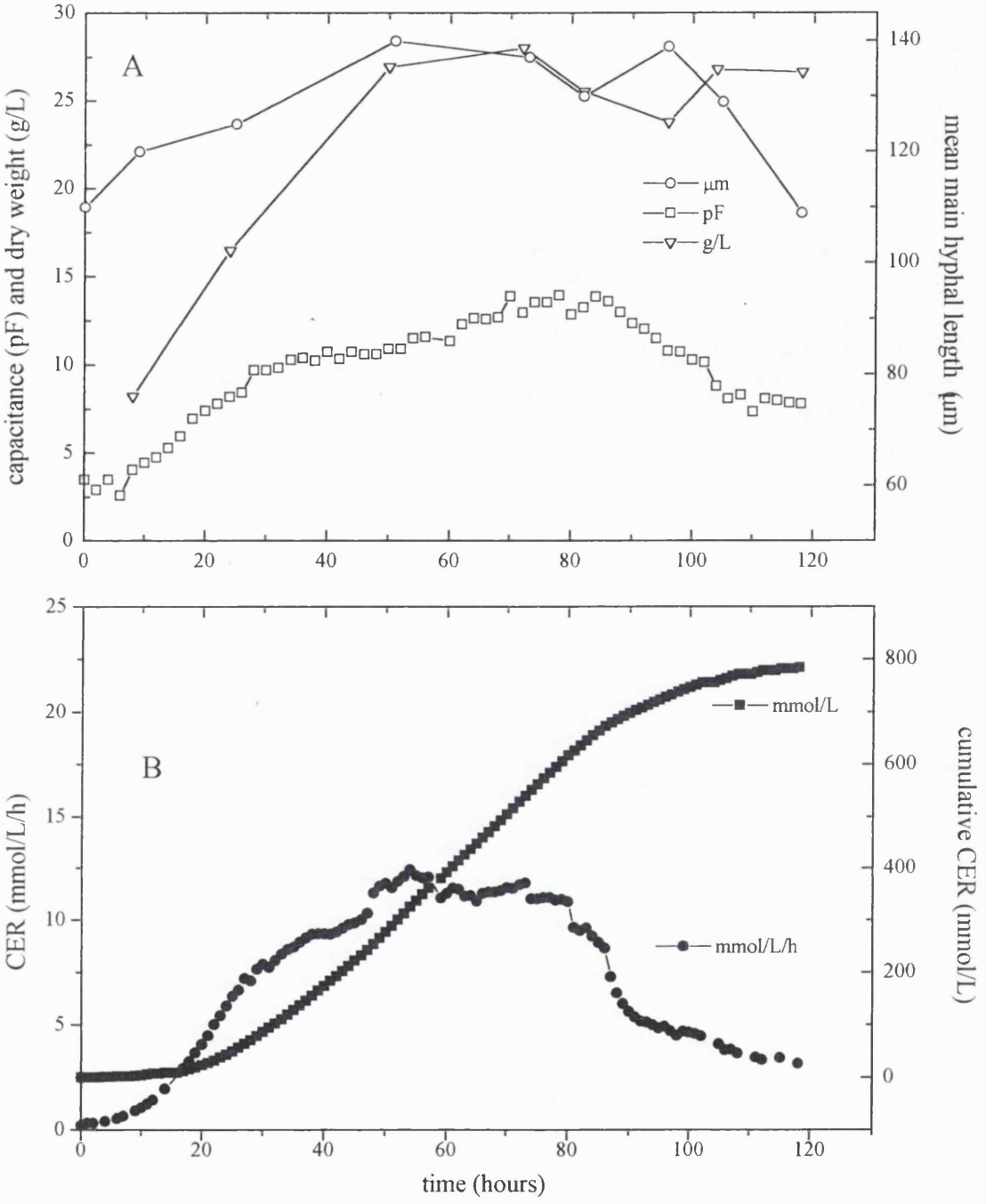


Figure 4.13 : capacitance (pF), dry weight (g/L), carbon dioxide evolution rate (CER, mmol/L/h), mean main hyphal length (μm) and cumulative CER (mmol/L) versus time
Penicillium chrysogenum defined media 5L fermentation PC1

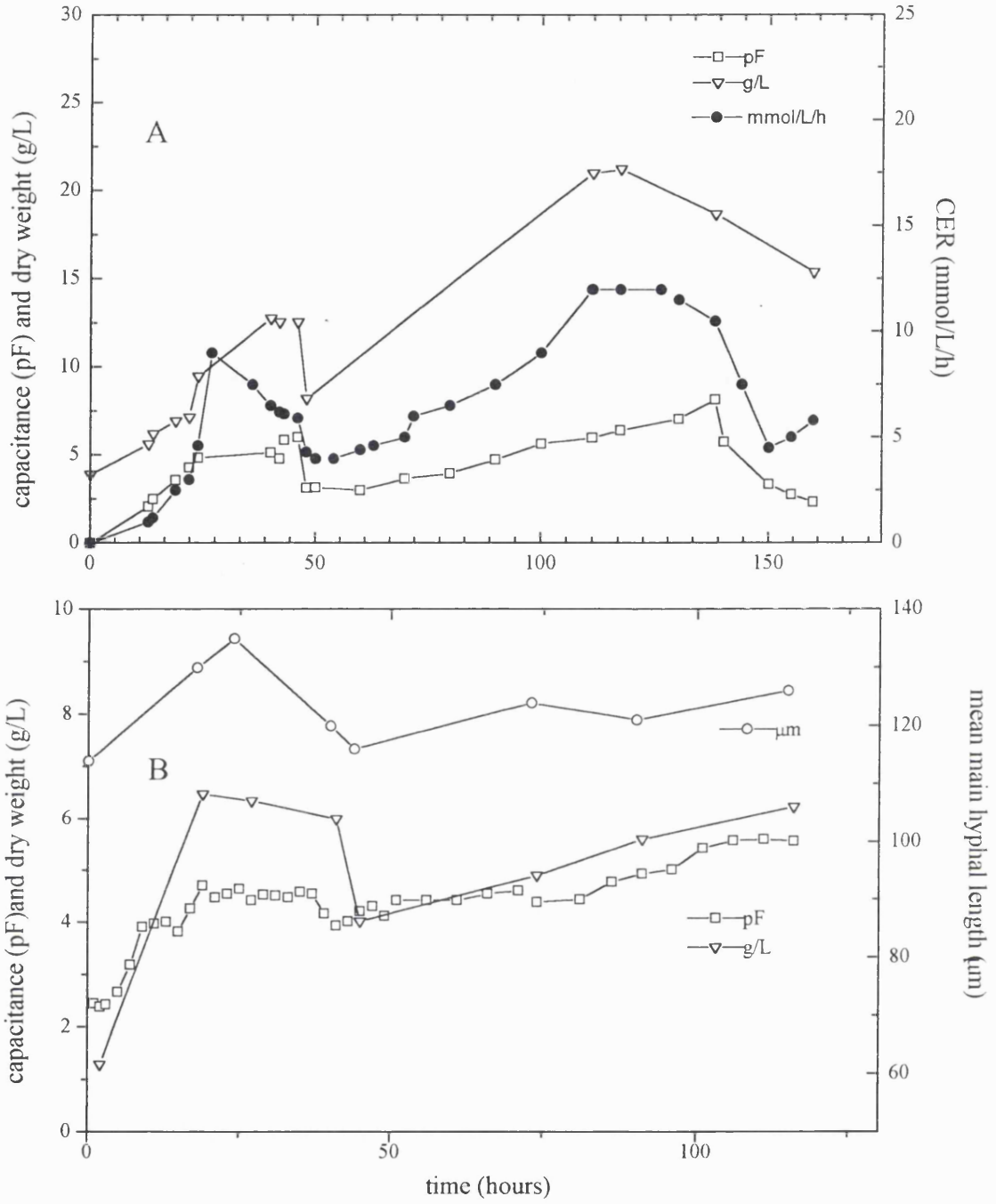


Figure 4.14a: capacitance (pF), carbon dioxide evolution rate (CER, mmol/L/h) and dry weight (g/L) versus time *Penicillium chrysogenum* defined media 5L fermentation PC2

Figure 4.14b: capacitance (pF), dry weight (g/L) and mean main hyphal length (µm) versus time *Pencillium chrysogenum* defined media 5L fermentation PC3

This is a probable indication of hyphal fragmentation and cell lysis, which is common in *P. chrysogenum* cultures of this age, although the off-line dry weight measurements did not reflect this.

Figures 4.14a and b shows the results from two further fermentations carried out using the same organism, conditions and vessel as above. The growth form in both these fermentations was diffuse mycelial. The differences in growth form for these fermentations was attributable to the effects of differences in the spore concentrations used to seed the inoculum for the first fermentation (Smith and Calam 1980). The growth curves for these fermentations showed the diauxic growth associated with the fermentation of *P. chrysogenum* P1 using two carbon sources. In both cases the initial growth phase ended around 25 hours as the sucrose was exhausted. This can be seen in the initial growth peaks of the CER, capacitance and dry weight in Figure 14a and in the capacitance, dry weight and mean main hyphal length in 14b. In both fermentations, capacitance peaked at around 5pF and only increased slightly over the longer, slower second growth phase utilising lactose. The fermentation in Figure 4.14b was terminated early at around 125 hours, thus the decline in capacitance seen in Fig. 14a after around 140 hours is not seen. This decline in capacitance in Figure 4.14a occurred after the metabolic activity of the culture had declined and this would seem to support the observation that the on-line capacitance measurement was detecting hyphal fragmentation and cell lysis in the later stages of these *P. chrysogenum* P1 fermentations.

To examine the relationship between the capacitance measured on-line and the other indicators of growth measured for this fermentation, linear plots of the data for these variables were carried out. Table 4.10 shows the correlation coefficients from linear plots of the data for these variables.

Table 4.10

Correlation coefficients for capacitance against various biomass measurements for *P.chrysogenum* P1 fermentations.

R^2 values versus capacitance

	CER	cCER	DW	MMHL
PC1	0.83	0.53	0.77	0.78
PC2	0.84	0.66	0.76	
PC3			0.84	0.62

For these fermentations, the variable which correlates best with capacitance throughout the fermentation is CER (R^2=0.83) although the low R^2 value indicates that it is not a strong correlation. All the other parameters have correlation coefficients of 0.7 or above except the cumulative CER measurements. The lack of on-line gas analysis in PC3 means that dry weight gives the best correlation with capacitance for this fermentation, although the R^2 value of 0.84 is as good as the correlations of capacitance with CER found in the other fermentations shown.

As the data yielded from the on-line capacitance measurements made during fermentations of *P. chrysogenum* P1 indicate that capacitance was related to biomass for fungal cultures but the correlations with the other indicator of growth were poor, work was carried out off-line to further examine the measurement of the capacitance of fungal cultures.

4.3.2 *P. chrysogenum* P1 homogenisation experiment

Experiments were carried out on a cell suspension of *P. chrysogenum* P1 harvested from a stationary phase fermenter culture, after 76 hours. The cell suspension was disrupted using a homogeniser at a range of pressures to simulate the fragmentation and lysis, as occurs in the fermenter in the later stages of the fermentations described above. The effects of these homogenisations on capacitance and other indicators of viability and cell integrity were examined . As a measure of the

background capacitance, the cell suspension was centrifuged pre and post homogenisation (3 passes at 1200 bar) to remove the cellular material and the capacitance of the supernatant measured.

4.3.2.1 *P. chrysogenum* P1 1400 bar homogenisation

Figure 4.15 showed the results from disrupting a *P.chrysogenum* cell suspension by homogenisation at a single pass using a range of pressures, and to achieve total homogenisation, 3 cumulative passes at 1200 bar.

The fall in capacitance after the homogenisation designed to achieve total homogenisation (3 x 1200 bar) gave a capacitance reading 0.6 pF higher than that of the suspending media, meaning the capacitance due to the cells in the suspension had fallen by 95%. In the experiment to examine the effect of homogenisation pressure on the capacitance signal from fungal biomass, the fragility of the fungal biomass is illustrated in that the fall in capacitance after the homogenisation at 200 bar was 62% of the pre-homogenisation value. Around a further 20% occurred after the homogenisation at 400 bar, thus indicating that the substantial falls in on-line capacitance seen in fermentation monitoring are probably due to mycelial fragmentation and lysis. However, the residual capacitance after the more extreme homogenisation also indicated that fully disrupted cell suspensions can and do make a contribution to the overall capacitance signal, which should be borne in mind when interpreting on-line data.

The correlation between protein release and fall in capacitance is reasonable. The fall in capacitance and the rise in protein are similar up to 400 bar and both tail off at the higher pressures. The experiment carrying out three passes at 1200 bar yielded a lower capacitance than the single pass at 1000 bar but this was not reflected in a higher protein release. This is an indication that the measure of capacitance in off-line situations is possibly a more accurate measure of cell homogenisation than conventional monitoring techniques as the variability of the protein release data at high pressures is possibly due to heat denaturation of the protein.

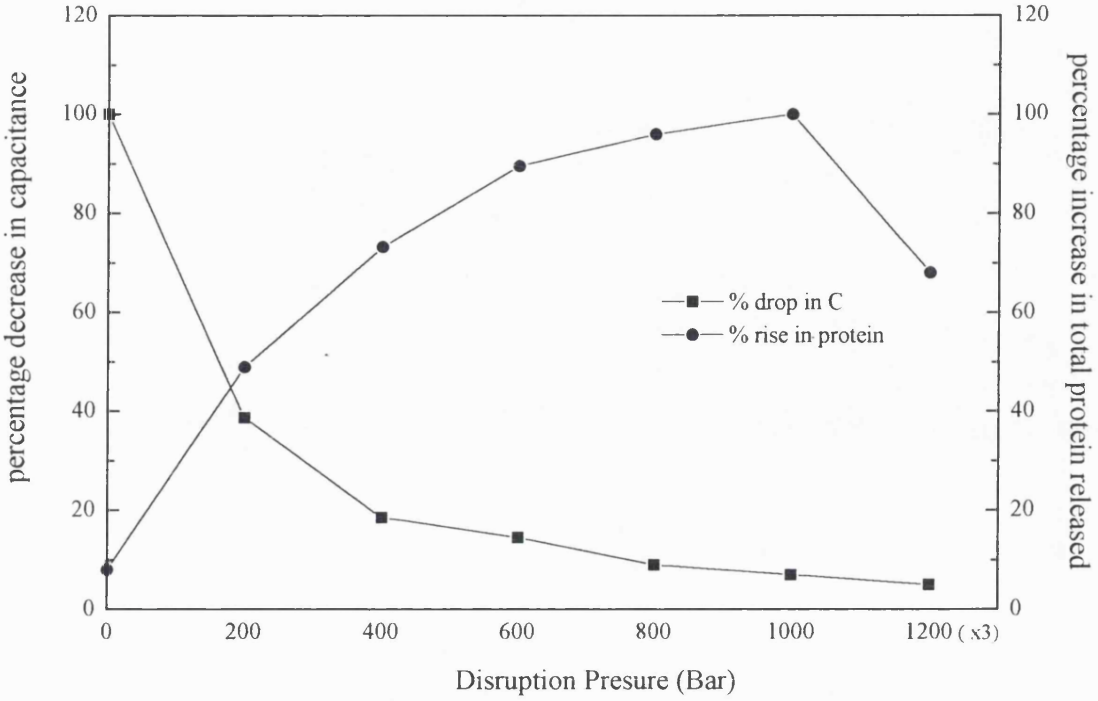


Figure 4.15: percentage decrease in capacitance and percentage increase in total protein released at a range of disruption pressures Varied pressure, single pass disruption of *Penicillium chrysogenum*

The data from these experiments indicated that the on-line and off-line measurement of capacitance was biomass-related in defined media, batch *P.chrysogenum* P1 fermentations. The next section will described the work carried out at SmithKline Beecham in measuring capacitance on-line in complex media, fed-batch fermentation of *P.chrysogenum.*

4.4 *P.chrysogenum* complex media, fed-batch fermentations.

To continue the investigation of the relationship between fungal biomass and capacitance, the on-line capacitance of a number of industrial *P.chrysogenum* fermentations was monitored. These were carried out at the SmithKline Beecham laboratories at Worthing and Irvine. The processes monitored were fed-batch fermentations using a complex media containing undissolved solids. The organism grown was an industrial strain of *P.chrysogenum.* All the processes monitored were routine fermentations, carried out under their normal conditions.

The processes were investigated at three different scales, laboratory, pilot and production scale. The differences in the processes of the fermentations monitored are as follows:

SB1 laboratory scale, normal feed regime.

SB2 laboratory scale, altered media composition and feed regime.

SB3 pilot scale, normal feed regime.

SB4 pilot scale, accelerated feed regime.

SB5 pilot scale, normal feed regime.

SB6 production scale, normal feed regime.

For reasons of confidentiality, all the measurements shown for these fermentations, with the exception of capacitance and mean main hyphal length, have had a scaling factor applied to them.

4.4.1 *P.chrysogenum* laboratory scale, complex media, fed-batch fermentations.

These fermentations were carried out at laboratory scale, using an industrial strain of the organism and a complex media under normal operational conditions for

these fermentations. Δ capacitance was monitored in two frequency mode using 0.4 MHz as the low frequency point. When available, on-line gas analysis was used to give exit CO_2 measurements and other fermentation parameters were logged as routine. Samples were taken at regular intervals for viscosity, dry weight and DNA content analysis.

Figure 4.16a shows capacitance and exit CO_2 versus time for SB1. There was a good correlation between capacitance and % exit CO_2 during the growth phase. Capacitance then rose faster than % exit CO_2 after the post-exponential stationary phase, at a rate similar to that of the measure of cumulative exit CO_2. The viscosity profile showed a growth peak around 50 hours, corresponding to the end of the rapid growth phase, then a decline, followed by an increase, as further growth occurred, to a level above that of the initial peak.

Figure 4.17a shows the measured parameters versus time for SB2. The process used for this fermentation is very similar to the standard process, the main difference is the addition of an extra carbon source at the start of the fermentation. The initial growth phase was faster and the peak and post-exponential plateau are at a higher level than SB1. As in the other fermentation shown above, the capacitance rose again after around 60 hours. The sharp drop in capacitance around 95 hours could be due to a harvest of 4% of vessel volume taken around 88 hours. The fermentation ran on until 180 hours but a power surge at 125 hours caused the capacitance signal to fall to a minus figure. Several clean pulses were applied but there was no change in the capacitance signal. At the end of the fermentation, the probe was removed and found to be coated with mycelia, especially the measuring pins. This was probably due to the agitation being off for a period. After cleaning, the probe was replaced into the vessel and gave a conductance reading similar to the last one before removal and a capacitance reading of 58.8 pF which is reasonable capacitance endpoint when related to the dry weights, but not when related to the viscosity. The viscosity profile showed a peak at the end of the initial growth phase. The values then dropped sharply, before rising again and then dropping to the end of the fermentation. This is an unusual profile, but the lack of off-gas analysis and the problems with the capacitance measurement make it difficult to assess the fermentation.

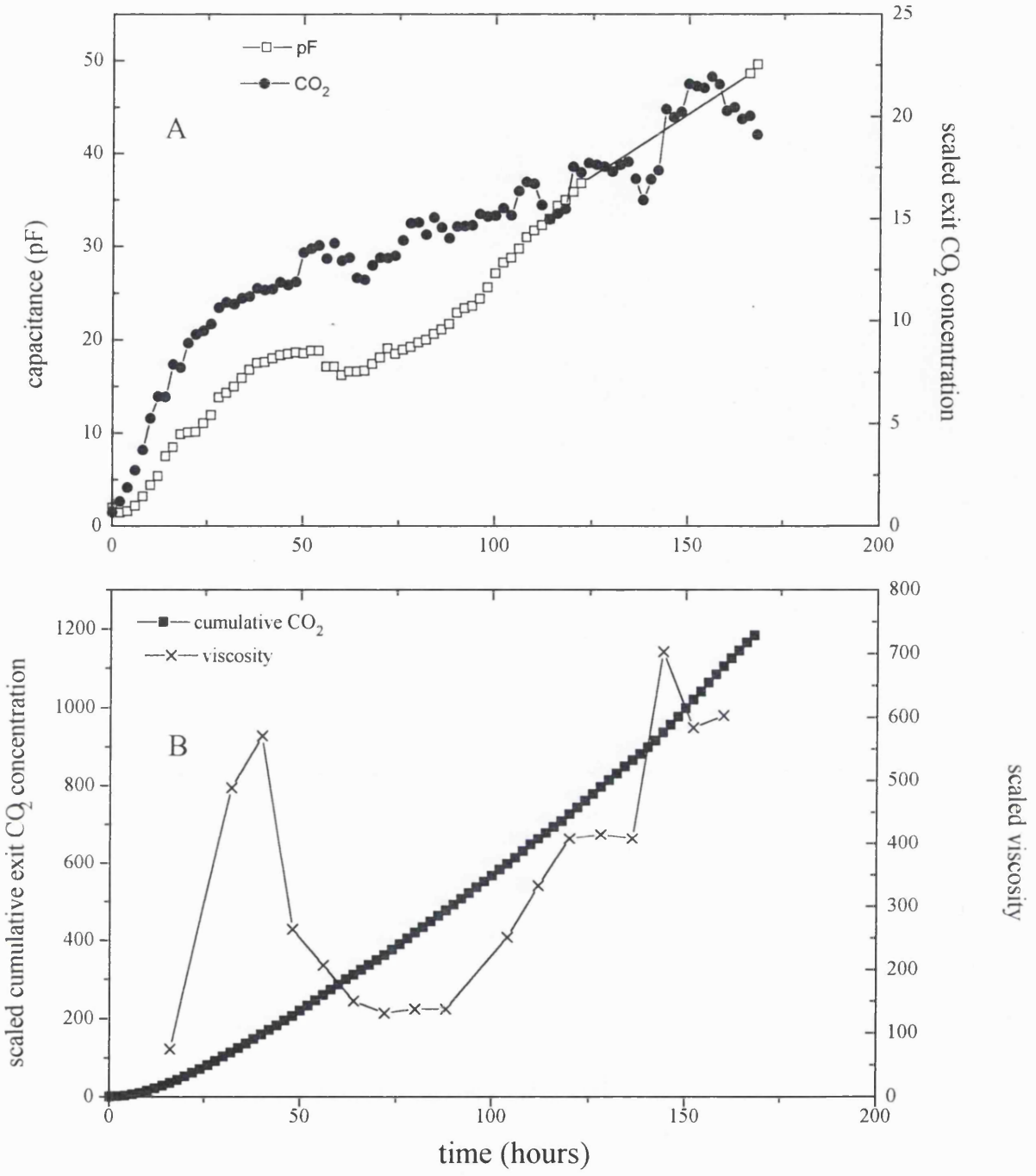


Figure 4.16: capacitance (pF), scaled exit CO_2, scaled cumulative exit CO_2 and scaled viscosity versus time

Penicillium chrysogenum 15L fermentation SB1

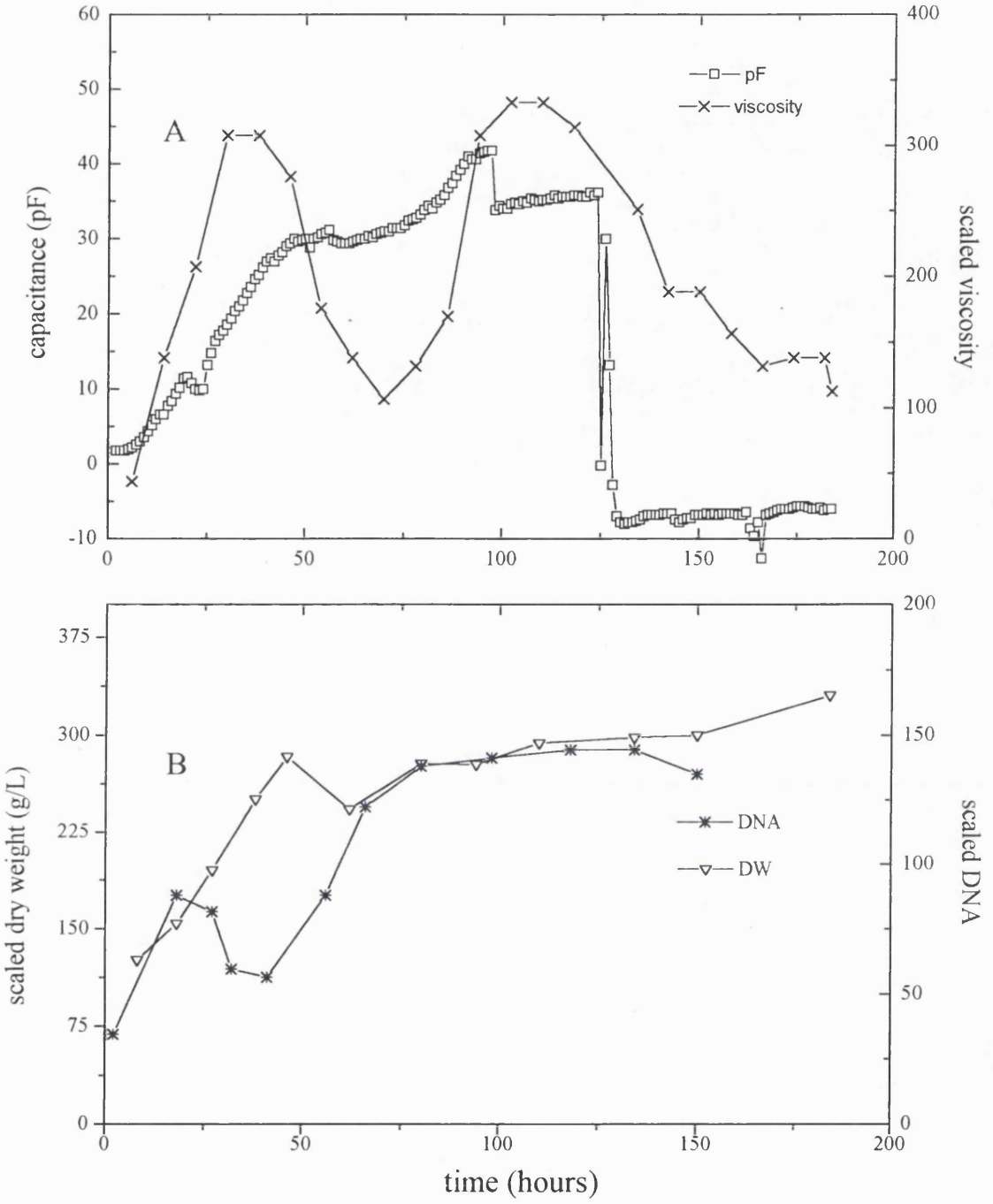


Figure 4.17: capacitance (pF), scaled dry weight (g/L), scaled DNA (g/L) and scaled viscosity versus time
Penicillium chrysogenum 15L fermentation SB2

The DNA content and dry weight profiles showed an initial peak, followed by a rise indicating further growth, which is largely in agreement with the capacitance profile.

Linear plots of the data from these fermentations were carried out to give correlation coefficients. Table 4.11 shows the correlation coefficients from linear plots of the data for these variables.

Table 4.11

Correlation coefficients for capacitance against various biomass measurements for laboratory scale *P.chrysogenum* fermentations.

R^2 values versus capacitance

	$C0_2$	$cC0_2$	DW	DNA	Visc.
SB1	0.91	0.96			0.29
SB2			0.95	0.77	0.47

The linear plots of the data for SB1 indicated that cumulative exit CO_2 has the best correlation with capacitance for this fermentation, R^2= 0.96. The correlations from the linear plots of the data from SB2 indicated that, in the absence of gas analysis, dry weight correlated best with capacitance for this fermentation. The R^2 value of 0.95 for dry weight was better than the correlations of capacitance and dry weight for the other fermentations, and as good as the best correlations with gas analysis data.

To further examine the usefulness of the BM in these fermentations. work was carried out on the pilot scale fermentations, running the same process.

4.4.2 *P.chrysogenum* pilot scale, complex media, fed-batch fermentations.

These fermentations were carried out at pilot scale using the same organism, conditions and media as the above fermentations. The capacitance probe was located in the lower third of the vessel and Δcapacitance was monitored in two frequency mode using 0.4 MHz as the selected low frequency point. The Δ

capacitance signals from the Biomass Monitor were logged using a 12 bit Squirrel data logger, as described in Section 3.1.6. The regime used was to read a value every 1 minute and then every 10 minutes, log an average of the readings obtained. The capacitance profiles show values taken from these data at hourly intervals. On-line gas analysis was used to give exit CO_2 measurements and other fermentation parameters were logged as routine. Samples were taken at regular intervals for viscosity, dry weight, DNA content analysis and morphological measurement.

The results shown in Figure 4.18 are for the normal *P.chrysogenum* process. Capacitance correlated well with exit CO_2 during the initial exponential growth phase of the fermentation, which ended around 20 hours. Both measurements plateaued after the initial growth phase and after this, they both began to rise again, although the initial correlation had broken down. The capacitance measurement rose faster than the exit CO_2 after the stationary phase around 75 hours, before it again levelled off around 125 hours and then rose again after 175 hours. Cumulative exit CO_2 shows that the culture continues to be metabolically active throughout the fermentation. The growth profile of this fermentation is similar to that for SB1, which indicates that the increase in scale does not significantly effect the profile of the fermentation. The capacitance values are higher in this fermentation than the values from the laboratory scale fermentations, although the exit CO_2 values are similar.

The other indicators of growth measured all show an initial growth phase peaking around 50 hours. Viscosity and mean main hyphal length both declined after this peak and levelled out around 150 hours, although viscosity showed an increase after around 175 hours till the end of the fermentation. Dry weight and DNA concentrations both fluctuate throughout the fermentation, precluding any detailed analysis.

Figure 4.19 gives the results for another pilot scale fermentation carried out in the same vessel, using the same organism, with another feed regime, known to give higher metabolic activity. Capacitance and exit CO_2 correlated during the initial growth phase and again diverged after 50 hours. The capacitance and exit CO_2 values are higher than those in Figure 4.18 showing that both capacitance and exit CO_2 are indicating a greater biomass level for this fermentation. The other indicators of growth showed varied trends.

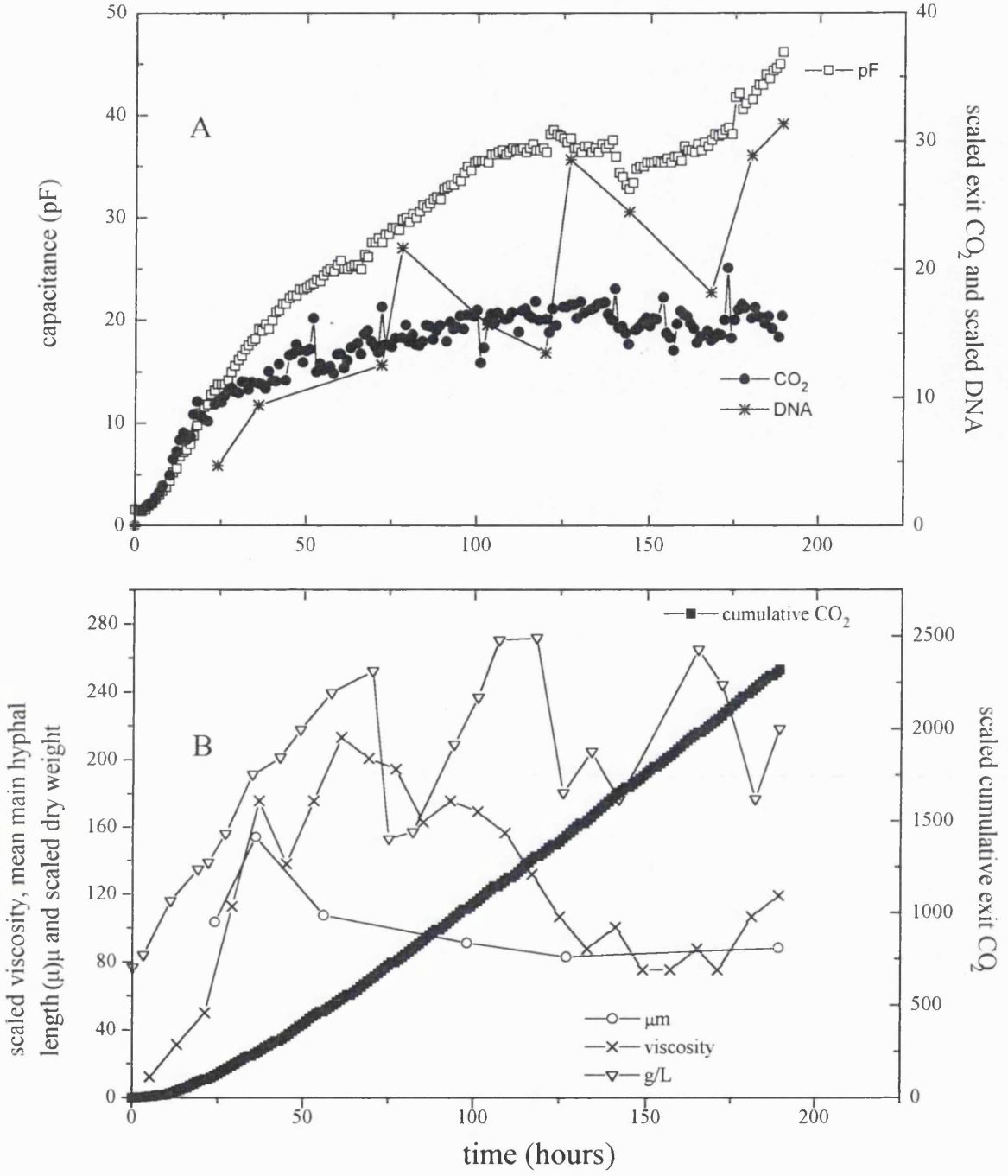


Figure 4.18 : capacitance (pF), scaled exit CO_2 ,
scaled cumulative exit CO_2, scaled DNA, scaled viscosity,
mean main hyphal length (μm) and scaled dry weight versus time
Penicillium chrysogenum pilot scale fermentation SB3

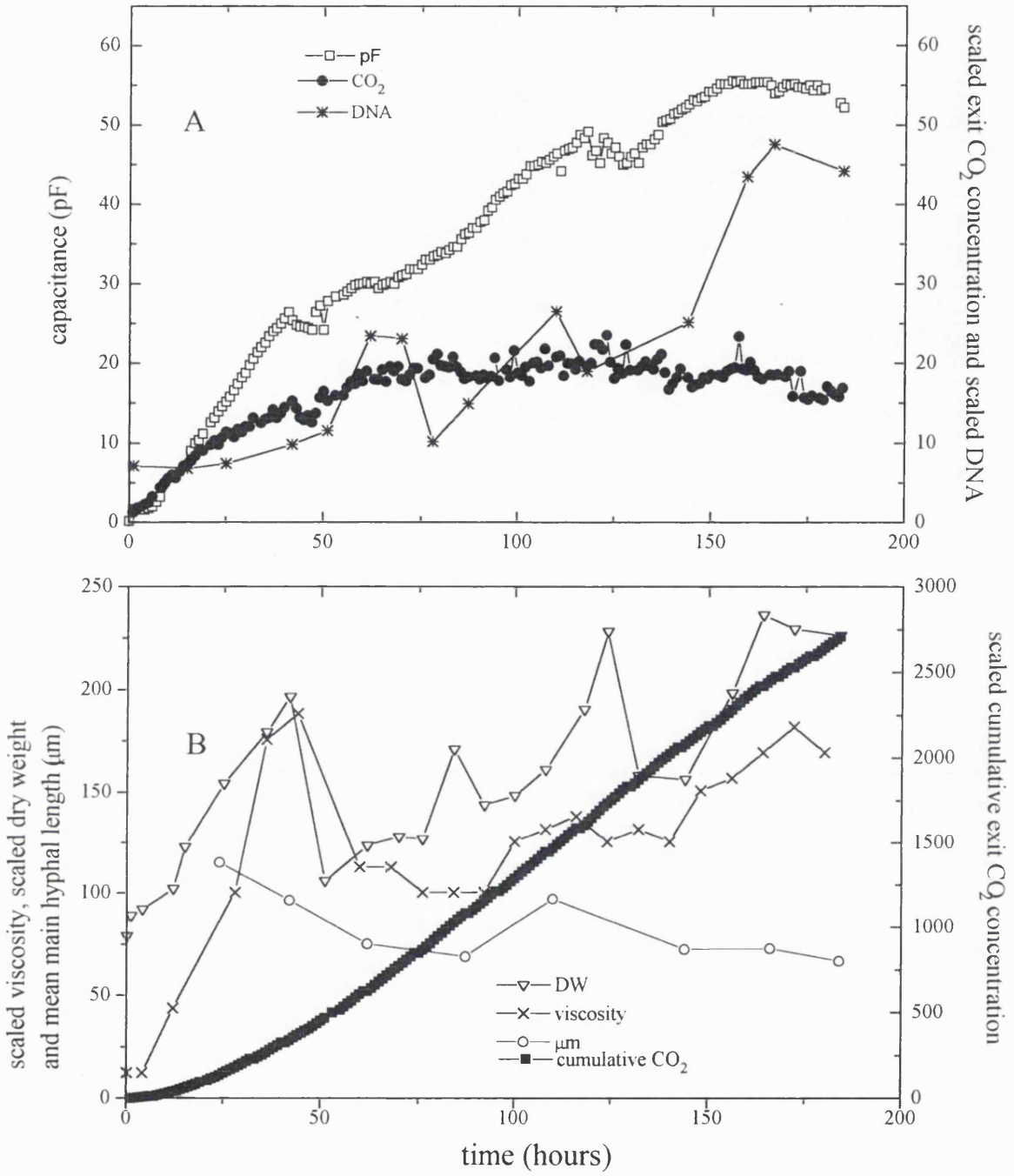


Figure 4.19: capacitance (pF), mean main hyphal length (μm), scaled exit CO_2, scaled cumulative exit CO_2, scaled DNA and scaled dry weight versus time
Penicillium chrysogenum pilot scale fermentation SB4

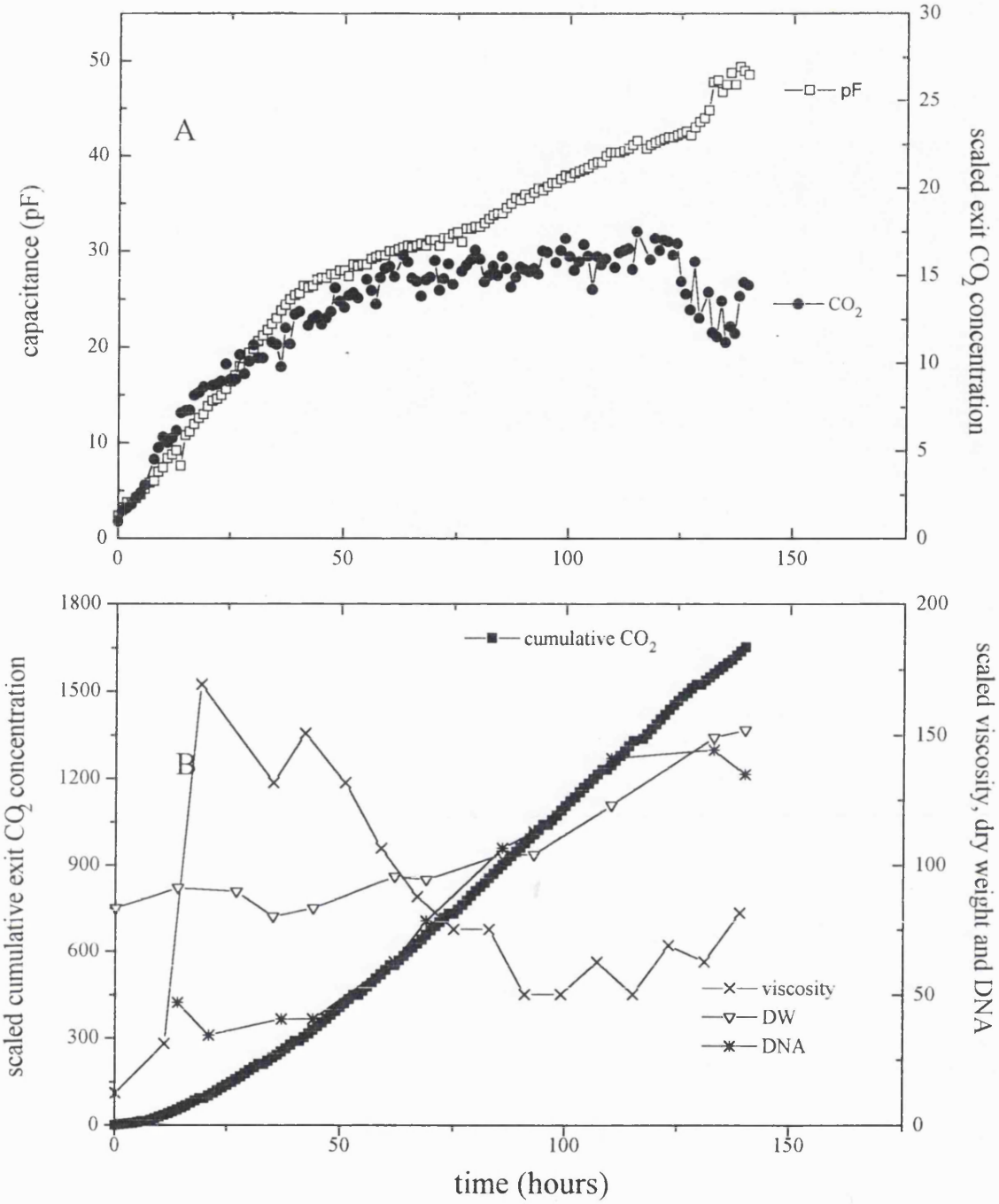


Figure 4.20: capacitance (pF), scaled exit CO_2, scaled cumulative exit CO_2, scaled dry weight, scaled DNA and scaled viscosity versus time

Penicillium chrysogenum pilot scale fermentation SB5

Mean main hyphal length peaked around 25 and then declined slowly. Viscosity peaked around 50 hours, declined to a plateau between 60 and 90 hours and rose slowly throughout the remainder of the fermentation, until it fell slightly at the end. Dry weight and DNA, again fluctuated, but overall showed and initial peak, followed by further growth.

Figure 4.20 shows the results of an attempted repeat of the fermentation shown in Figure 4.18. The fermentation shown in Figure 4.20 was found to be contaminated with yeast. The contamination was thought to be present from time of inoculation, but was not apparent until around 140 hours. The data is presented as a opportunity to look for differences in the capacitance measurements and in the relationship between capacitance and the other measured variables caused by the deviation from the previous experiment. Capacitance and exit CO_2 correlated during the initial growth phase and then diverged. The capacitance values for this fermentation were higher than those for the standard process shown in Figure 4.21, while the exit CO_2 values were not. The viscosity peaked around 25 hours at 175, 25 less than the other fermentations, and declined throughout the fermentation. The dry weight and DNA measurements showed no initial peak, and rose slowly throughout the fermentation.

In order to examine the relationship between the measured variables and the fermentations, linear plots of the data from these fermentations were carried out to give correlation coefficients. Table 4.12 shows the correlation coefficients from linear plots of the data for these variables.

Table 4.12

Correlation coefficients for capacitance against various biomass measurements for pilot scale *P.chrysogenum* fermentations.

R^2 values versus capacitance

	$C0_2$	$cC0_2$	DW	DNA	MMHL	Visc.
SB3	0.92	0.89	0.75	0.84	-0.65	0.27
SB4	0.79	0.95	0.77	0.80	-0.7	0.70
SB5	0.83	0.94	0.81	0.88		-0.08

The best correlations with capacitance are with exit CO_2, either the spot percentage value in SB3 (R^2= 0.92) or with cumulative exit CO_2 in SB4 and SB5. The correlations with the other measures of biomass such as dry weight and DNA were poor, reflecting the lack of accuracy of these measurements for these fermentations. The morphology based measurements, viscosity and mean main hyphal length (MMHL) show little or correlation with capacitance when viewed across the full range of fermentation time, but still seem to show event type correlations, possibly reflecting morphological changes in the cultures.

To examine these fermentations further, the specific growth rates (μ) were calculated for the exponential growth phases of these fermentations. Table 4.13 shows the μ values for these fermentations

Table 4.13

Specific growth rates for *P,chrysogenum* pilot scale fermentations, calculated from various biomass measurements.

parameters used to calculate μ

	Cap.	CO_2	cCO_2
SB3	0.14	0.15	0.20
SB4	0.13	0.16	0.20
SB5	0.09	0.24	0.24

From this data, the difference between the contaminated culture and the other fermentations can be seen, though it is odd that the capacitance specific growth rate is reduced by the presence of the contaminant while the metabolic rates are increased. The differences between SB3 and SB4 should be slight as they are largely the same prior to feeding, though it can be seen that the capacitance derived μ value is lower, while the metabolic data are the same. It can be seen from this data that capacitance is as good an on-line biomass measure as the metabolic data routinely used

The results obtained at pilot scale show no significant deviations from the profile found at laboratory scale and support the conclusion that, for *P.chrysogenum* fermentations, the Biomass Monitor is as good an on-line measure of biomass as exit CO_2, but that the capacitance measurement differs from the gas analysis data after the initial growth phase, giving a better indication of the biomass concentration during the latter parts of the fermentations.

It is difficult to come to any valid conclusions about the relationship between the pilot scale and laboratory scale work due to the limited number of fermentations monitored, but in general the trends appear to be very similar, while the capacitance values appear to be higher in the fermentations at pilot scale.

4.4.3 *P.chrysogenum* production scale, complex media, fed-batch fermentations.

To examine the effects of scale on the relationship between capacitance and biomass, a production scale fermentation of *P.chrysogenum* was monitored on-line. The capacitance probe was sited in a well-mixed region of the vessel, in the top third of the working volume. Figure 4.21 shows the results from this experiment plotted against time. Capacitance shows a correlation with dry weight and viscosity during the initial growth phase but not beyond that. The nutrient feeding pattern of this fermentation was not the normal one used after the initial growth phase. This could explain the plateau in capacitance between 30 and 60 hours. The increase at around 60 hours was due to the batched addition of feed media, and the subsequent rapid growth of the culture after this point can be seen in the rate of increase of capacitance. The fermentation was terminated after 96 hours as it had overgrown and was no longer acceptable as part of the production process. Linear plots of the data showed that the correlation with capacitance, taken over the full timescale of the fermentation, were poor, with dry weight having an R^2 value of 0.81 and viscosity an R^2 value of -0.08. The lack of gas analysis again makes a full comparison of this fermentation difficult. Nonetheless, it clearly demonstrated the successful use of the capacitance probe at this scale.

4.5 *Streptomyces sp.* complex media, fed-batch fermentations.

Fermentations: SB7, full nutrient
SB8, half nutrient

These fermentations were carried out at pilot scale. The processes used were the standard *Streptomyces sp.* fed batch, complex media processes, SB8 had half the initial concentration of nutrient than that used in SB7. The probe was situated in a port in the lower third of the vessel and was sterilised *in-situ*. Δcapacitance was monitored in two frequency mode. The low frequency point used was 0.5 MHz, which was chosen after off-line frequency scans on mid-run broth from a previous *Streptomyces sp* fermentation. Capacitance and conductance were recorded using a 12 bit Squirrel datalogger as described previously.

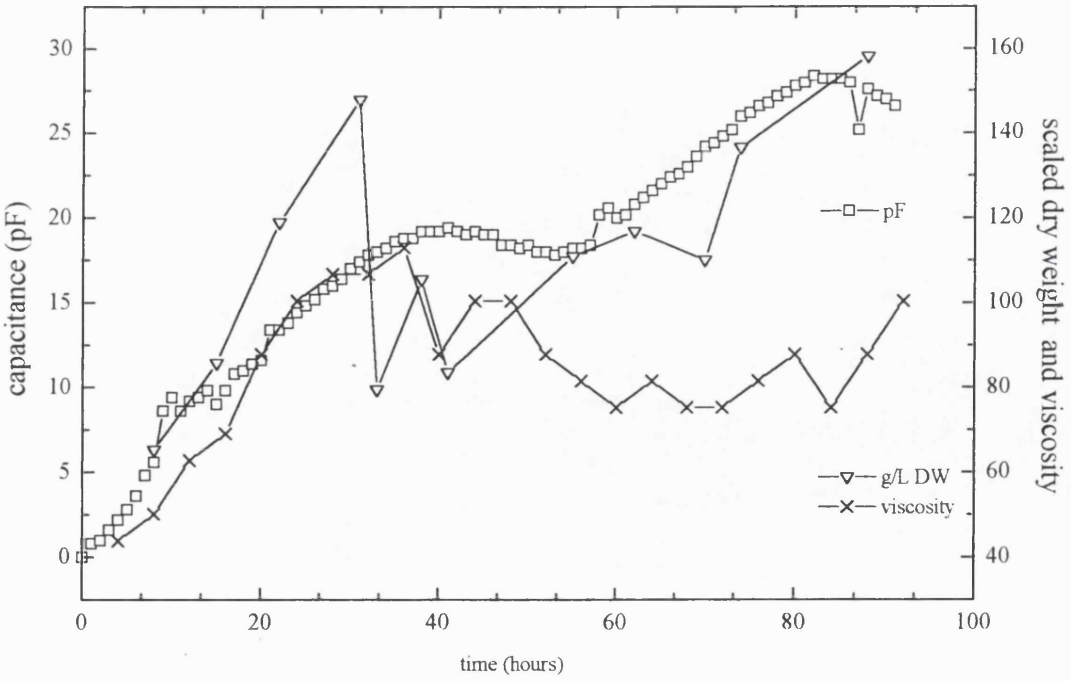


Figure 4.21: capacitance (pF), scaled dry weight and scaled viscosity versus time.
Penicillium chrysogenum production scale fermentation SB6

Exit CO_2 was also monitored on-line, as were the normal fermentation parameters. Samples were taken at regular intervals for viscosity measurement. Dry weight was attempted but was found to be very time-consuming and inaccurate.

Figure 4.22 shows capacitance and exit CO_2 versus time for SB7. Due to technical problems, some of the capacitance data is missing. However, the profiles indicate that exit CO_2 and capacitance measured different aspects of biomass. Exit CO_2 had a very short lag phase while capacitance did not begin to rise until around 8 hours. Exit CO_2 peaks at 13% (scaled) around 21 hours, fell before levelling out at 7.5% around 60 hours and remained relatively constant. The capacitance did not follow this pattern. Unfortunately, it is not possible to say where the capacitance peaked but it appeared to happen after the exit CO_2 had peaked. The capacitance then rose again before peaking at 9.5 pF around 70 hours and then fell until the signal was only 2 pF at the end of the fermentation. The capacitance and viscosity measured showed agreement over the full time course of the fermentation. Viscosity peaked initially at 280 after 45 hours, rose to a plateau at around 60 to 100, hours and then fell during the remainder of the fermentation. The dry weight results for this fermentation showed that the measurement is unreliable, due to the complex media.

Shown in Figure 4.23, SB8 has half the initial nutrient content in the media. Despite this, exit CO_2 had an even shorter lag phase and peaked slightly lower at 11.5% at around 22 hours. It then fell slightly and plateaued at 9%, higher than on full nutrient, before falling after 100 hours down to 6% at the end of the fermentation. Capacitance again had a lag phase of around 8 hours before it rose with growth. It peaked initially at 9pF in 40 hours before it rose again after 50 hours, peaking at 11pF after 60 hours. It then fell slowly down to 8pF at the end of the fermentation, thus showing both a greater peak and plateau value and a lesser fall in capacitance than the full nutrient fermentation. The half-nutrient fermentation had a viscosity peak and plateau which was slightly more than half that of the full nutrient fermentation, while the capacitance value is higher.

Linear plots of the data from these fermentations were carried out to give correlation coefficients. Table 4.14. shows the correlation coefficients from linear plots of the data for these variables.

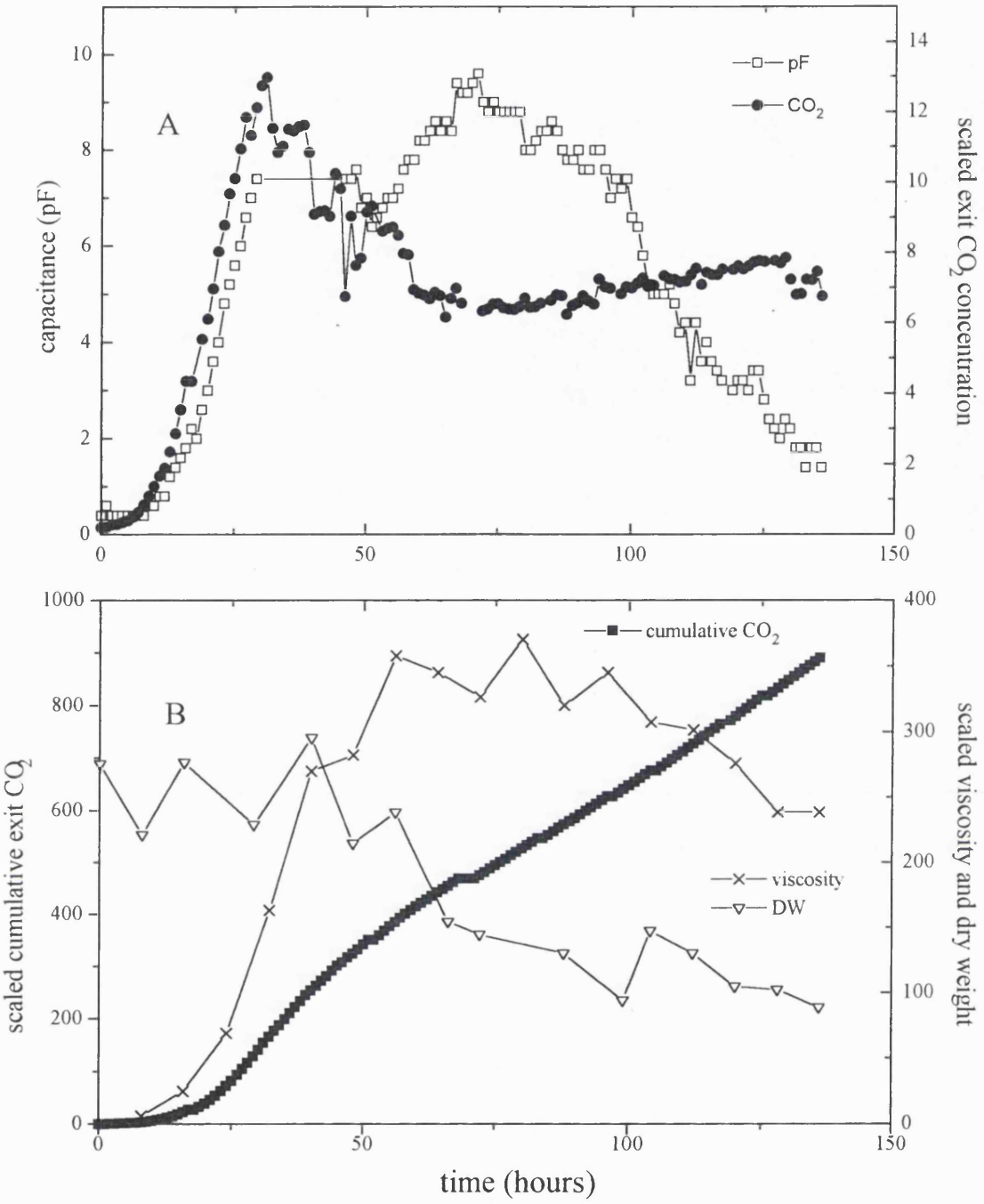


Figure 4.22: capacitance (pF), scaled exit CO_2 concentration, scaled cumulative exit CO_2, scaled viscosity and dry weight versus time

Streptomyces sp. pilot scale fermentation SB7

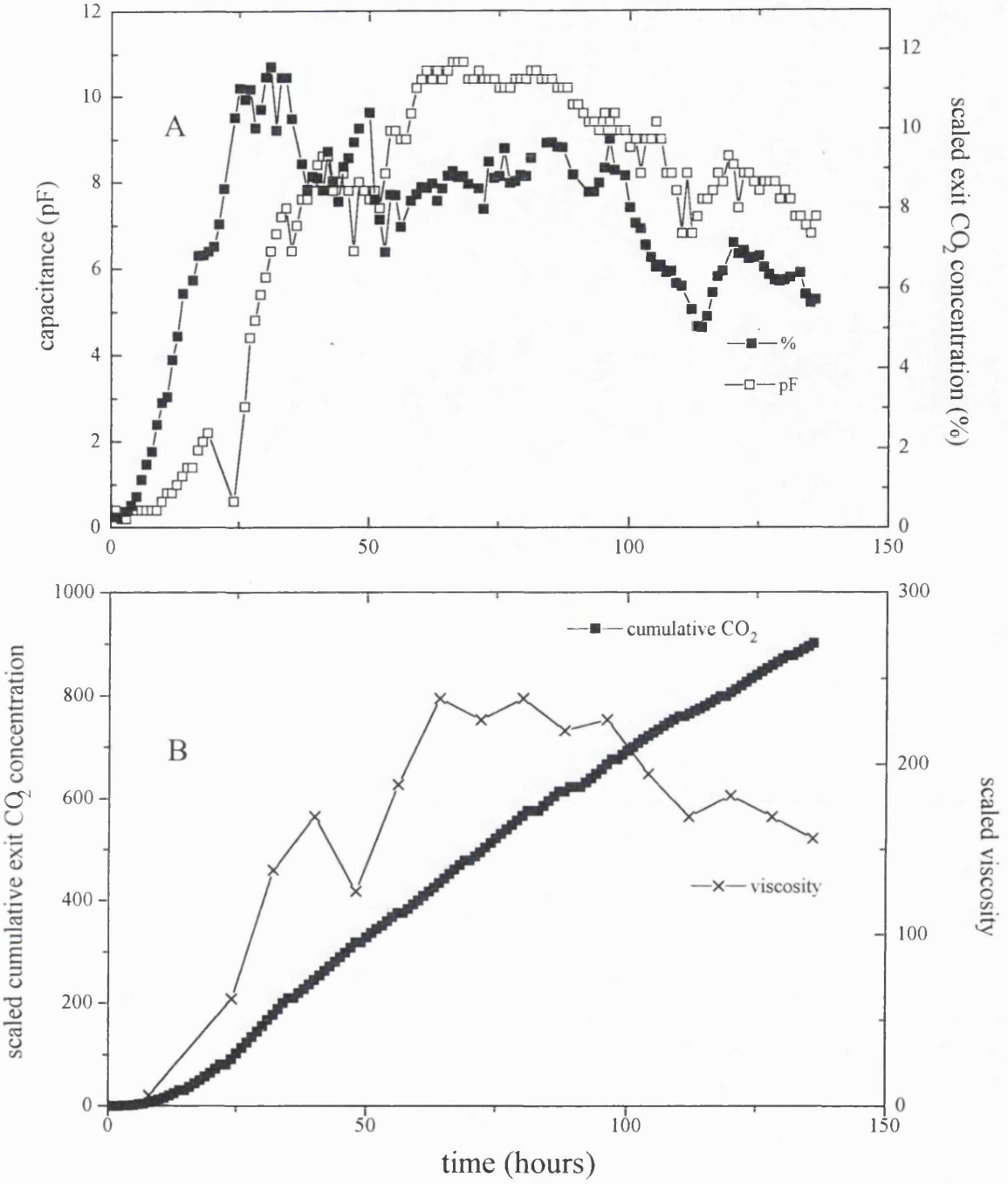


Figure 4.23: capacitance (pF), scaled exit CO_2 (%), scaled cumulative exit CO_2 and scaled viscosity versus time *Streptomyces sp.* pilot scale fermentation SB8

Table 4.14

Correlation coefficients for capacitance against various biomass measurements for laboratory scale *Streptomyces sp.* fermentations.

R^2 values versus capacitance

	$C0_2$	$cC0_2$	DW	Visc.
SB7	0.55	0.20	-0.16	0.71
SB8	0.62	0.65		0.95

Scaled viscosity showed the best correlation with capacitance for SB7 with an R^2 value of 0.71, a relatively weak correlation. The correlation between dry weight and capacitance (R^2= -0.16) showed the unreliability of the measurement for this type of fermentation and media. The linear plots of the data for SB8 showed that the correlation between capacitance and viscosity again was good, R^2= 0.95 and that the correlation between capacitance and the metabolic data was better for this fermentation than for the full nutrient fermentation

The specific growth rates (μ) of the initial exponential growth phase, calculated from semi-logarithmic plots of the measured variables of these fermentations are given in Table 4.15

Table 4.15

Specific growth rates for *Streptomyces sp.* fermentations, calculated from various biomass measurements.

parameters used to calculate μ

	Cap.	CO_2	cCO_2
SB7	0.15	0.20	0.21
SB8	0.22	0.26	0.32

In both cases, the values from the capacitance data are lower than those calculated from the metabolic data. The differences in the fermentations are clear with the half nutrient fermentation (SB8) having a faster specific growth rate than the full nutrient fermentation. It was also noted from the semi-logarithmic plots that the exponential growth phase started with no lag in the half-nutrient fermentation while the full nutrient fermentation showed a 7 hour lag phase before the start of exponential growth

The results described above for these *Streptomyces sp* fermentations seem to indicate that while capacitance is a biomass-related measure for the other fermentations, the correlations with viscosity for this organism point to there being a more complex relationship between capacitance and biomass with filamentous bacteria.

4.6 Results Summary

The data presented in this section has shown that capacitance, as measured by the Biomass Monitor, can be used to give a biomass-related measurement for yeast, bacterial, fungal and filamentous bacterial cultures, both off-line under ideal conditions for its use and on-line under normal fermentation conditions.

The capacitance measurements presented were, in general, as good indicators of biomass as the other main, on-line measurement used, exit gas analysis. The off-line measures of biomass and cell number were generally found to be inferior to the capacitance measurements, though correlations were found with capacitance. Work in trying to discern the nature of the capacitance measurement and its relationship with biomass and viability showed that it is strongly related to cell integrity and thus viability. This work has led to a conclusion that, as well as it use in fermentation monitoring, capacitance measurement could also be used as a rapid on-line indicator of homogenisation efficiency. The contribution to the overall capacitance signal by cell fragments was found in the off-line homogenisation work and has implications for interpretation of on-line capacitance measurements.

The next section will discuss these results in more detail and with reference to other work carried out using capacitance as a biomass measurement and to the theoretical basis of biomass related capacitance as discussed in Section 2.

5. DISCUSSION

The discussion of the results shown in the previous chapter will reflect the nature of the organism investigated. As an appraisal of the utility of capacitance measurement as a biomass measurement technique, the results of the capacitance measurements will be compared with the other on-line and off-line measures of biomass made for the experiments . It is proposed that this work also be discussed in comparison to other work carried out using capacitance measurement and with the theoretical capacitance values which can be calculated from the equations describing capacitance and its relationship to microbial biomass, as discussed in section 2.3.

After the sections relating to the different organisms, a short summary of the full discussion will aim to draw out the conclusions from the work, to be followed by a précis of these conclusions

5.1 Yeast results

5.1.1 Serial dilution experiments

The serial dilution experiments described in Section 4.1.1 were successful in that excellent correlations (R^2 =0.996) between dry weight of cells and capacitance were found. These results gave a specific capacitance value of 0.78pF/g/L dry weight, for this strain of *S. cerevisiae*. This is very similar to the values from the other work carried out using suspensions of this strain, discussed later. This off-line calibration shows that capacitance as measured by the BM is linear with biomass concentration up to 60g/L dry weight for *S. cerevisiae* and the excellent correlation and relatively low level of scatter on the plot indicates the accuracy of the capacitance measurement in relation to biomass, as measured by dry weight. This off-line work assumes that the cells used were 100% viable. The other suspensions of this strain used in the disruption experiments, in the same buffer, under similar conditions, were found to have a percentage viability of at least 99% and therefore it is reasonable to expect this suspension to have similar viability. The negative Y intercept value shows that even under ideal test conditions, the instrument can give incongruous results. The calibration curve shown in Figure

4.1 is typical of curves obtained for off-line measurements using packed yeast as an easily available test organism. Davey *et al.* (1992 and 1993) discuss at length the effects of high biomass concentrations on the linearity of capacitance versus biomass plots. The increase in the volume fraction *P* (values of >0.15, approximately 30g/L dry weight) at high biomass levels tends to cause a decrease in capacitance. This would not explain the negative Y intercept value, as correcting for this effect would cause an increase in the capacitance at the high biomass end of the plots. This would result in a tip down of the linear regression at the low biomass end, making the Y intercept more negative.

Similarly, the problem of electrode polarisation cannot be the cause as this gives an increase in capacitance, especially at low biomass levels, and correcting for this would further reduce the Y intercept. In the absence of a more complex explanation, the negative Y intercept is most likely due to the measurement threshold of the instrument at low biomass levels and would have to borne in mind with any on-line work.

This experiment showed that capacitance is an accurate measure of biomass for yeast suspensions under ideal conditions. To examine the effects of measuring capacitance in a more dynamic environment, the BM was used on-line in yeast fermentations.

5.1.2 Fermentation Experiments

5.1.2.1 Comparison of capacitance with other biomass measures

The three fermentations of *S.cerevisiae* GB4918 show the accuracy and utility of capacitance measurement by the BM to give an accurate estimate of biomass accretion on-line in yeast fermentations.

The two 14L fermentations (SC1 and SC2) illustrated in Figures 4.2 and 4.3 of the results section show that for two metabolically similar fermentations, the capacitance profile is the same. The two fermentations were reproducible in terms of growth, both having a peak exit CO_2 of 5.5%, 9 hours after inoculation and a μ of around 0.3 during the exponential (glucose) growth phase, calculated from the exit CO_2 concentration.

The capacitance profiles for these two fermentations were similar. The initial peak in capacitance was 5.5pF in both fermentations, also 9 hours after inoculation, corresponding to the initial growth peak in exit CO_2. The μ value calculated from the capacitance data was around 0.25 and 0.26 respectively for the two fermentations, showing the reproducibility of the capacitance measurements for the two fermentations.

In both cases, the capacitance continued to rise after the initial growth peak, presumably due to the culture metabolising the ethanol made during the growth on glucose. This is illustrated by the continued rise of the cumulative exit CO_2 measure after the growth peak, which shows a similar profile to capacitance, OD and dry weight. The most commonly used measures of biomass in fermentations, OD and dry weight show good agreement with capacitance throughout the time course of these fermentations and indicate that the on-line measure of capacitance is giving a good estimate of biomass in real-time in these fermentations.

Figures 4.2c and 4.3c show the measures of viable cell concentration (VCC), total cell number (TCN) and total cell volume (TCV) for these fermentations. While the cell number measured by particle counting shows the same profile as capacitance for both these fermentations, the VCC measurement, which should have the best overall correlation with capacitance, peaks at the growth peak and declines slightly in both fermentations. The VCC values are lower than those for TCN in fermentation SC2, and the correlation coefficient is higher between capacitance and TCN (R^2=0.98), than for capacitance versus VCC (R^2=0.94). The lower VVC values are not due to a significant level of dead cells as the level of stained cells (non-viable) observed in all cases was less than 1% of the total cells counted.

This is contrary to what would be predicted as the viability component of the capacitance measure should mean that VCC is a better fit than TCN. The lower VCC values and the slightly poorer correlation for the VCC measurements would seem to indicate that the more statistically robust particle counting method is more accurate than the microbiological method. The particle counting method is much less reliant on dilution which is well-known to introduce errors into measurements and also counts a far greater sample of cells, giving it greater statistical rigour. This suggests that the better correlations for capacitance with the cell number measurements made using the Elzone indicate the accuracy of the capacitance measurements.

Another difference noted between the SC1 and SC2 fermentations is that while the capacitance, dry weight, cumulative and spot exit CO_2 values are very similar for these two fermentations, the TCN and TCV values differ, with the second fermentation showing lower values.

The TCN and TCV values for the second fermentation are closer to those from the third fermentation, carried out at 5L scale (shown in Figure 4.4) which has a significantly lower capacitance. In fermentation SC3, the peak in capacitance is around 3pF after 10 hours. This corresponds to a peak in exit CO_2 of 3.5% at the same time point. The lower values suggests that the culture is not as metabolically active and thus has a lower biomass as seen by the measured capacitance. This lower biomass is also seen in the dry weight measurement, SC3 has a lower dry weight peak of 4g/L as opposed to 5g/L in the other two fermentations.

The TCN and TCV values for this fermentation are lower than those for other two but are as close to the values for fermentation SC2 as it's values are to fermentation SC1. The VCC value for fermentation SC3 is, again, lower than the TCN. VCC has a better correlation with capacitance (R^2=0.96) than TCN (R^2=0.92) in this fermentation. The poorer metabolic performance of this 5L fermentation is reflected in a lower biomass level seen by all the measured values of biomass, including capacitance which again shows a good correlation with cell viability.

In general, the on-line capacitance data from these fermentations are in agreement with the other measures of biomass and often give a better indication of the biomass of the cultures than the more conventional methods. There are some inconsistencies and slightly surprising results presented, but in general, the utility of the on-line capacitance measurement system has been proved for yeast fermentations and its data compares favourably with the other fermentation measures.

The results from fermentations SC1-3 and the off-line work showed that capacitance is strongly biomass related in yeast suspensions. To examine the effects of undissolved media solids and cell size on capacitance a further fermentation was carried out.

The nature and accuracy of capacitance and it's relationship with biomass, for another yeast fermentation is illustrated by the results from the 42L complex media fermentation of *S.cerevisiae* MC1 (SC4), which was known to be smaller than the strains used for the other fermentation and disruption experiments. The complex

media used was also known to contain undissolved solids, which can often be a problem for other on-line biomass measurement techniques.

In fermentation SC4, shown in Figure 4.4, capacitance showed a rise of 2.0pF during the growth of the culture. As this was a complex media fermentation, the sharp peak in exit CO_2 is not as obvious as in the defined media fermentation. It rose slowly to a peak of 1.7% at 9 hours and slowly declined over the rest of the fermentation time. Unlike the defined media fermentations, capacitance and the other biomass indicators do not show an initial peak corresponding to this peak in exit CO_2, but show an initial peak at around 11 hours, before continuing to rise to the end of the fermentation. Although the peak exit CO_2 spot value is lower than those seen in the defined media fermentations, the biomass produced in cells/mL is similar. The dry weight measurements indicate a dry biomass level of around 4-6 g/L, which is also similar to the values seen in the defined media fermentations. However, the presence of undissolved and unremoveable media solids in the fermentation samples may make these dry weight values unreliable. Despite the presence of these solids and the smaller cell size, the capacitance measurement still gave a biomass related measurement.

As discussed in Section 2, the effect of cell size on capacitance is theoretically very large. Figure 5.1 shows the mean cell diameter of the cells for each of these fermentations, plotted against fermentation time. As expected , fermentations SC1-3 of the same strain show similar mean cell diameters of around 6μm. The MC1 strain grown in fermentation 4 was found to have a mean cell diameter of around 4.2 μm. In general, the fermentations all show the same size distribution patterns over time. The mean cell size increases with the initial growth phase, before declining after the growth peak, often to a value less than that at the time of inoculation. These results are consistent with those of other workers investigating cell size during growth (Ranzi *et al.* 1986, Alberghina et al., 1983) and also with results in other work with the BM (Markx *et al.* 1991).

The effects of these cell sizes on capacitance can be seen from examining the Total Cell Volume (TCV) plots for all 4 fermentations. In fermentations SC1 and 2, the peak capacitance was 5.5pF in both cases, while the peak TCV differed slightly, with fermentation SC1 having a peak of around $3x10^{10}$ μm^3/mL and fermentation SC2 having a peak of $1.3x10^{10}$ μm^3/mL. In fermentation 3, the peak capacitance was around 3pF and the peak TCV was around 1 $x10^{10}$ μm^3/mL.

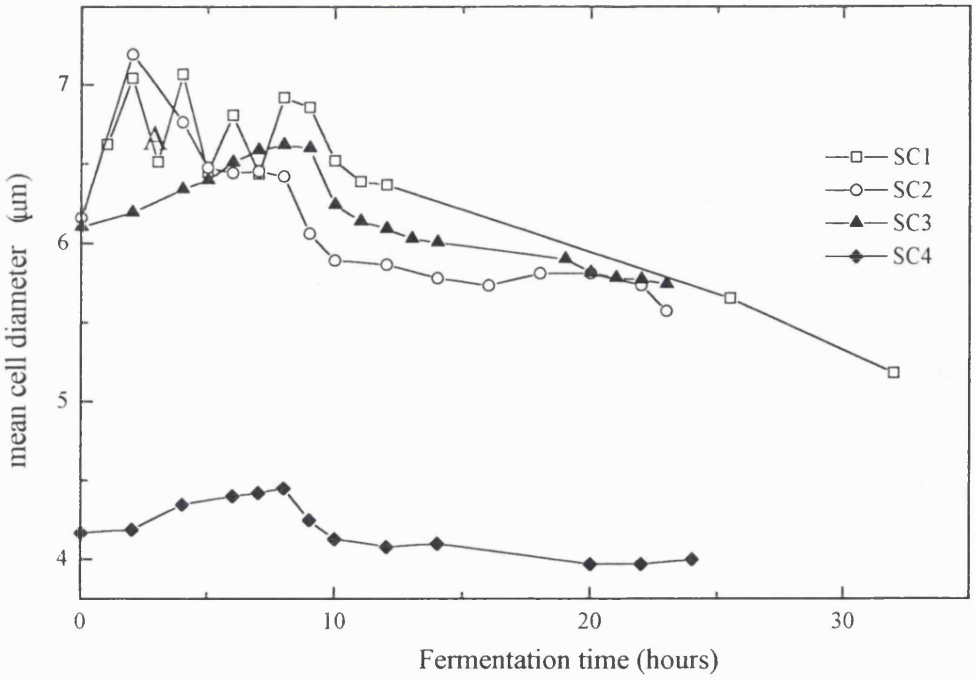


Figure 5.1: mean cell diameter (μm) versus fermentation time *Saccharomyces cerevisiae* fermentations SC1-4

The values and the relationship between TCV and capacitance are similar for these fermentations as the same strain was grown in each case and the average cell sizes are similar, as shown in Figure 5.1. It is interesting to note that SC2, which has a similar capacitance to that of its partner SC1 has both a lower cell count and a smaller average cell size. It also maintains this cell size after the growth peak more consistently than the other two fermentations of this strain, which decline in size after the growth peak. This shows that even for metabolically similar fermentations of the same strain of the same organism there can be differences on the capacitance values generated.

In fermentation SC4, of MC1 the smaller strain, the peak of capacitance was around 2pF and the peak TCV was 1.3 $x10^{10}$ μm^3/mL. From the TCV values, the smaller average size of the MC1 strain during the fermentation seems to have little effect on the measured TCV values, which are similar to those seen for the larger strain. From the TCV values, the predicted capacitance should be higher than the 2pF attained in this fermentation, and should be similar to the capacitance values found in the other fermentations if total cell volume or biovolume were the only factors in determining capacitance. This would seem to indicate that what determines capacitance is more than merely cell volume when comparing different strains. Looking at fermentations of the same strain, there is little in the size difference of the cells or in the TCV values to account for the differences in capacitance between fermentations SC1, 2 and fermentation SC3. Fermentation SC2 in particular has a peak capacitance of 2.5pF higher than SC3 while its TCV values are only 0.3 $x10^{10}$ μm^3/mL higher. This suggests that the relationship between capacitance, cell number and cell volume is more complex than anticipated.

A possible contributing factor in the case of SC3 is the fermenter volume. Relative gas hold-up volume is greater in a 5L vessel than in a 15L vessel. As capacitance can broadly be regarded as a measure of the volume bound within the cell membrane as a fraction of the total volume of the suspension, and air bubbles are non-conducting, therefore effectively decreasing the total volume of the fermenter as seen by the Biomass Monitor, this may contribute to the differences between the 5L and 14L fermentations of the same strain. Fauvre *et al.* (1993) used this phenomena to accurately measure gas hold up times and volumes in stirred

fermenters by measuring the changes in capacitance due to changes in aeration and agitation.

5.1.2.2 Comparison with other workers results

In order to further examine the data from these fermentation experiments, plots of specific capacitance (QC) calculated using dry weight, cell number, viable cell concentration and total cell volume are shown in Figures 5.2 and 5.3. Other workers have routinely calculated specific capacitance values, using dry weight, for single time points (Markx *et al.* 1991, Austin *et al.* 1995) and throughout a fermentation (Fehrenbach *et al.* 1991). Fehrenbach *et al.* (1991) proposed a new constant for use in comparing capacitance measurements, Cs. This is given by the formula:

$$Cs = Cr/X = f(r,v) \qquad \textbf{Equation 5.1}$$

where Cr is the relative capacitance at one or more appropriate frequencies as measured on-line

X is the biomass concentration probably in g/L

r is the equivalent cell radius

v is percent viability

They also note that for microorganisms whose size is difficult to determine (i.e. flocs or mycelial cultures) the BM can be used to give and estimate of changes in cell size providing viability remains unchanged.

This constant Cs will not be used in this work for a variety of reasons. Primarily, it is because the aim of this work is to examine the applicability of the BM and the nature of the relationship between capacitance and biomass. In order to do this, the data should be analysed from capacitance wherever possible. The Specific Capacitance constant values, calculated by dry weight (QCdw) shown here have not been corrected for the term *r*, to show the effect of cell size on the capacitance. As the viability of these fermentations was known at least 99% when measured by methylene blue staining, the viability term in this equation can be regarded as unimportant for this work, though it undoubtedly is a factor in the make up of a capacitance measurement, as will be seen in later sections.

These figures provide a means of assessing the relative accuracy of the capacitance measurements for these fermentations, both internally and compared with specific capacitance values from published work using the BM. Table 5.1 shows specific capacitance, QC (in pF/g/L) values from other work with yeast cells, including the mean cell diameter MCD (in μm), if available. The organism is *S.cerevisiae* unless stated. As a comparison, Table 5.2 shows the same data from the experiments described in this work. On comparing the values in Figure 5.2a with those in Table 5.1, it is clear that the values for SC1 and SC2 are generally higher than those found in the literature. It is possible that this may be caused by the BM used to make the measurements. This would be reflected in the cell constant of the instrument used. The cell constant of the BM used for this work was 0.93 cm^{-1}, which is not sufficiently different from these values in the literature to have caused the high QCdw values. The mean cell diameter for the cells in the fermentations in this work are larger than most of the cells used to derive the values shown in Table 5.1 and this is more likely to be the cause of the higher QCdw values, though it doesn't explain the higher values still, seen in fermentation SC2.

In Figure 5.2a, the QC dry weight values vary with fermentation time in manner very similar to the mean cell diameter values shown in Figure 5.1. In general, the value rises during the initial growth phase, declines slightly then levels out, which is in contrast to the mean cell diameter values which continue to fall, in all but SC2.

Fehrenbach *et al.* (1991) also found that the QCdw values of their *S.cerevisiae*, *P.pastoris* and *St.virginiae* fermentations showed a fall after the growth peak. They attributed the fall in specific values to a fall in average cell size due the cells ageing and the growth rate diminishing, which is mostly in agreement with the results from this work.

Table 5.1:

specific capacitance (QC) and mean cell diameter (MCD) values, calculated from dry weight, from the published literature.

authors	QCdw (pF/g/L)	MCD (μm)
Fehrenbach *et al* 1991	0.62-0.38	4.5-3.5
", *P.pastoris*	0.17	2.5-1-5
Markx *et al* 1991	0.43	6.5-5
Kronlof 1991	0.5-1.5	n/a
Austin 1995	0.62-1.2	n/a
Boulton *et al* 1989	1.1-0.46	n/a
Salter *et al* 1990	0.6	n/a

Table 5.2

Specific capacitance (dry weight) and mean cell diameter (MCD) values from the experiments reported in this work

Experiment	QCdw (pF/g/L)	MCD (μm)
Fermentation SC1	1.8-1.25	6.7-5.6
Fermentation SC2	2-1.25	6.7-5.5
Fermentation SC3	1-0.6	6.6-5.7
Fermentation SC4	0.4-0.3	4.5-4.0
Disruption 1	0.66-0.74	6-4.0
Disruption 2	0.76	6-4.0
Disruption 3	0.82	6-4.0

The differences between the fermentations are clearly illustrated in Figure 5.2a. The two identical replicates show very similar QCdw values throughout the course of the fermentation, although it is interesting to note that the values for SC2 are consistently higher than that for SC1.

Fermentation 3, the 5L repeat of fermentations 1 and 2, shows a lower specific value, calculated with dry weight. This may be due to the poorer growth and metabolic performance in this fermentation causing the lower capacitance with less concomitant lowering of dry weight. It may also be that the change of scale could have an effect. Fehrenbach *et al* (1991) found that their Cs values, calculated as described above, were slightly lower for the same strain in laboratory scale fermentations than the values found in pilot scale fermentations. This may be due to the comparatively greater effect of factors such as gas hold-up on volume and its relationship to capacitance in smaller scale fermentation vessels, as discussed above.

Fermentation SC4 has the lowest QCdw of the fermentations examined, probably due to the smaller cell size, although it is possible that the over-estimated dry weights in this fermentation may have an effect.

Figure 5.2b shows the Specific Capacitance constant values calculated with total cell number (QCtcn). In general, the QCtcn values show similar patterns versus fermentation time as the dry weight calculated values, there is an increase associated with growth then a decline to a plateau. In this case, there is a very obvious difference between SC1-SC3 and fermentation SC4, again showing the impact of cell size. This shows that although the cell number of fermentation SC4 is similar, the reduction in cell size results in these lower specific capacitance values. There are some slight differences between fermentations SC1-SC3, with SC2 again having the highest values.

Figures 5.3a and b show the specific capacitance calculated from viable cell concentration (QCvcc) and from total cell volume (QCtcv). Again, they illustrate small differences between fermentations SC1-SC3 and a much larger difference between those fermentations and SC4.

This is what would be predicted, both from the knowledge of the smaller cell size in SC4 and from the range of specific values seen for the different strains of yeasts measured in the literature and summarised in Table 1. The specific values vary

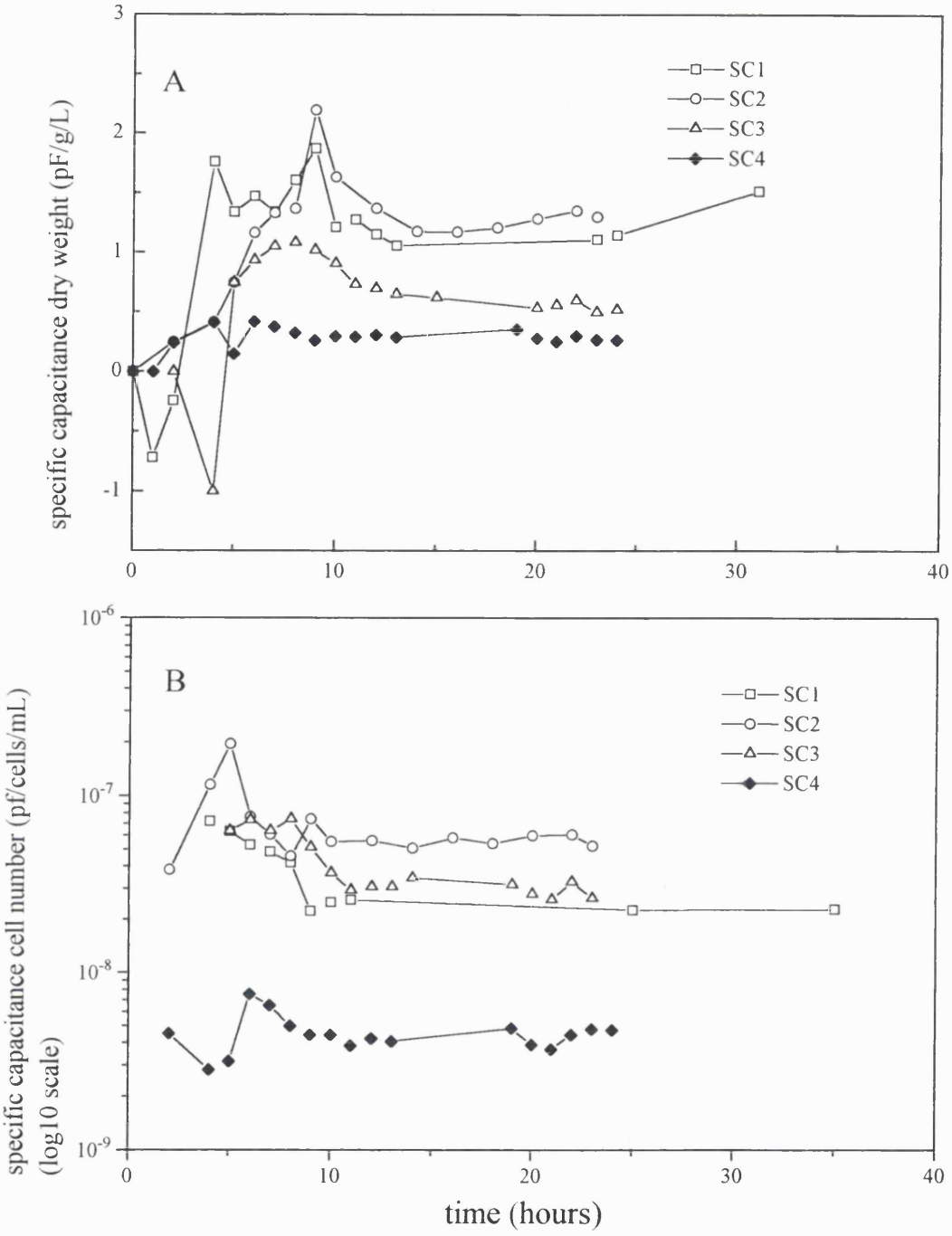


Figure 5.2a and b: specific capacitance dry weight (pF/g/L) and specific capacitance cell number (pF/cells/mL) versus time Replotted from *S. cerevisiae* fermentations

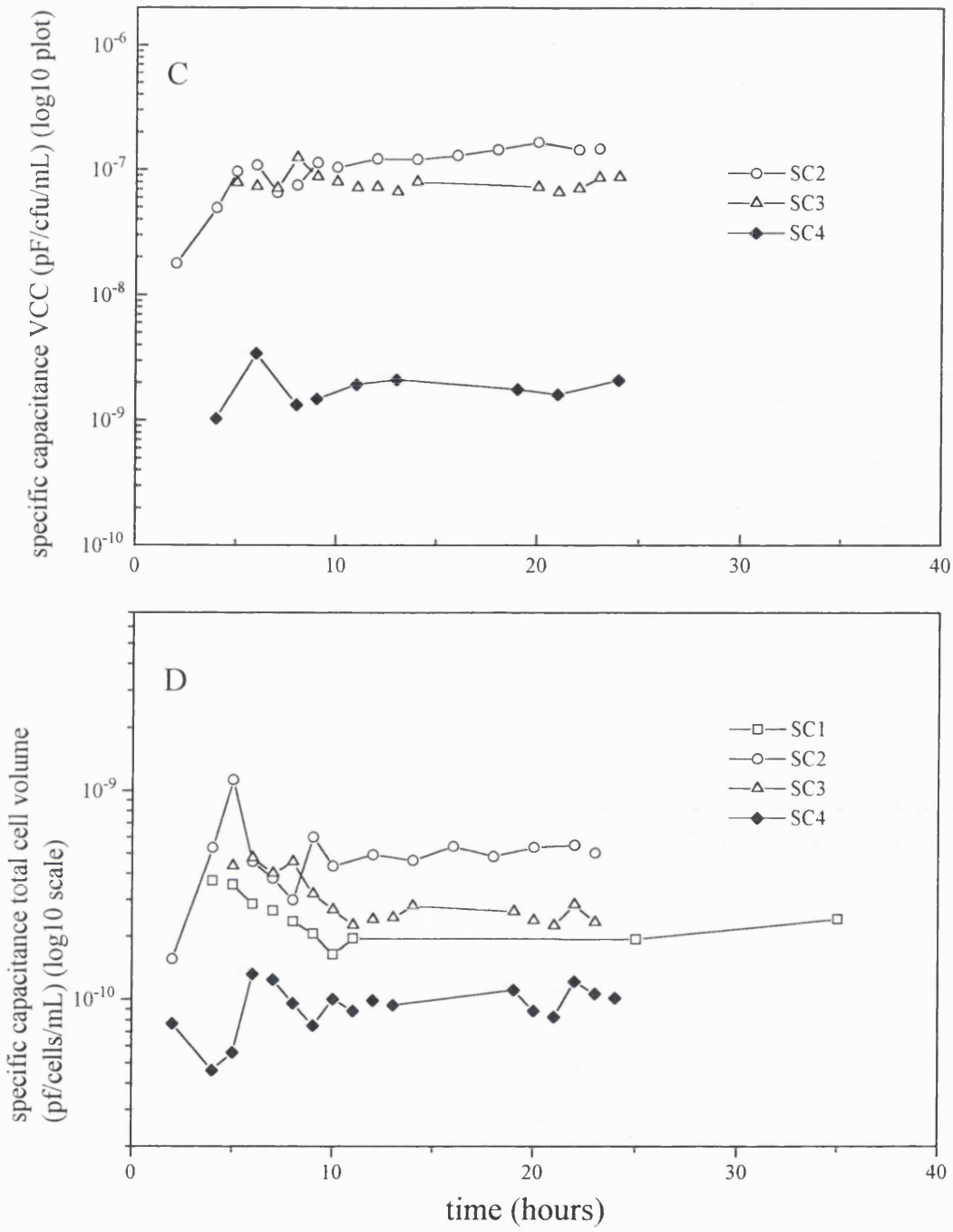


Figure 5.3a and b: specific capacitance VCC (pF/cfu/mL) and specific capacitance total cell volume (pF/υm^3/mL) versus time Replotted from *Saccharomyces cerevisiae* fermentations

considerably depending on the strain used and this would mean that a substantial database of knowledge would have to be built up for a particular strain before any conclusions could be drawn as to the causes and consequences of changes in specific values.

In general, the profiles of the values in Figures 5.3a and b are similar to those for the other specific capacitance values. However, the QCtcn and QCvcc values show some differences for the three fermentations of the same strain. The QCtcn values generally fall after the growth peak in the fermentation suggesting a decline in specific capacitance, which is not seen in the QCvcc values which hold a steady plateau after the growth peak. This may be an indication of the viability measurement capabilities of the capacitance measurements over the cell number measurements. If the apparent cell number, as measured by particle counting is dropping while the number of viable cells remains relatively more constant, this would explain this phenomena. If QCdw, QCtcn and QCtcv rise fall then plateau while QCvcc rises then plateau's, this shows that VCC has the best correlation with capacitance as it must be increasing at the same rate as capacitance, especially in the ethanol growth phase where there will be a decline in the viability and cell size as the culture runs out of available nutrients. QCtcn falls because the number of apparent cells is increasing at a rate faster than capacitance is increasing, as this does not take in to account viability.

Looking at the data for the MC1 strain fermentation, the lower QCtcn value for fermentation SC4 could be a result of the smaller cell size. This is further supported by the data shown in Figure 5.2a and 5.2b which show the specific capacitance values are significantly lower than the other fermentations of the larger strain. The data in Figure 5.3b seems to support the impact of the cell size as the calculated QCtcv values, which includes a component related to cell size, are closer to the values of the other fermentations than the other specific capacitance values which are independent of cell size.

Figure 5.4 shows an attempt to illustrate this phenomena. If the QCdw values, which do not take into account variations in cell size, are divided by biovolume, calculated from the cell number and cell size data, this should allow the calculation of a size independent specific capacitance value for these fermentations. This is obviously a rather crude measure as it is merely dividing the QCdw by the average cell count multiplied by the average cell size.

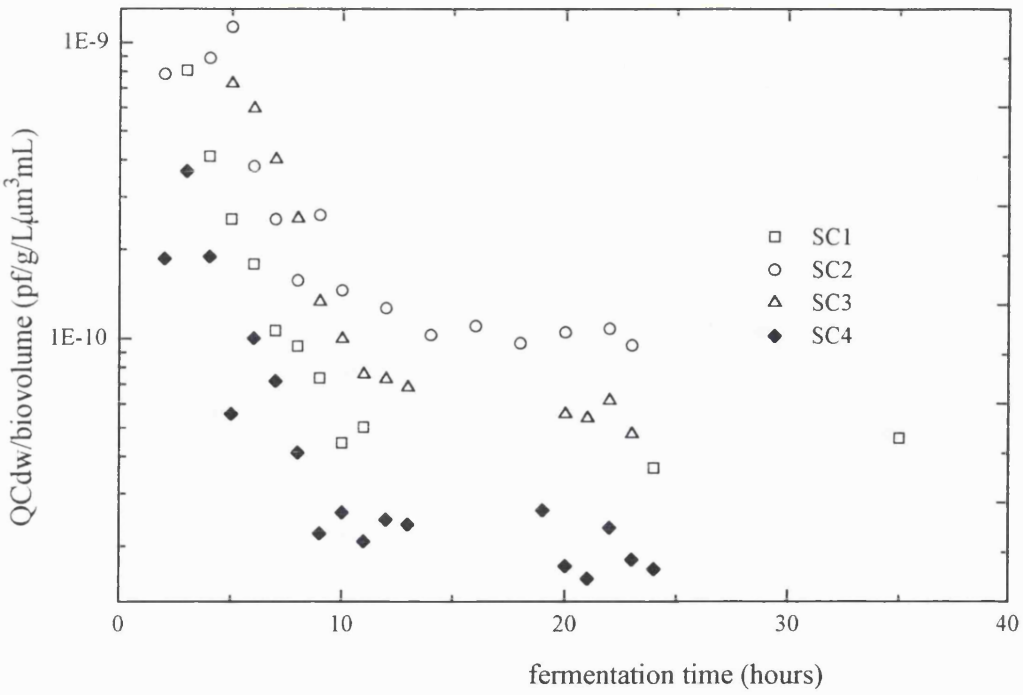


Figure 5.4: Specific capacitance dry weight divided by biovolume versus fermentation time
replotted from *Saccharomyces cerevisiae* fermentations SC1-4

It can be seen that the fermentation results of the MC1 strain are broadly similar in terms of capacitance generated per gram dry weight and unit volume to those for the larger strain grown in fermentations SC1-3, when the effect of cell size is negated. The MC1 fermentation still has the smallest specific capacitance value, but it is much closer to the values for the other fermentations than it is in the other specific capacitance figures. It is interesting to note that the differences in SC1 and SC2 are again marked. Although they are metabolically identical, there are differences in their capacitance and cell number values. In Figure 5.4, SC2 again seems to have the greatest capacitance generating ability of the other fermentations, mirroring the other sceptic values calculated for these fermentations. The relative lack of data means that the conclusions which can be drawn from this information must be tentative, however, it seems clear that capacitance monitoring of yeast fermentations certainly gives on-line biomass measurements, but may also give information about the metabolic state of the culture. This is an area of further work which could lead to much greater levels of understanding and subsequently control of yeast fermentations.

As is mentioned above, as well as comparing the values form this yeast work, it is feasible to compare the values found in this work with theoretical values and this is covered in the next section.

5.1.2.3 Comparison with theoretical capacitance values

To further and fully interpret the results of the experiments described in this work, it is necessary to relate them to the theory of capacitance measurement. To do this, the most useful method is to use the equations describing capacitance and relating it to biomass to give a theoretical capacitance value for each cell type and experiment, related to cell size and number and compare these with the actual values obtained in the experiments. The method used here to calculate the theoretical capacitance value for a given biomass of the organisms measured in this work is taken from the Biomass Monitor Source Book (Davey 1993). As discussed in Section 2.3, the capacitance measured by the Biomass Monitor is related to biomass by the following equation:

$$\Delta\varepsilon = 9PrC_m / 4\,\varepsilon_0$$ **Equation 2.3**

where:

$\Delta\varepsilon$ = the dielectric increment, change in permittivity from low to high frequency

P = the volume fraction of the biomass, i.e. the fraction of the total volume of the suspension which is enclosed by the plasma membranes of the cells.

r = the cell radius (m)

C_m = membrane capacitance of the cell per unit of membrane area (Fm^{-2})

ε_0 = permittivity of free space (a constant, 8.854 10^{-12} F m^{-1})

In order to simplify this equation to allow it to be immediately useful, some assumptions have to be made. Davey (1993) converted the above equation to the following simplified version

$$\Delta C = 225Pr/ k$$ **Equation 5.2**

where:

ΔC is the increase in capacitance attributable to the presence of the cells, in pF

P is the volume fraction attributable to the cells

r is the equivalent cell radius (μm)

k is the cell constant of the Biomass Monitor used, in cm^{-1}.

This assumes that the cells measured have a plasma membrane capacitance per unit area of 0.01 F. m^{-2} and an internal conductivity of 0.5S.m^{-1} (Davey and Kell 1993).

This work by Davey (1993) as gives methods for calculating the theoretical volume fraction , P. This is given in two forms as shown below:

For biomass expressed in cells per mL

$$P = 4.1888 .10^{-12} . r^3 .N$$ **Equation 5.3**

where:

P is the volume fraction

r is the equivalent cell radius (μm)

N is the number of cells per mL

For ellipsoid shape cells, the equivalent cell radius is calculated using the following equation:

$$r = [(L/2) (S/2)^2]^{1/3}$$ **Equation 5.3.1**

where:

r is the equivalent cell radius

L is the diameter of the long axis of the cell in μm

S is the diameter of the short axis of the cell in μm

For biomass expressed in g/L: wet weight

$$P = W/1000$$ **Equation 5.4**

where:

P is the volume fraction

W= wet weight in g/L

The methods given above allow the calculation of a theoretical capacitance value for a given biomass based on knowledge of the cell size or biomass. Since the principle focus of this section of work is on showing the impact of cell size on capacitance, the equation converting cell number and radius to a capacitance value is the most useful. Using the cell number and cell size data from fermentation SC1, it is possible to obtain an estimate of the theoretical capacitance and thus check the theoretical validity of the actual measured capacitance for this fermentation. Figure 5.5a shows the actual fermentation data of cell number and cell diameter as measured by the Elzone particle sizing equipment. Figure 5.5b

shows the actual measured Δcapacitance for the fermentation, and the theoretical capacitance for both spherical yeast cells of the size and cell number measured during the fermentation and that of ellipsoid yeast cells whose short axis is an invariant 4μm and whose long axis varies around 6μm, according to the size variations measured during the fermentation. The ellipsoid shape was chosen for reasons outlined below. As can be seen from the results, the theoretical capacitance values for the different cell shapes are quite marked, and the theoretical values for the ellipsoid cells much more closely resemble those of the actual data. This serves to emphasise the impact of both cell size and morphology on biovolume and therefore on capacitance as measured by the Biomass Monitor. The effects of yeast cell shape on theoretical and measured capacitance values have been modelled and studied by Gheorghiu (1996) and Asami and Yonezawa (1995[a], 1995[b]). The work by Gheorghiu (1996) is primarily theoretical and illustrates that for growing yeast cells, the cell shape is usually non-spherical as a consequence of budding and cell division. This means that at any given time in an asynchronously dividing yeast fermentation, the majority of the cells will not be spheres but will be ellipsoid in shape. Asami and Yonezawa (1995[b]) illustrated this with permittivity measurements of a synchronously dividing yeast fermentation, in which the change in shape and the point of cell division can be detect as stepwise changes in permitivitty corresponding to these events. These findings seem to support the results from the plot of theoretical capacitance illustrated above and add to the evidence as to the profound effects of biovolume on capacitance. As biovolume is very closely related to cell integrity and hence cell viability, the next section of this work is concerned with a further examination of the viability measurement capabilities of the Biomass Monitor.

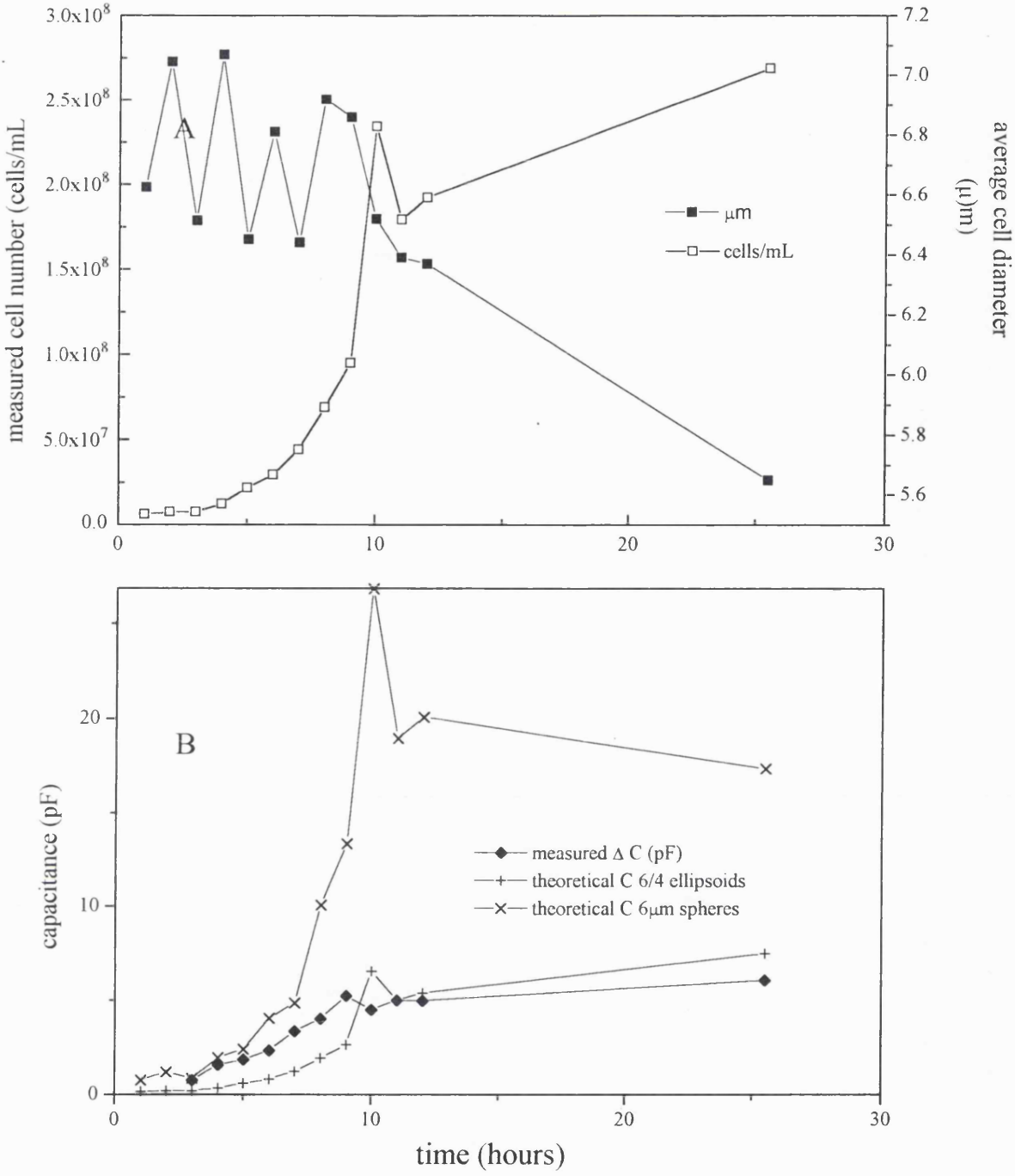


Figure 5.5: measured cell number, cell diameter, Δcapacitance and theoretical capacitance versus time
Replotted from *Saccharomyces cerevisiae* fermentation SC1

5.1.3 Yeast disruption experiments

To examine the role cell integrity and viability play in determining the capacitance of a yeast cell suspension, a further examination of the results from the disruption experiments carried out would be appropriate.

The results of the disruption experiments are shown in Figures 4.6-4.9 of the Results section. From these results, the decreases in capacitance and the other biomass related variables were calculated as a percentage of the initial values to allow comparison both between the experiments and between the variables. The QCdw and QCvcc values were derived from these experiments to compare them to those from the fermentations experiments.

The organism used for these disruption experiments was packed yeast (*S.cerevisiae*, The Distillers Co.) which is similar to the strain used in the fermentation experiments. The average size of the cells of the packed yeast was between 4-6 μm in each experiment, which is smaller than the strain used for the fermentations SC1-SC3.

The initial disruption experiment, three passes through the homogeniser at 1400 bar, two repeated experiments, showed good agreement between total protein release, the conventional indicator of disruption, and fall in capacitance. The protein assay data indicated that disruption was maximal after the third pass in both experiments. The variation between the repeats was lower between the capacitance measurements than between the protein measurement, indicating that capacitance decline is as good an indicator of disruption as the conventional method, protein release, and may indeed be better. Table 5.3 below summarises the results in terms of specific capacitance values from these experiments.

Table 5.3

1400 bar homogenisations summary

	% drop in C	% drop in dw	QCdw pre	QCdw post	QCvcc pre	QCvcc post
expt 1	96.2	91.2	0.66	0.29	$1.6e\ 10^{-9}$	$6.1e\ 10^{-9}$
expt 2	96	89.5	0.74	0.18	$1.5e\ 10^{-9}$	$5.3e\ 10^{-9}$

In both cases, the drop in capacitance is 96%, while the protein release data indicates maximal disruption and the VCC data show a drop of around 99% in viable cells . Based on microscopic examination, there were sufficient, apparently intact, cells present to give a residual capacitance of 0.7-0.9% of the initial values. The dry weight values indicate that there is sufficient cell debris present to give a dry weight of around 10% of the pre-homogenisation values. The 5% residual capacitance after total disruption must then be due to intact but non viable cells and cell fragments present in this residual dry weight. The pre-disruption QCdw values of 0.66 and 0.74 are lower than those for the fermentation experiments, probably due to the smaller size of the cells, but are similar to the published data given in Table 5.1. The decrease in these values after disruption indicates that although some material is left after disruption, it is not generating a capacitance equivalent to the same amount of intact cells.

The experiment to examine the effects of single pass at a range of pressures worked well, as indicated by the results shown in Figure 4.8. The capacitance and protein release data show agreement and indicate that the drop in capacitance is due to, initially, cell leakage and at higher pressures cell breakage. The decrease in capacitance is linear with pressure from 100-1000 bar while the increase in protein released loses linearity at 700 bar, but continues to rise before peaking and levelling out at 1400 bar. Comparing these results with those for VCC and dead cell concentration (DCC, defined here as cells wholly stained with methylene blue but retaining their shape) as measured by direct count microscopy show some

interesting phenomena. The DCC rises to a plateau between 500 and 700 bar then decreases with the remaining pressure increases. These results seem to suggest that the increase in protein release and the capacitance drop up to around 700 bar is primarily due to cell damage and leakage rather than cell breakage, while from 1000-1600 bar, the protein release and drop in capacitance is due to cell breakage. Methylene blue staining of yeast cells functions as a measure of viability in that the dye is taken up by all yeast cells but it is only decolourised by metabolically active cells.

Figure 5.6 shows these results expressed in percentage terms to facilitate easier comparisons. From this, the drop in capacitance and the drop in VCC can be seen to be very similar, with both being virtually linear between 100 and 1000 bar, though the drop in VCC is generally more pronounced than that for capacitance. This shows that capacitance is a good indicator of viability and that the influence of the DCC is perhaps not as strong as the other figures would seem to suggest.

It is interesting to note that the capacitance levels after maximal disruption, as indicated by protein release, are around 15% of the initial capacitance while VCC is lower than this at around 5%. The QCdw was 0.76pF/g/L and the QCvcc was 5.1×10^{-9} for the cell suspension pre-homogenisation. These values are broadly in agreement with those for the previous disruption experiment, indicating no significant changes in the dielectric characteristics of the yeast used.

The final experiment in this work on the effects of disruption on the capacitance signal from suspensions was carrying out multiple low pressure disruptions. The results from this experiment are given in Figures 4-9 in the Results section. This data is replotted as percentage values in Figure 5.7a and the calculated Specific capacitance values are plotted in Figure 5.7b.

One pass at 500 bar decreases the capacitance by 35%, and the VCC by 10% while the protein release is 60% of the maximum. The DCC value also rises sharply, indicating that the decrease in capacitance and increase in protein release from 1 pass at 500 bar is due to cell leakage not breakage. With the next pass at 500 bar, the DCC value declines as does the VCC and capacitance values, indicating that the drop in capacitance is now more due to cell breakage. The rise in protein release and fall in VCC reach plateau levels after the third pass at 500 bar, while the capacitance value continues to fall until the 5th pass at 500 bar. Despite the VCC and protein release values levelling off, indicating maximal disruption, the

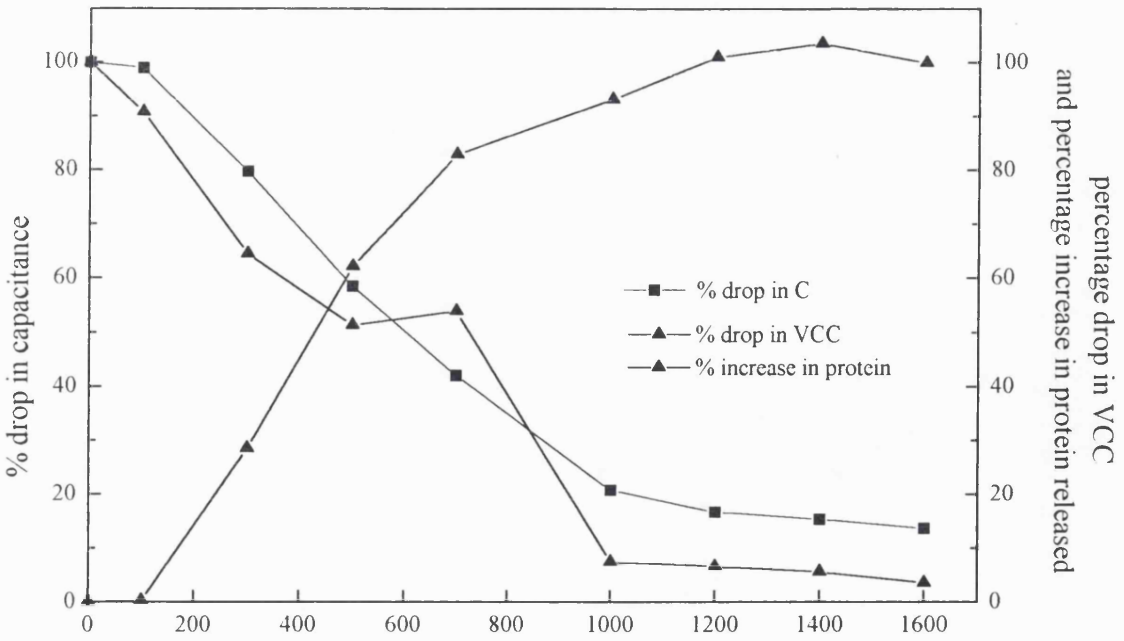


Figure 5.6:
Percentage decrease in capacitance and viable cell concentration and increase in total protein versus disruption pressure 100-1600 Bar disruption (single pass) of *Saccharomyces cerevisiae*

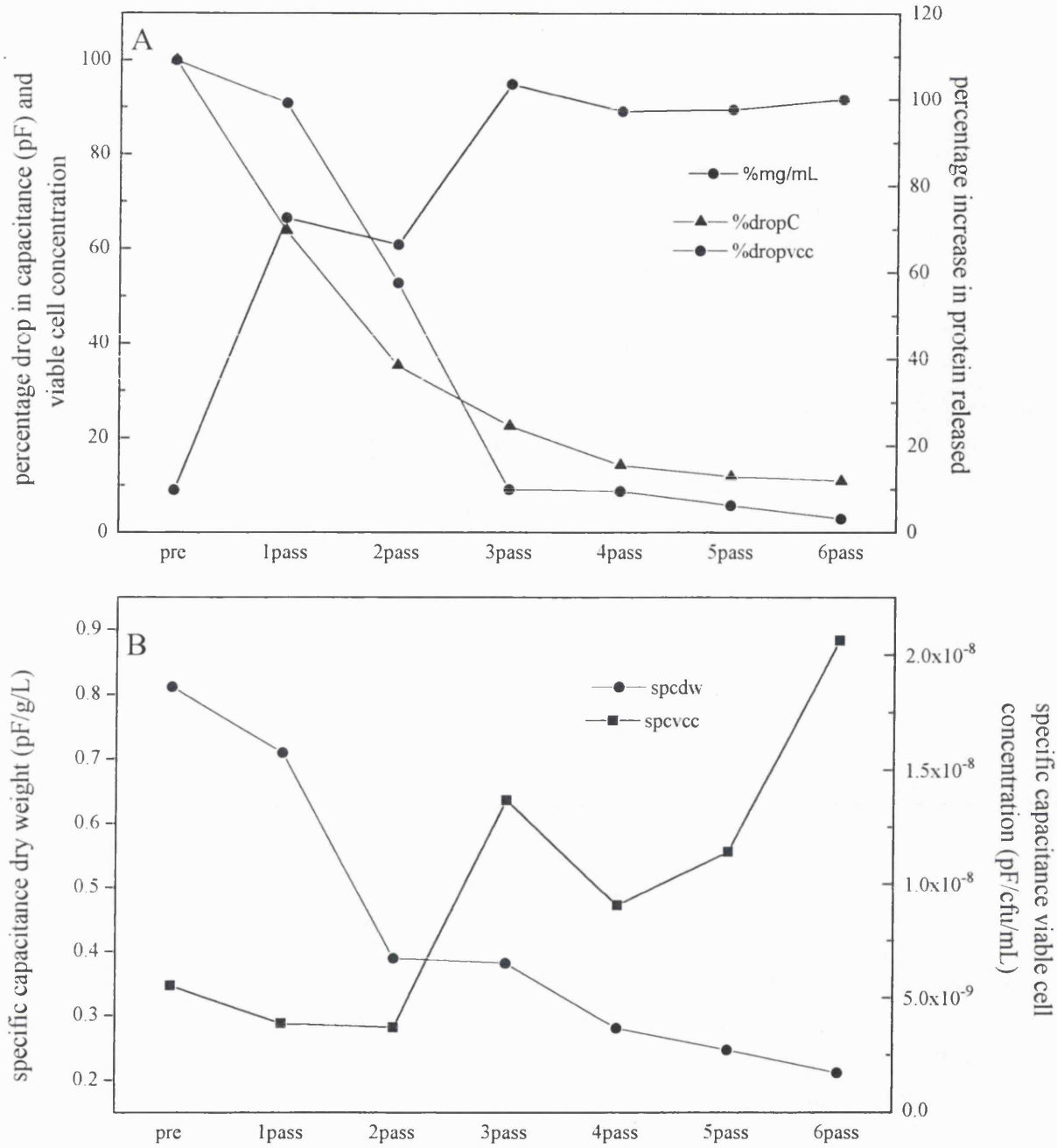


Figure 5.7:

A: percentage decrease in capacitance and viable cell concentration
percentage increase in total protein released
versus number of passes through homogeniser

B: Specific capacitance dry weight and viable
cell concentration
versus number of passes through the homogeniser
multiple passes at 500 Bar
disruption of *Saccharomyces cerevisiae*

suspension after 6 passes through the homogeniser at 500 bar still has a capacitance around 5pF more than the suspending buffer or the pre-homogenisation cell supernatant. This represents a residual capacitance of around 6% of the original capacitance value after maximal disruption is indicated by the other measures used. The results of these disruption experiments all show that capacitance is not only biomass related but can be used to measure viability and cell integrity for suspensions of yeast cells. It is interesting to note that the residual capacitance values for each of the fully disrupted suspensions are all similar and indicate that cell debris and non-viable but intact cells make a contribution to the overall capacitance. This is a phenomena which is worth bearing in mind when interpreting capacitance measurements of active yeast suspensions, that around 5 % of the capacitance could be due to the presence of cell fragments and non-viable cells.

In summary, the results from the experiments with yeasts show that capacitance is strongly related to viable biomass, is significantly influenced by cell size and shape, is a good indicator of cell integrity and may be a potential indicator of the metabolic state of the yeast suspension. These correlations were found to hold true in both ideal off-line situations and on-line in agitated, aerated fermentations in both defined media and in media containing undissolved solids.

5.2 Bacterial Results

As discussed in Section 2.5.2, very little work has been published on the use of the BM to measure capacitance in bacterial cultures. This makes it difficult to analyse the results from the bacterial experiments presented here in the context of their perceived accuracy, so it will need to be sufficient to examine their internal reproducibility and their relationship to the other measures of biomass used in these experiments.

5.2.1 Fermentation experiments

The results from the fermentation experiments, growing *P. putida* ML2 in a defined media and measuring the capacitance on-line with the BM, are shown in Figure 4.13 and 4.14 in the Results section. Despite a longer lag phase in

fermentation PP2, the two fermentations are generally reproducible in terms of metabolic activity, both having an exponential growth phase which last around 9 hours, and give a μ value of 0.22-0.24, calculated from the metabolic data. PP2 has a slightly lower peak CER value and peak VCC value than PP1, indicating slightly less growth, probably due to some of the available carbon in the carbon limited medium being expended for maintenance during the longer lag phase. The capacitance data for these two fermentation show good agreement with the other measures of biomass and good reproducibility between the two reproducible fermentations. The capacitance growth peaks in both fermentations coincide with the growth peaks in CER and VCC. The μ values are also similar at 0.21 and 0.25 for the two fermentations. The fall in metabolic activity shown in the sharp decline in CER is mirrored in a decline in the other measures of biomass. The two variables which should show greatest agreement; capacitance and VCC show very similar profiles, with both declining after the growth peak indicating a degree of cell lysis at the end of the fermentations. This agreement is reflected in the correlations between capacitance and VCC for these fermentations which are R^2= 0.97 and R^2=0.98, respectively. The slightly lower growth in PP2 is also reflected in the slightly lower capacitance value attained in this fermentation. In general, the capacitance measurements made by the BM on-line in these fermentations are seen to be as good indicators of biomass as the conventional methods employed and give an on-line indication of viable biomass in these bacterial fermentations.

However, the small size of these bacterial cells mean that the capacitance yielded from this, a fermentation with good growth is only in the region of a maximum of 3.5pF. While the instrument is obviously sensitive enough to measure this rise, and is reported to have a sensitivity of 0.1 pF (Aber Instruments, 1992), the manufacturers recommend that a minimum Δcapacitance of at least 5pF should be available before attempting on-line biomass measurement as the small signal from bacterial fermentations can easily become swamped by noise from the effects of agitation, aeration and changes in conductance. Despite this caveat, the fermentation results shown here do indicate that it is possible to accurately measure below the recommended minimum of 5pF.

As discussed above, the reason that bacterial cultures give such low capacitance values is their small size; in this case *P. putida* ML2 is a rod-shaped cell typically 1μm by 2μm (Paleroni 1975). To illustrate the effect of bacterial cell size on

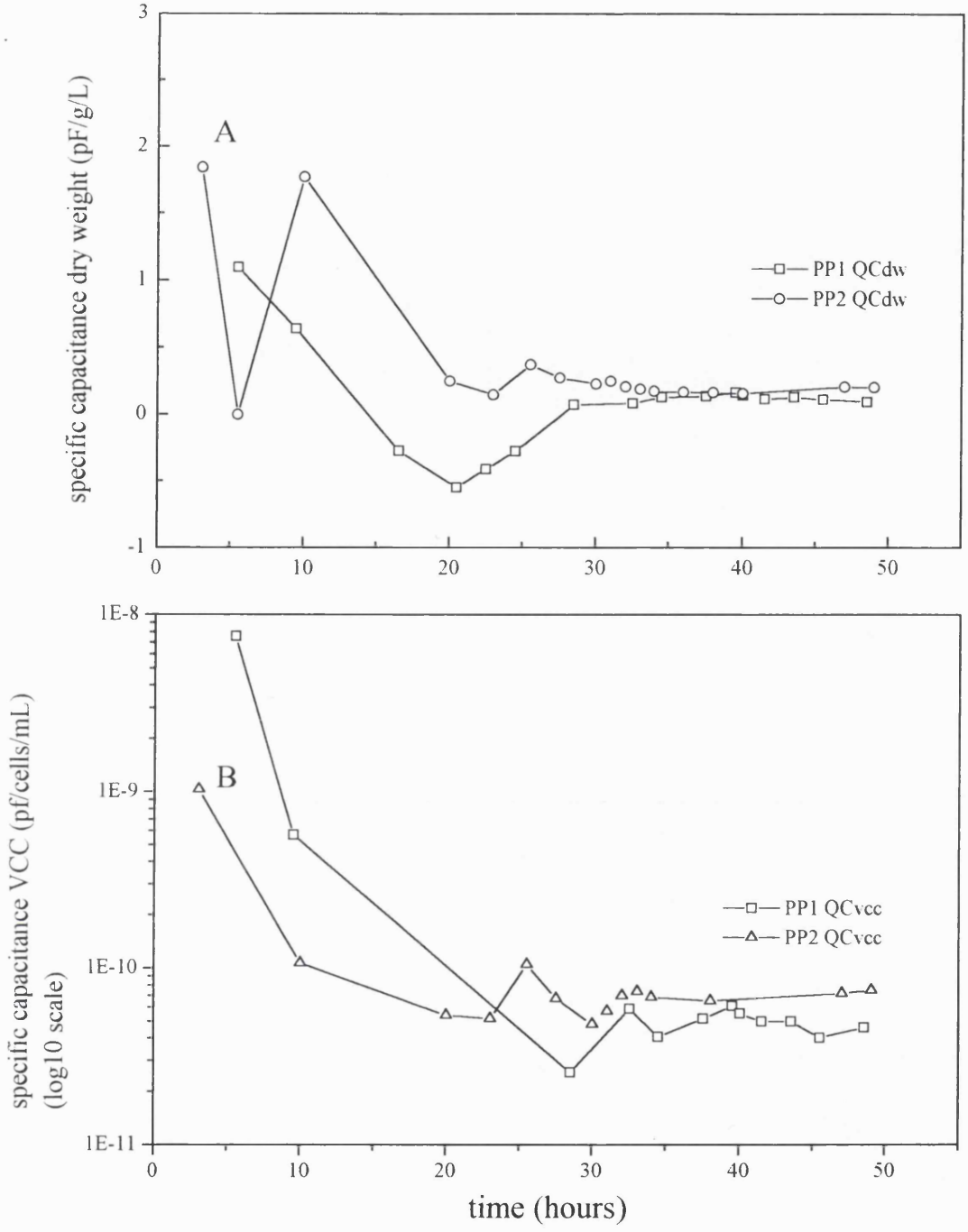


Figure 5.8: specific capacitance dry weight (pF/g/L) and specific capacitance VCC (pF/cells/mL) versus time Replotted from *Pseudomonas putida*ML2 fermentations

capacitance, Figure 5.8 shows the QCdw and QCvcc values from these fermentations. The QCdw values were calculated by converting the OD measurements to dry weight using the linear regression plot of dry weight against OD shown in Figure 5.9, which is derived from work by Hornby (1995).

The QCdw and QCvcc values show similar profiles against fermentation time as those seen with the yeast fermentations. The values are initially high (possibly an artefact) and then fall before rising again at the beginning of the growth phase, reaching a plateau at the end of growth. The plateau values for both fermentations are around 0.15-0.25 pF/g/L and $4x10^{-10}$ which are lower than the values seen for the larger cells in the yeast fermentations. It is surprising that there is a significant difference between the QCdw values for these fermentations during the growth phase as they are metabolically very similar. It is possible that this difference may be due to some differences in the growth of the two fermentations as the values show good agreement out of the growth phase, when the values for both fermentations plateau at around 0.2 pF/g/L.

The plateau in these specific values, while the capacitance and VCC values are falling, further supports the contribution of a viability component to the capacitance measurement. As the viability of the culture is falling, the plateau in the specific values indicates that capacitance is falling at a similar rate to the decline in viability.

5.2.2 Off-line experiments

The experiment shown in Figure 4.15 to give an extended biomass range shows that the BM seems to work better at higher bacterial biomass levels as the higher biomass samples are closer to the linear regression line than those at lower biomass levels. This is to be expected, from the manufacturers advice that 5pF is the minimum capacitance for useful operation of the instrument. Figure 5.10 shows the QCdw values calculated from this off-line high biomass experiment. The data from this experiment is broadly in agreement with that from the fermentation experiments. The range of values are between 0.19 and 0.23 pF/g/L, with the exception of the lowest biomass value, which has a higher specific capacitance. The higher specific values seen at low biomass levels are most likely to be artefacts

caused by the decrease in accuracy of the instrument at low capacitance levels rather than real results.

The data from the disruption experiments also give a similar specific values, and these are shown in Table 5.3

Table 5.3

Specific capacitance dry weight (pF/g/L) pre and post disruption, two repeated experiments

	QCdw pre	QCdw post
C1	0.28	0.09
C2	0.20	0.06

The specific values in C1 are higher, and this is reflected in the higher fall in capacitance in the disruption experiment. The values are broadly in agreement with those for the fermentation and high biomass experiment, indicating that the dielectric characteristics of the cells were relatively constant.

In the disruption experiments, for both replicates, the remaining Δcapacitance after the protein release and VCC data indicated total disruption was around 20% of the initial capacitance value. This is a substantially higher residual capacitance to that seen in the yeast disruption experiments, which was typically around 5%. There are a number of possible explanations for this. As discussed above, the capacitance measured values for these bacterial cells are low and thus are perhaps not as accurate as those for the yeast disruptions. Another possible explanation is that the bacterial cells are stronger than the yeast cells and therefore not fully disrupted by three passes at 1200 bar. This is not borne out by the protein release data, which indicates maximal disruption, or by the VCC data which gave no growth of cells post disruption at any dilution. Another possibility is that the more complex cell membranes of the gram negative bacteria give a higher residual capacitance after disruption than other organisms, potentially by the formation of liposome like structures which can retain a more significant capacitance.

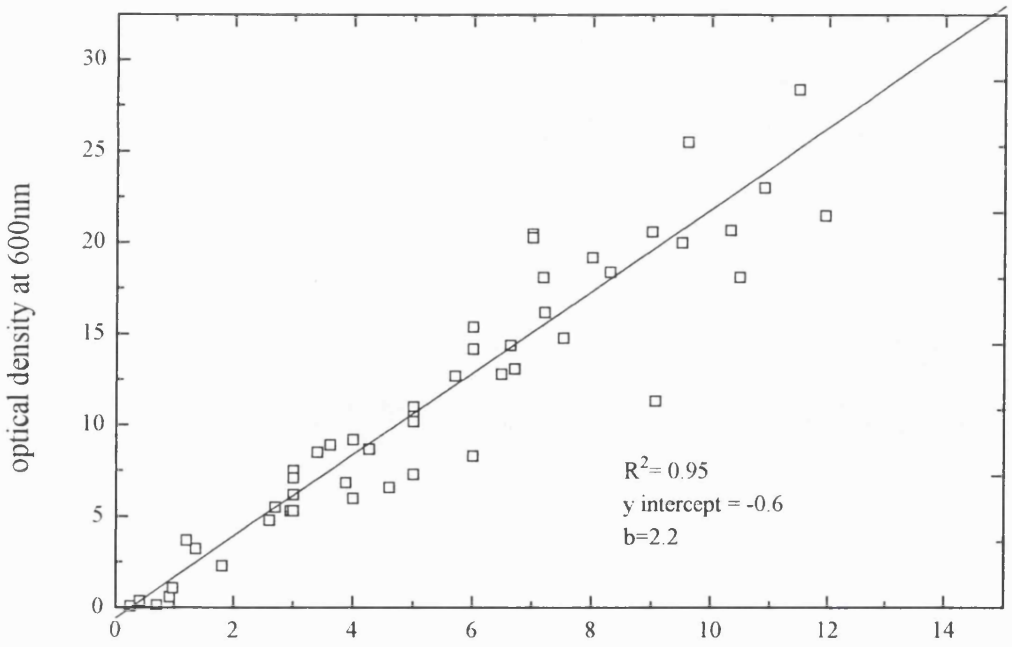


Figure 5.9: Optical density at 600nm versus dry weight (g/L) data replotted from *Pseudomonas putida* ML2 fermentations from Hornby (1995)

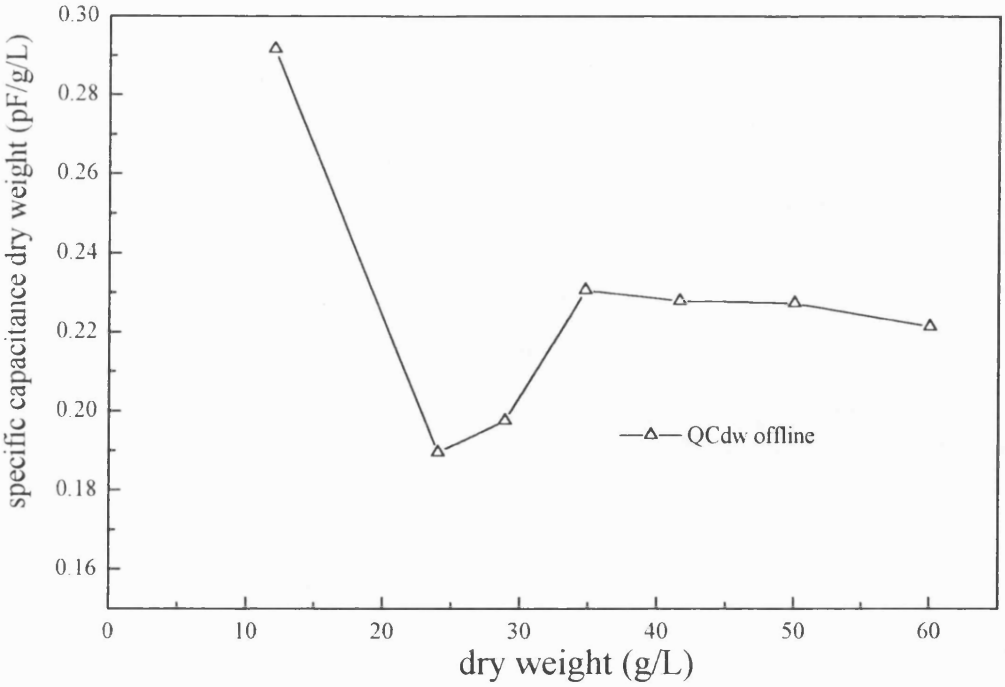


Figure 5.10: specific capacitance dry weight (pF/g/L) versus dry weight Replotted from *Pseudomonas putida* ML2 offline work

5.2.3 Comparison with other workers and theoretical values

As mentioned in Section 2.52, there is a relative lack of published work using the Biomass Monitor and other capacitance measuring methods to detect bacterial biomass. Mishima *et al.* (1991b) report a QCdw of 0.49 pF/g/L for a strain of *E.coli*, with a correlation coefficient of 0.993 for the work, measuring capacitance off-line at 0.3MHz. Matanguihan *et al.* (1994) showed good correlation between capacitance measured on-line with the KSCP, on-line optical density measured with a laser turbidimeter and off-line dry weight and derived a QCdw of 0.8 pF/g/L, measuring at 0.3 MHz. This value seems rather high and could be artificially boosted by the nature of the capacitance measuring system. In order to further assess the relative accuracy of the biomass related capacitance for the bacterial results described in this work, a calculation of theoretical values would be helpful.

The work by Davey (1991) used in Section 5.1.2.3 to derive the theoretical values for yeast suspensions can also be used to derive values for bacterial suspensions. For rod-shaped cells, the equivalent cell radius can be calculated thus:

$$r = [\, 0.75\ Rc^2\ Lc]$$ **Equation 5.6**

where

r is the equivalent cell radius in μm

Rc is the radius of the rod in μm

Lc is the length of the rod in μm

From this equation, the equivalent cell radius can be input into Equation 5.3 to calculate the volume fraction P

For *P.putida*,

$$r = [\, 0.75 \times 0.5^2 \times 2\,]^{1/3}$$

This gives an equivalent cell radius of 0.72

$$P = 4.1888 .10^{-12} . r^3 .N$$ **Equation 5.3**

where:

P is the volume fraction

r is the equivalent cell radius (μm)

N is the number of cells per mL

If we take 1 x 10^{10} as a representative biomass level and r as calculated above as 0.72,

$$P = 4.1888 \times 10^{-12} \times 0.72^3 \times 1 \times 10^{10} = 0.0156$$

This gives a value of P = 0.0156 for 1 x 10^{10} *P.putida* cells. If we then input this P value in to Equation 5.2, this will give us the change in capacitance which can be theoretically expected using the Biomass Monitor set-up used in these experiments.

$$\Delta C = 225Pr/ k$$ **Equation 5.2**

where:

ΔC is the increase in apacitance attributable to the presence of the cells, in pF

P is the volume fraction attributable to the cells

r is the equivalent cell radius (μm)

k is the cell constant of the Biomass Monitor used, in cm^{-1}.

This assumes that the cells measured have a plasma membrane capacitance per unit area of 0.01 F. m^{-2} and an internal conductivity of 0.5S.m^{-1}. (Davey and Kell 1993)

$$\Delta C = 225 \times 0.0156 \times 0.72/ 0.93 = 2.72pF$$

This gives a value of 2.72 pF for 1 x 10^{10} *P.putida* cells. This is close to the values seen in the experimental work on-line and off-line and supports the hypothesis that these measured values accurately reflect the level of bacterial biomass in these situations.

In summary, these results have shown that the Biomass Monitor can be used to give on-line biomass measurements in fermentations of *P.putida.* The capacitance measurements made have been shown to correlate with viable biomass, reflect the loss of viability seen after disruption and are in agreement with the theoretical values calculated for cells of this size.

5.3 Fungal and filamentous bacteria results

In this section, the results from both the defined media batch fermentations and the off-line work with *P.chrysogenum* P1 will be discussed initially. The industrial fed-batch complex media fermentations of *P.chrysogenum* will be dealt with after this and in the light of the points raised in the first section.

Subsequent to the discussion of the fungal results, the results of the work with *Streptomyces* will be discussed. It is appropriate to discuss the theoretical aspects of capacitance monitoring of *Penicillium* and *Streptomyces* together as they are both filamentous organisms and therefore have a similar shape and structure, which impacts considerably on their capacitance measurement characteristics.

In order to have an understanding of the results presented here it is best to leave the discussion of the theoretical aspects of capacitance monitoring until the results of both types of the filamentous organisms investigated have been discussed in terms of their relationship to other measures of biomass.

5.3.1 *P.chrysogenum* P1 batch fermentations and off-line work.

The defined media batch fermentation results shown in Section 4.3.1 indicate that the BM can be used to give an on-line estimate of biomass in fungal fermentations. In these fermentations, the variable which was found to correlate best with capacitance was CER, which had correlations of R^2= 0.83 and 0.84; though in the fermentation without gas analysis, dry weight was found to have a correlation of

R^2=0.84 with capacitance. These relatively poor correlations indicate that while capacitance is biomass related in fungal cultures, the agreement with conventional biomass estimation methods is not as good as those seen in the experiments with yeast and bacterial cultures.

The work carried out in disrupting a fungal suspension off-line was more successful, in that it gives a good indication of the accuracy and utility of the capacitance measurement for this organism. The effect of a single pass at 200bar through the homogeniser was sufficient to cause a drop in capacitance of around 70% from the pre-homogenisation level. This was accompanied by a rise in protein release of 40% of the maximum, indicating that capacitance is a much more sensitive measure of fungal disruption than protein release. In common with the other disruption experiments, the disruption method which gives maximal protein release, indicating maximal disruption still leaves a residual capacitance attributable to the cells. In this case, the maximal protein release is with a single pass at 1000 bar and this leaves a residual capacitance of 7% of the original value. The method which should give maximal protein release, 3 passes at 1200 bar has a lower protein release value, though this may be due to some heat denaturation of the protein. Again, this leaves a residual capacitance value of 5% of the original capacitance value. These residual values are in agreement with the work in disrupting yeast, but are lower than the residual values from the bacterial work. Again, the most likely explanation of this are the more complex cell wall structures of Gram negative bacteria yielding a greater residual capacitance when disrupted.

In general, the utility of capacitance measurement in monitoring disruption efficiency seems to be excellent, and the data from the work shown here suggests that it is more sensitive and reproducible than protein assay data, for fungal suspensions.

This work seems to support the interpretation of some of the on-line work that changes in the fungal morphology and integrity can be detected in the capacitance profiles of fungal cultures, this is a topic which will be discussed more fully in the other sections of this discussion. However, it is worthwhile to note here that while the linear correlations shown in Table 4.10 between capacitance and the measure of mean main hyphal length (MMHL) are reasonable, the event correlations are good. In both cases, the shape of the curve of MMHL against time resembles that of the capacitance profile and the decline in capacitance seen after around 90 hours in

fermentation PC1 is mirrored in a decline of MMHL. MMHL is a measure of the fungal morphology, growth and, to a certain extent, cell integrity. From this, and in light of the results seen above with the yeast experiments, it is entirely consistent that a relationship between MMHL and capacitance should be found. Perhaps, it is curious that it is not a stronger relationship, though that may be a result of the relative lack of data shown.

Figure 5.11 shows the QCdw values replotted from the fermentations PC1-3. In general the profiles for these values seem to indicate a rise with growth of the culture, a fall to a plateau during the typical production phase of these fermentations and then a fall towards the end of the fermentations, possibly indicating morphological changes and cell lysis.

As Fehrenbach *et al.* (1992) noted, in cultures whose size is difficult to determine, capacitance measurement can give an indication of changes in cell size. However, the problem in correlating capacitance in this case is the lack of viability measurement for fungal and filamentous cultures which makes it difficult to correlate capacitance properly for these fermentations.

The mean main hyphal length (MMHL) measurements also plotted were an attempt to find the points in these cultures where the changes in morphology were occurring . The data shown here is inconclusive as the MMHL falls at the end of the fermentation in one culture, correlated with a drop in QCdw. The other fermentation, however shows a rise in both MMHL and QCdw, possibly indicating a link, though further work would be required to illustrate this. The technique of estimation of fungal biovolume from selectively stained samples of fungal biomass which are then measured by image analysis (Packer *et al.,* 1994) would be useful as a means of correlating with capacitance for cultures such as these. This would allow a direct comparison between to measures of the same property, the volume of viable cytoplasm bounded within the selectively permeable cell membrane. A correlation between this measure and capacitance would be of some significance in analysing industrial cultures of fungal strains. The biovolume, in terms of vacuolisation and morphology has been shown to correlate with production efficiency and product yield, (Justen *et al.,*1998, Righelato *et al.*,1968 and Makagiansar, 1991). Thus, a correlation of biovolume with capacitance in these cultures would effectively allow an on-line measure of expected productivity.

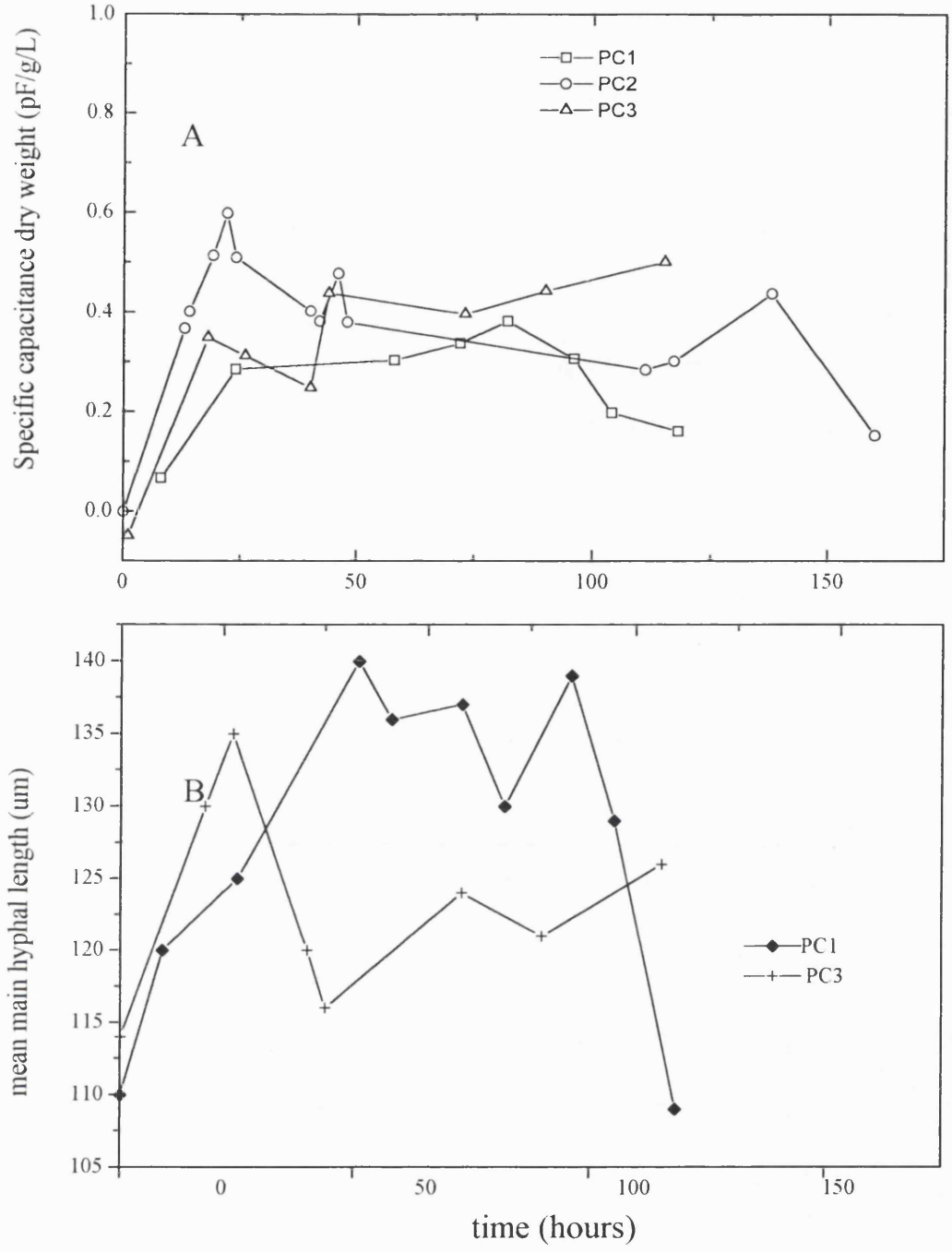


Figure 5.11: Specific capacitance dry weight (pF/g/L) and mean main hyphal length (υm) versus time *Penicillium chrysogenum* P1 5L fermentations PC1-3

5.3.2 *P.chrysogenum* industrial fed-batch fermentations

The work in using the BM to measure capacitance on-line in industrial fermentations of *P.chrysogenum* was successful and showed that the capacitance measurements correlated with the other measures of biomass in these industrial processes, despite the use of complex media containing undissolved solids and fed-batch techniques, which may alter the volume in the vessel.

The measurements were carried out on fermentations at laboratory, pilot and production scale and some differences were noted with the changes of scale. At the pilot and industrial scale, the signal was much more stable, probably reflecting the poorer mixing in the larger vessels and hence less turbulence. The capacitance values appeared to be relatively higher in the larger fermentations, a phenomena noted in the work with yeasts probably related to the lessening of gas hold-up effects on volume, and thus capacitance, in the larger vessels.

Capacitance was found to correlate best with the cumulative exit CO_2 measurements for these fermentations, which is a reflection of the nature of the fed-batch fermentations and because capacitance is also a cumulative measurement. The metabolic activity of a fed-batch fermentations is maintained throughout the fermentation due to the feeding of nutrients. The exit CO_2 spot values correlated well with capacitance during the initial exponential growth phases of the fermentations, and this was reflected in the similar specific growth rates calculated from these data. However, as the fermentations progressed, the cumulative measure correlated better with capacitance as they are both cumulative measurements reflecting the total metabolic activity of the fermentation and biomass produced over all the preceding fermentation time, and not merely the activity and biomass at a particular moment.

Correlations with dry weight were generally poor, around R^2=0.80, but this is probably due to the presence of media solids in the samples. Some technical problems were encountered in these experiments, but in general the BM performed well in the harsh environment of the industrial pilot plant. The technical problems were associated with the mycelia becoming entangled on the measuring pins of the probe and have resulted in the manaufacturers investigating a flush version of the probe to avoid this problem. The fermentations shown in section 4.4 of the results section are all of the same organism and very similar processes (see section 4.4 for

full details). The three pilot plant fermentations were the most successful, and will be examined first.

The capacitance data for these fermentations shows good correlations with metabolic activity as measured by the exit CO_2 values. In the absence of a true viability measurement, these correlations can be taken to indicate that for these fungal cultures, where metabolic activity should mirror biomass, capacitance is a good on-line indicator. The correlations with dry weight and viscosity are poor and those with the measure of cell morphology, mean main hyphal length, were non-existent when plotted as linear correlations. The two values which should be indicative of culture morphology, viscosity and mean main hyphla length show little similarity. This suggests that the relative accuracy of these measurements may be poor and this would explain the lack of a correlation with capacitance.

For reasons of confidentiality, the other biomass related values for these fermentations have had a scaling factor applied to them. Despite this, the profiles of the specific capacitance values should be useful in interpreting the results, although the QC values are obviously meaningless. The QC values calculated from the scaled dry weight and scaled viscosity measurements for the pilot plant fermentations are shown in Figure 5.12.

The contaminated fermentation SB5 stands clearly apart from the other two fermentations, having the highest values of QCdw and QCv. The difference in the QCdw profile for SB5 is obvious relatively quickly, within 50 hours and is noticeable after 100 hours in the QCv profile. Fermentation SB4, which was found to have a higher biomass due to the accelerated feed regime used in this experiment, also has a higher QCdw and QCv than the normal fermentation for most of the fermentation time. However, the effects of this can be seen at the end of the fermentation, when the values fall rapidly, probably due to lysis and fragmentation. The QCdw and QCv values for the other fermentations SB1,2 and 6 are shown in Figure 5.13. The values are very similar to those for the pilot scale fermentations, indicating that the capacitance to (scaled) dry weight and viscosity relationship is similar despite the changes in scale of fermentation. As the same strain was used for all these experiments, this would have been predicted and the demonstration of this across six fermentations at a range of scales gives a good indication of the accuracy and reproducibility of capacitance for these fungal fermentations.

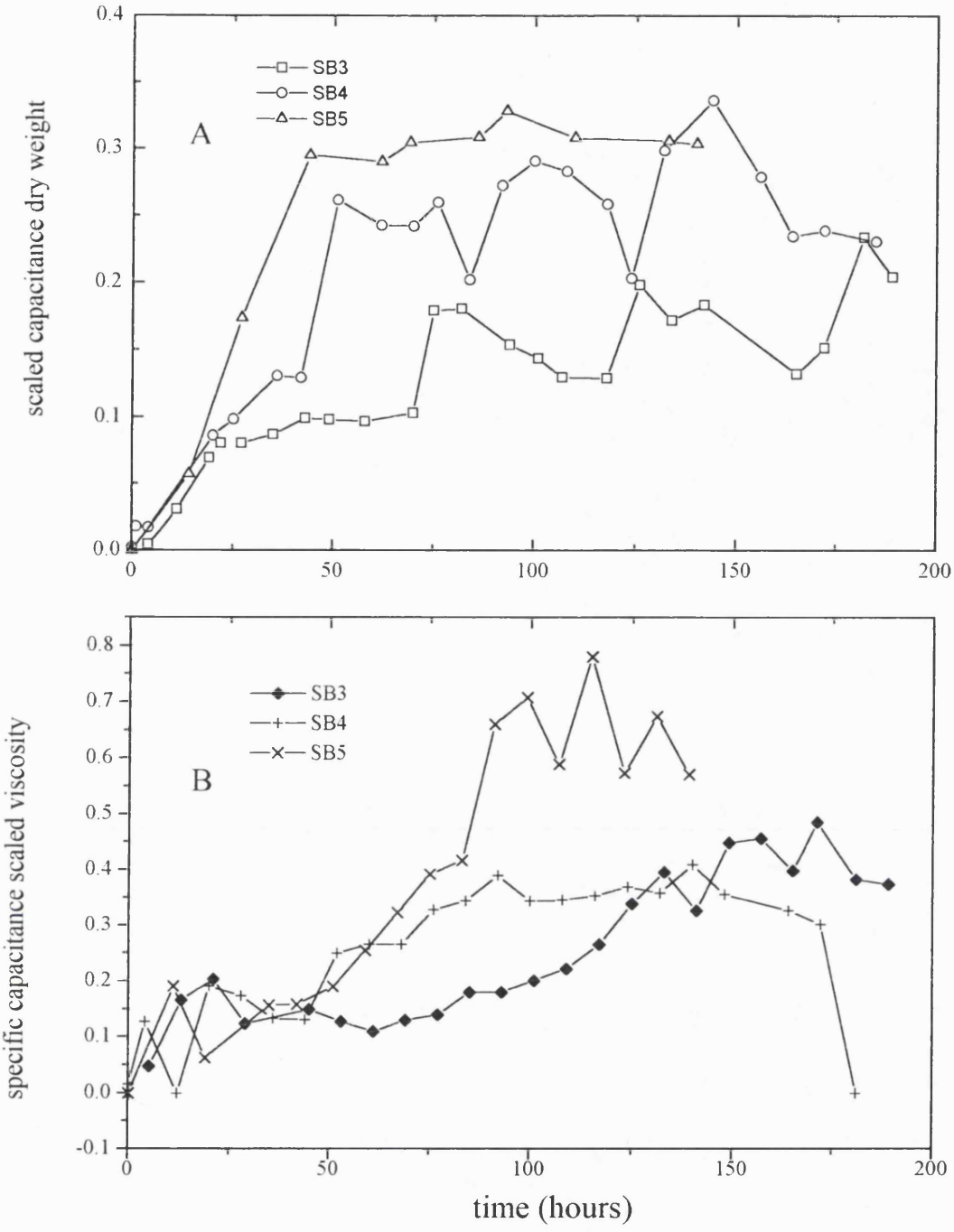


Figure 5.12: specific capacitance, scaled dry weight
and scaled viscosity versus time
Penicillium chrysogenum pilot scale fermentations SB3,4 and 5

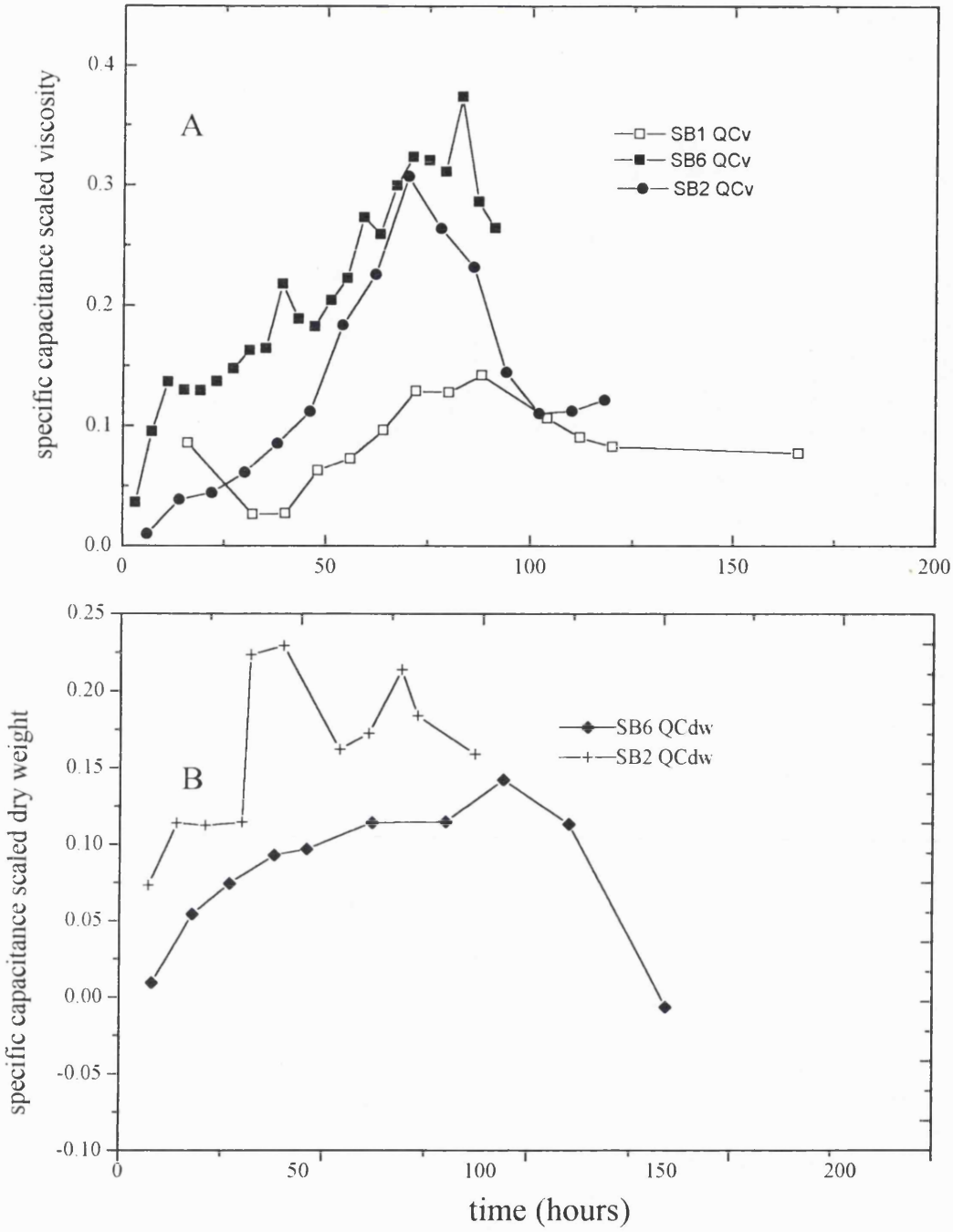


Figure 5.13: specific capacitance scaled dry weight and specific capacitance scaled viscosity versus time *Penicillium chrysogenum* laboratory and production scale fermentations SB1,2 and 6

5.3.4 *Streptomyces* industrial fed-batch fermentations

The work in using the BM to measure capacitance on-line in industrial fermentations of Streptomyces was successful and showed that the capacitance measurements correlated with some of the other measures of biomass in these industrial processes.

As in the fungal fermentations, this was despite the use of complex media containing undissolved solids and oils and despite the use of fed-batch techniques, which may alter the volume in the vessel.

The measurements were carried out on fermentations at pilot scale and therefore the signal was more stable, as seen in the work with the fungal fermentations. Some technical problems were encountered in these experiments, carried out in the harsh environment of the industrial pilot plant. The first fermentation had a problem with the power supply due to the elevated ambient temperature in the pilot plant. This resulted in some data being lost at the end of the growth phase of the fermentation. In the second fermentation, some data was lost at the start of the fermentation due to the probe giving a fixed negative reading. Normal operation was restored on the application of several clean pulses to the probe, indicating that the problem may have been mycelia adhering to the measuring pins.

In both these fermentations, capacitance was found to correlate best with the scaled viscosity measure. The viscosity measurement is a rough indicator of both biomass and morphology, and thus can be broadly regarded as a measure of biovolume. As discussed above for the fungal fermentations, this correlation would be expected given the operational theory of the Biomass Monitor. The SB8 fermentation had an excellent correlation of capacitance with viscosity (R^2=0.96) and the relatively poorer correlation found in SB7 may, in part be due to the missing capacitance data from the growth phase for that fermentation.

The excellent correlations seen with both spot and cumulative exit CO_2 measurements for the fermentations of the other organisms, are generally not seen with these fermentations. The spot exit CO_2 measurements show a good correlation during the exponential growth phase at the start of the fermentation. Some of this must be inferred due to the missing data, but the trends for both these

variables seem to agree, and both measures peak around the same timepoint at the end of the first growth phase in the two fermentations.

In the absence of another accurate biomass measure, it is difficult to determine the accuracy of the capacitance measurement in these fermentations, with regard to its relationship with viable biomass throughout the fermentation. After the initial growth peak, the breakdown in the agreement between capacitance and exit CO_2 suggests that the metabolic activity of these fermentations after the initial growth, is not strongly growth related but is possibly indicative of antibiotic synthesis.

An interesting phenomena was noted when comparing these two fermentations. Despite having half the initial nutrient content of SB7, SB8 has a higher capacitance reading and a faster growth rate. This is not reflected in the metabolic data or the other biomass related, with SB7 having a greater exit CO_2 output and a higher viscosity. It was also noted that in SB8, there was no observable lag phase while in SB7, there was a lag of around 7 hours.

All these factors seem to suggest that it is preferable to start these fermentations with half the standard nutrient input, a potential financial saving. The data on antibiotic titres for these fermentations was not made available due to its commercially sensitive nature, but it would be interesting to see if the differences in capacitance were reflected in the commercial productivity of the fermentations.

For reasons of confidentiality, the other biomass related values for these fermentations have had a scaling factor applied to them. Despite this, the profiles of the specific capacitance values could be useful in interpreting the results, although the QC values are obviously meaningless. The QC values calculated from the scaled viscosity measurements for the two fermentations are shown in Figure 5.14. In light of the discussion above regarding the differences in the nutrient concentrations of the two fermentations, it is interesting to note the shapes of the specific capacitance viscosity profiles for these two fermentations.

If viscosity is taken to represent biomass for these fermentations, the specific capacitance for the full nutrient fermentation peaks with the growth peak and then declines sharply, fluctuates about a plateau then declines again at the end of the fermentation. This would suggest that the full nutrient fermentation seems to be inherently less stable, the fall in the specific capacitance indicating changes in morphology and possibly cell fragmentation and lysis. However, the linear relationship between capacitance and viscosity for this fermentation is not as good

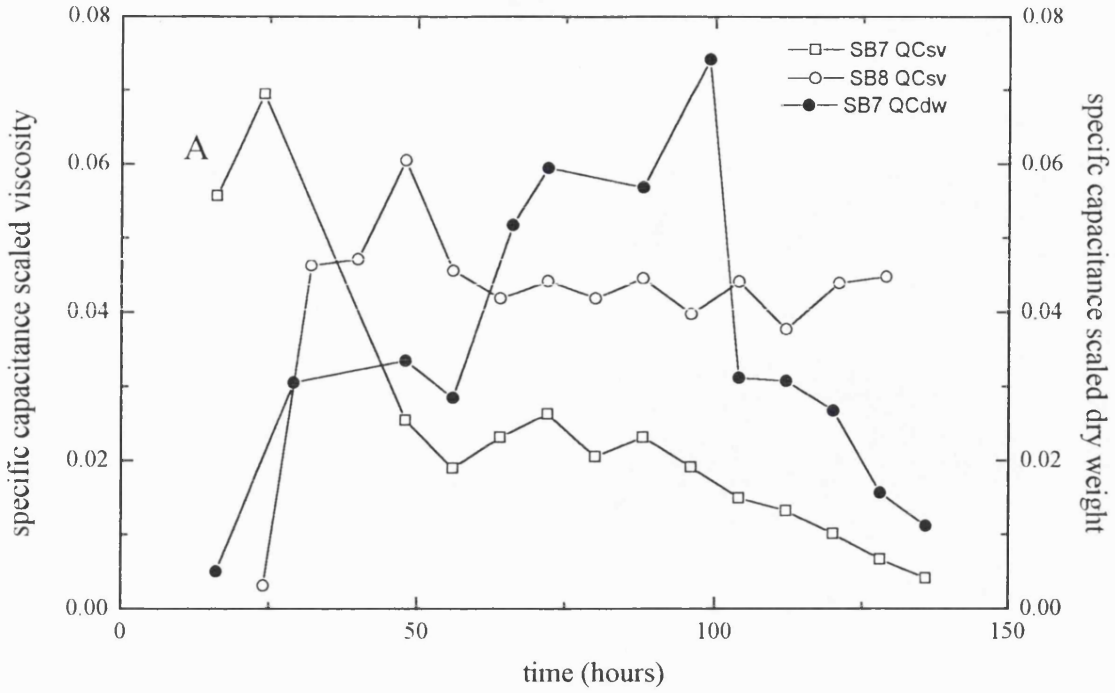


Figure 5.14: specific capacitance scaled viscosity and specific capacitance scaled dry weight versus time *Streptomyces sp* pilot scale fermentations SB7 and 8

as that for SB8, and it may be this relationship which is depicted here. Once again, the impact of cell morphology, can be clearly seen on the capacitance measurements. The absence of another biomass measuring technique or any other morphological measurements mean that it is difficult to interpret these results, but it seems clear that capacitance is measuring more than biomass for the fermentations of this filamentous bacteria.

On a simple and more obvious note, if the capacitance measurements from the fungal fermentations are compared to those for these fermentations, the effect of the larger cell size can be clearly seen with the capacitance values for the larger fungal hyphae being considerably larger than those for the smaller filamentous bacteria. This is reflected in the specific capacitance viscosity values. As both values have had the same scaling factor applied to them, it is feasible to compare them. The penicillium cultures all have values of about 5 times that for the Streptomyces, and the values seem to be inherently more stable over fermentation time, certainly when compared to SB7.

5.3.5 Comparison with other workers

As there are very little other reported results using capacitance measurement in fungal cultures, it is difficult to make valid comparisons. As discussed above, it is appropriate to consider the *Penicillium* and *Streptomyces* fermentations together as theoretically they are identical for the purposes of capacitance measurement from these cultures.

The structure and shape of filamentous organisms and the lack of full size details for the cultures shown in these results means that, unlike the yeast and bacterial work present here, there is no means of estimating theoretical capacitance values for these fermentations. All of these factors mean that the discussion of this interesting extension to the use of capacitance measurement will be limited to the available data generated from these fermentation and the little other work done in this field.

The most relevant and comprehensive work is that of Fehrenbach *et al.* (1992). Their work monitored capacitance in a pilot scale batch fermentation of *Streptomyces virginiae*, grown on a complex medium containing undissolved particulates. A good correlation was reported between capacitance and both dry

weight and packed mycelial volume. The capacitance profile showed a marked decline at the end of the fermentation, probably indicative of cell lysis. Replotting their data as QCdw values, they found that the values fluctuate around 0.5pF/g/L for the majority of the fermentation before falling to 0.4pF/g/L at the end of the fermentation. They postulate that this fall may be due to morphological changes, though it is interesting to note that this value changes little while the actual capacitance value fell the end of the fermentation, indicating that although the cells are lysing, the relationship between capacitance and biomass remains relatively constant, with only slight changes.

Comparing this data with that for the filamentous fermentations described in this work, the main similarity, beyond the uniformly good correlation between capacitance and the other measures of biomass, is in the detection of cell lysis at the end of the fermentations and in the off-line disruption experiment. Similar to these results, Mishima *et al* (1991a) measured capacitance off-line in cultures of *Aspergilus niger* and found good correlations between capacitance and dry weight. This work also reported the loss of this capacitance after homogenisation, leaving a residual capacitance equivalent to the residual values seen in the disruption experiments performed on the fungal cultures in this work. Work by Sarra *et al.* (1996), monitoring the capacitance of *Saccharopolyspora erythraea*, a filamentous bacteria, showed similar results to those reported here. Excellent correlations (R^2 = 0.97 or above) between capacitance and dry weight were found during the growth phase of the cultures. Other correlations (R^2=0.96) were found between capacitance and dry weight during the decline phase of these cultures. The slope of the line varied with agitation speed of the fermentation, with greater rate of decline correlating with higher agitation speed.

These results show that capacitance is a measure of cell integrity for cultures of filamentous organisms, and show that capacitance can be used to give indications of cell lysis, fragmentation and death on-line in fermentations of these organisms.

5.4 Summary of discussion and conclusions

5.4.1 The use of the Biomass Monitor in fermentation vessels.

As was set out in Section 2.5.6, the rationale of this work was to attempt to use the Biomass Monitor as an on-line capacitance measurement system in the fermentations of a group of organisms representative of those commonly used in industrial fermentations. The intention was firstly to ascertain whether this was feasible, in terms of accuracy and operability of the system. The results detailed and discussed above have shown that in general the Biomass Monitor can be used on-line in fermentation vessels successfully. This has been demonstrated at a full range of scales from 5L to an industrial production scale of tens of thousands of litres. It has also been demonstrated for yeast, bacterial, fungal and filamentous bacterial cultures, in defined and complex media and batch and fed-batch modes of operation. From this, it can be concluded that the Biomass Monitor is an instrument which is suitable for use on-line in fermentation vessels.

The second goal of this work was to examine the nature of the capacitance measurements obtained for these various cultures and compare them against the other, more commonly used measures of biomass for these fermentations. This was the inital goal of this work, however, once it became clear that capacitance was biomass related, this was extended, where possible, to compare the values obtained in this work with those found by other workers and by comparison with the theoretical values calculated for capacitance measurements of these organisms. As with the work described above, this summary will deal with each of the types of organism individually at first.

5.4.2 Summary and conclusions on the yeast work

The yeast results obtained were excellent and showed good correlations with capacitance against all the other measures of biomass used and with cumulative metabolic data but not instantaneous metabolic data. This was in agreement with the results obtained by other workers looking at the biomass related capacitance of yeast cultures. The calculation of specific capacitance measures showed that the results were consistent for the strains used and showed good reproducibility.

The importance of cell morphology on biovolume and its effects on theoretical and actual capacitance values was demonstrated in this work and this was in agreement with the work of Asami and Yonezawa (1995[2]), and their work demonstrating that cell division can be shown on-line by capacitance monitoring of synchronously dividing yeast cultures. The potential uses of these sorts of results included a greater level of understanding and control of yeast fermentations, once their dielectric characteristics are known. The theoretical values calculated for these yeast cultures were in broad agreement with the actual measured values, and indicated that the results of them experimental work were accurate and consistent with the theory of biomass-related capacitance and its relationship with cell viability and biovolume .

The use of off-line capacitance monitoring of homogenised yeast cultures showed that capacitance is a better and more sensitive measure of cell integrity after processing than protein release. It is also an on-line and real-time measure. This suggests that a capacitance measurement of a harvested yeast culture before and after a processing operation would allow the impact of that processing on the culture to be assessed and any damage or loss of viability to be monitored on-line and in real-time. As many bioprocessing operations are time-dependant and rely on cell integrity to protect the intracellular product, this is very useful information. Other operations involve cell breakage and therefore need to know that the maximal loss of integrity has been achieved, and the real-time, on-line measure of capacitance would also provide this, without the need for a time dependant assay.

In summary, the work described above as carried out on yeast cultures has shown the accuracy and utility of capacitance measurement for yeast cultures and advanced the use of capacitance measurement into its potential use in other bioprocessing operations other than fermentation.

5.4.3 Summary and conclusions on the bacterial work

The results obtained with the bacterial cultures were good, especially given the recomendations of Aber Instruments that a Δcapacitance of at least 5pF should be achievable as a minimum before using the instrument on-line. In the fermentation experiments, the capacitance attributable to the bacterial cells was less than this but excellent correlations between capacitance and the off-line measures of biomass

were found. As with the yeast cultures, the instantaneous metabolic measures did not correlate with capacitance, but the cumulative measure did.

The results of the homogenisation experiments showed that the capacitance measured correlated with cell integrity and viability, and indicated that the points discussed above about the use of capacitance to monitor disruption efficiency can be extended to include bacterial cultures. It was noted that the residual capacitance attributable to the remaining cell debris was, in percentage terms, greater than that seen for the yeast cultures. This was attributed to the presence of lipopolysaccharides in the more complex bacterial cell walls generating more residual capacitance once the cells were disrupted.

The theoretical values estimated for bacterial cells of the shape, size and cell number of those measured were in broad agreement with actual measured values. This indicates that, despite the advice of the instrument manufacturers, it is possible to accurately measure the low capacitance levels generated by bacterial cultures on-line. The specific capacitance measures calculated from the on-line and off-line data were consistent across each of the experiments and support the conclusion that the instrument performed well with these bacterial cultures and allowed the accurate measurement of viable biomass using this technique.

5.4.3 Summary and conclusions on the fungal and filamentous bacteria work

The results obtained with these cultures were good, showing biomass related increases in capacitance on-line for both the fungal and Streptomyces fermentations and in the fungal off-line work. The correlations with capacitance were closest for the metabolic data, possibly reflecting the relative inaccuracy of the other biomass related measures for these fermentations. The lack of a viability measurement means that the viability component of capacitance measurement must be inferred from the metabolic and cell integrity data, which is clearly demonstrated in the off-line work monitoring the effects of disruption on the capacitance of a fungal culture.

The results of the homogenisation experiments showed that the capacitance measured correlated with cell integrity and viability, and indicated that the points discussed above about the use of capacitance to monitor disruption efficiency can

be extended to include fungal cultures and perhaps be used on-line to detect cell lysis and morphological changes. The attempts to show a correlation between capacitance and fungal and filamentous morphology were mixed. The theoretical relationship between capacitance and biovolume, which was used successfully to estimate capacitance values for the other organisms could not be used in these experiments due to the lack of accurate size data for these cultures. In some instances, it could be postulated that event type correlations could be detected. These are instances where changes in morphology as measured by viscosity or morphological measurements seem to correlate to a change in the capacitance or specific capacitance profiles, occurring at the same time in the fermentation. Although, theoretically, this should be feasible, no unambiguous indications of these events could be identified. This is an area that would benefit from further study, especially in the development of a capacitance predicting model based on biovolume data. The potential applications of this data would be advances in the control of these types of fermentations to improve productivity by controlling feed-rates and aid in the prediction of optimum harvest windows for maximal productivity and product recovery.

5.4.4 Further work

It is clear that capacitance measurement has the potential to be a useful tool in the monitoring of cell cultures. In order that it may be used effectively, the dielectric characteristics of the strain of organism to be monitored need to be studied and understood, in the system to be monitored and controlled

A higher level of knowledge and understanding, could lead to the potential application of this technique in high-level metabolic control and piloting of fermentations to improve productivity, optimise harvest point, detect problems and atypical fermentations quickly, and monitor and control subsequent bioprocessing of these cultures to aid product recovery and quality.

As has been outlined in this work, the body of published knowledge and understanding of the dielectric characteristics of organisms of interest in biotechnology is relatively scant. The extension of this knowledge is beginning to happen and it is only through the greater use of this technique, the accumulation of knowledge and the application of the theoretical background of biological

dielectrics to this knowledge that the potential uses of these techniques can be fully realised.

Appendix 1: Abbreviations

Δ	delta, signifying change in
BM	Biomass Monitor
Cap.	Capacitance
CER	carbon dioxide evolution rate
CCER	cumulative CER
CFU	colony forming units
CCO_2	cumulative carbon dioxide
Cs	specific capacitance constant as defined by Fehrenbach *et al* 1991
DCC	dead cell concentration
DO_2	dissolved oxygen
DW	dry weight
Hz	Hertz
KSCP	Kobe Steel Capacitance Probe
MCD	mean cell diameter
MHz	mega Hertz
MMHL	mean, main hyphal length
NIR	near infra red spectroscopy
OUR	oxygen uptake rate
OD	optical density
PI	proportional and integrating
PID	proportional, integrating and differentiating
PMV	packed mycelial volume
RO	reverse osmosis
RPM	revolutions per minute
RQ	respiratory quotient
TCN	Elzone total cell number
TCV	Elzone total cell volume
VCC	viable cell concentration
VVM	volume per volume per minute
QCdw	specific capacitance constant calculated against dry weight
	Other QC values as per the relevant abbreviations

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