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Oscillatory, stochastic and chaotic growth rate fluctuations in permittistatically controlled yeast cultures

Hazel M. Davey[a], Christopher L. Davey[a], Andrew M. Woodward[a], Andrew N. Edmonds[b], Alvin W. Lee[b], Douglas B. Kell*[a]

[a]*Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed SY23 3DA, UK*
[b]*Prophecy Systems Ltd, Haydon House, Station Rd, Woburn Sands, Bucks MK17 8RX, UK*

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Abstract

We describe a continuous culture system related to the turbidostat, but using a feedback system based on biomass estimation from the dielectric permittivity of the cell suspension rather than its optical density. It is shown that this system provides an excellent method of maintaining a constant biomass level within a fermentor. The computer-controlled system was able to effect the essentially continuous registration of growth rate by monitoring the rate of medium addition via the time-dependent activity of the pump. At some biomass setpoints for aerobically grown cultures of baker's yeast substantial time-dependent fluctuations in the growth rate of the culture were thereby observed. At some biomass setpoints, however, or under anaerobic conditions, or when using a non-Crabtree yeast, the growth rate was constant, indicating that the fluctuations were inherent to the biological system and not simply a property of the fermentor and control system. A variety of time series analyses (Fourier transformations, Hurst and Lyapunov exponents, the determination of embedding dimension, and non-linear time series predictions based on the methodology of Sugihara and May) were used to demonstrate, for the first time, that as well as stochastic and periodic components these fluctuations exhibited deterministic chaos. 'Trivial predictors' were unable to give accurate predictions of the growth rate in these cultures. The growth rate fluctuations were studied further by means of offline measurements of changes in percentage viability, bud count, and in the external ethanol and glucose concentrations; these data and other evidence suggested that the growth rate fluctuations were closely linked to the primary respiro-fermentative metabolism of this organism. The identification of chaotic growth rates in cell cultures suggests that there may be novel methods for controlling the growth of such cultures.

Keywords: Dielectric spectroscopy; Biomass estimation; Cellular growth rate; Chaos; Non-linear prediction; Control

1. Introduction

In general, one may distinguish two types of continuous cell culture, the chemostat and the turbidostat (Anderson, 1956; Tempest, 1970). In the chemostat, the dilution rate is fixed by the experimenter (and equal to the growth rate), and the medium is designed such that growth is nutrient-limited (Monod, 1950; Novick and Szilard, 1950; Herbert, 1958; Novick, 1958). By contrast, in the

* *Corresponding author*, Tel.: +44 1970 622334; Fax: +44 1970 622354; Email: DBK@ABER.AC.UK

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turbidostat the medium is designed to be rich in all nutrients (*sensu lato*), growth is not nutrient-limited, and the growth rate is the maximum growth rate of which the organism is capable under the prevailing conditions; to effect this, the biomass level is constantly monitored, and only when it exceeds the setpoint is fresh growth medium pumped in, i.e. there is a feed-back loop (Munson, 1970).

The turbidostatic growth regime has the advantage that the volumetric productivity in biomass terms is higher than for any other culture regime, the culture is stable at dilution rates approaching μ_{max} (Watson, 1972), and, of particular importance for the production of improved strains, the selection pressures in the turbidostat are very high (Bryson and Szybalski, 1952; Fraleigh et al., 1989). Usually, selection will be for the organism that will grow at the fastest rate in the conditions provided (as the cells with slower growth rates will tend to be washed out of the fermentor before they divide), but conditions can be envisaged whereby other selection pressures could be imposed. Since the turbidostat is by its very nature a self-stabilising system, it is possible to use it to select for organisms capable of improved growth rate in the presence of a toxic substance (Brown and Oliver, 1982; Aarnio et al., 1991; Kell and Salter, 1995).

Despite the advantages offered by a turbidostatically controlled fermentation, the method remains under-exploited in microbial physiology, with the less-stable chemostat being the more widely used option. There are several problems associated with turbidostatic control that may in part account for this (Martin and Hempfling, 1976). In conventional turbidostats, as the name suggests, the biomass estimation for feed-back control is determined by turbidity (optical density) measurements (Myers and Clark, 1944). There are several disadvantages associated with optical biomass measurement (see e.g. Harris and Kell, 1985; Kell et al., 1990; Sonnleitner et al., 1992, Junker et al. 1994), the most important of which are that optical density is linear with biomass only over a very narrow range of low biomass concentrations, such that for most organisms the O.D. fails to be linear at concentrations well below those at which they would be of industrial interest. In addition, such measurements are prone to sensor fouling by the microorganisms that they attempt to measure, due to biofilm formation on the relevant optical surfaces (Anderson, 1953; Northrop, 1954; Watson, 1969). A third problem with turbidity measurements is that it is not only *bio*mass that is measured, since *necro*mass, particulate solids, and gas bubbles will all contribute to the optical density to some extent. For these reasons, novel methods for the on-line and real-time measurement of the biomass content of industrial fermentations have long been an area of interest (Harris and Kell, 1985; Clarke et al., 1986; Kell et al., 1990; Sonnleitner et al., 1992).

An instrument that monitors biomass via the radio-frequency electrical capacitance of the cell suspension has been developed (and commercialised by Aber Instruments Ltd., Science Park, Cefn Llan, Aberystwyth, SY23 3AH, UK as the Biomass Monitor; (see e.g. Harris et al., 1987; Kell et al., 1987, 1990; Davey, 1993a,b)). Continuous cultures in which the biomass is set by the Biomass Monitor have been called 'permittistats' because the biomass is kept at a constant level by a feed-back mechanism based on the dielectric permittivity of the suspension, and this approach has previously been used with great success for controlling anaerobic yeast cultures (Markx et al., 1991a).

Continuous culture methods are frequently used for studying 'steady-state' microbial growth, although fluctuations in NADH levels and the respiration rate of bacteria (Degn and Harrison, 1969; Harrison, 1970), and of various metabolic intermediates and fermentation parameters in cultures of yeast (Satroutdinov et al., 1992; Ölz et al., 1993) have been reported in these systems, and numerous studies have shown that the restricted (Rieger et al., 1983; Sonnleitner and Käppeli, 1986) respiro-fermentative *metabolism* of baker's yeast is capable of oscillations (Porro et al., 1988; Richard et al., 1994) and indeed chaos (Markus et al., 1985). Oscillations in the biomass content of yeast in a chemostat have been reported (Strässle et al., 1988, 1989; Chen et al., 1990a,b; Münch et al., 1992; Auberson et al., 1993), although of course the overall growth rate of such cultures remains constant, whilst (possibly entrained) growth

rate oscillations were also recently observed in fed-batch cultures of a recombinant strain of *Escherichia coli* (Ye et al., 1994). In contrast to the chemostat, however, the turbidostat-type continuous culture described herein permits the direct observation of fluctuations in the growth rate of the culture. It was therefore of interest to determine whether oscillatory behaviour could be observed in the growth rate of a continuous culture and whether the control strategy offered by the Biomass Monitor would provide a suitable means for its study. During the permittistatically controlled fermentations described herein, we indeed observed substantial oscillations in the growth rate of the yeast cultures and showed that at certain biomass set-points these fluctuations contained periodic, stochastic and deterministically chaotic components. Whilst the detailed origin of the observed oscillations could not be accounted for in full, they do appear to be linked to the central metabolic pathways of the cell.

2. Theoretical basis of the dielectric estimation of biomass

When an electric field is applied to an ionic solution the ions in that solution are forced to move. The positively charged ions are pushed in the direction of the field while the negatively charged ions are pushed in the opposite direction. However, if cells are present in the ionic solution then many of the ions both inside and outside of the cells can move only so far before they meet the cell's plasma membranes and are prevented from moving any further. The result of this is that there develops a charge separation or polarisation at the poles of the cells. The extent of the field-induced charge separations is measured by the capacitance (in Farads, F) of the suspension. As the volume fraction of cells increases, the amount of membranes polarised increases and so the capacitance of the suspension increases. Thus by the measurement of the capacitance of the suspension, one can monitor its biomass content (Harris et al., 1987; Kell et al., 1990; Davey, 1993a,b).

By reversing the field direction, the polarity of the charge separations is reversed, but the magnitude (and hence the capacitance of the suspension) remains unchanged. One can, of course, also change the *rate* at which the field changes direction, i.e. its frequency (in Hz). Frequency has a marked effect on the capacitance of a cell suspension, because a finite time is required for the charge separations to be induced (Pethig, 1979; Foster and Schwan, 1986; Pethig and Kell, 1987). Fig. 1 shows diagramatically how the capacitance of a typical cell suspension changes with frequency. At low frequencies (A) many ions have time to reach the plasma membranes and polarise them before the field changes direction and moves the ions the opposite way. Thus, the capacitance of the cell suspension is high. At higher frequencies (B) fewer ions have time to reach the membranes and so the extent of the induced transmembrane polarisation is less and the capacitance of the suspension is also lower. At very high frequencies (C),

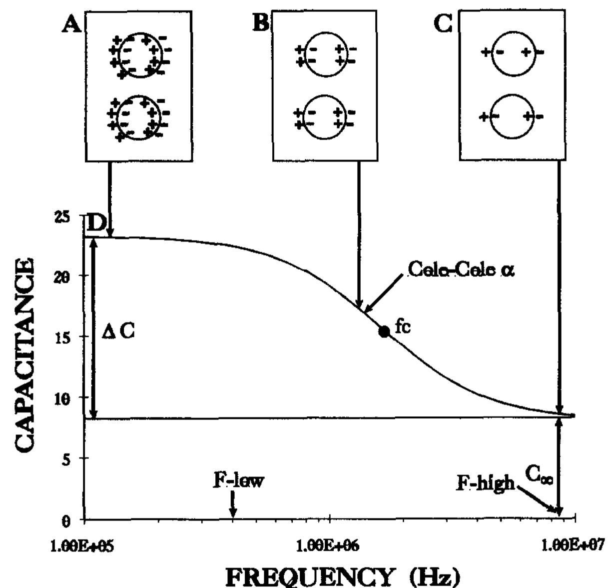


Fig. 1. Diagramatic representation of the β-dispersion of cell suspensions. Polarisation of the cell membranes involves movement of ions and so it takes a finite time for charge separations to be induced. At low frequencies (A) the cells are maximally polarised, at medium frequencies (B) there are fewer charge separations, and at high frequencies (C) the cells are not polarised at all. The curve that describes this relationship is known as the β-dispersion (D). For clarity in A–C, the charges are shown for just one field direction. The annotations are explained in the text.

very few ions have time to move to and polarise the membranes before the field changes direction, and so the induced membrane polarisation is very small. At these frequencies, the cell's contribution to the capacitance of the suspension is very small and one just measures the background capacitance of the medium (which is mainly due to water dipoles). Thus, the capacitance of the suspension (D) goes from a high-capacitance plateau at low frequencies (maximal cell polarisation) to a low-capacitance plateau at high frequencies (minimal cell polarisation). This fall in capacitance of a suspension due to the loss of induced membrane polarisation with increasing frequency is called the β-dispersion (Pethig, 1979; Foster and Schwan, 1986; Pethig and Kell, 1987; Davey and Kell, 1994). The residual high frequency capacitance due to the medium is called C_∞ and the height of the low-frequency plateau above this is the ΔC (capacitance increment) of the β-dispersion. The frequency at which the fall in capacitance is half completed (i.e. the frequency when capacitance equals $C_\infty + (\Delta C/2)$) is called the critical frequency (f_c). The Cole-Cole α is related to the steepness of the fall in capacitance with increasing frequency (see later), whilst the capacitance C and permittivity ϵ at any given frequency are related by the formula $CK = \epsilon\epsilon_r$, where ϵ_r is the permittivity of free space ($= 8.854.10^{-12}$ F.m^{-1}) and K is a so-called cell constant with the dimensions length^{-1} (see e.g. Kell, 1987).

As one is aiming to measure the biomass content of a cell suspension, one needs to see what effect this has on the β-dispersion curve shown in Fig. 1. Fig. 2 shows the β-dispersion of hypothetical cell suspensions with different biomass contents. The figure shows that the f_c is not changed by the biomass content, nor is C_∞. What does change as a function of biomass content is the magnitude of ΔC, which increases with the biomass. Thus, the problem of measuring the biomass content of a cell suspension reduces to one of measuring the magnitude of the ΔC of the β-dispersion.

As mentioned above, one of the problems associated with using optical density for the control of continuous cultures is that dead cells, gas bubbles and non-biomass solids all interfere with the measurements, so their effects on the dielectric method of biomass estimation must be considered. If the cells in a suspension have badly ruptured plasma membranes then the ions in the solution just move through the holes in the membranes and fail to cause polarisation of the membranes even at low frequencies. Thus, dead cells do not normally have a significant β-dispersion. If one uses the ΔC of the β-dispersion rather than the more conventional turbidity measurements to measure the biomass content of a fermentor, one can expect to measure only cells with intact plasma membranes (i.e. living cells) as opposed to ruptured (dead) cells (Stoicheva et al., 1989; Salter and Kell, 1992; Davey et al., 1993).

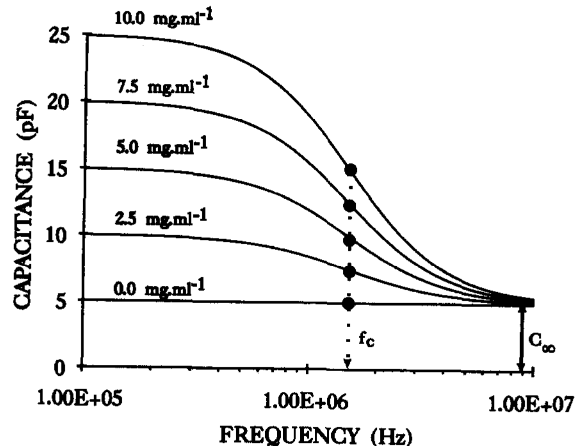


Fig. 2. The effect of increasing biomass concentrations (in mg wet weight·ml^{-1}) on the β-dispersion of a series of hypothetical cell suspensions of different biomass content (and in which their radius and the conductivities of the medium and cytoplasm are held constant).

If non-biomass solids, oil droplets or gas bubbles are present in the medium, then their effect on the ΔC of the β-dispersion must be considered. The ions in a growth medium either travel straight through the non-biomass material if it is permeable to ions or just move round it if it is not. In neither case are significant charge polarisations induced (since there are no plasma membranes) and so these materials will not tend to produce a significant ΔC term. Thus, non-biomass materials are not expected to contribute significantly to the ΔC of the β-dispersion in a real fermentation medium, nor to the feedback signal used to maintain constant biomass.

Turbidity measurements often fail as a result of sensor fouling; with the Biomass Monitor this problem can be solved by use of electrolytic cleaning pulses which may be applied to the electrodes in situ. It should also be noted that under certain circumstances the electrodes themselves can display a substantial, and frequency-dependent capacitance; artefacts of this type are minimised by using a four-terminal electrode system (Kell, 1987).

It was stated earlier that the biomass measurement problem reduces to one of the estimation of the ΔC of the β-dispersion. Thus, one needs a convenient means of measuring ΔC during a fermentation. There are two ways of achieving this. Fig. 1 showed a β-dispersion and marked on it were two spot frequencies labelled *f*-low and *f*-high. The capacitance at *f*-high is approximately equal to C_∞ whilst that at *f*-low approximately equals ($C_\infty + \Delta C$). Thus, one can see that if one measures the capacitance at *f*-high and *f*-low 'simultaneously' (or more practically in rapid succession), and then subtracts the capacitance at *f*-high (C_∞) from that at *f*-low ($\Delta C + C_\infty$) one gets ΔC, and thus a measure of biomass concentration. This is the principle of dual-frequency biomass measurements. The second method of estimating ΔC, and hence biomass concentration, uses the capacitance at *f*-low alone. At zero biomass concentration, the capacitance at *f*-low equals C_∞ (see Fig. 2); thus, one can measure the capacitance of the medium at *f*-low prior to inoculation and then back off this capacitance to zero (i.e. set C_∞ to zero). This means that any change in capacitance at *f*-low during a fermentation must reflect changes in ΔC and hence biomass concentration. For the present work, dual-frequency measurements were used throughout as these may be expected to be more stable to long-term instrumental drift (Davey, 1993a) which may occur within the time scale (typically 2 months) of the continuous culture fermentations that were carried out (since any changes that may occur will tend to affect measurements at both frequencies to a similar degree).

Dual- and single-frequency measurements using a Biomass Monitor (BM) have been successfully used to monitor the biomass concentrations in a variety of systems (Kell et al., 1987). These include bacterial and yeast cultures (e.g. Harris et al., 1987; Boulton et al., 1989; Ferris et al., 1990; Austin et al., 1994), bacterial biofilms (Markx and Kell, 1990), cultured cells (Markx et al., 1991c,d, Cerckel et al., 1993, Degouys et al., 1993), human blood (Beving et al., 1994), immobilised cells (Salter et al., 1990) and filamentous cells in liquid and solid substrate fermentations (Davey et al., 1991; Penaloza et al., 1991, 1992; Fehrenbach et al., 1992). Using a different instrument, Mishima and colleagues (Mishima et al., 1991a,b) have made two-terminal capacitance measurements as an attempted method for biomass estimation.

3. Materials and methods

A yeast clone was isolated from dried bakers yeast by repeated streaking onto agar, followed by growth in broth. The medium used was YPG which contained (all w/v): glucose (BDH) 5%, yeast extract (Oxoid) 0.5% and bacteriological peptone (Oxoid) 0.5%. The pH was set at 4.5 prior to autoclaving for 15 min at 121°C. Solid plates were prepared by adding 1.5% Lab M agar to the same medium save that the pH was set to 5.0. Plates and broth cultures were incubated at 30°C.

For permittistatic culture the yeast were grown in a 1-l fermentor (LH Engineering) with a working volume of 750 ml. The top of the fermentor was adapted to allow the insertion of the Biomass Monitor's probe (standard 25 mm probe, cell constant 1.18 cm^{-1}). The temperature was controlled at 30°C with a LH-503 temperature controller and the pH was controlled at 4.5 with a LH-505 pH controller using 2 M KOH and 2 M HCl. The culture was run under ambient lighting conditions. Filtered and wetted air was pumped through the fermentor at a rate of approximately 1.5 vol./min, and the contents of the fermentor were stirred at 450 rev./min. The Biomass Monitor was interfaced, via a set of amplifiers, to a 386SX IBM-compatible computer containing a DT2811-PGH 12-bit analog/digital I/O board (Data Translation Ltd. Wokingham, Berkshire, UK).

A program (PERMSTAT.EXE) was designed and written in-house in Microsoft QuickBASIC v4.5 for monitoring and control of the biomass

content of the fermentor. In brief, the program set the measuring frequencies (0.4 MHz and 9.5 MHz) on the Biomass Monitor, read back the resulting capacitances, calculated the difference between the capacitances (ΔC), and switched the medium pump on and off appropriately to control the biomass level. At user-defined intervals, the capacitance and conductance of the suspension at each frequency were recorded to a file. The volume of medium required to control the biomass was also recorded on an hourly basis. Since the dilution rate is equal to the specific growth rate, and the dilution rate (in h^{-1}) is equal to the volume of medium pumped per hour divided by the working volume, and the biomass is controlled at a constant level, the volume of medium pumped per hour gives a straightforward measure of both the growth rate and the specific growth rate. Finally, since the mixing time for a fermentor of this type (with an impellor turning at 450 rev./min) and scale (< 1 l) is a second or two (much quicker than typical probes respond, but comparable with the time constant of the dielectric biomass probe), we did not resort to more complex control regimes such as PID (see also, Markx and Kell, 1995).

Samples were removed from the permittistat periodically for the determination of wet weight, dry weight, viability, budding index and ethanol and glucose concentrations as described below. Off-line dielectric scans were also carried out as described below.

3.1. Wet and dry weight measurements

A pre-weighed 25 mm diameter, 0.2 μm pore size, Whatman filter (WCN type, cellulose nitrate, plain white) was wetted with distilled water and placed under gentle vacuum. A sample (typically 5 ml) of the diluted cell suspension was then pipetted onto the filter and sucked 'dry'. The cell pellet was then washed with 1 ml of distilled water and again sucked 'dry'. The filter was then reweighed to give the wet weight of cells per ml. The filters and yeast were then dried using a Sartorius drying machine set at 80°C using the auto-shutoff feature which dries the sample until no further weight change is detected. Drying the yeast samples in this way took about 10 min.

3.2. Microscopy

Viability was estimated by staining the (dead) cells with methylene blue. The methylene blue stain was prepared as described previously (Stoicheva et al., 1989). To ascertain the viability of the sample, the yeast suspension, methylene blue and fresh growth medium were combined in the ratio 1:4:40. The prepared samples were then examined immediately by light microscopy at a magnification of $\times 400$. Cells appearing deep blue were scored as dead while unstained cells and slightly coloured (grey) cells were scored as alive (Davey et al., 1993). At least 100 cells were scored for each sample and the percentage of viable cells was calculated. The percentage of cells with buds was determined by light microscopy by scoring at least 100 cells. The cells examined were either unstained or were the sample stained with methylene blue.

3.3. Determination of ethanol and glucose concentrations

Samples for determination of ethanol concentration were placed into Eppendorf tubes and centrifuged at 13 000 rev./min for 5 min in an Eppendorf-type centrifuge. The supernatants were removed and frozen until the day of assay. Ethanol concentrations were then determined using Sigma Procedure 332-UV. This assay involves alcohol dehydrogenase (ADH) which catalyses the oxidation of alcohol to acetaldehyde with the simultaneous reduction of NAD to NADH, giving an increase in absorbance at 340 nm that is directly proportional to the concentration of alcohol in the sample. The thawed supernatants (diluted where appropriate) were added to the NAD-ADH single assay vials together with glycine buffer (pH 9) and incubated at room temperature for 10 min. The samples were then transferred to plastic cuvettes and their absorbance at 340 nm was measured versus a blank which contained distilled water in place of the supernatant. Samples of known ethanol concentration were measured in the same manner and the concentration of ethanol in the supernatants was determined.

Samples for determination of glucose concentra-

tion were centrifuged and stored as described for the ethanol determination above. Glucose concentration was then determined using Sigma Procedure number 510. This assay is based on the conversion of glucose to gluconic acid by glucose oxidase, a reaction that also produces hydrogen peroxide. The hydrogen peroxide then oxidises colourless *o*-dianisidine to the brown oxidised form. The intensity of the brown coloration after 45 min incubation at room temperature (which is proportional to the glucose concentration in the original sample) is then measured versus a blank at 450 nm. Samples of known glucose concentration were measured in the same manner and the concentration of glucose in the supernatants thereby determined.

3.4. Off-line dielectric measurements

Cell samples removed from the fermentor were placed into a 'Mexican Hat' (Woodward and Kell, 1990) electrode attached to a Biomass Monitor. This type of electrode has been especially constructed to hold a volume of exactly 1 ml. The capacitance of the suspension was measured at 12 frequencies between 0.2 and 9.5 MHz evenly distributed on a logarithmic scale. For each cell suspension scanned, a polarisation control was carried out by adjusting its supernatant to the same conductance at 0.2 MHz as that of the cell suspension by the addition of KCl or distilled water (Davey et al., 1990, 1992).

4. Results

Fig. 3 shows the ΔC of a culture of baker's yeast during aerobic growth in permittistatic culture. The cells were initially grown as a batch culture (i.e. no medium inflow), and then at the point indicated by the arrow, permittistatic control of the biomass level was initiated. It can be seen that the Biomass Monitor provided generally excellent control of the biomass level in the fermentor, in this case, for a period in excess of 2 months. In other fermentor runs, the setpoint was changed periodically across a range of capacitance values, and with the exception of a few cases where too high a setpoint was chosen, the new capacitance setpoint was achieved and well maintained (data not shown).

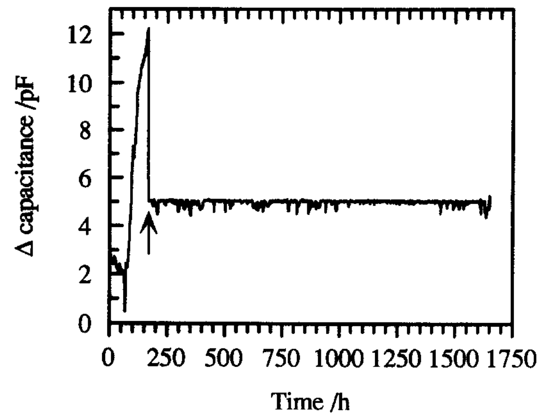


Fig. 3. The delta capacitance of a yeast culture during growth in a permittistat. Initially, yeast were grown in batch and then permittistatic control of the biomass concentration was initiated at the time indicated by the arrow. The ΔC was estimated from dual-frequency measurements as described in the 'Materials and methods' and when it exceeded the setpoint (5 pF) medium was pumped in to dilute the yeast to the correct biomass. The steady-state biomass level was approximately 8 $mg \cdot ml^{-1}$.

Permittistatic cultures of this type allow one to log the rate of medium inflow on a continuous basis and given the excellent control of the biomass concentration offered one might expect the volume of medium pumped into the fermentor per unit time either to be constant during permittistatic culture, or, possibly, to increase monotonically as faster-growing strains (or mutants) take over (Bungay et al., 1981; Fraleigh et al., 1989, 1990). However, as can be seen in Fig. 4, which shows the pump activity during the permittistat run represented in Fig. 3, this is not the case at all, since the pump rate (and hence the growth rate) exhibited dramatic time-dependent changes. Because the overall pattern could be seen by eye to vary rather significantly over the run, we analysed hours 200–600, 600–1000 and 1000–1400 separately. To establish the extent to which the oscillatory behaviour of the growth rate of this culture was in fact periodic the data were Fourier-analysed; the resulting power spectrum of the data for 1000–1400 h from Fig. 4C is shown in Fig. 5. The power spectrum shows that whilst period-

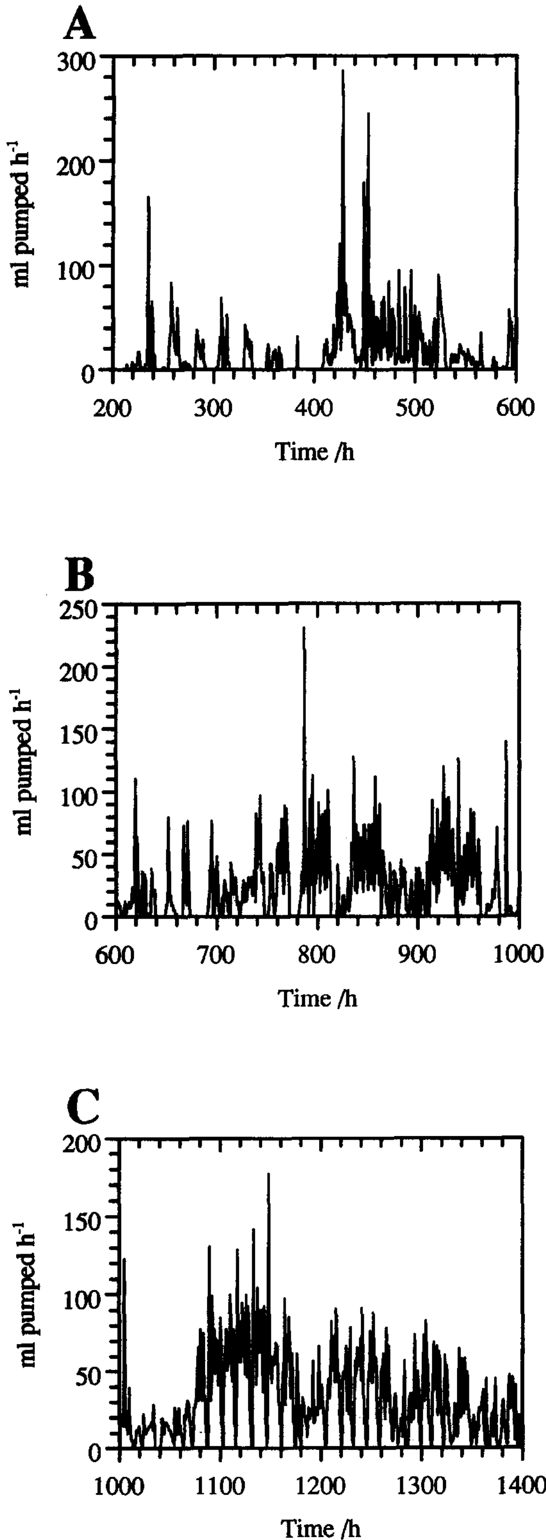


Fig. 4. Time-dependent growth rate changes in a permittistat culture. Measurements were made as described in 'Materials and methods'. Each time the pump was switched on to control the biomass, the length of time the pump was on was recorded to a file. The volume of medium pumped per hour was then calculated and these data points are displayed at hourly intervals. Three 400-h blocks of data are shown: A 200–600 h, B 600–1000 h, C 1000–1400 h. The data presented are for the permittistat run shown in Fig. 3.

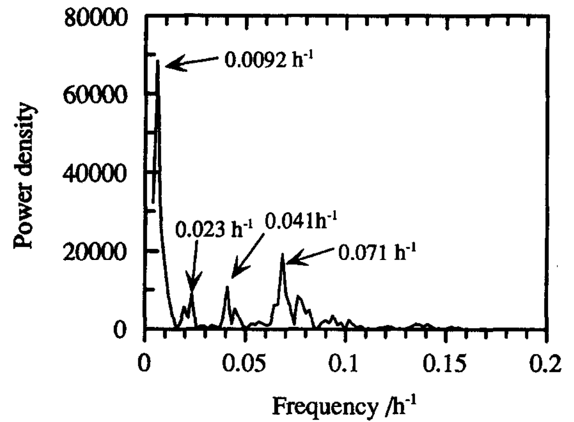


Fig. 5. Power spectrum of the medium-inflow data shown in Fig. 4C. To minimise aliasing effects, the raw data were mean filtered using a five-point moving average and power spectra were obtained via the fast Fourier transform using a minimum 3-term Blackman-Harris window (see Kell, 1987; Woodward and Kell, 1990).

icities are present, they occur at many frequencies, though the data from 1000 to 1400 h contain two major peaks (at frequencies of 0.0092 and 0.071 h^{-1}) representing underlying cycles of 108.7 and 14 h duration. A minor diurnal peak (at 0.041 h^{-1}), and another minor peak at 0.023 h^{-1} or 42.3 h may also be observed. All told, however, it is evident that the data are neither purely periodic nor purely stochastic (white noise), and might be considered to be deterministically chaotic. Indeed, all of the peaks in Fig. 5 except the (narrow) 108 h one are broad, suggesting pseudoperiodicity rather than pure periodicity, as might be expected towards the onset of chaos.

Fig. 6 shows the pump rate for a different permittistat run in which the setpoint was 4 pF. In this case, no oscillations in the growth rate were observed. This important control shows that the growth rate fluctuations are of biological origin,

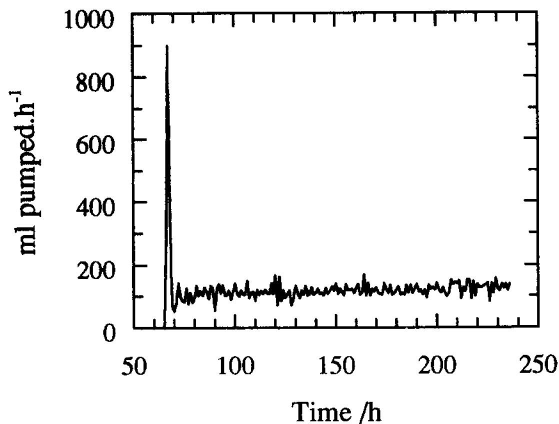


Fig. 6. A permittistat culture exhibiting an essentially uniform pump rate throughout an extended period. Following the initial high pump rate when permittistatic control was initiated the amount of medium inflow required to maintain the setpoint was approximately constant. Measurements were made exactly as described for Fig. 4, save that the setpoint was 4 pF.

and not simply a consequence of instability in the control system used (see also Locher et al., 1993b). The fact that prominent oscillations were observed at some setpoints but not at others, together with the changing growth rate seen in Fig. 4, suggested the possibility that in some circumstances the pump rate (and thus culture growth rate) was indeed deterministically chaotic. The changing pattern of fluctuations within one fermentation and at one setpoint (Figs. 4A–C) also suggest a high-degree of sensitivity to initial conditions, another known characteristic of chaotic processes. Whilst the time series presented in Fig. 4 undoubtedly contains both periodic and stochastic elements it was of great interest to establish whether the time-dependent growth rate changes were also of a deterministically chaotic character, since chaotic growth rates have not previously been observed in any axenic microbial culture. Although at the margin the distinction is perhaps a little arbitrary, many methods have been suggested for distinguishing chaotic data sets from (coloured) noise (e.g. Grassberger and Procaccia, 1983; Farmer and Sidorowich, 1987; Lapedes and Farber, 1987; Casdagli 1989; Sugihara et al., 1990; Sugihara and May, 1990, Kennel and Isabelle, 1992; Provenzale et al., 1992; Stone, 1992; Theiler et al., 1992; Tsonis and Elsner, 1992; Abarbanel et al., 1993; Holton and May 1993; Rapp, 1993; Lloyd and Gravenor, 1994; Skinner, 1994; see also a useful collection of reprints in Ott et al., 1994). However, there is little consensus as to which approaches are the most suitable, particularly when chaotic fluctuations may be present together with an unknown amount of genuine, stochastic behaviour (noise) within a single time series. For this reason, further attempts to analyse the pump data for the whole permittistat run represented in Figs. 3 and 4 were made using a battery of approaches, including calculation of the Hurst (1951; Peters 1991) and Lyapunov exponents (Wolf et al., 1985) and of the embedding dimension (Petigen et al., 1992; Kennel et al., 1992), as well as by using the Sugihara-May (1990) non-linear prediction method. An overview of these findings is provided in Table 1.

Positive first Lyapunov exponents of a non-linear time series, which estimate the mean exponential divergence or convergence of nearby trajectories in phase space, are widely regarded as indicative of the presence of real dynamical chaos (Eckmann and Ruelle, 1985; Brown et al., 1991; Wilson and Rand, 1993). Table 1 shows that as judged by this criterion, with the first Lyapunov exponent determined using the numerical method of Wolf et al. (1985), each of our time series would indeed be construed to be deterministically chaotic. The Hurst exponent (see Hurst, 1951; Peters, 1991) is another measure of the degree of randomness of a discrete time series, purely stochastic processes having a Hurst exponent of 0.5 whilst fully deterministic time series have a Hurst exponent of 1. It may again be observed (Table 1) that our data are characterised by Hurst exponents which are consistent with the view that they are in large measure deterministic.

Another persuasive set of approaches to deciding whether a given data series is deterministic or chaotic (rather than stochastic) are based on the idea that one should be better able to predict deterministically chaotic than stochastic time series. 'Embedded' time series are frequently used with neural networks or other supervised learning algorithms to generate predictions (Weigend and Gershenfeld, 1994). The process of 'embedding' as a means of illuminating the inner dynamics of chaotic time series was devised by Ruelle (1980),

Table 1
Non-linear time series analyses of growth rate data of yeast in a permittistatically controlled continuous culture

Times series data (h)	200–1400	200–600	600–1000	1000–1400
Lyapunov exponent	0.510	0.316	0.147	0.510
Calculated minimum embedding dimension	5	10	8	5
Calculated minimum embedding separation	2	2	1	2
Hurst exponent	0.755	0.680	0.747	0.773
Correlation coefficient predicted vs. actual from Sugihara-May method	0.974	0.959	0.827	0.679
Correlation coefficient predicted vs. actual from 1st-order trivial predictor	0.436	0.800	−0.050	0.214
Correlation coefficient predicted vs. actual from 2nd-order trivial predictor	0.865	0.894	0.901	0.872
Correlation coefficient predicted vs. actual from 6th-order autoregressive model	0.219	—	—	—
RMS error of predictions vs. actual from Sugihara-May method	6.749	8.38	17.65	13.44
RMS error of predictions vs. actual from 1st- and 2nd-order trivial predictors	26.11	17.63	45.67	22.74
RMS error of predictions vs. actual from 6th-order autoregressive model	26.53	—	—	—
Average number of ml added during the prediction period	109.46	21.43	47.77	24.22

Measurements were performed as described in the legend to Fig. 4. Lyapunov exponents were obtained numerically using the method described by Wolf et al. (1985). Hurst exponents were determined as explained in Hurst (1951). The embedding dimension (and separation values) were determined according to the average auto-mutual information method described by Kennel et al. (1992) (see also Kennel 1992; Abarbanel et al., 1992, 1993). The fit to the non-linear predictor was obtained as follows, using a methodology derived from the work of Sughara and May (1990). The embedding dimension and separation values previously obtained were used to generate embedded vectors from the first 80% and last 10% of the relevant time series. A database of these vectors was formed and the exponential interpolation regime described by Sughara and May was used to generate one-step-ahead predictions for the data points in the test set (the points between 80 and 90%) using nearest neighbours determined by Euclidean distance. The 1st-order trivial predictor is that in which the prediction for a given data point is the actual value of the previous data point whilst the 2nd-order trivial predictor is that in which the value of data point $n + 2$ is that for point n plus twice the signed difference between points $n + 1$ and n. Autoregressive models were performed using the TSP software (Quantitative Micro Software, Irvine, CA); that of order six was found to give the best predictions. In the Sugihara-May and trivial predictor methods, the inputs were the differences from the previous time series data points, but the RMS errors relate to the absolute values.

whilst Takens (1981) showed that, given a proper embedding, future values of a time series could be predicted to an arbitrary accuracy with an unstated smooth function. The process of embedding a single time series is as follows: the scalar series is converted to a vector $X_t = x_t, x_{d+t}, x_{2d+t}, x_{3d+t} \ldots x_{(n-1)d+t}$, where d is the separation and n the embedding dimension. A supervised learning algorithm is then trained with pairs of data in which the vector is the input and the next datapoint, x_{nd+t+1}, the output. The question then is to calculate the optimum embedding dimension. We used the approach described by Kennel et al. (1992) (and see Abarbanel et al., 1993) in which, broadly, the separation is found using the so-called auto-mutual information technique, in which two copies of the time series of interest have their mutual information calculated and the separation between the time series is increased by a single step at a time. The optimal separation is

identified as the first minimum on a plot of automutual information vs. offset. The optimal embedding separations and dimensions are given in Table 1. For each block of data (200–600, 600–1000, 1000–1400 and 200–1400 h), the Sugihara-May (1990) algorithm was then used to generate one-step-ahead predictions with a training set of the first 80% and last 10% of each data block, the model so formed being tested on the datapoints between 80 and 90% of the way through the time series (in each case, the updated prediction was based on the previous true data points in the test data series). Fig. 7(A,B) shows the actual and predicted data for the 80–90% segment of the entire dataset, and it may be observed both in Fig. 7 and in Table 1 that the fits are remarkably good, fully consistent with the view that despite their rather stochastic appearance the data are in fact representative of a deterministically chaotic time series.

It is worth mentioning that given a *perfect* measure of embedding, one would expect the dimension of the whole series to be the maximum one of that seen in the subsections (in contrast to what is shown in Table 1). The technique of false nearest neighbours works by finding points in the embedded series that appear to be close in n dimensions but are not close when a further dimension is added. An approximate measure of the radius of the attractor is formed and the definition of 'close'

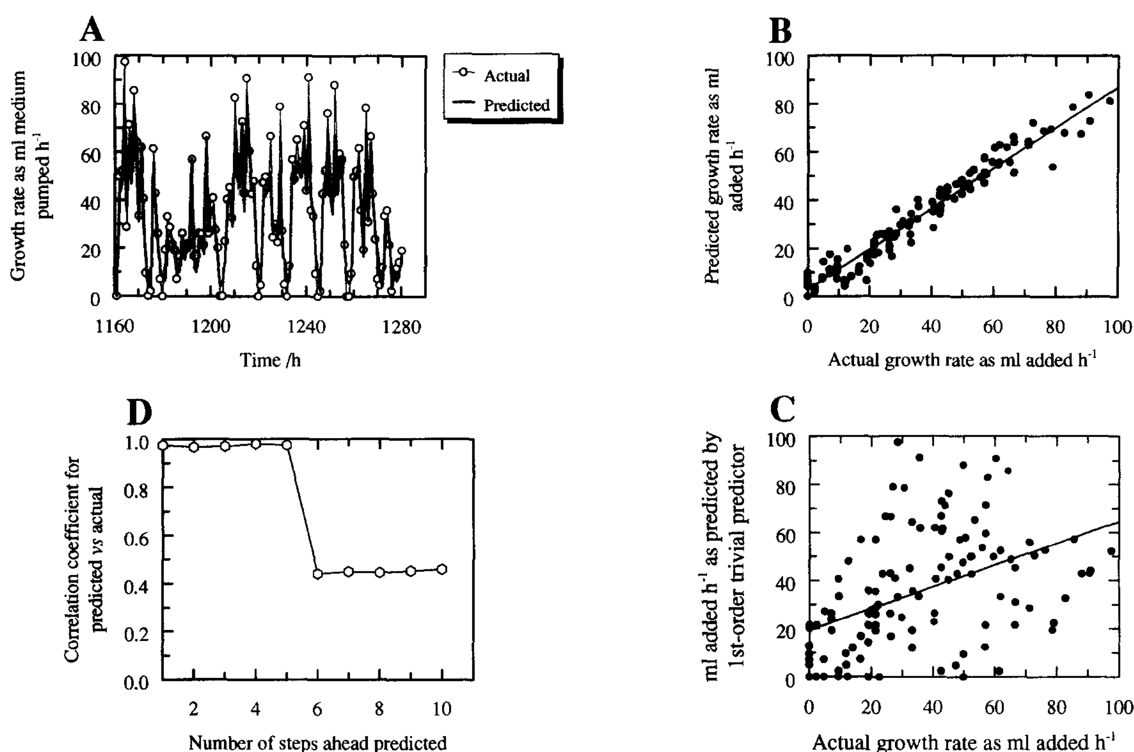


Fig. 7. Non-linear time series analyses of growth rate data of yeast in a permittistatically controlled continuous culture. Growth rate measurements were performed as described in the legend to Fig. 4. Non-linear predictions were carried out as described in the legend to Table 1. (A) Estimated volume (one-step-ahead predictions) of culture broth added per hour and actual volumes added vs. time for the non-linear predictor. (B) Estimated volume of culture broth added per hour vs. actual volumes added for the non-linear predictor. The line shown is the best linear fit and has a slope of 0.84 and an intercept of 2.84. (C) Estimated volume of culture broth added per hour vs. actual volumes added for the first-order trivial predictor. The line shown is the best linear fit and has a slope of 0.45 and an intercept of 19.3. (D) The effect of n on the correlation coefficient between estimated and actual data for n-step-ahead predictions of the same data, according to the Sugihara-May non-linear prediction method.

is scaled accordingly. We then decide that we have sufficiently embedded the series when the percentage of false neighbours falls below a certain value. If the sections of series with higher dimension had some particularly 'knotty' portions, the algorithm will increase the dimension until they are resolved; if the attractor is more dense in those sections the definition of 'close' would be more stringent and more false neighbours would be generated. When the whole series is considered, however, these knotty sections will be swamped, and a lower dimension estimate results.

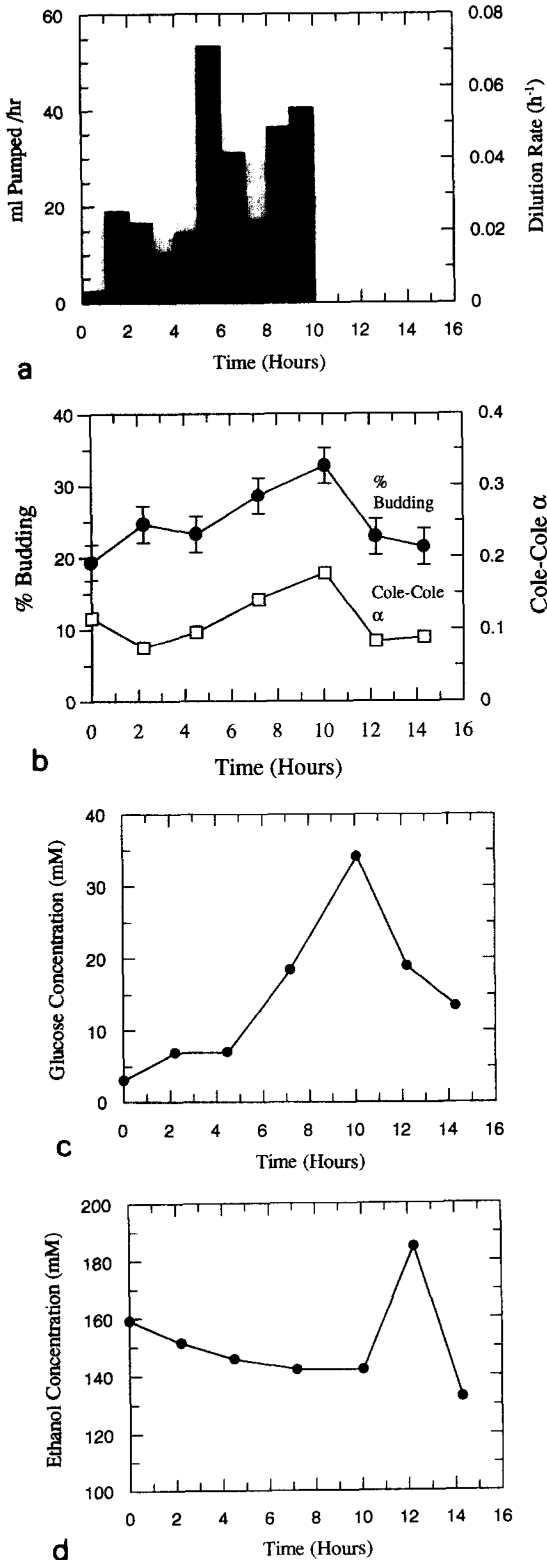
Since it might be argued that a 'trivial predictor' (in which the one-step-ahead prediction for time $t + 1$ is simply the value at time t) would also give good predictions, Table 1 therefore additionally shows the correlation coefficients and RMS errors determined for both the 1st- and 2nd-order trivial predictor, where the 1st-order trivial predictor is that in which the prediction for a given data point is the actual value of the previous data point, whilst the 2nd-order trivial predictor is that in which the value of data point $n + 2$ is taken as that for point n plus twice the signed difference between points $n + 1$ and n. Fig. 7C shows the 1st-order trivial predictor for the same data as in Fig. 7B. It may be observed from this and from Table 1 that the Sugihara-May predictions, and especially the RMS errors in all cases, are substantially better than those generated by any of the trivial predictors.

Further, to check that our data were indeed chaotic, we also studied pure autoregressive models as a means of fitting our time series. That of order six gave the best predictions, but the correlation coefficient was very poor and the RMS error of predictions no better than that of the trivial predictors. Application of the Box-Pierce statistic (Box and Pierce, 1970) showed that there were no significant autocorrelations in the residuals from the AR [6] model (data not shown), indicating that a linear model was incapable of giving adequate predictions for this time series.

Finally, further to bolster the view that we are indeed studying a deterministically chaotic process, we used the argument of Sugihara and May (1990) that the n-step-ahead prediction in a deterministically chaotic system is likely to get worse as n increases, but that for a purely stochastic system (white noise) it is likely to be essentially independent of n. Fig. 7D shows that the correlation coefficient drops dramatically as n increases above a value of 5. (Although the exact structure of the chaos obviously differed from run to run, it is worth mentioning that visually observable chaos was seen in seven independent start-ups of a culture.)

Given what is known about the oscillatory behaviour of metabolic fluxes in yeast growing respiro-fermentatively (see above), it was assumed that our fluctuations were also related to the major respiro-fermentative pathways of metabolism. A plausible scenario suggests that glycolytic ethanologenesis leads to a build-up of toxic ethanol and/or acetaldehyde, due to the limited respiratory capacity of *S. cerevisiae* (Rieger et al., 1983; Käppeli, 1986; Sonneitner and Käppeli, 1986; Alexander and Jeffries, 1990; Locher et al., 1993a), which then inhibit glycolysis and growth (Ingram and Buttke, 1985; Jones and Greenfield, 1986; van Uden, 1989; Jones, 1990; Wills, 1990; Markx et al., 1991b; Bruce et al., 1991; Alexandre et al., 1993). After a certain period, a combination of respiration, yeast acclimation, dilution (when medium begins to be added) and (possibly) evaporation removes the toxicity, and glycolysis and growth can then resume, producing more ethanol/acetaldehyde, and so on. Each of these metabolic processes is of course known to be non-linear. Consistent with this overall picture are the observations that neither *S. cerevisiae* growing anaerobically (Markx et al., 1991a), nor the Crabtree-negative yeast *Kluyveromyces marxianus* growing aerobically (unpublished observations; data not shown), could be induced to produce any marked oscillations in growth rate. In an attempt to gain an understanding of the processes underlying the shorter of the two oscillations seen in Fig. 4C, samples were removed from the permittistat approximately every 2 h for a 15-h time period. The pump activity during that 15-h period of the study is shown in Fig. 8A, and may be summarised as a 10-h period of medium input followed by a 5-h period during which no input of medium occurred.

Fig. 8B shows the percentage of budding cells present in the permittistat during this time. It can



be seen that the percentage of cells with buds increases during the part of the oscillation in which medium input is occurring and decreases towards the initial level when medium input ceases. Also shown in Fig. 8B are the data for the Cole-Cole α. The Cole-Cole α was calculated from the off-line dielectric scans of the yeast suspension (see Markx et al., 1991b) by fitting the data to the Cole-Cole equation (Cole and Cole, 1941):

$$\epsilon'_{\omega} = \frac{\Delta\epsilon'\left[1 + \left(\frac{f}{f_c}\right)^{1-\alpha}\sin(0.5\alpha\pi)\right]}{1 + 2\left(\frac{f}{f_c}\right)^{1-\alpha}\sin(0.5\alpha\pi) + \left(\frac{f}{f_c}\right)^{2-2\alpha}} + \epsilon'_{\infty} \quad (1)$$

where ϵ'_{ψ} is the permittivity of the suspension at a given frequency, ϵ'_{∞} is the permittivity at a high frequency with respect to the β-dispersion, $\Delta\epsilon'$ is the dielectric increment of the β-dispersion, f is the frequency in Hertz, f_c is the critical frequency of the β-dispersion (see Fig. 1), and α is the Cole-Cole α. GraFit v 2.0 (Erithacus Software Ltd., PO. Box 35, Staines UK) was used to fit the equation to each data set following the subtraction of its

Fig. 8. Short term changes in a permittistatic culture. (A) The flow rate required to maintain the biomass at the setpoint was measured as described in the legend to Fig. 4. For a 10-h period, a variable amount of medium input was required to maintain the setpoint, but over the next 5 h the biomass in the permittistat never exceeded the setpoint and so no medium input occurred. (B) Changes in the percentage of budding cells during the oscillation were reflected in the Cole-Cole α of the yeast suspension. Off-line scans and curve fitting for calculation of the Cole-Cole α were carried out as described in the text. An increase in both the budding index and the Cole-Cole α was seen during the phase of the oscillation where medium input was occurring and when medium input ceased the magnitude of both decreased. (C) Changes in glucose concentration during the oscillation. Glucose concentration was measured as described in the Methods. During the period where medium inflow is occurring the glucose concentration steadily increases, before beginning to fall again when medium input ceases. (D) Changes in ethanol concentration during the oscillation. Ethanol concentration was measured as described in the Methods section. During the period of medium inflow the ethanol concentration remained quite stable; however, when medium input ceased, a transient increase in ethanol concentration was seen.

polarisation control (Davey et al., 1992; Kell and Davey, 1992). Fig. 8B shows that changes in the percentage of budding cells appears to be reflected in the curve for the Cole-Cole α; although the mechanisms underlying the large Cole-Cole α values of biological systems are not well understood (Markx et al., 1991b; Davey and Kell, 1994), they may indeed in part reflect morphological polydispersity.

Fig. 8C shows the changes in glucose concentration occurring in the permittistat during the fluctuation. By comparison of this figure with Fig. 8A it can be clearly seen that during the phase of the oscillation where medium is being pumped in to the fermentor the glucose concentration increases, before falling back towards the base level in the period when there is no pump activity. Fig. 8D shows the changes in ethanol concentration over the same time period. In contrast to the glucose concentration, the ethanol concentration falls slightly as medium is pumped into the fermentor, and rises when medium input ceases. However *before* medium input restarts the ethanol concentration falls back to the initial level. Given the relatively low budding index, which shows that the population was highly polydisperse with respect to its growth (Kell et al., 1991), it is likely that only a fraction of the population switches to growth on (or metabolism of) ethanol as the carbon source when the glucose concentration falls.

5. Discussion

The measurement of biomass on-line and in real-time in fermentors is a necessity if turbidostatically controlled continuous cultures are to be used, and the development of sensing devices capable of this task has long been an area of interest (Harris and Kell, 1985; Clarke et al., 1986; Kell et al., 1990, Sonnleitner et al., 1992). An ideal sensing device for measuring the biomass in a fermentor should be sensitive to changes in biomass, but be insensitive to both non-biomass solids and *necro*mass. In addition the signal should be linear with biomass to high volume fractions. The dielectric system that we have described fulfils these criteria.

Much of microbial physiology is based on measurements made at 'steady state' in continuous cultures, yet the oscillatory behaviour of microbes has been reported in continuous cultures by several groups (see, e.g. Degn and Harrison, 1969; Harrison, 1970; Cunningham and Nisbet, 1983; Satroutdinov et al., 1992; Ölz et al., 1993). Steady state conditions are normally assumed to have been reached once a rather arbitrary five volume changes have elapsed following a change in dilution rate (but cf. Rutgers et al., 1987). In the permittistat cultures described, however, substantial fluctuations in growth rate may be apparent even after 50 volume changes have elapsed, though one may state that the Biomass Monitor has proved to be an effective tool not only for controlling the biomass level in continuous cultures but also for the induction of fluctuations in growth rate under these conditions. It is therefore desirable that one should be able properly to identify and characterise oscillatory and chaotic behaviour in continuous culture systems.

Whilst accepting that the presence of noise will tend to distort or obscure the characteristic patterns which are used in the identification of periodic and chaotic data sets, we applied a battery of analyses to the growth rate data observed. These included calculation of the Hurst and Lyapunov exponents, and of the embedding dimension, as well as by using the Sugihara-May (1990) non-linear prediction method. In each case, it was demonstrated that the time series contained periodic, deterministically chaotic and stochastic elements. Although with the hourly measurements of growth rate described herein, several months of experimentation yield a few thousand data points, we recognise that although this number is much greater than those usually available in ecological time series, this is considered by some to be a rather small number for the reliable identification of chaotic behaviour (Ruelle, 1990). One possible approach to this problem is modelling of an appropriately parameterised and simplified version of the system in order to obtain virtually unlimited noise-free time series; while this has been done for cell-free extracts of yeast (Markus et al., 1985), in the present case we cannot yet define a subsystem

of appropriate simplicity and accuracy. However, the low dimensionality of the phase space embedding that we have used to generate successful predictions (Table 1) suggests that the dimensionality of the biochemical processes generating the chaotic behaviour is also very low.

The fact that the origins of the chaotic fluctuations lie in the biology and not in the dynamics of the pump system, etc., comes from three main lines of argument: (a) with the same organism and set-point but under *anaerobic* conditions there is no chaos (Markx and Kell 1991); (b) with the same organism under aerobic conditions but at a slightly different set-point there is no chaos; and (c) with a non-Crabtree yeast, *Kluyveromyces marxianus*, there is no chaos observed aerobically at any set-point tested. In each of these cases, the pump system, etc. was the same as in the case where chaos was observed. These controls, which were explicitly designed exactly to ensure that we were indeed observing chaos of biological origin, would seem to make it hard to argue that the chaos has an abiological origin under these circumstances.

Offline measurements of the macroscopic behaviour of the yeast culture have suggested a metabolic origin for the oscillations with a switch by some fraction of the population from growth on glucose to growth on ethanol (or indeed to mixotrophy). Oscillations in the concentration of NAD(P)H have been reported to be much more prolonged in yeast cells harvested from batch cultures at the point of switching from growth on glucose to growth on ethanol when compared to cells growing on either carbon source alone (Richard et al., 1993).

While macroscopic measurements provide us with some insight to the observed oscillatory behaviour of the yeast cells, it is likely that the behaviour is in part linked to the heterogeneity of the population. Measurement of the properties of individual cells using techniques such as flow cytometry (see Kell et al., 1991; Lloyd, 1993; Shapiro, 1994) can identify and quantify the heterogeneity present within nominally steady state cultures. One of the advantages offered by flow cytometry is that measurements can be made simultaneously on several different parameters of the cell. Recently, flow cytometry has been used to determine the size, DNA content and the number of bud scars of individual cells in batch and continuous cultures of yeast (Münch et al., 1992a,b), and such techniques will be of importance in determining to what extent differences between cells can be accounted for by their position within the cell cycle.

In conclusion, however, the ability to effect the permittistatic control of yeast cell cultures has allowed us for the first time both to induce and to demonstrate the periodic, stochastic and, most interestingly, the deterministically chaotic behaviour of the macroscopic growth rate of microbial cell cultures. Although chaotic behaviour has been widely reported at the metabolic, physiological and ecological levels in biological systems, we believe this to be the first demonstration of deterministically chaotic growth rates in an axenic biological system. Finally, a corollary of the identification of deterministically chaotic behaviour in the growth rate of biological cells is that novel methods for improving the control of such growth rates may become available, both for raising and lowering them, since a number of authors (e.g. Ditto et al., 1990; Ott et al., 1990; Garfinkel et al., 1992; Schiff et al., 1994, Skinner, 1994, and see Ott et al., 1994) have indeed shown how a knowledge of the non-linear dynamics of such low-dimensionally chaotic systems may be exploited to gain a very effective control of them.

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