Real-time monitoring of the accretion of *Rhizopus oligosporus* biomass during the solid-substrate tempe fermentation

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We describe a novel method for the real-time estimation of the accretion of biomass during the solid-substrate tempe fermentation of soy beans, lupins and quinoa by *Rhizopus oligosporus* Saito. The method is based on measurements of the dielectric permittivity at radio-frequencies, using a four-terminal instrument (the Bugmeter). In all cases, excellent linearity is observed during the growth phase between the dielectric permittivity and the hyphal length as determined microscopically.

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Solid-substrate fermentation processes using moulds are traditionally exploited in the manufacture of a wide variety of oriental foods, including miso, sufu, shoyu, tempe and others. According to Mudget (1986) solid substrates are characterized as gas–liquid–solid mixtures, in which the tiny aqueous phase is intimately associated with both the solid surface and the gaseous environment. Mycelial growth takes place at the interface between the solid and aqueous phases. In solid-substrate fermentations many parameters and variables may have considerable effects on the aqueous phase and therefore on the fungal physiology, primarily the rate of mycelial development on the solid particles. As the mycelia are firmly attached to the substrate (Jurus & Sundberg 1976), there is no practical way to separate fungal biomass from the residual substrate solids. Whilst the real-time estimation of microbial biomass during biotechnological processes remains a thorny problem generally (Harris & Kell 1985; Blake-Coleman *et al.* 1986; Clarke *et al.* 1986; Clarke 1987; Kell *et al.* 1990) this feature in particular has been the major hindrance to the measurement of biomass accretion during solid-substrate fermentations. Although a knowledge of mycelium concentration is essential in planning the koji process for example (Arima & Uozumi 1967), quantitative process optimization and scaling-up have been limited.

Tempe, a typical example of a solid-substrate fermentation, is traditionally a soya bean product fermented by *Rhizopus oligosporus* (Hesseltine *et al.* 1963; Ko & Hesseltine 1979; Steinkraus 1983; Nout & Rombouts 1990). In the vegetarian market, tempe is a substitute for meat. Therefore it is commercially produced not only in Indonesia, where it originated, but also in the USA, Canada and the Netherlands (Liem *et al.* 1977; Samson *et al.* 1987).

Wang and Hesseltine (1966) and Hesseltine *et al.* (1967) extended the tempe concept to include the fermentation by *R. oligosporus* of different cereals, including wheat, barley, oats, rye and corn. Tempe has also been made using garbanzos (chick peas) and broad beans (Robinson & Kao 1977), sweet lupin (Kidby *et al.* 1977), bitter lupin (Chavez & Peñaloza 1988), quinoa (Robalino & Peñaloza, 1988) and mixtures of beans and cereals (Blakeman *et al.* 1988).

The quantitative analysis of moulds in foods and feedstuffs has been a serious

problem (Jarvis *et al.* 1983). The determination of glucosamine and ergosterol have been suggested as means of measuring fungal invasion in grains and processed foods (Seitz *et al.* 1977, 1979; Cousin *et al.* 1984). Nevertheless, in common with all chemical approaches (Harris & Kell 1985), such fungal-specific constituents do not correspond strictly to mycelial biomass but vary considerably with the age of the culture (Arima & Uozumi 1967; Cousin *et al.* 1984). Recently, a mechanical device that measures the units of force necessary to pull a pin across the solid substrate, as an indirect assessment of biomass production during the tempe fermentation, has been described by Blakeman *et al.* (1988).

Thus, the direct measurement of microbial biomass, on-line and in real time, has been a major problem in liquid-substrate fermentation, and virtually impossible in those employing solid substrates. In the former case, we have shown that it is possible to measure biomass accumulation by exploiting the radio frequency dielectric properties of cellular suspensions (Harris *et al.* 1987). The purpose of the present article is to describe how we have been able to exploit this same approach for measuring the accretion of fungal biomass during solid-substrate fermentations, using tempe as a model system. An accompanying article (Peñaloza *et al.* 1991) describes the exploitation of the present approach in the demonstration of an unusual potassium requirement by R. *oligosporus* during the fermentation of debittered lupins.

Theoretical Background

The non-faradaic or 'passive' electrical properties of a system, such as a cell suspension, may be completely characterized by its macroscopic capacitance (in Farads) and conductance (in Siemens). These depend in part upon the size and geometry of the electrodes, and reflect, respectively, the system's intrinsic properties: permittivity (ability to store electrical energy) and conductivity (ability to dissipate it) (see Kell 1987; Pethig & Kell 1987). Conductivity has the units Siemens/m, whilst permittivity is dimensionless. For plane-parallel electrodes of area A separated by a distance d, the relationship between the conductivity σ' and conductance G is $\sigma' = G(d/A)$, where (d/A) is known as the cell constant and has units of length^{-1}. The capacitance C is similarly related to the permittivity ε' by $\varepsilon' = C(d/A\varepsilon_0)$, where ε_0 is an experimental constant equal to $8.854.10^{-12}$F/m, such that a cubic electrochemical cell of unit dimensions containing water [which has a permittivity ('dielectric constant') of 78.4 at 298 K] has a capacitance of some 6.94 pF.

The passive, non-faradaic electrical or 'dielectric' properties of cellular suspensions themselves (as opposed to those of the suspending medium or the electrodes) are generally characterized by three major areas of frequency dependence or 'dispersion', known (in order of increasing frequency) as the α-, β- and γ-dispersions (Pethig & Kell 1987). The β-dispersion, centred in the radio-frequency region of the electromagnetic spectrum, is caused by the charging of the rather large membrane capacitance C_m displayed by all intact cells (Kell & Harris 1985). This is typically of the order 1 μF cm^{-2}, and is due to the possession by cells (and by nothing else likely to be found in a fermentation system) of non-micellar phospholipid membranes of molecular thickness. For spherical cells of radius r, present at a volume fraction P, the permittivity at *low* radio frequencies exceeds that of the background by a value given to a close approximation by $9PrC_\mathrm{m}/4\varepsilon_0$. For non-spherical cells the factor 9/4 is different. For extremely high volume fractions, there is a slight nonlinearity between the permittivity (or, more strictly, the dielectric increment $\Delta\varepsilon = \varepsilon_\mathrm{low} - \varepsilon_\mathrm{high}$) and the volume fraction (Harris & Kell 1983). As will be seen below, however, this effect was not significant, probably due in part to the changes in conductivity that were observed during the fermentation. Thus, by measuring the dielectric permittivity of cell suspensions

at low radio frequencies, it is possible to design a biomass probe that is specific for viable cells (Harris *et al.* 1987; Kell & Todd 1989) (since necromass, particles, emulsions and gas bubbles do *not* have intact bilayer-type cell membranes).

Based on these principles, we have developed a biomass probe (the Bugmeter) suitable for the real-time estimation of biomass in fermenters *in situ* (Harris *et al.* 1987; Kell & Todd 1989; Kell *et al.* 1990). The (steam-sterilizable) probe consists of four gold electrodes in an insulating matrix suitable for insertion in a standard 25 mm port. The outer two electrodes apply alternating current of a suitable frequency in the range 0.1 to 10 MHz whilst the inner two pick up the alternating potential difference, an arrangement that more-or-less completely avoids artefacts due to electrode polarization phenomena (Kell 1987; Kell & Davey 1990). Biofouling is obviated by the manual or automatic application of electrolytic cleaning pulses. A similar system *without* cleaning pulses may be used to assess the extent of such biofouling (Markx & Kell 1990). The output of this device may be chosen in terms of absolute capacitance, capacitance *minus* that upon inoculation, or (via a previously determined calibration) mg/ml. An output of the conductance of the broth is also provided. Previous work has shown that the Bugmeter is suitable for use in a variety of situations, including pitching control in breweries (Boulton *et al.* 1988), plant cells (unpublished data), animal cells (Davey *et al.* 1988), immobilized cells (Salter *et al.* 1990) and (since it measures biomass) in assessing cytotoxicity (Stoicheva *et al.* 1989). Thus, particularly since the method registers biomass, i.e. those cells with intact plasma or cytoplasmic membranes, it was of great interest to seek to apply the method to a solid-substrate fermentation.

Materials and Methods

Soya beans were prepared for tempe production using the basic laboratory methods described elsewhere (Ko & Hesseltine 1979; Steinkraus 1983). Briefly, beans were soaked overnight, dehulled, cooked in boiling water (100°C) for 45 min, cooled, superficially dried in a drying cabinet at 40°C to remove the excess of water from the surface of the beans, and chopped in a liquidizer to a particle size of 3 to 4 mm.

The Andean bitter lupins (*Lupinus mutabilis* Sweet) were soaked overnight, cooked in excess water at 100°C for 45 min, debittered in running tap water for 1 week to remove the alkaloids and their bitter taste, dehulled, cooked again at 100°C for 15 min, and chopped as above.

Quinoa seeds (*Chenopodium quinoa* Willd) were scarified in a modified rice milling machine to remove saponins, cooked in a 6-fold volumetric excess of boiling water (100°C) for 12 min, cooled and surface-dried until an overall moisture content of 60 to 65% was obtained. Lupins and quinoa seeds were kindly supplied by Latinreco SA, Quito.

Inoculation

A freeze-dried starter culture of *R. oligosporus* NRRL 2710, containing 3×10^7 c.f.u./g was added at 1% (w/w) of the wet substrate and mixed thoroughly for 3 min. The inoculated substrates were packed into sterile plastic petri plates (diameter 87 mm, depth 12.5 mm), each plate containing approximately 42 g.

Fermentation

The Bugmeter probe ($d = 25$ mm) was carefully introduced through a central perforation in the lid of the petri dish into the substrate, in such a way that the four gold pins and probe body were 3 to 7 mm into the substrate. It was necessary to place the probe near the centre of these small petri plates since a reduction in the capacitance was seen when the probe was placed towards the edges, due to edge effects. Under these circumstances, the cell constant, as measured using

10 mM KCl, was 0.65 cm^{-1}. A pH electrode and a semiconductor sensor for temperature were inserted into an adjacent plate. Silicone rubber was used to make a moisture-proof seal between probes and the lids of the petri plates. Probes and plates were clamped and placed in an incubator at 31°C and a relative humidity of 70 to 85%. Probes were connected as appropriate to a digital pH meter, a Bugmeter (Aber Instruments Ltd, Science Park, Cefn Llan, Aberystwyth, Dyfed SY23 3DA, UK), and thence to a Blackstar 2308 analog-to-digital interface. Data were logged into an Opus PC-II (XT-compatible) microcomputer. At the start of incubation, the frequency of operation of the Bugmeter was set at 0.30 MHz, the capacitance (pF) backed off to zero and the increments or delta capacitance logged every 30 min together with the conductance (mS), pH, tempe temperature and incubator temperature. A diagram of the monitoring system is given in Figure 1. At the end of the incubation, data were recovered and analysed using a spreadsheet method (Davey *et al.* 1990). Although tempe requires only a 24 h incubation, the course of the fermentation was followed for up to 2 to 5 days. Samples were removed periodically from replicate plates for off-line analysis of pH and moisture content. Hyphal length and dry weight losses were also determined.

For hyphal length measurements, agar films were prepared by a modification of the Jones & Mollison (1948) technique. Briefly, 5 g of fresh tempe was homogenized for 60 s with 45 ml sterile distilled water using a Polytron top
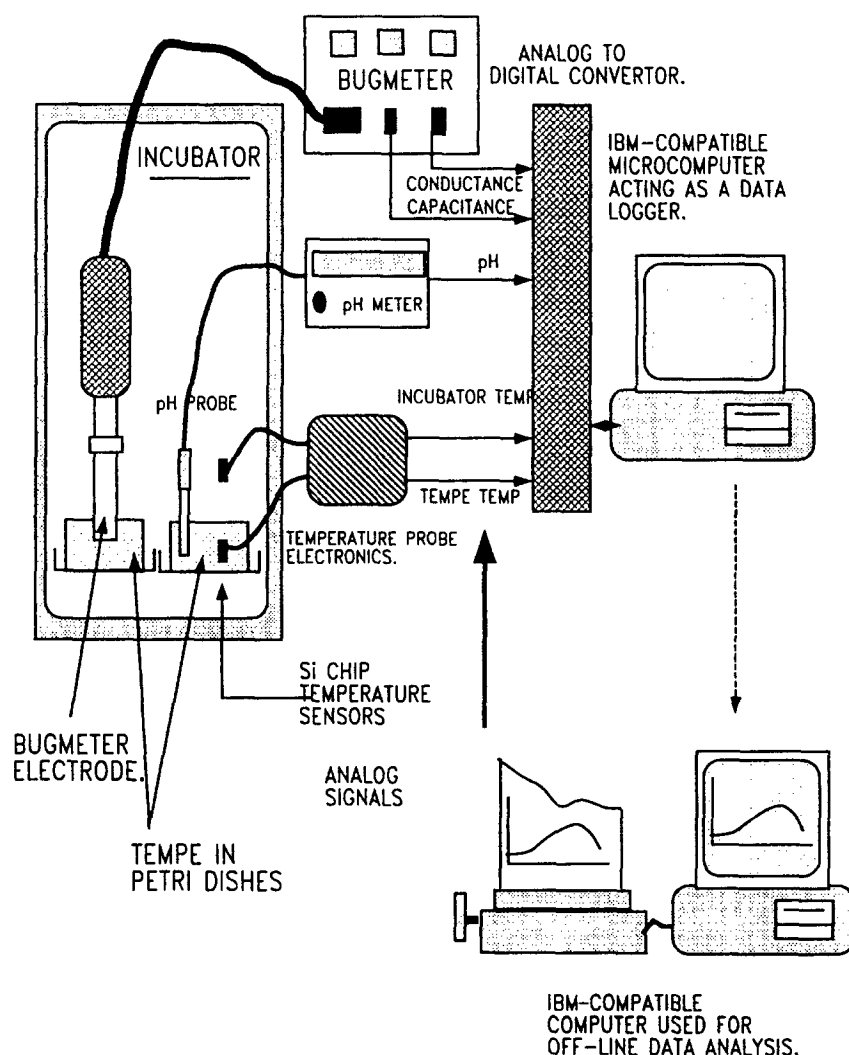

Figure 1. Diagram of the computerized arrangement used for the on-line and real-time monitoring of the capacitance and conductance, pH and incubator and tempe temperatures during the solid-state fermentation at 31°C of soya bean, lupin or quinoa to produce tempe.

homogenizer (Kinematica, CH-6010 Kriens-Lu, Switzerland) at position 4.5. A second homogenate was prepared from the first one. Then 0.5 ml from the second homogenate was mixed with 1 ml of 3% (v/v) molten water agar at 60°C and 'whirlimixed' for 10 s. Mod-Fuchs Rosental chambers of depth 0.2 mm were used for the preparation of agar films. They were recovered, mounted and stained with phenol aniline blue for 30 min. Finally the measurements of hyphal length were performed with an image analysis system, consisting of a drawing tube attached to a Jenaval microscope (Carl Zeiss, Jena, Germany) at 250× magnification, a Summagraphics Bit Pad 2 digitizer tablet, BBC microcomputer and Digit software (Brian Hayes & Fred Fitze, 1987, Institute of Ophthalmology, Judd St, London WC1H 9QS, UK). The length of all hyphal bits contained in 20 random fields per agar film, two agar films per sample, were determined and expressed in km/g of dried tempe.

Results

Tempe prepared from soya beans, debittered lupins and quinoa was consistently engulfed and bound firmly together by the mycelium. After 24 h of incubation sporulation became evident as a thin black ring, which grew thicker as the incubation time increased. Quinoa tempe showed a later and lower sporulation than did beans.

The time course of the fermentation of soya beans and lupins is essentially the same (Figures 2, 3), whilst that of quinoa was somewhat different (Figure 4). The fermentation of soya beans and lupins for tempe can roughly be divided into two major phases: first a growing phase, taking place during the first two days of incubation at 31°C and in which capacitance, as an on-line measurement of biomass, increases monotonically (and, for a long period, more or less linearly), and second a non-growing or lytic phase, characterized by a continuous decrease of capacitance with time (Figure 3). The (extracellular) conductance measured at
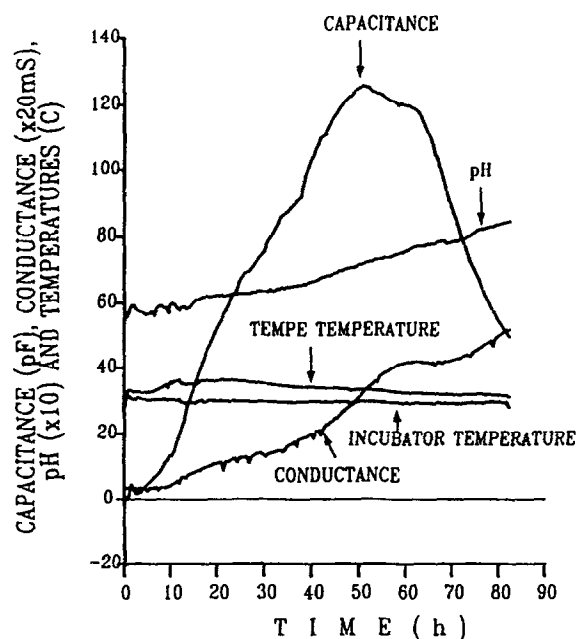

Figure 2. Time course of the capacitance, conductance, pH and temperature of soya beans, and the incubator temperature, during the tempe fermentation. Experiments were carried out at 31°C using *R. oligosporus* NRRL 2710, as described in the Materials and Methods section, using the arrangement illustrated in Figure 1.
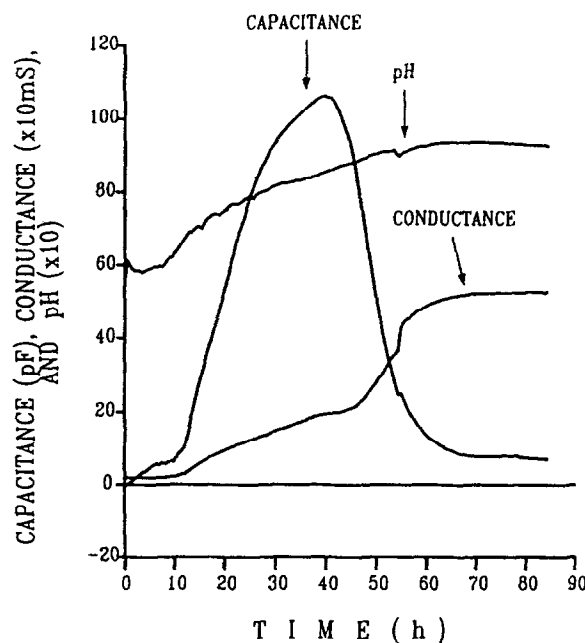
Figure 3. Time course of the capacitance, conductance, and pH of lupins incubated at 31°C and at a relative humidity of 75 to 90%. Other experimental conditions as described for Figures 1 and 2.

0.3 MHz increased slightly during the growing phase and sharply in the lytic phase, when cytoplasmic material is released from the burst hyphae. The pH of soya bean and lupin tempe increased from 5.5 to 6.9 in about 24 h, and up to 8.5 after 3 days of incubation (when the release of ammonia was evidenced by its smell).

The time course of the quinoa fermentation was quite different. Lower increments of capacitance and conductance were observed; nevertheless the maximum capacitance attained (and thus the cellular viability) remained almost constant for a longer period of time than for the bean fermentation. A slight fall of capacitance in quinoa fermentation was noticed only after some 60 h of incubation. The time course of the pH of the quinoa fermentation was characterized by three phases. At first, the pH dropped sharply during the first 8 to 12 h after inoculation, followed by an increase of pH between 25 and 50 h. Finally, the pH decreased again (Figure 4).

The results of off-line analyses for moisture, dry weight loss and hyphal length for a soya bean fermentation are shown in Figure 5. Moisture and dry weight losses increase more or less linearly with the incubation time, as reported elsewhere (Smith *et al.* 1964; Hesseltine 1965; Steinkraus 1983; Blakeman *et al.* 1988). Hyphal length increases sharply during the growing phase and very slowly afterwards. Similar patterns are shown for both lupin and quinoa fermentations in Figures 6 and 7.

The on-line and off-line data for the different solid substrates after 24 h of incubation are summarized in Table 1, where it may be observed that there is an excellent linear relationship between the capacitance change in pF and the hyphal length in km/g of dry tempe. During the growing phase, individual correlation coefficients (r^2) are above 0.92, although they fall dramatically if data from the lytic phase (more than 48 h of incubation) are also included.

Discussion

Tempe can be made from different legumes and cereals (e.g. Steinkraus *et al.* 1960; Hesseltine *et al.* 1963; Martinelli & Hesseltine 1964; Wang & Hesseltine 1966; Hesseltine *et al.* 1967; Kidby *et al.* 1977; Robinson & Kao 1977; Steinkraus 1983; Gandjar 1986). The versatile enzymatic systems of R. *oligosporus* can metabolize several types of substrate constituents (Wagenknecht *et al.* 1961; Hesseltine *et al.* 1963; Wang & Hesseltine 1965; Sorenson & Hesseltine 1966; Van Buren *et al.* 1972; Wang *et al.* 1972; Ellis *et al.* 1974). Thus the major chemical constituents of the substrate have a definite effect on the pattern of changes occurring during tempe fermentation (Steinkraus *et al.* 1960; Wang & Hesseltine 1966; Blakeman *et al.* 1988). Soya beans and lupins are rich in fat and proteins (Murata *et al.* 1967; Hill 1977; Williams 1989). Moreover, the effects on the beans of preparation is such that the growing mycelium has immediate access to nutrients in the cotyledons. Therefore, during the fermentation the increase of pH, hyphal length and biomass (measured as capacitance) are higher in the beans than in the quinoa.

Quinoa is a 'pseudocereal', which has a very high carbohydrate content and relatively little protein and fat (Risi & Galwey 1984; Koziol 1990). During the fermentation of quinoa the pH drops to 4.5 or lower due to the enzymatic conversion of starch into sugars and organic acids (Wang & Hesseltine 1966; Blakeman *et al.* 1988). A low pH could be a very interesting advantage in industrializing the quinoa tempe fermentation. Firstly, it is an unfavourable environment for spoilage bacteria that have been found in commercial soya bean tempe (Tanaka *et al.* 1985; Nout *et al.* 1985, 1987; Samson *et al.* 1987). Secondly, the heat treatment required to kill the potential contaminants of fresh tempe is lower at pH 4.5 than at pH 6.5 to 7.0. The increase of hyphal length is obviously
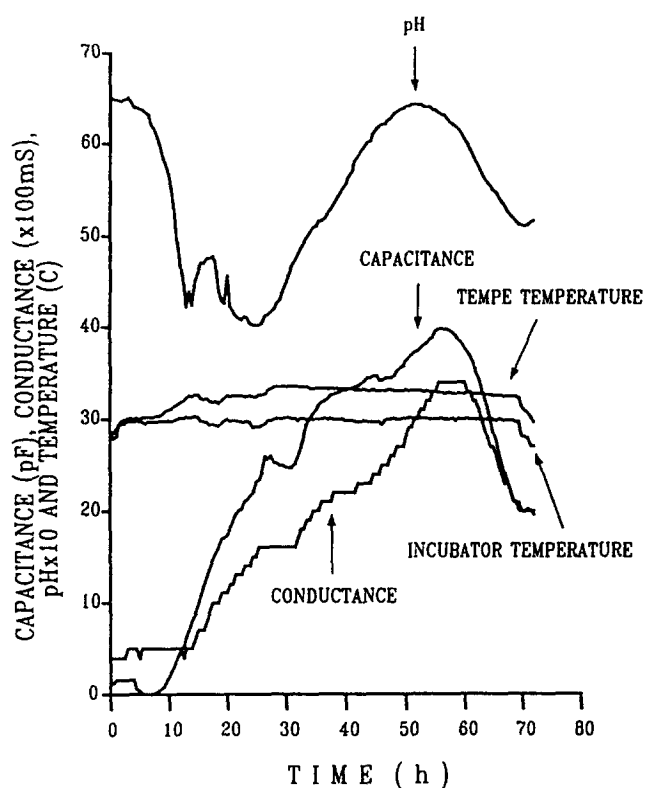
Figure 4. Time course of the capacitance, conductances pH and temperature of quinoa, as well as incubator temperature, when quinoa inoculated with *R. oligosporus* was incubated at 31°C and 75 to 90% relative humidity for tempe. Experiments were performed as described in the legend to Figure 2.
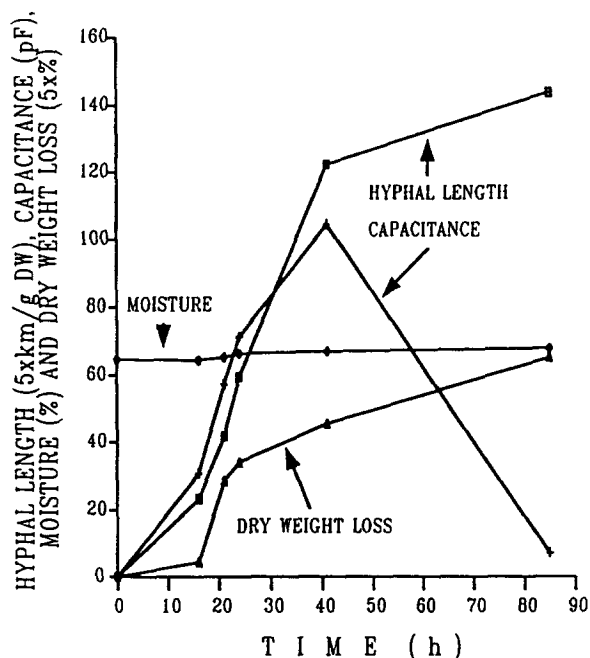
Figure 5. Variation of hyphal length in km per gram of dried tempe, capacitance in pF, moisture and dry weight loss of soya beans during the tempe fermentation described in the legend to Figure 2 and in Materials and Methods. Capacitance data were read from the on-line output corresponding to that given in Figure 2, but only at the time when replicate samples were taken out for off-line analysis of hyphal length, moisture and dry weight loss.

Figure 6. Changes in hyphal length, capacitance, moisture and dry weight loss during the growth of *R. oligosporus* mycelium on lupins during a tempe fermentation. Data were obtained both from the on-line and off-line analyses, exactly as described for Figure 5.
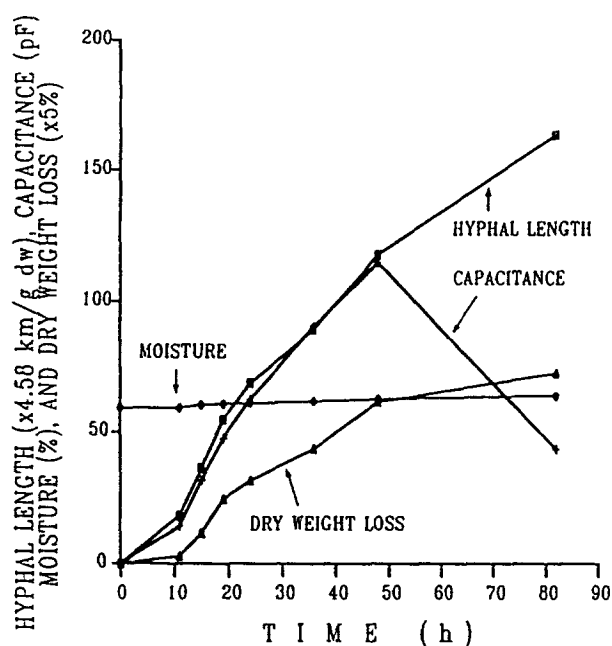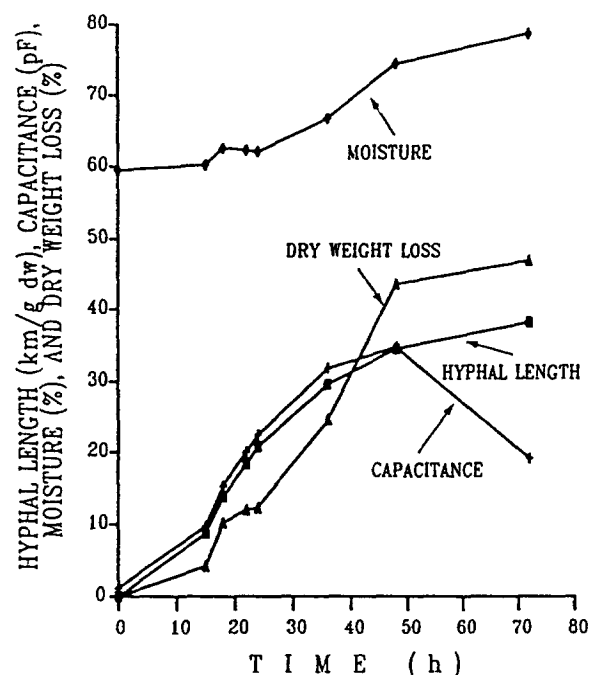
Figure 7. Changes in hyphal length, capacitance, moisture and dry weight loss during the growth of *R. oligosporus* mycelium on quinoa at 31°C. Data from analysis of duplicate samples taken out periodically, during the incubation of quinoa, are compared with discrete values of the on-line capacitance readings at the same incubation time. The latter correspond to the trace displayed in Figure 4. NB: the hyphal length on the ordinate should be divided by 2.5.

Table 1. Characteristics of each of the tempe types studied during two separate fermentations (R1 and R2).

	Tempe					
	Soya bean		Lupin		Quinoa	
	R1	**R2**	**R1**	**R2**	**R1**	**R2**
Moisture (%)						
Initial, at 0 h	59.2	59.2	66.4	64.1	59.5	64.1
Final, at 24 h	61.7	61.0	67.2	66.3	62.1	69.6
pH						
Initial, at 0 h	5.8	5.7	5.1	5.4	6.5	6.6
Final, at 24 h	6.8	6.3	7.2	7.7	4.1	4.5
Dry weight loss (%)						
Final, at 24 h	8.6	6.3	5.3	6.8	12.3	14.5
Hyphal length (km/g dry weight)						
Final, at 24 h	14.8	15.0	8.6	11.9	8.7	7.6
Capacitance (pF)						
Final, at 24 h	90.7	62.4	57.0	71.4	22.5	24.7
Conductance (mS)						
Final, at 24 h	0.8	0.6	1.0	1.2	0.24	0.24

Soya bean, lupin and quinoa were inoculated with *R. oligosporus* as described in the Materials and Methods section. Capacitance (pF) and conductance (mS) were read immediately after inoculation and after 24 h of incubation at 31°C, using the arrangement shown in Figure 1. pH was determined both on- and off-line, the latter in a tempe homogenate diluted 10-fold in distilled water. Moisture (%), dry weight loss (%) and hyphal length (km/g of dried tempe) data were obtained from off-line analysis of replicate samples taken at 24 h of incubation at 31°C and 75 to 90% of relative humidity. Further experimental details are given in the Materials and Methods section. The best fits by linear regression for the relationship between capacitance (C, in pF) and hyphal length (HL, in km/g) were:

Soya bean fermentation

R1: $C = 3.09 + 6.10\ HL$; $r^2 = 0.99$.
R2: $C = -3.40 + 4.58\ HL$; $r^2 = 0.99$.

Lupin fermentation

R1: $C = 13.30 + 4.01\ HL$; $r^2 = 0.95$.
R2: $C = 12.00 + 4.13\ HL$; $r^2 = 0.93$.

Quinoa fermentation

R1: $C = 1.31 + 2.39\ HL$; $r^2 = 1.00$.
R2: $C = 1.73 + 3.18\ HL$; $r^2 = 0.99$.

associated with the increasing mycelial colonization of the solid substrate. During the non-growing phase the dry weight losses account for the slight hyphal length increase. Hyphal lengths as high as 35 km/g dry weight were typical of over-fermented tempe in both soya bean and lupin fermentations, whereas in quinoa tempe hyphal length is maintained at around 10 km/g.

The time course of the capacitance is the most interesting aspect of the present work. After spore germination, i.e. after some 12 to 14 h of incubation at 31°C, the capacitance increases by as much as 100 pF, and more in the beans during the second day of incubation, after which autolysis of hyphae associated with high pH values of 6.5 to 8.3 are reflected in the dramatic fall of capacitance (Figures 2 and 3). At this stage, mycelial death was visible as a 'shrinkage' away from the

Figure 8. Time-course of the capacitance of two runs of a solid-state fermentation of quinoa for tempe. Incubation was at 31°C and at 75 to 90% relative humidity. In order to obtain a high reproducibility of capacitance, the Bugmeter probe was inserted into the inoculated quinoa before the incubation in an identical manner, i.e. the cake depth below the probe surface was 10 mm and the packing density was 42 g of inoculated quinoa per sterile standard petri plate of 8.7 × 1.25 cm. Other experimental conditions were as described in the legend to Figure 4 and in the Materials and Methods section.
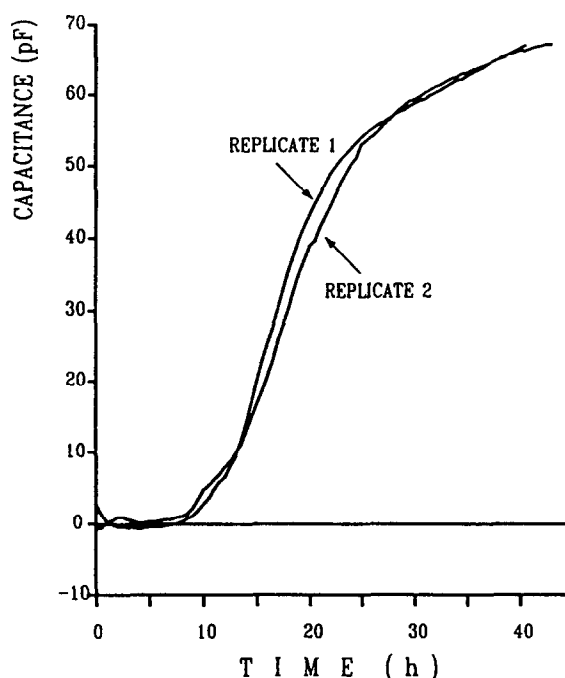
beans. The capacitance of biomass in quinoa tempe does not experience the above-mentioned fall during the non-growing phase, probably due to the more favourable pH (4.5 to 5.0), which might be responsible for preventing any significant reduction in viable biomass. Therefore, capacitance is a unique technique for measuring biomass on-line and in real time in solid-state fermentations as well as those in the solution phase.

As described above, the same dielectric theory has proved useful in biomass determinations in liquid-substrate fermentations (Harris *et al.* 1987; Davey & Kell 1990; Kell *et al.* 1990). Even given the drawbacks that they suffer, which have been pointed out elsewhere (Jones & Mollison 1948; Arima & Uozumi 1967; Sharma *et al.* 1977; Seitz *et al.* 1977, 1979; Bishop *et al.* 1982; Jarvis *et al.* 1983; Cousin *et al.* 1984), none of the previous methods used for monitoring solid-substrate fermentations can differentiate biomass from necromass.

By contrast, capacitance and hyphal length during the growing phase are closely correlated, the correlation coefficients for each replicate being close to unity. Nevertheless, differences between replicates do arise, and could be explained by a number of possible factors unique to solid-state fermentations. At the present stage of the development of dielectric measurement of biomass, the extent of insertion of the probe into the solid substrate, at the beginning of the incubation, is critical, since it affects the effective cell constant. The cake depth under the probe surface (varying from 0.5 to 0.9 cm in these experiments) and, to some degree, the cake compactness or packing density, cause the variations observed between replicates. When the cake depth was kept constant at 1.0 cm and packing density at 42 g per sterile plastic petri dish (1.25 × 8.7 cm), the time course of the capacitance between replicates was highly reproducible, as shown for quinoa in Figure 8. If the depth of the cake was varied, the capacitance signal was independent of the depth of the (uninoculated) substrate below the probe, provided that this exceeded 1.5 cm.

We conclude that capacitance provides a unique, reliable, reproducible and above all on-line measurement of biomass in solid-substrate fermentations, namely tempe in the present study. It has the added benefit that it also provides a measurement of possible conductance changes in the substrate. Thus a more

complete approach to solid-state fermentation process development is now possible, by measuring and controlling the temperature, humidity, O_2 and CO_2 levels in the gas phase (Mudget 1986), coupled with a computerized system (Figure 1) for measuring biomass (capacitance), conductance, pH and other variables. Indeed, capacitance could be the most precise method for studying the effect of variables on biomass formation during process optimization of both solid- and liquid-state fermentations.

Acknowledgements

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