The Use of Dielectric Permittivity for the Control of the Biomass Level during Biotransformations of Toxic Substrates in Continuous Culture

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Since the permittivity signal of a cell suspension measured using dielectric spectroscopy at radio frequencies is essentially determined only by viable (intact) cells, it can be used to monitor the concentration of viable cells in a fermentor in which a large proportion of the cells is nonviable. This could be used to select for organisms that are highly resistant to stress, for example from toxic chemicals used in biotransformations. We sought to control the concentration of viable yeast cells in a fermentor by adding small amounts of benzaldehyde, thus imposing a selection regime for cells highly resistant to benzaldehyde. However, after the addition of benzaldehyde, an increase in the permittivity is seen first followed by a decrease, thus making the control of biomass using a standard on–off controller difficult. It is shown that it is possible effectively to control the level of viable biomass in the fermentor in the presence of a large concentration of necromass using a combination of an inverse response compensator and a PID controller.

Introduction

Dielectric spectroscopy is a technique that can be used to estimate biomass levels in cell suspensions (Harris et al., 1987; Kell et al., 1990; Markx et al., 1991a–c; Fehrenbach et al., 1992; Austin et al., 1994). The method is based on the measurement of charge separations induced across the (intact) cell membrane by an applied RF electric field, and the theory of the dielectric properties of biological materials has been described in several reviews (Kell & Harris 1985; Pethig, 1979; Pethig & Kell, 1987, Kell, 1987; 1988; Kell & Davey, 1990). Using a feedback loop, similar to the turbidostatic approach (Anderson, 1956; Dykhuizen & Hartl, 1983), biomass levels in a fermentor can be controlled at a predefined level using permittivity measurements instead of turbidity (Markx et al., 1991b; Austin et al., 1994). The dielectric properties of cells at radio frequencies change considerably when the cell membrane is permeabilized (Huang et al., 1992). As a result, the contribution of nonviable cells to the permittivity signal is negligible, and an excellent correlation exists between cell viability and the permittivity signal; the method of dielectric spectroscopy has already been used in the measurements of the cytotoxicity of organic solvents (Stoicheva et al., 1989; Salter & Kell, 1992; Davey et al., 1993) and the effects of shear stress (Markx et al., 1991c). The contribution of noncellular particles to the permittivity signal is, in most cases, also negligible.

The fact that nonviable cells are not measured could be used to advantage in fermentation process optimization and continuous selection. Oliver and co-workers (Brown & Oliver, 1982; Wiebe et al., 1992, 1993) developed the technique of interactive continuous selection in which the evolution of a microbial population grown in a continuous culture is directed by monitoring the desired phenotype on-line and controlling the selection pressure in response to the culture's performance. Since the chemicals used in biotransformations and biodegradation are often cytotoxic (Brink & Tramper, 1985; Stoicheva et al., 1989; Osborne et al., 1990), one would prefer to work with an organism that has a high resistance to the chemicals involved. By using on-line permittivity measurements to estimate the concentration of viable cells in a continuous culture, it would be possible not only to work at the highest concentration of the cytotoxic chemical allowed but also to impose a selection regime for the most tolerant phenotype while not killing the culture completely.

We describe here how we used permittivity measurements in the control of a permittistatic culture of baker's yeast in which the biomass level was controlled by the addition of small amounts of the benzaldehyde. Although benzaldehyde can also be transformed by yeast to (phenylacetyl)carbinol (Long & Ward, 1989; Ward & Young, 1990; Nikolova & Ward, 1992 a–c), here it is transformed primarily to benzyl alcohol.

Materials and Methods

Yeast. The yeast studied was baker's yeast (*Saccharomyces cerevisiae*) described previously (Markx et al., 1991b).

Toxicity Studies. For the toxicity studies, a system similar to that described previously was used (Stoicheva et al., 1989; Davey et al., 1993; Salter & Kell, 1992). Suspensions were made of baker's yeast in water, with a differential permittivity value at 0.4 MHz (i.e., exceeding that of water) of approximately 150 (equivalent to approximately 10 mg of dry wt mL^{-1}) in a volume of 75 mL, and various amounts of benzaldehyde were added. The electrode used for the toxicity studies had a cell constant of 0.830 cm^{-1}.

Continuous Culture. The fermentation system used is illustrated in Figure 1. The fermentor used was a

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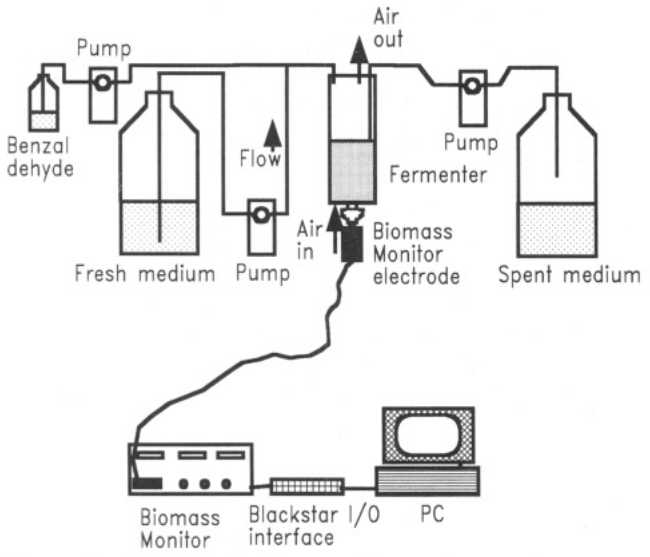

Figure 1. Outline of the fermentation system used.

bubble column with a liquid volume of 300 mL. Fresh medium and spent suspension were pumped in and out of the fermentor using Watson-Marlow peristaltic pumps. The medium had the following composition (w/v): 5% glucose (BDH), 1% yeast extract (Sigma), and 1% bacterial peptone (lab M). Medium pH prior to sterilization was 4.5. A pumping rate of 1.1 mL min^{-1} was used (dilution rate, 0.22 h^{-1}). Benzaldehyde was obtained from Sigma. It was pumped undiluted into the fermentor using a Pharmacia PP1 peristaltic pump.

The biomass levels were measured using a dielectric spectrometer, Biomass Monitor Model 214A (Aber Instruments, Unit 5, Science Park, Cefn Llan, Aberystwyth, Dyfed SY23 3AH, UK). Biomass concentrations were determined from the difference between the permittivities at frequencies of 0.4 and 9.5 MHz. The electrode used had a cell constant of 0.803 cm^{-1}. Data acquisition and control were performed as described before (Markx et al., 1991b). The addition of benzaldehyde was started when the difference in the permittivity reached a value of 132. A hysteresis of 1 permittivity unit was used.

Experiments and Results

Toxicity Experiments. To measure the toxicity of benzaldehyde, yeast suspensions were made and the change in the permittivity after the addition of a known amount of benzaldehyde was measured in time. Typical curves obtained with different concentrations of benzaldehyde are given in Figure 2. At first an increase in the permittivity is observed, followed by a decline in the permittivity. This phenomenon has been reported before with other chemicals (Stoicheva et al., 1989; Chanturiya, 1990) and is probably due to an increase in the membrane capacitance as the chemical enters the hydrophobic interior of the membrane (Seeman, 1972). A first-order rate equation was fit to the secondary decline in permittivity, and in Figure 3 the rate constants are plotted as a function of the benzaldehyde concentration added. The results clearly show that a threshold exists in the concentration of benzaldehyde needed. A threshold in the effect of the cytotoxic chemical benzyl alcohol on erythrocytes has been reported before by Colley et al. (1971), and thresholds of this type are best interpreted in terms of the requirement for a critical concentration of solvent to have dissolved in the membrane.

Continuous Culture. In the next experiment (Figure 4), the yeast was first allowed to attain a high concentration in batch culture. Continuous culture was then started by switching on the pump (dilution rate 0.22 h^{-1}), and at the arrows indicated, small amounts of benzal-

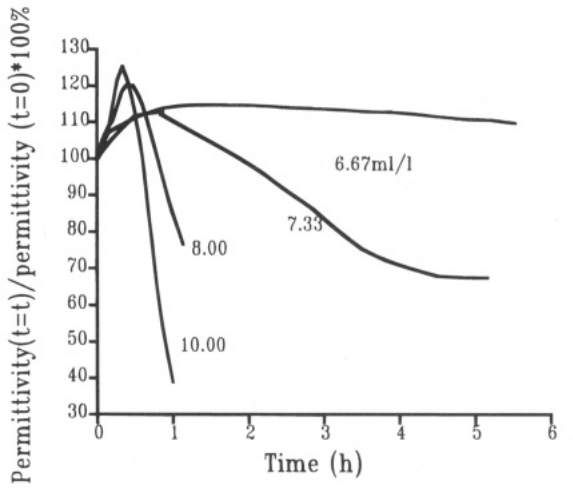

Figure 2. Time dependence of the permittivity of a baker's yeast suspension after the addition of final benzaldehyde concentrations (in mL l^{-1}) shown. An increase in permittivity is seen after the addition of benzaldehyde to the suspension, followed by a decrease at higher benzaldehyde concentrations.

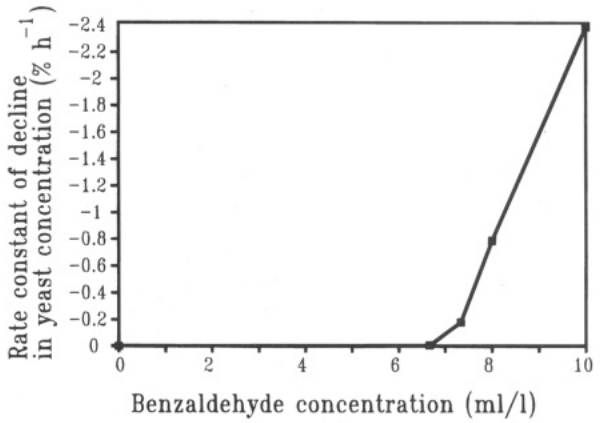

Figure 3. Rate constants of the decline in the permittivity after the addition of benzaldehyde in different final concentrations. Data were obtained from the experiments displayed in Figure 2.

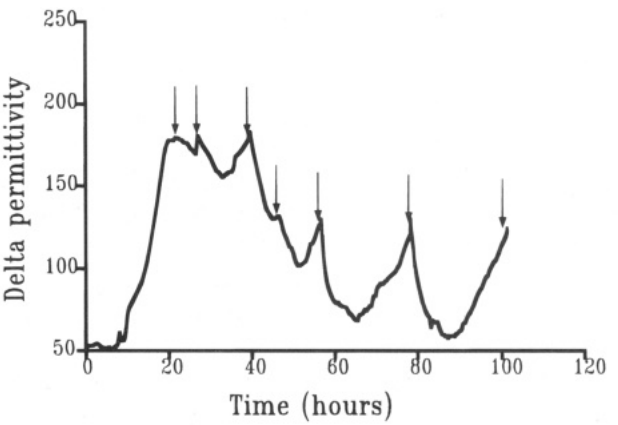

Figure 4. Time dependence of the permittivity (biomass concentration) of a continuous culture of yeast. At the first four arrows, small amounts of benzaldehyde were added to the suspension in the fermentor. Unlike previous experiments, growth resumed after an initial decline in the biomass signal. At the three later arrows, the addition of benzaldehyde was performed automatically. During the last addition the tube of the benzaldehyde pump ruptured, and data after this point were not considered valuable.

dehyde (1 mL doses), which were large enough to kill part of the yeast population but small enough to keep a proportion of it viable (so that growth could be reestablished), were injected into the suspension. When this proved successful, the control system was switched to automatic, such that the addition of benzaldehyde occurred at a Δ permittivity set point value of 132 (and stopped when the Δ permittivity was below 131). Although the trace of the experiment (Figure 4) shows that it is possible to keep the viable yeast concentration below a certain level and yet maintain the viability of the yeast in culture high enough to allow regrowth, the control of the level of viable biomass in the fermentor is not optimal, as large dips in the permittivity are seen after the addition of the cytotoxic chemical before growth is regained. In the next section of this paper, this phenomenon is explained and a way to solve this problem is proposed.

Modeling and Discussion

In the toxicity experiments (Figure 2), we had already seen that after the first addition of benzaldehyde an *increase* in the permittivity can occur and only then followed by a decrease due to a drop in cell viability. As a consequence, in the continuous culture, the flow of benzaldehyde into the fermentor goes on for longer than is necessary, the viable yeast concentration decreases by more than is desirable, and a long time is needed before the viable yeast concentration reaches its set point again. We will now model this situation to find a control mechanism that can overcome these problems.

Model for the Effect of Cytotoxic Chemicals on Cell Viability. In the model we assume that of the total volume, V_{tot}, the volume occupied by the yeast (V_{yeast}) is much smaller than that occupied by the medium (V_{med}), so that $V_{med} = V_{tot}$. We also assume that all of the yeast is viable at the start and that the concentration of the toxic compound in the yeast ($X_{toxyeast}$) and the medium (C_{toxmed}) is zero.

The rate of uptake of the toxic compound is most likely determined by mass transfer. The flow of toxic compound, J_{tox} (in g of toxic compound s^{-1} (m of yeast membrane)$^{-2}$ from the medium into the yeast is given by

$$J_{tox} = k_{mass}(C_{toxmed} - C_{tox}^*) \quad (1)$$

where k_{mass} is a mass transfer coefficient. C_{tox}^* is the concentration of the toxic compound in the medium that is in equilibrium with the concentration of the toxic compound inside the yeast, $X_{toxyeast}$:

$$C_{tox}^* = X_{toxyeast}/K \quad (2)$$

where K is the partition coefficient.

The total surface area in the suspension, A, is directly related to the amount of yeast present:

$$A = k_1 V_{tot} C_{yeast} \quad (3)$$

A balance of the flow of the toxic compound then gives

$$dC_{toxmed}/dt = k_1 V_{tot} C_{yeast} k_{mass}(C_{toxmed} - C_{tox}^*)/V_{med} = k_1 C_{yeast} k_{mass}(C_{toxmed} - C_{tox}^*) \quad (4)$$

if $V_{yeast} \ll V_{med}$ and

$$dX_{toxyeast}/dt = k_1 k_{mass}(C_{toxmed} - C_{tox}^*) \quad (5)$$

The yeast dies as a consequence of the presence of an overdose of the toxic compound in its structures. The death rate of the yeast is dependent on the concentration of the toxic compound in the yeast, where a critical amount is necessary to start killing the yeast. The rate of decline in the yeast concentration could be described by the equation

$$dC_{yeastviable}/dt = -k_2 C_{yeastviable}(X_{toxyeast} - [\text{threshold}]) \quad (6)$$

In our experiments, we used permittivity as an estimate of the concentration of viable biomass in the suspension. The addition of a toxic compound enlarges the permittivity. Thus, the total permittivity of the yeast could be described by the equation

$$\text{permittivity} = k_3 C_{yeastviable} + k_4 X_{toxyeast} C_{yeastviable} \quad (7)$$

Numerical simulation of eqs 1−7 for the above actions of a cytotoxic chemical on the permittivity of cell suspensions was performed, and the results are shown in Figure 5. A curve very similar to those in the experiments (Figure 2) at high benzaldehyde concentrations is seen, including the increase in the permittivity after the addition of the toxic compound.

Modeling the Situation in the Continuous Culture. We assume that the yeast grows on a substrate, s, with Monod kinetics in a chemostat with dilution rate D_1, and we start adding the cytotoxic chemical with a dilution rate D_2 when the permittivity measured reaches a level, Permset, plus some hysteresis. We can set up the following balances to describe a continuous culture of an organism in which the biomass level is controlled by the addition of a cytotoxic compound:

$$dC_s/dt = D_1 C_{sin} - (D_1 + D_2)C_s - (r_{max} C_{yeastviable} C_s)/(K_m + C_s) \quad (8)$$

$$dC_{yeastviable}/dt = -(D_1 + D_2)C_{yeastviable} - k_2 C_{yeastviable}(C_{toxyeast} - [\text{threshold}]) + Y_1(r_{max} C_{yeastviable} C_s)/(K_m + C_s) \quad (9)$$

$$dC_{yeastdead}/dt = k_2 C_{yeastviable}(C_{toxyeast} - [\text{threshold}]) - (D_1 + D_2)C_{yeastdead} \quad (10)$$

$$dC_{yeast}/dt = dC_{yeastviable}/dt + dC_{yeastdead}/dt \quad (11)$$

Further balances are

$$dC_{toxmed}/dt = D_2 C_{toxin} - (D_1 + D_2)C_{toxmed} - k_1 C_{yeast} k_{mass}(C_{toxmed} - C_{tox}^*) \quad (12)$$

if $V_{med} = V_{tot}$, and

$$dX_{oxyeast}/dt = (-X_{toxyeast} dC_{yeast}/dt + k_1 C_{yeast} k_{mass} \times (C_{toxmed} - C_{tox}^*) - (D_1 + D_2)X_{toxyeast}) \quad (13)$$

Of course eq 7 is still valid.

The permittivity in the continuous culture is controlled at a certain level by the controlled addition of the toxic chemical:

$$D_2 > 0 \quad \text{when} \quad \text{Perm} > \text{Permset (+some hysteresis)}$$

$$D_2 = 0 \quad \text{when} \quad \text{Perm} < \text{Permset}$$

Equations 6−13 were used in the next simulation (Figure 6A) in which the the yeast concentration at which

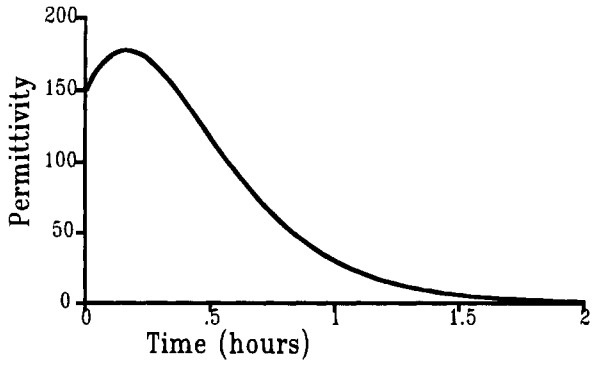

Figure 5. Results of the numerical simulation of the influence of benzaldehyde on the permittivity signal of a yeast suspension. The trace is similar to experimentally obtained traces at high concentrations of the cytotoxic chemical. The parameters used are as follows: C_{toxmed} at start = 0.007 g L^{-1}, $C_{yeastviable}$ at start = 10 g L^{-1}, K = 1000, [threshold] = 0 g L^{-1}, k_{mass} = 0.05 L s^{-1} m^{-2}, k_1 = 5 m^2 g^{-1}, k_2 = 5000 L g^{-1} h^{-1}, k_3 = 15 L g^{-1}, k_4 = 20 000 L^2 g^{-2}.

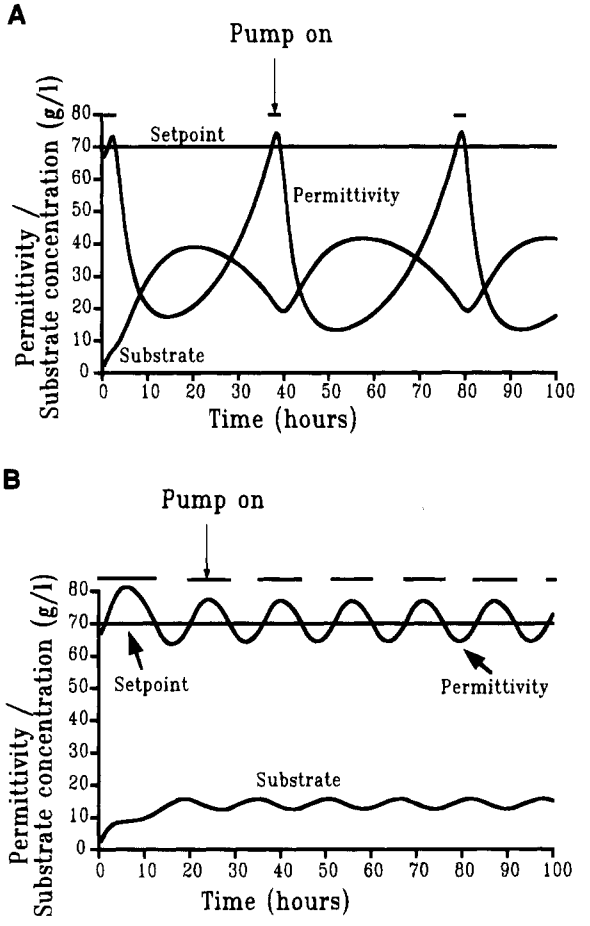

Figure 6. (A) Numerical simulation of a continuous culture, in which biomass level is controlled by the addition of benzaldehyde. The parameters used are as follows: $C_{yeastviable}$ at start = 4.5 g L^{-1}, r_{max} = 0.3 h^{-1}, K_m = 0.5 g L^{-1}, Y_1 = 0.17, D_1 = 0.22 h^{-1}, D_2 = 3.10^{-7} h^{-1}, C_s at start = 0 g L^{-1}, C_{sin} = 50 g L^{-1}. The other parameters were as for Figure 5. (B) Decreased rate of addition (3×10^{-8} h^{-1}) of the cytotoxic chemical to the culture. The size of the dips is decreased.

the fermentor started was increased to a level just below the set point, as was the case in our experiment (see Figure 4). The simulation shows that the yeast stays alive and that its concentration can be maintained intermittently around the set point, but the same large dips in the yeast concentration occur that were seen

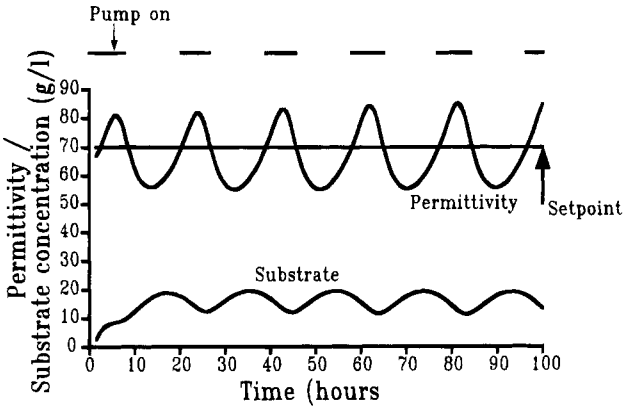

Figure 7. Same as Figure 6, but a PID controller was used instead of the simple on–off control used previously. Biomass level control is improved. The relevant control parameters for the PID controller were as follows: K_p (constant for proportional control) = 5×10^{-9}, K_i (integrational control) = 1×10^{-10}, K_d (differential control) = 2×10^{-11}.

during the real experiment. The large dips in the biomass concentration can partly be overcome by decreasing the rate of the addition of benzaldehyde. The effect of such a decreased rate of addition is shown in Figure 6B. However, even then the control is somewhat usatisfactory, and a reduced bioconversion may be the result.

An Improved Control Algorithm. During the experiment, a simple on–off control was used or, in other words, the pump was switched on when the permittivity exceeded a certain set point and switched off when it fell below another, slightly lower set point. A more advanced control mechanism than the one used would obviously be helpful.

The control of chemical processes and fermentation processes is the subject of many publications (e.g., Jacobs, 1974; Stephanopoulos, 1984; Leigh, 1987; Bastin & Dochain, 1990; Van de Vegte, 1990), and a large number of control mechanisms are available (e.g., Miller et al., 1969). A common improved control mechanism is the use of a so-called PID controller, where PID stands for proportional, integral, and derivative, which are based on, respectively, the distance between the set point and the actual signal, the integration in time over the difference between the set point and the actual signal, and the derivative of the difference between the set point and the actual signal with respect to time.

An optimally tuned PID controller was used for Figure 7 to control the biomass level instead of an on–off control. A PID controller itself can control our system, but did not seem to be able to do it very adequately [see also Waller and Nygardas (1975)]. An alternative method was proposed by Iinoya and Altpeter (1962), who used an inverse response compensator which is related to a dead-time compensator or Smith predictor (Smith, 1967). In Figure 8 the numerical simulation of such an approach is shown. To prevent the overshoot, a period is introduced after each pulse of cytotoxic compound during which no cytotoxic compound may be added. The dips in the biomass level are largely reduced. Alternatively, one could make use of eq 7 and try to separate from the permittivity signal the contribution of the toxic compound in the cell and obtain that of the viable yeast itself. However, this would demand a better knowledge of the history of the signal and very good estimates of the parameters involved. Self-tuning and adaptive control (Wellstead & Zarrop, 1991; Isermann et al., 1992) are also a possibility, but outside the scope of the the present investigations.

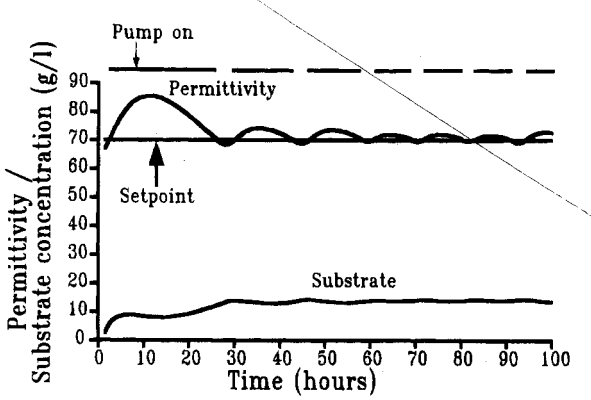

Figure 8. Same as Figure 7, but the addition of the cytotoxic chemical is only for a short period (0.1 h), followed by a period during which no cytotoxic chemical is added (1.4 h). The large dips do not occur with this improved control regime.

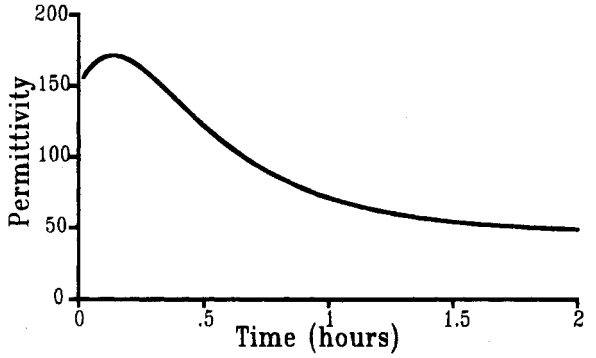

Figure 9. Same as Figure 5, but the cytotoxic chemical is also converted by the yeast ($k_5 = 3\ h^{-1}$). Trace is now also similar to those observed at lower benzaldehyde concentrations.

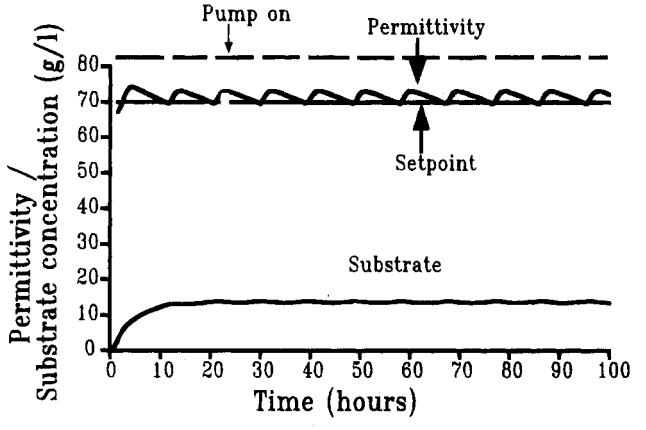

Figure 10. Same as Figure 8, but the cytotoxic chemical is also converted by the yeast ($k_5 = 3\ h^{-1}$). The duration of the pulse can be longer this time (0.5 h) and the time between pulses shorter (0.05 h).

Refinement of the Model. Thus far, we have assumed that the cells do not modify the benzaldehyde. However, the benzaldehyde is converted by the yeast into another compound. This situation can be simulated by the introduction of a term for the metabolism of benzaldehyde. As the benzaldehyde is only converted once it has entered the yeast, the conversion of the benzaldehyde will occur inside the yeast only:

$$-dX_{toxyeast}/dt = k_5 X_{toxyeast} \quad (14)$$

Numerical modeling of this situation is given in Figure 9. The traces are now more similar to those observed experimentally at lower benzaldehyde concentrations, as at these concentrations the benzaldehyde was insufficient to kill all of the yeast.

Figure 10 shows the numerical modeling of the continuous culture controlled by the pulsed addition of the cytotoxic chemical. When the cytotoxic chemical is converted by the yeast, it is possible to add more of the cytotoxic chemical. The model could be further refined by the inclusion of expressions for the inhibitory nature of the cytotoxic chemicals (Andrews, 1968). Also, the different cytotoxic characters of the products of the biotransformation (Lucchini et al., 1990) could easily be incorporated.

Conclusions

Studies of the change in the permittivity of a yeast cell suspension after the addition of various concentrations of benzaldehyde have shown that a threshold exists in the lethality of the compound and that an increase in the permittivity precedes the drop in the permittivity due to the loss in cell viability. We have shown that it is possible to grow yeast in a continuous culture in which the biomass level is controlled by the addition of the cytotoxic chemical benzaldehyde, using dielectric spectroscopy as an estimate of the viable biomass concentration, but that the initial increase in the permittivity after the addition of benzaldehyde tends to cause too much of the benzaldehyde to be added and large dips occur in the biomass concentration. The control of the addition of the benzaldehyde can be improved by the use of lower flow rates for the addition of the cytotoxic chemical, the use of PID controllers, and in particular the introduction of lag times after a pulse of the cytotoxic chemical during which none is added to the culture.

Notation

dC_s/dt	rate of change in total amount of substrate over total volume (g L^{-1} h^{-1})
dC_{yeast}/dt	rate of change in total amount of biomass (viable and nonviable) over total volume (g L^{-1} h^{-1})
$dC_{yeastviable}/dt$	rate of change in total amount of viable yeast over total volume (g L^{-1} h^{-1})
$dC_{yeastdead}/dt$	rate of change in total amount of nonviable biomass over total volume (g L^{-1} h^{-1})
$dX_{toxyeast}/dt$	rate of change in total amount of cytotoxic chemical in the yeast over total amount of yeast (g L^{-1} h^{-1})
dC_{toxmed}/dt	rate of change in total amount of cytoxic chemical in the medium over total volume of medium (g L^{-1} h^{-1})
C_{yeast}	total amount of yeast over total volume (g L^{-1})
$C_{yeastviable}$	total amount of viable yeast over total volume (g L^{-1})
$C_{yeastdead}$	total amount of nonviable yeast over total volume (g L^{-1})
C_{sin}	substrate concentration in substrate feed (g L^{-1})
C_s	substrate concentration in fermentor (g L^{-1})
C_{toxmed}	total amount of cytotoxic chemical in the medium over total volume of medium (g L^{-1})
C_{toxin}	concentration of cytotoxic chemical in feed of cytotoxic chemical (g L^{-1})
$X_{toxyeast}$	concentration of cytotoxic chemical in yeast (g L^{-1})
D_1	dilution rate of biomass/substrate (h^{-1})
D_2	dilution rate of cytoxic chemical (h^{-1})
K	partition coefficient
K_m	Monod saturation constant (g L^{-1})

k_{mass}	mass transfer coefficient susbstrate between medium and yeast (L s^{-1} m^{-2})
k_1	conversion factor for calculation of the surface area of yeast from the volume of yeast (m^2 g^{-1})
k_2	rate constant for death of yeast caused by the presence of the cytotoxic chemical in the yeast (L g^{-1} h^{-1})
k_3	conversion factor for permittivity from yeast concentration (L g^{-1})
k_4	constant for calculation of permittivity increase caused by presence of the cytotoxic chemical in the yeast (L^2 g^{-2})
k_5	rate constant for the conversion of the cytotoxic chemical in the yeast (h^{-1})
Perm	permittivity
Permset	permittivity set point
r_{max}	maximum specific growth rate (h^{-1})
V_{tot}	total volume of suspension in fermentor (L)
V_{med}	volume of medium in fermentor (L)
V_{yeast}	volume of yeast in fermentor (L)
[threshold]	threshold concentration of cytotoxic chemical in yeast: above this concentration the yeast starts to die (g L^{-1})
Y_1	yield coefficient of substrate in biomass (g g^{-1})

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