Periodic Operation of a Continuous Culture of Baker's Yeast

E-M. Abulesz* and G. Lyberatos†
Department of Chemical Engineering, University of Florida, Gainesville, Florida 32611

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The possibility of enhancing the biomass productivity of a continuous culture of *Saccharomyces cerevisiae* growing on a glucose-limited medium is addressed. An unstructured Monod-type model is first identified using steady-state data. The culture is subjected to step changes in dilution rate, and it is seen that the Monod model is unable to predict even qualitatively the dynamic response of the culture. Incorporation of a time delay allows significant improvement in the transient fit. It is found that the culture has a time lag of about 3 h in adapting its growth rate. Cycling the dilution rate with a period of 3 h leads to substantial improvement in the average biomass productivity.

INTRODUCTION

Compared to batch and semibatch processes, little work has been done with continuous-fermentation processes. However, over the past few years continuous fermentations have increased in significance, and their optimization has become important.

If some manipulated process variables are allowed to vary with time, very often superior productivity or even a change in culture fate is possible when compared to steady-state operation.[1,2] A significant amount of work has been undertaken[3–9] to establish periodic operations that maximize the yields and/or selectivities of chemical reactors.

A few workers have conducted experiments to observe the behavior of continuous chemostat cultures when subjected to cyclic operation. The major references are summarized in Table I.[10–16] It is hard to draw general conclusions from those first attempts to assess the effect of cycling experimentally. In many cases, complex (e.g., undefined) growth media like molasses were used or some quantities, such as yield factors, have either been not well defined or defined differently. Nonetheless, those early works suggest that there is a lot of opportunity for process improvement through periodic operation. In these experiments the feed-limiting substrate concentration or the dilution rate served as the manipulated process variable. Picket, Bazin, and Topiwala[10] (using s^0, the feed-limiting substrate concentration as control variable) found that at some frequencies average biomass productivities are superior to those for steady-state operation for a continuous *Escherichia coli* culture. Moreover, the observed improvement in these frequency ranges was found to increase with increasing cycling amplitudes.

In some cases it may be desirable to improve not the productivity of cell mass but the protein productivity. It has been known for some time that the concentration of some important metabolites inside the cell is not constant during a dynamic process. It has also been observed that the average macromolecular composition of the cell changes under cyclic conditions.[12] So it was found that the fraction of protein and RNA is higher when periodic operation is employed. Especially in the case where total protein is the product of interest, this result is of considerable significance.

In a previous work[1] it was theoretically established that improvement in the yield of biomass through cyclic reactor operation is possible only when there exists a time lag in the adaptation of specific growth rate to environmental changes. The purpose of this work is to study the behavior of a glucose-limited continuous culture of *S. cerevisiae* undergoing square-wave forcing in the dilution rate. After a steady-state model has been obtained from steady-state data, a dynamic time delay model is found to perform better than an unstructured Monod-type model in predicting the response of the system to step changes in the dilution rate. Then experiments are performed to observe the behavior of the culture under periodic conditions.

In the next sections the experimental apparatus and materials and methods will be described. Subsequently the steady-state as well as the dynamic response experiments are described. The last part of this article is concerned with the periodic operation of the bioreactor.

EXPERIMENTAL APPARATUS

A schematic representation of the experimental system is given in Figure 1. The Bioengineering KLF 2000 fermentor with a 3-L glass cylinder serves as chemostat. A CD motor driven from below rotates axially two flat-blade agitators. Mixing is enhanced by stream breakers inside

* Current address: Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045.
† To whom all correspondence should be addressed.

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Table I. References on experiments involving periodic reactor operation.

Reference no.	Culture type	Control variable	Waveform	Variation of	Effect on average biomass yield
12	*Escherichia coli*–glucose	s_0	Square	Frequency	Higher than steady state in some frequency ranges
10	*E. coli*–glucose	s_0	Square	Amplitude	Increasing function of amplitude
11	*Saccharomyces cerevisiae*–molasses	s_0	Square + sinusoidal	Amplitude and frequency	Small effect if any
15	*E. coli*–glucose	s_0	Sinusoidal	Frequency	Not reported
13	*S. cerevisiae*–molasses	D	Varying	Form of oscillation	None
14	*K. aerogenes*–glucose	D	Sinusoidal	Frequency	Not reported
16	*S. cerevisiae*–glucose	D	Sinusoidal	Amplitude	Not reported

the vessel. The agitation system is connected with the control panel for speed measurement and setting. The rotation speed was maintained at 500 rpm. The top of the vessel has access ports which are used for pH measurement and control and addition of the growth medium and an additional port for reentry of a recycle loop for the turbidity measurement. The bottom plate of the fermentor has two ports, one for harvesting and one for the recycle loop.

The temperature is controlled via a temperature controller located in the control panel. A Pt 100 temperature sensor monitors the fermentation temperature continuously. An 800-W heating finger keeps the temperature at 30°C, the optimal growth temperature for yeast.

The pH controller, also located at the control panel, is a quasi-steady-state three-point eliminator. A silver–silver chloride electrode is inserted through the top of the vessel and monitors the pH continuously. The controller keeps the pH inside the fermentor at 4.0 through actuating two peristaltic pumps that deliver either NaOH or HCl as needed.

The available pumps are constant-flow-rate pumps. In continuous steady-state operation, the harvesting pump is set for a certain flow rate and not controlled; the feed pump is controlled by a load-cell control system (on–off).

A turbidity measurement device is employed for the continuous measurement of the optical density, from which the concentration of biomass can be obtained. The Bioengineering TMK turbidity monitor is equipped with a flow-through cuvette through which the recycle stream is guided. Since the residence time in the recycle stream is

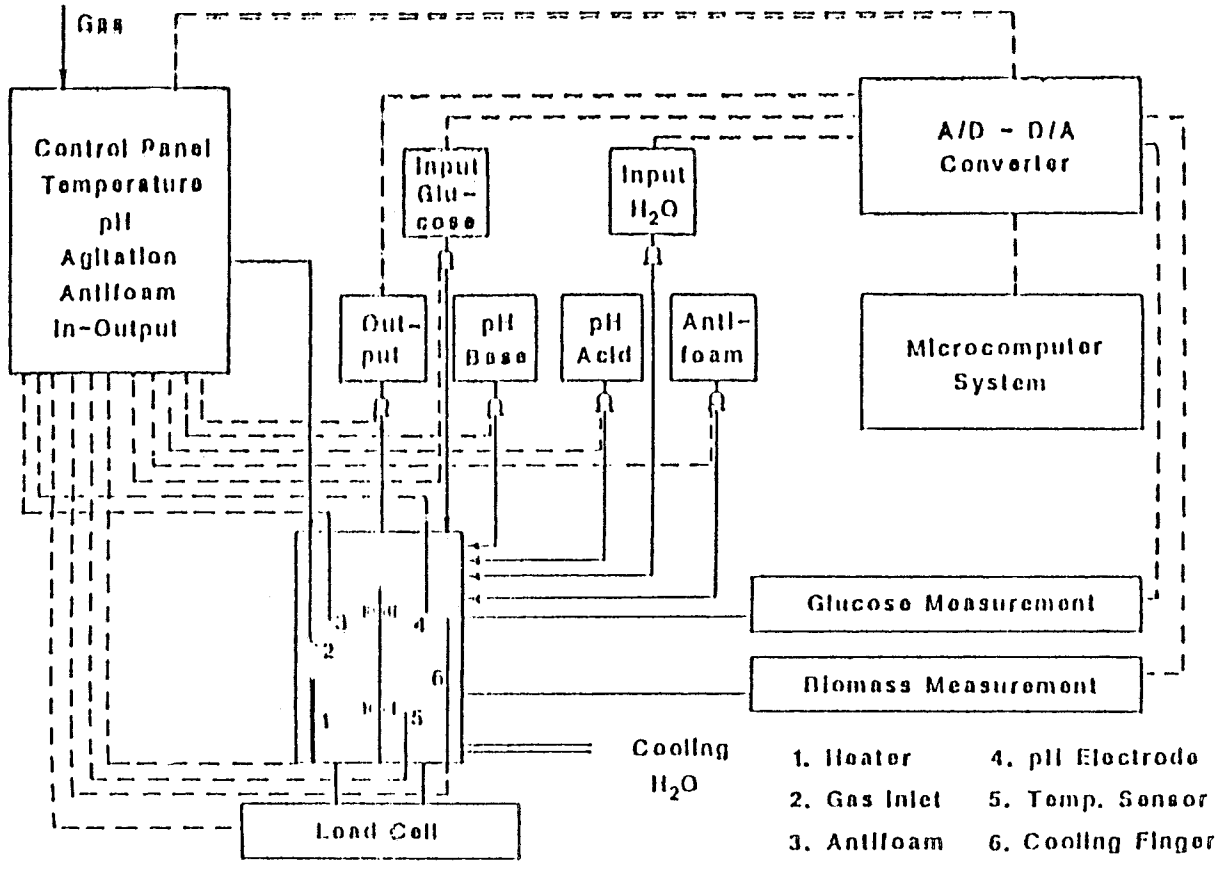

Figure 1. Schematic representation of experimental system.

maintained at less than 1 min, no influence on the reactor dynamics is expected.

A continuous glucose analyzer from Analytical Research was used for continuous glucose measurement. Every minute a small amount of the medium is sampled and led to the analyzer. At the end of an electrochemical sensor rests an immobilized enzyme membrane. The enzyme catalyzes the reaction between the sugar and oxygen to form H_2O_2, which is detected by the sensor.

A PDP 11/23 microcomputer equipped with a 10M hard disk drive, a graphics terminal, and an LA 100 printer together with Data Translation AD/DA converters comprises a complete data acquisition, manipulation, and actuation system used on-line for direct digital control. For the case of periodic square-wave forcing the computer has been programmed to control the harvest pump in an on–off manner. The control panel adjusts the feed pump to keep the reactor volume constant.

MATERIALS AND METHODS

Saccharomyces cerevisiae was obtained from the Department of Microbiology at the University of Florida. Stock cultures were maintained on nutrient broth agar at 8°C. The composition of the feed medium for continuous operation is listed in Table II. To avoid the possibility of a change in the feed composition through a chemical reaction, batches I and II were autoclaved separately and mixed under UV light. For the start-up proceedings batch II was sterilized in the fermentor at 121°C for 30 min while batch I was autoclaved and used as a seed flask for the reactor.

Dry weights of cell mass were obtained by heat-shocking 50 ml of sample for 6 minutes to inhibit further growth, followed by centrifugation (30 minutes at 1200 rpm). The liquid was decanted and the cells washed with distilled water. After suspending them in 2 ml of distilled water they were poured in open petri-dishes and the water was gently evaporated.

ESTABLISHMENT OF STEADY-STATE MODEL

The simplest possible type of model, an "unstructured model" has two inherent assumptions: that the cell is in a state of "balanced growth"[18] and that there does not exist a time lag between a change in the environmental conditions and the adaptation of the growth rate thereafter. Following these assumptions, the dynamics of the chemostat can be described by the following two-dimensional system of equations:

$$\frac{dx}{dt} = \mu(s,x)x - Dx \tag{1}$$

$$\frac{ds}{dt} = -\frac{1}{Y}\mu(s,x)x - D(s^0 - s) \tag{2}$$

where x and s are the combinations of biomass and limiting substrate, respectively, μ is the specific growth rate, and Y is the yield factor

$$Y = \frac{\text{grams biomass produced}}{\text{grams limiting substrate used}} \tag{3}$$

By far the most commonly used model is that due to Monod.[19] It assumes the following functionality for the specific growth rate:

$$\mu(s) = \frac{\mu_m s}{k_s + s} \tag{4}$$

where μ_m is the maximum growth rate at the culture temperature and pH and k_s is a constant. Upon rearrangement, equations (1) and (4) at steady state yield

$$\frac{1}{D} = \frac{k_s}{\mu_m}\cdot\frac{1}{s} + \frac{1}{\mu_m} \tag{5}$$

This expression can be used to determine the constants μ_m and k_s (Lineweaver–Burk plot). Table III summarizes the steady-state behavior of the system. Figure 2 shows the Lineweaver–Burk plot obtained from the steady-state data. Given the culture conditions described in the previous section and using linear regression, the constants were found to be $\mu_m = 0.5574\ h^{-1}$ and $k_s = 2.0535$ g/L. The yield factor Y was determined to be 0.175.

RESPONSE OF SYSTEM TO STEP CHANGES IN DILUTION RATE

Although unstructured models succeed in describing the chemostat at steady state, in most cases they are expected to fail to predict dynamic responses accurately. Figure 3 proves this to be true for the system at hand. The response of the biomass in grams per liter is followed over time after a step change in the dilution rate from 0.2 to 0.1 has been performed. As can be seen clearly, the Monod model is

Table II. Composition of growth medium.

	Substance	Concentration (g/L)
I	Glucose	2
	Yeast extract	0.2
	$MgSO_4 \cdot 7H_2O$	0.4
II	Potassium phosphate monobasic	5
	Ammonium sulfate	2

Table III. Summary of steady-state behavior.

Dilution rate (h^{-1})	Biomass concentration (g/L)	Substrate concentration (g/L)
0.100	0.275	0.460
0.104	0.290	0.403
0.116	0.268	0.530
0.154	0.173	0.802
0.200	—	1.196
0.209	0.133	1.181

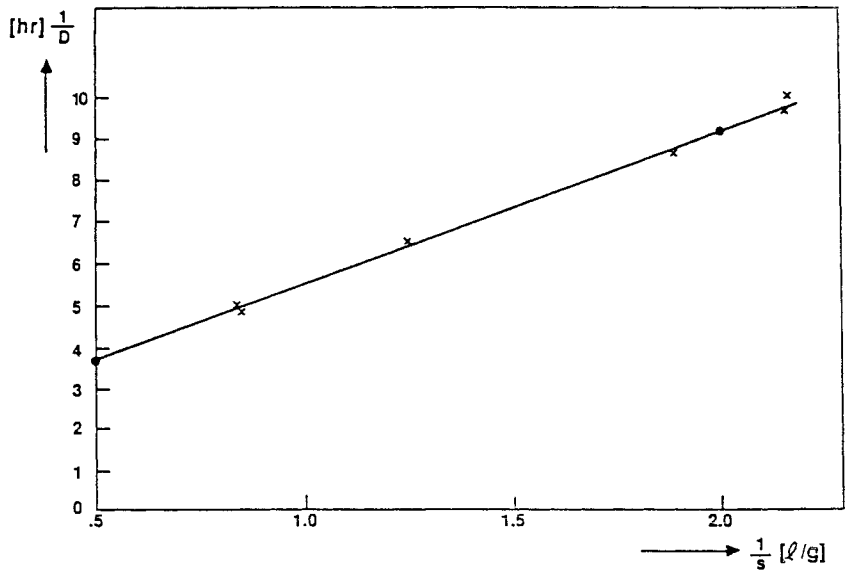

Figure 2. Lineweaver–Burk plot for determination of constants in Monod model.

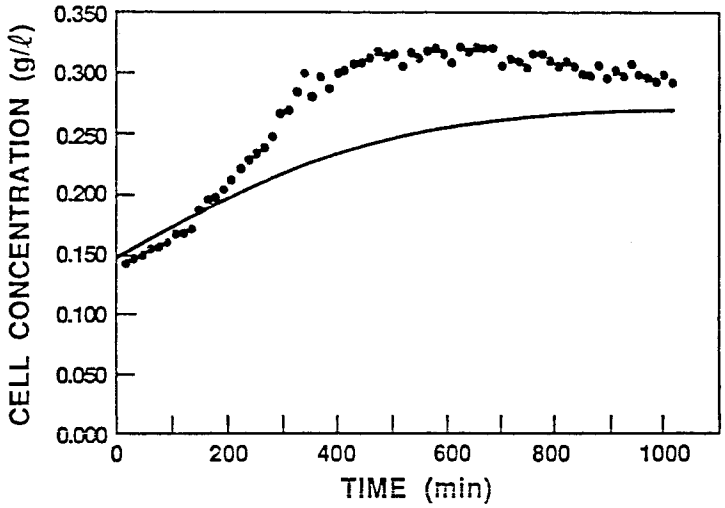

Figure 3. Response of biomass to step change in dilution rate from 0.2 to 0.1 and predictions of Monod model.

not only too sluggish to portray the fast initial response of the culture but also fails to predict the overshoot and subsequent return to the steady-state value corresponding to the new dilution rate. This is due to the assumption that there does not exist a time lag between a change in the substrate concentration and adaptation of the culture for growth at the new level. To relax this assumption, one may assume that the specific growth rate is a function not only of the present substrate level but also of previous levels in a weighed manner. In this case the growth rate is taken to be a function of a new variable z instead of s, where

$$z = \int_{-\infty}^{t} s(\tau)F(t - \tau)\,d\tau \qquad (6)$$

where $F(t - \tau)$ is a memory function that weighs appropriately previous substrate levels. The advantage of this approach is that the steady-state model form can be identical to that of the unstructured model while the dynamic behavior is better predicted.

The case of infinite delay results when the memory function $F(\gamma) \equiv 0$, resulting in the variable γ and thus also the growth rate $\mu(z)$ being a constant. In this case the culture will remain growing at the growth rate corresponding to the conditions before the step change, and no adaptation will occur. This situation is shown in Figure 4 where unlimited exponential increase in the biomass concentration is predicted. Comparing Figures 3 and 4 to the experimental data clearly shows the existence of a finite time delay. Several time delay models were investigated, and the "pure delay" model was identified as fitting the experimental data best. Here growth depends not on a weighted average of previous substrate levels but on the level at a

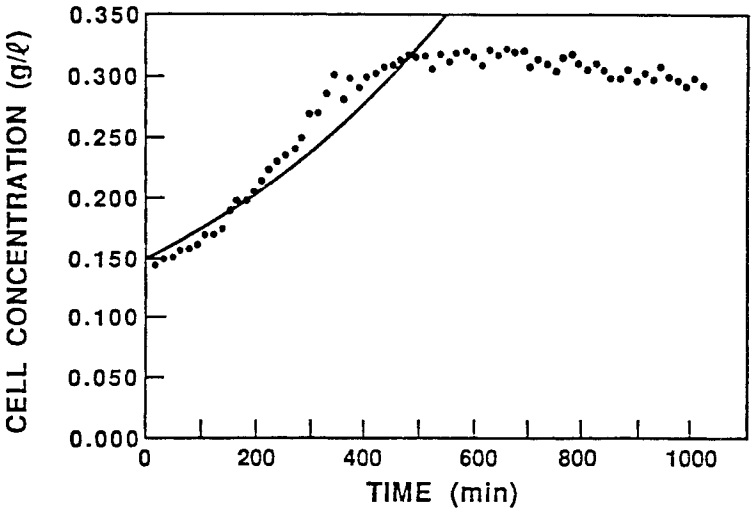

Figure 4. Response of biomass to step change in dilution rate from 0.2 to 0.1 and predictions of infinite-delay model.

particular instant in the past. The memory function takes the form

$$F(\gamma) = \delta(\gamma) \tag{7}$$

where δ is the Dirac delta function. This leads to the following set of delay-differential equations:

$$\dot{x} = \frac{\mu_m s(t-\tau)x}{k_s + s(t-\tau)} - Dx \tag{8}$$

$$\dot{s} = -\frac{1}{Y}\frac{\mu_m s(t)x}{k_s + s(t)} + D[s^0 - s(t)] \tag{9}$$

The method proposed in ref. 20 was used for numerical integration of these equations. The least-squares value of the delay τ was found to be approximately 3.3 h. Figure 5 shows that the response is in qualitative agreement with the data. Features like the overshoot and the fast initial rise in the biomass concentration are correctly predicted.

Neither the Monod model (Fig. 6), nor the infinite-delay model (Fig. 7), nor the model as described by equations (7)–(9) (Fig. 8) describes adequately the observed response of the culture when a step change in the dilution rate from 0.1 to 0.2 was performed. After analyzing the data it was concluded that after the dilution rate was stepped up, all growth had stalled. As Figure 9 proves, the drop in the biomass concentration corresponds exactly to the one expected for zero growth rate, leaving only the hydraulic effect of the culture being washed out of the fermentor.

It might seem surprising at first that growth should be arrested following a step up in the dilution rate, but this type of shock can be compared to the one where an inoculum is placed into new medium. Again, the culture reacts by entering a lag phase to adapt to the new environment. As the previous figures indicate, growth starts to recur after a certain time. This time is slightly higher than 3 h, which is similar to the length of the time lag for a step

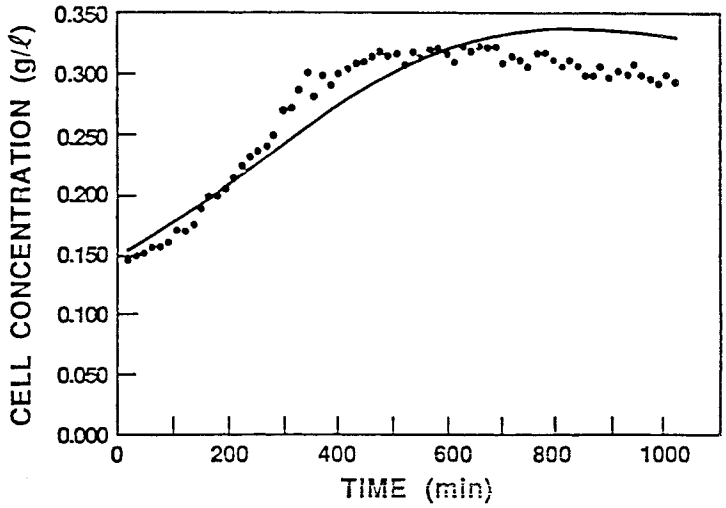

Figure 5. Response of biomass to step change in dilution rate from 0.2 to 0.1 and predictions of delay model (τ = 3.3).

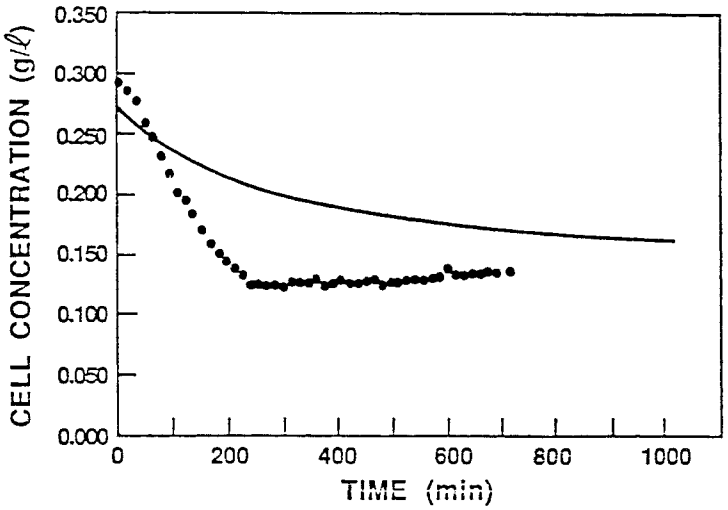

Figure 6. Response of biomass to step change in dilution rate from 0.1 to 0.2 and predictions of Monod model.

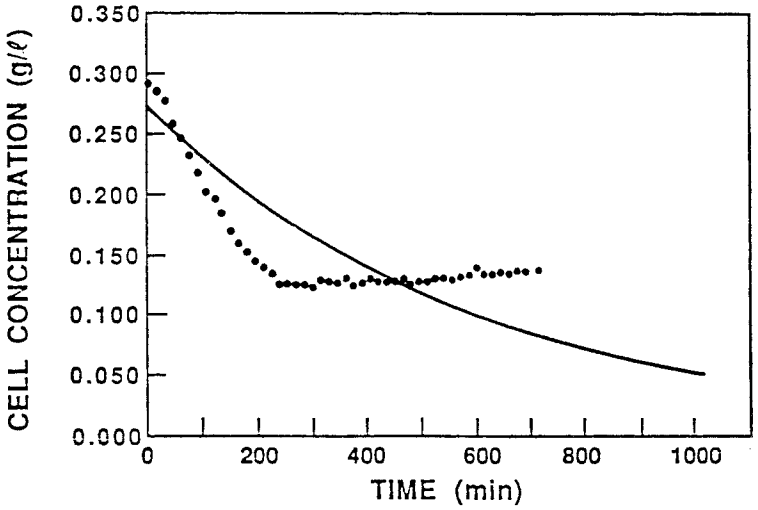

Figure 7. Response of biomass to step change in dilution rate from 0.1 to 0.2 and predictions of infinite-delay model.

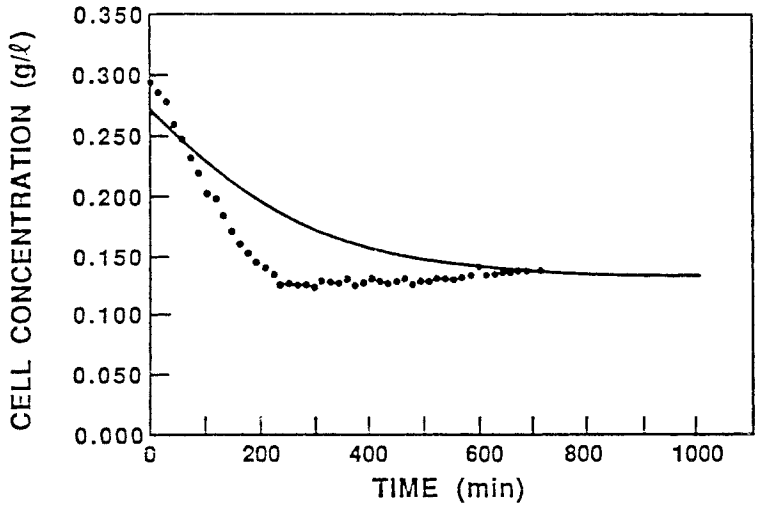

Figure 8. Response of biomass to step change in dilution rate from 0.1 to 0.2 and predictions of delay model (τ = 3.3).

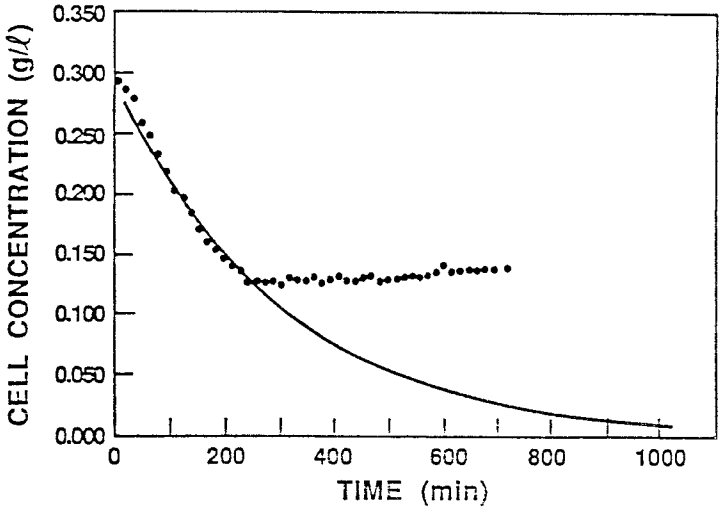

Figure 9. Response of biomass to step change in dilution rate from 0.1 to 0.2 and predictions for behavior of system at zero growth rate (washout).

change down in the dilution rate. Complete stalling of growth is most likely only to be expected for big sudden changes in the dilution rate.

There are several possible explanations for the observed time lag during transient operation. One relies on the fact that the length of the budding phase of the life-cycle of yeast does not depend on the growth conditions.[21] Consequently, the fraction of cells at that phase would not adapt quickly to changes in the growth environment. An alternate possibility is that growth is partly on glucose directly and partly on the produced ethanol.[22] Thus under transient conditions glucose may be converted to ethanol first, which may be later consumed for growth. Finally any catabolic or anabolic step may be rate limiting, causing a delay in the growth rate adaptation. Further research would be needed to establish the exact cause of the observed time lag.

RESPONSE OF SYSTEM TO PERIODIC VARIATIONS OF DILUTION RATE

Starting from a steady state corresponding to a dilution rate of 0.1, the system was subjected to square-wave variations in the dilution rate of different periods. The amplitude was equal to this dilution rate, i.e., switches from 0.2 to 0.0 h^{-1} (batch operation) were implemented, so that the total amount of substrate over a period was equal to the one at steady-state operation at a dilution rate of 0.1 h^{-1}.

Cycling with too high a period cannot result in improved reactor performance because, in essence, that means that the culture is operating at one steady state for half the time and at the other steady state for the remainder of the time. On the other hand, cycling at too low a period does not allow enough time for adjustment of the growth rate and thus is expected to give results close to those at steady-state operation. Unfortunately, as discussed in the previous section, a time delay model able to adequately fit both step-up and step-down experiments was not found. Consequently the theory developed in ref. 1 cannot be directly used to predict the optimal operating frequency. The clear presence, though, of a time delay in growth rate adaptation suggests that, according to ref. 1, periodic operation could indeed enhance the biomass productivity. From the step response experiments, which indicated a time lag of about 3 h, it is anticipated that a cycling period in the vicinity of 3 h would be expected to influence the performance of the system.

Figures 10–12 present the results (after all transients have subsided). Performance at each cycling period was repeated in two different runs to check the reproducibility of the results. While cycling with a period of either 2 or 4 h does not produce as much biomass as steady-state operation, cycling with a period of 3 h results in significantly improved reactor performance. Table IV lists the concentrations during the cycle and the productivities.

The improvement observed at a cycling period of 3 h (Fig. 11) is quite remarkable. During the half of the cycle when the dilution rate is equal to zero (essentially batch growth), the culture clearly is in a state of exponential growth, and during the remainder, when the dilution rate is high, growth has stalled. This is in agreement with the observations made in the step change experiments. The mag-

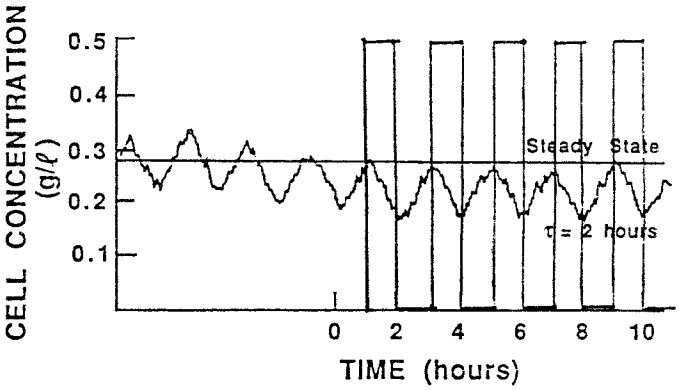

Figure 10. Biomass concentration vs. time for cycling period of 2 h. Dilution rate is varied between 0 and 0.2.

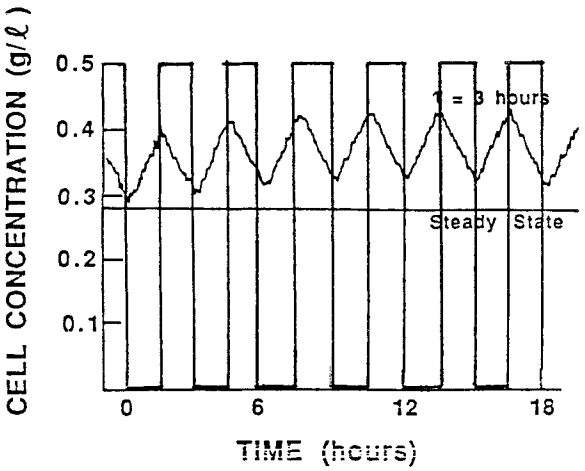

Figure 11. Biomass concentration vs. time for cycling period of 3 h. Dilution rate is varied between 0 and 0.2.

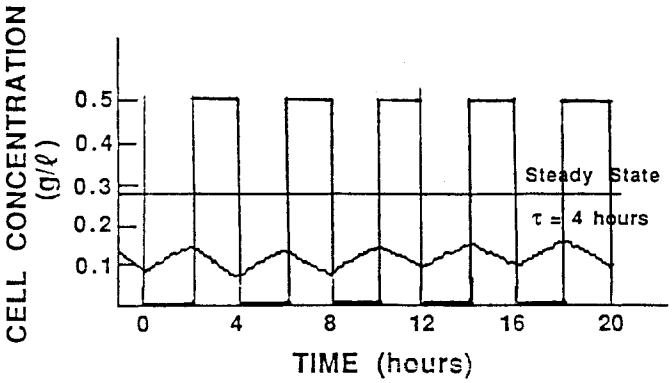

Figure 12. Biomass concentration vs. time for cycling period of 4 h. Dilution rate is varied between 0 and 0.2.

Table IV. Biomass concentrations and average productivities during cyclic reactor operation.

	Concentration (g/L)		
τ	Low	High	Productivity (g/h)
2	0.15	0.22	0.0518
3	0.29	0.40	0.0952
4	0.11	0.18	0.0435
Steady state (D = 0.1)	0.275	0.275	0.0756

nitude of the improvement might be explained by the fact that, in this case, a high (exponential) growth rate coincides with a high concentration of biomass at a time where there is ample substrate present.

At a cycling period of 4 hours, as Figure 12 shows, the culture is not in a state of exponential growth during the half of the cycle when batch operation prevails. The leveling-off of the slope indicates inhibition or limitation of growth, while once again, no growth is found for the rest of the cycle. The culture thus switches between a slow-growing state and a lag phase, not optimal conditions under which to operate. This results in low productivity at this cycling frequency. The reduced productivity at a period of 2 h (Fig. 10) might be caused by the fact that the culture is subjected to a change in the environmental conditions too frequently and does not have time to recover during the repeated shocks.

CONCLUSIONS

Periodic dilution rate variation of continuous yeast cultures can indeed result in enhanced average biomass productivities. The optimal cycling period is of similar magnitude to the time delay observed in growth rate adaptation when the culture is subjected to step changes in the dilution rate. Periodic operation allows high growth rates when the biomass concentration is high, something not possible under steady-state operation where high biomass concentrations are accompanied by low substrate concentrations and as a result low specific growth rates. Further work is needed to establish the exact causes of the observed time delay in the growth rate adaptation.

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NOMENCLATURE

- D dilution rate (h^{-1})
- F memory function
- k_s half-saturation constant (g/L)
- s substrate concentration (g/L)
- s^0 feed substrate concentration (g/L)
- t time (h)
- Y yield factor
- x biomass concentration (g/L)

Greek letters

- γ dummy variable
- δ Dirac delta function
- μ specific growth rate (h^{-1})
- μ_m maximum specific growth rate (h^{-1})
- τ time lag (h)

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