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# Process analysis in disturbed environment during oscillatory metabolism of *Saccharomyces cerevisiae*

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The yeast *Saccharomyces cerevisiae* exhibits sustained oscillations under noise-free controlled conditions in continuous cultures. The regular periodicity of the oscillations and synchrony between different metabolic variables may be described by mathematical models and model-based control. Large bioreactors, however, are prone to disturbances or noise in the feed stream, which may alter the oscillatory behaviour. This aspect has been investigated. Time-dependent Gaussian noise was applied to the substrate feed rate, and it was seen through simulations that while periodicity in the cell mass concentration was lost, other variables were less severely affected. A corollary observation was that the earlier synchrony among different variables, some intra-cellular and some extra-cellular, remained neither complete nor constant, indicating that intra-cellular processes are affected by external disturbances. Thus, deterministic models and control policies based on them may not be suitable in realistic industrial conditions, where intelligent heuristic approaches are more appropriate.

**Keywords**: *Saccharomyces cerevisiae*, inflow disturbances, oscillatory metabolism, process analysis.

**IPC Code: Int.Cl.[7]**: C12C11/00; C12R1:865

## Introduction

Inflow disturbances are an undesirable but common feature of microbial fermentations under production conditions. While batch fermentations are less likely to be affected by disturbances, kinetic and metabolic considerations favour fed-batch or continuous operation[1]. Inhibitions by glucose (beyond a certain concentration) and overflow metabolism are two key features, and their effects vary with both space and time in nonhomogeneous broths. Nevertheless, nonhomogeneity is unavoidable in large bioreactors and may even be beneficial under controlled situations[2,3].

White the size of the vessel, the type of impeller, the presence or absence of two or more phases and the rheological properties of the broth determine the spatial variations; the position and the mode of addition of the substrate influences the spatial distributions of cells[4] as well as the intra-cellular compositions and genetic responses to extra-cellular variations[5]. With the increasing use of control methods based on cell physiology and artificial intelligence, classic methods of substrate feed, such as the linear or polynomial or exponential rate, are being replaced by nonlinear rates that are more closely linked with metabolic and hydrodynamic changes while they occur[6,7]. Whereas, such feeding methods enable accurate control and high productivities, they also introduce rapid changes in the mixing patterns, which in turn affect substrate consumption, cell movements and growth, and intra-cellular metabolism.

Experimental data of feed rates and concentrations in bioreactors with spatial gradients[4,8,9] and of industrial scale fermentations subject to inflow disturbances[10,11] indicate that often it may be difficult to differentiate between fluctuations introduced by deliberate control actions and those arising through environmental disturbances. Therefore, not surprisingly, it has been difficult to propose simple and accurate dynamic models that are amenable to easy automation for complex microbial processes. Lumped models do not include all relevant features and are not sufficiently flexible, while distributed models employing fluid dynamic equations are too complex for on-line applications[12].

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Although, model-independent methods, such are expert systems, neural networks and genetic algorithms, provide one answer to the model-building problem for large bioreactors, they are essentially 'black box' devices that may not always have a strong fidelity to the physical and physiological processes. They can also be computationally demanding. So, a good compromise seems to be to combine mathematical models with the artificial intelligence methods mentioned above[13,14]. However, even for well-studied organisms, such as *Saccharomyces cerevisiae*[15] and *Penicillium chrysogenum*[16], different configurations of 'grey box' models, i.e. combinations of mathematical equations and artificial intelligence components, seem to work comparably well in the laboratory but it is not known whether these performances will be sustained under nonideal conditions. These observations underscore the need to analyze bioreactor performance adequately before developing hybrid models and control strategies.

A fundamental issue in any modeling effort is the identification of the principal variables and their behavior under the conditions of interest. However, for bioreactors subject to the disturbances possible in industrial operation, this aspect has received less attention than the kinetics and dynamics of 'clean' small-scale fermentations and their use for model-based control and applications of artificial intelligence. Since process identification is at the core of any bioreactor optimisation, this aspect is the subject of the present study. The more complex the fermentation, the more important it is to understand its behaviour before optimisation and control are attempted. The cultivation of *S. cerevisiae* for ethanol production was chosen from this perspective. It is an industrially important organism with a complex physiology, which displays spontaneous oscillations under certain conditions. The nature and existence of oscillations depend on the operating conditions[10,17] as well as the genetic make-up of the cells[18,19], and the exact reasons for the appearance and disappearance of oscillations are still under debate.

## Materials and Methods

### Fermentation Description and Data Generation

*S. cerevisiae* exhibits sustained oscillations in the cell mass concentration and those of key intra-cellular and extra-cellular components under certain conditions in continuous cultures. Owing to its industrial importance, complex but fascinating physiology, and both the need and difficulty of controlling oscillatory behaviour in production-scale bioreactors, different mechanisms and models have been proposed. The model used herein captures, without being too complex, most of the features observed in laboratory-scale fermentations.

Unlike the mechanistic approach of most workers, Jones and Kompala[17] proposed a cybernetic model. For the purpose of present study, only a brief outline of the model development will suffice. The cybernetic approach, first proposed by Ramkrishna[20], hypothesizes that a microorganism utilizes the available resources in such a way as to maximize its own survival. Mathematically this is usually expressed by maximizing the growth rate.

On this basis, Jones and Kompala[17] extended an earlier model[21] to include variations in dissolved oxygen concentration. Previous studies have shown that, with glucose as the main carbon source, there are three metabolic pathways. One is glucose fermentation, which produces a high growth rate and ethanol production. The second pathway is followed when glucose concentration is low; here the cells consume ethanol oxidatively. While these two metabolic routes are common to both batch and continuous cultures, a third, glucose oxidation is observed only in continuous operation. According to Jones and Kompala[17], the pathways follow Monod kinetics.

(a) Glucose fermentation

$$r_1 = \mu_1 e_1 \left( \frac{G}{K_1 + G} \right) \qquad \ldots(1)$$

(b) Ethanol oxidation

$$r_2 = \mu_2 e_2 \left( \frac{E}{K_2 + E} \right) \left( \frac{O}{K_{O_2} + O} \right) \qquad \ldots(2)$$

(c) Glucose oxidation

$$r_3 = \mu_3 e_3 \left( \frac{G}{K_3 + G} \right) \left( \frac{O}{K_{O_3} + O} \right) \qquad \ldots(3)$$

As seen above, each pathway has a 'key enzyme', $e_1$ or $e_2$ or $e_3$. The cybernetic method postulates that

the growth rate $r_i$ along a metabolic path is maximized when two sets of cybernetic variables, $u_i$ and $v_i$ (i = 1, 2, 3), follow the equations given below. A detailed explanation of the basis of these equations is too elaborate and not relevant to the present requirement. Kompala *et al*'s[21] paper may be consulted for more information.

$$u_i = \frac{r_i}{\sum_j r_j}; \quad i = 1,2,3 \qquad \ldots(4)$$

$$v_i = \frac{r_i}{\max_j r_j}; \quad i = 1,2,3 \qquad \ldots(5)$$

Briefly, the $u_i$'s control the enzyme synthesis rates and the $v_i$'s govern their activities.

Kompala *et al*[21] identified 8 key variables. These were the concentrations of cell mass, glucose, ethanol, oxygen and intra-cellular carbohydrate, and the activities of the three key enzymes. Storage carbohydrates, notably trehalose and glucogen, are accumulated inside the cells when there is deficiency of glucose and ethanol, and they are consumed if either of these substrates is present in appreciable quantities[17]. In a continuous culture, the dynamic mass balances for these variables follow the equation given below[17].

$$\frac{dX}{dt} = \left( \sum_i r_i v_i \right) - D \right) X \qquad \ldots(6)$$

$$\frac{dG}{dt} = (G_0 - G) D - \left( \frac{r_1 v_1}{Y_1} + \frac{r_3 v_3}{Y_3} \right) X - \phi_4 \left( C \frac{dX}{dt} + X \frac{dC}{dt} \right) \qquad \ldots(7)$$

$$\frac{dE}{dt} = -DE + \left( \phi_1 \frac{r_1 v_1}{Y_1} - \frac{r_2 v_2}{Y_2} \right) X \qquad \ldots(8)$$

$$\frac{dO}{dt} = k_L a (O^* - O) - \left( \phi_2 \frac{r_2 v_2}{Y_2} + \phi_3 \frac{r_3 v_3}{Y_3} \right) X \qquad \ldots(9)$$

$$\frac{de_i}{dt} = \alpha u_i \frac{S_i}{K_i + S_i} - \left( \sum_j (r_j v_j) + \beta \right) e_i + \alpha^* \qquad \ldots(10)$$

$$\frac{dC}{dt} = \gamma_3 r_3 v_3 - (\gamma_1 r_1 v_1 + \gamma_2 r_2 v_2) C - \sum_i (r_i v_i) C \qquad \ldots(11)$$

The term $\alpha^*$ in Eq. (10) was included on the recommendation of Turner and Ramkrishna[22], who showed that a small constitutive synthesis term was required in order to predict correctly the induction of enzymes that have been repressed for long durations.

The model is completed by adding the equations for the specific growth rates contained in Eqs (1)-(3).

$$\mu_i = \mu_{i,max} \left( \frac{\mu_{i,max} + \beta}{\alpha + \alpha^*} \right) \qquad \ldots(12)$$

Previous studies[10,11,23,24] have shown that the performances of continuous and fed-batch bioreactors are sensitive to disturbances in the inflow rate of the substrate, which is also a manipulated variable for control[25]. These disturbances may be modelled satisfactorily by Gaussian distributions with means equal to the instantaneous flow rates and time-dependent variances, optimised such that a prescribed objective function is maximized. In the cybernetic approach employed here for *S. cerevisiae*, the objective, as explained earlier, is the growth rate of biomass. So, to generate data mimicking a large-scale fermentation with disturbances in the feed stream, Gaussian noise of the kind described here was added to the dilution rate D in Eqs (6)-(8) and the full model, Eqs (1)-(12), was solved over the same duration of time, *i.e.* 76 hrs, as employed by Jones and Kompala[17]. The values of the parameters were also taken from their work and are listed in Table 1.

The use of simulated data generated, as described above from kinetic models developed experimentally in the laboratory, has been justified by many authors[13,14,26,27] for two reasons. Firstly, it circumvents the practical and proprietary restrictions on the availability of industrial data covering different scenarios. Secondly, simulations allow exploration of reactor performance under different conditions without damaging plant operation so as to evolve suitable optimisation and control policies for implementation[11,12].

**Results and Discussion**

Figs 1-3 show the time-domain profiles of biomass, glucose and ethanol, (a) when there are no disturbances in the feed stream and (b) when

persistent noise is present. A significant difference between Fig. 1 and the other two figures is that noise destroys the regular periodicity of the cell mass profile but not those of glucose and ethanol. The profiles for carbohydrates and dissolved oxygen (not shown) were also not significantly affected by the inflow noise.

These observations have important implications for the identification of process characteristics[28], mechanism of oscillations and control of the bioreactor. It is evident that cell mass concentrations are the most sensitive to the presence of noise. On comparing the deterministic (noise-free) plots of this variable with those of others (Figs 1-3), at least three differences may be noted. Firstly, the cell mass concentrations are one to three orders of magnitude larger than other concentrations. Secondly, the variations in the cell mass concentrations without noise, measured in terms of the changing amplitude as a percentage of the mean value, are much smaller than for other variables. Thirdly, cells are discrete entities, whereas ethanol, glucose, dissolved oxygen and carbohydrate (within the domain of a cell) are continuous variables. The first two differences and similar results for another ethanol-producing organism, *Zymomonas mobilis*, which also exhibits oscillations in continuous cultures[29], suggest that low-amplitude oscillations whose mean values are large compared to those of other variables in the system are more likely to be destabilized by inflow disturbances. This inference, however, requires further substantiation.

The difference in the effect of variations in the substrate feed rate on the performance variables were corroborated by their fractal dimensions in the case of *Z. mobilis*[30]. Similar support has been provided here for *S. cerevisiae* through periodograms and statistical measures. The closeness of the noise-free and noise-affected profiles in Figs 2 and 3 and the preservation of periodicity are reflected in the narrow and practically indistinguishable periodograms (Figs 5 & 6). On the contrary, the two periodograms for cell mass concentration (Fig. 4) are wider and clearly separated. Similar inferences also follow from the statistics in Table 2. Although, the Pearson's correlation coefficients[31] are positive for all variables, the value for the cell mass is a quarter of the other values. Moreover, the P-value for cell mass is larger than 0.05, whereas the others are nearly zero. These observations suggest that inflow disturbances upset the deterministic periodic changes in the cell mass concentrations more strongly than they do for glucose and ethanol.

Table 1 — Values of the parameters (from Jones and Kompala[17])

| Parameter | Units | Value |
|---|---|---|
| $\alpha$ | $g\ g^{-1}\ hr^{-1}$ | 0.3 |
| $\alpha^*$ | $g\ g^{-1}\ hr^{-1}$ | 0.1 |
| $\beta$ | $hr^{-1}$ | 0.7 |
| $\mu_{1,\max}$ | $hr^{-1}$ | 0.44 |
| $\mu_{2,\max}$ | $hr^{-1}$ | 0.19 |
| $\mu_{3,\max}$ | $hr^{-1}$ | 0.36 |
| $\phi_1$ | $g\ g^{-1}$ | 0.403 |
| $\phi_2$ | $mg\ g^{-1}$ | 2000.0 |
| $\phi_3$ | $mg\ g^{-1}$ | 1000.0 |
| $\phi_4$ | $g\ g^{-1}$ | 0.95 |
| $\gamma_1$ | $g\ g^{-1}$ | 10.0 |
| $\gamma_2$ | $g\ g^{-1}$ | 10.0 |
| $\gamma_3$ | $g\ g^{-1}$ | 0.8 |
| $G_0$ | $g\ l^{-1}$ | 28.0 |
| $K_1$ | $g\ l^{-1}$ | 0.05 |
| $K_2$ | $g\ l^{-1}$ | 0.01 |
| $K_3$ | $g\ l^{-1}$ | 0.001 |
| $K_{o_2}$ | $mg\ l^{-1}$ | 0.01 |
| $K_{o_3}$ | $mg\ l^{-1}$ | 2.2 |
| $k_La$ | $hr^{-1}$ | 225.0 |
| $O^*$ | $mg\ l^{-1}$ | 7.5 |

For fermentations without disturbances in the feed stream, some research workers[32,33] have explained the observed oscillations in continuous cultures of *S. cerevisiae* by attributing them to cell cycle synchrony. However, the annihilation of periodicity in cell mass concentration, while largely preserving this feature for other variables when disturbances occur, suggests that other causes are possible. Jones and Kompala[17] also

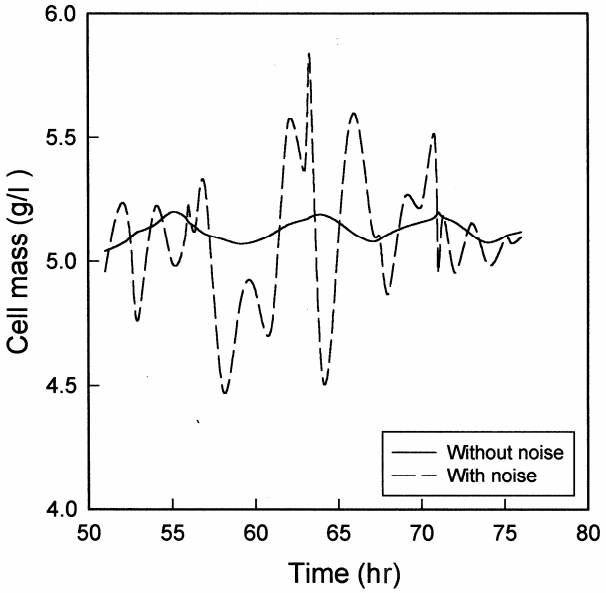


Fig. 1 — Profiles of cell mass concentration without noise and with Gaussian noise in the substrate flow rate.

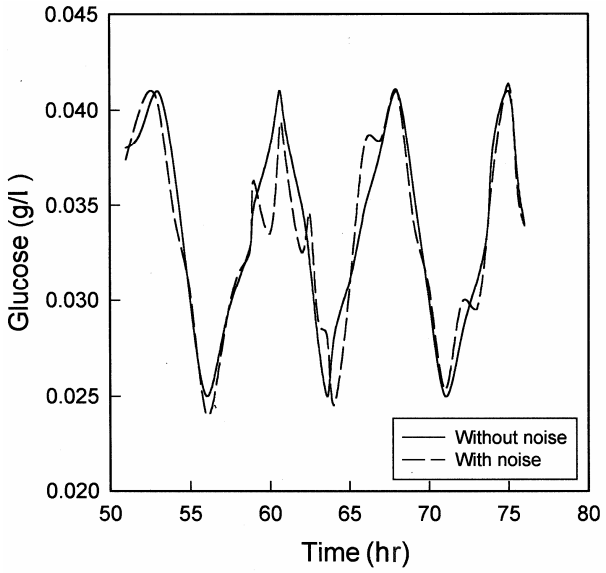


Fig. 2 — Profiles of glucose concentration without noise and with Gaussian noise in the substrate flow rate.

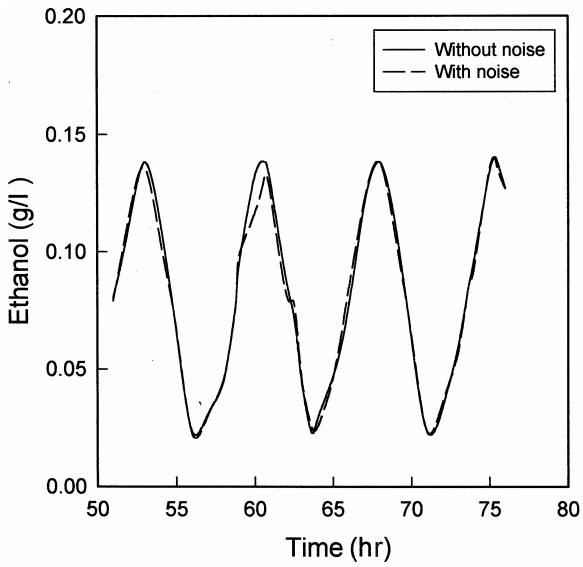


Fig. 3 — Profiles of ethanol concentration without noise and with Gaussian noise in the substrate flow rate.

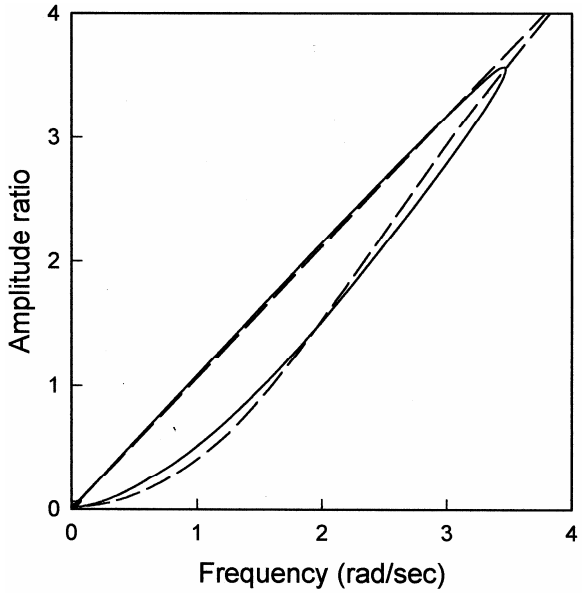


Fig. 4 — Periodograms for cell mass concentration. The continuous plot is without noise, while the discontinuous plot is for a noise-affected fermentation.

shared this view and proposed that dynamic competition between the three metabolic pathways—glucose fermentation, ethanol oxidation and glucose oxidation—is the main cause of oscillations. Fluctuations in the feed stream cause rapid changes in the relative values of the fluxes through these pathways, and the present results indicate that the cells are not able to respond and adapt fast enough to these changes. This leads to a weakening of cell cycle synchrony (Fig. 1). A similar loss of periodicity was also observed for the specific growth rate (not shown), which is in consonance with Jones and Kompala's[17] thesis that a cyclic variation in the growth rate is the main driving force for cell cycle synchrony.

The inability of the cells to keep pace with rapid changes in their environment has also been proposed to explain the behavior of *Escherichia coli*

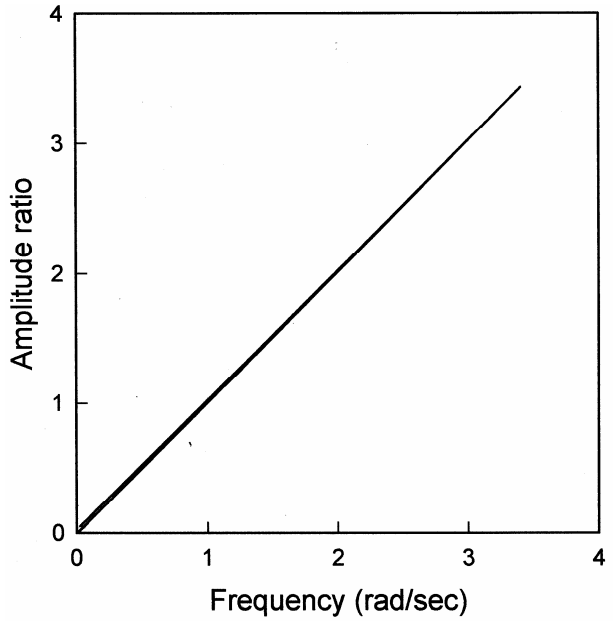


Fig. 5 — Periodograms for glucose concentration. The two periodograms, with noise and without noise, are almost coincident and therefore indistinguishable.

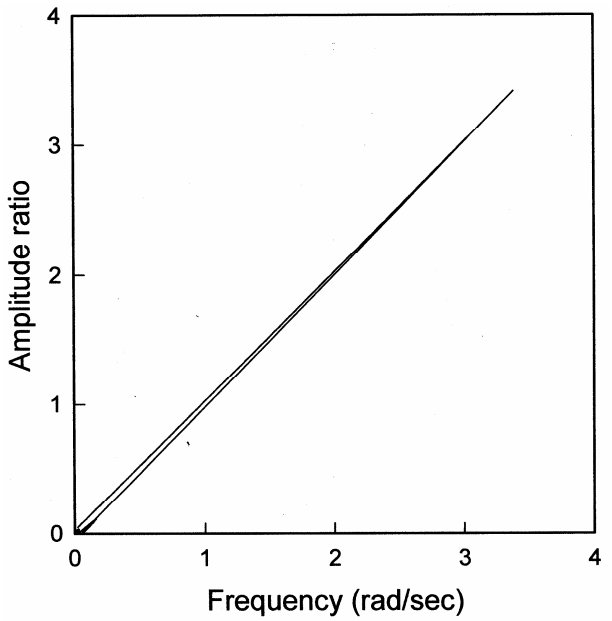


Fig. 6 — Periodograms for ethanol concentration. As in Fig. 5, the disturbance-free and disturbance-affected periodograms are indistinguishable.

fermentations subjected to either deliberate cycling in the feed rate[34] or environmental disturbances affecting the feed rate[24]. Since both *E. coli* and *S. cerevisiae* exhibit overflow metabolism and anaerobic metabolism, and are, therefore, sensitive to excess glucose as well as oxygen limitation[1,8]; the differences in the effects of feed rate fluctuations on cell growth and on glucose and oxygen concentrations in the broth have important implications for bioreactor control. Although, a discussion of the control of oscillatory fermentations is not within the scope of the study, it is useful to recognize some implications of the preceding observation:

(1) The changes in periodic behaviour induced by disturbances may render any model-based deterministic control algorithm inappropriate or non-optimal.

(2) Hybrid control strategies should be preferred under such nonideal conditions[7,13]; however, differences in the time-dependences of different variables (Figs 1-3) require unequal sampling intervals and piecewise optimisation over successive time slices[35].

(3) While the flow rate of glucose is an established manipulated variable[25], the dissolved oxygen concentration may be controlled through the airflow rate or the stirring speed. Since these two variables maintain substantial synchrony between them (Figs 2 & 3) even in the presence of noise but lose synchrony with the cell mass (Fig. 1), any control system should accommodate this kind of partial and variable synchrony. Intelligent heuristic systems are better equipped to handle such situations than model-based controllers[35,36].

Table 2 — Statistical measures correlating noise-free and noise-affected concentrations

| Concentration variable | Pearson's correlation coefficient | P-value |
|---|---|---|
| Cell mass | 0.262 | 0.135 |
| Dissolved oxygen | 0.975 | $10^{-22}$ |
| Glucose | 0.937 | $10^{-15}$ |
| Carbohydrate | 0.992 | $10^{-28}$ |
| Ethanol | 0.994 | $10^{-31}$ |

## Conclusions

Under disturbance-free laboratory conditions, the yeast *S. cerevisiae* shows steady oscillations with time

in continuous cultures. While some authors[32,33] have attributed this to cell synchrony, others[17,21] consider dynamic competition between three metabolic pathways to be the cause and cell synchrony a consequence of this.

As important as the debate on the mechanism of metabolic oscillations is its manifestation under the disturbed conditions that occur in industrial environments. Since it is known that bacterial fermentations, both oscillatory[29,30] and non-oscillatory[8,24], perform differently in 'ideal' conditions and while disturbances occur, the present investigation has been applied to oscillating continuous cultures of a budding yeast. To simulate industrial conditions, Gaussian noise was added to the substrate flow rate as explained in the text.

For a fermentation displaying periodic variations in the concentrations of cell mass, substrate (glucose), product (ethanol), storage carbohydrate and dissolved oxygen, the onset of disturbances destroyed periodicity in the cell mass concentration but did not severely affect the other variables. This is not to suggest that the other concentrations were unaffected but it indicates that the cell mass is the most sensitive variable to be monitored and controlled. These differences were corroborated by periodograms and statistical measures. While the noise-free and noise-affected periodograms of the cell mass concentration were wide, conspicuously elliptical and distinguishable, these pairs for the other concentrations were narrow and indistinguishable.

Since the metabolic processes involve interactions among all variables, the differences in the effects of feed fluctuations have important implications for bioreactor monitoring and control. The complexity of yeast metabolism and the time-varying degrees of synchrony among the oscillations in different variables suggest that intelligent heuristic controllers will work better than model-based controllers in production conditions[7,35,36].

## Nomenclature

- $C$ intra-cellular storage carbohydrate concentration (g $g^{-1}$ biomass)
- $D$ dilution rate ($hr^{-1}$)
- $e_i$ key enzyme concentration for i-th pathway (g $g^{-1}$ biomass)
- $E$ ethanol concentration (g $l^{-1}$)
- $G$ glucose concentration (g $l^{-1}$)
- $G_0$ inlet glucose concentration (g $l^{-1}$)
- $K_i$ Michaelis constant for i-th pathway (g $l^{-1}$)
- $K_{o_2}$ saturation constant for ethanol oxidation (mg $l^{-1}$)
- $K_{o_3}$ saturation constant for glucose oxidation (mg $l^{-1}$)
- $k_La$ oxygen mass transfer coefficient ($hr^{-1}$)
- $O$ dissolved oxygen concentration (mg $l^{-1}$)
- $O^*$ dissolved oxygen solubility limit (mg $l^{-1}$)
- $r_i$ cell growth rate for i-th pathway ($hr^{-1}$)
- $S_i$ carbon substrate concentration for i-th pathway (g $l^{-1}$)
- $t$ time (hr)
- $u_i$ i-th pathway cybernetic variable controlling enzyme synthesis (–)
- $v_i$ i-th pathway cybernetic variable controlling enzyme activity (–)
- $X$ cell mass concentration (g $l^{-1}$)
- $Y_i$ yield coefficient for i-th pathway (g biomass $g^{-1}$ substrate)

## Greek letters

- $\alpha$ specific enzyme synthesis rate ($hr^{-1}$)
- $\alpha^*$ constitute enzyme synthesis rate ($hr^{-1}$)
- $\beta$ specific enzyme degradation rate ($hr^{-1}$)
- $\phi_i, \gamma_i$ stoichiometric parameters (g $g^{-1}$)
- $\mu_i$ specific growth rate for i-th pathway ($hr^{-1}$)
- $\mu_{i,max}$ maximum specific growth rate for i-th pathway ($hr^{-1}$)

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