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"Modeling Interpretation of Microbe Metabolism Detected by Nuclear Magnetic Resonance"

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Modeling Interpretation of Microbe Metabolism Detected by Nuclear Magnetic Resonance

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A new modeling approach is discussed for the analysis of microbial dynamics. It is based on structurally non linear compartmental models and on the dynamics of the substrate and product. Experimental data were acquired by NMR spectroscopy which is a non-invasive technique. This combined approach was tested with the fermentation of glucose to ethanol by *Saccharomyces cerevisiae*. The model was fitted with the experimental data to obtain the values of the parameters of the model. Similar processes can be analyzed and compared using this approach.

Classical methods of biological investigation have revealed the general trends of metabolic pathways. The information that can be obtained by traditional experimental approaches is not sufficient when we want to clarify the relationships between the various components of a system. For example, microbe metabolism is generally analyzed using Michaelis-Menten dynamics to describe experimental data. This type of analysis only gives information on the speed of the reaction and the rate of degradation of the substrate, which is insufficient when we need information about interactions between cells, precursors and products.

Although much data are now available on the solution structures of different biological components, such as proteins (1-2), nucleic acids (3) and membranes, little is known about the details of cell organization and the role of each constituent in modulating the chemical properties of bio-structures. Future research should aim to define chemical properties at molecular level, and to clarify the upper levels of biomolecular organization, namely, that of cells and organisms. The biological significance of each bio-constituent can be completely understood only by considering the complex network of interactions of micro and macro components that take place within the system. Tracer techniques and mathematical modeling, applied to pharmacokinetics and physiology, can be used to study microbial metabolic processes (4).

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In this study we analyzed the metabolism of the yeast *Saccharomyces cerevisiae* by ^{13}C-NMR *in vivo* spectroscopy using the compartmental models proposed by Odum (5). These models have been successfully used for the description of macroscopic ecological systems using the concept of energy flows through different compartments. We therefore used a given structure of the model to fit the experimental data and to estimate the model parameters.

Our interest on the metabolism of *Saccharomyces cerevisiae* is due to its efficiency in converting glucose to ethanol. Bioethanol produced by sugar fermentation is a current biofuel, and the investigation of biological processes for biofuel production from agricultural residues such as sugar, starch, cellulose and hemicellulose is of considerable interest. The replacement of fossil fuels with biofuels is an urgent priority in efforts to stabilize fundamental parameters of the biosphere such as carbon dioxide (CO_2) concentration and global temperature.

We believe that our investigation will contribute to a better understanding of biological organization and can be of some utility for biotechnological applications.

MATERIALS AND METHODS

^{13}C-NMR spectra were recorded with a Varian XL-200 spectrometer operating at 200.085 MHz and 50.288 MHz for proton and carbon respectively. Carbon spectra were recorded under broad-band proton decoupling conditions using a low power MLEV-16 pulse in order to avoid sample temperature effects. 10 mm coaxial tubes, containing 99.75% D_2O in the outer part, were used for the NMR measurements. The ^{13}C-NMR were recorded in blocks of 10 minutes until the end of the fermentation process.

Saccharomyces cerevisiae strain KL-144A was grown at 31 °C in a liquid medium containing 6 g yeast extract, 0.5 g L-cysteine HCl, 5.6 g KH_2PO_4, 7 g K_2HPO_4, 1.0 g/l $NaHCO_3$, 1.5 g $(NH_4)_2SO_4$, 0.15 g $MgCl_2 \cdot 6H_2O$, 0.01 g $FeSO_4 \cdot 6H_2O$, 3 g/l sodium citrate$\cdot 2H_2O$. A density of 2.5×10^9 cell/ml was used for each sample analyzed. A pH of 6.5 was used in all determinations and kept constant during the fermentation process. The initial glucose concentrations were 85 and 200 g/l. The number of transients necessary for a spectrum with a high signal to noise ratio was reduced by adding 5 g/l of [1-^{13}C] 90% enriched glucose (from Stohler Isotopic Chemicals) to each sample.

MODEL

The model we used to describe this process (Figure 1) consists of storages of: glucose, the substrate; ethanol, the main reaction product; active cells, that transform glucose; "inhibited cells", the yeast cells inhibited by ethanol as its concentration increases. The model considers

only ethanol production, neglecting glycerol (1-2 % the total end products), which we assumed not able to effect the metabolic process.

Glucose consumption is described as the overlap of two kinetic processes: an autocatalytic one (box 1), and the other dependent only on the active cell concentration (box 2). Yeast activity and fermentation can be modeled as flows from the interactions between glucose and the active cells, meaning that part of the glucose is used by the yeast for the production of ethanol (respiration), and the remainder for feeding cell activity. Inhibition of the ability of yeast to convert glucose, due to the presence of ethanol, has been modeled as an outflow from the storage of active to the inhibited cells. This is proportional to the ethanol concentration which acts as controller (which is the meaning of the box under the storage symbol).

The differential equations derived from the model are:

$$\frac{dG}{dt} = -k_{1d} \cdot G \cdot C - k_{2d} \cdot C$$

$$\frac{dC}{dt} = k_{1a} \cdot G \cdot C + k_{2a} \cdot C - k_i \cdot C \cdot E$$

$$\frac{dE}{dt} = k_{1p} \cdot G \cdot C + k_{2p} \cdot C$$

where G is glucose concentration (g/l), C is an index related to the metabolic efficiency of the active cells, and E is ethanol concentration (g/l). k_{1d}, k_{1a} and k_{1p} are respectively, in the autocatalytic pathway, the kinetic constants of the glucose degradation, of the activation of the yeast cells and of the ethanol production; k_{2d}, k_{2a} and k_{2p} have the same meanings in the pathway dependent only on the cell activity; k_i is the kinetic constant of the ethanol inhibition affecting cell activation. It is evident that the autocatalytic part is relatively more important at the beginning of the transformation process, in relation to the quantity of glucose in the system. The part that is proportional to the number of cells becomes dominant with respect to the other towards the end, meaning that part of the glucose is consumed (and thus part of the ethanol is produced) as a "required minimum" for the cells.

In developing the model, all the parameters $k_{j\alpha}$ (j=1,2; α=d,a,i,p) were constrained to be non negative. For energy reasons there is the requirement (to be tested *a posteriori*) that k_{1d} must be greater than (k_{1a} + k_{1p}) and k_{2d} greater than (k_{2a} + k_{2p}), i.e. products cannot have greater energy contents than the substrates.

RESULTS AND DISCUSSION

The present metabolic model was tested in relation to experimental results. The experimental description of the metabolic process was obtained using in vivo NMR spectroscopy and ^{13}C enriched substrate. The NMR approach provides a step-by-step description of how the ^{13}C-labeled atom is transferred along the metabolic pathway and precise information on the amount of substrate consumed and ethanol yielded (6-7).

Two samples containing 85 and 200 g/l of sugar substrate respectively, were analyzed. In this range of concentrations, the cell culture behaved in a homogeneous manner. Higher (250-280 g/l) and lower (5-20 g/l) substrate concentrations were avoided to eliminate effects such as changes in the physical condition of the sample (viscosity or quenching of the metabolic rates caused by too low initial substrate concentrations, etc.).

Figure 2 shows the ^{13}C-NMR spectra which describe the sugar metabolization process (A) and ethanol production (B). It emerges that the [1-^{13}C] glucose labeled carbon is transferred to the methyl carbon of the ethanol.

A nonlinear least-squares estimation technique (8) as implemented in the MLAB computer program (9) (using the Marquardt-Levenberg method) was used to estimate the unknown parameters $k_{j\alpha}$ (j=1,2; α=d,a,i,p). The data sets of glucose and ethanol obtained in the two experiments were simultaneously used in the parameter estimation (the use of individual set of data failed to estimate properly model parameters). The results of the fitting procedure follow the experimental data very closely, as shown in Figures 3 and 4. A small bias can be recognized in the fit of data from experiment 1 (initial sugar concentration equal to 85 g/l). However, the goal of the study was not to find the best model to fit the experimental data, but the best estimate of parameters having a precise physiological meaning.

Table 1 shows the optimal values of the estimated parameters and their coefficient of variation (CV). R^2 was 0.997. The values of the parameters were determined utilizing only the glucose and ethanol data from experiment 1 and 2, since the true number of active cells and the real level of activation of the cells can not be measured. The model reflects the activity of the yeast which increases and then decreases when the effect of inhibition caused by ethanol becomes dominant. The results reported in Table 1 are useful for interpreting the yeast biochemical process. It is important to note the null value of k_{2a}, meaning that no further cells

are activated when the amount of glucose is close to zero and the minimum quantity of sugar available is used only for respiration. This is an important result of the model and it is not due to external constraints to the fitting procedure. Other parameters, e.g. k_{2d} and k_{2p} reported in Table 1, have precise biophysical meaning: the rate of consumption of glucose per cell and the rate of production of ethanol per cell respectively, when the amount of glucose available is close to zero. From Table 1 the requirement that $k_{jd} > (k_{ja} + k_{jp})$ is also verified.

A close correlation between each component of the model and the biochemical function of the cells could be identified. In particular the constituents of box1 and box2 show the existence of "biological effectors", constituted by single proteins, enzyme systems, or bio-structures with a complex dynamics, to be identified.

The flexible structure of this model enables it to be applied in many similar cases. The constants of the model can constitute a useful tool for the comparison of microorganism performances, along the different pathways, thereby helping those scientists who try to improve the performances of engineered microbes.

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Table 1

Estimated values and coefficients of variation (CV) of the kinetics parameters.

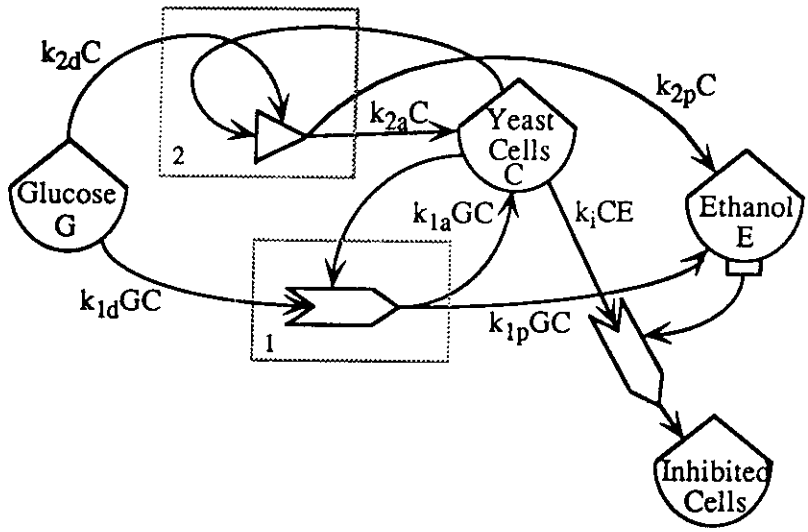
Kinetic Constant	Glucose 85-200 g/l	CV
k_{1d}	3.992E-4	7%
k_{2d}	4.253E-2	3%
k_{1a}	1.284E-5	13%
k_{2a}	0	-
k_i	1.471E-5	43%
k_{1p}	2.058E-4	7%
k_{2p}	9.019E-3	10%

Captions to figures

Figure 1. Diagram of the model of the fermentation process.

Figure 2. NMR spectra of glucose and ethanol in relation to time.

Figure 3. Results of the simultaneous fit of data from experiment 1 (initial glucose concentration of 85 g/l) and 2 (initial glucose concentration of 200 g/l). Panel A; experiment 1: comparison of experimental data (ethanol=closed triangles; glucose=open triangles) and results of fit (continuous lines). Panel B; experiment 2: comparison of experimental data (ethanol=closed circles; glucose=open circles) and results of fit (continuous lines).


$k_{2d}C$
$k_{2a}C$
$k_{2p}C$
Yeast Cells C
Glucose G
Ethanol E
$k_{1a}GC$
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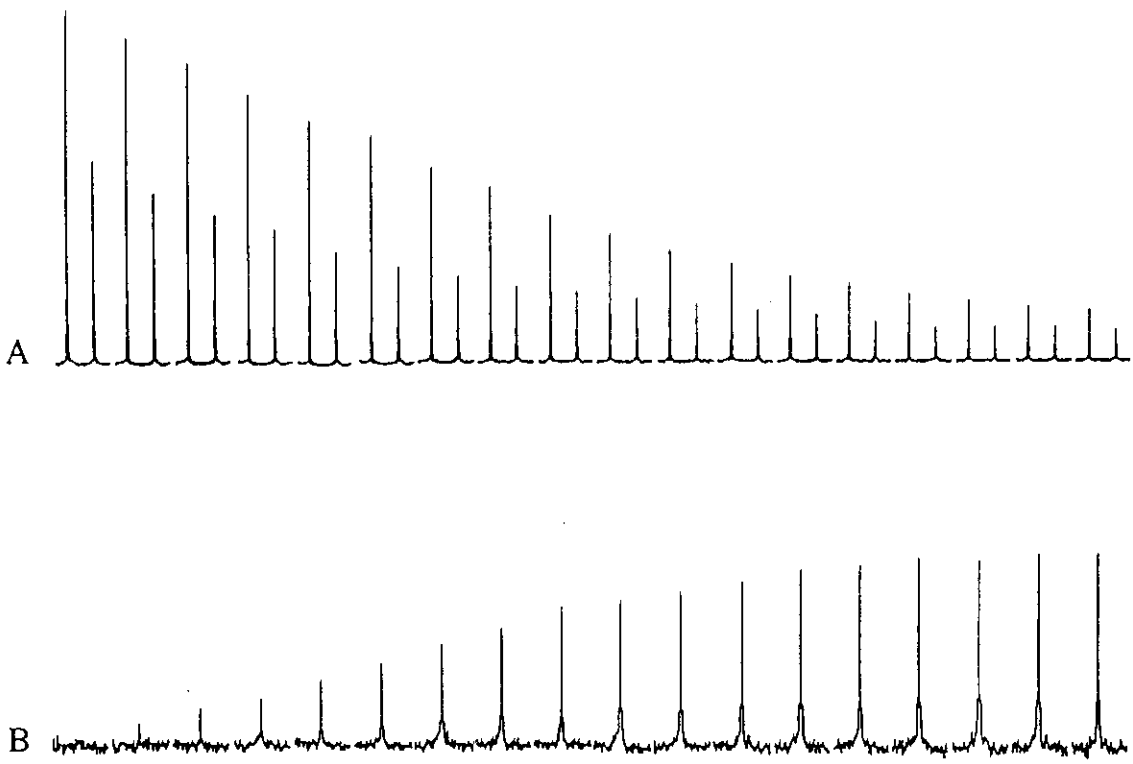
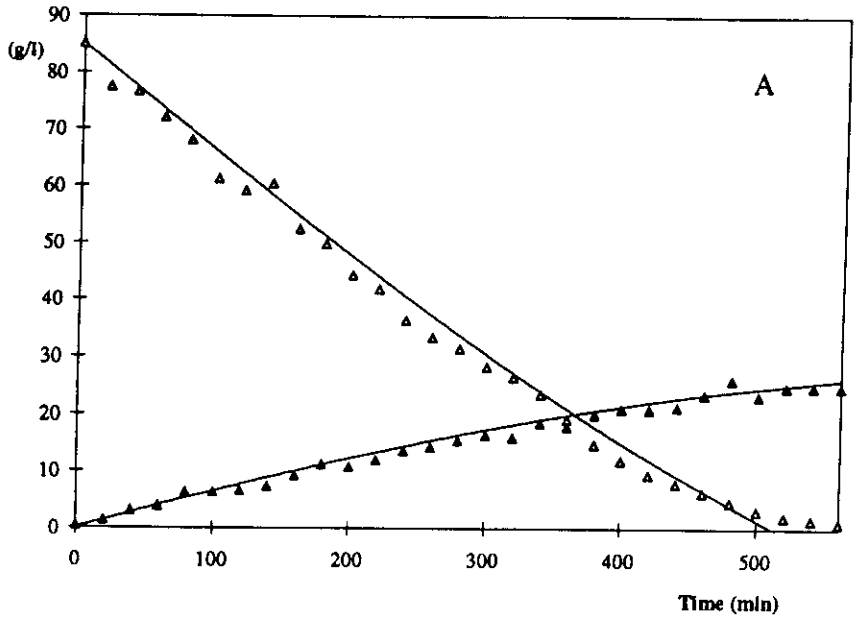
$k_{1d}GC$
$k_{1p}GC$
2
1
Inhibited Cells


A
B


(g/l)
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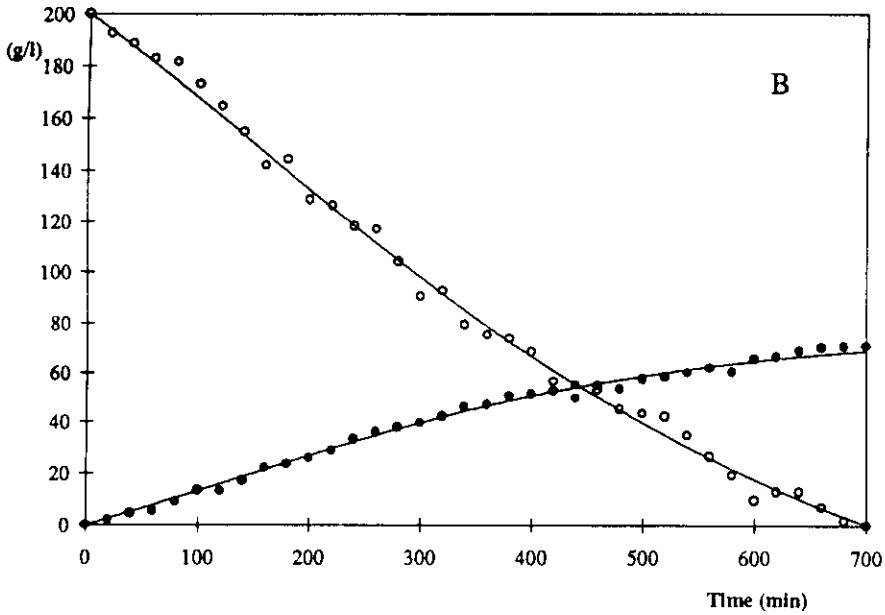
60
50
40
30
20
10
0
A
0
100
200
300
400
500
Time (min)


(g/l)
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B
0
100
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500
600
700
Time (min)