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Combined Spectroscopy and Modelling Analysis of Sugar-Ethanol Bioconversion

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SUMMARY

In this paper we analyzed the metabolism of mesophilic *Saccharomyces cerevisiae* yeast by combining experimental ^{13}C-NMR spectroscopy results with a modelling approach based on energy flows through different compartments. The growth of the yeast is described as the combination of an autocatalytic process driven by a limited source and a flow from the sugar storage proportional to the number of cells. The inhibition of glucose convertion, due to the presence of the ethanol, is an outflow from the storage of yeast cells, proportional to the quantity of ethanol, that acts as a controller.
Inserting the NMR data in the model gave us the values of the constants which describe the flows of energy.
This approach was used for investigating glucose fermentation by *Saccharomyces cerevisiae* for a wide-range of initial substrate concentrations. The methodology was then extended to the more complex metabolization of xylose by a strain of *Klebsiella planticola*.

INTRODUCTION

The cell is the fundamental constituent of higher organisms and in unicellular species it still contains sub-cellular structures with their specialized functions. Different approaches have been used for investigating cell organization and functions. These include study of cell cycles, uptake processes, energy metabolism, communications with other cells, storage of genetic information and so forth. These studies have mainly been based on biochemical, chemical and physical methodologies. Many elegant experiments have revelead in detail how enzymes work and the cooperative interactions between macromolecules and subcellular structures. Crystallographic and spectroscopic investigations have also clarified the molecular organization of proteins, nucleics acids, polysaccharides and other biostructures[1-6]. In many cases, knowledge of structure has revealed biological role and function; for instance, the resolution of the crystal structure of DNA revealed the genetic role of nucleic acids and how they work. However, besides the investigation of the cell organization at molecular level, methods of studying a second level of biomolecular organization of the cell need to be developed. In fact, the biological significance of each cellular bio-constituent can only be completely understood by considering the complex network of interactions that take place within the system[7].
Cells are dynamical systems, maintained far from equilibrium by external energy sources and by their ability to dissipate the entropy produced during the metabolic process outside the system[8]. The use of classical tools, such as thermodynamics, does not allow complete understanding of the dynamical equilibrium that occurs in a cell. Innovative points of view and methodologies are needed to investigate the global organization of highly complex systems and their behaviour in response to external stimuli.
In this paper we investigate the anaerobic metabolism of a mesophilic yeast, *Saccharomyces cerevisiae* KL 144A, by combing modelling analysis and experimental results. Metabolic processes involve the transformation of energy through different metabolic pathways. These processes can be described by the compartmental models proposed by Odum[9]. The present approach has been successfully used to describe the behaviour of macroscopic ecological systems through energy flows between different compartments. The model used to study *Saccharomyces cerevisiae* metabolism studies describes energy flows between system compartments to produce new energy structures through structurally non linear processes[10].
The experimental data was obtained by "in-vivo" carbon-13 Nuclear Magnetic Resonance Spectroscopy of the yeast, cultured in the presence of selectively carbon-13 enriched substrates. An extension of this methodology for the investigation of xylose fermentation to ethanol by *Klebsiella planticola* G11 was also considered[11].
Our interest in ethanol production by fermentation of sugar lies in its potential as an alternative to fossil fuels to slow down atmospheric accumulation of CO_2. This research involves efforts to obtain better yields in the fermentation process and to identify of appropriate microrganisms. The present investigation should help to provide a new view of the metabolic processes and to define kinetic parameters useful for identifying the best conditions of fermentation, namely those under which energy dissipation during bioconversion is reduced.

MATERIALS AND METHODS

Sample Preparation

Saccharomyces cerevisiae strain KL-144A was grown at 31°C in a liquid medium containing 6g yeast extract, 0.5g L-cysteine.HCl, 5.6g KH_2PO_4, 7g K_2HPO_4, 1.5g $(NH_4)_2SO_4$, 0.15g $MgCl_2$-$6H_2O$, 0.01g $FeSO_4.6H_2O$ and 3g sodium citrate.$2H_2O$ per liter. Samples for spectroscopy analysis had a cell density of $2x10^9$ cells/ml, a pH of 6.5 and glucose concentration of 85, 200 and 280 g/l respectively. Each sample contained 5 g/l of [1-^{13}C] 90% enriched glucose (from Stohler Isotopic Chemicals) in order to reduce the number of transients necessary for spectra with high signal-to-noise ratios. ^{13}C-selective enrichment of the substrate also allows the transfer process of ^{13}C isotopes to the end-products to be followed.

Klebsiella planticola G11 was isolated from soil samples collected near Siena (Italy) in an area where corn has been cultivated for 30 years[11].

Several procedures were used to identify the strain. These included the Enterobacteriaceae identification test, "rapid ID32E2" (BioMerieux, Lyon-France) with API automatic detection (BioMerieux, Lyon-France). Growth at 10°C, gas production at 44.5°C and melizitose fermentation tests were positive, negative and negative, respectively, leading to the final identification of the bacterium as a strain of *Klebsiella planticola* according to Bergey's classification[12].

Klebsiella planticola G11 was grown in flasks at 35°C in a nitrogen atmosphere. The culture medium consisted of 5.25 g/l KH_2PO_4, 6.85g/l K_2HPO_4, 5g/l $NaHCO_3$, 0.1 g/l $MgSO_4$, 0.1 g/l NaCl, 0.2 g/l $(NH_4)_2SO_4$, 0.3 g/l urea, 0.02g/l $CaCl_2$, 0.2 g/l yeast extract and 10 g/l xylose unless otherwise stated. The following trace elements were also present: Fe, Cu, Co, Mo and Mn. The pH of the medium was adjusted to 7.5. Growth was monitored in terms of spectrophotometric optical density (O.D.) at 660 nm.

^{13}C-NMR spectra were recorded with a Varian XL-200 spectrometer operating at 200.058 and 50.288 MHz for proton and carbon nuclei respectively. Carbon spectra were recorded under conditions of broad-band proton decoupling. In order to avoid temperature changes, decoupling was obtained using the Waltz-16 pulse sequence. The microbatch for the spectroscopy measurements was placed in a coaxial tube with 100% D_2O as NMR lock signal in the external section. All chemical shifts were expressed in relation to tetramethylsilane. Identification of the end-products of fermentation was based on carbon chemical shift considerations.

The Model

The model used to describe sugar metabolization by *Saccharomyces cerevisiae* and *Klebsiella planticola* is shown in Figure 1. The model consists of four storage structures or "tanks" for sugar substrates, ethanol, active cells and the inhibited cells, (cells inhibited by the end-products as they increase).

The model is only concerned with the production of ethanol, the main product of glucose and xylose fermentation by *Saccharomyces cerevisiae* and *Klebsiella planticola* respectively.

The sugar metabolization is defined as the result of two simultaneous processes, an autocatalytic step (route 1) and route 2 which only depends on the number of active cells. The model takes account of the fact that an energy flow from the sugars (glucose and xylose respectively) to the active cells in the presence of sugar substrates is promoted. Part of the energy flow is used for the survival, maintenance and respiration processes of the microrganisms (route 2) and the remainder to increase the concentration of active cells (reproduction and activation processes, route 1).

Ethanol results from both the autocatalytic step and route 2 (respiration). It also acts as controller of the number of active cells, through an outflow from the active to the inhibited

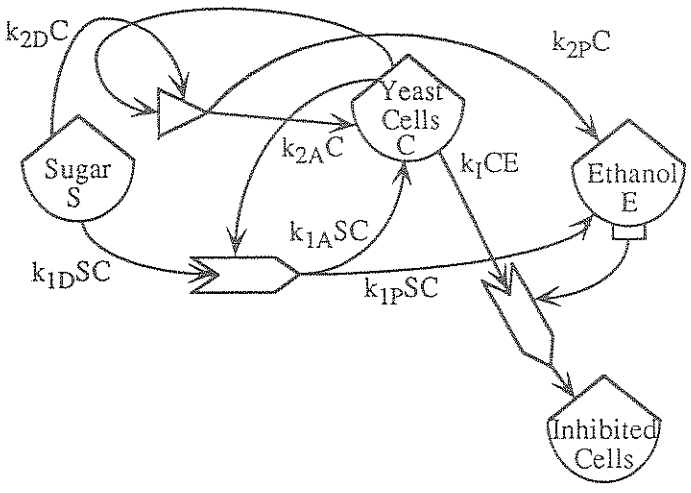

Figure 1: Energy flow diagram of the sugar metabolization processes.

cells tank. This is indicated by the box under the ethanol storage symbol.
In mathematical language the model can be summarized by the following set of differential equations:

$$\frac{dS}{dt} = -K_{1D}SC - K_{2D}C \qquad [1]$$

$$\frac{dC}{dt} = K_{1A}SC + K_{2A}C - K_{I}CE \qquad [2]$$

$$\frac{dE}{dt} = K_{1P}SC + K_{2P}C \qquad [3]$$

where S is the sugar concentration (g/l), C is an index of active cells (number of active cells/ml) and E is the ethanol concentration (g/l).
On the basis of equations [1], [2] and [3] it can be concluded that routes 1 and 2 have different weights at different stages of the fermentation processes.
The autocatalytic route 1 predominates at high sugar concentrations (initial metabolization steps), whereas route 2 which is independent of sugar concentration becomes dominant at the end of the process. No specific external constraints were used in the fitting procedure for the calculations of the kinetic constants, except that the K_{ij} must be non-negative and for thermodynamics reasons, the end-products cannot have greater energy than the sugar-substrates.

The differential equations [1], [2] and [3] were solved by curve fitting of the experimental data. The fitting procedure was possible using the MLAB program (MLAB: Mathematical Modelling System: Civilized Software Inc., Bethesda, MD).

RESULTS AND DISCUSSION

Carbon-13 NMR spectroscopy together with selective 13C-sugar substrate enrichments were used to study yeast and bacteria metabolization processes. Due to its "non-invasive" nature, NMR spectroscopy can be useful in kinetic studies in which several samplings are required during the metabolic activity. This technique also reveals the metabolic route of the enriched carbon-13 nuclei from the substrates to the end-product.
In this study we compared the results of modelling analysis of the experimental data obtained in cell culture samples showing homogeneous behaviour with the data obtained at higher substrate concentrations, when yields and efficiency were reduced. The same fitting procedure was then used for the analysis of xylose fermentation by *Klebsiella planticola* G11.
Figure 2 shows the ^{13}C-NMR spectra which describe the metabolization process of glucose at 85g/l by *Saccharomyces cerevisiae* which transfers the [1-^{13}C] glucose labeled carbon nuclei to the methyl group of the end-product, ethanol.
With fitting procedure we estimated the values of the kinetics constants of the model expressed in equations [1]-[3].

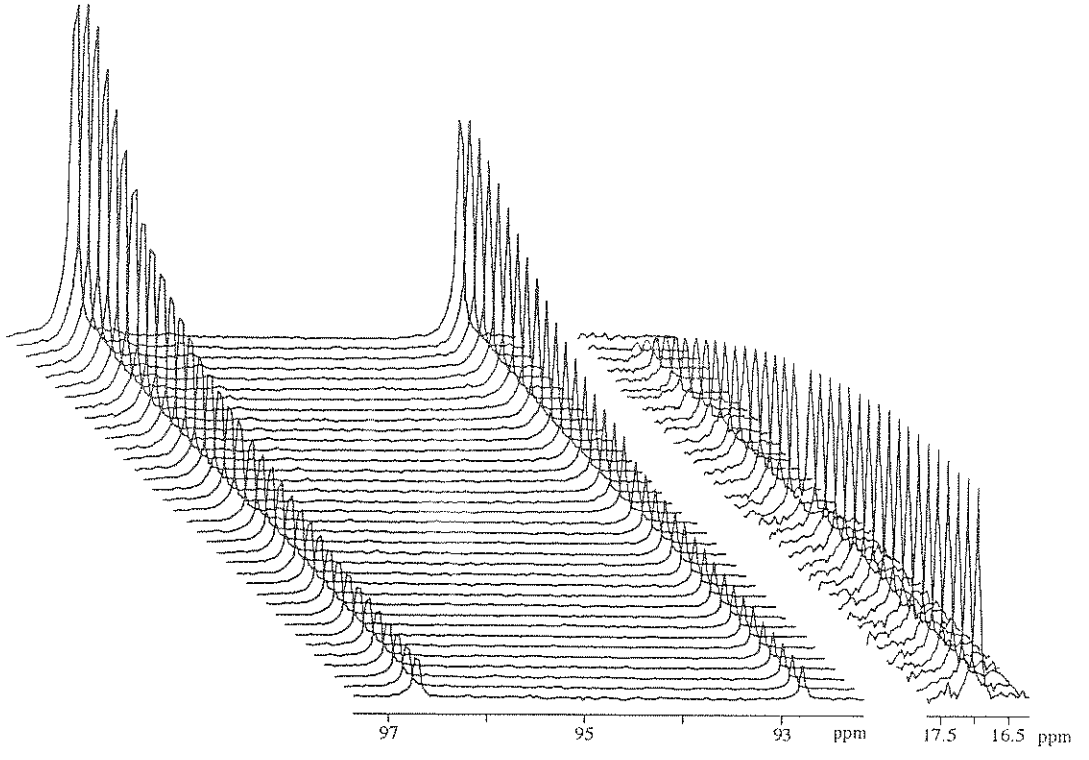

Figure 2: ^{13}C-NMR spectra obtained at 20 minutes intervals during of glucose fermentation to ethanol by *Saccaromices cerevisiae* . The ^{13}C-NMR spectra show the signals of [1-^{13}C]-enriched glucose (β) 96.7 ppm and (α) 92.8 ppm and the ethanol methyl carbon. Initial glucose concentration was 85 g/l .

A unique set of K_{ij} (i=1.2; j=a,d,i,p) constants were estimated using the glucose and ethanol concentrations obtained in the NMR experiments at 85 and 200 g/l of glucose. Another set of constants was calculated by a similar procedure for the metabolization of 280 g/l of glucose by *Saccharomyces cerevisiae*.

Figure 3 shows the experimental glucose and ethanol concentrations at different stages of the fermentation process for systems containg 85 and 200 g/l of sugar. The solid line represents the simulated curves. Figure 4 shows the results for the system with an initial glucose concentration of 280 g/l. The constants are reported in Table 1. Figures 3 and 4 shows the good quality of the fit and give positive indications on the reliability of the metabolic model.

Table 1. Estimate Values of the Kinetic constants of the Metabolic Model

	S.cerevisiae	S.cerevisiae	K.planticola
Kinetic Constants	Glucose 85-200 g/l	Glucose 280 g/l	Xylose 10 g/l
K_{1D}	3.99E-4	2.826E-4	9.150E-4
K_{2D}	4.253E-2	1.621E-2	0
K_{1A}	1.284E-5	6.011E-6	3.288E-4
K_{2A}	0	0	0
K_I	1.471E-5	1.471E-5	2.044E-4
K_{1P}	2.058E-4	5.416E-5	1.815E-4
K_{2P}	9.017E-3	8.835E-3	0
R^2	9.966E-1	9.987E-1	9.982E-1

As the quantity of active cells is not experimentally determined, the model reflects the tendency of this parameter to increase in the first part of the fermentation and to decrease when the effect of the inhibition of the ethanol becomes dominant. The experimental data showed that the initial slope of the glucose degradation curve at 200 g/l was greater than at 85 g/l. The same pattern is shown by the corresponding ethanol production curves. In the range 85-200 g/l the same quantity of glucose is metabolized with higher yields and efficiency at higher substrate concentrations, until the effect of inhibition of the ethanol becomes dominant.

The kinetic parameters determined independently for the *Saccharomyces cerevisiae* systems showed similar values and K_{2A} was zero in both systems. This means that through this pathway no more cells are activated and the energy flow is used to maintain minimum cell activity.

The constants for the 280 g/l system were slightly lower than those obtained for the 85-200 g/l systems. This suggests that at high glucose concentrations, the physical chemistry properties of the system change and the metabolization proceeds more slowly and less efficiently.

At 280 g/l, K_I was assumed to have the same value as for *Saccharomyces cerevisiae* cell cultures in the 85-200 g/l of glucose range.

The kinetic constants reported in Table 1 may have precise biochemical meaning of great utility to compare the efficiency of a metabolic process in different species or to identify biological functions. In the systems analyzed, K_{2D} and K_{2P} assume specific metabolic meaning, representing the rate of glucose degradation and ethanol production per cell, respectively, at low substrate concentrations.

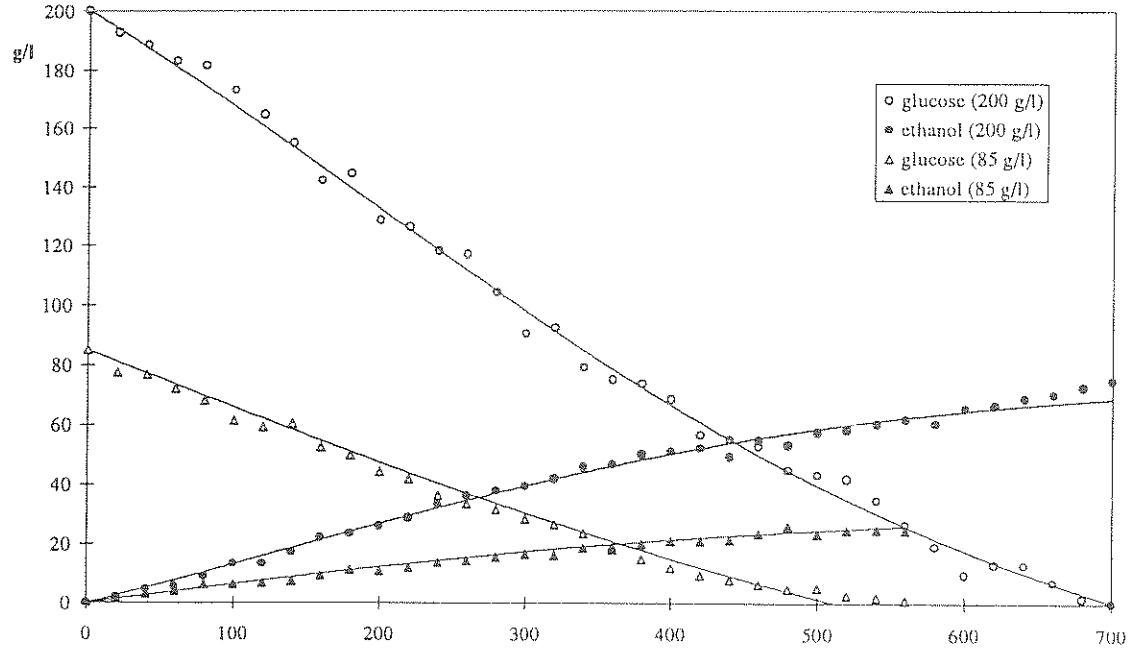

Figure 3: Time course of glucose consumption (85 and 200 g/l respectively) and ethanol production during *Saccaromices cerevisiae* fermentation, together with the curves simulated by the model.

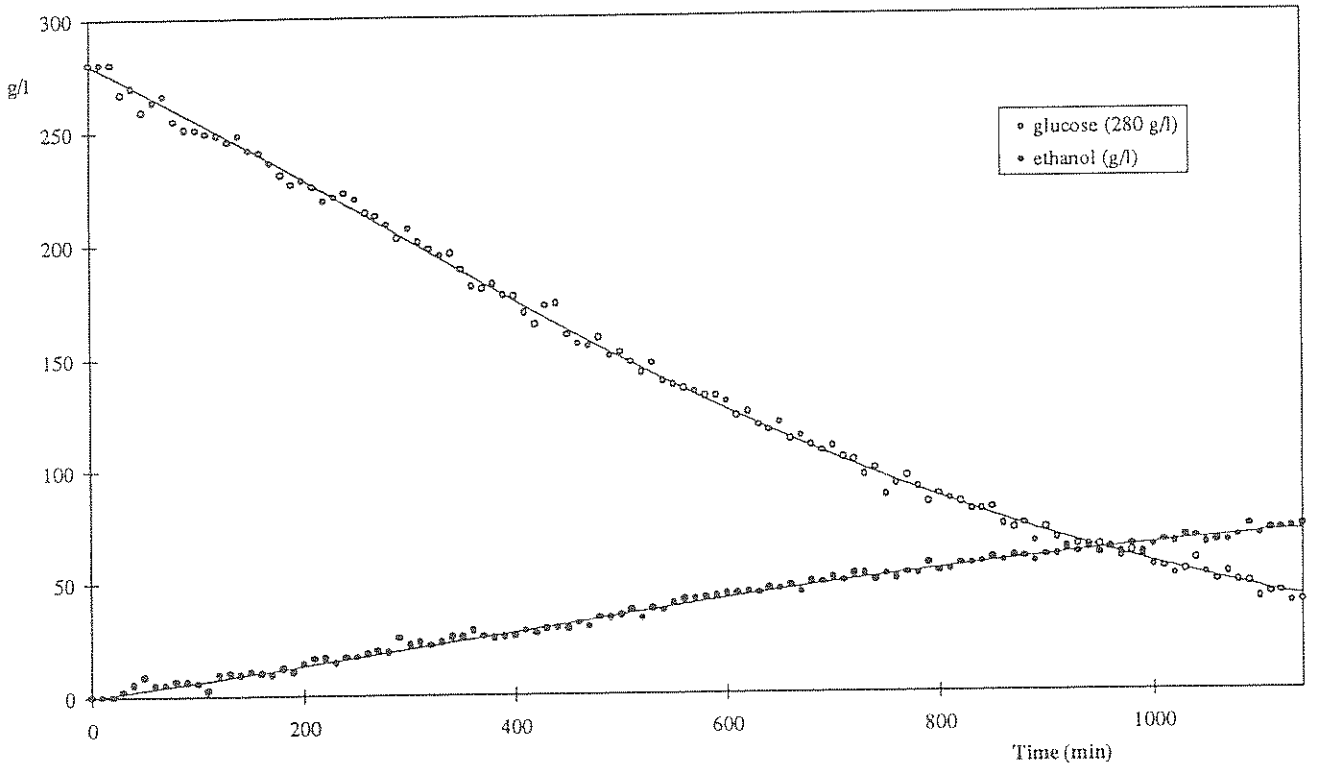

Figure 4: Experimental and theoretical behaviour of the *Saccaromices cerevisiae* fermentation process at 280 g/l of glucose.

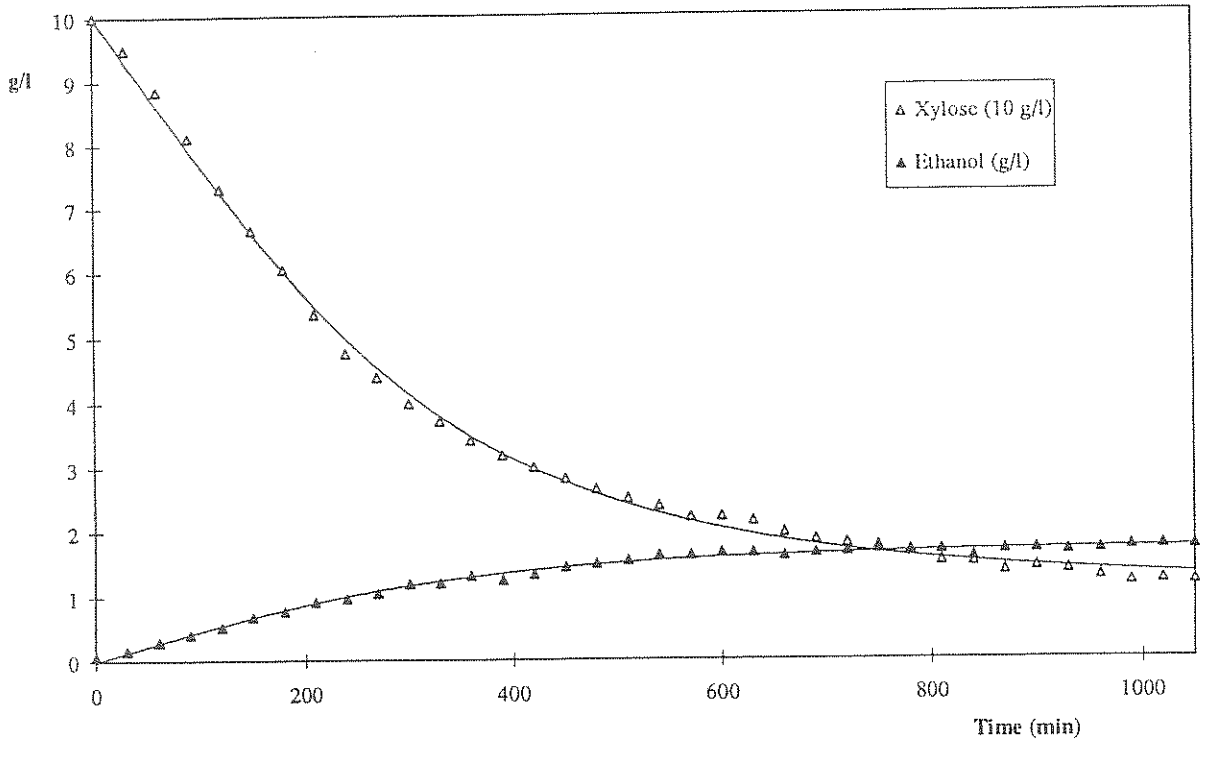

Figure 5: Experimental and theoretical behaviour of the *Klebsiella planticola* fermentation process at 10 g/l of xylose.

The good fit of the model with experimental data and its ability to suggest specific biochemical behaviour, encouraged us to use the same approach for studying the more complex sugar metabolization process involving *Klebsiella planticola* G11. We used xylose as substrate energy source and monitored the ethanol production. It was only assumed that less concentrated by-products of fermentation, such as succinic acid, lactic acid, acetic acid and formic acid, in the buffered culture medium, cannot cause stronger inhibition than ethanol. The experimental results of xylose degradation and ethanol production and the fitting curves are shown in Figure 5. The resulting kinetic parameters are reported in Table 1.

Comparison of these results with those obtained in the *Saccharomyces cerevisiae* experiments show the potential of this methodology for biochemical and biophysical interpretation of metabolic processes in cells and more complex systems. In this case, using no initial constraints, the results obtained from the fitting procedure gave K_{2D}, K_{2A} and K_{2P} values of zero whereas some minor changes in the other kinetic parameters were observed.

This suggests that under these experimental conditions does route 2 not exist or is not utilized in *Klebsiella planticola*. Below a threshold of sugar concentration, the cells fermentation processes no longer have the capability to uptake and metabolize xylose. Several biochemical possibilities to be confirmed by further experimental data are suggested by these results. One is that route 2 is only activated for metabolizing glucose due to the ubiquity of this sugar in the environment.

It is interesting, that the in *Klebsiella planticola* system, K_{1A} has a higher value than in the *Saccharomyces cerevisiae* systems. This agrees with the observed increase in the biomass content of *Klebsiella planticola* culture from 0.5 O.D., at the beginning, to 4.0 O.D., at the end of the fermentation process, whereas that of *Saccharomyces cerevisiae* was smaller.

We regard this model as only the first stage of the metabolic modelling procedure which should include more experimental data and biochemical information. The kinetic constants obtained have biochemical meaning and can be useful for defining the metabolic features of living organisms with a view to biotechnological applications.

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