In vivo ^{13}C-NMR and modelling study of metabolic yield response to ethanol stress in a wild-type strain of *Saccharomyces cerevisiae*

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Abstract In this paper the combined use of in vivo ^{13}C-nuclear magnetic resonance spectroscopy and mathematical modelling allowed the analysis of the response to ethanol stress in a wild-type strain of *Saccharomyces cerevisiae*, in terms of a reduced metabolic activity. The model developed succeeded in describing and interpreting the effects of increasing concentrations of exogenous ethanol. In particular, the ratio between the kinetic constants associated with ethanol production and glucose consumption gave the estimation of the metabolic yield of the processes in perfect agreement with experimental results.

Key words: Metabolism; Modelling; Inhibition; In vivo nuclear magnetic resonance; *Saccharomyces cerevisiae*

1. Introduction

Due to the exposure to specific environmental stresses, microorganisms have to provide adaptive responses [1–3]. *Saccharomyces cerevisiae* represents an ideal eukaryotic model system to investigate the effects of stress conditions on metabolic processes. Among a great variety of physical, chemical, and biological injuries which yeast cells can experience, ethanol represents the main source of stress during fermentation [4,5]. The toxic effects of ethanol on *S. cerevisiae* cells are known, and involve the modification of membrane lipid composition, the synthesis of stress proteins, modulation of ion exchange processes, as well as a reduction of metabolic activity which causes the inhibition of glucose uptake, decreasing of growth rate, and product formation [6,7].

The literature provides a large number of studies which explain the processes taking place at the molecular level during *S. cerevisiae* adaptive efforts in response to external stimuli [5,8–11]. Nevertheless, an approach able to describe and understand the complex network of interactions among the different components of the system needs to be developed.

Because of its 'non-invasive' and 'non-destructive' characteristics, nuclear magnetic resonance (NMR) spectroscopy has been used to perform in vivo experiments using enriched substrates.

This technique allows the mapping of the fate of metabolites during fermentation, tracing the different pathways which lead to the formation of the end-products of the process. These features enable the investigation of chemical equilibria, as well as the kinetics of metabolisation processes such as substrate degradation and end-product formation [12–14].

In this study we propose a novel approach in order to investigate the effects of the presence of external stress on *S. cerevisiae* glucose degradation. In particular, the combined use of in vivo ^{13}C-NMR spectroscopy and mathematical modelling allowed the evaluation of the changes in the metabolic yield of the fermentation process in a wild-type strain of *S. cerevisiae* due to the presence of exogenous ethanol.

Experimental NMR data related to glucose consumption and ethanol production were used in the present work to build a mathematical model able to simulate the yeast metabolism through fluxes of energy and matter within the system [15–22].

We also adopted the formalism introduced by H.T. Odum, modified in order to take into account increasing levels of complexity due to stress factors such as the presence of exogenous ethanol [23–25].

This approach was conceived to develop a flexible compartmental model, providing several new points of view on the complex behaviour of cell metabolism: (i) the metabolisation process was described using concise symbols for energy and fluxes within the system and all main events related to the glucose metabolism that occur in the cell culture; (ii) all the parameters describing the state of the system were treated as kinetic parameters and non-linear equations were developed in order to describe their evolution in time; (iii) specific kinetic constants associated with the main processes taking place within the system were calculated, each one having a specific biological meaning [26].

The model includes different compartments: the carbon source (e.g. glucose), the main fermentation product (e.g. ethanol), and the number of active yeast cells (i.e. the number of cells capable of degrading glucose), which interact exchanging energy and matter during the biochemical phenomena involved in the degradation processes (i.e. glucose degradation, ethanol production, and feedback inhibition due to the presence of both endogenous and exogenous ethanol). As stated above, one of the main features of this approach is the calculation of kinetic parameters which describe the main processes taking place during the fermentation, having a precise

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Abbreviations: EMY, experimental metabolic yield; Y_M, yield obtained from mathematical model

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biological meaning [15–18,26]. The calculation of the kinetic constant associated with the degradation of glucose, k_D, and that related to the ethanol production, k_P, allowed the estimation of the metabolic yield response of *S. cerevisiae* cells to the inhibition effects due to an exogenous stress within the system. In fact, the presence of ethanol affected the growth rate of yeast cells, with a decreased amount of ethanol produced at the end of fermentation.

We found that increasing concentrations of exogenous ethanol lowered the metabolic yield of the yeast from 88% in the absence of ethanol to about 55% in the presence of 50 g/l of exogenous ethanol.

2. Materials and methods

S. cerevisiae strain K310 was isolated from spontaneous wine fermentation [5,27]. K310 was grown in YPD (yeast peptone dextrose) at 30°C with rotary shaking up to saturation (cell concentration 1×10^4 cells/ml). An aliquot of the saturated culture was inoculated in YPD, adjusted to final pH 4.5 by adding 0.2 M citrate-phosphate buffer and containing 100 g/l unlabelled glucose. The cell suspension was incubated at 25°C without shaking (semiaerobiosis) for 10 h.

Stirring or shaking during cell growth should provoke oxidation due to air oxygen that would introduce an additional undesirable shock. It is well known that one of the main effects of fermentation is a sort of oxidation which is exclusively due to ethanol production, oxygen-derived oxidation being ruled out just because of semiaerobiosis (growth without shaking) [5]. Reproducibility of our experimental system was warranted by the monitoring of many parameters during growth and the model proved to be reproducible since it was adopted in several previous studies [4,5,27].

Moreover, proteome maps, produced with protein extracts from the same yeast strain grown within NMR tubes and larger batches under the same semiaerobiosis conditions, showed no significant difference (data available upon request), thus proving that protein expression is unaffected. This indicated that at least the majority of the cells are subjected to the same treatment in both narrow and larger containers, where, theoretically, the impact of ethanol could be expected to be different. This further supported the reproducibility of our experimental system.

Samples were then collected and the growth steps were monitored by measuring the absorbance of the culture at 660 nm. For NMR measurements, K310 cell suspension at 1×10^6 cells/ml, indicating an early log phase of growth, was centrifuged for 5 min at room temperature and $3000\times g$ in a Beckman centrifuge model J2-21 equipped with a JA10 rotor.

The supernatant was discarded and the pellet was resuspended in the same medium containing 100 g/l of [1-^{13}C]glucose. 20% (v/v) of D_2O was added. Three sets of experiments were conducted. In each set the cells were placed in the same batch conditions except for the exogenous ethanol dilution rate which was 0, 20 and 50 g/l respectively. ^{13}C spectra were collected with a Bruker Avance DRX 600 spectrometer operating at 600 and 150 MHz for proton and carbon nuclei, respectively. Carbon spectra were recorded under continuous broad-band decoupling conditions at time intervals of about 3 min. During the experiment the NMR probe was thermostated at 30°C. The substrate and the end-product concentrations were calculated from the area of NMR peaks through an appropriate calibration. The total ethanol concentration was estimated by an enzymatic assay by Sigma-Aldrich (kit code 332-C). The fitting procedure of experimental data was performed using the program MLAB [28]. A parallel growth was carried out during yeast growth under the NMR monitoring. Samples were collected at time 0 and after 12, 16, 19, 22, 27, 36, 44, 50 and 62 h. At each sampling the pH of the cell suspension was checked and growth was monitored by measuring the absorbance of the culture at 660 nm (results not shown). The pH (4.5) never changed during cell growth, as also already reported [5].

All high purity reagents were from Sigma-Aldrich (Milan, Italy), Merck Eurolab (Milan, Italy), Carlo Erba (Rodano, MI, Italy), Serva (Heidelberg, Germany). All the water used was Milli-Q quality (Millipore, Bedford, MA, USA). [1-^{13}C]Glucose was from Cambridge Isotope Laboratories (Andover, MA, USA).

3. Results and discussion

Fig. 1 reports the carbon-13 spectra displayed at time intervals of about 20 min showing the metabolisation process of 100 g/l of glucose by *S. cerevisiae* which transfers the [1-^{13}C]glucose-labelled carbon nuclei to the methyl group of the end-product (i.e. ethanol).

Due to the high initial concentration of glucose, we assumed that the main metabolic pathway for the degradation of this substrate was the fermentation. In fact, when yeast grows in the presence of high glucose concentrations, the negative impact exerted by the sugar on the other metabolic pathways makes the fermentation the sole degradation pathway. Thus, we considered glucose as the unique carbon source and ethanol as the main end-product of the fermentative process [29,30].

The left panel of Fig. 2 shows the experimental data of

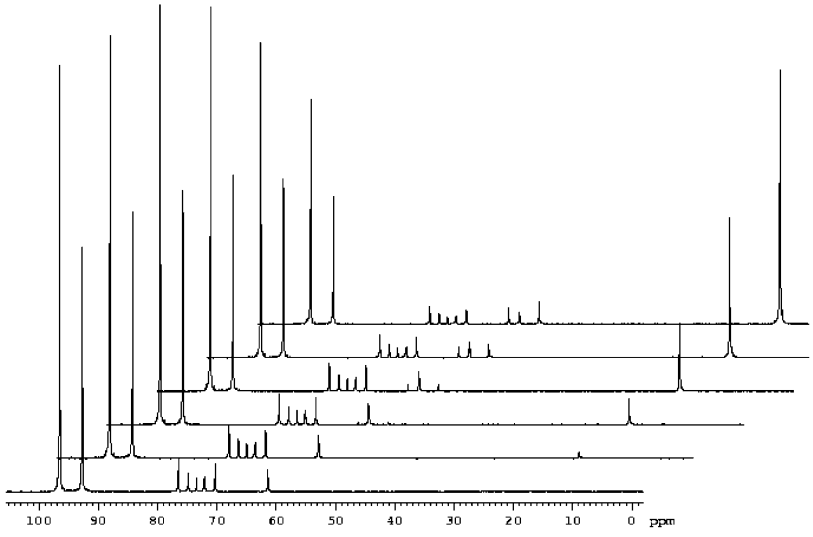

Fig. 1. ^{13}C-NMR spectra obtained at 20-min intervals during glucose fermentation by *S. cerevisiae*.

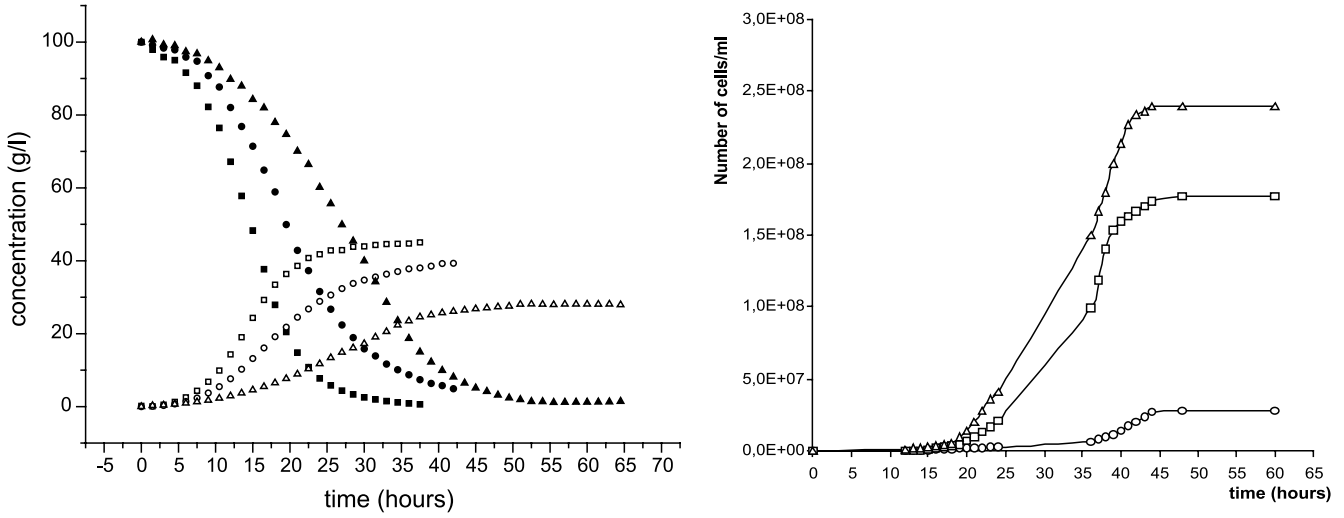

Fig. 2. Left: Data comparison of glucose and ethanol concentration collected in the presence of increasing exogenous ethanol concentrations. Experiments were performed with 0 g/l of exogenous ethanol [glucose (■) and ethanol (□)], 20 g/l of exogenous ethanol [glucose (●) and ethanol (○)] and 50 g/l of exogenous ethanol [glucose (▲) and ethanol (△)]. Right: Yeast growth expressed as number of cells per ml in the absence of ethanol (○), in the presence of 20 g/l of exogenous ethanol (□) and 50 g/l (△).

glucose and ethanol concentrations in relation to time for three different experiments in which the fermentation process of 100 g/l of glucose was carried out with 0, 20, and 50 g/l of exogenous ethanol. Data show that the kinetics of glucose degradation and ethanol production were strongly affected by the presence of the external perturbation. In fact, in the absence of exogenous ethanol, the fermentation process ended after about 38 h to yield 45 g/l of endogenous ethanol, while in the presence of 20 and 50 g/l ethanol, glucose consumption ended after 43 and 55 h respectively, producing 41 and 28 g/l of endogenous ethanol. The right panel of Fig. 2 reports the growth of the yeast strain in the three different experimental conditions. Data showed a change in the cell growth depending on the concentration of exogenous ethanol within the system. In particular, the rate of growth, as well as the number of cells at the end of the process, decreased with increasing ethanol concentration, following the same trend as the production of endogenous ethanol.

These results clearly showed the main effects emerging upon addition of ethanol: a lowering of the fermentation rate, a decrease in the final concentration of the end-product, and consequently a reduced efficiency of the fermentation process.

3.1. The modelling approach

The complex network of interactions between the components of the systems under study can be understood by using a theoretical approach able to describe and interpret the biochemical phenomena occurring during the metabolisation process. In this work we used a compartmental analysis which implied the adoption of a phenomenological and macroscopic point of view in modelling the different processes taking place in the system [23–25].

The experimental evidence obtained from in vivo NMR data was the basis for developing the energy system diagram of the glucose conversion by *S. cerevisiae* reported in Fig. 3. The model is composed of a few elements: glucose concentration, total ethanol concentration (which involves two distinct compartments referring to exogenous and endogenous ethanol concentrations), and an index related to the number of active cells subject to a modulation due to the inhibition processes taking place because of the presence of ethanol. An important feature of the model is represented by the possibility of considering the extent of the damage induced by the addition of exogenous ethanol on the cell culture at time zero. In fact, the effective number of cells able to carry out the fermentation changes depending on the amount of exogenous ethanol added to the system at the beginning of the degradation process. The dynamics of glucose metabolisation were assumed to be the result of an autocatalytic process which depends on the concentration of the available carbon source and on the cellular activity. The presence of the sugar promotes an energy flow to the active cells which allows the cell culture to grow.

The interaction between the total ethanol and the number of actual active cells takes into account the inhibition effects which produced the amount of inhibited cells during the fermentation.

The entire system is represented by a set of non-linear differential equations as follows:

$$\frac{dG}{dt} = -k_D[G][C] \quad (1)$$

$$\frac{dE}{dt} = k_P[G][C] \quad (2)$$

$$\frac{dC}{dt} = k_A[G][C] - k_I\{[E_{exo}] + [E_{endo}]\}[C] \quad (3)$$

$$[C]_{t=0} = [C_0]\frac{1}{1 + h[E_{exo}]} \quad (4)$$

where [G], $[E_{endo}]$ and $[E_{exo}]$ are the concentrations of glucose, endogenous and exogenous ethanol, respectively. [C] is the cellular activity during fermentation, $[C]_{t=0}$ is the initial cellular activity, and $[C_0]$ is the cellular activity in the absence of exogenous ethanol which we assumed to be equal to 1. In this work we consider the 'cellular activity' as an index related to the concentration of cells capable of carrying out the fermentation process. At time zero, in the presence of exogenous

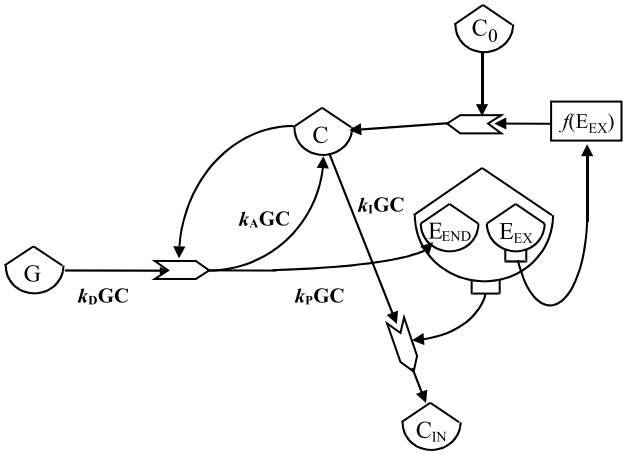

Fig. 3. Energy system diagram of the model of the fermentative process.

ethanol, the initial concentration of cells is the same as in the absence of ethanol, but the concentration of 'active cells' (i.e. the cellular activity) decreases according to Eq. 4.

Both the rates of glucose degradation and ethanol production are related to the glucose concentration and to cellular activity (Eqs. 1 and 2). Ethanol acts as an inhibition factor modulating the cellular activity [C] (Eq. 4).

k_i (with $i = D, A, P, I$) represents the kinetic constants associated with the degradation of glucose (k_D), production of ethanol (k_P), cellular activation (k_A) promoted by the substrate, and with cellular inhibition (k_I) due to the total ethanol within the system.

The constant h (Eq. 4) refers to a function which controls the initial concentration of active cells ($[C]_{t=0}$) in the presence of exogenous ethanol which reduces the cellular activity at the beginning of the fermentation.

In the absence of exogenous stress, the initial cellular activity is assumed to be 1; as the concentration of the stress source increases, the value of $[C]_{t=0}$ decreases. Cellular activity [C] includes both the inhibitory effects exerted by exogenous ethanol at the beginning of the process and the negative feedback due to the increasing amount of ethanol during fermentation.

Fig. 4 shows the results of the fitting procedure performed on the three sets of experimental data reported in the left panel of Fig. 2.

A non-linear least-squares estimation that makes use of the Marquardt–Levenberg method allowed the calculation of the constants k_D, k_A, k_I, k_P and h, fitting all data simultaneously, shown in Table 1. The analysis of the values assumed by the kinetic constants associated with the different processes highlights some interesting aspects of the phenomena under study. In particular, the constant related to the degradation of glucose, k_D, assumes the same value for the three sets of data. This suggests that the lowering of the fermentation rate observed in the presence of exogenous ethanol was due to a decrease of the cellular activity at the beginning of the fermentation. In fact, experimental data showed that the yeast cells were able to completely degrade the substrate even in the presence of the stress source, implying that the concentration of active cells represented the variable which strongly affected the rate of glucose degradation.

The kinetic constant associated with ethanol production, k_P, represents an index related to the efficiency of ethanol production in the presence of exogenous ethanol. As noted above, the yielded ethanol at the end of the fermentation was different, depending on exogenous ethanol concentration. According to the experimental results, the calculated k_P values decreased with increasing exogenous ethanol concentration; this suggests that the stress induced by the presence of ethanol subtracts fermentative ability that reduces the production rate and the final concentration of ethanol.

The results reported in Table 1 also show that k_A assumes a

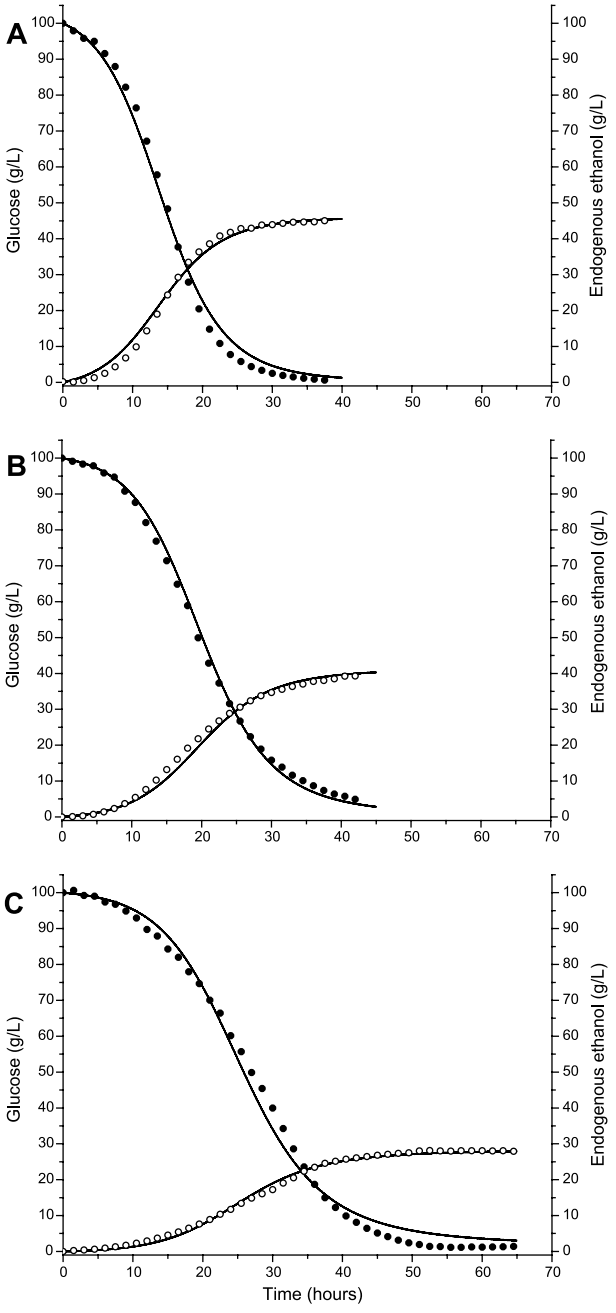

Fig. 4. Results of the simultaneous fit of data from experiments performed at increasing ethanol concentrations. A–C: Experimental time course of ethanol (○) and glucose (●) is compared with model simulation (continuous line). In A *S. cerevisiae* was grown in the absence of exogenous ethanol. In B and C the concentrations of exogenous ethanol were 20 g/l and 50 g/l respectively.

Table 1
Estimated values and coefficients of variation (CV) of the kinetics parameters

Constant	Value	CV (%)
k_D	8.78×10^{-3} (s^{-1})	1.2
k_P (ethanol = 0 g/l)	4.05×10^{-3} (s^{-1})	1.1
k_P (ethanol = 20 g/l)	3.64×10^{-3} (s^{-1})	1.1
k_P (ethanol = 50 g/l)	2.53×10^{-3} (s^{-1})	1.1
k_A	2.26×10^{-3} (s^{-1})	0.4
k_I	0.77×10^{-3} (s^{-1})	1.3
h	8.60×10^{-2} (l/g)	1.3

higher value than k_I, suggesting that the ability of glucose to promote the fermentation process is greater than the feedback inhibition due to the presence of ethanol.

Let us consider the bioconversion of glucose into ethanol occurring during the fermentation:

$$1\ \text{Glucose} \rightarrow 2\ \text{Ethanol} \quad (5)$$

The theoretical metabolic yield of the process can be calculated from the ratio between the weight of two moles of ethanol (i.e. 92 g) and one mole of glucose (i.e. 180 g), which gives a value of 0.51 which represents the maximum achievable yield.

The experimental metabolic yield (EMY) for the three different systems can be obtained from the ratio of the concentration of produced ethanol to the concentration of glucose. Fig. 5 reports the plot of endogenous ethanol vs glucose concentration in the absence of exogenous ethanol (a) and in the presence of 20 g/l (b) and 50 g/l of ethanol (c). The slopes of the straight lines represent the values of EMY for the fermentation process carried out in different stress conditions; they are also reported in Fig. 5. Results show that the yield of the fermentation process decreased with increasing exogenous ethanol concentration, and reaches the minimum efficiency (about 28%) in the presence of 50 g/l of ethanol.

The analysis of the values of the kinetic parameters obtained by mathematical modelling allowed the interpretation

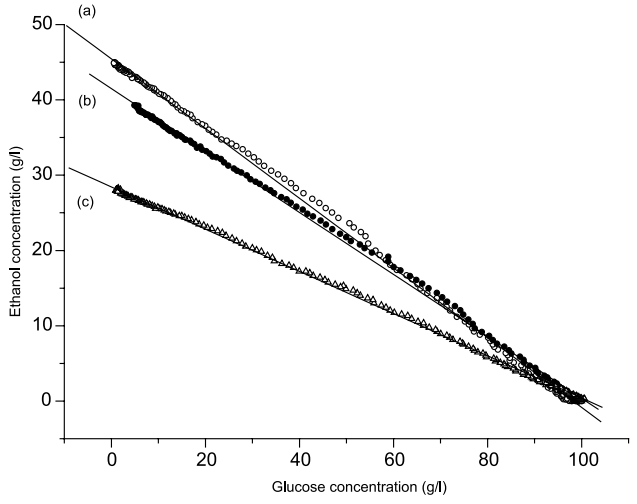

Fig. 5. Plot of endogenous ethanol vs glucose concentration for the fermentation process of 100 g/l of glucose: (a) in the absence of exogenous ethanol, (b) in the presence of 20 g/l of ethanol and (c) in the presence of 50 g/l of ethanol. The slope of each line calculated by linear regression of experimental data is also reported as EMY_0, EMY_{20} and EMY_{50}.

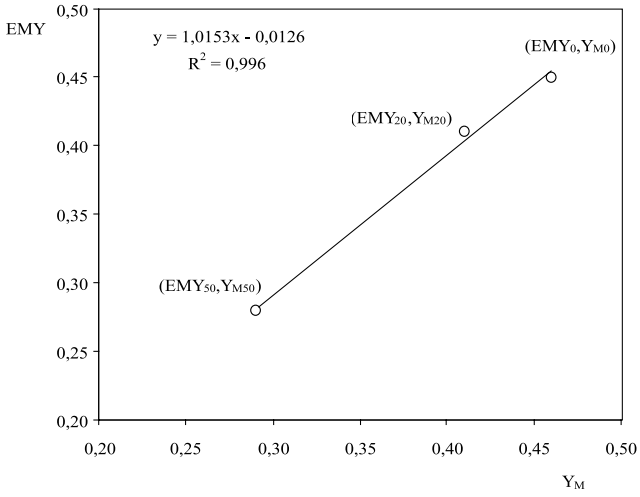

Fig. 6. Linear relationship between metabolic yield values obtained from experimental results (EMY), and from mathematical modelling (Y_M) for the fermentation of 100 g/l of glucose in the absence of ethanol (EMY_0, Y_{M0}), in the presence of 20 g/l of (EMY_{20}, Y_{M20}), and 50 g/l exogenous ethanol (EMY_{50}, Y_{M50}).

of the responses of the yeast to the stress conditions applied to the systems as well as the estimation of the metabolic yield of the three processes. For this purpose, we can use Eqs. 1 and 2 in order to calculate the yield of the fermentation from the ratio between the amount of ethanol produced and the glucose consumed during the degradation process:

$$dG = -k_D[G][C]dt$$

$$dE = k_P[G][C]dt$$

then

$$\frac{dE}{dG} = -\frac{k_P}{k_D}$$

The ethanol produced and the glucose consumed by fermentation can be explicited in terms of finite variations:

$$\frac{\Delta E}{\Delta G} = \left|-\frac{k_P}{k_D}\right| = Y_M \quad (6)$$

This relation shows that it is possible to derive the metabolic yield of each process simply by calculating the ratio between the kinetic constants associated with ethanol formation and glucose consumption. These results, obtained from the mathematical model (Y_M), were compared with the values calculated from experimental data (EMY) in the plot shown in Fig. 6. The linear relationship existing between the two sets of results as well as the slope of the straight line (1.01) suggest that the model developed is able to interpret coherently the behaviour of the yeast cells in the presence of different concentrations of exogenous ethanol and to describe the stress effects in terms of decreased metabolic activity in perfect agreement with the experimental results.

4. Conclusions

The metabolic behaviour of complex systems such as eukaryotic cells is often difficult to analyse, since the information that can be obtained from traditional studies is sometimes inadequate to clarify and understand the relationship

between the various components of the system. For this reason, the development of new approaches able to deal with the complexity due to a large number of interactions has great importance. In this paper, we applied an approach based on in vivo NMR experiments and mathematical modelling in order to analyse the metabolic response to ethanol stress of a wild-type strain of *S. cerevisiae*. Considering the cellular metabolic processes resulting from activation, inhibition and feedback activities, we developed a model capable of considering higher levels of complexity due to increasing concentrations of the stress source in the system. Kinetic model analysis highlighted that increasing concentrations of exogenous ethanol produced two important effects: a lowering of the degradation rate of glucose and a decreased production of endogenous ethanol, i.e. a reduced metabolic activity. The ratio between the kinetic parameters related to ethanol production (k_P) and glucose consumption (k_D) provided a theoretical estimation of the metabolic yield of the fermentation process in relation to exogenous ethanol concentration in the yeast. Calculated yield values decreased with increasing exogenous ethanol concentration, in perfect agreement with experimental results. These results point out that the kinetic model is able to describe and interpret the extent of the stress effects induced by the presence of ethanol on the fermentative ability of *S. cerevisiae*.

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