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Determination and modeling of yeast viability under stress conditions

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Abstract

In this work the effect of the total pressure and the oxygen concentration in the gas phase on the growth and viability of *Saccharomyces cerevisiae* is studied. Cell viability was measured staining with methylene blue and assessed using digital image processing. A model that takes into account cell viability was developed and used for the correlation of the measured data. A dependence of the death rate, K_D, with the total pressure and the oxygen concentration is proposed. Its behavior indicates that opposing effects between oxygen availability and baric and oxidative stresses are present on the system. It is shown that the proposed model can, with this extra parameter, successfully describe not only the traditional X-P-S evolution but also the ratio of viable cells with time.

Keywords: modeling, digital image processing, cell viability, yeast

1. Introduction

Literature models for biological reactors usually consider biomass as a homogeneous entity making no distinction between the different conditions that the microorganisms may display. However behind this simple picture lays a complex world of cells some dead, others alive but not active, some others active and undergoing reproduction, all with different ages and sizes. Usually, the traditional studies quantify the cells on its different life stages and conditions and most of the process models used are non-structured, their kinetics being a function of the total biomass concentration. Although a successful approach to the correlation of fermentation data these models are unsatisfactory due to its poor description of the underlying complexity of the system.

On the other side, there are many structured models for various microorganisms presented in the literature, showing different levels of complexity and robustness to describe cell dynamics, either for a physiological or a morphological description (Bizukocj and Ledakowicz, 2003; Henson, 2003). Few studies (Oliveira *et al.*, 2000) are related to the loss of cell viability despite of its importance on industrial productivity. The loss of cell activity is well known in bioreactors, part due to nutrient limitation or toxin accumulation in the broth, but other types of stress may also contribute to cell deactivation, like pressure, oxidative or mechanical stresses.

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In this work, the loss of viability of *S. cerevisiae* under distinct pressures (1 to 15 bar) and gas composition (air, oxygen or nitrogen) is determined using automatic image analysis. A structured model is developed to incorporate a parameter relating the effects of total pressure of the system and oxygen content in gas composition on cell viability.

2. Experimental

2.1 Yeast strain and medium

The strain *Saccharomyces cerevisiae* ATCC 32167 was used. It was stored at – 80°C in liquid medium with 20% (v/v) glycerol. From these stock cultures, agar plates (20 g L^{-1} agar) were inoculated and colonies from agar plate were used to obtain the inoculum for batch experiments under hyperbaric conditions. The liquid medium was composed by 0.4 g L^{-1} $MgSO_4.7H_2O$, 2.0 g L^{-1} $(NH_4)_2SO_4$, 5.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} yeast extract, 5.0 g L^{-1} glucose. The pH was adjusted to 4.0 prior autoclaving. Inocula were grown in 250-mL shaker flask containing 50-mL of medium at 30°C and 120 rpm in an orbital incubator for 15 h.

2.2 Batch and fed-batch cultivation

The experiments were carried out in a 600-mL stainless steel reactor (Parr 4563) at a temperature of 30°C, stirring rate of 400 rpm and a gas flow-rate of 1 sLpm. The manipulation of the compressed pressure (inlet gas) and the regulatory valve position in the exit gas line set the operating pressure. The reactor was equipped with a pressure transducer to monitor total internal pressure. In the batch experiments, the medium employed in pressured reactor experiments had the same composition as the medium used for the inoculum preparation. The total batch time was of about 8 hours.

For fed-bath experiments, the medium used had the same composition as the medium used for the inoculum preparation with the exception of 60-g glucose L^{-1}. The medium was pumped into the reactor using a high-pressure pump (Jasco 880-PU) with flow rates between 1 and 25 mL h^{-1} to allow the volumetric cell mass concentration to increase exponentially. The total fed-batch experiment takes circa 30 hours. Detailed information about the fed-batch procedure adopted can be found in Belo *et al.* (2003).

2.3 Analytical methods:

Cell concentration was estimated through optical density at a wavelength of 620 nm, previously correlated to dry weight determination. Glucose was measured by 3,5-dinitrosalycilic acid method (Miller, 1959). Ethanol was quantified by HPLC. Cell viability was determined through methylene blue staining and the percentage of viable and non-viable cells were estimated using digital image processing.

2.4 Image Analysis Procedure

Image acquisition was conducted in an optical microscope (Axioscop, Zeiss) with 400x magnification coupled with a 3CCD DXC-9100P camera (Sony) and linked to a microcomputer by a frame grabber (DT2851, Data Translation, Inc.). The colour RGB images were decomposed in its blue channel. To take into account irregularities in the background, an image without cells was employed (background), and its background divided by the decomposed image. A binarization step was carried on and, afterwards,

the application of morphological operations, like erosion, to remove small debris, and reconstruction, gave the number of non-viable cells. To obtain the number of total cells a similar procedure was employed except for the additional procedure of hole-fill step after reconstruction. This image processing, developed with Matlab v.6.1 (The Mathworks Inc.) package is fully automated. Performing such analysis, an average deviation inferior to 5% is obtained when compared to the traditional manual counting.

3. Model Formulation

The usual model structure employed in many mathematical representations of macro-kinetic variables in biological systems was the starting point for the formulation of the model herein proposed. The influence of environment conditions in cell viability was incorporated by the assumption that the total cell population is divided in two categories, viable and non-viable cells, which are inactive or non-growing but nevertheless intact cells. It means that cellular lysis is not considered in this formulation. Since this work presents experimental data for cell viability, it is possible to show that the viability (γ) was not constant throughout the experiments. The loss of viability is given by an additional parameter, K_D, which assumes that the rate of transformation into the nonviable form is directly proportional to the viable cell concentration. This parameter relates the influence of two opposite phenomena: the higher oxygen availability in pressurized systems and the baric and oxidative stresses.
The overall model is composed by four ordinary differential equations relating the kinetic rates for cell growth, cell viability, substrate consumption and product formation for both batch and fed-batch systems, as described by

$$\frac{dX_V}{dt} = -\frac{F}{V}X_V + \mu_R\left(\frac{S}{K_S + S}\right)X_V - K_D X_V \tag{1}$$

$$\frac{dX_{NV}}{dt} = -\frac{F}{V}X_{NV} + K_D X_V \tag{2}$$

$$\frac{dS}{dt} = \frac{F}{V}(S_f - S) - \frac{1}{Y_{X/S}}\mu_R\left(\frac{S}{K_S + S}\right)X_V \tag{3}$$

$$\frac{dP}{dt} = -\frac{F}{V}P + Y_{P/X}\,\mu_R\left(\frac{S}{K_S + S}\right)X_V \tag{4}$$

The solution of the system of ordinary differential equations and parameters evaluation was performed using Matlab v.6.1 (The Mathworks Inc.). The implicit 3th order Runge–Kutta method for non-stiff ODEs was applied to find the solution of the system. The parameters were found by means of the optimization procedure joining the elements of Nelder-Mead simplex procedures. Other parameters, namely $Y_{X/S}$ and $Y_{P/X}$, were directly calculated from the experimental data.

4. Results

The K_D values obtained for different operational conditions are presented in Table 1. The model description of some of the experimental data is presented in Figures 1 to 3. To verify the statistical significance of the parameters, analyses through t-Student distribution were performed with 95% confidence. In all cases, the hypothesis of parameter equal to zero was rejected indicating that they are significant even being a small number.

Table 1- Values of K_D for the Batch and Fed-batch systems studied and deviations between experimental values for Fed-batch and Equation (5)

P_R / bar	% O_2	K_d Batch	K_d Fed-batch	Deviations (%)
1	0	0.0152		
6	0	0.0140		
1	21	0.0351	0.0470	1.8
6	21	0.0090	0.0299	2.7
10	21		0.0401	3.5
15	21		0.0425	0.1
1	100		0.0544	9.1
3	100	0.0254	0.0397	14.5
5	100	0.0075	0.0316	7.0

The proposed model provides a good description of the experimental data available not only in terms of X, P, S but allowing also the determination of the viability profile along the experiments. The K_D values obtained show a complex pattern relating the influence of the increase in both oxygen content and pressure. In fact, a pressure increase leads to a favorable effect in cell viability until pressures of about 10 bar. This may be due to an increment in the oxygen availability at high pressure. It was shown by Belo *et al.* (1999) that up to 10 bar the intracellular ATP concentration increases, driving the cell metabolism to an oxidative regime more efficient energetically.

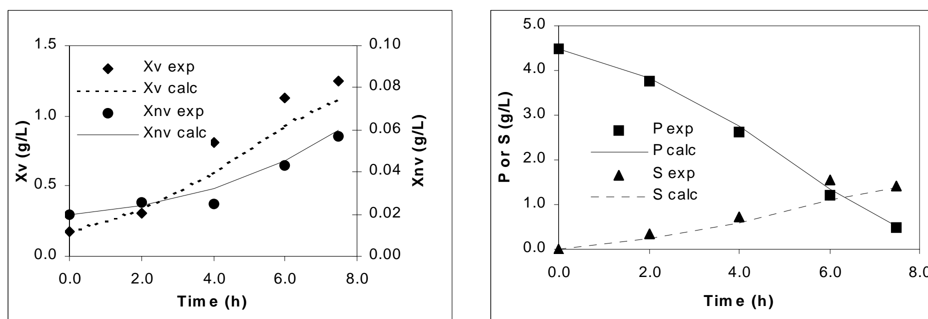

Figure 1 – Kinetic data and model description for a batch experiment with air at 6 bar

On the other hand, higher levels of oxygen surely provoke an oxidative stress to the cells, denoted by the activity of anti-oxidant enzymes like superoxide dismutase and catalase (Belo *et al.*, 1999). These enzymes are recognized to act as a cellular defense

against the oxygen reactive species. Some authors (Bartlett, 1992) describe a negative effect of the pressure raise in cell viability, mostly due to a pressure effect on cell membrane reducing its fluidity. For the range of pressure values used, the effects of oxygen partial pressure are the first cause of cell damage, besides the total air pressure raise.

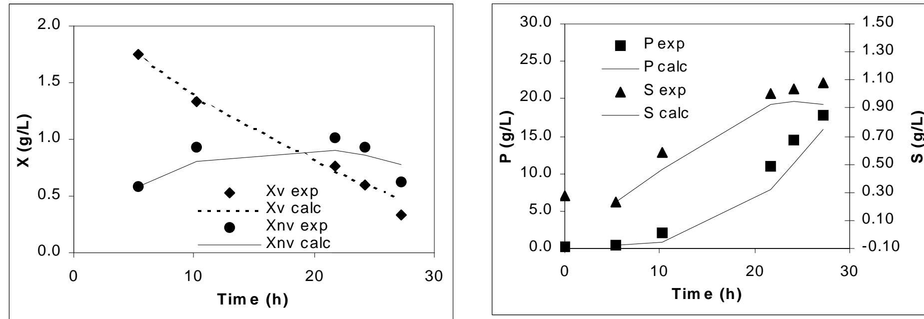

Figure 2 – Kinetic data and model description for a batch experiment with O_2 at 3.2 bar

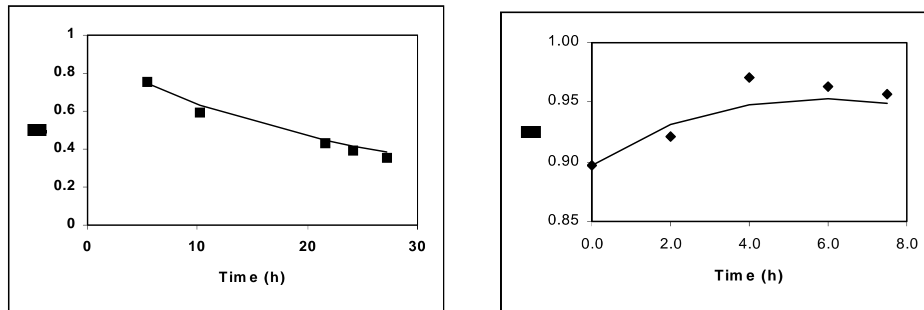

Figure 3 – Viability profiles for the a) Batch system with air at 6 bar and b) Fed-batch system with O_2 at 3.2 bar

Face to these process characteristics, a function is proposed to correlate the influences of the raise of both oxygen content and pressure on K_D, given by equation (5). The parameter values obtained are presented in Table 2 and were estimated to adjust for batch and fed-batch experimental conditions.

$$K_D = \frac{a\, x_{O_2}}{(b + c\, x_{O_2})}\left[\exp(-d\, P_R) + e\, P_R\right] \quad (5)$$

Table 2 – Parameter values estimated for Equation (5)

a	b	c	d	e
0.065	0.029	0.013	0.246	0.051

The deviations between the values obtained using equation (5) and those derived from experimental values are reported in Table 1 and demonstrate the applicability of such

correlation to predict the cell viability under different oxygen content and pressure conditions.

5. Nomenclature

F– flow rate (L h^{-1})
K_S –sugar saturation constant (g L^{-1})
K_D – specific rate of transformation of viable cells into nonviable cells (h^{-1})
P – product concentration (g L^{-1})
P_R – pressure (bar)
t – time (h)
S – substrate concentration (g L^{-1})
S_f – substrate concentration in the feed stream (g L^{-1})
V – reactor volume (L)
X_{O2} – oxygen content (%)
X_t – total cell concentration (g L^{-1})
X_V – viable cells concentration (g L^{-1})
X_{NV} – non-viable cells concentration (g L^{-1})
$Y_{X/S}$ – biomass to substrate yield coefficient (g g^{-1})
$Y_{P/X}$ – product to biomass yield coefficient (g g^{-1})
γ – cell viability (-)
μ_R – real specific biomass growth rate (h^{-1})

6. Acknowledgments

MAZ Coelho is grateful for the financial support given by CAPES and CNPq (Brazil) for this work development.

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