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Mathematical Modeling as a Tool to Describe and Optimize Heterologous Protein Production by Yeast Cells in Aerated Fed-Batch Reactor

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In this work, two recombinant yeast strains, the prototrophic non-conventional *Zygosaccharomyces bailii* [pZ$_3$KIIL-1β] and the auxotrophic *Saccharomyces cerevisiae* BY4741[PIR4-IL1β], both producing human interleukin-1β, have been cultured in aerated fed-batch using glucose as limiting substrate. A mathematical model of the fed-batch reactor has been developed, based on mass balance equations of the main process variables -biomass, glucose and product- and implemented with kinetic expressions to explain the yeast behaviour within the aerated fed-batch reactor. In the case of *Z. bailii*, the mathematical model evidenced the suitability of the fermentative inoculum with respect to the respiratory one at the start of the exponential feeding. In the case of the auxotrophic *S. cerevisiae* BY4741, the modellistic approach has permitted to highlight a strong deviation from the expected behaviour and quantify the glucose amount that is spent for maintenance rather than for growth, thus impairing the outcome of the bioprocess.

1. Introduction

The aerated fed-batch is the cultural system mainly employed in the production of recombinant proteins with glucose-sensitive yeasts (Mendoza-Vega et al., 1994; Porro et al., 2005). Indeed, fed-batch provides, through limited supply of one nutrient (generally the carbon and energy source), a suitable strategy to avoid over-flow metabolism, promote fully respiratory pathway and high yield of biomass and product of interest. Furthermore, fed-batch mode allows the proliferating biomass to be accumulated. This is a prerequisite to maximize volumetric productivity *i.e.* the amount of biomass and/or product in a given volume within a certain time, which is the most plausible target for optimization.
Mathematical modeling of bioprocess is an useful tool to describe microbial cell growth, product formation and to optimize culture conditions. General attempts to model fed-batch processes have been described (Sinclair et al., 1987). Notwithstanding this, modeling of yeast high-cell density cultures and optimization of recombinant protein production need to be further developed, considering the peculiar environment represented by the aerated fed-batch reactor. In this concern, unstructured and non-segregated models, which describe the rate of growth based on the availability of a single substrate, may be easier and faster to develop and optimize with respect to the more sophisticated structured and segregated models.
In this work, an unstructured non-segregated model has been developed to describe the fed-batch cultures of two glucose-sensitive yeast strains, the non-conventional prototrophic *Zygosaccharomyces*

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bailii, and the auxotrophic *Saccharomyces cerevisiae* BY4741, both engineered for interleukin-1β (IL-1β) production. In all the experiments, a medium properly formulated has been used, and the feeding strategy consisted in an exponentially increasing feed covering the entire run, which allowed the yeast strain to grow at a constant value of specific growth rate. The proposed unstructured and non-segregated model proved to be able of accurately describing and predicting key aspects of the fermentations, experimentally observed during yeast proliferation in the fed-batch reactor.

2. Materials and Methods

2.1 Strains

The strain *Z. bailii* [pZ$_3$KIIL-1β], kindly provided by prof. D. Porro, (UNIMIB-Italy), carried the plasmid pZ$_3$KIIL-1β containing the human *IL-1β* gene expressed under the constitutive *S. cerevisiae TPI* promoter and, as selective marker, the resistance to geneticin (G418) (Vigentini et al., 2005). The *S. cerevisiae* BY4741[PIR4-IL1β] strain was obtained according to Paciello et al. (2010) by transformation of *S. cerevisiae* BY4741 *(MATa,ura3Δ0, leu2Δ0, met15Δ0, his3Δ1)* with the expression vector pIA1, containing *URA3* as selectable marker and the human *IL-1β* gene functionally fused with a portion of *PIR4* ORF.

2.2 Inocula preparation

Strain samples, from the frozen cultures (-80 °C in 12.5% (v/v) glycerol), was grown at 30 °C in 500 ml flasks containing 100 ml of a defined mineral medium (Verduyn et al., 1992), pH 5.0. and 1% (w/v) casamino acids (BD Bacto™ Casamino Acids, BectonDickinson & Co., Sparks, MD 21152 USA) and made selective with 200 mg L^{-1} G418 in the case of *Z. bailii* [pZ3KIIL-1β]. Initial α-D glucose concentration was 5 and 2% w/v for *Z. bailli* and *S. cerevisiae* respectively.

2.3 Fed-batch cultures

Fed-batch cultures have been performed at 30 °C in a 2.0 L working volume of a stirred fermenter, Bioflo 110 (New Brunswick Scientific). The fermenter initially contained 1 L of the defined mineral medium above mentioned. The fermenter was inoculated to give an initial O.D.$_{590}$ of 0.04. As regards *S. cerevisiae*, fed-batch culture started after 15 h when glucose in the batch was exhausted, whereas for *Z. bailli*, fermentative and respiratory inocula were obtained with 18 and 30 h of batch phase, respectively. Then, an exponentially increasing feed was applied to allow the biomass to proliferate with a constant value of specific growth rate (0.13 h^{-1} and 0.16 h^{-1} for *Z. bailii* [pZ$_3$KIIL-1β] and *S. cerevisiae* BY4741[PIR4-IL1β], respectively), lower than the 60% of the maximum specific growth rate of the strain (Enfors, 2001). The feeding solution contained glucose (50% w/v), salts, trace elements, glutamic acid, vitamins, and casamino acids, the concentration of which was calculated according to Paciello et al. (2010), taking into account the value of biomass yield for the given amino acid under aerobic conditions (Pronk, 2002). Oxygen was supplied by air sparging (DOT 30% air saturation).The culture pH was maintained at 5.0 by automatic addition of 2 N KOH during batch phase and 10% v/v NH_4OH during exponential phase. The foam level in the fermenter was controlled by the automatic addition of the antifoam B (Sigma Aldrich) (dil. 1:10).

2.4 Determination of biomass, cell viability and specific death rate

Total biomass was determined by optical density (O.D.$_{590}$) and dry weight. The calibration curve relating O.D.$_{590}$ values to biomass density provides a correlation factor of 2.0, and 2.45 O.D.$_{590}$ per mg mL^{-1} for *Z. bailii* [pZ$_3$KIIL-1β] and *S. cerevisiae* BY4741 [PIR4-IL1β], respectively.
Viable cell density during fed-batch runs was determined by viable count (in triplicate) on YPD (1%Yeast Extract, 2% Peptone, 2% w/v Destrose) agar plates incubated at 30 °C for 48 h, and calculated according to:

$$X(t) = X_0 \cdot \frac{(CFU/ml)_t}{(CFU/ml)_{t=0}} \quad (1)$$

It was assumed that, at the start of feeding (t = 0), all the yeast cells were viable. The specific death rate (k_d,) was evaluated as a first order kinetic constant by plotting the ratio CFU mL^{-1}/ O.D.$_{590}$ *vs.* time, where CFU corresponds to the colony forming units originated by viable cell count.

2.5 Analyses

Samples withdrawn from fed-batch cultures were filtered on 0.45 µm GF/A Millipore filters and analyzed to determine residual glucose, ethanol and IL-1β concentrations in the culture medium (Paciello et al., 2010). All samples were analyzed in triplicate and the values of standard deviation obtained varied between 1 and 2%.

3. Mathematical model

The unstructured non-segregated mathematical model was developed on the basis of component mass balances, starting from the differential equation written below which describes the change with time of the variable of interest (y, concentration, g L^{-1}).

$$\frac{dy}{dt} = \frac{F(t)}{V(t)}[y_i - y(t)] \pm q_y \cdot x(t) \quad (2)$$

q_y is the specific rate of production or consumption referred to the generic variable y. The differential equation was numerically solved, by Eulero method, starting from given initial values. The first mass balance developed was that on the biomass x(g L^{-1}):

$$\frac{dx}{dt} = -\frac{F(t)}{V(t)} \cdot x(t) + q_x \cdot x(t) \quad (3)$$

The specific rate q_x (h^{-1}) includes the specific growth rate (μ, h^{-1}) and the specific death rate (k_d, h^{-1}) (Tab.1). This equation was combined with the mass balance on glucose, the limiting substrate:

$$\frac{ds}{dt} = \frac{F(t)}{V(t)}[s_i - s(t)] - q_s \cdot x(t) \quad (4)$$

Glucose specific consumption rate (q_s, h^{-1}) is the overall specific rate of glucose consumption, including consumption for both growth (q_g, h^{-1}) and maintenance (q_m, h^{-1}). IL-1β production was modeled, considering that it is a growth-linked product :

$$\frac{dp}{dt} = -\frac{F(t)}{V(t)} \cdot p(t) + q_p \cdot x(t) \quad (5)$$

where q_p (h^{-1}) is the IL-1β specific production rate. This latter is represented by the product between specific growth rate (μ) and product yield coefficient on biomass ($Y_{p/x}$).

Table.1: Kinetic expressions for fed-batch reactor with recombinant yeast strains

Strain	q_x	q_s	q_p	Validity range
Z. bailii **[pZ$_3$KIIL-1β]**	μ	$q_g = \frac{\mu}{Y_{x/s}}$	$\mu \cdot Y_{p/x}$	$\forall\ t$
S. cerevisiae **BY4741 [PIR4-IL1β]**	$\mu - k_d$	$q_g + q_m$	$\mu \cdot Y_{p/x}$	$0 \le t < t_1$
	$\mu \cdot \exp[-\tau(t-t_1)] - k_d$	$q_g \cdot exp[-\theta(t-t_1)] + [\alpha(t-t_1)^2 + \beta(t-t_1) + \gamma]$	$\{\mu \cdot \exp[-\tau(t-t_1)]\} \cdot Y_{p/x}$	$t \ge t_1$

Model parameter $\left(\mu,\ t_1,\ k_d,\ q_g,\ Y_{x/s},\ Y_{p/x}\right)$ (Tab.1) values were obtained setting up *ad hoc* experiments. Particularly, the µ value was evaluated as the derivative of biomass profile over time; t_1 was the time at which the specific growth rate and the glucose consumption began to vary; q_g value was evaluated from the ratio between μ value and $Y_{x/s}$; k_d was evaluated by viable count on agar plate assuming that the death kinetics was a first order kinetics; the yield coefficients $Y_{x/s}$ and $Y_{p/x}$ were determined experimentally from biomass or product obtained per glucose unit consumed. To assign other parameter ($\tau, \theta, \alpha, \beta, \gamma, q_m$) values, simulations were compared to the experimental data, so as to find a parameter set which gave the best fit of the model to the experimental data. Fitting

evaluation was done by minimizing the sum of squared errors between the model and the experimental data. The exponential profile of flow rate $F(t)$ was obtained from the mass balance on limiting substrate and calculated according to Enfors and Haggstrom (1998) throughout the assumption of a quasi-steady state on the glucose balance.

3.2 Nomenclature

- α constant for parabolic increase of specific glucose consumption for maintenance for $t \geq t_1$ [h^{-3}]
- β constant for parabolic increase of specific glucose consumption for maintenance for $t \geq t_1$ [h^{-2}]
- γ constant for parabolic increase of specific glucose consumption for maintenance for $t \geq t_1$ [h^{-1}]
- F glucose feed rate at time *t* [L h^{-1}]
- θ constant for exponential increase of specific glucose consumption rate for growth [h^{-1}]
- k_d specific death rate [h^{-1}]
- s residual glucose concentration at time *t* [g L^{-1}]
- s_i glucose concentration in the inlet [g L^{-1}]
- μ specific growth rate [h^{-1}]
- p interleukin-1β concentration at time *t* [g L^{-1}]
- q_g specific glucose consumption rate for growth [g g^{-1} h^{-1}]
- q_m specific glucose consumption rate for maintenance [g g^{-1} h^{-1}]
- t time [h]
- t_1 time at which the variation of specific growth rate and glucose consumption starts [h^{-1}]
- τ constant for exponential decrease of specific growth rate [h^{-1}]
- V_0 initial culture volume [L]
- V culture volume at time *t* [L]
- x viable biomass concentration at time *t* [g L^{-1}]
- x_0 initial viable biomass concentration [g L^{-1}]
- y concentration of variable of interest at time *t* [g L^{-1}]
- y_i concentration of variable of interest in the inlet [g L^{-1}]
- $Y_{x/s}$ biomass yield on glucose
- $Y_{p/x}$ IL-1β yield on biomass

4. Results

4.1 Modeling of aerated fed-batch culture with *Z. bailii* [pZ$_3$KIIL-1β]

The model developed to describe growth and IL-1β production with *Z. bailli* [pZ$_3$KIIL-1β] (Table 1) considered that yeast cells remained viable over the entire fermentation run. This assumption was supported by the experimental determination of k_d which resulted zero. The unfitting between the

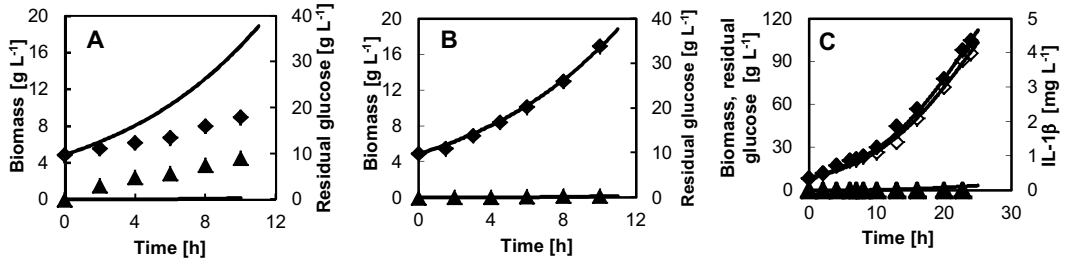

Figure 1: Z. bailii [pZ$_3$KIIL-1β] growing in the aerated fed-batch reactor: respiratory inoculum (A), fermentative inoculum (B), and run of 24 h with fermentative inoculum (C). Simulation curves (continuous lines) and experimental data refer to biomass (full rhombus), residual glucose (full triangle), and product (empty rhombus) concentrations x_0 = 4.9 $g\,L^{-1}$, s_0 = 0 $g\,L^{-1}$, p_0 = 0.35 $mg\,L^{-1}$, $Y_{x/s}$ = 0.37, $Y_{p/x}$ = 4 × 10^{-5}.

simulation curves and the experimental data regarding biomass and residual glucose (Figure 1A, B) highlighted that μ of *Z. bailli* [pZ$_3$KIIL-1β] kept constant at the given value (0.13 h^{-1}) only when the inoculum came from a fully fermentative batch culture (Figure 1A). During the fed-batch run carried out with the fermentative inoculum (Figure 1C), a good fitting between simulation curves and experimental data was observed. Glucose did not accumulate in the medium and ethanol was not produced (data not shown), indicating that *Z. bailii* [pZ$_3$KIIL−1β] displayed a fully respiratory metabolism. A cell density of more than 100 g L^{-1} and a IL-1β concentration of 4 mg L^{-1} was achieved after 24 h, with a IL-1β productivity of 0.15 mg L^{-1} h^{-1}.

4.2 Modeling of aerated fed-batch culture with *S. cerevisiae* BY4741[PIR4-IL1β]

Differently from the bioprocess carried out with *Z. bailii*, the model for *S. cerevisiae* BY4741 (Table 1) considered that yeast cells did not remain viable over the entire fermentation run, since k_d was significantly high (k_d = 0.028 h^{-1}). Figure 2 shows a good agreement existing between experimental data and simulation curves. It is evident that the specific growth rate (μ) chosen to build up the exponential feeding profile, was maintained in the time interval 0 ≤ t< t_1 and exponentially decreased when t ≥ t_1 (see Table 1), where t_1 corresponds to 7 h of feeding. Maximum of IL-1β productivity was achieved after 17 h (0.08 mg L^{-1} h^{-1}) of feeding, then it diminished because cell density diminished, due

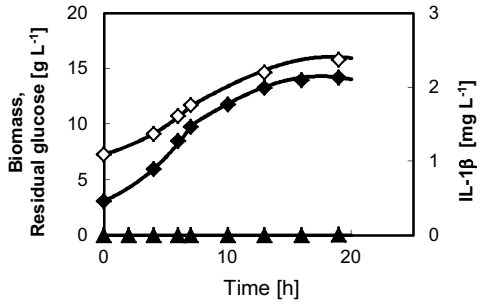

Figure 2: S. cerevisiae BY4741[PIR4-IL1β] growing in the aerated fed-batch reactor: simulation curves (continuous lines) and experimental data refer to biomass (full rhombus),residual glucose (full triangle), and product (empty rhombus) concentrations. x_0 = 3.1 $g\ L^{-1}$, s_0 = 0 $g\ L^{-1}$, p_0 = 1.1 $mg\ L^{-1}$, $Y_{x/s}$ = 0.50, $Y_{p/x}$ = 1 × 10^{-4}

to both cell death and culture dilution which prevailed on proliferation. The final biomass was significantly lower (14 g L^{-1}) than that achieved by the prototrophic *Z. bailii*. Glucose was completely consumed (Figure 2), without ethanol production (data not shown). In an attempt to describe the complete glucose consumption coupled with the lowering of specific growth rate, modeling considered that the specific glucose consumption rate (q_s) was split into two components, q_g and q_m (Table 1), which account for energy and precursors for growth and energy demand for maintenance, respectively. More precisely, mathematical model considered that q_g was constant during the first seven hours of run, then it decreased exponentially with time, similarly to what happened to the specific growth rate (Table 1), whereas the specific glucose consumption for maintenance (q_m), negligible in the first seven hours, increased according to a parabolic law (Table 1).
As expected, IL-1β profile follows that of biomass and IL-1β concentration achieved a maximum of 2.4 mg L^{-1} (Figure 2).

5. Discussion

The unstructured non-segregated mathematical model developed in parallel with the experimental runs carried out with the two producer strains examined, *Z. bailli* [pZ$_3$KIIL-1β] and *S. cerevisiae* BY4741 [PIR4-IIL1β], revealed to be of basic importance for both bioprocess optimization and understanding the physiological characteristics of the strains. In fact, with *Z. bailii* [pZ$_3$KIIL-1β], the mathematical

model gave the possibility to optimize the bioprocess achieving a significantly high productivity, provided that a fermentative inoculum is employed. Contrarily to *Z. bailii* [pZ$_3$KIIL-1β], the viability of auxotrophic *S. cerevisiae* BY4741[PIR4-IIL1β] drastically reduced during the run and growth unavoidably arrested. The mathematical model, based on the viable biomass, lead to the specification and quantification of the maintenance coefficient (q_m), which increased over time. Apparently, unlike the robust prototrophic *Z. bailii*, the auxotrophic *S. cerevisiae* strain used most of the available carbon source to satisfy an increasing energy demand for maintenance during the fermentation run, thus impairing the overall biomass/product productivity. This behaviour was ascribed to the peculiar environment which arose in the aerated fed-batch reactor, able to affect the overall performance of the auxotrophic strain. In conclusion, the modellistic approach presented in this work revealed of fundamental importance to understand the strain physiology and consequently to predict its performance under conditions which resemble those of production.

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