Trehalose accumulation in *Saccharomyces cerevisiae* cells: experimental data and structured modeling

Juan S. Aranda [a,*], Edgar Salgado [a], Patricia Taillandier [b]

[a] *Department of Bioengineering, Unidad Profesional Interdisciplinaria de Biotecnologıa, Instituto Politécnico Nacional (UPIBI-IPN), Av. Acueducto s/n, 07340 Mexico, D.F., Mexico*

[b] *Laboratoire de Génie Chimique, ENSIACET-INP, 18 route de Narbonne, 31077 Toulouse Cedex, France*

Abstract

Several studies have shown that the reserve disaccharide trehalose also protects yeast cells under environmental stress. Besides, it is acknowledged that trehalose improves yeast viability so its synthesis has become paramount in baker's yeast industry, as well as in wine and beer production. In this work, starving carbon or nitrogen conditions were imposed to *Saccharomyces cerevisiae* growing in fed-batch cultivations in order to induce biosynthesis and accumulation of trehalose. Our experimental results show that yeast can accumulate trehalose up to 13% of biomass dry weight ($0.13\,g_{trehalose}\,g^{-1}_{biomass}$) under carbon or nitrogen starvation. A compartmentalized and biochemically structured model is proposed in order to mathematically describe these experimental findings. The model predictions were compared with literature results obtained under growth conditions different from those established in our experiments, resulting in a very good agreement and thus indicating that the proposed hypotheses are essentially correct.

Keywords *Saccharomyces cerevisiae*; Trehalose; Fed-batch cultivation

1. Introduction

Trehalose (α-D-glucopyranosyl-1, 1-α-D-glycopyranoside) is a non-reducing disaccharide composed of two molecules of glucose linked by a α-(1,1)-glycosidic bond. Several lines of evidence suggest that trehalose has at least two important metabolic roles in *Saccharomyces cerevisiae* cells: on the one hand it would be a reserve carbohydrate [1,2] and, on the other hand, it would also protect cytosol components against adverse growth conditions such as heat shock, osmotic shock or starvation [3,4]. Following this latter function, it is generally accepted that trehalose improves yeast viability [5–7] in commercial yeast production processes. So, understanding trehalose formation and accumulation has become a main subject in baker's yeast industry, in so far as in winemaking and brewing industries.

The biosynthesis of trehalose occurs entirely in the cytosol [8]. Uridyl-glucose diphosphate (UDPG) is the nucleotide that donates the glucose moiety that reacts with glucose-6-P to form trehalose-6-P in a reaction catalyzed by the enzyme trehalose phosphate synthase (6-phosphate transglucosidase, EC 2.4.1.15). Then, a specific phosphatase (trehalose-6-P phosphohydrolase, EC 3.2.1.28) cleaves off phosphate from trehalose-6-P to form trehalose. Trehalose phosphate synthase and trehalose-6-P phosphohydrolase are associated in an enzymatic complex called TPS complex. Trehalose accumulation is known to happen under two circumstances: slow growth [7] or environmental stress [9]. Slow growth occurs in the stationary phase [9,23] of batch cultures and in continuous or feed-batch cultures with small dilution rates [10,8], whereas heat or osmotic shocks [1,2], nutrient depletion [8,9,11] and other adverse environmental conditions put the cells under stress inducing trehalose accumulation.

Works on cell protection and viability improvement by trehalose had led to applications in cryogenic preservation of yeast strains, as well as in brewing, winemaking and dry yeast production. However, intracellular accumulation of trehalose from a modeling perspective has been barely studied. In this paper, we investigate how trehalose content in yeast cells evolves in fed-batch fermentations under carbon or nitrogen starvation conditions, and we propose a biochemically structured model for explaining our results. The modeling approach is interesting for gaining novel insight into the regulation of trehalose synthesis and degradation—particularly in the quantitative level. Besides, a model could be a useful way to get a narrow estimate of trehalose content in cells during yeast production.

* Corresponding author. Tel./fax: +52-5729-6000x56338.
E-mail address jaranda@acei.upibi.ipn.mx (J.S. Aranda).

doi:10.1016/S1369-703X(03)00148-7

Nomenclature

A	co-repressor concentration in cells (M)
ApoR	aporepressor protein concentration in cells (M)
cAMP	intracellular cAMP concentration (M)
e_H	TH fraction (inactive conformation) ($g_{TH}\, g_{biomass}^{-1}$)
e_S	TPS fraction (inactive conformation) ($g_{TPS}\, g_{biomass}^{-1}$)
E_H	TH fraction (active conformation) ($g_{TH}\, g_{biomass}^{-1}$)
E_S	TPS fraction (active conformation) ($g_{TPS}\, g_{biomass}^{-1}$)
E_{TH}	neutral trehalase fraction in the cells ($g_{TH}\, g_{biomass}^{-1}$)
E_{TS}	trehalose phosphate synthase fraction in the cells ($g_{TPS}\, g_{biomass}^{-1}$)
f_v	feed volumetric flow ($l\, h^{-1}$)
F	function to be minimized for parameters estimation (–)
G_H	unrepressed gene concentration for TH synthesis in cells (M)
G_S	unrepressed gene concentration for TPS synthesis in cells (M)
G_{TH}	total concentration gene for TH synthesis in cells (M)
G_{TS}	total concentration gene for TPS synthesis in cells (M)
$G_H R$	repressed gene concentration for TH synthesis in cells (M)
$G_S R$	repressed gene concentration for TPS synthesis in cells (M)
k_A	production constant for cAMP ($M\, g_{biomass}\, g_{substrate}^{-1}$)
k_{eqA}	equilibrium constant for the reaction $e_H + \text{cAMP} \leftrightarrow E_H$ (M^{-1})
k_{eqH}	equilibrium constant for the reaction $G_H + R \leftrightarrow G_H R$ (M^{-1})
k_{eqI}	equilibrium constant for the reaction $E_S + 2\text{cAMP} \leftrightarrow e_S$ (M^{-2})
k_{eqR}	equilibrium constant for the reaction $\text{ApoR} + A \leftrightarrow R$ (M^{-1})
k_{eqS}	equilibrium constant for the reaction $G_S + R \leftrightarrow G_S R$ (M^{-1})
k_{EH}	reaction constant for constitutive production of TH ($g_{TH}\, g_{biomass}^{-1}$)
k_{ES}	reaction constant for constitutive production of TPS ($g_{TPS}\, g_{biomass}^{-1}$)
k_H	reaction constant for trehalose hydrolysis ($g_{trehalose}\, g_{TH}^{-1}\, h^{-1}$)
k_{PH}	reaction constant for TH lysis (h^{-1})
k_{PS}	reaction constant for TPS lysis (h^{-1})
k_S	reaction constant for trehalose synthesis ($g_{trehalose}\, g_{TPS}^{-1}\, h^{-1}$)
k_1	reaction constant for inducible TPS synthesis ($g_{TPS}\, g_{biomass}^{-1}\, M^{-1}\, h^{-1}$)
k_2	reaction constant for inducible TH synthesis ($g_{TH}\, g_{biomass}^{-1}\, M^{-1}\, h^{-1}$)
m	number of model variables (x, v, w_T, E_{TS}, E_{TH}, cAMP) (–)
n	number of experimental data (–)
$\boldsymbol{p}$	vector of estimated model parameters (–)
q_A	specific rate of intracellular cAMP synthesis ($M\, h^{-1}$)
q_{EH}	specific rate of TH synthesis ($g_{TH}\, g_{biomass}^{-1}\, h^{-1}$)
q_{ES}	specific rate of TPS synthesis ($g_{TPS}\, g_{biomass}^{-1}\, h^{-1}$)
q_{HT}	specific rate of trehalose hydrolysis ($g_{trehalose}\, g_{biomass}^{-1}\, h^{-1}$)
q_P	specific rate of total TH and TPS proteolysis ($g_{enzyme}\, g_{biomass}^{-1}\, h^{-1}$)
q_{PH}	specific rate of TH proteolysis ($g_{TH}\, g_{biomass}^{-1}\, h^{-1}$)
q_{PS}	specific rate of TPS proteolysis ($g_{TPS}\, g_{biomass}^{-1}\, h^{-1}$)
q_s	specific substrate consumption rate ($g_{substrate}\, g_{biomass}^{-1}\, h^{-1}$)
q_{ST}	specific rate of trehalose synthesis ($g_{trehalose}\, g_{biomass}^{-1}\, h^{-1}$)
R	repressor protein concentration (M)
s	substrate concentration in the bioreactor ($g_{substrate}\, l^{-1}$)
s_0	substrate concentration in the fed medium ($g_{substrate}\, l^{-1}$)
t	cultivation time (h)
TH	neutral trehalase (–)
TPS	trehalose phosphate synthase (–)
v	working volume in the bioreactor (l)

w_C	other cellular constituents fraction (different from w_T and w_E) in the cells ($g_{biomass}\, g^{-1}_{biomass}$)
w_E	enzymes TPS and TH fraction in the cells ($g_{enzymes}\, g^{-1}_{biomass}$)
w_T	trehalose fraction in the cells ($g_{trehalose}\, g^{-1}_{biomass}$)
x	biomass concentration in the bioreactor ($g_{biomass}\, l^{-1}$)
$\boldsymbol{y}^{exp}$	$n \times m$ matrix of experimental data (–)
$\boldsymbol{y}^{mod}$	$n \times m$ matrix of model numerical values (–)
Y_x	biomass yield ($g_{biomass}\, g^{-1}_{substrate}$)
$\boldsymbol{z}$	vector of model variables (–)

Greek letters

μ	specific growth rate (h^{-1})
σ_{exp}	standard deviation of experimental data ($g\, l^{-1}$)

2. Mathematical model

2.1. Biomass structure

The proposed model for trehalose accumulation comprehends three compartments, as seen in Fig. 1.

The trehalose compartment corresponds to the concentration of the disaccharide in yeast cells. In the enzymatic compartment are gathered the enzymes that modify intracellular trehalose content, specifically trehalose phosphate synthase complex (TPS) and neutral trehalase (TH). The cellular compartment represents all other cellular components, including regulating proteins and other molecules that modify TPS and TH enzymatic activities. Therefore, in this structured modeling approach, there are three macroscopic (abiotic) variables (x, s, v) and five intracellular (biotic) variables (w_T, w_E, w_C, E_{TS}, E_{TH}). It has to be noted that:

$$w_T + w_E + w_C = 1$$

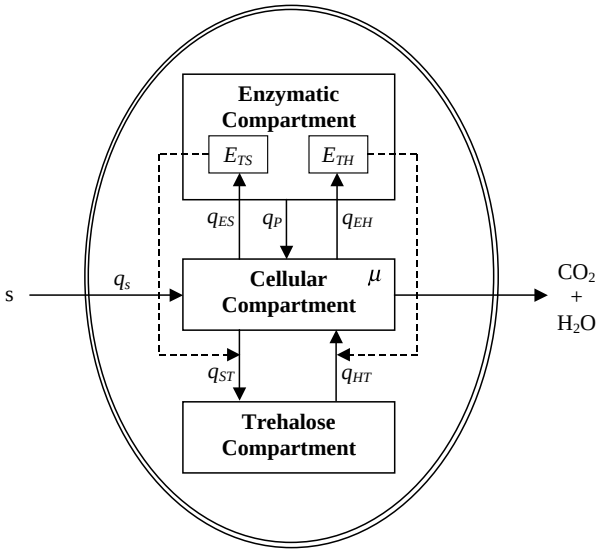

Fig. 1. Biomass structure.

and

$$w_E = E_{TS} + E_{TH}$$

It is also considered that the enzymatic fraction of TPS (E_{TS}) is present in cytoplasm under two conformational structures, one of them is an active conformation (E_S), and the other is the inactivated enzyme (e_S). So

$$E_{TS} = E_S + e_S \tag{1}$$

Similarly, for the neutral trehalase enzymatic fraction (E_{TH}), this is:

$$E_{TH} = E_H + e_H \tag{2}$$

The proposed biomass structure is evidently incomplete, as much as no model can totally describe the real conformation of the cell. However, it captures the key features of the studied phenomenon, so it should be helpful in understanding and estimating the trehalose accumulation in *S. cerevisiae*. The equations relating the biotic and abiotic variables of the model will be described in the following sections.

2.2. Evolution of abiotic variables

In fed-batch cultures of yeast, changes in x, s and v can be stated from

$$\frac{dx}{dt} = \left(\mu - \frac{f_v}{v}\right) x \tag{3}$$

$$\frac{ds}{dt} = \frac{f_v}{v}(s_0 - s) - q_s x \tag{4}$$

$$\frac{dv}{dt} = f_v \tag{5}$$

In a fed-batch cultivation, substrate concentration can be kept near to zero by feeding such a small amount of substrate that it is depleted almost instantaneously. Under such conditions, substrate accumulation can be neglected, and, assuming $s \approx 0$, Eq. (4) can be rewritten as

$$q_s = \frac{f_v}{vx} s_0 \tag{6}$$

Eq. (6) is useful for the estimation of the specific growth rate (μ):

$$\mu = Y_x q_s \tag{7}$$

Eq. (7) assumes a constant yield coefficient of biomass on the substrate, meaning that the energy needed for cell maintenance is neglected. According to values reported elsewhere, the maintenance of yeasts is small as compared to that in most bacteria [39]. In *S. cerevisiae*, even with specific growth rates as low as $0.05\,h^{-1}$, the effect of maintenance cannot be observed [40]. Hence, the influence of the maintenance on bioenergetics of yeast appears to be small as already discussed by various authors [41,42], and then assumption on Eq. (7) seems to be justified.

2.3. Evolution of biotic variables: trehalose compartment

Trehalose accumulation in cells depends on its rates of synthesis and hydrolysis. In order to increase the trehalose compartment, meaning a more elevated trehalose concentration in yeast cells, the synthesis rate must be higher than that of hydrolysis. This can be mathematically established as follows:

$$\frac{dw_T}{dt} = q_{ST} - q_{HT} - \mu w_T \tag{8}$$

where μw_T is the dilution term due to expansion of the biomaterial (cell growth) [12]. The biosynthesis of trehalose can be simplified as the condensation of glucose 6-P and the glucosyl group from UDPG:

$$\text{glucose 6-P} + \text{UDPG} \xrightarrow{\text{trehalose phosphate synthase}} \text{trehalose-P}$$
$$\xrightarrow{\text{trehalose phosphatase}} \text{trehalose}$$

The specific rate for this reaction is a function of TPS enzyme and substrates concentrations, so defining high order kinetics. However, assuming that both reactants are in excess, the specific rate of trehalose synthesis will depend solely on the TPS active fraction (E_S), that is:

$$q_{ST} = k_S E_S \tag{9}$$

In that case, the reaction rate is then taken as pseudo-first-order kinetics. Under a similar hypothesis, the specific rate of trehalose hydrolysis is given as

$$q_{HT} = k_H E_H \tag{10}$$

In Eqs. (9) and (10), k_S and k_H are pseudo-first-order kinetic constants because they take account of excess reactant concentrations.

2.4. Evolution of biotic variables: enzymatic compartment

The enzyme fractions E_{TS} and E_{TH} change in time within the cell cytoplasm as a result of their production, accordingly to the expression of, respectively, G_S and G_H genes, and of their rates of degradation by endogenous proteolysis. Thus, the variation of TPS and TH in the cells can be expressed by

$$\frac{dE_{TS}}{dt} = q_{ES} - q_{PS} - \mu E_{TS} \tag{11}$$

$$\frac{dE_{TH}}{dt} = q_{EH} - q_{PH} - \mu E_{TH} \tag{12}$$

where μE_{TS} and μE_{TH} are the cellular dilution terms.

Although TPS and TH enzymes are normally present in the cells, under certain culture conditions the enzyme production is increased [13,7], meaning that TPS and TH are both partially inducible enzymes. This experimental evidence for induction suggests that the observed rate of enzyme production is composed by constitutive and inductive production, i.e.,

$$q_{ES} = q_{ES,1} + q_{ES,2} \tag{13}$$

The first term on the right side of Eq. (13) is the specific rate of TPS synthesis associated only to normal growth:

$$q_{ES,1} = k_{ES}\mu \tag{14}$$

and the second one represents induced production of TPS. An operon repression model is involved in TPS induced production, and cAMP has been postulated as a main co-repressor molecule [14–17]. The structural gene for the TPS (G_S) is able to synthesize the enzymatic complex only if an active repressor protein (R) does not block it. When the co-repressor (A) combines with an aporepressor (ApoR) to get the active repressor, a complex ($G_S R$) is formed which blocks the expression of the structural gene by binding the operator gene. This can be stated as follows:

$$\text{ApoR} + A \overset{k_{eqR}}{\leftrightarrow} R$$

The equilibrium constant for this reaction is

$$k_{eqR} = \frac{[R]}{[\text{ApoR}][A]}$$

so the repressor concentration is written as

$$R = k_{eqR}[\text{ApoR}][A] \tag{15}$$

If the repressor protein stops the structural gene expression, then:

$$G_S + R \overset{k_{eqS}}{\leftrightarrow} G_S R$$

Assuming that this is an equilibrium reaction, the equilibrium constant is expressed as

$$k_{eqS} = \frac{[G_S R]}{[G_S][R]}$$

and the gene–repressor complex concentration is

$$G_S R = k_{eqS}[G_S][R] \tag{16}$$

The total structural gene (G_{TS}) in cells is considered to exist under repressed and unrepressed conformations, that is:

$$G_{TS} = G_S + G_S R \tag{17}$$

By combining Eqs. (16) and (17) one obtains:

$$G_S = \frac{G_{TS}}{k_{eqS}[R] + 1} \tag{18}$$

Eq. (18) allows us to estimate the structural gene that is expressed to synthesize the TPS enzyme [18]. Specific rate of inducible TPS synthesis ($q_{ES,2}$) should be proportional to the expressing structural gene (G_S), so:

$$q_{ES,2} = k_1[G_S] \tag{19}$$

and substituting Eq. (18) into Eq. (19):

$$q_{ES,2} = \frac{k_1[G_{TS}]}{k_{eqS}[R] + 1}$$

Finally, the global specific rate of TPS synthesis, including growth associated and induced productions, is, from Eq. (13):

$$q_{ES} = k_{ES}\mu + \frac{k_1[G_{TS}]}{k_{eqS}[R] + 1} \tag{20}$$

An equivalent expression for the specific rate of TH production shall be:

$$q_{EH} = k_{EH}\mu + \frac{k_2[G_{TH}]}{k_{eqH}[R] + 1} \tag{21}$$

The proteins and enzymes no longer useful for cell metabolism are hydrolyzed in a reaction that follows a pseudo-first-order rate expression [19,20]:

$$q_{PS} = k_{PS}E_{TS} \tag{22}$$

$$q_{PH} = k_{PH}E_{TH} \tag{23}$$

where k_{PS} and k_{PH} are reaction constants.

2.5. Evolution of biotic variables: cellular compartment

The cellular compartment includes cellular components other than trehalose and enzymes TPS and TH. The regulatory molecules that modify TPS and TH activities are also included in it. Although there is a number of stimulatory and inhibitory mechanisms on those enzymes, a variation of the cAMP intracellular concentration is considered as a major feature in the presented model because the cAMP present in the cell plasma significantly modifies the TPS and TH enzymatic activities [21–23]. Apparently, the cAMP cytoplasmic concentration is related to extracellular substrates [24,25]. Besides, the intracellular adenosine-phosphate compounds diminish concomitantly with extracellular glucose [7,26,27] and q_s is proportional to extracellular glucose too, so intracellular cAMP could be proportional to the limiting substrate consumption:

$$\frac{\mathrm{d(cAMP)}}{\mathrm{d}t} = q_A - \mu(\mathrm{cAMP}) \tag{24}$$

where

$$q_A = k_A q_s \tag{25}$$

The cAMP cytoplasmic concentration effects on TH and TPS enzymatic activities can be modeled as follows. There are two coexisting conformations of the TPS, one of them is active the other is not. The inactive fraction is due to the presence of cAMP, hence, the enzyme can be inhibited when cAMP is present in the cytoplasm [28]:

$$E_S + 2\mathrm{cAMP} \overset{k_{eqI}}{\leftrightarrow} e_S$$

The equilibrium constant for this reaction is

$$k_{eqI} = \frac{[e_S]}{[E_S][\mathrm{cAMP}]^2}$$

which can be rewritten as

$$e_S = k_{eqI}[E_S][\mathrm{cAMP}]^2 \tag{26}$$

By substituting Eq. (26) into Eq. (1) one obtains:

$$E_S = \frac{E_{TS}}{k_{eqI}[\mathrm{cAMP}]^2 + 1} \tag{27}$$

Concerning the trehalase, the effect of cAMP is the opposite as it activates the enzyme following

$$e_H + \mathrm{cAMP} \overset{k_{eqA}}{\leftrightarrow} E_H$$

and the constant equilibrium is defined by

$$k_{eqA} = \frac{[E_H]}{[e_H][\mathrm{cAMP}]}$$

from which

$$e_H = \frac{E_H}{k_{eqA}[\mathrm{cAMP}]} \tag{28}$$

By substituting Eq. (28) into Eq. (2) one can finally obtain:

$$E_H = \frac{k_{eqA}[E_{TH}][\mathrm{cAMP}]}{k_{eqA}[\mathrm{cAMP}] + 1} \tag{29}$$

Table 1 presents all the equations of the structured model.

3. Materials and methods

3.1. Microorganism

The microorganism used in this work was a *S. cerevisiae* EC1118 strain commercialized by Lallemand Inc. (Montreal, Canada). The microorganism was maintained on slants (glucose 20 g l^{-1}, yeast extract 10 g l^{-1}, agar-agar 20 g l^{-1}) at 4 °C. Periodic inoculations were made in new slants every 4 months ca.

Table 1
Model equations

No.	Equation	Physical meaning
Abiotic variables		
(3)	$\frac{dx}{dt} = \left(\mu - \frac{f_v}{v}\right)x$	Biomass accumulation in the reactor
(5)	$\frac{dv}{dt} = f_v$	Variation of working volume in the bioreactor
Biotic variables		
(8)	$\frac{dw_T}{dt} = q_{ST} - q_{HT} - \mu w_T$	Variation of intracellular fraction of trehalose
(11)	$\frac{dE_{TS}}{dt} = q_{ES} - q_{PS} - \mu E_{TS}$	Variation of intracellular fraction of TPS
(12)	$\frac{dE_{TH}}{dt} = q_{EH} - q_{PH} - \mu E_{TH}$	Variation of intracellular fraction of TH
(24)	$\frac{d(\text{cAMP})}{dt} = q_A - \mu(\text{cAMP})$	Variation of intracellular concentration of cAMP
Kinetic equations		
(6)	$q_s = \frac{f_v}{vx}s_0$	Specific substrate consumption rate
(7)	$\mu = Y_x q_s$	Specific growth rate
(9)	$q_{ST} = k_S E_S$	Intrinsic specific rate of trehalose synthesis
(27)	$E_S = \frac{E_{TS}}{k_{eqI}[\text{cAMP}]^2 + 1}$	TPS fraction (active conformation)
(10)	$q_{HT} = k_H E_H$	Intrinsic specific rate of trehalose hydrolysis
(29)	$E_H = \frac{k_{eqA}[E_{TH}][\text{cAMP}]}{k_{eqA}[\text{cAMP}] + 1}$	TH fraction (active conformation)
(20)	$q_{ES} = k_{ES}\mu + \frac{k_1[G_{TS}]}{k_{eqS}[R] + 1}$	Intrinsic specific rate of TPS synthesis
(21)	$q_{EH} = k_{EH}\mu + \frac{k_2[G_{TH}]}{k_{eqH}[R] + 1}$	Intrinsic specific rate of TH synthesis
(25)	$q_A = k_A q_s$	Specific rate of intracellular cAMP synthesis
(22)	$q_{PS} = k_{PS}E_{TS}$	Specific rate of TPS proteolysis
(23)	$q_{PH} = k_{PH}E_{TH}$	Specific rate of TH proteolysis

3.2. Fermentation medium

The effect of a nutrient depletion on intracellular trehalose content can only be done by using a chemically well-defined fermentation medium. In regard of this, we have chosen a synthetic medium [29,30] for our experimental purposes. The medium composition is glucose 50 g l^{-1}, KH_2PO_4 7 g l^{-1}, $CaCl_2{\cdot}2H_2O$ 0.25 g l^{-1}, NaCl 0.5 g l^{-1}, $MgCl_2{\cdot}6H_2O$ 6 g l^{-1}, minerals solution 10 ml l^{-1}, vitamins solution 10 ml l^{-1}. Five hundred milliliters of minerals solution contain $FeSO_4{\cdot}7H_2O$ (278 mg), $ZnSO_4{\cdot}7H_2O$ (288 mg), $CuSO_4{\cdot}5H_2O$ (7.5 mg), $Na_2MoO_4{\cdot}2H_2O$ (25 mg), $MnSO_4{\cdot}H_2O$ (169 mg), H_2SO_4 a few drops. Five hundred milliliters of vitamins solution are prepared with biotin (1.5 mg), calcium pantothenate (20 mg), inositol (125 mg), pyridoxine-HCl (25 mg), thiamine-HCl (50 mg).

3.3. Inoculum development

Inoculum was grown in a 1 l flask containing 500 ml of the synthetic medium at 30 °C and 150 rpm over 24 h. The bioreactor was inoculated with the obtained biomass and then a batch fermentation was carried out on a 6 l work volume. The fed-batch cultivations were initiated after 10 h of the previously established batch cultivation, until a final volume between 12 and 13 l was reached.

3.4. Experimental conditions

A 15 l bioreactor (Applikon Z81315 M607) was used for all fed-batch experiments. The experimental conditions were: temperature 30 °C, pH 5.0, air flow 450 l/h, dissolved oxygen 10% of saturation value (0.8 mg_{O_2} l^{-1} ca). The pH of the culture was controlled with ammonia–water (20% (v/v)) and this solution was the only nitrogen source. The flow of carbon substrate was a function of the respiratory quotient (RQ) of the culture as calculated from effluent gas composition data. The glucose concentration in the working liquid in the bioreactor was always kept near to zero. In order to investigate the effect of nutrient depletion on trehalose content in cells, fed-batch cultures with a final phase of starvation (depletion period) in carbon or nitrogen were also carried out. Carbon starvation was imposed to cells by stopping the carbon substrate feed, and switching the alkaline

solution for pH control from ammonia–water to NaOH 3 N did the nitrogen depletion. All experiments were triplicated to check out the reproducibility of data.

3.5. Analytical methods

Trehalose. Samples of 20 mg dry yeast were extracted twice with 3 ml of 0.05 M trichloroacetic acid in continuous orbital shaking during 40 min each time. Trehalose was then determined by the anthrone method [6].

Biomass. The yeast growth was followed by measuring the optical density of the culture at 620 nm with an UV-Vis spectrophotometer (Hitachi U-2000). A correlation between dry weight and optical density was previously established.

Glucose. The glucose concentration was determined by the glucose oxydase method with an automatic analyzer (YSI 2700 Select).

4. Results and discussion

4.1. Experimental results

S. cerevisiae responds to a carbon depletion phase in fed-batch cultivation by increasing cytoplasmic trehalose (see Fig. 2). The trehalose accumulation induced by carbon starving is slightly a slower process than induction caused by other stress conditions. This assumption is coherent with previous experimental observations [31]. Compared with carbon starvation, a higher and faster trehalose accumulation was observed when yeast cells were depleted on nitrogen source (see Fig. 3). Depletions on carbon or nitrogen produced a 4-fold increase of intracellular trehalose content through the fed-batch cultivations. The intracellular trehalose content in yeast is normally about 0.025–0.035 $g_{trehalose}\, g_{biomass}^{-1}$ (2.5–3.5% of biomass dry weight), through the fed-batch cultures trehalose concentration in yeast cells attained almost 0.13 $g_{trehalose}\, g_{biomass}^{-1}$ (13% of biomass dry weight) at the end of the culture. Similar results were reported elsewhere [8,9].

Since the trehalose accumulation depends on the enzymatic activity of trehalase and trehalose phosphate synthase, and so on the intracellular concentration of cAMP, it can be hypothetically stated that nitrogen starvation could originate a reduction of cAMP in the cells and therefore a greater trehalose accumulation. There is some evidence supporting this hypothesis. It has been reported that the activity of adenyl cyclase (the enzyme that catalyses the synthesis of cAMP) is enhanced when a membrane associated Ras system is active [23]. Like some other G-proteins, the activity of the Ras proteins is controlled by the guanine nucleotide being inactive when bounded to GDP and active when bound to GTP [32]. During nitrogen starvation, the synthesis of guanine nucleotides is stopped [38], the Ras system becomes inactive and cAMP synthesis could be stopped or, at least, decreased. Thus, low intracellular levels of cAMP may bring, as a consequence, a significant increment of trehalose in cells cytoplasm.

4.2. Parameter estimation and model results

The model parameters and model initial conditions are shown, respectively, in Tables 2 and 3. Nine model parameters have been estimated by minimizing residuals between model numerical values and experimental data according to:

$$F(\boldsymbol{p}) = \sum_{j=1}^{m}\sum_{i=1}^{n}(y_{i,j}^{\exp} - y_{i,j}^{\mathrm{mod}})^2 \tag{30}$$

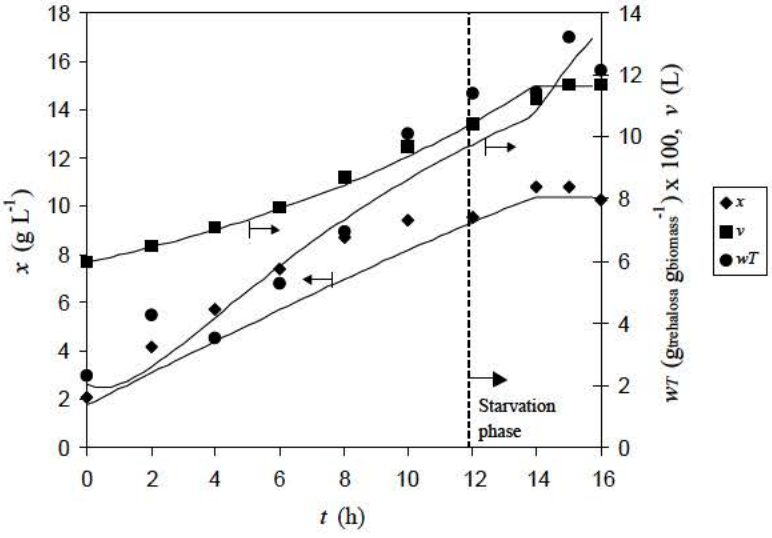

Fig. 2. Comparison between model results (solid lines) and experimental results (marks) for a fed-batch cultivation of yeast cells with a final carbon starvation period.

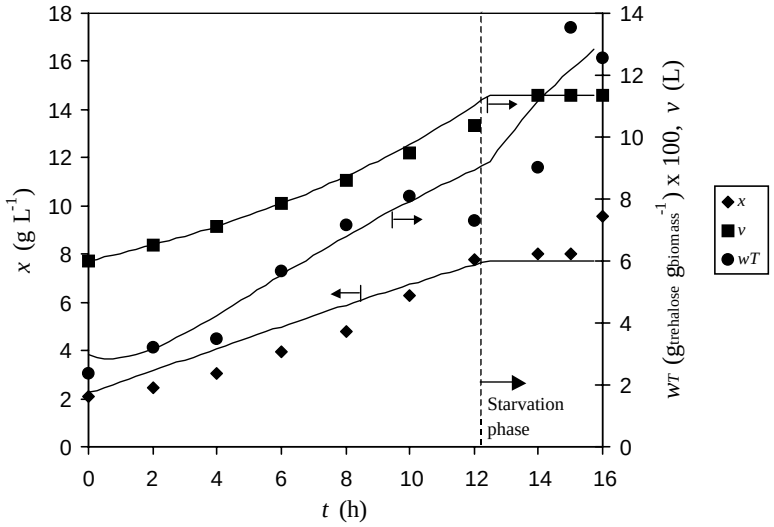

Fig. 3. Comparison between model results (solid lines) and experimental results (marks) for a fed-batch cultivation of yeast cells with a final nitrogen starvation period.

Table 2
Model parameters

Model parameter	Numerical value		Reference
	Limiting C	Limiting N	
$f_v = f_v(t)$	$0.11\exp(0.08t)$	$0.113\exp(0.1t)$	This work
Y_x	0.43	0.34	This work
s_0	45.1	43.1	This work
k_S	1.4328 ± 0.262		This work
k_H	0.5198 ± 0.158		This work
k_{ES}	0.0156 ± 0.0034		This work
k_{EH}	0.0218 ± 0.0018		This work
k_{eqS}	2×10^9		[34–36]
k_{eqH}	2×10^9		[34–36]
k_{eqR}	0.1269 ± 0.0127		This work
k_A	0.0023 ± 0.00014		This work
k_{eqI}	0.01×10^{-6}		[18]
k_{eqA}	11.9483 ± 2.309		This work
k_1	3.0650 ± 0.240		This work
k_2	2.8327 ± 0.194		This work
k_{PS}	0.09		[20]
k_{PH}	0.07		[20]
G_{TH}	4×10^{-9}		[34,35]
G_{TS}	4×10^{-9}		[34,35]
ApoR	2×10^{-8}		[34,35]

Table 3
Model initial conditions

Initial conditions	Limiting substrate		Reference
	C	N	
x_0 ($g\,l^{-1}$)	1.769	2.243	This work
v_0 (l)	5.96	6.00	This work
w_{T0}	0.0205	0.0298	This work
E_{TS0}	0.001	0.001	This work
E_{TH0}	0	0	This work
$cAMP_0$ (mM)	0.2	0.2	[34,37]

where F is the function to be minimized, $\boldsymbol{p} = [k_S k_H k_{ES} k_{EH} k_{eqR} k_A k_{eqA} k_1 k_2]$ is the vector of model parameters, $\boldsymbol{y}^{exp}$ is an $n \times m$ matrix of experimental data, $\boldsymbol{y}^{mod}$ is an $n \times m$ matrix of model numerical values corresponding to $\boldsymbol{y}^{exp}$, n is the number of experimental points and m is the number of model variables. The $\boldsymbol{y}^{mod}$ values were calculated by integrating model equations with a fourth order Runge–Kutta method. The research of the minimum has been accomplished with a Nelder–Mead simplex method. All calculations were performed with MATLAB®. Figs. 2 and 3 show model behavior and experimental data for carbon and nitrogen depletion experiments.

Confidence intervals for the estimated parameters were calculated by a Monte Carlo approach. Basically, it was assumed that the structured model is consistent enough to represent the trehalose accumulation in yeast cells, so the estimated parameters from experimental data can be used to produce synthetic data sets by adding white noise to model numerical results. With those synthetic data sets one should be able to produce a population of estimated parameters whose standard deviation is a good estimate of the confidence intervals. The confidence intervals reported in Table 2 have been calculated with fifty synthetic data sets and within a confidence level of 60% of a Student's t distribution.

Figs. 4 and 5 present model results and confidence intervals for trehalose accumulation for experiments with carbon and nitrogen as limiting substrates.

In order to investigate if the structured model could represent trehalose accumulation in yeast under different growth conditions, the results obtained by Jørgensen et al. [33] were contrasted to model predictions, as shown in Figs. 6 and 7.

Jørgensen et al. [33] carried out their experimental design under the following growth conditions. After an initial 1 h batch phase, an exponential feed of medium corresponding to a specific growth rate of $0.2\,h^{-1}$ was started. After 20 h of

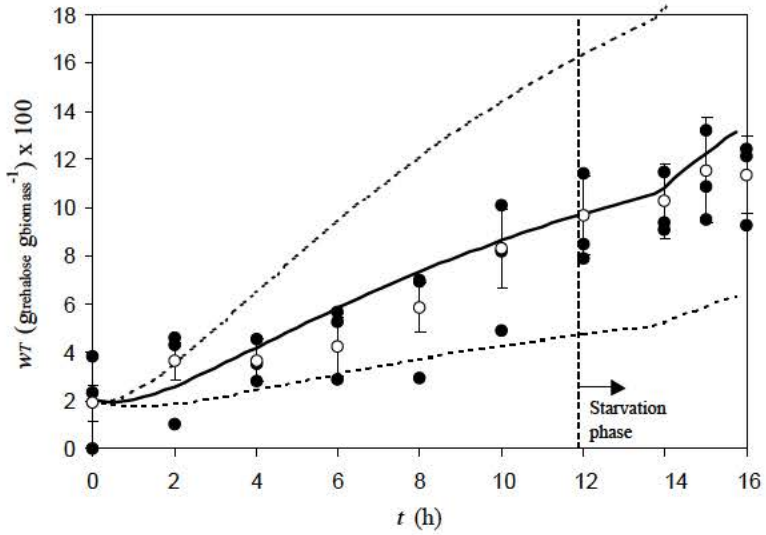

Fig. 4. Comparison between experimental data (filled marks) and model results (solid line) for intracellular trehalose content in a fed-batch yeast culture with a final depletion period (carbon source). Empty marks are experimental averages with a σ_{exp} error bar, and dotted lines are confidence intervals for trehalose.

fed-batch operation, the addition of complete medium was shift to the starvation medium and the feed-rate was reduced to 50% after 1 h and to 25% after 2 h of starving conditions. It can be seen that the model is able to describe acceptably well the trehalose accumulation in yeast cells. The fitting of experimental data by Jørgensen et al. to the model predictions could be interpreted somehow as an experimental validation of the proposed approach, showing that it can be useful in trehalose enriched yeast production, in spite of its intrinsic conceptual limitations. As can be seen in Table 4, about half of the estimated parameters with the data of Jørgensen et al. fall within the 60% confidence limits of the parameters obtained with the data from the present work. This suggests that the model is correct enough to describe the trehalose accumulation in yeast cells, and differences are either attributable to unaccounted strain-related phenomena or to insufficient, in quality or quantity, data.

4.3. *Sensitivity analysis*

Model equations for biotic and abiotic phases depend implicitly on estimated parameters, so an analysis of sensitivity coefficients can be carried out. Let $\boldsymbol{z}$ be a vector defined by model biotic and abiotic variables

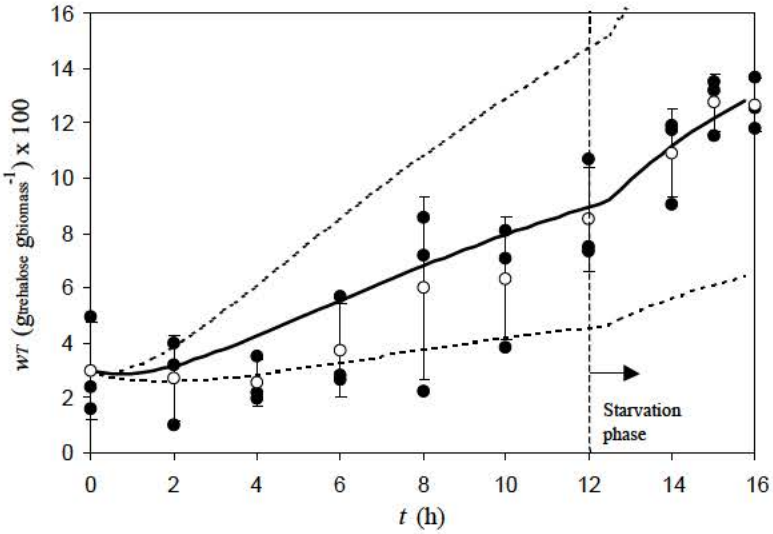

Fig. 5. Comparison between experimental data (filled marks) and model results (solid line) for intracellular trehalose content in a fed-batch yeast culture with a final depletion period (nitrogen source). Empty marks are experimental averages with a σ_{exp} error bar, and dotted lines are confidence intervals for trehalose.

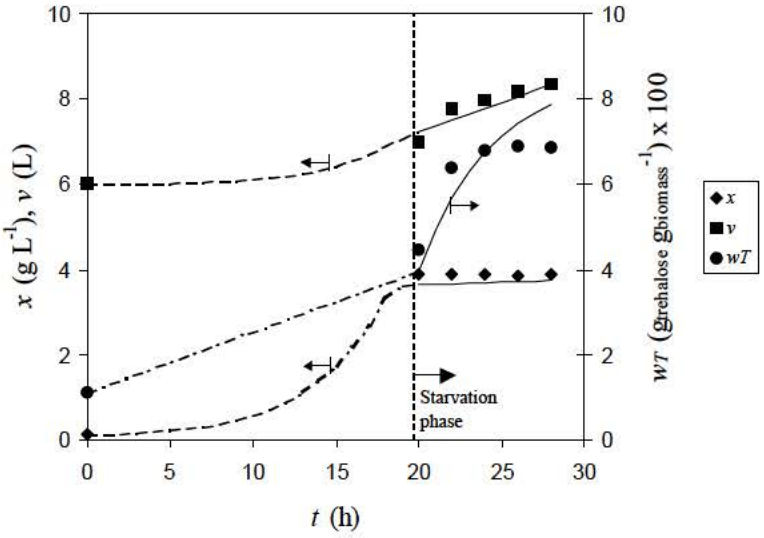

Fig. 6. Structured model behavior (solid lines) compared to experimental data (marks) by Jørgensen et al. [33] for a fed-batch yeast culture with a carbon depletion phase.

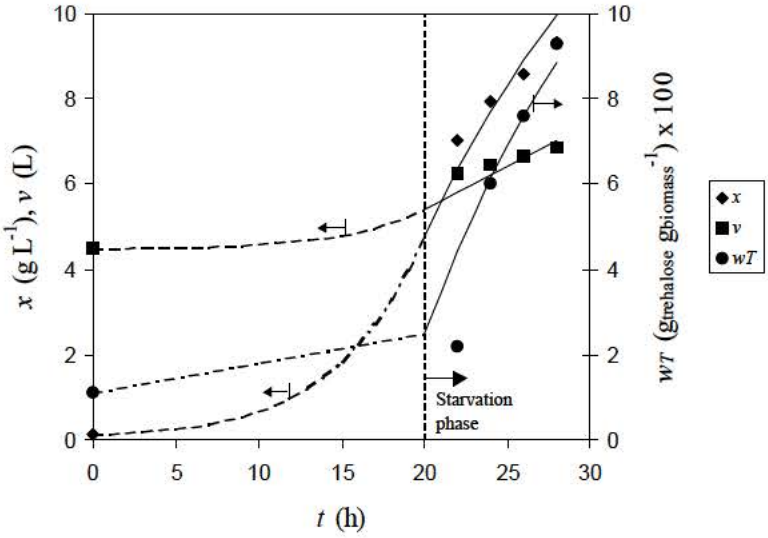

Fig. 7. Structured model behavior (solid lines) compared to experimental data (marks) by Jørgensen et al. [33] for a fed-batch yeast culture with a nitrogen depletion phase.

Table 4
Comparison of estimated parameters

Parameter	This work		Jørgensen et al. [33]		Relative deviation (%)	
	Numerical value	Confidence interval	C starving culture	N starving culture	C starving culture	N starving culture
k_S	1.4328	0.262	1.4486	1.4507	[a]	[a]
k_H	0.5198	0.158	1.1517	0.96533	32.3	19.2
k_{ES}	0.0156	0.0034	0.0169	0.01553	[a]	[a]
k_{EH}	0.0218	0.0018	0.0858	0.10255	70.0	74.9
k_{eqR}	0.1269	0.0127	0.1047	0.10496	[a]	−7.4
k_A	0.0023	0.00014	0.00139	0.001395	−51.1	−50.8
k_{eqA}	11.9483	2.309	12.117	12.108	[a]	[a]
k_1	3.0650	0.240	2.3973	2.3951	−11.5	−11.6
k_2	2.8327	0.194	2.7632	2.7724	[a]	[a]

[a] Within confidence interval.

Table 5
Maximum sensitivity coefficients

Sensitivity coefficient	Numerical value	Sensitivity coefficient	Numerical value
$\frac{\partial w_T}{\partial k_S}$	0.19153	$\frac{\partial E_{TH}}{\partial k_{eqR}}$	-1.1379×10^{-7}
$\frac{\partial w_T}{\partial k_H}$	−0.17485	$\frac{\partial w_T}{\partial k_{eqA}}$	0.002864
$\frac{\partial E_{TS}}{\partial k_{ES}}$	2.0878	$\frac{\partial \text{cAMP}}{\partial k_A}$	6.0225
$\frac{\partial E_{TH}}{\partial k_{EH}}$	2.0878	$\frac{\partial E_{TS}}{\partial k_1}$	9.6305×10^{-8}
$\frac{\partial E_{TS}}{\partial k_{eqR}}$	-1.2312×10^{-7}	$\frac{\partial E_{TH}}{\partial k_2}$	9.6305×10^{-8}

$\boldsymbol{z} = [xvw_T E_{TS} E_{TH}\text{cAMP}]$. The sensitivity coefficients are defined as

$$\frac{\partial \boldsymbol{z}}{\partial \boldsymbol{p}} = \begin{pmatrix} \frac{\partial z_1}{\partial p_1} & \cdots & \frac{\partial z_1}{\partial p_9} \\ \vdots & & \vdots \\ \frac{\partial z_6}{\partial p_1} & \cdots & \frac{\partial z_6}{\partial p_9} \end{pmatrix}$$

These coefficients provide valuable information on the behavior of dependent variables to changes on parameter values. So coefficient $\partial z_3/\partial p_1 = \partial w_T/\partial k_S$ states how much trehalose fraction in cells changes when the reaction constant for trehalose synthesis is modified. Table 5 includes maximum calculated numerical values for sensitivity coefficients of the structured model.

It should be noted from Table 5 that sensitivity coefficients of parameters k_{ES}, k_{EH} and k_A have high numerical values meaning that those parameters have a major influence on $\boldsymbol{z}$ vector, specially on biotic variables E_{TS}, E_{TH} and cAMP. Some of the sensitivity coefficients are equal, meaning that the corresponding model parameters are correlated. Mathematically, this signifies that an infinite number of linear combinations of them yields basically the same model behavior; physically, this implies that if a biological meaning were to be associated with the parameters, a careful experimental design should be devised to break up the correlation.

5. Conclusion

A biochemically structured model for trehalose accumulation analysis and description in yeast cells is presented. The model has been build up by taking into account variations on enzymatic activity of TH and TPS attributable to concentration changes on intracellular cAMP. The model integrates regulation of enzymes synthesis through genetic repression as well. Experimental data fit reasonably well to model results, so it could be established that the proposed model captures quite well the key features of trehalose accumulation in yeast, supporting the use of structured modeling as a more rational approach to move steadily towards a better understanding and exploitation of yeast metabolic behavior in bioprocess design.

The fitting of the proposed model to the experimental data and the results of the sensitivity analysis, suggest that there is a strong dependence of trehalose accumulation in cells on cAMP intracellular concentration, so trehalose accumulation will apparently be increased as much as intracellular cAMP diminishes. Thus, extracellular factors (such as carbon and nitrogen source starvation) leading to a cAMP decrease in cytoplasm will produce yeast biomass with a high intracellular trehalose content.

The presented results and the proposed model could be helpful to develop technological applications for yeast production on a more rational basis. By using the model, experiments shall be devised in order to increase the knowledge of the biochemical basis of trehalose accumulation, and to improve by the same way the proposed model.

Acknowledgements

The authors wish to thank the National Council of Science and Technology (CONACyT) of Mexico, the National Polytechnic Institute (IPN) of Mexico and the National Council for Scientific Research (CNRS) of France for financial support.

References

[1] T. Hottiger, P. Schultz, A. Wiemken, Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*, J. Bacteriol. 169 (1987) 5518–5522.
[2] J.G. Lewis, R.P. Learmonth, P.V. Attfield, K. Watson, Stress cotolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*, J. Ind. Microbiol. Biotechnol. 18 (1997) 30–36.
[3] A. Wiemken, Trehalose in yeast, stress protectant rather than reserve carbohydrate, A. van Leeuw, J. Microbiol. 58 (1990) 209–217.
[4] P.V. Attfield, S. Kletsas, B.W. Hazell, Concomitant appearance of intrinsic thermotolerance and storage of trehalose in *Saccharomyces cerevisiae* during early respiratory phase of batch-culture is CIF1-dependent, Microbiology 140 (1994) 2625–2632.
[5] H.H.W. Silljé, J.W.G. Paalman, E.G. ter Schure, S.Q.B. Olsthoorn, A.J. Verkleij, J. Boonstra, C.T. Verrips, Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*, J. Bacteriol. 181 (1999) 396–400.
[6] J.C. Slaughter, T. Nomura, Intracellular glycogen and trehalose contents as predictors of yeast viability, Enzyme Microb. Technol. 14 (1992) 64–67.
[7] J.M. Thevelein, Ciclyc-AMP content and trehalase activation in vegetative cells and ascospores of yeast, Arch. Microbiol. 138 (1984) 64–67.
[8] N. Ertugay, H. Hamamci, A. Bayindirli, Fed-batch cultivation of bakers' yeast: effect of nutrient depletion and heat stress on cell composition, Folia Microbiol. 42 (1997) 214–218.
[9] S.H. Lillie, J.R. Pringle, Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation, J. Bacteriol. 143 (1980) 1384–1394.
[10] N. Arneborg, M. Karskov Moos, M. Jakobsen, The effect of acetic acid and specific growth rate on acetic acid tolerance and trehalose content of *Saccharomyces cerevisiae*, Biotechnol. Lett. 17 (1995) 1299–1304.

[11] A.D. Panek, Trehalose synthesis during starvation of bakers' yeast, Eur. J. Appl. Microbiol. 2 (1976) 34–46.
[12] A.G. Fredrickson, Formulation of structured models, Biotechnol. Bioeng. 18 (1976) 1481–1486.
[13] J.J. Mansure, R.C. Souza, A.D. Panek, Trehalose metabolism in *Saccharomyces cerevisiae* during alcoholic fermentation, Biotechnol. Lett. 19 (1997) 1201–1203.
[14] J.L. Parrou, M.A. Test, J. François, Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for stress-induced recycling of glycogen and trehalose, Microbiology 143 (1997) 1891–1900.
[15] M. Geymonat, L. Wang, H. Garreau, M. Jacquet, Ssa1p chaperone interacts with the guanine nucleotide exchange factor of Ras Cdc25p and controls the cAMP pathway in *Saccharomyces cerevisiae*, Mol. Microbiol. 30 (1998) 855–864.
[16] J. Nikawa, S. Cameron, T. Toda, K.M. Ferguson, M. Wingler, Rigourous feedback control of cAMP levels in *Saccharomyces cerevisiae*, Gen. Dev. 1 (1987) 931–937.
[17] E. Boy-Marcotte, D. Tadi, M. Perrot, H. Boucherie, M. Jacquet, High cAMP levels antagonize the reprogramming of gene expression that occurs at the diauxic shift in *Saccharomyces cerevisiae*, Microbiology 142 (1996) 459–467.
[18] J.D. Chung, G. Stephanopoulos, On the physiological multiplicity and population heterogeneity of biological systems, Chem. Eng. Sci. 51 (1996) 1509–1521.
[19] O. Maaloe, N.O. Kjeldgaard, Control of Molecular Synthesis, Benjamin, New York, 1966.
[20] S.J. Coppella, P. Dhurjati, A mathematical description of recombinant yeast, Biotechnol. Bioeng. 35 (1990) 356–374.
[21] J.M. Thevelein, Regulation of trehalose mobilization in fungi, Microbiol. Rev. 48 (1984) 42–59.
[22] J.M. Thevelein, Activation of trehalase by heat shock in yeast ascospores: correlation with total cellular cyclic-AMP content, Curr. Microbiol. 10 (1984) 159–164.
[23] M. Werner-Washburne, E. Braun, G.C. Johnston, R.A. Singer, Stationary phase in yeast *Saccharomyces cerevisiae*, Microbiol. Rev. 57 (1993) 383–401.
[24] M. Russel, J. Bradshaw-Rouse, D. Markwarst, W. Heideman, Changes in gene expression in the Ras7 adenylate cyclase system of *Saccharomyces cerevisiae*: correlation with cAMP levels and growth arrest, Mol. Biol. Cell 4 (1993) 757–765.
[25] J.M. Thevelein, M. Beullens, Cyclic AMP and the stimulation of trehalase activity in the yeast *Saccharomyces cerevisiae* by carbon sources, nitrogen sources and inhibitors of protein synthesis, J. Gen. Microbiol. 131 (1985) 3199–3209.
[26] M.E. Smith, J.R. Dickinson, A.E. Wheals, Intracellular and extracellular levels of cyclic AMP during the cell cycle of *Saccharomyces cerevisiae*, Yeast 6 (1990) 53–60.
[27] I.L. Krallish, B.E. Damberga, M.J. Beker, State of adenosine phosphates during dehydration of yeast, Appl. Microbiol. Biotechnol. 31 (1989) 194–199.
[28] J.M. Thevelein, J.H. de Winde, Novel sensing mechanism and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*, Mol. Microbiol. 33 (1999) 904–918.
[29] G.M. O'Connor, F. Sanchez-Riera, Ch.L. Cooney, Design and evaluation of control strategies for high cell density fermentations, Biotechnol. Bioeng. 39 (1992) 293–304.
[30] J. Zhang, R. Greasham, Chemically defined media for commercial fermentations, Appl. Microbiol. Biotechnol. 51 (1999) 407–421.
[31] J.L. Parrou, B. Enjalbert, L. Plourde, A. Bauche, B. Gonzalez, J. François, Dynamic responses of reserve carbohydrates metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*, Yeast 15 (1999) 191–203.
[32] M. Barbacid, Ras genes, Annu. Rev. Biochem. 56 (1987) 779–827.
[33] H. Jørgensen, L. Olsson, B. Rønnow, E.A. Palmqvist, Fed-batch cultivation of baker's yeast followed by nitrogen or carbon starvation: effects on fermentative capacity and content of trehalose and glycogen, Appl. Microbiol. Biotechnol. 59 (2002) 310–317.
[34] S.B. Lee, J.E. Bailey, Genetically structured models for lac promoter–operator function in the *Escherichia coli* chromosome and in multicopy plasmids: lac operon function, Biotechnol. Bioeng. 26 (1984) 1372–1382.
[35] S.B. Lee, J.E. Bailey, Genetically structured models for lac promoter–operator function in the *Escherichia coli* chromosome and in multicopy plasmids: lac promoter function, Biotechnol. Bioeng. 26 (1984) 1383–1389.
[36] K. Toda, Dual control of invertase biosynthesis in chemostat culture, Biotechnol. Bioeng. 18 (1976) 1117–1124.
[37] M.L. Pall, Adenosine 3′,5′-phosphate in fungi, Microbiol. Rev. 45 (1981) 480–642.
[38] A.G. Moat, J.W. Foster, Microbial Physiology, 2nd ed., Wiley, New York, 1988.
[39] C. Verduyn, A.H. Stouthamer, W.A. Scheffers, J.P. van Dijken, A theoretical evaluation of growth yields of yeasts, A. van Leeuw. J. Microbiol. 59 (1991) 49–63.
[40] E. Postma, W.A. Scheffers, J.P. van Dijken, Adaptation of the kinetics of glucose transport to environmental conditions in the yeast *Candida utilis* CBS 621: a continuous-culture study, J. Gen. Microbiol. 134 (1988) 1109–1116.
[41] R. Lagunas, E. Ruiz, Balance of production and consumption of ATP in ammonium-starved *Saccharomyces cerevisiae*, J. Gen. Microbiol. 134 (1988) 2507–2511.
[42] J.A. Roels, Biochemically structured balances of microbial metabolism, in: Energetics and Kinetics in Biotechnology, Elsevier, Amsterdam, 1983, pp. 99–128.