A model of energy limitation and population structuring to estimate phototrophic growth of industrially significant *Halobacterium salinarum* strains

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Dynamics of energy consumption during the growth of photosynthetic *Halobacterium salinarum* in populations of various age structures has been studied. Changes in the biosynthesis of bacteriorhodopsin, a key retinal containing protein of *H.salinarum* photosystem, were revealed. For several industrially significant strains, exponential accumulation of nonproliferating halobacterium cells, whose growth is limited by different modes of light exposure, was calculated. Various effects of proliferating and nonproliferating cells on bacteriorhodopsin biosynthesis rate were shown. Necessity to prevent influences of other growth limiting factors, except for energy consumption, during halobacterium cultivation was proved using cell population structuring model.

Keywords: *Halobacterium salinarum*, halobacteria, model, cell population, growth dynamics, specific and absolute biomass growth rate, substrate utilization, metabolite biosynthesis, bacteriorhodopsin.

INTRODUCTION

In the last decade *Halobacterium salinarum*, a phototrophic halobacterium, has become a frequent object of research in biotechnology. Previously, interest in the unique properties of this extreme bacterium was aroused basically due to phototrophic character of its cell growth. In the early 1970s it was shown that *H. salinarum* cell membranes synthesize bacteriorhodopsin, a 7-α-helical retinal containing protein capable of providing a significant portion of energy due to proton potential formation, $\Delta\mu_{H}+$, Lanyi et al. (2001). Many diverse studies of bacteriorhodopsin now provide significant insight into the mechanisms underlying biosynthesis regulation of the protein, formation of its tertiary structure in the so-called purple membranes, and of the cyclic conformational changes that allow photodependent proton transport Osterchelt (1999), Birge et al (1997)

Expansion of knowledge about the biosynthetic properties of *H. salinarum* has drawn increased attention to the study of its biotechnological potential, Skladnev et al. (2008). In this study, highly productive derivatives of widely known genetically differing halobacterium strains constructed in our laboratory were used. In our opinion, these strains have the greatest biotechnological prospects. All the selected *H. salinarum* cultures possessed high growth properties. *H. salinarum* wild strain ST033 is a highly active producer of bacteriorhodopsin, Tjurin et al (2008). Mutant strain D96N synthesizes a modified form of the protein with a slowed photocycle. The cells of colorless strain JW5 have a disturbed synthesis of retinal and, accordingly, synthesize inactive bacteriorhodopsin. Thus, photoenergetic biosystems of all three halobacterium strains essentially differ in the effectiveness: from normal (ST033), to weakened (D96N) and inactivated (JW5). Data about the growth characteristics of another *H. salinarum* strain of a wild type (*H. salinarum* strain UM17) were taken from the literature, Kaljonov et al 2006 a,b.

Studies on microorganism growth and biosynthesis of metabolites have caused occurrence

of many mathematical models describing the dynamics of biomass growth and nutrient substrate consumption. Many models of microorganism growth are based on J.Monod's growth model or its variants, Pirt (1995), Bailey et al. (1986). The basic idea of these models consists in the restriction of the specific growth rate of a cell population either by the concentrations of a limiting substrate or of a biosynthetic end-product. However, the limited application areas and numerous exceptions are usual drawbacks of such models that necessitate the construction of a new model.

The basic disadvantage of the J.Monod model as described in Bailey et al. (1986) is an ambiguity about the physical sense of its parameters (or even their absence). According to the Monod model, the limitation of cell growth by substrates is determined by the constants $\mathbf{K_s}$ **,** a key substrate concentration at which the specific growth rate of the culture is equal to the half of μ_{max}. However, in case of phototrophic microorganisms (in which cell growth depends directly on light exposure or more precisely on the energy of quanta captured by the photobiosystem) it is pointless to talk about any limiting concentration of energy substrates. In other words, for phototrophic microbes, the limitation of cell growth depends mainly on the rate of quantum energy capture, and only secondly on any specific nutrient. If the nutrient composition of a medium for cultivation of photosynthetic organisms is unbalanced or inappropriate, the initial energy consumption is quickly counteracted by the lack of minor nutrients or substrates needed to synthesize cellular components. In case of large-scale cultivation, this fact indicates the necessity for a correct of nutrient medium composition since incorrect medium composition will usually quickly inhibit culture growth. Such situations will be analyzed in the examples given below.

An unstructured microorganism growth model was proposed earlier Derbyshev et al (2001). The model describes energy limitation during cultivation of both aerobic and facultatively anaerobic bacteria. The main point of the model assumes that if growth limitation by nutrient elements (or any other growth inhibiting factors) is avoided, energy substrate use during the growth inhibition phase (GIP) would be constant. In this case, the absolute and specific growth rates of the biomass would decrease linearly and hyperbolically, respectively.

A biotechnological method providing optimum conditions for cell growth has been used for many years. This method suggests that during cultivation microorganism cultures receive additional feeding from a balanced nutrient medium concentrate. The inflow rate and amount of the feeding concentrate exclude both the required nutrients and the inhibition of growth by excess of corresponding nutrient components while the oxygen mass exchange rate in the culture remains constant and the oxygen concentration approaches zero.

The proposed model suggests an absolutely new view of the behavior of growing microbial populations and the biosynthesis of various metabolites. The model enables closer conformity between theoretical predictions and experimental observations.

Pirt-Marr's equation

$$Q = -dS/d\tau = adX/d\tau + mX \quad (1)$$

for the calculation of energy substrate consumption rate has been modified for the analysis of energy consumed by cells for growth and viability maintenance. This was done by expressing Q through oxygen mass exchange rate as follows:

$$Q = JQ_{O2} \quad (2).$$

This yields the initial equation (3) for the unstructured model of cell growth limited by oxygen consumption was obtained. All the equation terms are expressed in energy units:

$$JQ_{O2} = adX/d\tau + mX \quad (3).$$

The solution of basic equation (3) allows for formulation of fundamental laws of culture growth when the oxygen supply rate is limited. Unlike earlier conceptions, this model shows that a linear decrease in absolute growth rate of the biomass and hyperbolic reduction in specific growth rate is a function of biomass concentration, and that the energy substrate consumption rate specified by oxygen mass exchange rate is constant. Methods are provided to define parameters for the unstructured model proposed, these include growth efficiency and energy substrate consumption (***m***, ***a*** and ***A = m/a***), which were not previously used in any practical way to estimate periodic culture growth. Parameter ***A*** describes a delay of the biomass growth rate.

Studies on the effect of *Salmonella* culture growth rate on cell survival under adverse external influences Klykov et al (1996) showed that during GIP, if there is lack of dissolved oxygen then stable cells accumulate at constant specific rate equal to that of the growth delay (***A = m/a***). The share of stable cells within a population is obviously equal to that of nonproliferating cells, which consume energy only for viability maintenance. These results allow for some speculations about the age structure of microbial populations limited by lack of oxygen in terms of energy consumption and the maintenance of cell viability. On the base of these speculations a structured cell population growth model was proposed Klykov et al (1996); Klykov et al (2003); Derbyshev et al (2003); Klykov et al (2009); Klykov et al (2011) that describes the consumption of substrates utilized for cell construction and synthesis of metabolites in the cultures consisting of two groups of cells differing in their rates of energy consumption. Methods are described for defining parameters of the structured model for substrate consumption and metabolite biosynthesis by using preliminary counted parameters of the unstructured model.

Thus, the proposed structured model assumes that within a growing population there are two groups of cells essentially differing in their physiology. Group I represents newly generated (young) cells and Group II contains the cells being in the state of active proliferation. Although the cells of Group I are often called “quiescent” Pirt (1995), in our opinion these are the cells of “zero age” Bailey et al (1986) , i.e., the cells being in phase G_1 or in phase B as designated for eukaryotes and prokaryotes, respectively. Group I cells exhibit minimal physiological functions, and for each cell these functions are constant. A characteristic feature of these cells is that they consume energy substrates only for their viability maintenance. We call these cells “stable”.

The growth of *Halobacterium* and consumption of nutrient medium components during fermentation of *Halobacterium* have been studied previously, Rodriguez-Valera (1995), Manikandan et al (2009), Robinson et al (2005), but the works are not widely available. Most studies on this theme deal with the metabolism of halobacterial cells , Margesin et al (2001), Tebbe A et al (2009), Gonzalez O. et al (2008), Lange et al (2007), Mitra et al (1993), Baliga et al (2002), and of bacteriorhodopsin production Gonzalez et al (2009), Lee et al (2006), Schafer et al (1999), Ghasemi et al (2008).

In this study we attempted to analyze phototrophic halobacterium growth and bacteriorhodopsin biosynthesis using both the proposed unstructured and structured models that have been tested on other microbial species and genera as mentioned above. There is a believe that this attempt is justified since the laws underlying the energy consumption limitation during cell growth under oxygen deprivation should also be observable during the growth of phototrophic halobacteria when energy consumption is limited by low light intensities. Theoretical preconditions like those described by equation (3) were used: it is obvious that the left term of this equation expressed in the units of energy transformed during "usual" aerobic processes can be replaced for equivalent "light" energy component characteristic of photosynthetic processes and equal to E:

$$E = adx/d\tau + mX = mX_p \qquad (3a).$$

This energy is received by cells from light and transformed during biosynthesis. All the results obtained for halobacteria are similar to those presented in [Klykov SP et al (2011)].

Thus, the objectives of the present paper are as follows:

1) To describe the new unstructured and structured models of cell growth and biosynthesis based on the well-known laws about cell viability maintenance using preliminary estimated phototrophic halobacterium growth and bacteriorhodopsin biosynthesis as an example.

2) To show potentialities of the used halobacterium strains estimated by the method described.

MATERIALS AND METHODS

Halobacterium salinarum cells were cultivated in a liquid medium containing (g/l): NaCl 250 $MgSO_4 \cdot 7H_2O$ 20, KCl 2, $CaCl_2$ 0,065, sodium citrate 0,5 (pH 7,2). Peptone (7 g/l) and yeast extract (2 g/l) were used as carbon and nitrogen sources. Cultivation was performed at 37 °C and at constant lighting by daylight lamps LD40. For bacteriorhodopsin isolation *H. salinarum* cells were harvested by centrifugation (7.000 min^{-1}, for 15 min) and then were osmotically ruptured in distilled water with DNA-ase I added (0,5 mkg/ml). The suspension obtained was incubated at 10 °C under intensive stirring for 1 h. The purple membranes were precipitated by centrifuging at 50.000 g for 1 h. The precipitate was twice washed with distilled water, and the supernatant was separated in the same regimen. Bacteriorhodopsin preparation purity was controlled spectrophotometrically according to ratio $A_{\lambda 280}/A_{\lambda 568}$ (e_{280} = 1,1 · 10^{-4} Mcm and e_{568} = 6,3 · 10^{-5} Mcm). Highly productive *H. salinarum* cultures used were selected initially as derivatives having high growth properties: wild type strains ST033; strain D96N synthesizing a mutant form of bacteriorhodopsin characterized by slow photocycle and strain JW5 with disturbed retinal synthesis and inactive bacteriorhodopsin. Description of model equations used for halobacteria growth and biosynthesis estimation.

The equations previously considered in Derbyshev et al (2001), Klykov et al (2011) are presented in Table

Equations for the unstructured model

Equations (1) - (6), except for (3a), were deduced in Derbyshev et al (2001)and represent the basic equations of the unstructured model of cell growth.

Experimental biomass concentration parameters are used for estimation of biomass growth according to the unstructured model. Sampling is performed in identical time intervals $\Delta\tau = \tau_{i+1} - \tau_i = const$, during which the biomass concentration changes, $\Delta X_\tau = X_{\tau+\Delta\tau} - X_\tau$, occur.

According to equation (3) for GIP the following is deduced:

$$X_{\tau+\Delta\tau} = X_\tau + \Delta X \qquad (25)$$

$$X_\tau + \Delta X = X_p - (X_p - X_\tau)^* \exp(-A^*\Delta\tau) \qquad (26),$$

or $$X_{\tau+\Delta\tau} = X_p - (X_p - X_\tau)^* \exp(-A^*\Delta\tau) \qquad (27)$$

$$\Delta X = X_p - X_\tau - (X_p - X_\tau)^* \exp(-A^*\Delta\tau) \qquad (28)$$

$$\Delta X = (X_p - X_\tau)(1 - \exp(-A^*\Delta\tau)) \qquad (29)$$

at $\Delta\tau = const$ (30) equation (29) represents a linear regression function of X_τ

$$\Delta X = (1 - \exp(-A^* \Delta\tau))X_p - (1 - \exp(-A^*\Delta\tau))X_\tau \qquad (31)$$

In the point of intersection with the ordinate axis, at x = 0, equation (31) is converted as follows:

$$\Delta X = (1 - \exp(-A^* \Delta\tau))X_p = \Delta^o X \qquad (32),$$

where $\Delta^o x$ is the point where the regression line intersects the ordinate axis (31).

From this it follows that

$$1 - \Delta^o X / X_p = exp(-A \cdot \Delta\tau) \quad (33).$$

Expression (33) can be presented in the following form:

$$A = -[ln(1 - \Delta^o X / X_p)] / \Delta\tau \quad (34).$$

Similarly to formula (34) for A of logarithmic growth phase (LGP), equations for μ_{max} can be deduced: $X_{\tau+\Delta\tau} = X_\tau + \Delta x = X_\tau \, exp(\mu_{max} \cdot \Delta\tau)$ (35),

from which $\Delta X = [exp(\mu_{max} \cdot \Delta\tau) - 1] X_\tau$ (36),

and $\mu_{max} = [Ln(1+M)] / \Delta\tau$ (37),

where $M = [exp(\mu_{max} * \Delta\tau) - 1]$ is inclination angle tangent of the straight line $\Delta X = f(X)$ for LGP.

In the given work, μ_{max} was calculated by the standard technique [8]. In this case, dependence of LGP natural logarithms, X, on time τ was built and the tangent of inclination angle of the obtained straight line was determined. These values were compared to the results obtained with equation (37).

For the analysis of biomass growth, the following parameters are determined first: biomass concentration, X_{lim}, and time, τ_{lim} , corresponding to LGP termination and beginning of GIP; hypothetical maximum biomass concentration, X_p, when all amount of energy transformed by the system is consumed for biomass viability maintenance; $A = m/a$ is the specific growth delay rate of the biomass in GIP.

Equations for the structured model

Equation (7) presented for the first time in Klykov et al (1996), was theoretically proved in Klykov et al (2009). This equation indicates that in any population limited in energy consumption, X^{st}, a specific rate of accumulating nonproliferating cells, which consume energy only for viability maintenance, is constant and equal to A. This is due to the fact that the energy consumed for cell viability maintenance causes total reduction in the growth rate of proliferating cells, X^{div} , and, consequently, of the population growth rate as a whole. In this case, nonproliferating cell accumulation is directly proportional to concentration X^{st} at any instant time.

Equation (8) represents an integrated form of equation (7). For the first time it was used in Klykov et al (1996) and theoretically proved in Klykov et al (2003) and Klykov et al (2009). This equation shows exponential character of X^{st} accumulation in time.

Equations (9) - (16) were deduced in Klykov et al (2003) and Klykov et al (2009).

Equations (9) and (10) represent a share of nonproliferating cells in the population. In Klykov et al (2009) and Klykov et al (2011) it was shown that $R = X^{st}/X$ also describes the degree of synchronization of zero age cultures. All physiological functions of zero age cells are minimal, and cell resistance to adverse external influences is maximum owing to the temporarily inhibited metabolism. It is shown Klykov et al (2009) and Klykov et al (2011) that if biomass growth is limited only by energy inflow, all proliferating cells are converted into stable ones after which further proliferation ceases. Thus, the whole amount of energy is consumed for cell viability maintenance. It is obvious that for this case equality $R = 1$ is true.

The physical meaning of parameter K in equation (11) consists in the fact that with growth limitation strengthening (increase the duration of growth phase), the effect from the same work of biochemical mechanisms underlying cell processes continuously decreases, i.e., the number of proliferating cells reduces over time. At the same time, there is an accelerated increase in the quantity of stable cells consuming energy only to maintain cell viability (see equations 7 and 8). The latter statement should probably be understood to reflect the increase of stable cell number due not only to the termination of early cell proliferation cycles, but also to the progressive failure of stable cells to proliferate; otherwise, the cells could start proliferating again in the absence of limits.

Equations (12) - (16) describe the time of culture growth termination and specific growth rate at the moment, when the unstructured model of cell growth and biosynthesis in GIP presented in Table 1 does not work. In this case, other equations described below are required.

Equations (17), (18) and (19) - (21) were considered in Klykov et al (2003), Klykov et al (2009) and Klykov et al (2011). **The** physiological processes occurring in proliferating and stable cells differ greatly and are diametrically opposed. Therefore, subdivision of population cells into proliferating (X^{div}) and stable (X^{st}) ones makes it possible to use equations (17, 18, Table 1) to describe the consumption rate of substrates utilized for cell construction and metabolite synthesis:

$$dP(or\ -S)/d\tau = k^{div}_{P,S} X^{div} + k^{st}_{P,S} X^{st}.$$

We assume that metabolites are synthesized only by proliferating cells. Nonproliferating cells, as a rule, destroy these products. Therefore, the signs for the constants for metabolite synthesis and degradation are opposite. The same should be stated for substrates utilized for cell construction.

If stable cells do not influence the synthesis of metabolites (or substrate utilization), i.e., $k^{st}_{P,S} = 0$, then the synthesis is carried out by proliferating cells and can be described by the integrated equation (19). If both proliferating cells and zero age cells participate in the synthesis, then the accumulation of metabolites is described by equation (20). The similar equation (21) is proposed for the consumption of substrates used for cell construction, an analysis of equations (17) - (21) shows that metabolite synthesis proceeds with rate constants, whose signs are opposite to each other if the influence of the stable

Table 1 Basic model equations for growth inhibition phase

Function form	Equation	##
1	2	3
Q = - $\frac{dS}{d\tau} = f(X)$	$Q = a\frac{dX}{d\tau} + mX$	(1)
Q = $f(Q_{O2})$	$Q = JQo2$	(2)
Q = f(X)	*$JQ_{O2} = adX/dr+mX = mX_p$*	(3)
Q=E	*E = adX/dr+mX= mX_p*	(3a)
$\frac{dx}{d\tau} = f(X)$	$\frac{dX}{d\tau} = A(X_p - X)$	(4)
$\mu = \frac{dX}{d\tau} \times \frac{1}{X} = f(X)$	$\mu = A(\frac{X_p}{X} - 1)$	(5)
$X = f(\tau)$	$X = X_p - (X_p - X_{Lim})\exp[-A(\tau - \tau_{Lim})]$	(6)
$\frac{dX^{st}}{d\tau} \times \frac{1}{X^{st}}$	$\frac{dX^{st}}{d\tau} \times \frac{1}{X^{st}} = \frac{m}{a} = A$	(7)
$X^{st} = f(\tau)$	$X^{st} = X_{Lim}^{st}\exp[A(\tau - \tau_{Lim})]$	(8)
$R = f(\mu)$	$R = \frac{K}{A^2 x_p^2} \times \left(\frac{\mu}{A} + \frac{A}{\mu} + 2\right)$	(9)
$R = f(X)$	$R = \frac{K}{A^2(X_p X - X^2)}$	(10)
K	$K = \frac{dX}{d\tau} \times \frac{dX^{st}}{d\tau}$ *$=A^2 X^{st}_{Lim} \times (X_p - X_{Lim})$*	(11)
τ_{final}	$\tau_{final} = \frac{1}{A}\ln\frac{X_p - X_{Lim}}{X_p - Xfinal} + \tau_{Lim}$	(12)
τ_{final}	$\tau_{final} = \frac{1}{A}\ln\frac{X_{final}}{X_{Lim}^{st}} + \tau_{Lim}$	(13)
X_{final}	$X_{\text{final}} = \frac{X_p}{2} + \frac{1}{2}\sqrt{X_p^2 - 4X_{Lim}^{st}(X_p - X_{Lim})}$	(14)
X_{final}	$X_{final} = \frac{X_p + \frac{1}{A}\sqrt{A^2 X_p^2 - 4K}}{2}$	(15)
μ_{final}	$\mu_{final} = A(\frac{X_p}{X_{final}} - 1)$	(16)

cells is not equal to 0. This means that groups of proliferating and stable cells behave differently during biosynthesis: the former group synthesizes metabolites while the latter destroys them.

It was found out that, if the cell growth process is limited not only by energy consumption but also by any substrate required for cell construction, than the increase of biomass yield may suddenly stop. Studies

Table 1 continues

$\frac{dS}{d\tau} = f(X^{st}, X^{div})$ ***- rate of consumption of substrate utilized for cell construction***	$-\frac{dS}{d\tau} = k_s^{st} X^{st} + k_s^{div} X^{div}$	**(17)**
$\frac{dP}{d\tau} = f(X^{st}, X^{div})$ ***metabolite synthesis rate***	$\frac{dP}{d\tau} = k_p^{st} X^{st} + k_p^{div} X^{div}$	**(18)**
$P = f(X)$ ***- metabolite concentration***	$P = P_{Lim} + (\frac{k_p^{div}}{A})\left[X_p \ln\frac{X_p - X_{Lim}}{X_p - X} - (X - X_{Lim})(1 + \frac{X_{Lim}^{st}}{X_p - X})\right]$	**(19)**
$P = f(X)$ ***metabolite concentration***	$P = P_{Lim} + (\frac{k_p^{div}}{A})\left\{X_p \ln\frac{X_p - X_{Lim}}{X_p - X} - (X - X_{Lim})\right\} + \frac{1}{A}(k^{st} - k^{div}) X_{Lim}^{st} \frac{X - X_{Lim}}{X_p - X}$	**(20)**
$S = f(x)$ ***- rate of consumption of substrate utilized for cell construction***	$S = S_{Lim} - (\frac{k_s^{div}}{A})\left\{X_p \ln\frac{X_p - X_{Lim}}{X_p - X} - (X - X_{Lim})\right\} - (\frac{1}{A})(k_s^{st} - k_s^{div}) X_{Lim}^{st} \frac{X - X_{Lim}}{X_p - X}$	**(21)**
q =f(R)	$q = k_p^{div} + (k_p^{st} - k_p^{div})R$	**(22)**
X^{st}_{Lim}	$X^{st}_{Lim} = 2(X^2_{Lim}/X^2_p)(X_p - X_{Lim})$	**(23)**
X^{st}_{Lim}	$X^{st}_{Lim} = (X_p - X_{final})X_{final}/(X_p - X_{Lim})$	**(24)**

on the dynamics of biomass accumulation limited by substrates utilized for cell construction showed that in equation (7) this parameter can be presented as:

$R_{final} = k^{div}/(k^{div} - k^{st})$ (38).

The required final value of R_{final} is expressed by the identical equation if it is necessary to terminate biosynthesis process in order to avoid the destruction of metabolite products by active cells when $k^{st}_P \neq 0$. The situation when maximal biomass accumulation does not coincide with that of metabolite accumulation, i.e., when the latter occurs earlier than the former, is actually rather common. For this case, equation (15) would have the following appearance:

$$X_{final} = \frac{X_p + \frac{1}{A}\sqrt{A^2 X_p^2 - (1/Rf_{inal}) * 4K}}{2}$$

(39).

In the studies of growing cultures, especially, of *Pseudomonas* or *Yersinia pestis*, by cytorefractometric methods, Fikhman (1967), we have repeatedly noticed that a certain number of nonproliferating cells are always present in the exponential growth phase. As the cell growth reaches GIP, the number of such cells increases significantly.

Equations (12), (13), (14) and (16) then can be transformed according to (39).

In the given work biosynthesis parameters were determined for cells in GIP. The calculated values were used to estimate the conformity of the data obtained for the cells in GIP. As is well known, biosynthesis of metabolites often occurs during GIP. In Klykov et al (2003), Klykov et al (2009) and Klykov et al (2011), the factors affecting cell population structuring during LGP were investigated. Equations describing metabolite accumulation during this growth phase are as follows:

$X^{st} = 2X_l(1 - X_l/X)$ (40),

$R = 2X_l(1/X - X_l/X^2)$ (41)

and $P = P_0 + (k^{div}/\mu_{max})(X - X_l) + 2[(k^{st} - k^{div})/\mu_{max}]\, X_l\, [\ln(X/X_l) + X_l/X - 1]$ (42), where

$X_l = X_{Lim}^2/X_p$ (43)

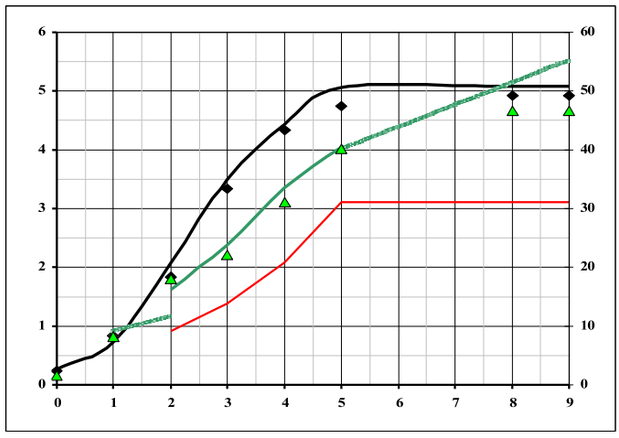

Figure 1 Initial data of halobacterium biomass, ***X***, strain ST033, zero age cells, X^{st}, and synthesis of bacteriorhodopsin. Data calculated according to the presented models. The abscissa axis is Growth time, days. The ordinate axis 1 is ***X*** and X^{st}, g/l. The ordinate axis 2 is bacteriorhodopsin concentration **,** ***P***, mg/l. Biomass concentration, g dry weight/L, experimental data; Biomass concentration calculated according to the unstructured model, g dry weight/L; Zero age cells, X^{st} , g dry weight/L; Bacteriorhodopsin concentration, mg dry weight/L, experimental data; Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L; . Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L. Parameters of GIP were used for calculation of bacteriorhodopsin concentrations in last period of LGP and for predictions of bacteriorhodopsin concentrations after GIP.

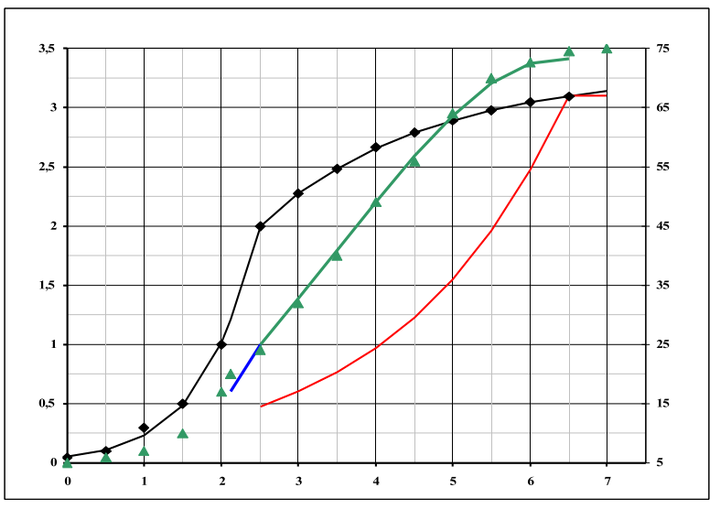

Figure 2 Initial data of halobacterium biomass ,***X***, strain UM17, zero age cells,X^{st}, and synthesis of bacteriorhodopsin. Data calculated according to the presented models. The abscissa axis is Growth time, days. The ordinate axis 1 is ***X*** and X^{st}, g/l. The ordinate axis 2 is bacteriorhodopsin concentration **,** ***P***, mg/l. Biomass concentration, g dry weight/L, experimental data; Biomass concentration calculated according to the unstructured model, g dry weight/L; Zero age cells, X^{st} , g dry weight/L; Bacteriorhodopsin concentration, mg dry weight/L, experimental data; Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L; Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L. Parameters of GIP were used for calculation of bacteriorhodopsin concentrations in last period of LGP.

is the initial biomass concentration during LGP, corresponding to the beginning of cell population structuring.

Technique for estimation of halobacteria growth and bacteriorhodopsin biosynthesis.

Parameters X^{theor} for LGP were calculated according to standard exponential equation [8]:

$$X^{theor} = X_0 * exp[\mu_{max} * \tau],$$

where μ_{max} and X_0 were determined from $LnX^{experiment} = f(\tau)$ (see Fig.6).

μ_{max} was also calculated from equation (37) and compared with the parameter calculated according to the above equations. For this purpose ΔX for the cells during LGP was estimated according to experimental data $X^{experiment}$ from which corresponding values ΔX were calculated for each of the specified sampling interval. For each previous

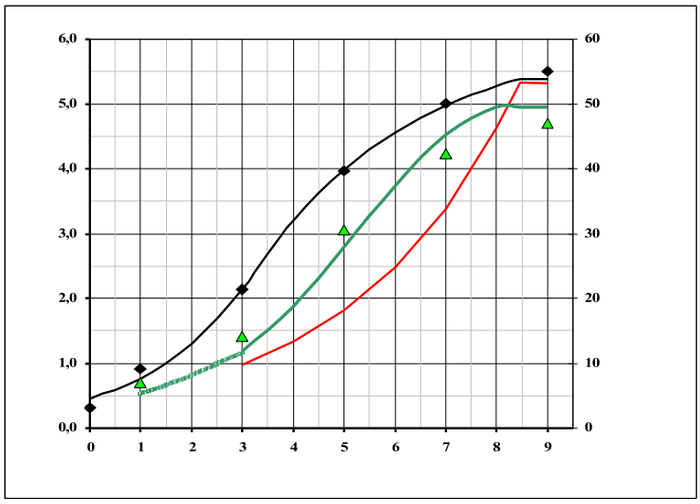

Figure 3 Initial data of halobacterium biomass , ***X***, strain D96N, zero age cells, X^{st}, and synthesis of bacteriorhodopsin. Data calculated according to the presented models. The abscissa axis is Growth time, days. The ordinate axis 1 is ***X*** and X^{st}, g/l. The ordinate axis 2 is bacteriorhodopsin concentration **,** ***P***, mg/l. Biomass concentration, g dry weight/L, experimental data; Biomass concentration calculated according to the unstructured model, g dry weight/L; Zero age cells, X^{st} , g dry weight/L; Bacteriorhodopsin concentration, mg dry weight/L, experimental data; Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L; . Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L. Parameters of GIP were used for calculation of bacteriorhodopsin concentrations in last period of LGP.

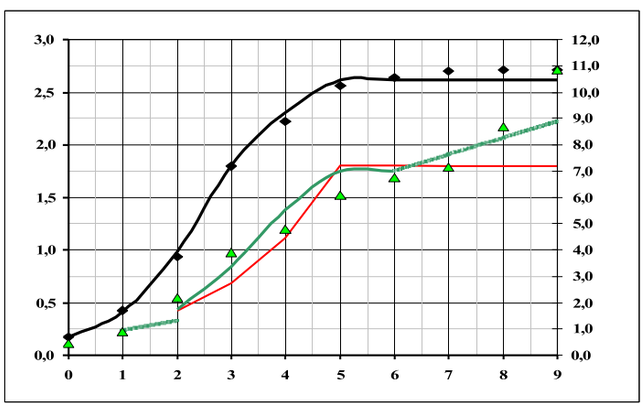

Figure 4 Initial data of halobacterium biomass , ***X***, strain JW5, zero age cells, X^{st}, and synthesis of bacteriorhodopsin. Data calculated according to the presented models. The abscissa axis is Growth time, days. The ordinate axis 1 is ***X*** and X^{st}, g/l. The ordinate axis 2 is bacteriorhodopsin concentration **,** ***P***, mg/l. Biomass concentration, g dry weight/L, experimental data; Biomass concentration calculated according to the unstructured model, g dry weight/L; Zero age cells, X^{st} , g dry weight/L; Bacteriorhodopsin concentration, mg dry weight/L, experimental data; Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L; . Bacteriorhodopsin concentration calculated according to the structured model, mg dry weight/L. Parameters of GIP were used for calculation of bacteriorhodopsin concentrations in last period of LGP and for predictions of bacteriorhodopsin concentrations after GIP.

value of experimental biomass concentration change, ΔX , i.e. $\Delta X_\tau = X_\tau + {}_{\Delta\tau} - X_\tau$. Dependence $\Delta X_\tau = f(X_\tau)$ was then built according to equation (36) and μ_{max} was determined from (37) (see Fig. 5).

Parameters X^{theor} for GIP were calculated on the base of experimental data $X^{experiment}$, according to which values ΔX for sampling within the specified time $\Delta\tau$, were obtained as a difference between previous and subsequent X , i.e. $\Delta X_\tau = X_{\tau + \Delta\tau} - X_\tau$.

Then, the dependence $\Delta X_\tau = f(X_\tau)$ was drawn, and the corresponding constants $\Delta^o X$, X_p, A were determined. $\Delta^o X$ is the intersection of the regression line (31) with the ordinate axis; X_p is an intersection of regression line (31) and abscissa axis; A is calculated from formula (34) (see Fig. 5). The boundary point of the two growth phases (X_{Lim}, τ_{Lim}) is determined from the intersection of the straight lines (31) and (36) (see Fig.5).

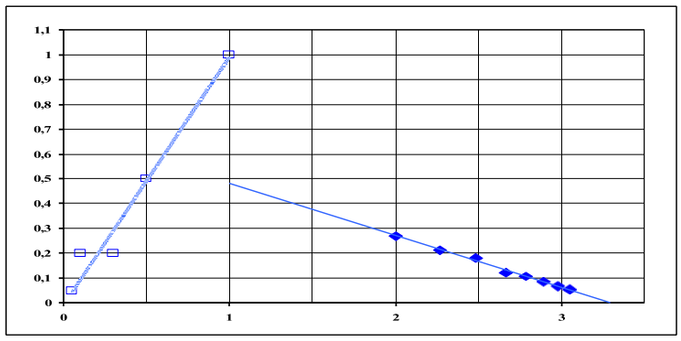

Figure 5 Calculation of halobacterium cell growth parameters, strain UM17: ***A***, X_p, X_{Lim}, τ_{Lim},μ_{max}- (see Table 2). The abscissa axis is biomass, ***X***. g /l. The ordinate axis is ΔX for 0,5 days, g /(l*0,5 days). Straight line for ***LGP*** is $\Delta X_{LGP} = 0{,}9852X$. Straight line for ***GIP*** is $\Delta X_{GIP} = -0{,}2097X + 0{,}6908$. Experimental data, GIP; Experimental data, LGP; , Linearization of LGP data; Linearization of GIP data.

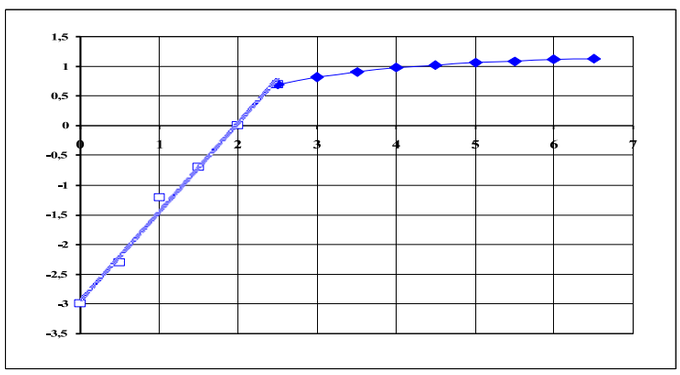

Figure 6 Calculation of maximal specific growth rate, $\mu_{max,}$, of halobacterium, strain UM17, according to Pirt (1995): μ_{max}=1,478 days^{-1} The abscissa axis is Growth time, days. The ordinate axis is ***LnX***. Experimental data, LGP; Experimental data, GIP; linearization of LGP data.

Biosynthesis parameters are calculated from equations (10) and (22). For this purpose X_{Lim}^{st} is first estimated by equation (23). Then values of biosynthesis specific rate are calculated. $(q_P)_\tau$ is estimated from $[\Delta P/\Delta\tau]_\tau = [(P)_{(\tau i+\Delta\tau)}-(P)_\tau]/\Delta\tau$ and

$(q_p)_\tau = (1/X_\tau)[\Delta P/\Delta\tau]_\tau$. On the base of X_p, X_{Lim}, ***A***, τ_{Lim} previously estimated from equation (8) dependence $X^{theor} = f(\tau)$ is deduced. X_{Lim}^{st} calculated above makes it possible to obtain dependence $R = f_1(\tau) = f_2(X)$. Then straight line $(q_P)_\tau = k_P^{div} + (k_P^{st} - k_P^{div})R_\tau$, at $R = 0$, cuts off a line segment equal to k_P^{div} on the ordinate axis. The inclination angle tangent of this line is equal to **kPst-kPdiv,** from which **kPst** can be calculated.

These processes are characteristic of H. salinarum strains that synthesize bacteriorhodopsin. We show

The integrated accumulation of metabolite **Ptheor** is estimated from equation (19) or (20): if **kPst** = 0, then equation (19) is used, if **kPst ≠ 0**, equations (20) and (21) are suitable.

Experimental results on halobacteria cultivation ***X***, bacteriorhodopsin synthesis ***P*** and the corresponding calculated parameters X^{theor} and P^{theor} for LGP were compared with the model values. The latter were obtained from the calculated parameters for GIP, X_p, X_{Lim}, X_{Lim}^{st}, ***A*** , k_P^{div}, k_P^{st} , and from μ_{max} determined preliminary for LGP. Equation (40) for calculating the number of stable cells was used. For the description of metabolite accumulation during LGP, equation (42), where P_0 is the product concentration at the moment, when the biomass structuring supposed to occur during LGP, is used.

As it was noted above, often there is a situation, when cell biomass stops growing while metabolite biosynthesis continues for some time. here that an absolute rate of bacteriorhodopsin synthesis can be described by equation (17). However, if no changes of **R** occur, then **X, Xst**, and **Xdiv** remain constant. The integrated accumulation

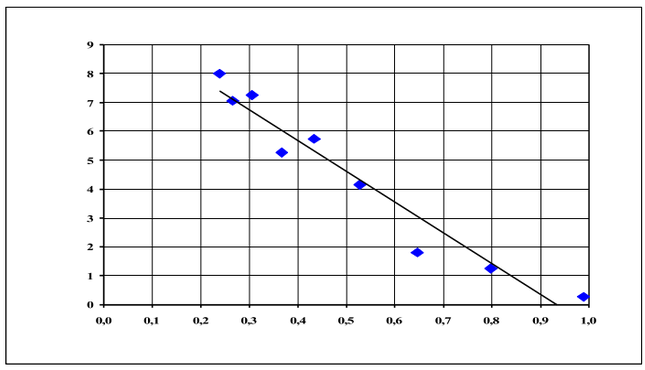

Figure 7 Calculation of metabolite synthesis constant, k^{div} , and metabolite degradation constant, k^{st}: k^{div} =9,944 mg***P***/(g ***X****days) and k^{st} =-0,716 mg***P***/(g ***X****days),strain UM17. The abscissa axis is ***R***, parts of 1. The ordinate axis is ***q*** , mg***P***/(g***X****day). Straight line is ***q = -10,66R + 9,944***. ◆ Experimental data; ▬ Linearization of experimental data according to equation (22).

of biosynthesis metabolites in this case can be expressed by the following equation:

$$P = P_{final\ GIP} + k^{div} * X^{div}_{final\ GIP} * (\tau - \tau_{final\ GIP}) + k^{st} * X^{st}_{final\ GIP} * (\tau - \tau_{final\ GIP}) \quad (44),$$

where $P_{final\ GIP}$ is the final concentration of the product from equation (20), Table 1, and where $X^{div}_{final\ GIP}$ and $X^{st}_{final\ GIP}$ are the corresponding final concentrations that can either be calculated using equations (6), (8), (38) and (39) or determined experimentally.

RESULTS AND DISCUSSION

Experimental results on halobacteria cultivation ***X***, bacteriorhodopsin synthesis ***P*** and corresponding calculated parameters X^{theor} and P^{theor} are presented in Fig. 1 - 4. The changes of halobacteria biomass yield and bacteriorhodopsin concentrations calculated according to the presented model are also depicted in these figures.

Physiological parameters of growth and biosynthesis of four halobacteria strains (Table 2) were determined from the experimental results. The order of physiological parameter calculations demonstrated under item 2.3. is presented in Table 2.

Figures 5-7 demonstrate the calculation of the parameters presented in Table 2 using strain UM17 as an example. For other strains the order of the calculations was similar. On the basis of the obtained calculations mathematical models describing biomass growth and bacteriorhodopsin accumulation were constructed and graphically presented in Fig. 1 - 4 and Fig. 8. These figures also show predictive curves for biomass growth and and Figure 1-4 it is apparent that the mentioned strains estimated according to energy consumption limiting and on the base of the structured model differ in the physiological parameters that also allow certain conclusions to be drawn.

bacteriorhodopsin synthesis. Parameter X_{Lim}^{St} for strain UM17 was calculated according to equation (24) since it was the only case when a sharp almost 2-fold decrease of absolute growth rate ***dX/dτ*** was observed within a rather short interval (Figure 5). When a step-by-step transition from LGP to GIP, which is characteristic of most of cell populations, occurs, it can be expected that equation (23) will give the same result as equation (24). Biomass growth parameters for the other three halobacterial strains studied here also fit both of these equations.

Figure 8 shows differences among the four studied strains in their absolute bacteriorhodopsin biosynthesis rate ***dP/dτ*** (solid lines) and increase of the amount of bacteriorhodopsin ΔP during a constant time $\Delta\tau$ -$\Delta P/\Delta\tau$ (dotted lines).

The growth of the phototrophic halobacteria, strains D96N, JW5 and ST033, was estimated from a limited number of points (from 3 to 5). Therefore, the accuracy of the estimates may be limited. For LGP, the calculation of μ_{max} by two known ways showed different values (Table 2). For further calculations equation (8) was used because the number of estimation points is small and does not allow for using (34). However, even with the given accuracy of our experimental data, the description of growth and biosynthesis during both GIP, and LGP seems to be reasonable and reflect the differences among these strains and the conditions of their cultivation. Besides it is evident that strains JW5 and ST033 - unlike D96N and UM17 - have obviously expressed a stationary growth phase starting on day 5. Model parameters calculated for GIP describe acurately the biomass growth and bacteriorhodopsin biosynthesis during both GIP and LGP (Figure 1, 4). From Table 2

Knowing the amount of energy input (light exposure rate), ***Xp***, expressed in terms of heat of biomass combustion, it is easy to count specific viability maintenance ***m*** from Equation 3 (a). It would serve a good help for estimation of not only strains, but also quality of the used nutrient medium.

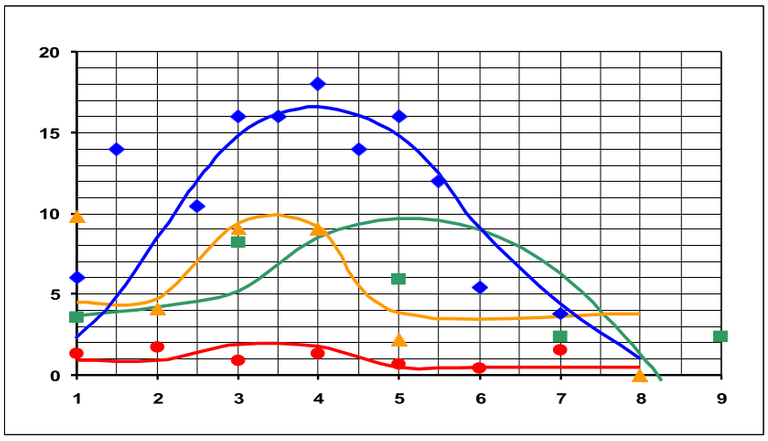

Figure 8 Model calculations of absolute metabolite synthesis rates ***dP/dτ*** (solid lines) and increase of the amount of bacteriorhodopsin ***ΔP*** for constant time ***Δτ -ΔP/Δτ*** (dotted lines), different halobacterium strains. The abscissa axis is Growth time, days. The ordinate axis is ***dP/dτ*** and ***ΔP/Δτ***, mg/(l*day). D96N, JW5, ST033, UM17 (solid lines- model calculations, dotted lines- experimental data)

Comparison of growth parameters of highly productive H.salinarum cultures made using the proposed mathematical model have shown appreciable distinctions in the level of biotechnological potential of the halobacterium strains studied:

1) Strain D96N has a lower specific growth rate μ_{max}, than strains JW5, ST033 and UM17;

2) Strain D96N has a lower specific rate of nonproliferating cell growth inhibition and accumulation, **A**, than strains JW5, ST033 and UM17;

3) Strain D96N shows higher value of X_p, so $m = E/X_p$ (see equation 3a) is lower than that for other strains;

4) strains D96N and UM17 have higher values for parameter R_{final} (very nearly equal to equal to 1.0) that gives evidence for more balanced growth by these strains than for JW5 and ST033, and for the fact that biomass growth and bacteriorhodopsin biosynthesis are limited only by energy consumption;

5) For strains D96N and UM17 the constant of bacteriorhodopsin destruction is close to 0, that also confirms the above assumption on energy limitation;

6) Strains D96N and UM17, as the above conclusions stated, have more desirable profiles of absolute (total) biosynthesis rate of bacteriorhodopsin, Figure 8;

7) Strains JW5 and ST033, in comparison to strains D96N and UM17, are similar in their properties and behavior during cultivation with a high specific growth rate μ_{max}, and also the ability to synthesize bacteriorhodopsin at rather high rate in the absence of biomass growth and population structure changes (Figure 8) ;

8) Nutrient media for strains JW5 and ST033 in the estimated conditions appear to be nutritionally balanced.

CONCLUSIONS

1. The proposed unstructured and structured models based on energy limitation show a good conformity between the calculated and experimental data on both biomass yield and bacteriorhodopsin concentrations; these allow useful predictions about these biotechnological parameters.

2. The genetically modified, highly productive derivative of *H. salinarum* D96N strain used here has better biotechnological prospects than ST033 (in contrast to UM17) because of its high levels of biomass accumulation and bacteriorhodopsin synthesis.

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DESIGNATIONS.

LGP is the logarithmic growth phase;
GIP is the growth inhibition phase;
S is a substrate concentration, g/l;
X is a biomass concentration, g/l;
X^{theor} is a biomass concentration, g/l, were calculated according to the nonstructured model;
τ is a time, days;
P is products (metabolite) concentrations, mg/l;
dP/dτ is an absolute rate of product synthesis, mg/(l*day);

$Q = -dS/d\tau$ is an absolute rate of substrate consumption, g/(l*day);

Q_{O2} is oxygen mass exchange rate, mmoleO_2 / (l*min);

J is a stochiometric factor of energy substrate (***S***) oxidation,

Joule(or g)***S***/mmoleO_2;

μ is a specific growth rate of biomass ***X***, (hour or days)$^{-1}$;

$q = Q/X$ or $q = (1/X) *dP/d\tau$ is a specific rate of substrate utilization or product synthesis, mg***P***/(g***X****day);

a is a trophic coefficient, amount of energy substrate consumed for the synthesis of a biomass unit, Joule***S***/Joule***X*** or g***S***/g***X***;

f is an amount of energy substrate accumulated in biomass ***X*** during cultivation on a synthetic medium, Joule***S***/Joule***X*** or g***S***/g***X***;

m is an energy maintenance coefficient, the rate of substrate consumption for maintaining viability of one biomass unit per a unit of time, Joul ***S***/(Joul***X****day) or g***S***/(g***X****day);

A=m/a 1) Parameter describes a delay of the biomass growth rate;

2) Specific rate of accumulation of stable cells, (hour or days)$^{-1}$;

X_p is a maximum biomass concentration, when all the energy generated during cultivation is consumed for cell viability maintenance, g/l;

X_{Lim} is a biomass concentration in the end of exponential growth phase and beginning of growth inhibition phase, g/l;

Xst is a concentration of the biomass of zero age cells (stable), the content of «resting» cells, g/l;

X_{Lim}^{st} is a concentration of the biomass of zero age cells (stable) in the end of exponential growth phase and beginning of growth inhibition phase, g/l;

X^{div} is a concentration of proliferation biomass, g/l;

τ_{Lim} is the time of exponential growth phase termination, days;

R is a ratio of X^{st} to biomass ***X***, relative content of stable cells in the biomass, synchronization degree, parts of 1;

X_I is the initial biomass concentration in ***LGP*** corresponding the beginning of population structuring, g/l;

X_{final} is the final biomass concentration, at which R=1 (when energy consumption is limited), g/l;

k_p^{div}, k_p^{st}, k_s^{div}, k_s^{st} are the constants of metabolite and substrate biochemical reaction rates,

g of product (substrate)/ g of biomass per one hour or days;

P_{lim} is metabolite concentration at the end of ***LGP*** and beginning of ***GIP,*** g/l;

P_0 is metabolite concentration in LGP, when biomass structuring occurs at $X= X_I$, g/l.

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