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Modeling the growth of *Corynebacterium glutamicum* under product inhibition in L-glutamic acid fermentation

Noor Salam Khan [a,*], Indra Mani Mishra [a], R.P. Singh [b], Basheshwer Prasad [a]

[a] *Deapartment of Chemical Engineering, Indian Institute of Technology Roorkee 247667, India*
[b] *Department of Biotechnology, Indian Institute of Technology Roorkee 247667, India*

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Abstract

In the fermentation of L-glutamic acid by *Corynebacterium glutamicum*, the growth inhibition by the substrate (glucose) at higher concentrations, and by the product at almost all concentrations seem to occur. In order to identify the range of concentrations for substrate limitation/inhibition, the experiments were conducted separately with different initial glucose concentrations. Proof of growth inhibition by the product was established by analyzing the data obtained from the time course of batch fermentation. Based on the experimental observations, a product inhibition model has been developed by modifying the Monod's kinetic equation for cell growth. This model simulates the growth satisfactorily. The same model is also able to describe the experimental data for growth of *C. glutamicum* obtained from different investigators.

Keywords: L-Glutamic acid; Fermentation; *Corynebacterium glutamicum*; Monod's equation; Substrate limitation; Product inhibition; Modeling

1. Introduction

L-Glutamic acid (LGA) is commercially one of the most important amino acids produced chiefly by fermentation process. Its sodium salt, i.e. monosodium L-glutamate (MSG) is widely used as a flavour enhancer and the total estimated worldwide production in 1996 was one million tons [1,2]. A recent survey indicates the annual production level of around 1.5 million tons and at the moment, market is growing by about 6% per year [3]. Several strains of *Corynebacterium* and *Brevibacterium*, now collectively known as *Corynebacterium glutamicum* are used for industrial production [4].

In a batch fermentation process, the growth of the cells passes through a number of phases: lag phase, exponential phase, stationary phase and decline phase. The exponential and stationary phases are explained by the relation where specific growth rate is a function of substrate concentration (like in Monod's equation). Bona and Moser [5] reported that the growth of *Corynebaterium glutamicum* does not follow a simple Monod's kinetics, the growth inhibition by product seems to occur plausibly and the production follows a "formal" analogy according to the S-inhibition or repression type. Bona and Moser [6] attempted to model the growth of *C. glutamicum* under biotin limitation with a number of equations: bio(logistic) equation (their own modification of the logistic model), modification of Monod's model [7], and by these modifications with various extensions for the lag phase. They concluded that the growth of *C. glutamicum* could be satisfactorily represented only by using the various extension terms proposed by them [6] and by Bergter and Knorre [8] for the lag phase, incorporated with the modifications of Monod's model [7] and bio(logistic) equation [6].

In the present work, a series of batch experiments in shake flasks were conducted with different initial substrate (glucose) concentrations in order to identify its range for limitation and inhibition. The batch fermentation (for L-glutamic acid production) was carried out in a bioreactor with initial glucose concentration, which was substrate limiting. Product inhibition was established after analyzing the time course of batch fermentation data obtained from the bioreactor. The product inhibition model has been developed for simulation

* Corresponding author. Tel.: +91 1332 284253; fax: +91 1332 276535.
E-mail address: nskhan_786@rediffmail.com (N.S. Khan).

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Nomenclature

X	biomass (cell) concentration ($g\,l^{-1}$)
X_0	initial biomass (cell) concentration ($g\,l^{-1}$)
X_{max}	maximum biomass (cell) concentration ($g\,l^{-1}$)
S_0	initial substrate concentration ($g\,l^{-1}$)
P_0	initial product concentration ($g\,l^{-1}$)
P_{max}	maximum product (L-glutamic acid) concentration ($g\,l^{-1}$)
t	time (h)
dX/dt	biomass (cell) growth rate ($g\,l^{-1}\,h^{-1}$)
$Y_{X/S}$	yield coefficient (biomass from substrate) ($g\,g^{-1}$)
$Y_{P/x}$	yield coefficient (product from biomass) ($g\,g^{-1}$)
Y_{PS}	yield coefficient (product from substrate) ($g\,g^{-1}$)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
K_S	Monod growth constant for the substrate ($g\,l^{-1}$)
F	F-distribution
i	experimental data points, 1 to n
j	process variables, 1 to m
n	number of experimental data points
m	number of process variables
S_j	the variance of the error of residues

Greek symbols

η	toxic power
Δ_{ij}	difference between the model and experimental values
$\bar{\Delta}_j$	mean standard deviation
λ	the statistics

of the growth of *C. glutamicum*. Attempts have also been made to simulate the experimental growth data generated from Bona and Moser [9], and from Zhang et al. [10]. It is assumed that the growth is limited by the substrate (glucose): other components of the medium are in surplus and have no effects on fermentation kinetics.

2. Materials and methods

2.1. Microorganisms and inoculum

Corynebacterium glutamicum MTCC 2745 (wild type) supplied from the Microbial Type Culture Collection IMTECH Chandigarh, India was used in the present study. Inoculum (seed culture) was prepared by transferring cells from agar slant into 500 ml Erlenmeyer shake flask, containing 100 ml of the culture medium.

2.2. Agar slant and seed culture medium

The constitution of the medium for preparing agar slant was ($g\,l^{-1}$): beef extract, 1; yeast extract, 2; peptone, 5; sodium chloride, 5; agar, 15. pH was kept at 7.0 and incubated at 30 °C for at least three days depending upon the growth of the culture. The slants were preserved at 4 °C, and subcultured twice a month.

Seed culture medium was used with the composition ($g\,l^{-1}$): glucose, 50; urea, 5; corn steep liquor (CSL), 5 ($ml\,l^{-1}$); K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.4; $FeSO_4{\cdot}7H_2O$, 0.01; $MnSO_4\,H_2O$, 0.01; biotin (5 $\mu g\,l^{-1}$); thiamin HCl (80 $\mu g\,l^{-1}$). Biotin, thiamine–HCl and urea were sterilized by membrane filter (0.2 μm, Schleicher & Schull, Germany), whereas glucose and minerals were sterilized separately by autoclaving at 15 psi (121 °C) for 15 min. All components were mixed together aseptically. The initial pH was adjusted to 7.0 with potassium hydroxide and hydrochloric acid. The culture was incubated and shaken at 30 °C for 18 h in an orbital shaking incubator (CIS-24, Remi, India) at 120 rpm before transferring to the production medium.

2.3. Production medium (batch fermentation)

The composition of the production medium was same as the seed culture medium but without corn steep liquor; urea and biotin concentrations were 8 $g\,l^{-1}$ and 1 $\mu g\,l^{-1}$, respectively. Temperature, pH and sterilization conditions were also the same. Batch fermentation was conducted in a 2 l bioreactor (Biostat M, B. Braun, Germany) with a working volume of 1.8 l. The fermentation medium was inoculated with 2% of the inoculum. pH and foaming were controlled with 25% of ammonia solution and 10% solution of a commercial antifoam, respectively. Dissolved oxygen tension was kept at 30% of air saturation.

2.4. Separation of biomass (cells)

Cells were separated from the rest of the broth by using a table top centrifuge (R-24, Remi, India) at 10,000 rpm for 5 min. The clear supernatant was carefully decanted from the centrifuge tubes for analysis of sugar and L-glutamic acid.

2.5. Analytical methods

2.5.1. Estimation of cells

Bacterial growth was estimated by measuring the optical densities (absorbance) at 610 nm with the help of a spectrophotometer (Lambda 35, Perkin-Elmer, USA) between the absorbance 0.2 and 0.9 with the Beer's law being followed. Whenever required the samples were diluted with double distilled water for the attainment of desired range of absorbance. For estimation of cell dry weight (CDW), a known volume of the sample with the known absorbance was filtered by a filtration membrane (0.45 μm, Millipore, USA). Retained biomass was washed twice with double distilled water, and

thereafter, dried in an oven at 110 °C for 8 h [11]. The differential weight of the membrane gives the dry weight of cells. A standard graph was plotted for cell dry weight versus absorbance for further estimation of CDW.

2.5.2. Estimation of glucose and L-glutamic acid

Glucose was estimated by DNS method [12], while LGA was estimated by copper complex method [13] as also discussed in EICA [14].

2.6. Inhibition studies

2.6.1. Substrate (glucose) inhibition

In order to establish the proof of growth limitation and/or inhibition by the substrate (glucose) and the ranges of the concentrations in which they occur, a number of shake flask (Erlenmeyer, 500 ml) experiments were carried out with 50 ml of the fermentation medium, containing 2% of inoculum. Initial glucose concentration range was kept at 10–350 g l^{-1}. All other conditions of fermentation were the same. Samples were withdrawn from the incubator as the whole flask at desired time intervals and 0.5 ml of formaldehyde (37–40%, w/v) was added immediately to the flask and shaken well in order to inactivate the cells. The absorbance (optical densities) for cell growth was measured at 610 nm for starting few hours of fermentation and the data so obtained were plotted against time for different initial glucose concentrations.

2.6.2. Product (L-glutamic acid) inhibition

To study the growth inhibition by the product, the specific growth rates were calculated from the data obtained from the time course of batch fermentation, and plotted against the respective product concentrations.

3. Results and discussions

3.1. Inhibition studies

Fig. 1 shows the plots of optical density of the broths at 610 nm against the fermentation time for different initial glucose concentrations. Up to 50 g l^{-1}, the growth slightly increases with increasing substrate concentrations and that inhibition is not visible. Here, the substrate limitation may be thought of dominating. Above 50 g l^{-1}, the growth appears to decrease with the increasing glucose concentrations (Fig. 2). If we further go on increasing the initial glucose concentration, a point may be arrived at which the growth will be completely inhibited. The severe effect of growth inhibition has been shown by Fig. 3. Here, the growth is not visible when the initial glucose concentration is 350 g l^{-1}.

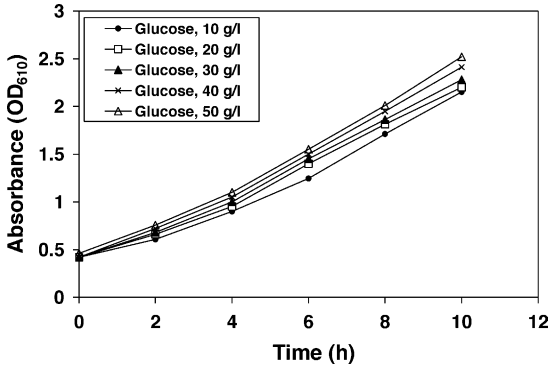

Fig. 1. Effect of initial glucose concentrations on the growth of *Corynebacterium glutamicum*.

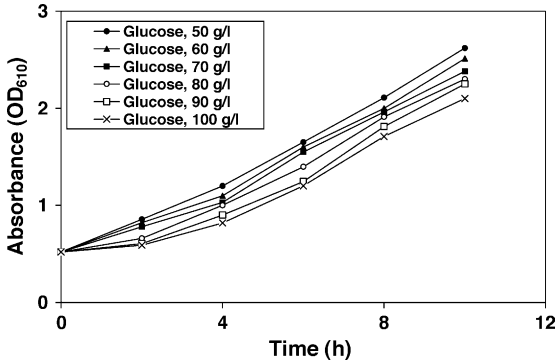

Fig. 2. Effect of initial glucose concentrations on the growth of *Corynebacterium glutamicum*.

In Fig. 4, the specific growth rate is continuously decreasing with the increasing L-glutamic acid concentration and becomes almost zero when the product concentration is highest at about 12 g/l. This shows that the cell growth is inhibited by the product. Bergter and Knorre [8] also arrived at the same conclusion through this approach. It may be that in the beginning when the product concentration is negligible or less the inhibition is not visible. As the acid accumulates the magnitude of inhibition gradually increases. The specific growth rate continuously decreases and becomes zero at the maximum concentration of L-glutamic acid. Here, the decrease is almost linear as evident from Fig. 4.

3.2. Model development

In fermentation processes, the growth of microorganisms is a very complex phenomenon. The specific growth rate μ

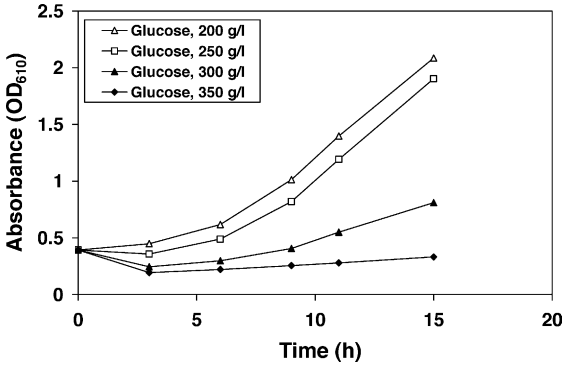

Fig. 3. Effect of initial glucose concentrations on the growth of *Corynebacterium glutamicum*.

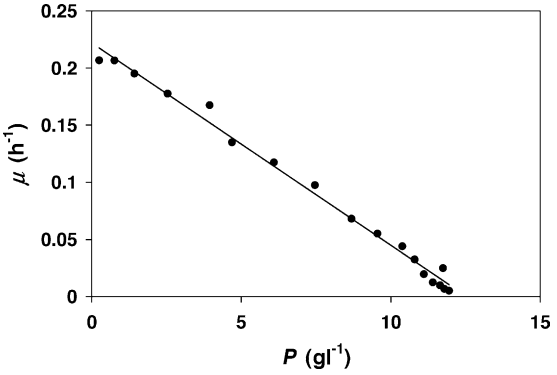

Fig. 4. Effect of product (L-glutamic acid) concentrations on the specific growth rate of *Corynebacterium glutamicum*.

is usually expressed as a function of the limiting substrate concentration S by a Monod type relationship:

$$\mu = \frac{\mu_{\max} S}{K_S + S} \tag{1}$$

where $\mu_{\max}$ and K_S are the maximum specific growth rate and saturation constant (Monod constant), respectively. The above equation is only applicable when the presence of substrate and product do not have any toxic (inhibitory) effects on growth. In order to demonstrate the inhibitory effect of the product, the Eq. (1) may be written as:

$$\mu = f(S, P) \tag{2}$$

The specific growth rate, therefore, can be represented by the equation:

$$\mu = \mu_{\text{obs}} \frac{S}{K_S + S} \tag{3}$$

where μ_{obs} is the maximum specific growth rate observed in the presence of inhibitory effects of substrate (S) and/or product (P). It depends on the concentrations of both or one based on the type of inhibition involved during the growth of the cells.

In the present study, an initial glucose concentration of 50 g l^{-1} has been used which comes in the range of substrate limitation (Fig. 1). Gradual decrease of specific growth rate with increasing product concentration is an indication of the growth inhibition by the product (Fig. 4). Based on these observations one can proceed with the concept of substrate limitation along with product inhibition. This is the situation where both the substrate availability and the product inhibition effect the specific growth rate. For such conditions, Levenspiel [15] proposed a generalized equation to account for the influence of product inhibition:

$$\mu_{\text{obs}} = \mu_{\max}\left(1 - \frac{P}{P_{\max}}\right)^{\eta} \tag{4}$$

where $P_{\max}$ is the maximum product concentration at which the growth is completely inhibited and η is the toxic power.

For the growth of *C. glutamicum*, the Monod's Eq. (1) under product inhibition conditions can be modified as follows:

$$\mu = \frac{\mu_{\max} S}{K_S + S}\left(1 - \frac{P}{P_{\max}}\right)^{\eta} \tag{5}$$

The final kinetic equation for growth is given below:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \frac{\mu_{\max} S}{K_S + S}\left(1 - \frac{P}{P_{\max}}\right)^{\eta} X \tag{6}$$

3.3. Estimation of model parameters

The optimal values of the parameters of the models are estimated by non-linear regression technique [16] with the help of computer programmes [17,18]. Model predictions for the differential equations were made by a software package "Polymath" version 5.1 (CACHE Corpn., USA) using the method RKF45. The optimization programme for direct search of the minimum of a multivariable function was based on the original method of Rosenbrock [19]. For minimizing the difference between the model generated values and the corresponding experimental data, the criterion of weighted sum of squares of residuals was used [17,18,20,21]:

$$\text{SSWR} = \sum_{i=1}^{n}\sum_{j=1}^{m} \frac{\Delta_{ij}^2}{w_j^2} \tag{7}$$

where SSWR is the sum of squares of weighed residues. n and m denote the number of experimental data points and the number of process variables, respectively. w_j is the maximal weight of the variable and Δ_{ij} represents the difference between the model and the experimental value of the jth variable in the ith experimental point.

The method recommended by Bard [16] was used for the evaluation of the degree of reliability of the hypothesis concerned with the model pertaining to the growth of *C. glutamicum* in L-glutamic acid fermentation. The hypothesis, whether the estimate of parameters guarantees the zero mean deviation between the model and experimental data was tested. The mean standard deviation ($\bar{\Delta}_j$) of the variable was calculated as follows:

$$\bar{\Delta}_j = \frac{1}{n}\sum_{i=1}^{n} \Delta_{ij}; \qquad j = 1, m \tag{8}$$

The variance of the error of residues (S_j) was further estimated as:

$$S_j = \frac{1}{n-1}\sum_{i=1}^{n} (\Delta_{ij} - \bar{\Delta}_j)^2; \qquad j = 1, m \tag{9}$$

The statistic 'λ' defined as:

$$\lambda = \frac{(n-m)n}{(n-1)m}\sum_{j=1}^{m} \frac{\bar{\Delta}_j^2}{s_j} \tag{10}$$

has the $F_{m,n-m}$ distribution and is used to find out the statistical adequacy for acceptance of the model.

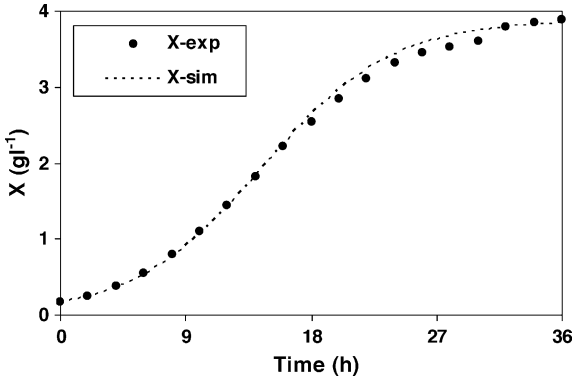

Fig. 5. Plot of simulation for growth of *Corynebacterium glutamicum* according to product inhibition model.

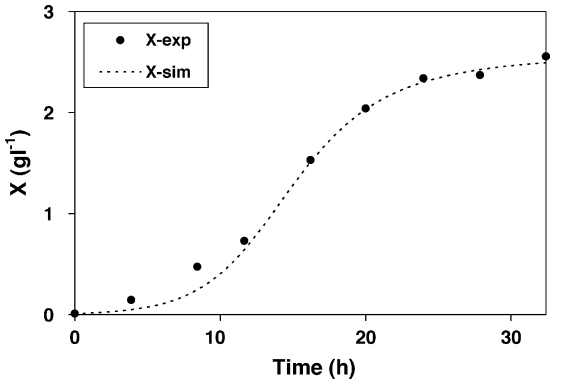

Fig. 6. Plot of simulation for growth of *C. glutamicum* (lab. data [10]) according to product inhibition model.

The graphical results of modeling for the growth of *C. glutamicum* with our experimental data and those of Bona and Moser [9], and Zhang et al. [10] are shown in Figs. 5–7. The experimental data and the kinetic parameters are given in Tables 1 and 2, respectively.

Fig. 5 is showing good agreement of the experimental data with the model. The statistic 'λ' is 6.77. This is lower than the value obtained for $F_{1,18}$ in the *F*-table for 99% confidence level.. All these evidences establish the accuracy of optimized

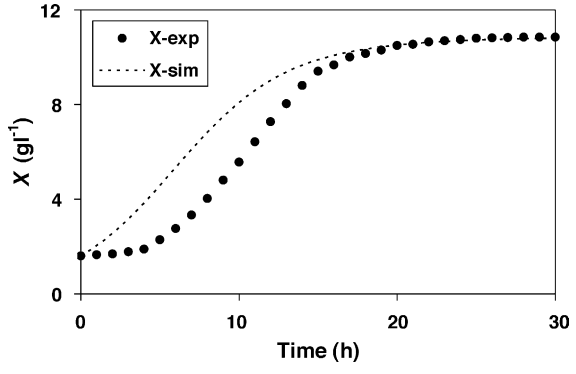

Fig. 7. Plot of simulation for growth of *C. glutamicum* (lab. data, Bona and Moser, 1997c) according to product inhibition model.

Table 1
Experimental data

Data source	Initial ($g\,l^{-1}$)	Final ($g\,l^{-1}$)	Figures
Present work	$X_0 = 0.164$ $P_0 = 0.00$ $S_0 = 49.87$	$X_{max} = 3.88$ $P_{max} = 11.529$ $S_{final} = 25.1$	Fig. 5
Zhang et al. [10]	$X_0 = 0.012$ $P_0 = 0.000$ $S_0 = 1.305$	$X_{max} = 2.555$ $P_{max} = 0.3045$ $S_{final} = 0.225$	Fig. 6
Bona and Moser (1997c)	$X_0 = 1.6$ $P_0 = 0.0$ $S_0 = 99.7$	$X_{max} = 10.85$ $P_{max} = 32.74$ $S_{final} = 7.78$	Fig. 7

Note: For Figs. 6 and 7, it was assumed that value of *P* corresponding to X_{max} as used by the workers is P_{max}.

parameters and validity of the model for the growth of *C. glutamicum* in the present work.

Fig. 6 represents the modeling of laboratory data for growth, generated from Fig. 1A of Zhang et al. [10]. It is clear from the graph (Fig. 6) that the laboratory data [10] for growth agree with the product inhibition model under the optimized values of model parameters as shown in Table 2. The statistic 'λ' is 0.59, which is less than the '$F_{1,8}$' value (obtained from *F*-table) for 95% confidence. This establishes the accuracy of the product inhibition model used for the growth of L-glutamic acid producing bacteria. Here the initial glucose concentration obtained from Fig. 1B of [10] is very low ($1.305\,g\,l^{-1}$), and as a result of that substrate limitation and product inhibition may be thought of prevailing and the data are modeled satisfactorily by the present model.

Modeling of experimental data for growth, generated from Fig. 11 of Bona and Moser [9] is shown in Fig. 7. A series of computer simulations were done in order to determine the values of kinetic parameters for the best simulating graph. Fig. 7 shows initial deviation from the model, which continues up to about 18 h of fermentation. After that the data agree well with the model. This may be due to high initial concentration of glucose (i.e. $100\,g\,l^{-1}$) used by Bona and Moser [9] causing substrate inhibition at the beginning of fermentation. As the concentration comes down within the substrate limitation range the product inhibition model is followed. At 18 h of fermentation, the concentration is about $46.89\,g\,l^{-1}$ as calcu-

Table 2
Parameters for dynamic simulations

Parameters for	Model parameters	Y's ($g\,g^{-1}$)	Figures
Present work	$\mu_{max} = 0.21\,h^{-1}$ $K_S = 0.8\,g\,l^{-1}$ $\eta = 1$	$Y_{X/S} = 0.149$ $Y_{P/x} = 3.216$ $Y_{PS} = 0.48$	Fig. 5
Zhang et al. [10]	$\mu_{max} = 0.534\,h^{-1}$ $K_S = 0.5748\,g\,l^{-1}$ $\eta = 1$	$Y_{X/S} = 2.354$ $Y_{P/x} = 0.119$ $Y_{PS} = 0.2819$	Fig. 6
Bona and Moser (1997c)	$\mu_{max} = 0.26\,h^{-1}$ $K_S = 5\,g\,l^{-1}$ $\eta = 1$	$Y_{X/S} = 0.10065$ $Y_{P/x} = 3.539$ $Y_{PS} = 0.356$	Fig. 7

Note: Y's have been calculated macroscopically directly from the experimental data.

lated from Fig. 1 of [9]. This concentration comes in the range of substrate limitation (as observed from our investigation, Fig. 1). Under these conditions of substrate concentrations, the model is able to describe the experimental growth data of Bona and Moser [9].

4. Conclusions

The growth of *Corynebacterium glutamicum* does not follow a simple Monod's kinetics. Growth inhibition by the product (L-glutamic acid) occurs during fermentation. Substrate (glucose) limitation at lower concentration and inhibition at higher concentrations are exhibited against growth. Modified form of Monod's equation along with product inhibition term proposed by Levenspiel [15] is able to define the growth kinetics at lower substrate concentrations. For higher substrate concentrations when substrate inhibition also occurs, the present modified model appears to fail. At very high concentrations, like 350 g l^{-1} of glucose, growth is almost zero, which may be due to severe substrate inhibition.

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