Modeling The Growth Of *Corynebacterium Glutamicum* In L-Glutamic Acid Fermentation

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Abstract
Biotin limitation plays a key role in production of L-glutamic acid by fermentation whereas the biomass concentration also shows plausible effects. Monod and logistic equations were modified by incorporating the specific biotin concentration term. The growth data were found to be satisfactorily simulated with the logistic model and the modified form of Monod and logistic models. Whereas, Monod equation itself was not able to explain the growth pattern. Broader applications of graphical representation and statistical estimates were also shown in support of modeling.

Keywords: *L-glutamic acid; fermentation; Corynebacterium glutamicum; Monod's equation; Logistic equation; Biomass growth; Modeling*

1. Introduction

L-glutamic acid (LGA) is commercially one of the most important amino acids. Monosodium glutamate (MSG), the sodium salt of LGA is widely used as a flavor enhancer throughout the world [1]. As per a recent report, the annual production level is more than 2 million tons [2] and the demand is increasing by about 6% per annum [3].

Biotin is used as a growth factor and its optimized supply and/or addition of penicillin or treatment with various surfactants are must for efficient production of LGA during fermentation. Inhibition of the growth of LGA-producing bacteria (i.e. *Corynebacterium glutamicum*) by the substrate at higher concentrations and by the product at almost all concentrations was observed and growth data were defined by product inhibition model proposed by Khan et al. [4].

Presently, the batch and fed-batch fermentation processes are commonly used for the commercial production of LGA. The growing market has led to the demand for improvement in bioprocess technology. Modeling and simulation are the important steps leading towards process development.

The logistic model describes the characteristic sigmoidal curve of biomass growth:

$$\frac{dX}{dt} = \mu_{\max} X \left(1 - \frac{X}{X_{\max}}\right) \qquad (1)$$

Monod's model correlates the growth to substrate concentration:

$$\frac{dX}{dt} = \frac{\mu_{\max} S}{K_S + S}.X \qquad (2)$$

where, μ_{max} and K_S can be determined by appropriate linearization of the equation according to Lineweaver–Burk plot or Eadie-Hofstee plot.

The logistic equation (1) was modified by Bona and Moser [5] in bio(logistic) form as:

$$\frac{dX}{dt} = \mu_{\max}.X.\left(1 - \frac{b_{\min}}{b}\right) * L \qquad (3)$$

where, L is a retardation term for lag phase.

The Monod-type growth model was modified by Yamashita et al [6] and used in the following form:

$$\frac{dX}{dt} = \mu_{\max}.X.\frac{(b-b_{\min})}{K_b+(b-b_{\min})}*L \qquad (4)$$

Here, b is the specific biotin concentration, defined as $b = A_0/X$ and $b_{min} = A_0/X_{max}$,where A_0 is the initial biotin concentration and X_{max} is the maximum cell concentration.

Bona and Moser [5] used four different expressions for the lag phase term L as given by Pirt [6] i.e. $L=(t-t_L)$, Bergter-Knorre [8] i.e. $L=(1-e^{-t/t_L})$, and their own arc tangent and hyperbolic tangent functions i.e. $L=\{a\tan[(t-t_L)\times 10^n]/\pi+0.5\}$ and $L=\{[\tanh(t-t_L)\times n]+1\}/2$, respectively; with the models (3) and (4) and found good agreement of the model predictions with experimental data for growth of the cells.

In the present study, the Monod and logistic equations have been further modified and satisfactorily used for explaining the growth of *Corynebacterium glutamicum*. Attempts have also been made to simulate the data with Monod and logistic equations in their original forms. The paper also deals with the estimation of model parameters using nonlinear regression technique [9]. The modeling and simulation have been explained graphically as well as statistically.

2. Materials and Methods

2.1. Microorganisms and Inoculum

Corynebacterium glutamicum MTCC 2745 supplied by 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 50 mL of the culture medium.

2.2 Agar Slant and Seed Culture Medium

The constitution of the medium for preparing agar slant in gl^{-1}: beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; sodium chloride, 5.0 and agar, 15.0. pH was kept at 7.0 and incubated at 30 ^{0}C for at least three days depending upon the growth of the culture. The slants were preserved at 4 ^{0}C, and subcultured twice a month.

Seed culture medium was used with the composition (gl^{-1}): glucose, 50; urea, 5.0; corn steep liquor (CSL), 5 mll^{-1} , K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4$ $7H_2O$, 0.4; $FeSO_4$ $7H_2O$, 0.01; $MnSO_4$ H_2O, 0.01; biotin, 5.0 μgl^{-1} and thiamin HCl (vitamin B1), 80 μgl^{-1}. Biotin, thiamin-HCl and urea were sterilized by membrane filter (0.2µm, Schleicher & Schull, Germany) whereas glucose and minerals were sterilized separately by autoclaving at 15 psi (121 ^{0}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 ^{0}C for 18 h in an orbital shaking incubator (CIS-24, Remi, India) at 120 rpm before transferring to the production medium.

2.3 Fermentation (Production) Medium

The composition of the production medium was same as the seed culture medium except that no corn steep liquor was used; urea and biotin concentrations were 8 g/l and 1 µg/l, respectively. Temperature, pH and sterilization conditions were also the same. Batch fermentation was carried out for 36 hours in a 2l bioreactor (Biostat M, B. Braun, Germany) with a working volume of 1.8l and a stirring speed of 250 rpm was maintained. The fermentation medium was inoculated with 2% of inoculum. pH and foaming were controlled with 25% of ammonia solution and 10% of commercial antifoam, respectively. Dissolved oxygen tension was kept at 30% of air saturation. Samples were withdrawn from the bioreactor at every two hours and used for analysis of cell, glucose and LGA concentrations.

2.4 Separation of Biomass (cells)

Cells were separated from 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 0.2–0.9 with the Beer's law being followed. Whenever required the samples were diluted with double distilled water for attainment of desired range of absorbance. For estimation of cell dry weight (CDW), known volume of the sample with known absorbance was filtered by a filtration membrane (0.45μm, Millipore, USA). Retained biomass was washed twice with double distilled water, and thereafter dried in an oven at 110 ^{0}C for 8 hours [10]. The differential weight of the membrane gives the dry weight of the 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 [11] while LGA was estimated by copper complex method [12] as also discussed in EICA [13]. All the results were reported as the average of three sets of experiments.

3. Results and Discussions

3.1 Models Used

In view of the very small lag phase observed during the course of experiments, the logistic equation (1) and the Monod equation (2) have been used in their original forms. In order to reflect the effect of biotin concentration, these two equations have been further modified. The logistic equation (1) is modified as:

$$\frac{dX}{dt} = \mu_{\max}.X\left(1-\frac{b_{\min}}{b}\right) \qquad (5)$$

Whereas the Monod equation (2) is modified as:

$$\frac{dX}{dt} = \mu_{\max}.X\,\frac{(b-b_{\min})}{K_b+(b-b_{\min})} \qquad (6)$$

There is no retardation term *L* used in the above equations for the lag phase.

The values of experimental data and the optimized kinetic parameters are given in Table 1. The validity of the models and accuracy of the optimized model parameters have been shown by graphs (Figures 1 to 6) with statistical error estimates (Tables 2 to 5).

3.2 Assumptions Made

(i) The model (1) was used with the assumption that biotin, glucose and other components in the medium are sufficiently available and the growth is controlled by the cell concentration itself.
(ii) While using the model (2), it was assumed that the growth is limited by the substrate (glucose).
(iii) Models (5) and (6) were used with the assumption that initial biotin concentration (A_0) was absorbed immediately after inoculation. During growth phase the mother cell divide and transfer biotin to the daughter cells for acceptance of the specific biotin concentration [6].

3.2 Estimation of Model Parameters

The optimal values of the parameters of all the models used are estimated separately by nonlinear regression technique [9] with the help of computer programmes [14, 15]. Model predictions for the differential equations were calculated 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 function was based on the original method of Rosenbrock [16]. For minimizing the difference between the model generated values and the corresponding experimental data, various error estimates were calculated as used by different researchers [15, 17, 18]. These include the criterion of the minimization of the weighted sum of squares of residues, SSWR; the mean standard deviation, $\bar{\Delta}_j$; the variance of error of residues, S_j; an error statistic, λ; and the root mean square error, RMSE. The weighted sum of squares of residues is defined as:

$$SSWR = \sum_{i=1}^{n}\sum_{j=1}^{m}\frac{\Delta_{ij}^{\ 2}}{w_j^{\ 2}} \qquad (6)$$

Where, 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 model and experimental value of the jth variable in the ith experimental point.

The method recommended by Bard [9] was used for the evaluation of the degree of reliability of hypothesis concerned with each model pertaining to the growth of *Corynebacterium glutamicum* in L-glutamic acid fermentation. The hypothesis, whether the estimate of parameters guarantees the zero mean deviation of the model and experimental data was tested. The mean standard deviation ($\bar{\Delta}_j$) of the variable was calculated as follows:

$$\bar{\Delta}_j = \frac{1}{n}\sum_{i=1}^{n}\Delta_{ij} \qquad j = 1, m \qquad (7)$$

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

$$S_j = \frac{1}{n-1}\sum_{i=1}^{n}(\Delta_{ij} - \bar{\Delta}_j)^2; \qquad j = 1, m \qquad (8)$$

The value of the statistic λ defined as

$$\lambda = \frac{(n-m)n}{(n-1)m}\sum_{j=1}^{m}\frac{\bar{\Delta}_j^2}{s_j} \qquad (9)$$

The statistic λ can be calculated by using Eqs. (8) and (9). It has the $F_{m,\,n-m}$ distribution and is used to find out the statistical adequacy for the acceptance of the model.

The root mean square error (RMSE), the commonly used estimate [19] to check the validity of the model for single variable can be determined as:

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(\text{Observed} - \text{Modelled})^2} \qquad (10)$$

Closer the values of SSWR, S_j and RMSE to zero, the better are the estimates of the model parameters.

Table 1. Parameters for dynamic simulation[a]

Experimental data			Optimized parameters
X_0 = 0.164 gl^{-1}	P = 0.00 gl^{-1}	$Y_{X/S}$ = 0.150 gg^{-1}	μ_{max} = 0.21 h^{-1}
S_0 = 49.87 gl^{-1}	P_{max} = 11.952 gl^{-1}	$Y_{P/S}$ = 0.4825 gg^{-1}	K_b = 0.25 µgg^{-1} CDW
X_{max} = 3.88 gl^{-1}	A_0 = 1.0 µgl^{-1}	$Y_{P/X}$ = 3.216 gg^{-1}	L = 1.0

a) Values of yield coefficients were directly calculated from the experimental data applying the macroscopic approach.

Figure 1 represents the agreement of the logistic model with the experimental data. Product-inhibition plausibly occurs with substrate repression in L-glutamic acid fermentation [4, 20]. Since the substrate concentration (50 gl^{-1}) used in the present work is lower than 100 gl^{-1} used by Bona and Moser [20], the substrate-repression may be neglected and product-inhibition may be thought of dominating. Cell growth and product formation in such situations are interrelated. Complete growth-inhibition occurs at P_{max}, and consequently at X_{max}. At P_{max}, the growth rate was found to be zero [4]. In other words the growth rate is zero at X_{max} as evident from the logistic Eq. (1). The validity of model has also been proven for μ_{max} = 0.21 h^{-1} with statistic λ = 0.106 as given in Table 2. The value of λ is lower than the value obtained for $F_{1,18}$ in the F-table for 99% confidence level. The accuracy of the optimized value of μ_{max} was also tested by varying it. It is shown graphically (Figure 1) as well as statistically (Table 2).

Table 2. Values of kinetic parameters and the error estimates for model Eq. (1) .

Model	Parametric value	S_j	λ	RMSE	Figure
(1)	μ_{max} = 0.19 h^{-1}	0.013	31.231	0.189	1
	μ_{max} = 0.21 h^{-1}	0.004	0.106	0.062	
	μ_{max} = 0.25 h^{-1}	0.039	29.403	0.313	

Figures 2 and 3 show comparison of Monod's model with the experimental data. In Figure 2, the graph with μ_{max} = 0.21 h^{-1} and K_S = 0.8 g/L, appear to follow Monod's kinetics initially and then deviates considerably as the time passes. This may be due to the accumulation of product which inhibits the growth [4]. The values of S_j, λ and RMSE as given in Table 3 are

Table 3. Values of kinetic parameters and the error estimates for model Euation (2).

Model with parameters	Parameters varied	S_j	λ	RMSE	Figure
(2) K_S = 0.8 g/L	μ_{max} = 0.07 h^{-1}	0.850	50.400	1.745	2
	μ_{max} = 0.089 h^{-1}	0.522	39.013	1.252	
	μ_{max} = 0.21 h^{-1}	3.808	28.132	3.041	
(2) μ_{max} = 0.21 h^{-1}	K_S = 0.8 g/L	3.810	28.132	3.041	3
	K_S = 10 g/L	3.51	16.190	2.910	
	K_S = 20 g/L	2.83	6.910	2.330	

considerably very high for all deviations. As a result, it is proved graphically as well as statistically that the growth of *C. glutamicum* in LGA fermentation does not follow Monod kinetics.

Figure 4 demonstrates the growth kinetics according to the modified form of logistic Equation (5). The graph shows that the data has good agreement with the model for μ_{max} = 0.21, where as the deviation from this value also shows deviation from the model. This is the optimized value of model parameter which have also got the least values of S_j, λ and RMSE in Table 4 . This has more than 99% confidence level for its validity in the F-distribution table.

Table 4. Values of kinetic parameters and the error estimates for model Equation (5).

Model Eq.	Parameters varied	S_j	λ	RMSE	Figure
(5)	μ_{max} = 0.19 h^{-1}	0.012	31.231	0.189	4
	μ_{max} = 0.21 h^{-1}	0.004	0.106	0.062	
	μ_{max} = 0.25 h^{-1}	0.039	29.403	0.313	

Figures 5 and 6 represent the plot of simulation according to the modified form of Monod Equation (6) under biotin limitation, with variation of μ_{max} and K_b, respectively. All figures show good agreement of the model for the experimental data with optimized values of kinetic parameters i.e. μ_{max} = 0.21 h^{-1} and K_b = 0.25 µg/g CDW. The values of S_j, λ and RMSE are also the least as shown in Table 5 as compared to the deviations.

Table 5. Values of kinetic parameters and the error estimates for model Eq. (6).

Model with parameters	Parametric variation	S_j	λ	RMSE	Figure
(4) K_b = 0.25 µg/g CDW	μ_{max} = 0.19 h^{-1}	0.012	28.774	0.176	5
	μ_{max} = 0.21 h^{-1}	0.004	1.271	0.070	
	μ_{max} = 0.25 h^{-1}	0.041	30.770	0.328	
(4) μ_{max} = 0.21 h^{-1}	K_b = 0.20 µg/g CDW	0.015	13.220	0.162	6
	K_b = 0.25 µg/g CDW	0.004	1.271	0.070	
	K_b = 0.30 µg/g CDW	0.008	26.324	0.085	

The values of λ for all optimized kinetic parameters fall below the value of $F_{1,18}$ in the F-table for 99% confidence level.

4. Conclusion

The optimized values of kinetic parameters in the models (1), (5) and (6) are numerically same. The values of λ in all the three cases are very small as compared to the values of $F_{1,18}$ obtained from the F-table for 99% confidence. This establishes the validity of the model. The variation of the model parameters around their optimized values is quite high as compared to the values for $F_{1,18}$ in the F-table for 95% and 99% confidence levels. This establishes the accuracy of the values of optimized parameters. Therefore, it may be concluded that the growth of *Corynebacterium glutamicum* in L-glutamic acid fermentation may be modeled and simulated well with the logistic model (1), modified form of logistic model (5) and modified form of Monod model (6). The Monod model (2) alone is not able to represent the growth kinetics of *Corynebacterium glutamicum* in L-glutamic acid fermentation. This inability of the model may be attributed to the inhibitory effect of the product (L-glutamic acid) concentration towards growth.

Nomenclature

b	Specific biotin concentration ($\mu g g^{-1}$ CDW)
b_{min}	Minimum specific biotin concentration ($\mu g g^{-1}$ CDW)
exp	Experimental
F	F-distribution
K_b	Monod constant for specific biotin concentration ($\mu g g^{-1}$ CDW)
S	Substrate concentration ($g l^{-1}$)
S_0	Initial substrate concentration ($g l^{-1}$)
Sim	Simulated
t	Time (h)
t_L	Lag time (h)
X	Biomass (cell) concentration ($g l^{-1}$)
X_0	Initial biomass concentration ($g l^{-1}$)
dX / dt	Biomass (cell) growth rate ($g l^{-1} h^{-1}$)
$Y_{X/S}$	Yield coefficient (biomass from substrate) ($g g^{-1}$)
i	Experimental data points, 1 to n
j	Process variables, 1 to m
μ	Specific growth rate (h^{-1})
π	Dimensionless number, numerical value = 3.14

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Figures:-

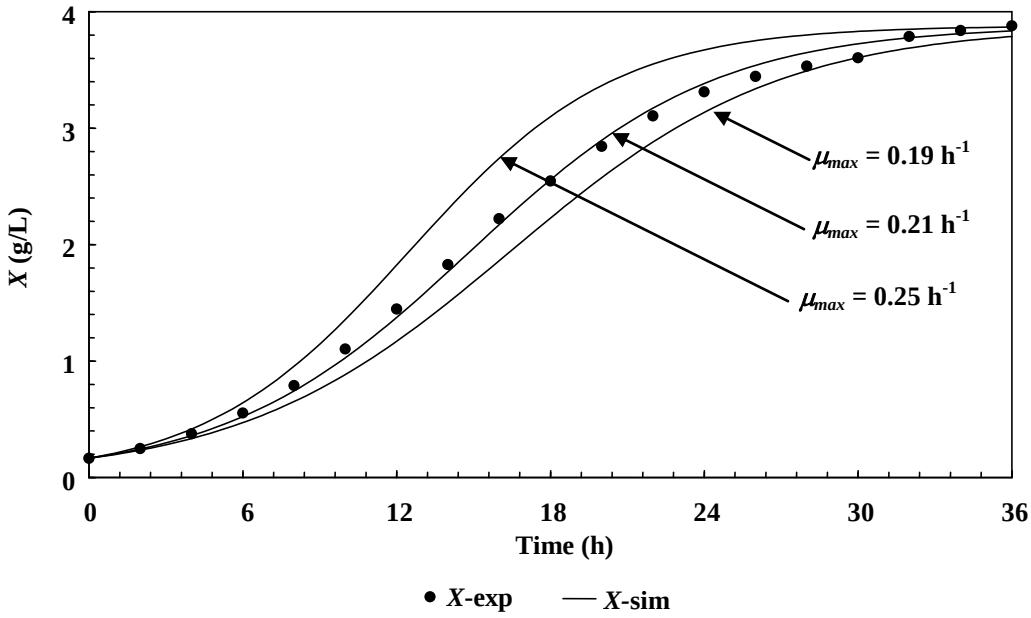

Figure 1. Simulation of the growth data according to the model Eq. (1) with variation of μ_{max}.

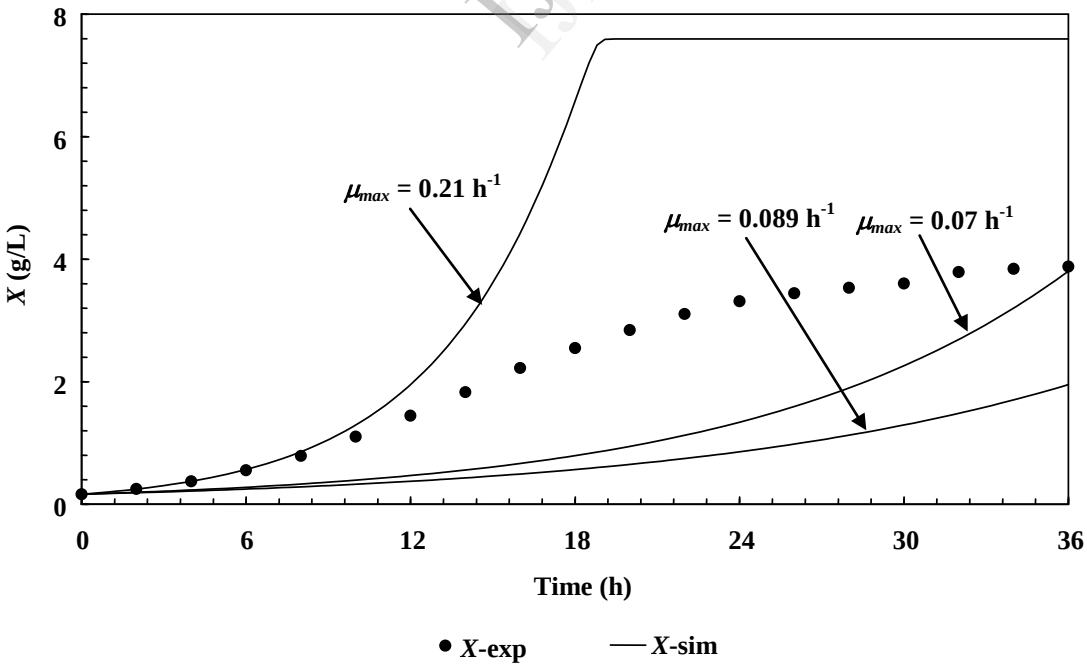

Figure 2. Simulation of the growth data with the model Eq. (2) for different values of μ_{max}, where K_S = 0.8 g/L.

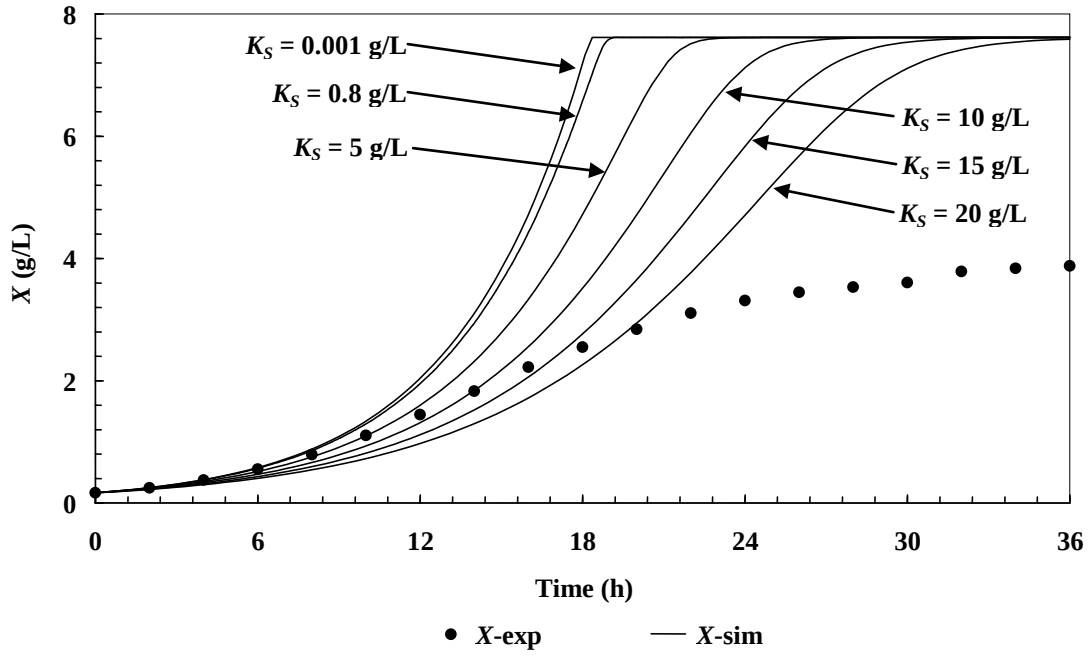

Figure 3. Simulation of the growth data according to the model Eq. (2) with variation of K_S, where μ_{max} = 0.21 h^{-1}.

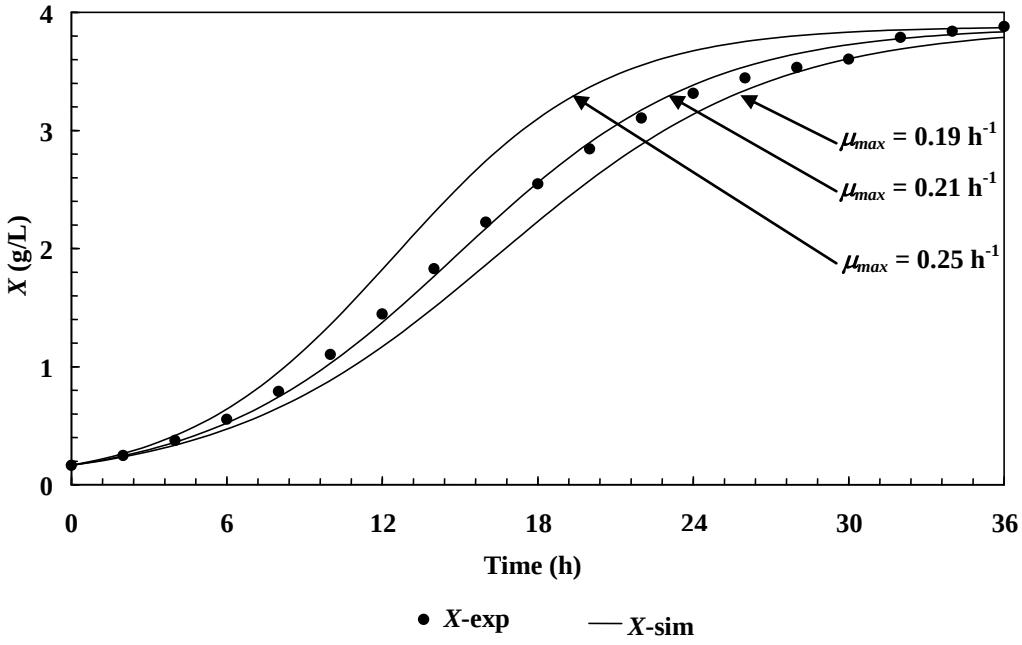

Figure 4. Simulation of the growth data according to the model Eq. (5) for different values of μ_{max}.

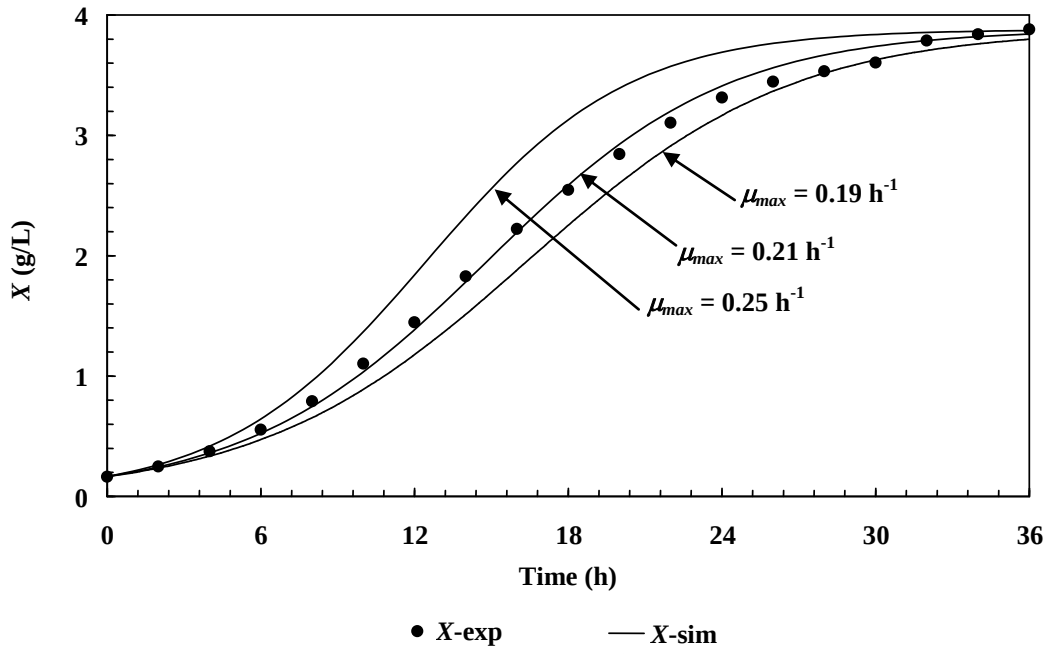

Figure 5. Simulation of the growth data with the model Eq. (6) for different values of $\mu_{max,}$ where K_b = 0.25 μg/g CDW.

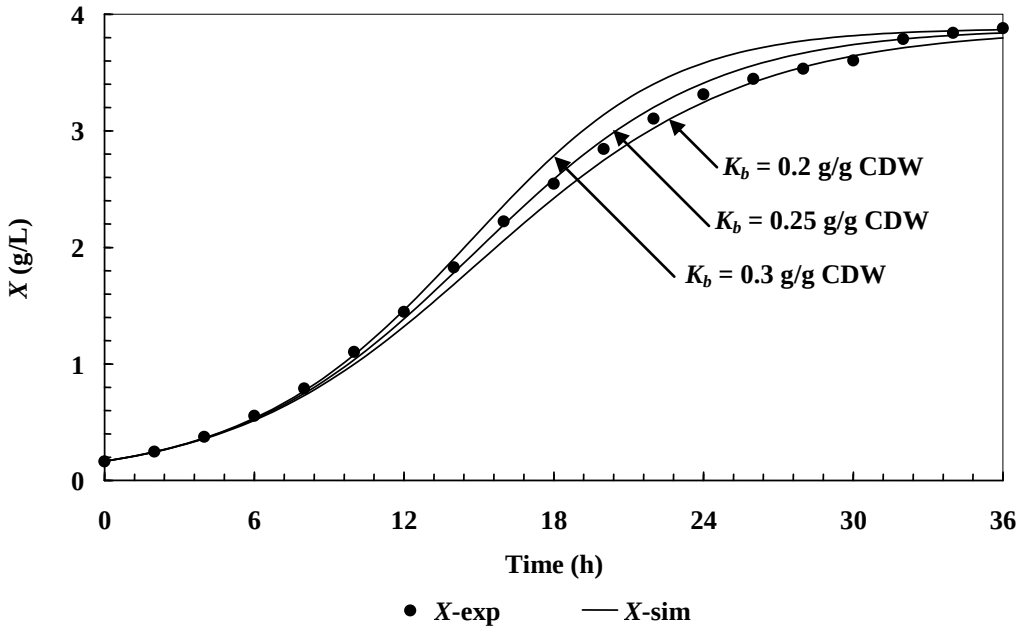

Figure 6. Simulation of the growth data according to the model Eq. (6) for different values of K_b, where μ_{max} = 0.21 h^{-1}.