MODELING THE FERMENTATIVE PRODUCTION OF L-GLUTAMIC ACID BY CORYNEBACTERIUM GLUTAMICUM IN A BATCH BIOREACTOR

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Abstract

The fermentation kinetics of L-glutamic acid by *Corynebacterium glutamicum* was studied in a batch bioreactor. Mathematical model using the logistic equation for growth, Leudeking-Piret kinetic equation for product formation and Leudeking-Piret like equation for substrate consumption was proposed. Based on the analysis of experimental data followed by computer simulation, the model seemed to provide a reasonable description for L-glutamic acid fermentation.

Keywords: L-glutamic acid; Fermentation; *Corynebacterium glutamicum*; Logistic equation; Growth–linked equation

1. Introduction

L-glutamic acid fermentation is a well known typical aerobic fermentation. Commercially, it is manufactured by the batch/fed-batch processes [11]. The rapid increase in the demand for its sodium salt (i.e. monosodium glutamate) as a flavour enhancer, both for food industry and for table use has been witnessed in recent years. The estimated worldwide production for monosodium glutamate (MSG) in 1996 was one million tons [10, 13]. As per a recent report, the annual production level is more than 2 million tons [26] and the market is growing by about 6 % per year [9]. Several strains of *Corynebacterium* and *Brevibacterium*, now collectively known as *Corynebacterium glutamicum* are used for the production of L-glutamic acid [12].

Bona and Moser [4] examined the mathematical modeling of the production of L-glutamic acid in batch fermentation under product inhibition and substrate repression along with biotin limitation. They summarized that the growth pattern was quite well represented by the formal model; the production behavior was still lacking appreciable modeling without identified reasons.

Bona and Moser [5] studied the modeling with biotin limitation following the formal kinetic approach. The mathematical model for bacterial growth was based on the Yamashita [25] growth model with Bergter-Knorre [3] extension for the lag phase and with production being proportional to biomass, including a maturation term. The model formulation was based on the assumptions that fermentation is biotin limited and substrate is surplus. For kinetically relevant variables, varying degrees of agreement was demonstrated between the experimental and simulated results. A perfect similarity was still lacking.

Bona and Moser [6] modeled the L-glutamic acid fermentation with *Corynebacterium glutamicum* under biotin limitation. In this study, they developed a kinetic model with the assumption of cell maturation for the production of L-glutamic acid in the batch process. It was also assumed that the substrate is sufficiently available and has no effect on the fermentation kinetics, whereas the role of biotin was considered to be significant in the bioprocess. Yamashita [25] growth model with Bergter and Knorre [3] extension was used for the lag phase.

Zhang et al. [27] proposed a number of empirically nonlinear and time dependent kinetic equations for cell growth, substrate consumption and product formation. All the three process variables (X, P and S) were modeled separately. They showed good agreement between the experimental and predicted values by comparing the data obtained from the laboratory and the factory. Although in the case of factory data, more error was shown as compared to the laboratory data. The statistics for defining the adequacy of the model was shown by calculating the root mean square error (RMSE).

In the present study, the time course of fermentation was analyzed macroscopically in order to determine the specific growth rates. The logistic equation for growth and growth-linked equation for product formation were

established on the basis of the evidences obtained from analysis of the experimental data. The equation for substrate consumption was based on the macroscopic balance concerning the flow of substrate entering the microbial system [16]. Kinetic parameters were determined by nonlinear regression technique assisted by computer programmes.

2. Materials and methods

2.1 Microorganisms and inoculum

Corynebacterium glutamicum MTCC 2745 (wild type) supplied by the Microbial Type Culture Collection IMTECH Chandigarh, India was used. 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): beef extract, 1; yeast extract, 2; peptone, 5; sodium chloride, 5; agar, 15 . 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 (g/l): glucose, 50; urea, 5; corn steep liquor (CSL), 5 ml/l; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4$ $7H_2O$, 0.4; $FeSO_4$ $7H_2O$, 0.01; $MnSO_4$ H_2O, 0.01; biotin, 5×10^{-6}; thiamin HCl, 8×10^{-5} . Biotin, thiamin-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 ^{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 Production medium (b*atch fermentation*)

The composition of the production medium was same as the seed culture medium. Corn steep liquor was not used in the production medium. 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 conducted in a 2 litre bioreactor (Biostat M, B. Braun, Germany) with a working volume of 1.8 litre. 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 0.2–0.9 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 (0.45 μm, Millipore, USA). Retained biomass was washed twice with double distilled water, and thereafter dried in an oven at 110 ^{0}C for 8 hours [18]. 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 [15] whereas LGA was estimated by copper complex method [22] as also discussed in EICA [21].

3. The kinetic models

The model employs time-dependent rate equations for cell (biomass) growth, product (L-glutamic acid) formation and substrate (glucose) consumption which describe the kinetics of the fermentation process.

3.1 Biomass (Cell) growth

The logistic equation (1) is a substrate independent kinetic model which can define the microbial growth very well in much fermentation [8, 20]. The equation is described as follows:

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

The above equation explains the characteristic sigmoidal curve of biomass growth in the batch culture and quantifies the change in biomass from the beginning of the exponential phase to the end of the maximum cell growth.

3.2 Product formation

Leudeking-Piret kinetic equation consisting of two-parameter kinetic expression has been proved to be useful and versatile in fitting product formation data in fermentation processes [1]. The equation is given below:

$$\frac{dP}{dt} = \alpha.\frac{dX}{dt} + \beta.X \qquad (2)$$

Whether the fermentation kinetics is growth associated or nongrowth associated, is decided by the values of α and β. If $\beta = 0$ and $\alpha \neq 0$, the equation (2) is growth associated. If $\alpha = 0$ and $\beta \neq 0$, it is nongrowth associated [1, 16, 20].

3.3 Substrate uptake

Glucose as a substrate is used to form the cell material, metabolic products and for the maintenance of the cells. The macroscopic balance equation concerning the flow of substrate entering the microbial system was proposed for substrate uptake [16]. The equation is as follows:

$$\frac{dS}{dt} = \frac{1}{Y_{X/S}}.\frac{dX}{dt} + m_S.X \qquad (3)$$

The above equation was used by many workers [7, 14] for substrate uptake in batch fermentation processes.

The system of differential balance equations from (1) to (3) were used for description of the fermentative production of L-glutamic acid in the present study.

4. Results and discussions

4.1 Estimation of model parameters

The optimal values of the parameters of the models are estimated by nonlinear regression technique [2] with the help of computer programmes [23, 24]. 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 [19]. For minimizing the difference between the models generated values and the corresponding experimental data, the criterion of weighted sum of squares of residuals was used [7, 17, 23, 24].

$$SSWR = \sum_{i=1}^{n}\sum_{j=1}^{m}\frac{\Delta_{ij}^{2}}{w_j^{2}} \qquad (4)$$

Where *SSWR* is the sum of squares of weighted 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 model predicted and experimental values of the *j*th variable in the *i*th experimental point.

The method recommended by Bard [2] was used for the evaluation of the degree of reliability of hypothesis concerned with each model pertaining to the growth of cells, product formation and substrate utilisation in L-glutamic acid fermentation with *Corynebacterium glutamicum*. The mean standard deviation ($\bar{\Delta}_j$) of the variable was calculated as follows:

$$\bar{\Delta}_j = \frac{1}{n}\sum_{i=1}^{n}\Delta_{ij} \qquad j = 1, m \qquad (5)$$

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

$$S_j = \frac{1}{n-1}\sum_{i=1}^{n}(\Delta_{ij} - \bar{\Delta}_j)^2; \qquad j = 1, m \qquad (6)$$

The value of the statistics λ defined as

$$\lambda = \frac{(n-m)n}{(n-1)m}\sum_{j=1}^{m}\frac{\bar{\Delta}_j^{2}}{s_j} \qquad (7)$$

was calculated. The statistics 'λ' 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 [27] was also applied to check the error with individual variable during modeling and simulation.

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(Observed - Modelled)^2} \qquad (8)$$

4.2 Modeling and simulation

In order to examine the validity of the logistic model, specific growth rates obtained from the experimental data were plotted against the corresponding cell concentrations and simulated with the model predictions as shown in Fig. 1. Here, the specific growth rate (experimental) decreases almost linearly with the cell concentration and becomes zero at the X_{max} (i.e. 3.88 kg/m^3). The model (Eqn. 1) predicted data shows good agreement with the experimental data. The optimized value of μ_{max} is given in the Table 1. All these establish the adequacy of the logistic equation to be used for representation of growth of *Corynebacterium glutamicum* in L-glutamic acid fermentation.

Table 1 Experimental data and parametric values for dynamic simulation

Experimental data		Parameters for dynamic simulations
Initial (kg/m^3)	Final (kg/m^3)	
X_0 = 0.164, P_0 = 0 S_0 = 49.87	X_{max} = 3.88 P_{max} = 11.53	μ_{max}= 0.21 h^{-1}, m_s = 0.07 kg.kg^{-1}.h^{-1} α = 3.23 kg.kg^{-1}, β = 0 kg.kg^{-1}.h^{-1} $Y_{X/S}$= 0.12 kg.kg^{-1}

The value of β is zero as obtained from Equation (2) leads to the establishment of growth linked (first part of the equation) product formation kinetics. Graphically, it was proved by plotting the rate of product formation against the rate of cell growth as demonstrated in Fig. 2. Here, the linear relation of product formation kinetics to the growth kinetics establishes the validity of growth associated product formation model. Satisfactory agreement of the model predictions with the experimental data (Fig. 2) further strengthens the present finding.

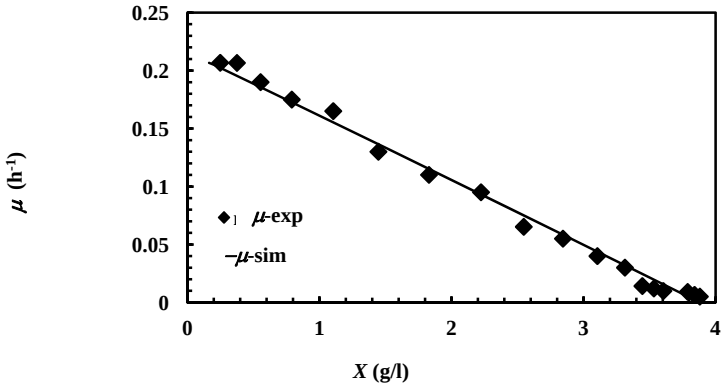

Fig. 1. Variation of the specific growth rate as a function of *X* during fermentation.

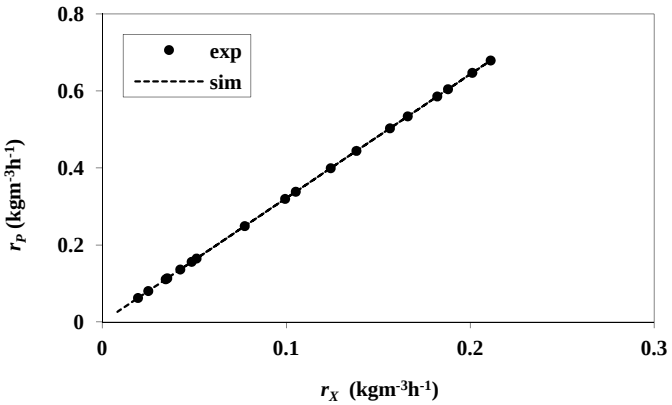

Fig. 2. Graphical dependencies between production rate and growth rate.

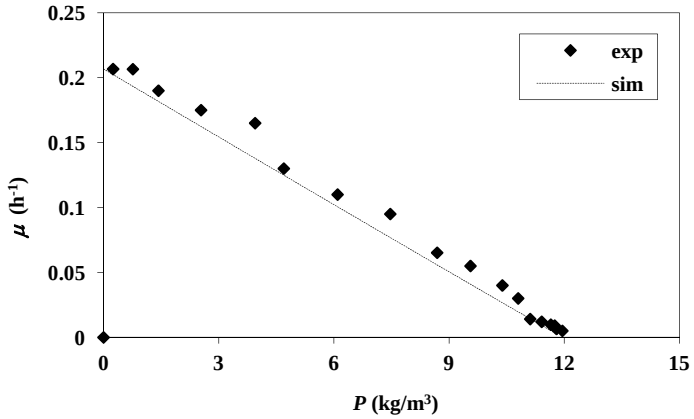

Fig. 3. Graphical dependencies between specific growth rate and product concentration.

The recognition of the first part of equation (2) for product formation in the present study was also supported by the comparison of time course of fermentation data Fig. 3 with the Fig. 5.4.1 [16] and Fig. 6.6 [20] for concentration/time plot of the three basic types of microbial product formation kinetics, i.e. growth associated, nongrowth associated and mixed growth associated.

The effect of product concentration on specific growth rate has been demonstrated by the Fig. 3. With the increasing concentration, specific growth rate decreases. This is an indication of growth inhibition by the product. The specific growth rate seems to be zero at a product concentration of 11.95 kg/m^3. The present findings also strengthen the investigation made by Bona and Moser [5] regarding growth inhibited product formation kinetics in L-glutamic acid fermentation.

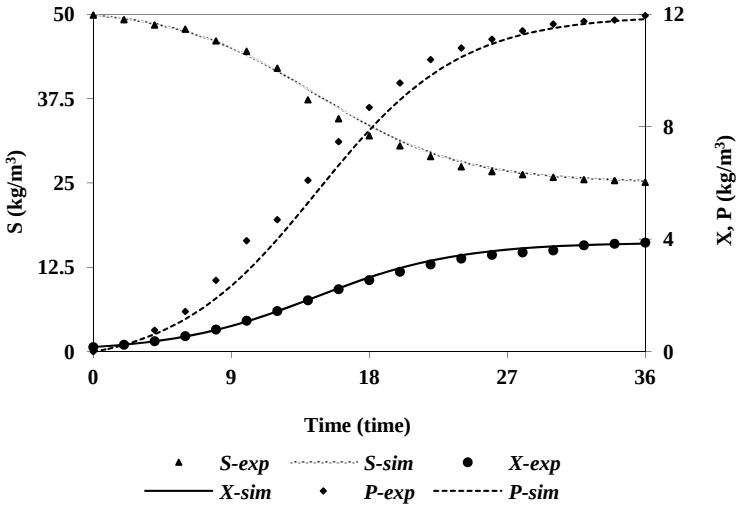

Fig. 4. Time course of original fermentation data and simulation for biomass, product and substrate.

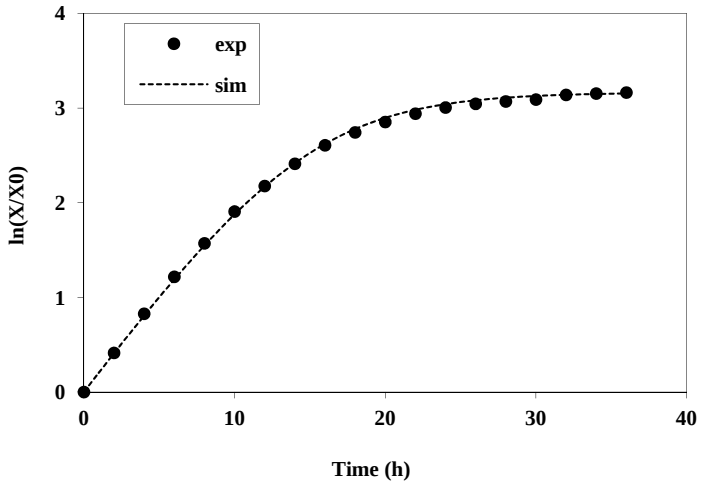

Fig. 5 Time course of the logarithmic growth curve .

Fig. 4 shows the time course of fermentation data and their simulation with the model (Eqns. 1 – 3) predicted values. The values of RMSE individually for *X*, *P* and *S* were found to be 0.06, 0.59 and 0.96, respectively. The graphical patterns are also showing varying degree of agreement with the model for biomass, product and substrate. The statistics 'λ' is 3.22, which is less than the $F_{3,16}$ value (obtained from F-table) for 95% confidence for the entire experimental set. The biomass has the best agreement with the model. Whereas, product formation and substrate consumption exhibited closer correlation.

Fig. 5 shows graphical comparison between experimental and simulated results for time course of logarithmic growth curve. There seems to be a very good agreement between the experimental data and model predictions.

5. Conclusions

The logistic equation can be used to model the growth of cells in L-glutamic acid fermentation. The product formation appears to follow growth associated kinetics. Growth inhibition by the product plausibly occurs in L-glutamnic fermentation. Leudeking-Piret like equation (3) can be applied to represent the consumption of the substrate.

Nomenclature

m_s	Maintenance energy constant, (kg/kg.h)
P	Product concentration, (kg/m^3)
r_s	Specific substrate consumption rate, (kg/m^3.h)
r_p	Specific product formation rate, (kg/m^3.h)
S	Substrate concentration, (kg/m^3)
S_j	Variance of the error of residues
t	Time, (h)
X	Biomass (cell) concentration, (kg/m^3)
$X_{\max}$	Maximum biomass (cell) concentration, (kg/m^3)
$Y_{X/S}$	Biomass yield with respect to substrate, (kg/kg)
$\frac{dP}{dt}$	Product formation rate, (kg/m^3.h)
$\frac{dS}{dt}$	Rate of substrate consumption, (kg/m^3.h)
$\frac{dX}{dt}$	Biomass (cell) growth, (kg/m^3.h)

Greek Symbols

α	Coefficient of proportionality between the rate of product formation and growth rate, (kg/kg)
β	Coefficient of proportionality between the rate of product formation and biomass concentration, (kg/kg.h)
λ	Statistics
$\bar{\Delta}_j$	Standard mean deviation
Δ_{ij}	Difference between the model generated and experimental values standard mean deviation
μ	Specific growth rate, (h^{-1})
μ_{max}	Maximum specific growth rate, (h^{-1})

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