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Kinetics investigation of cell growth, xanthan production and sugar cane molasses consumption by *Xanthomonas campestris*

A. Zakeri[1], M. Pazouki[1 *], M. Vossougi [2]

[1]Environmental Group, Energy Department, Material and Energy Research Center, Meshkin Dasht, Karaj, Iran

[2]Chemical and Petroleum Engineering Department, Sharif University of Technology, Tehran, Iran

Abstract

In this research, unstructured kinetic modeling for cell growth evaluation by X.campestris, xanthan production and sugar cane molasses consumption in a batch culture were investigated. Logistic model for biomass growth, Luedeking-Piret model for xanthan biopolymer production and modified Luedeking-Piret model for sugar cane molasses consumption provides an accurate prediction of the fermentation kinetics parameters with high coefficient of determination R^2 values. Luedeking-Piret model for xanthan biopolymer production in three different concentrations of sugar cane molasses (30, 60 and 90 g/L) as the sole carbon source substrate were studied. Good agreement between experimental and predicted values indicated that the unstructured models were able to describe this fermentation process successfully. The values of specific growth rate μ_{max} of logestic model for sugar cane molasses (30, 60 and 90 g/L) were 0.029, 0.031 and 0.032 1/h respectively. The values of α and β are 5.280, 6.594, 8.518 and 0.072, 0.066, 0.086 respectively, which shows that the xanthan production is growth associated since the value of the growth associated parameter α is much more than the value of nongrowth associated parameter β in Luedeking Piret model. Moreover, the values of γ and η in modified Luedeking-Piret model were obtained.

Keywords: *Xanthan, Logestic Model, Cell Growth, Luedeking-Piret Model, Kinetics*

* Corresponding author: mpazouki@merc.ac.ir

1. Introduction

Xanthan biopolymer is an important anionic hetero-polysaccharide produced by aerobic fermentation by gram negative strains of *Xanthomonas campestris* and is widely used in various industries [1]. Increasing use of xanthan in different industries is due to its unique properties such as high viscosity, excellent resistance to temperature and pH changes and good compatibility with many materials. The molecular weight of xanthan biopolymer is approximately 2 million g/mo1 but it can be more than 13–50 million g/mo1 [2]. The basic structure of this natural polymer consists of 1, 4 linked β-D-glucose residues, having a trisaccharide side chain of β-D-mannose-β-D-glucuronic acid-1, 2-α-D-mannose attached to alternate D-glucose units of the main chain [3]. The anionic specification of this polymer is due to the presence of both glucuronic acid and pyruvic acid groups in the side chain [4]. Typically, *X.campestris* is cultured in Erlenmeyer flasks or batch fermenter. The medium contains a carbohydrate source, a suitable nitrogen source and nutrient salts. In this study three different concentrations of sugar cane molasses (30, 60 and 90 g/L) were used as carbon source which is a co-product of sugar production, and is defined as the output syrup from the final stage of crystallization, from which further crystallization of sugar is uneconomical. Finally, when the fermentation has finished the broth is centrifuged and the xanthan biopolymer is recovered by precipitation with isopropanol. Then the polymer is dried, milled and packaged.

In order to find the optimal conditions for production of expensive biological materials, it is necessary to determine the kinetic models and mechanism of the reaction. Different authors have studied the unstructured and the structured kinetics models of xanthan biopolymer production in batch fermentation [5-8]. Many authors used the unstructured kinetic to model the synthesis of xanthan gum by *X.campestris* species. [9,10]. These unstructured kinetic models would include a balance on the biomass, the xanthan production, and the substrates consumption. In this work kinetic models for xanthan biopolymer synthesis have been implemented including *X.campestris growth*, xanthan formation and substrate consumption in a batch system. Three different concentrations of sugar cane molasses (30, 60 and 90 g/L) as the sole carbon source were used. Logistic model, Luedeking-Piret model and modified Luedeking-Piret model were used for kinetic parameters prediction. Finally, comparison between model prediction data and experimental data was performed.

2. Materials and methods

2-1. Microorganism and culture media

X.campestris IBRC-M 10644 was obtained from Iranian Biological Resource Center**.** Stock cultures of *X.campestris* IBRC-M 10644 were maintained on yeast extract malt agar slants, which contained 20 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 15 g/L agar [11]. The pH of the medium was adjusted to 6.0 and the culture media was sterilized at 121°C for 20 min. After 48 h of growth at 30°C, the culture was stored under sterile conditions at 4°C. Culture was transferred once every 2 weeks

to maintain better survival and stability for xanthan production [12].

2-2. Growth condition

The yeast extract malt broth medium used for preparation of the inoculums contained: 20 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, and 5 g/L peptone. The cells were grown in 100 mL inoculums at 30°C and 200 rpm for 24 h [13]. Fermentation was carried out in 250 mL Erlenmeyer flasks, each of which contained 100 mL of the sterile production medium. The medium was inoculated for 72 h at 30°C and 500 rpm with 5 (v/v%) of the inoculum culture. The medium used for xanthan biopolymer fermentation contained: [Sugar Cane Molasses (30, 60 and 90 g/L), KH_2PO_4 (5g/L), $MgSO_4$.$7H_2O$ (0.2 g/L), Citric Acid (2g/L), H_3BO_3 (0.006g/l), $FeCl_3.6H_2O$ (0.002 g/L), $CaCO_3$ (0.02 g/L), Glutamate (2 g/L)]. The medium was sterilized for 20 min at 121°C and medium initial pH adjusted was set to 7 [14].

2-3. Analytical methods

2-3-1. Determination of cell dry weight

For biomass determination, final fermentation medium was centrifuged in a Funke Gerber super varrio centrifuge at 6000 g for 30 min. The supernatant was used to extract xanthan biopolymer. Sediment cells were dried in an oven at 60°C for 24 h and weighed [15].

2-3-2. Xanthan biopolymer recovery

Xanthan biopolymer was extracted from the culture supernatant. The polysaccharide was precipitated with two volumes of isopropanol solvent in the presence of 1% KCl salt in supernatant. The mixture was kept for 24 h at 4°C to precipitate the xanthan biopolymer. Then, the supernatant was centrifuged at 6000 g for 30 min. Finally precipitate xanthan was dried in an oven at 60°C for 24 h and weighed [16].

2-3-3. Determination of total carbohydrate concentration

The culture supernatant was used for the determination of reducing sugars. The method is based on total reducing sugar by reagent of dinitrosalicylic acid (DNS) as described by Miller in 1959 [17]. Total carbohydrate standard curve was plotted by spectrophotometer at wavelength of 580 nm by using colorimetric method.

2-4. Kinetic study model

Kinetic studies are an important part of the overall research of xanthan biopolymer production. Various unstructured models were proved to be sufficient for characterizing the fermentation kinetics. In biopolymer fermentation, the kinetic model is significantly capable of predicting product formation. An effective unstructured model for xanthan biopolymer fermentation kinetics includes variable parameters of substrate, biomass and xanthan production.

2-4-1. Logistic equation for biomass growth

Biopolymer fermentation process is under special consideration. In specific cases, these fermentation processes do not usually follow the classical kinetic model of substrate-

limited biomass growth and product formation given by Monod in 1949 [18]. Batch fermenters generally contain approximately constant volume of broth and to simulate microbial growth in them the Logistic equation is commonly used [19]. Under optimal growth conditions in a batch fermentation, a simplified growth model of *X.campestris* (X), according to Malthus's Population theory, can be given as [1,3]:

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

Where X_{max} is the maximum attainable *X.campestris* (biomass) concentration (g cell [dry weight] 1/h) and μ_{max} is the maximum specific growth rate (1/h).The integrated form of the above equation using X=X_0*(t=0)* results in the following Logistic model equation which may represent both an exponential and stationary phase:

$$X(t) = \frac{X_0 e^{\mu \max t}}{1 - \frac{X_0}{X_{\max}}(1 - e^{\mu \max t})} \quad (2)$$

Equation 1, 2 was extremely useful in batch fermentation research, because it does not have any time dependent parameter, i.e. substrate concentration. Once the parametersX_0, X_{max} and μ_{max} are given, it simulates growth as a function of time only. This makes data analysis easier by eliminating the necessity of simultaneous solution methods [19].

Equation (2) was linearized as:

$$\mu_{\max} t = \ln\left(\frac{X_{max}}{X_0} - 1\right) + \ln(\frac{\bar{X}}{1-\bar{X}}) \quad (3)$$

Where $\bar{X} = \frac{X}{X_{max}}$ (4)

2-4-2. Luedeking-Piret equation for product formation

The kinetics of xanthan formation was based on Luedeking-Piret equation [18]. This model shows the linear dependency on *X.campestris* concentration and growth rate [20]. According to this model, the product formation rate (r_p) depends on both biomass concentration and the growth rate in a linear manner:

$$r_p = \frac{dP}{\mathrm{dt}} = \alpha \frac{dX}{dt} + \beta X \quad (5)$$

Where α is a constant obtained in product formation during growth of *X.campestris* and β is product formation activity/mass of cells [20] and may differ under different fermentation conditions [18]. To relate the xanthan formation with time, equation (3) is solved (where $P=P_0$ at t=0) by substitution of equations (1) & (2) into equation (3) yields:

$$\mathrm{P(t)} = \mathrm{P0} + \alpha\left(\frac{X_0 e^{\mu_{max} t}}{1-(X_0 - X_{max})(1-e^{\mu_{max} t})} - X_0\right) + \beta \frac{X_{max}}{\mu_{max}} \ln\left(1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max} t})\right) \quad (6)$$

By simplifying equation (4) with equation (2):

$$\mathrm{P(t)} - \mathrm{P0} - \beta \frac{X_{max}}{\mu_{max}} \ln\left(1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max} t})\right) = \alpha\,(\mathrm{x}(t) - X_0) \quad (7)$$

As β is non-growth associated (not dependent on growth phase) parameter, the value of β has to be evaluated with the help of stationary phase data (where $\frac{dX}{dt} = 0$) [3]:

$$\beta = \frac{\frac{dP}{\mathrm{dt}}}{\mathrm{x_{max}}} \quad (8)$$

To determine α, Equation 5 is plotted VS. The $(X(t) - X_0)$. The value of α is obtained by calculating the slope of the line.

2-4-3. Modified Luedeking-Piret equation for substrate consumption

Sugar cane molasses is used as substrate to form cell component and metabolic products for the maintenance of cells [20]. The substrate utilization kinetics is given by the Modified Luedeking-Piret equation, which considers substrate conversion to cell mass, to product and substrate consumption for maintenance [21,22].

$$-\frac{dS}{\mathrm{dt}} = \gamma \frac{dX}{dt} + \eta X \quad (9)$$

Where γ is a constant obtained in substrate consumption during growth of *X.campestris* and η is substrate consumption activity/mass of cells and may differ under different fermentation conditions. To relate the substrate consumption with time, equation (7) is solved (where $S=S_0$ at t=0) by substitution of equations (1) & (2) into equation (7) yields:

$$\mathrm{S(t)} = S_0 + \gamma \left(\frac{X_0 e^{\mu_{max} t}}{1-(X_0 - X_{max})(1-e^{\mu_{max} t})} - X_0\right) + \eta \frac{X_{max}}{\mu_{max}} \ln\left(1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max} t})\right) \quad (10)$$

By simplifying equation (8) with equation (2):

$$\mathrm{S(t)} - S_0 - \eta \frac{X_{max}}{\mu_{max}} \ln\left(1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max} t})\right) = \gamma\,(\mathrm{x}(t) - X_0) \quad (11)$$

Similar to product formation kinetics, nongrowth-associated constant, η, in substrate utilization kinetics is also calculated from stationary phase data [20]:

$$\eta = \frac{-\frac{dS}{\mathrm{dt}}}{\mathrm{x_{max}}} \quad (12)$$

To determine γ, Equation 9 is plotted VS. The $(X(t) - X_0)$. The value of γ is obtained by calculating the slope of the line.

Thus, equations (2), (6) & (10) can be solved simultaneously, using sufficient kinetic parameters to study the behavior of *X.campestris* growth, Xanthan biopolymer formation and Sugar cane molasses consumption, under batch fermentation conditions.

3. Results and discussion

In current research, Mathematical modeling approaches of batch fermentation of *X.campestris* for Xanthan biopolymer production were successfully developed. Also, for three different concentrations of sugar cane molasses as the sole carbon source (30,60 and 90 g/L) at 30°C and 500 rpm, growth kinetic parameters were evaluated from kinetic models. Logistic and Malthus models were used to predict cell growth and Luedeking-Piret and Modified Luedeking-Piret were employed for production formation and substrate consumption respectively.

3-1. Kinetic analyses of microbial growth

The purpose of kinetic analyses of microbial growth was to supply a simple autonomous biomass growth rate expression for batch fermentations. In this part of study, according to equation (3) numerical values for the

maximum specific growth rate μ_{max} and Initial biomass concentration X_0 were determined.

The term $ln(\frac{\bar{X}}{1-\bar{X}})$ was plotted versus time for 3 different substrate concentrations as shown in Fig. 1. The initial specific growth rate was obtained from the slope of this line. Intercept of line with t=0 axis was $-\ln\left(\frac{X_{max}}{X_0}-1\right)$ and Initial biomass concentrations X_0 was obtained from the intercepts (Table 1).

For determination X, equation (2) was solved after substituting these values of X_0, μ_{max} and experimental value of X_{max} (Table 1). For comparison, calculated X values (fit line) and experimental data point of X were plotted against time in Fig. 2. The results show that the calculated values for cell growth and specific growth rate were basically in good agreement with the experimental data and this model is applicable for predicting the experimental results.

3-2. Kinetic analyses of product formation

Various unstructured kinetic models were used to predict the kinetics of Xanthan biopolymer fermentation by *X.campestris*. Luedeking-Piret model for Xanthan biopolymer production provides an accurate approximation of the fermentation kinetics with high R^2 values. According to equation (7) and (8) the value of α Fig. 3 and β Fig. 4 were determined (Table 2).

Comparison between value of α and β shows that the Xanthan production *X.campestris* is growth associated since the value of the growth associated parameter α is much more than the value of nongrowth associated parameter β in Luedeking-Piret model. The product formation models were able to predict the kinetics of Xanthan biopolymer production during the exponential phase of the microorganism accurately.

Comparisons between model predictions and the experimental data for xanthan biopolymer production are given in Fig. 5. It is obvious that this model is very suitable for describing xanthan biopolymer yield.

3-3. Kinetic analyses of substrate consumption

In Xanthan biopolymer fermentation, increasing the concentration of biomass was accompanied by a decrease in sugar cane molasses concentration. The consumption of sugar cane molasses was to supply cell growth, cell maintenance, and product formation. Modified Luedeking-Piret model for sugar cane molasses consumption provides an accurate approximation of the fermentation kinetics with high R^2 values. According to equation (11) and (12) the value of γ Fig. 6 and η Fig. 7 were determined (Table 3).

Comparison between value of γ and η shows that the sugar cane molasses consumption is growth associated since the value of the growth associated parameter γ is much more than the value of nongrowth associated parameter η in modified Luedeking-Piret model. By increasing concentration of carbon source the amount of γ and η were increased. The rate of sugar cane molasses consumption was directly dependent on the cells concentration in the stationary phase. These models were able to predict the kinetics of substrate consumption

during the exponential phase of the microorganism accurately.

Comparisons between model predictions and the experimental data for sugar cane molasses consumption are given in Fig. 8. It is clear that this model is very suitable for describing substrate consumption.

Mathematical analysis of any fermentation data can convincingly explain the kinetics of biomass, substrate and product with unstructured models. In general, these models give us good approximation of parameter profiles even though the complete mechanism of microbial growth is not considered. Table 4 had shown a comparison of kinetic constant parameters under optimized conditions from modeling of Xanthan biopolymer and the literature on various carbon substrates using unstructured models [6,22,23].

Based on the model and literature kinetic parameters, xanthan biopolymer production and carbon substrate consumption is a close growth associated process and good agreement between experimental and predicted values indicated that the unstructured models were able to describe this fermentation process successfully.

4. Conclusions

In this study, unstructured modeling of batch fermentation of *X.campestris* for Xanthan gum production were studied. These used kinetic models of *X.campestris* growth, Xanthan gum production and sugar cane molasses consumption in a batch fermentation were successfully proven to be in good agreement with the experimental results. According to the reported results logistic model, Leudeking-Piret model and modified Leudeking-Piret model clearly represented the *X.campestris* growth, the xanthan gum production and the sugar cane molasses utilization respectively, with good R^2. The product formation models were able to predict the kinetics of xanthan biopolymer production during the exponential phase of the microorganism accurately. Based on the model, a higher level of xanthan biopolymer production was obtained in batch fermentation by increasing the specific growth rate. Also, xanthan biopolymer production and sugar cane molasses consumption is a close growth associated process.

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