Kinetics of Hyaluronic Acid Production by *Streptococcus Zooepidemicus*

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ABSTRACT

Kinetic studies of hyaluronic acid (HA) production by Streptococcus zooepidemicus were performed in an aerobic fermentation. An unstructured model taking into accounts the cell growth and HA production is proposed. It was found that the logistic model incorporated by Leudeking-Piret model described fairly well the experimental observations compared to Monod model. The model however is influence by the glucose limitation and by-product inhibition.

Keywords: *Hyaluronic acid, growth kinetics, kinetic studies, Streptococcus zooepidemicus*

Introduction

Hyaluronic acid (HA), a linear, unbranched polysaccharide consisting alternating N-acetyl-D-glucosamine and D-glucuronic acid, is a valuable biopolymer in the medicinal and cosmetic market (Radaeva et al., 1997; Lapeik et al., 1998; Fong Chong et al., 2005). Traditionally, it has been extracted from rooster combs and bovine vitreous humor (Reagan et al., 1994). However, due to limited tissue sources, risks of viral infection and high cost (Reagan et al., 1994; Crescenzi, 1995; Lerner, 1996), a new method have been developed to produce HA. Recently, HA from microbial sources through fermentation processes have received increasing attention especially using gram-positive bacterium *Streptococcus zooepidemicus* (Goh, 1998). This bacterial process presents the opportunity to optimize the product yield and quality through genetic engineering and control of culture conditions (Van Brunt, 1986).

A number of kinetic models have been proposed for different phases of fermentation. The kinetic of microbial growth, substrate uptake and product formation in a fermentation process can be described by using mathematic models (Guardiola et al., 1994). The logistic model and Luedeking-Piret equations have been widely used for cell growth and formation of desired metabolic products (Murat & Ferda, 1999; Jian et al., 2002). The structured models took into account some basic aspect of the cell structure, function and composition; while the unstructured models involve only the cell mass in order to describe the biological system (Gong, 1996).

Various structured and unstructured kinetics models have been reported for production of lactic acid either by bacteria or fungi. Unstructured models are much easier to use, and have proven to accurately describe lactic acid fermentation in a wide range of experimental conditions and media. Various kinetics models available in the literature have been explored in a search of the model which can give the best fit to the experimental data (Gu et al., 2006). In this study, an unstructured model for cell growth, product formation and glucose consumption was developed using batch production of HA by *Streptococcus zooepidemicus* ATCC 39920 in a shake flask culture.

Methods and Materials

Microorganism

The bacteria, *Streptococcus* equi subsp. *zooepidemicus* ATCC 39920 was obtained from American Type Culture Collection (Rockville, Md) as a freeze-dried culture in ampoules. The strains were maintained by weekly transfer on sheep blood agar (SBA) and stored at 4°C after

incubated at 37°C for 24 hr. Monthly subculture ensured the availability of sufficient stock cultures for experimental processes.

Production Medium

The composition of the medium used comprised of (gL^{-1}): glucose 30, yeast extract 10, KH_2PO_4 0.5, $Na_2HPO_4.12\ H_2O$ 1.5 and $MgSO_4.7H_2O$ 0.5 respectively. The medium was prepared and autoclaved at 121°C for 20 min. Glucose solution was autoclaved separately to avoid caramelisation, and mixed aseptically with other components on cooling.

Cell Suspension Preparation

Cell suspension for the inoculum was prepared by inoculating a stock culture of *Streptococcus zooepidemicus* onto SBA-plates and incubated overnight at 37°C. The colonies that were formed was punched by a sterile cork borer to obtain a round disk of 0.85cm in diameter. The disks (5) were then put into a sampling bottle containing 50ml of sterile distilled water. The sampling bottle was then vortexed for 3min so that the cells will be evenly distributed in the liquid.

Inoculum Preparation

Seed culture or inoculum was prepared by inoculating 15ml of cell suspension into a 500 ml Erlenmayer flask containing 135 ml of the fermentation medium. The flask was then incubated in a rotary shaker at 37°C for 2 h at a speed of 250 rpm. The inoculum was standardized by measuring the absorbance (optical density) at 600 nm using a spectrophotometer. 150 ml of inoculum with optical density within 0.5-0.9 was used to inoculate the fermentation medium.

Analytical Procedure

Samples were withdrawn at regular time interval and analyzed for cell, glucose, product and by-product concentration. Cell concentration was determined by measuring the optical density (OD) at 600_{nm} by a spectrophotometer and dry cell method. A correlation between cell dry weight and OD_{600} was established. Glucose concentration was measured using High Performance Liquid Chromatography (HPLC) (Model: Shidmadsu, Japan) equipped with Gel Permeation Chromatography (GPC) column and a guard column and monitored by a differential refractometer detector (RID). HA was determined using the method as described by Mashitah et al., (2002). The concentration of H_2O_2 produced was analyzed by spectrophotometeric method as suggested by Emiliani and Riera (1968) with slight modification.

Results and Discussion

Model Development

The kinetic model for batch fermentation was developed based on the report by Gu et al.,(2006). Kinetic model can divided into a growth model, a substrate model and a product model. Three different equations derived to describe the kinetics behavior of hyaluronic acid production by *Streptococcus zooepidemicus* will be analyzed in this study.

Microbial Growth

The most widely used unstructured models to described cell growth are the Monod and logistic equation. The logistic equation is substrate independent model which account for the inhibition growth, which occur in many batch processes. Logistic equations are a set of equations that characterizes the growth in terms of carrying capacity (Gu et al.,2006). It can be described as:

$$\frac{dX}{dt} = \mu_m X\left(1-\frac{X}{X_m}\right) \quad (1)$$

where, X = biomass concentration
T = fermentation time
μ_m = the maximum specific growth rate (h^{-1})
X_m = maximum biomass concentration (g dry $weight^{-1}$)

Integrating the equation with the initial condition, $X=X_0$ at $t=0$:

$$\mu_m t = \ln\left(\frac{X_m}{X_0}-1\right)+\ln\left(\frac{X}{X_m}-X\right) \quad (2)$$

or

$$X(t)=\frac{X_0 e^{\mu_m t}}{\left[1-\frac{X_m}{X_0}\left(1-e^{\mu_m t}\right)\right]} \quad (3)$$

From equation (2), it can be seen that by plotting $\ln\left(\frac{X}{X_m}-X\right)$ versus times, t, a straight line is obtained, with the slope of line equals to μ_m and intercept equals $-\ln\left(\frac{X_m}{X_0}-1\right)$ (shown in Figure (1)). By substituting the values of X_0, X_m and μ_m into equation (3) will give a model equation for the growth of *Streptococcus zooepidemicus* cell.

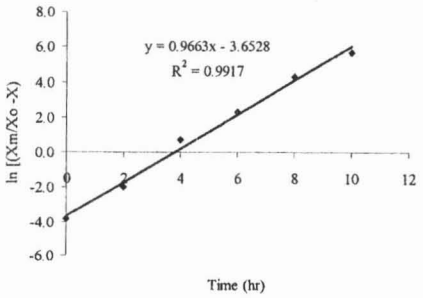

Figure 1: Evaluation μ_m

As shown in Figure 2, HA formation by *Streptococcus zooepidemicus* cell showed a classical growth trend. After a lag phase (about 2 hr), the cell enter an exponential growth phase, whereby cell growth and HA production occurs simultaneously. By substituting X_m = 0.58 g/L into equation 2, the values of X_o and μ_m are 0.0146g/L and 0.9663 h^{-1}, respectively. Results showed that the experimental data for biomass concentration fitted very well with those predicted by the logistic model.

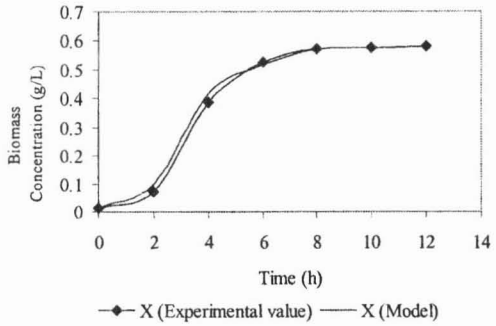

Figure 2: Experimental Data and Kinetic Model Prediction for Biomass Concentration as a Function of Time using Logistic Model.

Product Formation

Many models have been proposed for the production of various useful metabolites (Kono & Asai, 1969). The model for product formations that are often used is based on Gu et al., (2006). This model was originally developed for the formation of lactic acid by *Lactobacillus delbruckii.* The model is:

$$\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right)X + \beta X \tag{4}$$

where,

α = growth associated rate constant for product formation
β = non growth associated rate constant for product formation

The α and β values are empirical constant that vary with fermentation conditions (Temperature, pH etc) as well as with microbial strains. The production formation with time can be estimated by solving the Leudeking-Piret kinetics with the growth rate equations of logistic equation (Equation 1).

$$\frac{dP}{dt} = \alpha\mu_m\left(1-\frac{X}{X_m}\right) + \beta X \tag{5}$$

Integrating,

$$P(t) = P_0 + \alpha[X(t) - X_0] + \beta\frac{X_m}{\mu_m}\ln\left[\left(1-\frac{X_0}{X_m}\right)\left(1-e^{\mu t}\right)\right] \tag{6}$$

Simplify equation (6) gives

$$P(t) = P_0 + \alpha A(t) + \beta B(t) \tag{7}$$

Where $A(t) = X(t) - X_0$, $B(t) = (X_m/\mu_m)\ln\{1-(X_0/X_m)(1-\exp[\mu_0 t])\}$. By plotting $\frac{P(t)-P_0}{B(t)}$ versus $\frac{A(t)}{B(t)}$, a straight line will be obtained with the slope of the line equals to α and the intercepts equals to β. If $\alpha \neq 0$ and $\beta = 0$, the product formation is growth associated.

There had been only several models in hyaluronic acid production reported in the literature. Cooney et al., (1999) used a structured, two compartment model for HA fermentation under anaerobic and aerobic condition. Richard and Margaritis (2004) proposed empirical model and Huang et al., (2007) proposed delayed growth associated model for HA production.

In this study, the experimental data and those predicted by the kinetic model for HA production is shown in Figure 3. A large deviation between the experimental and predicted values might be due either to substrate, product or by-product inhibition. Huang et al., (2007) reported that HA formation is associated to delayed growth. Previously Mashitah et al., (2005) stated that H_2O_2 (a by-product) produced by *Streptococcus zooepidemicus* cell did not effect the cell growth but influenced HA production. Accordingly, H_2O_2 production took place during growth phase and HA production started after the growth had reached late exponential phase, that is, when H_2O_2 in the culture media was depleted. This is inline with the result obtained in this study (Figure 3 and 4), which showed that HA production is higher at lower H_2O_2 concentration in the media.

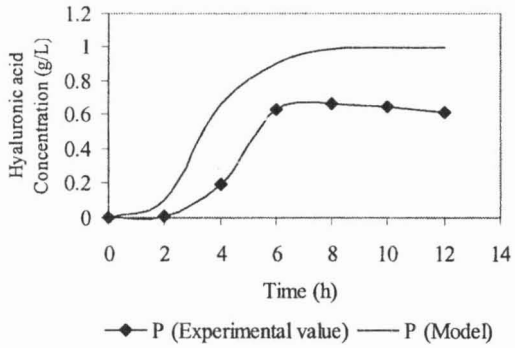

Figure 3: Comparisons of the Calculated values and Experimental Data of Hyaluronic Acid Production at for 30g/L of Initial Glucose Concentration.

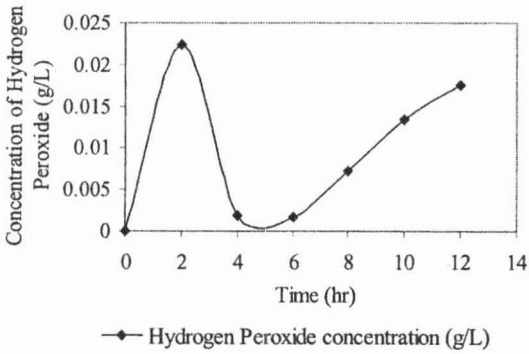

Figure 4: Accumulation of Hydrogen Peroxide in Batch Culture (Initial Glucose Concentration, $30gL^{-1}$) by *Streptococcus Zooepidemicus*.

Glucose Uptake

The substrate consumption model is applied from the Leudeking-Piret-like equation:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + K_e X \qquad (8)$$

where, $Y_{X/S}$ = yield of biomass (g)
$Y_{P/S}$ = yield of HA production (g)
S = concentration of substrate
K_e = maintenance constant

Substituting equation (4) into equation (8) gives,

$$-\frac{dS}{dt}=\left(\frac{\beta}{Y_{P/S}}+K_e\right)X+\left(\frac{1}{Y_{X/S}}+\frac{\alpha}{Y_{P/S}}\right)+\frac{dX}{dt} \tag{9}$$

or

$$\frac{dS}{dt}=-b_1X-b_2\frac{dX}{dt} \tag{10}$$

With $b_1 = \beta/Y_{P/S}+K_e$ and $b_2 = 1/Y_{X/S}+\alpha/Y_{P/S}$.

Integrating equation (10) will give

$$S(t)=S_0-b_2A(t)-b_1B(t) \tag{11}$$

By plotting $\frac{S_0-S}{B(t)}$ versus $\frac{A(t)}{B(t)}$, a straight line with slope equals to b_2 and intercepts equals to b_1 can be obtained (Figure 5). By substituting all the constants that obtained inside equation (11) will give a model equation for the substrate (glucose) consumption by *Streptococcus zooepidemicus*.

From Figure (6), it is found that the experimental data for glucose consumption fitted well with the predicted values with R^2 =0.9986. The result showed that the substrate concentration decreased gradually throughout the fermentation process.

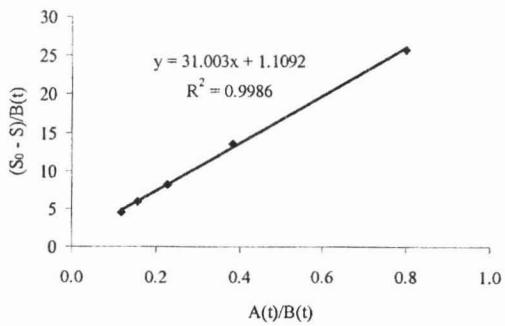

Figure 5: A Graph [(S0-S)/B(t)] versus A(t)/B(t) (b_2 is Détermined)

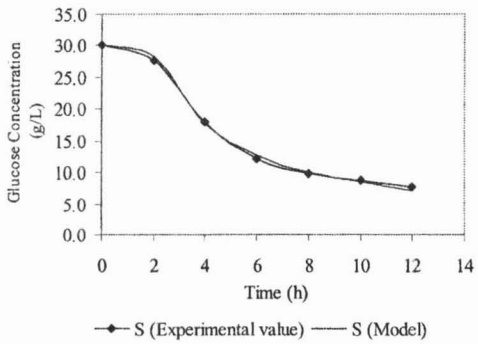

Figure 6: Comparisons of the Calculated values and Experimental Data of Glucose Consumption.

Conclusion

Logistic model fitted well with cell growth. For glucose consumption Leudeking-Piret-like equation is in agreement with experimental data. Leudeking-Piret equation however gave a negative impact to HA product. This might be only is due to by-product (H_2O_2) inhibition or presence of other components in the culture media.

Nomenclature

X = biomass concentration (g/L)
T = fermentation time (h)
μ_m = the maximum specific growth rate (h^{-1})
X_m = maximum biomass concentration ($g\ L^{-1}$)
α = growth associated rate constant for product formation gg^{-1}
β = non growth associated rate constant for product formation $gg^{-1}h^{-1}$
$Y_{X/S}$ = yield of biomass (g cell/g substrate)
$Y_{P/S}$ = yield of HA production (g product/ g substrate)
S = concentration of substrate
K_e = maintenance constant

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