JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Studies on Cultivation Kinetics for Elastase Production by *Bacillus* sp. EL31410

QI-HE CHEN,† GUO-QING HE,*,† AND PAUL SCHWARZ‡

Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China, and Department of Cereal Science, North Dakota State University, North Dakota 58105

It was the first time to study elastase batch cultivation kinetics. This paper discusses the growth kinetics, elastase production, and substrate consumption kinetics model of *Bacillus* sp. EL31410 in batch cultivation. A simple model was proposed using a logistic equation for growth, the Luedeking−Piret equation for elastase production, and the Luedeking−Piret-like equation for glucose consumption. The model appeared to provide a reasonable description for each parameter during the growth phase. This study could provide some support for studying elastase fermentation kinetics, especially for studying its singular growth phenomenon. However, the model for elastase production is not good for explaining the real process and is still up to research.

KEYWORDS: **Elastase; *Bacillus* sp. EL31410; batch cultivation; kinetic model; logistic equation; Luedeking−Piret equation**

INTRODUCTION

An enzyme that attacks and solubilizes elastin is called elastase. The elastase can degrade elastin (*1*), which other proteases cannot degrade. Consequently, it has broad applications in medical therapy, food processing, and daily chemical industry. In addition, elastase can also be applied to bioconversion and other applications.

Microbial elastase has been isolated from species of *Pseudomonas aeruginosa* (*2*), *Staphylococcus epidermidis* (*3*), *Flavobacterium* sp. (*4*), *Alkalophilis B.* strain Ya-B (*5*), and so on. Among these, only small part of strains had potential to industrial application. Up to date, it was reported that most *Bacillus* sp. strains isolated could be applied to use in large-scale cultivation because of its high yield and security. Previously, we have isolated a *Bacillus* sp. with high elastase production yield and engineered by using physical−chemical methods and employed the method of response surface design to improve medium compositions for elastase production by *Bacillus* sp. EL31410 in shake-flask experiments.

The development of a kinetic model for scale-up and bioreactor design is necessary for this important bioprocess. Nevertheless, no references exist in the literature on a kinetic model able to describe elastase production. The building of kinetic models consists of comparing assumed models with experimental data in order to obtain more relevant equations. Kinetic models enable the bioengineer to design and control the microbial process (*6*). In predicting the behavior of the process, mathematical models, together with carefully designed experiments, make it possible to evaluate the behavior of systems more rapidly than with laboratory experiments only. A mathematical model for a microbial process can be expressed using two different mechanisms: structured and unstructured models (*7*). Structured models take into account some basic aspects of cell structure, function, and composition. In unstructured models, however, only cell biomass is employed to describe the biological system. In this study, an unstructured model for cell growth, product formation, and glucose consumption was found to be convenient to characterize the fermentation process.

A knowledge and understanding of the kinetics of production of elastase are of great economic importance in view of the fact that elastase fermentation is a major industrial operation. Industrial fermentation is gradually moving away from the traditional and largely empirical approach toward a simpler and better-controlled process. The rational design and optimization of the latter required a quantitative understanding of production kinetics. However, the kinetics of elastase production is imperfectly understood due to the complex nature and little work has been done in order to understand the process. This report is the first to study the batch cultivation kinetic model in a 5 L fermentor. Also, the experimental data from batch fermentations of elastase (using defined medium) were examined in order to form the basis of a kinetic model of the process.

Kinetic Model. The model employs rate equations for biomass (x), elastase (P), and glucose (S) to describe the fermentation process.

Microbial Growth. The examination of the large number of experimental data showed that the Monod kinetic model was not applicable to this particular fermentation system. Therefore, the logistic equation that is a substrate-independent method was used as an alternative empirical function. In many fermentation

* To whom correspondence should be addressed. Fax: 086-571-86971166. E-mail: gqhe@zju.edu.cn.
† Zhejiang University.
‡ North Dakota State University.

10.1021/jf0303161 CCC: $27.50 © 2004 American Chemical Society
Published on Web 05/04/2004

systems such as polysaccharide fermentations by many types of microorganisms (*8*), cell growth has been characterized by the logistic equation. The logistic equation can be described as follows:

$$\frac{dx}{dt} = \mu_m\left(1 - \frac{x}{x_m}\right)x \quad (1)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) and x_m is the maximum attainable biomass concentration (g dry cell weight L^{-1}). The integrated form of eq 1 using $x = x_0$ ($t = 0$) gives a sigmoid variation of x as a function of t, which may represent both an exponential and a stationary phase (eq 2).

$$x = \frac{x_0 e^{\mu_m t}}{\{1 - (x_0/x_m)(1 - e^{\mu_m t})\}} \quad (2)$$

Rearrangement of eq 2 yields eq 3

$$\ln\frac{x}{(x_m - x)} = \mu_m t - \ln\left(\frac{x_m}{x_0} - 1\right) \quad (3)$$

Product Formation. The kinetics of product formation was based on the Luedeking−Piret equations. According to this model, the product formation rate depends on both the instantaneous biomass concentration, x, and the growth rate, in a linear manner.

$$r_p = \frac{dP}{dt} = \alpha\frac{dx}{dt} + \beta x \quad (4)$$

where α and β are the product formation constants, which may vary with fermentation conditions. The benefit of this model is that β can be found from stationary phase data. At stationary phase ($dx/dt = 0$) and ($x = x_m$), therefore, β can be obtained using the following equation:

$$\beta = \frac{(dP/dt)st}{x_m} \quad (5)$$

To express as a function of time, eq 4 is rearranged.

$$dP = \alpha\, dx + \beta\int(t)\, dt \quad (6)$$

To simplify the equation, we used the following model to express the product formation in which

$$A(t) = x_0\left\{\frac{e^{\mu_m t}}{[1 - (x_0/x_m)(1 - e^{\mu_m t})]} - 1\right\}$$

$$B(t) = \frac{x_m}{\mu_m}\ln\left[1 - \frac{x_0}{x_m}(1 - e^{\mu_m t})\right] \quad (7)$$

$$P = \alpha A(t) + \beta B(t) \quad (8)$$

Plotting $[P - \beta B(t)]$ against $A(t)$ gives the growth-associated product formation constant, α.

Glucose Uptake. A carbon substrate such as glucose is used to form cell material and metabolic products as well as the maintenance of cells. The glucose consumption equation given below is a Luedeking−Piret-like equation in which the amount of carbon substrate used for product formation is assumed to be negligible.

$$-\frac{dS}{dt} = \gamma\frac{dx}{dt} + \delta x \quad (9)$$

where $\gamma = 1/Y_{x/s}$ and $\delta = m_s$. These two parameters can be evaluated by the same method as stated in the product formation kinetics.

At stationary phase, $dx/dt = 0$ and $x = x_m$. Therefore, δ can be obtained using the following equation:

$$\delta = \frac{[-(dS/dt)]_{st}}{x_m} \quad (10)$$

To evaluate γ, eq 9 is rearranged.

$$-dS = \gamma\, dx + \delta\int(t)\, dt \quad (11)$$

The change of this equation and integrating yield the following equation:

$$S = S_0 - \gamma C(t) - \delta D(t) \quad (12)$$

where $S = S_0$ at $t = 0$.

$$C(t) = x_0\left\{\frac{e^{\mu_m t}}{[1 - (x_0/x_m)(1 - e^{\mu_m t})]} - 1\right\}$$

$$D(t) = \frac{x_m}{\mu_m}\ln\left[1 - \frac{x_0}{x_m}(1 - e^{\mu_m t})\right] \quad (13)$$

Plotting $S_0 - S - \delta D(t)$ vs $C(t)$ will gives a line of slope γ.

MATERIALS AND METHODS

Fermentation. The microorganism used in this study was *Bacillus* sp. EL31410 that was maintained at 4 °C. The defined medium was based on the described medium (*9*). It contained (g/L): 74, glucose; 11.3, casein; 6.16, corn steep flour; 2.06, K_2HPO_4; and 0.34, $MgSO_4 \cdot 7H_2O$ according to the previous research. Each flask (250 mL) containing 25 mL of fermentation medium was inoculated with 5% (v/v) seed culture. The 18 h shake-flask culture was used to inoculate a 5 L bioreactor (with 3.5 L working volume) at 5% (v) level. The monitoring and/or control of various parameters, such as temperature, pH, and dissolved oxygen concentration, were performed in a PID control unit. The bioreactor was operated at 37 °C with aeration of 1.5 L/min, and agitation was changed according to experiment. The pH of the medium was initially adjusted to 7.5 and then allowed to follow its natural course. Adding antifoam agent achieved foam control.

Analyses. The cell growth was measured as the dry cell weight. The culture sample (10 mL) was centrifuged at 400*g* for 10 min, and the cell pellet was washed twice with distilled water, dried to constant weight at 80 °C, and then weighed. The glucose concentration in the medium was measured using the DNS method (*10*). Elastase activity was determined as described by Sachar (*11*). The volumetric oxygen uptake rate was determined using the dynamic gassing-out method.

RESULTS AND DISCUSSION

Microbial Growth. The most popular kinetic expression for microbial growth is the Monod equation. However, the examination of the large number of experimental data (not shown) obtained from batch fermentations showed that this model is not suitable for this particular system. Therefore, the logistic rate equation was chosen as an alternative empirical equation.

Elastase fermentation by *Bacillus* sp. EL31410 in batch cultures using the optimized medium showed a classical growth

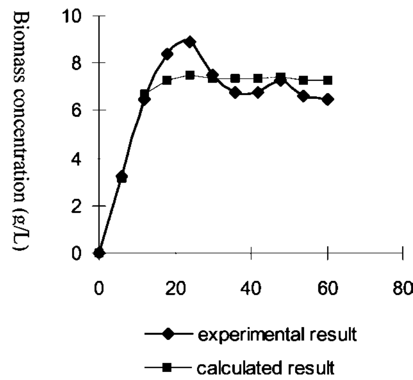

Figure 1. Comparison of eq 2 with the experimental biomass concentrations.

trend. After a short lag phase, the cells entered the exponential growth phase. The production of elastase started when the cells entered the exponential phase. The elastase production is very low at the lag phase. When the cell reached the maximum, the elastase production did not enter the maximal point. This means that elastase production did not overly associate with cell growth. Furthermore, the cell appeared the secondary metabolism at 42 h culture time. The cell growth begins to increase from 42 to 48 h culture time. This phenomenon had been studied in a previous study. As a result, the growth of *Bacillus* sp. EL31410 did not follow the typical pattern. In this case, the logistic equation was used to express the cell growth model, taking x_{max} = 9 dry weight (g/L) from the preliminary study and using the DPS software to analyze the model parameters. The analyzing results are as follows: $x_0 = 0.372$ (g/L), $\mu_m = 0.443$ (h^{-1}). The calculated value of x_0 lowers that of the experimental value. This can perhaps be attributed to the viability of the cells. The model parameter preciseness was checked by a *F*-test, which shows that the maximum cell growth concentration is very significant. The chosen model significance was also checked by Fisher's test. The checking result demonstrated that this model is very applicable to predict the experimental results ($p = 0.009906 < 0.01$). The chosen model also can be depicted in **Figure 1**.

Product Formation. Product formation parameters were obtained from eq 8 and the form of the stationary phase where cell growth rate (dx/dt) is zero. The two parameters, α and β, plus x_0, x_m, and μ_m, were used to calculate the product formation rate. The values of α and β were 38 150 U elastase activity (g cells)$^{-1}$ and −48 U elastase activity (g cells h)$^{-1}$, respectively. A comparison of calculated function $P(t)$ with the experimental data is given in **Figure 2**. The comparison of the calculated data with the experimental results is not in good agreement. This may be explained that this enzyme exits by another metabolic way. This model is suitable for predicting the experimental data before 18 h culture time.

Substrate Uptake. In elastase fermentation, the increase in biomass concentration was accompanied by a decrease in residual glucose concentration. The glucose consumption was used to supply the cell growth and cell maintenance, and litter was used to supply for product formation.

Glucose consumption may be represented with eq 12. The initial glucose was $S_0 = 6.61$ g 100 mL^{-1}. The parameters of this model were optimized using DPS soft, and the results were given in the following **Table 1**. A comparison of the experimental data with calculated function $S(t)$ is then made using these two parameters (see **Figure 3**). From the figure, it is obvious that this model is very suitable for describing glucose

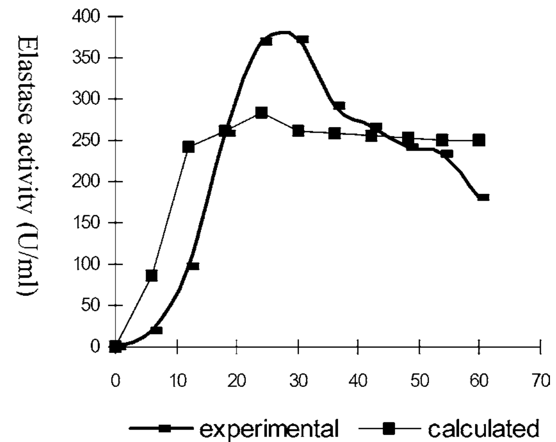

Figure 2. Comparison of calculated product formation with the experimental results.

Table 1. Parameter Values Used for Testing the Model

parameter	value	unit
X_0	0.37	g/L
x_m	9	g/L
μ_m	0.44	h^{-1}
α	38150	U elastase activity (g cells)$^{-1}$
β	−48	U elastase activity (g cells h)$^{-1}$
γ	3.01	g glucose (g cells)$^{-1}$
δ	0.06	g glucose (g cells h)$^{-1}$

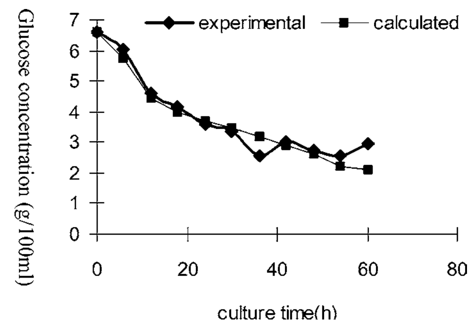

Figure 3. Comparison of calculated substrate consumption with the experimental results.

consumption. The parameters provided by the model were as follows: $\gamma = 3.01$ g glucose (g cells)$^{-1}$, $\delta = 0.06$ g glucose (g cells h)$^{-1}$.

All of the parameters were listed in the **Table 1**. The predicted models for cell growth, substrate consumption, and elastase production all were given in the following eq 14.

$$X = \frac{9 \times e^{0.44t-3.15}}{1 + e^{0.44t-3.15}}$$

$$P = 14\,120 \times \left(\frac{e^{0.44t}}{1 - e^{0.44t}} - 1\right) - 1020 \times \ln(0.96 + 0.04e^{0.44t})$$

$$S = S_0 - 1.11 \times \left(\frac{e^{0.44t}}{1 - e^{0.44t}} - 1\right) - 1.20 \times \ln(0.96 + 0.04e^{0.44t}) \quad (14)$$

Testing the Model. The microbial growth, product formation, and substrate models were tested using the parameters evaluated above. A summary of these parameters is listed in **Table 1**.

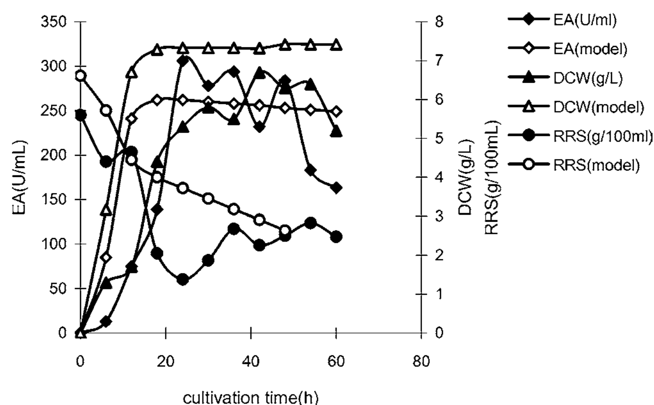

Figure 4. Comparison of calculated values and the experimental data for another experiment performed in a batch system.

A comparison of the calculated functions of $x(t)$, $P(t)$, and $S(t)$ is given in **Figure 4**. The results showed that the substrate consumption model is very suitable, and elastase production only can be used before the exponential phase.

In conclusion, fermentation is a very complex process, and it is often very difficult to obtain a complete picture of what is actually going on in a particular fermentation. The model presented in this work provides a good description of biomass and substrate concentrations vs batch fermentation time. However, the model of product formation is not well suitable. Reasonable but occasionally less satisfactory correspondence, particularly for product formation, between the model and experimental data is found. This could perhaps be due to the complex of elastase biosynthesis and also to environmental conditions that change during cultivation. At the same time, this strain in the defined culture medium had some different growth properties, such as the second growth characterization and high dissolved oxygen requirement. Currently, it is difficult to fully describe its growth model. All of these cause the product formation model to be very unstable. If the elastase biosynthesis mechanism and its metabolic pathway were elucidated, it is possible to precisely express its model.

One of the important limitations of this model is the maximum biomass concentration since it was found experimentally. Therefore, to predict the parameter values of the model, an accurate value of maximum biomass is required. With the model proposed here, all model parameters may be evaluated from a single batch of data using simple graphical methods requiring neither estimation techniques nor simultaneous solution approaches. Up to the limitations of these models appeared, much more work has to be designed and carried out.

NOMENCLATURE

Greek letters: α, growth-associated product formation coefficient (mg/g); β, nongrowth-associated product formation coefficient (mg/g h); γ,δ, parameters in Luedeking−Piret-like equation for substrate uptake [g S(g cell)$^{-1}$, g S(g cell)$^{-1}$ h^{-1}, respectively]; μ, specific growth rate (h^{-1}); μ_{max}, maximum specific growth rate (h^{-1}).

LITERATURE CITED

(1) Morihara, K. Elastolytic properties of various proteases from microbial origin. *Arch. Biochem. Biophys.* **1967**, *120*, 68−78.
(2) Tsuzuki, H.; Oka, T. *Pseudomonas aeruginosa* elastase: isolation, crystallization and preliminary characterization. *J. Biol. Chem.* **1965**, *8*, 3295−3303.
(3) Teufel, P.; Gotz, F. Characterization of an extracellular metalloprotease with elastase activity from *Staphylococcus epidermidis*. *J. Bacteriol.* **1993**, *13*, 4218−4224.
(4) Ozaki, H.; Shiio, I. Purification and properties of elastolytic enzyme from *Flavobacterium immotum. J. Biochem.* **1975**, *77*, 171−180.
(5) Tsai, Y.-CH.; Jung, R.-Y.; Lin, S.-F.. Production and further characterization of an alkaline elastase production by alkalophilic *Bacillus* strain YaB. *Appl. Environ. Microbiol.* **1988**, *1*, 3156−3161.
(6) Moser, A.; Steiner, W. The influence of the term k_d for endogenous metabolism on the evaluation of Monod kinetics for biotechnological processes. *Eur. J. Appl. Microbiol.* **1975**, *1*, 281−289.
(7) Sinclair, C. G.; Kristiansen, B. *Fermentation Kinetics and Modeling*; Open University Press: Milton Keynes, London, 1987; p 156.
(8) Malchandani, A.; Luong, J. H. T.; Leduy, A. Batch kinetics of microbial polysaccharide biosynthesis. *Biotechnol. Bioeng.* **1998**, *32*, 639−646.
(9) Chen, Q. H.; He, G. Q. Optimization of medium composition for the production of Elastase by*Bacillus* sp. EL31410 with Response Surface Methodology. *Enzyme Microbiol. Technol.* **2002**, *5*, 667−672.
(10) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426−427.
(11) Sachar, L. A. Photometry method for estimation of elastase activity. *Proc. Soc. Exp. Biol. Med.* **1955**, *90*, 323−325.

Received for review April 28, 2003. Revised manuscript received February 3, 2004. Accepted February 26, 2004.

JF0303161