

Kinetic Modeling of Cell Growth and Product Formation in Submerged Culture of Recombinant *Aspergillus niger*

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*The kinetics of cell growth and protein production for the recombinant *Aspergillus niger* strain AB4.1[pgpdAGLAGFP]#11 were investigated. An unstructured kinetic model was developed to describe the cell growth and product formation mathematically. The dynamics of glucose consumption and biomass production can be well-described by the model. The assumption that the degradation of GFP by proteases is a first-order reaction can express the trend of the GFP profile reasonably well. The dynamics of protease production is also described reasonably well by considering the inhibition effect of glucose on protease production. Because of the high initial glucose concentration and keeping the pH value of the broth at 6, the protease activity could be kept low most of the time during the fermentation. The increase of protease activity near the end of the culture might be caused by cell lysis because the intracellular protease activity was much higher than the extracellular activity.*

Keywords *Aspergillus niger*; Fermentation; Kinetics; Recombinant protein

Introduction

Filamentous fungi have been recognized as promising hosts for recombinant proteins due to their advantages in growth characteristics and protein secretion and their ability to perform post-translational processing. However, their application is hindered by problems such as proteolytic degradation of heterologous proteins. The strategies of morphology control (Xu et al., 2000) and pH control (O'Donnell et al., 2001) to inhibit extracellular protease activity and thereby increase heterologous protein production have been reported earlier. A fungal fermentation system is a complicated multiphase, multicomponent system. Further understanding of the system can be helped by studying the kinetics of cell growth and product formation using a mathematic model.

In this work, the cell growth and product formation kinetics of the recombinant *Aspergillus niger* strain AB4.1[pgpdAGLAGFP]#11 were investigated. The strain carries a glucoamylase-green fluorescence protein (GFP) fusion gene that is under

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the control of an *Aspergillus nidulans* *gpdA* promoter. The fusion protein is cleaved in the secretion pathway (Gouka et al., 1997).

Materials and Methods

Fungal Strains and Medium

The recombinant *A. niger* strain AB4.1[*pgpdAGLAGFP*]#11 carries a plasmid containing the glucoamylase-GFP fusion gene driven by the constitutive *gpdA* promoter. This strain produces two types of glucoamylase, the plasmid encoded type, which is part of the fusion protein, and a homologous type produced by the host. The strain AB4.1 is a *pyrG1* derivative of N402 (Van Hartingsveldt et al., 1987) and N402 is a *cspA1* derivative of strain ATCC 9029 (Bos et al., 1988). The medium for *A. niger* cultures was Vogel's minimum medium (Vogel, 1956).

Culture Conditions

Experiments were carried out in a 22 L B. Braun Biostat C bioreactor. The fermentation conditions were as follows: agitation rate 300 rpm; aeration rate 1 vvm; temperature 25°C. The broth pH was kept at 6 by adding 4 N NaOH. This higher pH was reported to help inhibit protease activities (O'Donnell et al., 2001). Spores for inoculation were obtained from wort agar plates incubated at 27°C for five days. The spores were harvested by adding 20 mL of sterilized water to the plates. The standard inoculation level was 2.5×10^5 spores/mL. The parameters such as dissolved oxygen (DO) level, volume of NaOH added, and the airflow rate were recorded by a control software package called LabVIEW (National Instruments, Austin, Tex.). LabVIEW was installed on a PC linked to the bioreactor's controller via a serial interface (Huang et al., 2005). During the cultivation period, samples with a volume of about 90 mL were withdrawn from the reactor every 12 h. One-third of the sample was filtered through a pre-weighed filter paper to measure the biomass concentration, and one-third was centrifuged to collect the supernatant to measure the residual glucose, extracellular protease activity, GFP level, and glucoamylase concentration. The remaining one-third was used to measure the intracellular protease activity, GFP level, and glucoamylase concentration. This was done by filtering the sample, washing the biomass, resuspending it in pH 7 phosphate buffer solution (PBS) with the same volume of the sample, dismembrating the biomass ultrasonically in an ice-water bath, and finally collecting and measuring the supernatant.

Sample Analysis

The methods for quantifying biomass, glucose, glucoamylase, and GFP concentrations have been reported elsewhere (O'Donnell et al., 2001).

Protease Assay

The protease assay method is based on Smith et al. (1991). A reaction solution (1.1 mL final volume) containing azocasein (5 mg/mL) in sodium acetate buffer (1 mL, 0.1 M, pH 5.0) was mixed with a suitably diluted culture filtrate (0.1 mL). It was incubated for 1 h at 50°C. After incubation, the reaction was terminated by adding TCA (0.255 mL, 10% (w/v)). After mixing on a vortex, the tubes were

centrifuged for 20 min at 600 rpm using a micro-centrifuge (Model 59A, Fisher Scientific, Pittsburgh). The supernatant (1.0 mL) was mixed with 0.5 M sodium hydroxide (0.1 mL), and the optical density was measured at 450 nm. One unit of activity is the amount of enzyme that causes an increase of one absorbance unit per unit of digestion under the conditions given.

Assays for Intracellular Enzymes

Cells were disrupted using an ultrasonic dismembrator (Model F60, Fisher Scientific) to release intracellular proteins. A 20 mL sample was withdrawn from the reactor. Biomass was separated from the broth by vacuum filtration, washed twice with deionized water, and then resuspended in PBS buffer (pH 7.4). The mixture was then soaked in an ice-water bath and treated with the ultrasonic dismembrator at 25 W for 10 min. The supernatant collected from the centrifugation was assayed for GFP, glucoamylase, and protease activity. Assay methods were the same as mentioned above.

Results and Discussion

Cell Growth and Product Formation

Cell growth with an initial glucose concentration of 46.9 g/L is illustrated in Figure 1. Spores began to germinate about 12 h after inoculation, followed by a rapid exponential growth phase, which lasted 30–40 h. In this phase, the dissolved oxygen concentration decreased and the consumption of NaOH increased rapidly in order to maintain the pH at 6 (Figure 1). The growth rate then decreased and proceeded to

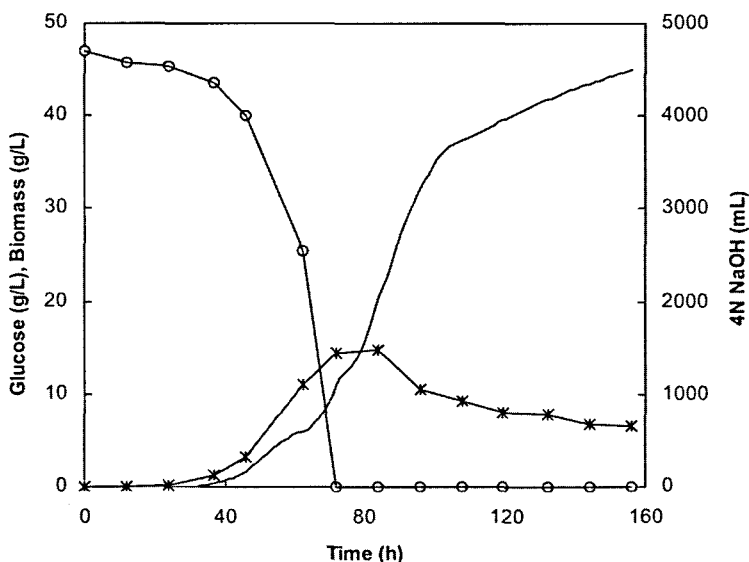


Figure 1. Cell growth and glucose and NaOH consumption in recombinant *A. niger* fermentation. Conditions: initial glucose concentration 46.9 g/L, agitation rate 300 rpm, temperature 25°C, pH 6 (by addition of 4 N NaOH). DO level was adjusted by changing airflow rate. Glucose concentration (○), biomass (*), and 4 N NaOH consumption (—).

a stationary phase lasting approximately 15 h. The dissolved oxygen tension began to increase, while the consumption of NaOH decreased. After that, the growth cycle proceeded to the death phase, in which cell lysis occurred and the cell mass decreased quickly, indicating the end of cultivation.

The expression of the fusion gene GLAGFP is under the control of a constitutive promoter *gpdA* from *A. nidulans*. The constitutive expression of genes under the control of a *gpdA* promoter was found to be growth-associated (Punt et al., 1990, 1991). It was revealed in the experiments that the production of the heterologous glucoamylase was mainly in the first two days, during the exponential cell growth phase. It was reported that *A. niger* excreted glucoamylase predominantly through the hyphal tip in a growth-associated manner because the growing hyphal tips were more porous (Wörsten et al., 1991). However, it was also shown that some glucoamylase seemed to leak from some older cell walls of nongrowing parts of the fungus. It was concluded that the glucoamylase release from this area originated from enzyme earlier secreted into the wall during apical exocytosis (Wörsten et al., 1991). In this study, experimental results revealed that the extracellular glucoamylase accumulated mainly during the stationary phase, although the intracellular glucoamylase concentration began to decrease after the cell growth ceased (Figure 2). The excretion was likely caused by the leakage of enzyme from some older cell walls of nongrowing parts of the fungus. It was also possible that the increase of glucoamylase (GLA) concentration near the end of fermentation was caused by cell lysis.

The production process of GFP was more complicated than that of the glucoamylase because of the proteolytic degradation. The extracellular protease activity could be lowered by several fold when the cultivation was properly controlled, e.g., through pH and morphology controls (Xu et al., 2000; O'Donnell et al., 2001). However, the intracellular protease activity was still high (Figure 3). In Figure 2, it was shown that the concentration of intracellular heterologous glucoamylase was

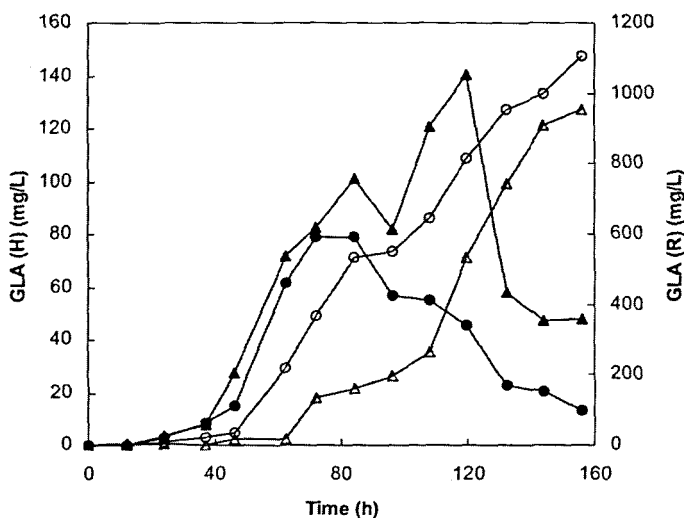


Figure 2. Glucoamylase production in recombinant *A. niger* fermentation. Conditions: same as in Figure 1. Extracellular (○) and intracellular (●) homologous glucoamylase; extracellular (△) and intracellular (▲) recombinant glucoamylase.

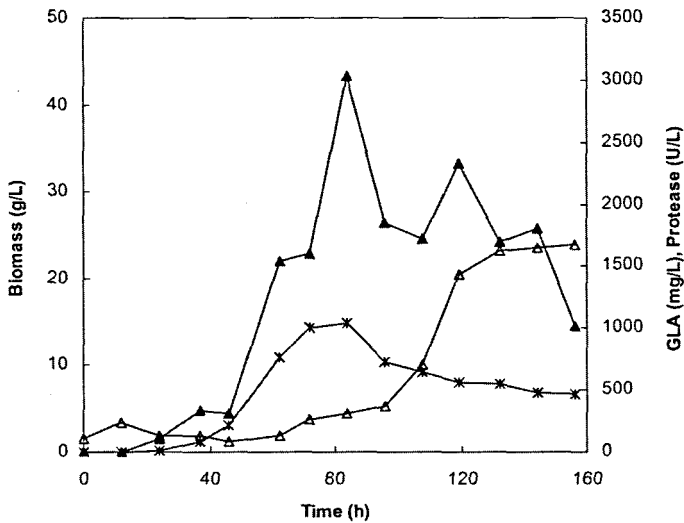


Figure 3. Protease production in recombinant *A. niger* fermentation. Conditions: same as in Figure 1. Biomass (*), intracellular (▲), and extracellular (△) protease activity.

much higher than that of the extracellular glucoamylase during the growing phase. In the GFP production profile (Figure 4), the concentrations of intra- and extracellular GFP were almost the same during the growth phase. A possible explanation was that more GFP was degraded intracellularly since the intracellular protease activity was much higher. Another possibility is that the GFP molecule is easier to be secreted since it is much smaller (27 kDa) than the glucoamylase molecule (84 kDa).

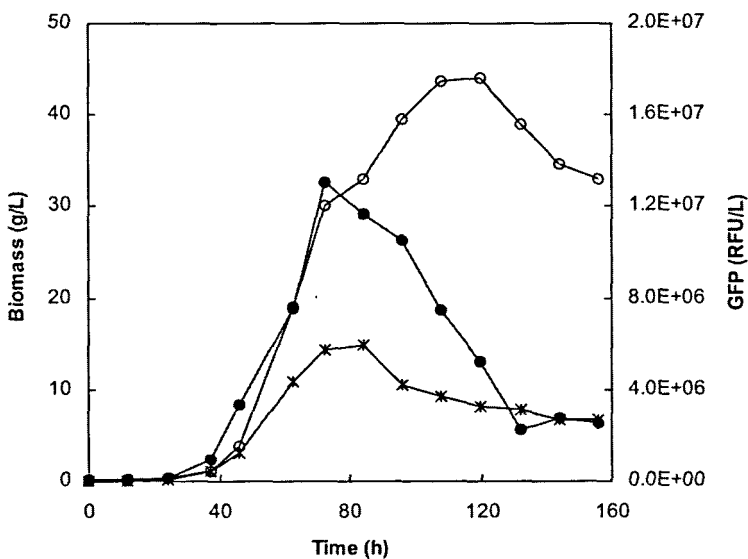


Figure 4. GFP production in recombinant *A. niger* fermentation. Conditions: same as in Figure 1. Biomass (*), intracellular (●), and extracellular (○) GFP concentration.

According to the literature (Van den Hombergh et al., 1997), protease secretion is regulated by the carbon and nitrogen sources. It was found that all the extracellular proteases are expressed only when the preferred carbon and nitrogen sources are not available to the cell (Van den Hombergh et al., 1997). The extracellular fungal proteases are also strictly pH regulated, e.g., acid proteases are expressed only at an acidic pH (Denison, 2000). *A. niger* predominantly produces aspartic (acid) proteases. Thus, it was not surprising that the extracellular protease activity began to increase only after the depletion of glucose in the broth. When the cultivation pH was kept at pH 6, which was not the optimal pH value for acid proteases, the extracellular protease activity could be kept at quite low levels throughout the cultivation process. However, there was an increase of protease activity near the end of cultivation when the cell mass decreased sharply. This increase might be due to cell lysis, which could release some intracellular enzymes into the broth.

Kinetic Model Development

An attempt was made to describe the cell growth and product formation mathematically by developing an unstructured kinetic model. Modeling the growth and product formation characteristics of microorganisms is a very challenging task. There are many different approaches to modeling microbial kinetics (Nielsen and Villadsen, 1992). Growth mechanisms and primary metabolism for fungi are more complex than for bacteria. The growth mechanism of filamentous fungi is unique even among fungi. The fungal cells are connected in the so-called hyphal structure, which is completely different from that of unicellular microorganisms, including yeast. In the literature, various structured fungal growth models have been proposed. In a morphologically structured model of the growth of *Aspergillus oryzae* (Agger et al., 1998), a hypha was divided into three parts: the hyphal region, the active region, and the extension zone. Nestaas and Wang (1983) described a simple morphologically structured model for penicillin fermentation. Three morphological forms were considered: apical cells, hyphal cells, and degenerated or inactive cells. Tholudur et al. (1999) structured their kinetic model for *Trichoderma reesei* by dividing the cell mass into three categories: primary mycelia, secondary mycelia, and spores. Compared to the structured models, the unstructured models (e.g., incorporating Monod kinetic terms or some other expressions) do not offer much in terms of elucidating the exact nature of the fermentation processes. However, more structured models often involve introducing process variables that cannot be estimated reliably.

In the experiments above for the fermentation of the AB4.1[pgpdAGLAGFP] #11 strain, pellets were generally formed. Since information about how the recombinant fusion protein is expressed and secreted and whether the expression of the recombinant proteins affects the cell growth is still lacking, an unstructured model was developed. It was based on a model for cellulase protein production from *Trichoderma reesei* (Tholudur et al., 1999), modified by adding a substrate inhibition effect for protease production and a degradation term for the GFP production.

The differential equations describing the cell growth and protein production are as follows:

$$\frac{dS}{dt} = -\mu \frac{X}{Y_{X/S}} \quad (1)$$

$$\frac{dX}{dt} = \mu X - k_d X \quad (2)$$

$$\frac{dP}{dt} = r_P X \quad (3)$$

$$\frac{dG}{dt} = r_G X - k_{dG} G \quad (4)$$

In the equations above, S , X , P , and G are the glucose, cell mass, protease, and GFP concentrations, respectively, μ is the specific growth rate, $Y_{X/S}$ is the biomass yield on glucose, k_d is the cell death rate, r_P and r_G are the specific production rates of proteases and GFP, respectively, and k_{dG} is the GFP degradation rate due to proteases. The Michaelis-Menten kinetics can usually be applied to enzyme-catalyzed reactions. It is expressed by the following equation:

$$\nu = \frac{\nu_{max} S_e}{K_m + S_e} \quad (5)$$

This expression was obtained by assuming that the substrate concentration was much higher than that of the enzyme. This assumption may not be appropriate for the degradation of GFP by proteases in the bioreactor. Previously reported data (Xu et al., 2000) showed that the degradation curve deviated considerably from a Michaelis-Menten form, thus Equation (5) should not be used. It was assumed that the degradation reaction could be considered a first-order reaction in this work, as shown in Equation (4).

Following are the specific growth rate and the production rates for proteases and GFP:

$$\mu = \frac{\mu_m S}{K_S + S} \quad (6)$$

$$r_P = (\alpha_P \mu + \beta_P) \frac{K_{sp}}{K_{sp} + S} \quad (7)$$

$$r_G = \alpha_G \mu + \beta_G \quad (8)$$

Parameters in the differential equations were estimated by a nonlinear least-squares routine called *lsqnonlin.m* in the Optimization Toolbox of Matlab software (Version 6.6.0.88, MathWorks Inc., Natick, Mass.). The initial guesses of the parameters were entered and then the nonlinear least-squares routine was called to optimize the square of the residuals between predicted and experimental values. The predicted values were obtained by calling the Matlab solver *ode45* to solve the ordinary

Table I. Estimated parameter values for the kinetic model

Parameter	Value	Parameter	Value
μ_m	0.23/h	$Y_{X/S}$	0.37 g/g
K_S	18.5 g/L	k_d	0.012/h
α_P	16.7	β_P	2.68/h
K_{sp}	0.019 g/L	α_G	193
β_G	6.5×10^4 /h	k_{dG}	0.037/h

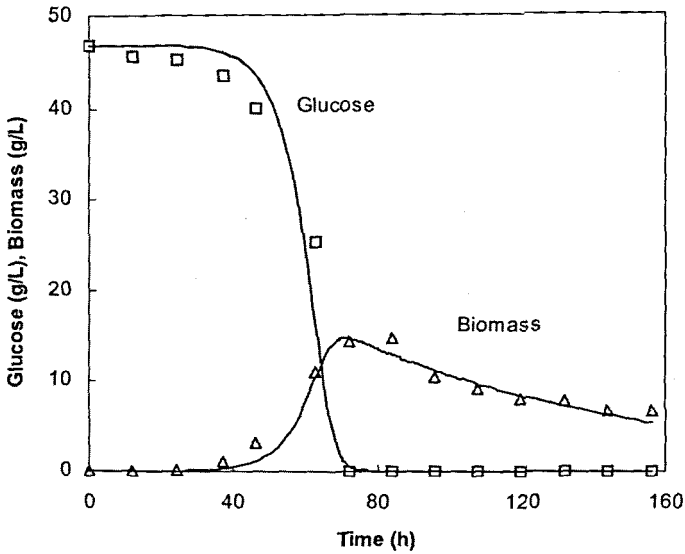


Figure 5. Growth kinetics predicted by the kinetic model.

differential equations. The initial conditions were given according to the experimental data, which were: glucose 46.9 g/L, biomass 0.001 g/L, proteases 110 U/L, and GFP 60000 RFU/L (the RFU reading of fresh medium). The estimated parameters are listed in Table I. The maximum specific growth rate was estimated to be 0.23/h, which was consistent with the values (0.19–0.23/h) reported in the literature (Favela-Torres et al., 1998; Pedersen et al., 2000a,b).

The performance of this model is illustrated in Figures 5 and 6. Figure 5 shows that the dynamics of glucose consumption and biomass production can be well

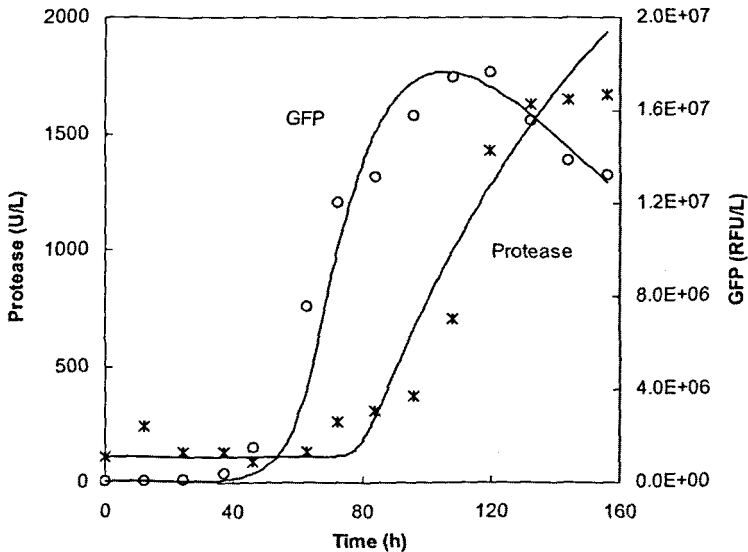


Figure 6. Protein production kinetics predicted by the kinetic model.

described by the model. The assumption that the degradation of GFP by proteases is a first-order reaction can express the trend of the GFP profile reasonably well. Figure 6 shows that the dynamics of protease production is also described reasonably well by considering the inhibition effect of glucose in the model. It was mentioned earlier that the protease production was affected by the carbon and nitrogen sources, and the conditions of fermentation, e.g., pH value of the broth. Because of the high initial glucose concentration and keeping the pH value of the broth at 6, the protease activity was kept low most of the time during the fermentation. The increase of protease activity near the end of the culture might be caused by cell lysis because the intracellular protease activity was much higher than the extracellular protease activity. This may explain the drop in protease activity after 120h in Figure 6 that was not predicted by the model.

Conclusions

This work presented an unstructured model that described the cell growth and protease and GFP concentrations in the fermentation of a recombinant *A. niger* strain reasonably well. The degradation GFP by proteases was modeled successfully based on a first-order reaction. The inhibition effect of glucose on the protease production was incorporated in the model.

Nomenclature

G	GFP concentration, RFU/L
K_S	saturation constant, g/L
K_{sp}	inhibition coefficient, g/L
k_d	death rate, h^{-1}
k_{dG}	GFP degradation rate by proteases, h^{-1}
P	protease concentration, U/L
r_P	specific protease production rate, h^{-1}
r_G	specific GFP production rate, h^{-1}
S, S_e	substrate concentration, g/L
t	time, h
X	biomass concentration, g/L
$Y_{X/S}$	biomass yield coefficient, dimensionless

Greek Letters

α_P, α_G	model constants, dimensionless
β_P, β_G	model constants, h^{-1}
μ	specific growth rate, h^{-1}
μ_m	maximum specific growth rate, h^{-1}
ν	enzyme reaction rate, h^{-1}
ρ	density, kg/m^3

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