

A Kinetic Study for Exopolysaccharide Production in Submerged Mycelial Culture of an Entomopathogenic Fungus *Paecilomyces tenuipes* C240

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The unstructured model was tested to describe mycelial growth, exopolysaccharide formation, and substrate consumption in submerged mycelial culture of *Paecilomyces tenuipes* C240. The Logistic equation for mycelial growth, the Luedeking-Piret equation for exopolysaccharide formation, and Luedeking-Piret-like equations for glucose consumptions were successfully incorporated into the model. The value of the key kinetic constants were: maximum specific growth rate (μ_m), 0.7281 h⁻¹; growth-associated constant for exopolysaccharide production (α), 0.1743 g(g cells)⁻¹; non-growth associated constant for exopolysaccharide production (β), 0.0019 g(g cells)⁻¹; maintenance coefficient (m_s), 0.0572 g (g cells)⁻¹. When compared with batch experimental data, the model successfully provided a reasonable description for each parameter during the entire growth phase. The model showed that the production of exopolysaccharide in *P. tenuipes* C240 was growth-associated. The model tested in the present study can be applied to the design, scale-up, and control of fermentation process for other kinds of basidiomycetes or ascomycetes.

Key words · Exopolysaccharide, Kinetic model, Logistic equation, Luedeking-Piret equations, *Paecilomyces tenuipes*

Many types of exopolysaccharide produced by mushrooms and/or insect-born (entomopathogenic) fungi have been studied and are currently used in a wide range of industries due to their diverse functional and pharmacological activities[1,2]. Recently, several investigators have attempted to obtain optimal submerged culture conditions for exopolysaccharide production from those higher fungi [3,4].

Paecilomyces tenuipes is one of the medicinal entomopathogenic fungi belonging to the family Clavicipitaceae. Recently, some constitutions from *P. tenuipes* and evaluation of their antimycobacterial, antiplasmodial activities have been reported[5-7].

Kinetic studies supported by mathematical models are a vital part of the overall investigation of cell growth and product formation in most fermentation processes. The model allows easy data analysis and provides a strategy for solving problems encountered at the design stage. Knowledge and an understanding of the kinetics of exopolysaccharide production are of great economic importance in view of the fact that exopolysaccharide production is one of major industrial subjects in many fermentation processes.

Industrial fermentation is gradually moving away from the traditional and largely empirical approach towards simpler and better-controlled process[8]. The rational design and optimization of the latter required a quantitative understanding of production kinetics.

A mathematical model for a microbial process can be expressed using two different mechanisms: structured and unstructured models[9,10]. Structured models take into account some basic aspects of cell structure and intracellular metabolic pathways. In unstructured models, however, only cell mass is employed to describe the biological system. Hence, unstructured model seems easier for normal use, and has proven to accurately describe many fermentation processes [11-13]. Kinetic studies for several extracellular microbial polysaccharides has been reported[14,15]. However, little studies have been carried out on the unstructured kinetic model for exopolysaccharide production in higher fungi such as basidiomycetes and ascomycetes even though suitable prediction tools are required for understanding their prolonged and complicated fermentation process[16].

In the present study, the unstructured model was tested for describing cell growth, exopolysaccharide formation, and substrate consumption in submerged mycelial culture of *Paecilomyces tenuipes* and examined its feasibility by comparing the predicted data with experimental data.

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Materials and Methods

Kinetic model

Nomenclatures

m_s	maintenance coefficient (g substrate (g cells h) ⁻¹)
P	exopolysaccharide concentration at time t (g l ⁻¹)
P_0	initial exopolysaccharide concentration (g l ⁻¹)
S	glucose concentration at time t (g l ⁻¹)
S_0	initial glucose concentration (g l ⁻¹)
t	fermentation time (h)
X	cell concentration at time t (g l ⁻¹)
X_m	maximum cell concentration (g l ⁻¹)
X_0	initial cell concentration (g l ⁻¹)
$Y_{X/S}$	yield coefficient for cells on carbon substrate (g cells (g substrate) ⁻¹)

Greek letters

α	growth-associated product formation coefficient (g g ⁻¹)
β	non-growth-associated product formation coefficient (g g ⁻¹ h ⁻¹)
γ	parameter in Luedeking-Piret-like equation for substrate uptake (g S (g cells) ⁻¹)
μ_m	maximum specific growth rate (h ⁻¹)

Fungal growth

The most widely used unstructured models for describing cell growth are the Monod kinetic model[17], the logistic equation[18], and the Haldane model[19]. The approximation used for cell growth is the logistic equation that characterizes cell growth in terms of the maximum value obtained[20]. The logistic equation can be described as follows:

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

where μ_m is the maximum specific growth rate (h⁻¹) and X_m is the maximum attainable biomass concentration (g dry weight l⁻¹). The integrated form of Eq. (1) using $X=X_0$ ($t=0$) gives a sigmoidal variation of X as a function of t which may represent both an exponential and a stationary phase (Eq. (2)):

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

Rearrangement of Eq. (2) yields Eq. (3):

$$\ln \frac{X}{X_m - X} = \mu_m t - \ln \left(\frac{X_m}{X_0} - 1\right) \quad (3)$$

The value of X_m is evident from the experimental data. A plot of $\ln [X/(X_m - X)]$ versus time (t) will give a line of slope μ_m and y intercept equal to $\ln [X/(X_m - X_0) - 1]$, from which the initial viable inoculum size (X_0) can be found.

Product formation

The kinetics of exopolysaccharide formation was based on the Luedeking-Piret equation. This model was originally developed for the formation of lactic acid by *Lactobacillus delbrueckii*[21]. According to this model, the product formation rate depends upon both the instantaneous biomass concentration (X), and growth rate (dX/dt) in a linear manner:

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

Where α and β are the product formation constants which may vary with fermentation conditions. In order to express P as a function of time, substituting of Eq. (1) in Eq. (4) and integrating yields the following equation:

$$P(t) = \alpha A(t) + \beta B(t) + P_0 \quad (5)$$

In which,

$$A(t) = X(t) - X(0) \quad (6)$$

$$B(t) = \frac{X_m}{\mu_m} \ln \left[1 - \frac{X_0}{X_m} (1 - \exp(\mu_m t))\right] \quad (7)$$

The benefit of this model is that β can be found from stationary phase data. At stationary phase ($dX/dt=0$) and ($X=X_m$), β can be obtained using the following equation (8):

$$\beta = \frac{(dP/dt)_{st}}{X_m} \quad (8)$$

Plotting $[P(t) - P(0) - \beta B(t)]$ against $A(t)$ gives the growth-associated product formation constant, α .

Glucose consumption

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.

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

Where $\gamma = 1/Y_{X/S}$.

Substituting of Eq. (1) into Eq. (9) and integrating yield the following equation:

$$S(t) = S_0 - \gamma A(t) - m_s B(t) \quad (10)$$

In which,

$$A(t) = X(t) - X(0) \tag{6}$$

$$B(t) = \frac{X_m}{\mu_m} \ln \left[1 - \frac{X_0}{X_m} (1 - \exp(\mu_m t)) \right] \tag{7}$$

At stationary phase, $dX/dt=0$ and $X=X_m$. Therefore, m_s can be obtained using the following equation (11):

$$m_s = - \frac{(dS/dt)_{st}}{X_m} \tag{11}$$

Plotting $[S_0 - S(t) - m_s B(t)]$ versus $A(t)$ will give a line of slope γ . The EXCEL 2000 (Microsoft) was used for the estimations of the set of parameter values from all batch fermentation data determined from a modeling program written in VBA (Visual Basic for Application, Microsoft) working in EXCEL 2000 (Microsoft)[22].

Experimentals

Microorganism and media

P. tenuipes C240 was kindly provided by Dr. J.M. Sung in Kangwon National University, Chuncheon, Korea and was used throughout the study. The stock culture was maintained on potato dextrose agar (PDA) slant. Slant were incubated at 25°C for 6 d and stored at 4°C. The seed culture was conducted in a 250 ml flask containing 50 ml of YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose) and cultured on a rotary shaker incubator at 25°C and 150 rev min⁻¹ for 4 d. The flask culture experiments were performed at the same conditions as noted above after inoculating with 4% (v/v) of the seed culture.

Fermentation conditions

The fermentation medium (3% glucose, 0.4% KNO₃, 0.1% K₂HPO₄, and 0.1% MgSO₄ · 5H₂O) was inoculated with 4% (v/v) of the seed culture and then cultivated for 12 days at 28°C in a 5 l (3 l working volume) stirred tank bioreactor (KF250, KoBioTech Co., Seoul, Korea) equipped with pH, dissolved oxygen, temperature sensors and automatic controls. The pH, aeration rate, and agitation speed were controlled at 4.0, 2 vvm, and 150 rpm, respectively[23].

Analytical methods

Samples collected at various intervals from the bioreactor were centrifuged at 10,000 × g for 15 min, and the resulting supernatant was filtered through a membrane filter (0.45 μm), followed by mixed with four volumes of absolute ethanol, stirred vigorously and left overnight at 4°C. The precipi-

tated exopolysaccharide was centrifuged at 10,000 × g for 15 min, discarding the supernatant. The residue was re-precipitated with four volumes of ethanol and the precipitate of exopolysaccharide sample was lyophilized, and the weight of the polysaccharide was noted. Dry weight of mycelium was measured after repeated washing of the mycelial pellet, obtained after centrifugation with distilled water and drying at 90°C for 12 h to a constant weight. The filtrate from a membrane filtration (0.45 μm) was analyzed by high performance liquid chromatography (HPLC) using an Aminex HPX42C column (0.78 × 30 cm, Bio-rad) equipped with a refractive index detector (Shimadzu Co, Kyoto, Japan) for quantitative analysis of residual sugar concentration[24].

Results and discussion

Microbial growth

Fig. 1 shows the typical time courses of mycelial growth and exopolysaccharide production in a 5-l stirred-tank bioreactor under optimal culture conditions. After a lag phase (about 1-2 day), the cells entered the exponential growth phase, thereby starting to form exopolysaccharides. Taking $X_m=18.34$ g dry weight l⁻¹ obtained from the Fig. 1, a plot of Eq. (3) yielded $\mu_m=0.7298$ (h⁻¹) and an intercept of -3.3428, corresponding to $X_0=0.626$ g dry weights (Fig. 2). The calculated value of X_0 was lower than that of the experimental value that was 0.92 g dry weights. This can presumably be attributed to the viability of the cells. Less than 100% viability may yield an X_0 value less than the measured initial cell concentration. The biomass concentration (X) was calculated according to Eq. (2) using the values of X_0 and μ_m determined previously, and the value of X_m which

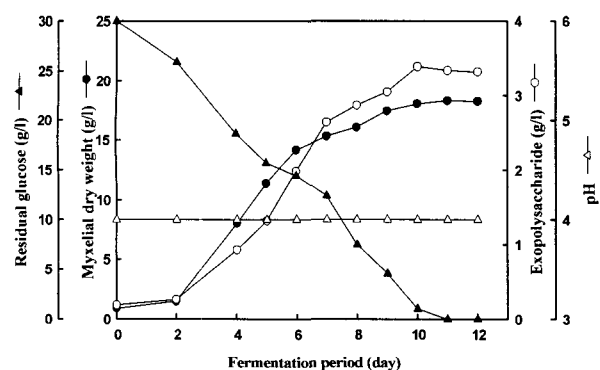


Fig. 1. Typical time courses of the mycelial growth and exopolysaccharide production in a 5-l stirred tank bioreactor. (●) mycelial dry weight, (○) exopolysaccharide, (▲) Residual sugar, (△) pH.

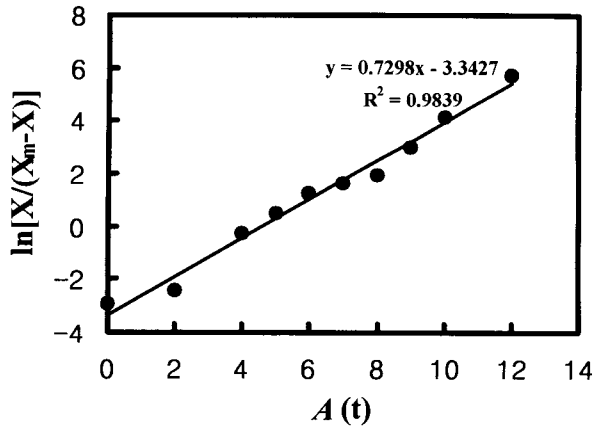


Fig. 2. Determination of X_0 and μ_m using Eq. (3).

was known from the experimental data. The result is depicted in Fig. 3.

Product formation

Product formation parameters were obtained from Eqs. (5) and (8) during the stationary phase where cell growth rate (dX/dt) is zero: $\beta = (dP/dt)_{st}/X_m = 0.0019$ g exopolysaccharide (g cells-d^{-1}). As stated earlier, plotting $[P-P_0-B(t)]$ against $A(t)$ will give the growth-associated product formation constant, α . The slope of the line in Fig. 4 provided $\alpha = 0.1743$ $\text{g}(\text{g cells})^{-1}$. When $\alpha = 0$, $\beta = 0$, the product formation is growth-associated. The value of β is 0.0019 (close to 0) g exopolysaccharide (g cell-d^{-1}), which indicate that the production of exopolysaccharide was growth-associated [16,25]. These two parameters (α and β), X_0 , X_m and μ_m were used to calculate the product formation rate. A comparison of calculated function $P(t)$ with the experimental data is given in Fig. 5.

Substrate uptake

In exopolysaccharide fermentation, the increase in biomass concentration was accompanied by a decrease in residual glucose concentration. Glucose was depleted after 11 d of fermentation (Fig. 1). Glucose consumption may be represented with Eq. (10). The initial glucose concentration (S_0) was 30 g l^{-1} . Eq. (11) provided the parameter m_s as given below: $m_s = (dS/dt)_{st}/X_m = 0.0571$ g glucose (g cells-d^{-1}). A plot of Eq. (10) in Fig. 6 yielded a slope of $\gamma = 1.1660$ g glucose ($\text{g cells})^{-1}$. A comparison of the experimental data with calculated function $S(t)$ was then made using these two parameters (Fig. 7).

Conclusions

To evaluate the model, the calculated values from the

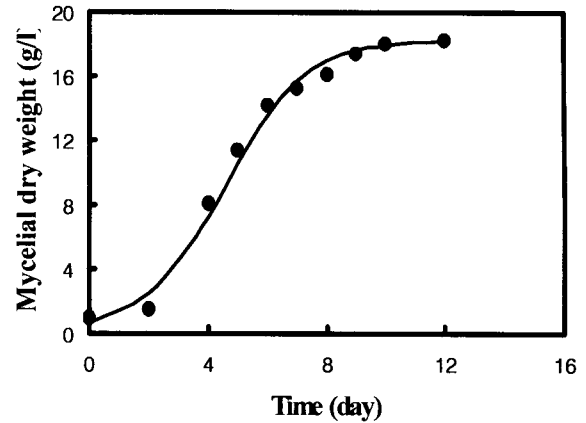


Fig. 3. Comparison of Eq. (3) with the experimental biomass concentrations (●) Experimental, (—) Calculated.

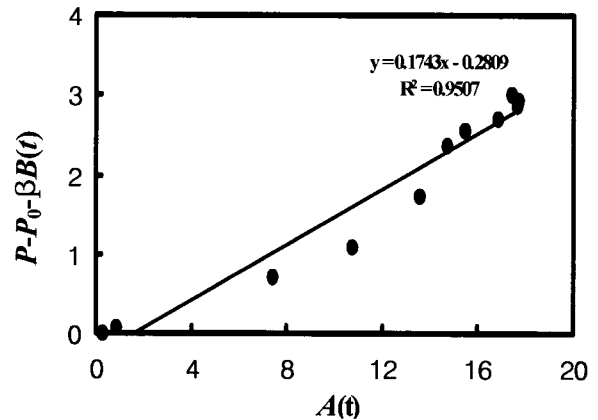


Fig. 4. Evaluation of α using Eq. (5).

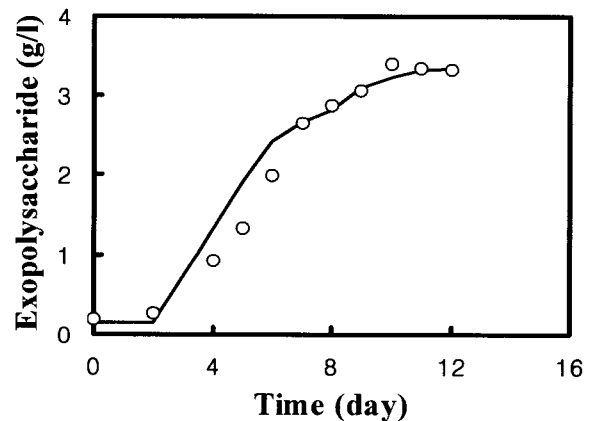
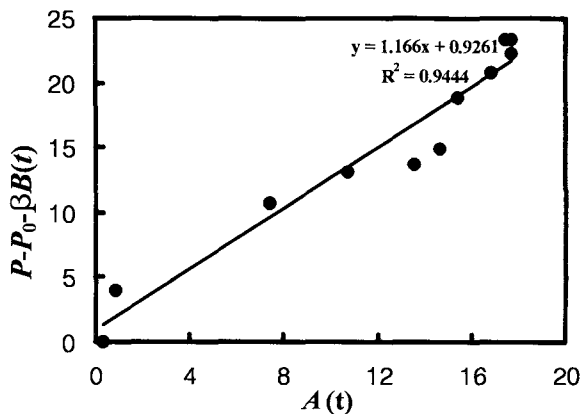
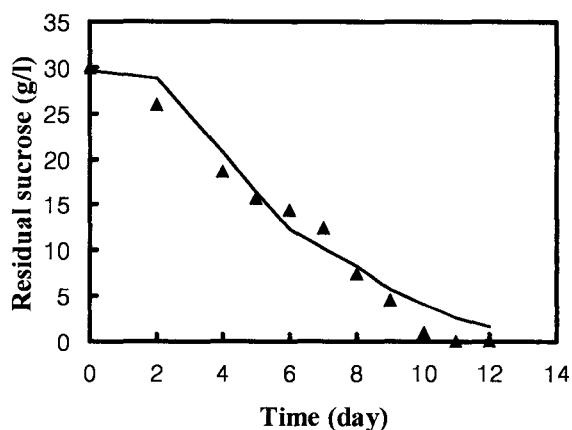
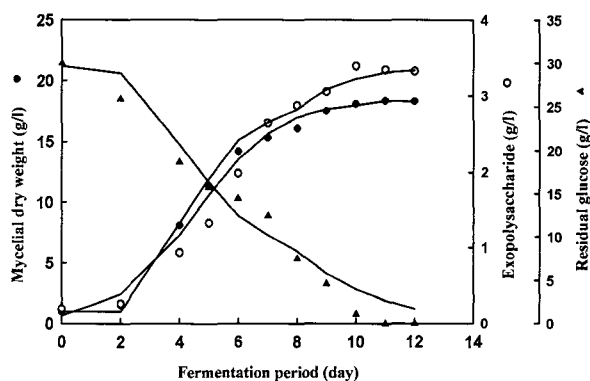


Fig. 5. Comparison of Eq. (5) with the experimental data. (○) Experimental, (—) Calculated.

kinetic model with experimental data obtained in a 5-l bioreactor were compared (Fig. 8). Each profile of mycelial growth, exopolysaccharide formation, and substrate uptake were well fitted within the error ranges $<10\%$, suggesting that the model can possibly describe the submerged culture

Fig. 6. Evaluation of γ using Eq. (10).Fig. 7. Comparison of Eq. (10) with the experimental data. (\blacktriangle) Experimental, (—) Calculated.Fig. 8. Comparison of calculated values (lines) and the experimental data (symbols) for an experiment performed in a batch system. (\bullet) mycelial dry weight, (\circ) exopolysaccharide, (\blacktriangle) Residual sugar. Solid lines indicate predicted values.

process of *P. tenuipes* C240. The simplicity, feasibility, accuracy and generality of the model give rise to a very useful tool for further design, scale-up, and control of the submerged culture process of *P. tenuipes* C240.

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초록 : 동충하초 *Paecilomyces tenuipes* C240의 균사체 배양에 의한 세포외 다당체 생산의 동력학적 연구

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동충하초 *Paecilomyces tenuipes* C240의 균사체 배양과정에서 균사체 성장, 세포외 다당체 생산, 기질감소 속도를 표현할 수 있는 동력학적 모델을 제시하였다. 균사체 성장은 Logistic 식을, 세포외 다당체 생산은 Luedeking-Piret 식을, 기질소모는 Luedeking-Piret 유사식을 각각 적용함으로써, 전체 균사체 배양과정을 예측할 수 있었다. 모델식에서 사용된 주요 kinetic constant들은 다음과 같다: 균사체의 최대 비성장속도(μ_m), 0.7281 h^{-1} ; 다당체 생산에서의 growth-associated constant (α), $0.1743 \text{ g (g cells)}^{-1}$; non-growth-associated constant (β), $0.0019 \text{ g (g cells)}^{-1}$; maintenance coefficient (m_s), $0.0572 \text{ g (g cells)}^{-1}$. 5L 발효조에서 얻은 균사체 성장, 세포외 다당체 생산, 기질감소 속도자료들을 모델에서 예측한 결과와 비교한 결과 서로 잘 일치하는 것으로 보아, 본 연구에서 제안된 모델식은 이 동충하초 균사체 배양공정의 scale-up 등의 프로세스 설계에 응용가능 할 것이며, 다른 종류의 동충하초 균사체 배양공정에도 적용가능 할 것으로 판단된다.