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Kinetic Studies of Gluconic Acid Fermentation, Using *Aspergillus niger*

Yoshiharu Miura, Katsumi Tsuchiya, Hiroko Tsusho and Kazuhisa Miyamoto

(Department of Chemical Engineering, Faculty of Pharmaceutical Sciences, Osaka University, Toyonaka, Osaka)

Abstract

Kinetic studies of gluconic acid fermentation have been conducted at pH 6.5, using *Aspergillus niger* 5131. The formulae for the kinetic model of the fermentation processes were obtained by assuming that the limiting step of the fermentation processes is the transport of glucose through the cell membrane. It was found through the experiments that these formulae predicted the rate processes of the fermentation from the exponential to the stationary phase.

Introduction

Kinetic studies are necessary for basic understanding of fermentation processes and for rational design and control of industrial fermentation processes. Although kinetic investigations on fermentation processes have been widely reported[1~3,5,7,12~14], kinetic comparisons between fermentation reactions *in vivo* and *in vitro* have been little researched. In the present study, the kinetics of glucose consumption by *Aspergillus niger* were compared with that of glucose oxidase to determine the rate controlling step throughout the fermentation.

The gluconic acid fermentation, particularly that using a homofermentative organism such as *Aspergillus niger*, is well suited for basic fermentation studies, for its rate processes are relatively simple. In this fermentation glucose is converted to a single product (gluconic acid), with negligible amounts of intermediate and side products[6,9], so the attention may be focused on three primary processes: the rates of nutrient disappearance, product formation, and cell growth. In order to investigate the applicability of Monod's equation[10] to the growth rate of this microorganism and the controlling step in the fermentation, theoretical formulae for the rates of consumption of glucose and production of gluconic acid were derived and compared with experimental data.

Experimental Equipment and Procedures

In the present study, a jar fermentor of stainless steel and glass was employed; it was constructed to be quite similar to the one used in our previous work[8].

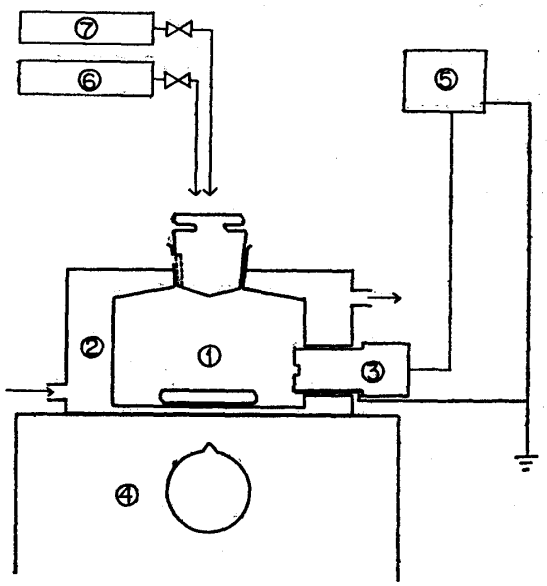
Its total capacity was fifteen liters. The organism used in our experiments was a strain of *Arpergillus niger*, 5131, contributed by the Department of Fermentation Technology of Osaka University. A synthetic medium was used for the present experiments because of good reproducibility; its composition is shown in Table 1, where the composition of the medium for preculture is also shown.

Table 1.

Medium composition for seed culture.	
Glucose	7.5 %
Ammonium phosphate, dibasic	0.2 %
Magnesium sulfate	0.07%
Potassium phosphate, monobasic	0.12%
Medium composition for product culture.	
Glucose	5.0 %
Ammonium phosphate, dibasic	0.05%
Magnesium sulfate	0.01%
Potassium phosphate, monobasic	0.02%

Preculture was carried out in a 500 ml shaker-flask on a reciprocal shaker at a speed of 120 rpm with a 5 cm amplitude at 30°C for one day. The preculture thus obtained was inoculated into the jar fermentor, in which the culture broth was maintained at pH 6.5 and 30°C. The aeration rate was 1.5 v.v.m. (15 *l*/min), and the speed of agitation was 430 rpm. In the course of the fermentation the concentration of dissolved oxygen in the medium was kept constant by introducing oxygen into the aeration gas and the medium was continuously adjusted to pH 6.5 by titration with aqueous solution of sodium carbonate.

The cell concentration was obtained by withdrawing 50-ml samples and filtering, washing, and drying the cells to a constant weight at 45°C under vacuum. The concentration of diammonium hydrogen phosphate, the limiting substrate, was analyzed by the Micro-Kjeldahl method, and glucose analyses were made by Somogyi's method on the clear broth from the filtering operation. The concentration of gluconic acid produced was evaluated through the volume of sodium carbonate required to maintain constant pH in the fermentation broth. Glucose oxidase in the cell was extracted by a phosphate buffer solution of pH 5.8 from cells crushed by a crushing machine at 4°C. The activity of the glucose oxidase in the buffer solution was quite stable for three weeks under low temperature conditions. The glucose oxidase in the buffer solution was poured into the apparatus shown in Fig. 1, where the con-



① Reactor ② Isothermal jacket
③ Electrode for meassuring concentration of dissolved oxygen
④ Magnetic stirrer
⑤ Analyzer for measuring concentration of dissolved oxygen
⑥ Oxygen cylinder ⑦ Nitrogen cylinder

Fig. 1. Schematic diagram of experimental apparatus for measuring activity of glucose oxidase.

centration of dissolved oxygen was increased by aeration and the surface was covered to prevent surface aeration. The activity of the oxidase was evaluated by measuring the rate of decrease of dissolved oxygen concentration through a gold cathode and a silver anode with a teflon membrane (Beckman's model 777).

Results and Discussion

1. Growth rate Assuming Monod's model for the growth of a microorganism from the exponential phase to the stationary phase in a batch culture, the growth rate is expressed by the following equation (for definitions of terms see below, "Nomenclature"):

$$\frac{dx}{d\theta}=\frac{\mu_m S}{K_s+S}\cdot x \quad \cdots\cdots (1)$$

Assuming that the rate of consumption of the limiting substrate is proportional to the growth rate of the cells, since the limiting substrate is consumed to constitute the cells and maintain the life of the organism, this rate of consumption may be experessed by Equation (2).

$$\frac{dS}{d\theta}=-\frac{1}{Y_S}\frac{dx}{d\theta} \quad \cdots\cdots (2)$$

Solving Equation (2) so as to satisfy the initial conditions of Equations (3), Equation (4) may be obtained.

$$x=x_0,\quad S=S_0 \quad \text{at} \quad \theta=0 \quad \cdots\cdots (3)$$

$$S=S_0-\frac{1}{Y_S}(x-x_0) \quad \cdots\cdots (4)$$

By substituting Equation (4) into Equation (1), Equation (5) may be derived.

$$\frac{dx}{d\theta}=\frac{\mu_m\left\{S_0-\frac{1}{Y_S}(x-x_0)\right\}}{K_S+S_0-\frac{1}{Y_S}(x-x_0)}\cdot x \quad \cdots\cdots (5)$$

Solving Equation (5) so as to satisfy the initial conditions of Equations (3), Equation (6) may be obtained.

$$\theta=\frac{K_2}{K_1}\ln(K_1-1)-\frac{K_2}{K_1}\ln(K_1-X)+\left(\frac{K_2}{K_1}+K_3\right)\ln X \quad \cdots\cdots (6)$$

where

$$\left.\begin{aligned} X&=\frac{x}{x_0}, & K_1&=1+\frac{Y_S S_0}{x_0}\\ K_2&=\frac{K_S Y_S}{x_0\mu_m}, & K_3&=\frac{1}{\mu_m}\end{aligned}\right\} \quad \cdots\cdots (7)$$

Equation (6) indicates the relation between the cell concentration and the culture time from the begining of the exponential phase. The parameters K_1, K_2, and K_3 were obtained from experimental results by the nonlinear least square method and μ_m, K_S, and Y_S were calculated through K_1, K_2, and K_3; the results are shown in Table 2. The changing patterns of cell concentrations estimated by Equation (6), using the average values of these parameters, were compared with the experimental findings. The results are shown in Fig. 2, which indicates good agreement between the experimental and calculated values of cell concentration from the exponential phase to the stationary phase.

Table 2. Values of parameters for Eqs. (6) and (7), determined by the nonlinear least square method.

	μ_m (1/hr)	K_s (g/l)	$Y_s(-dx/dS)$
Run 1	0.206	0.075	1.99
Run 2	0.206	0.060	2.00
Run 3	0.200	0.117	2.45
Average	0.204	0.086	2.13

The relation between the concentrations of limiting substrate and cells was estimated by Equation (4), using the average value of the yield constant Y_S, and the estimated values were compared with the experimental values. The results are shown in Fig. 3, which indicates good agreement between the estimated and experimental values.

It was concluded from the results shown in Fig. 2 that Monod's Equation (1) is applicable for obtaining the growth rate of *Aspergillus niger* from the exponential phase to the stationary phase.

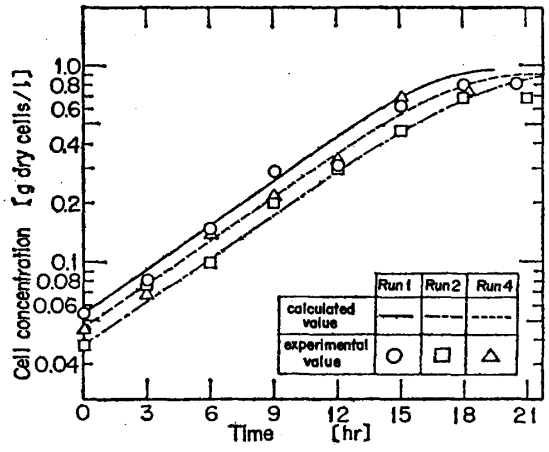


Fig. 2. Comparisons of calculated growth rate with experimental growth rate for *Aspergillus niger*.

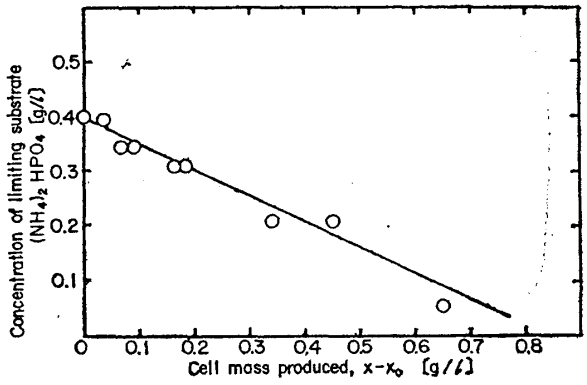


Fig. 3. Relation between cell mass produced and concentration of limiting substrate. The solid line indicates the estimated values and the symbol ○ indicates experimental values.

2. The rate of gluconic acid production The reactions in the cell for forming gluconic acid are expressed by the following equations:

$$\beta\text{-D-glucose} + \text{glucose oxidase-FAD} \rightleftarrows \delta\text{-D-gluconolactone} + \text{glucose oxidase-FADH}_2 \quad \cdots\cdots (8)$$

$$\delta\text{-D-gluconolactone} + H_2O \rightleftarrows \text{gluconic acid} \quad \cdots\cdots (9)$$

$$\text{glucose oxidase-FADH}_2+O_2 \rightleftarrows \text{glucose oxidase-FAD}+H_2O_2 \quad \cdots\cdots(10)$$

$$H_2O_2 \xrightarrow[\leftarrow]{\text{catalase}} H_2O+\frac{1}{2}O_2 \quad \cdots\cdots(11)$$

$$\beta\text{-D-glucose}+\frac{1}{2}O_2 \xrightarrow[\leftarrow]{\text{glucose oxidase}} \text{gluconic acid} \quad \cdots\cdots(12)$$

It was reported by Moyer[9] and by Hsieh *et al.*[6] that the accumulation of δ-D-gluconolactone was negligible. Therefore, the oxidization of β-D-glucose to δ-D-gluconolactone, represented by Equation (8), is considered to be the controlling reaction in gluconic acid production by glucose oxidase in *Aspergillus niger*.

The relation between the consumption of glucose and the production of gluconic acid is shown in Fig. 4. It was found from the results shown in Fig. 4 that the rate of gluconic acid production was proportional to the rate of glucose consumption, as in Equation (13):

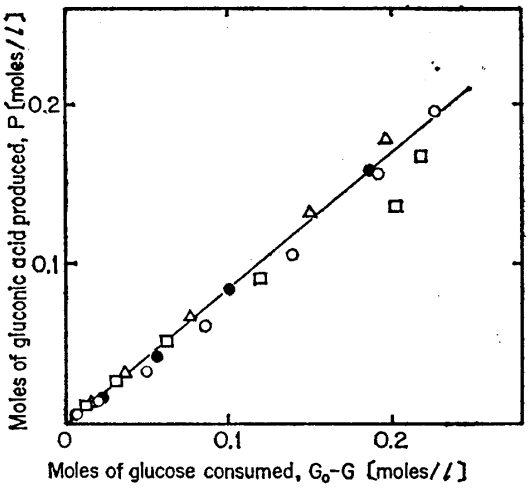


Fig. 4. Moles of gluconic acid produced vs. moles of glucose consumed per unit volume of medium. The solid line indicates the calculate values and symbols of ○, ●, □, and △ indicate experimental data for runs 1, 2, 3 and 4, respectively.

$$\frac{dP}{d\theta}=-b\frac{dG}{d\theta} \quad \cdots\cdots(13)$$

Solving Equation (13) so as to satisfy the initial conditions of Equations (14), Equation (15) may be obtained.

$$P=0, \quad G=G_0 \quad \text{at} \quad \theta=0 \quad \cdots\cdots(14)$$

$$P=b(G_0-G) \quad \cdots\cdots(15)$$

The experimental values of b are shown in Table 3. The rate of gluconic acid production may be estimated from the rate of glucose consumption through Equation (15).

Table 3. Values of the productivity constant, b, of gluconic acid.

	b $(-dP/dG)$
Run 4	0.806
Run 5	0.88
Run 6	0.81
Run 7	0.90
Average	0.85

The changing pattern of activity of glucose oxidase throughout this fermentation course is shown in Fig. 5. The activity of glucose oxidase reached a maximum in the early stages of the exponential phase.

De La Fuente *et al.*[4] and Okada *et al.*[11] have reported that the transport of the substrate through the cell membrane is often a controlling step in the rate pro-

cesses of fermentations such as glucose fermentation. It is generally considered that a large molecule such as glucose is transported by some carriers such as permease (active transport), and therefore, that the consumption rate of substrate by cell converges to a definite value with higher concentration of the substrate.

Fig. 6 shows experimental evidence for the above mentioned mechanism, i.e. saturation kinetics, for glucose consumption in this fermentation. The consumption rates of glucose were obtained by the following method: (1) Exponentially growing cells in the culture broth were transfered into phosphate buffer solution (pH 6.5) to stop the growth; (2) 20 ml of the cell suspension thus obtained was added to 380 ml of glucose phosphate buffer solution of pH 6.5, in which dissolved oxygen was maintained at a constant concentration by aeration and agitation; and (3) the rate of consumption of glucose by the cells was obtained by measuring the initial rate of glucose consumption. The rates of glucose oxidization by glucose oxidase were obtained by a similar method, except that enzyme solution was used instead of cell suspension.

Fig. 7 shows the Lineweaver-Burk plot of glucose oxidization by glucose oxidase. Table 4 gives the apparent saturation constant, K_G, for glucose consumption by the cells and the Michaelis-Menten constant, K_m, for glucose oxidization by glucose oxidase. Fig. 8 shows the influence of pH on the rates of glucose consumption by the cells and glucose oxidization by glucose oxidase. If glucose oxidization by glucose oxidase

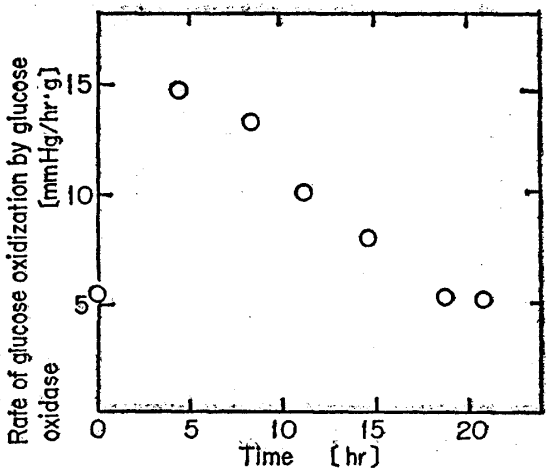


Fig. 5. Rate of glucose oxidization by glucose oxidase in the course of fermentation.

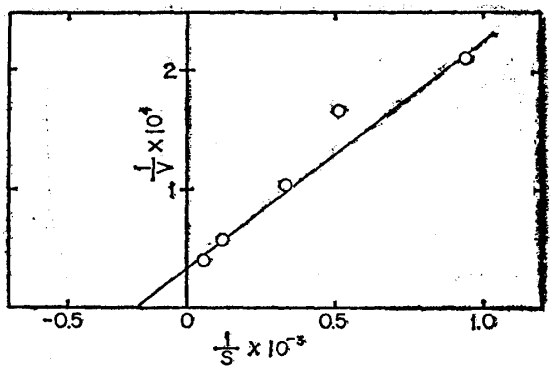


Fig. 6. Double reciprocal plot for rate of glucose consumption by cells.

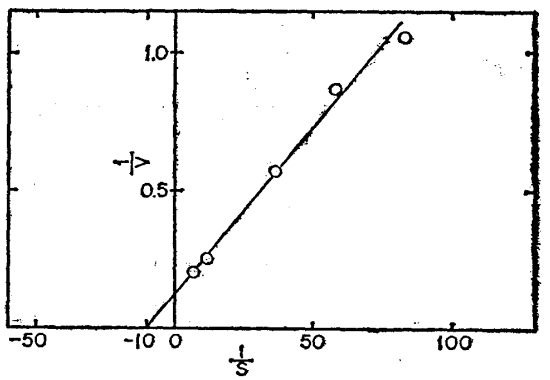


Fig. 7. Lineweaver-Burk plot for rate of glucose oxidization by glucose oxidase.

Table 4. The values of the saturation constant, K_G and the Michaelis constant, K_m.

K_G (mol/l)	5.9×10^{-3}
K_m (mol/l)	1.0×10^{-1}

were the controlling step in this culture, the value of K_m would be equal to the value of K_G and the optimum pH for glucose oxidization and consumption would coincide. However, as shown in Table 4 and Fig. 8, the values of K_G and K_m and the optimum pH for glucose oxidization and consumption did not coincide. The maximum rate of glucose consumption by the cells was much lower than that of glucose oxidization by glucose oxidase, as shown in Figs. 6 and 7. It is considered from above results that the transport of glucose through the cell membrane is the controlling step in this fermentation.

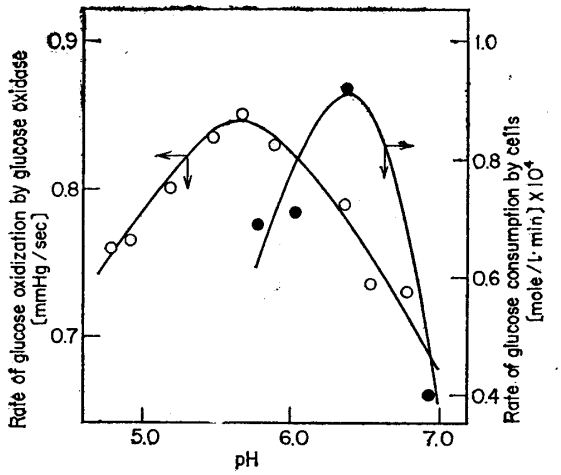


Fig. 8. Effect of pH on glucose oxidization by glucose oxidase and glucose ccnsumption by *Aspergillus niger*.

The active transport of glucose in the cell membrane is expressed by the following equation:

$$G_e + E \rightleftarrows G_e\text{-}E \longrightarrow G_i + E \quad \cdots\cdots(16)$$

In above equation G_e and G_i indicate the glucose outside and inside the cell, respectively, and E indicates the permease in the cell membrane.

The rate of glucose consumption by the cells is expressed by the following equation:

$$-\frac{dG}{d\theta} = \frac{VG}{K_G + G} \cdot x \quad \cdots\cdots(17)$$

Dividing Equation (17) by Equation (5), Equation (18) may be obtained:

$$-\frac{dG}{dx} = \frac{VG\left\{K_s + S_0 - \frac{1}{Y_s}(x - x_0)\right\}}{\mu_m (K_G + G)\left\{S_0 - \frac{1}{Y_s}(x - x_0)\right\}} \quad \cdots\cdots(18)$$

Solving Equation (18) so as to satisfy the initial condition of Equation (19), Equation (20) may be obtained.

$$G = G_0, \quad \text{at} \quad x = x_0 \quad \cdots\cdots(19)$$

$$-\frac{\mu_m K_G}{V}\ln\frac{G}{G_0} - \frac{\mu_m}{V}(G - G_0) = (x - x_0) - Y_s K_s \ln\frac{S_0 - \frac{1}{Y_s}(x - x_0)}{S_0} \quad \cdots\cdots(20)$$

The parameters in Equation (20) were obtained by the nonlinear least square

method and the results are shown in Table 5. In the present fermentation, the value of K_G is very low and the first term of the left side of Equation (20) is negligible compared with the other terms. Equation (20) may, therefore, be simplified to Equation (21):

Table 5. The values of the parameters of Eq. (20).

K_G (mol/l)	5.9×10^{-3}
K_s (g/l)	8.0×10^{-2}
V (mol/l·hr)	4.5×10^{-2}
b	8.5×10^{-1}
μ_m (1/hr)	2.0×10^{-1}
Y_s	2.13

$$G=G_0-\frac{V}{\mu_m}(x-x_0)+\frac{VK_sY_s}{\mu_m}\ln\frac{S_0-\frac{1}{Y_s}(x-x_0)}{S_0} \quad \cdots\cdots\cdots\cdots\cdots\cdots(21)$$

The changing patterns of glucose concentration predicted by Equation (21) were compared with the experimental patterns. The results of this comparison are shown in Fig. 9, which indicates good agreement between calculated and experimental values. Using the values of glucose concentration estimated by Equation (21), the gluconic acid concentrations estimated by Equation (15) were also compared with the experimental values. Those comparisons, too, indicate good agreement between estimated and experimental values, as shown in Fig. 9.

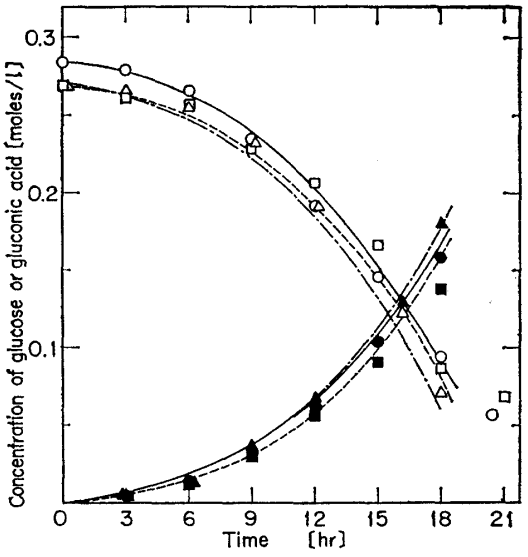


Fig. 9. Comparisons between calculated and experimental values of concentrations of glucose and gluconic acid throughout the fermentation.

	Glucose			Gluconic acid		
	Run 1	Run 2	Run 4	Run 1	Run 2	Run 4
Calculated value	——	- - -	- - -	——	- - -	- - -
Experimental value	○	□	△	●	■	▲

Conclusions

Monod's Equation may be applied to the growth rate of *Aspergillus niger* from the exponential phase to the stationary phase. The rate of consumption of glucose was proportional to the rate of gluconic acid in gluconic acid fermentation using *Aspergillus niger*. It may be considered that the transport of glucose in the cell membrane is the controlling step in this fermentation, and that the transport is an active transport. It was found that Equation (20) for glucose concentration, derived by taking into account the active transport of glucose, was valid as shown in Fig. 9, the values of gluconic acid concentration predicted by Equations (15) and (20) agree well with the experimental values, as also shown in Fig. 9.

Nomenclature

b : moles of gluconic acid produced per mole of glucose consumed, $-dP/dG$
G : concentration of glucose, mole/l
G_0 : concentration of glucose at $\theta=0$, mole/l
K_G: saturation constant for fermentation, mole/l
K_m: Michaelis constant for the oxidization of glucose, mole/l
K_s: Monod's constant, g/l
P : concentration of gluconic acid, mole/l
S : concentration of limiting substrate, g/l
S_0 : concentration of limiting substrate at $\theta=0$, g/l
V : maximum rate of glucose consumption by cells, mole/g·hr
Y_s : yield of growth per mass of limiting substrate, $-dx/dS$
x : concentration of cells, g dry weight/l
x_0 : concentration of cells at $\theta=0$, g dry weight/l
θ : fermentation time from beginning of exponential phase, hr
μ_m : maximum specific growth rate, hr^{-1}

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