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Estimation of Residual Biomass, PHB, and Nutrient Concentrations by Supplied Amount of Ammonia Solution in Fermentation of *Alcaligenes latus*

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Abstract A novel estimation method was investigated for determining the concentrations of residual biomass, poly-3-hydroxybutyrate (PHB), and main nutrients including carbon and nitrogen sources, phosphate, and mineral ions from the supplied amount of ammonia solution used for a pH-control solution and nitrogen source in a PHB fermentation. The estimation equations for a batch culture and a fed-batch culture were derived from the relationship between the growth rate of residual biomass and the feed rate of the pH-control solution, and then were applied to the batch culture and the fed-batch cultures of *Alcaligenes latus*. This method was successfully applied to estimate the concentrations of residual biomass, PHB, and nutrients.

Key words: Estimation method, pH-control, *Alcaligenes latus*, ammonia solution, poly-3-hydroxybutyrate (PHB), pH-stat fermentation.

The on-line measurement of cell and nutrient concentrations in a bioreactor are one of the most important subjects in biotechnology, because this gives information on the *in situ* state of cultivation and creates a possibility of framing a real-time control strategy for the highest activity of microorganisms. On-line monitoring in a microbial culture, however, has so far been restricted to mainly measurements of pH, temperature, dissolved oxygen concentration (DO), or redox potential. The main problems for on-line monitoring are on-line sampling conditioning, calibration and blank measurements, and the lack of biosensors for measuring concentrations [15].

Recently, the laser turbidity method [21] and induced fluorescence method [17] have been applied to directly measure the concentrations of microorganisms. Although these methods are attractive, the instruments are complicated and expensive. In the latter case, it is also greatly affected by the culture conditions.

In PHB fermentation processes, PHB content has to be identified in real time to control the processes effectively. There are, however, difficulties in measuring PHB content on-line, since PHB is an intracellular metabolite. Thus, an off-line assay by a gas chromatography [2] is currently the most popular method for measuring PHB content.

The pH of broth usually drops gradually when ammonium compound is used as a nitrogen source during fermentation. To maintain the pH in a bioreactor, an alkaline solution like KOH or NaOH is usually automatically fed by a pump linked to a pH-controller which is set to the optimal pH for cell growth. There is a pH-stat culture technique in which this mechanism is applied to the substrate feeding method in a fed-batch culture or a continuous culture. As cell growth proceeds with substrates being consumed, an alkaline substrate, *viz.*, an ammonia solution, in place of the KOH or NaOH solution, is supplied automatically to maintain the pH, and acts not only as a pH-control solution but also as a nitrogen source. Especially in a high-cell-density culture, the pH-stat technique is an alternative to avoid the dilution of the cell concentration and damage to the cells due to a pH-control solution [3, 7, 9, 16, 19, 20]. However, the theoretical validity of pH-stat, *viz.*, the correspondence between the feed rate of the alkaline substrate by pH-stat and the consumption rate of the substrate during cultivation, is still not clarified.

In this article, a relationship between cell growth and the supplied amount of the pH-control solution is suggested, and the estimation equations of the residual cell mass, PHB, and nutrients concentrations in batch and fed-batch cultures are then derived from this relationship. These estimated equations were applied to batch and fed-batch cultures of *Alcaligenes latus*, a growth-associated PHB producer.

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RELATIONSHIP BETWEEN CELL GROWTH RATE AND FEED RATE OF pH-CONTROL SOLUTION

If it is assumed that the pH change of the medium is proportional to cell growth rate during fermentation, the growth rate can be expressed as follows:

$$\mu X=\frac{F_B S_{BF}}{HV} \tag{1}$$

where F_B is the feed rate of the pH-control reagent [l/h], S_{BF} is the concentration of the pH-control solution [mol/l], and H the required amount of the pH-control solution to maintain the pH per increased cell weight [mol of alkaline pH-control solution/g dry cell weight]. Equation (1) is similar to that proposed by Rice and Hempfling [13]. The proposed equation, however, does not have a relationship between the cell concentration and buffering capacity of the medium in the reservoir as previously suggested, but instead applies a relationship between the cell growth rate and the feed rate of the pH-control solution.

When the ammonia solution is used for pH control, it is also utilized as a nitrogen source. In microbial PHB production, nitrogen is exclusively incorporated into the residual biomass. If the feeding of the ammonia solution is well balanced with its incorporation into the residual biomass, H is correlated with the nitrogen content of the residual biomass, [g-nitrogen/g residual biomass], or residual biomass yield based on nitrogen, $Y_{X/N}$, as

$$H=\delta/14=1/(14Y_{X/N}) \tag{2}$$

Equation (2) is useful to estimate the value of H.

ESTIMATION EQUATIONS FOR MICROBIAL PHB PRODUCTION

It is well recognized that *A. latus* synthesizes PHB associating to cell growth under no limitation of any nutrients [1]. From the data analysis of Braunegg and Bogensberger's culture of *A. latus* [1], a linear relationship between the residual biomass growth rate (X) and PHB production rate (qX) was derived (data not shown). Hence, this relationship can be expressed by a linear equation as:

$$qX=a+b\mu X \tag{3}$$

where μ and q are the specific growth rate of the residual biomass and the specific PHB production rate, respectively. a and b are the coefficients of the equation.

Batch Culture

The model suggested by Lee and Yoo [8] was considered for the cultivation of *A. latus*, except that the stoichiometry for the nitrogen source was substituted by that for each nutrient such as a nitrogen source, phosphate, and minerals.

After the model equations are combined with equations (1) and (3), the mass balance equations for the residual biomass, carbon source, and other nutrients are

$$\frac{dX}{dt}=\mu X=\frac{F_B S_{BF}}{HV} \tag{4}$$

$$\frac{dP}{dt}=qX=a+b\frac{F_B S_{BF}}{HV} \tag{5}$$

$$\frac{dC}{dt}=-\left(\frac{\mu X}{Y_{X/C}}+\frac{qX}{Y_{P/C}}\right)=-\left(I+J\frac{F_B S_{BF}}{HV}\right) \tag{6}$$

$$\frac{dN_i}{dt}=\frac{\mu X}{Y_{X/Ni}}=-\frac{F_B S_{BF}}{HVY_{X/Ni}} \tag{7}$$

Where,

$$I=\frac{a}{Y_{P/C}} \tag{8}$$

$$J=\frac{1}{Y_{X/C}}+\frac{b}{Y_{P/C}} \tag{9}$$

It is assumed in equations (6) and (7) that the carbon source is consumed for the biosynthesis of both the residual biomass and PHB, but other nutrients only for the biosynthesis of the residual biomass. The broth volume change due to the supplied pH-control solution and evaporation accompanying exhaust gas is neglected in a batch culture. The definitions of $Y_{X/C}$ and $Y_{P/C}$ in equation (6) are described afterward in the section "Determination of Yields". Equations (4) through (7) were integrated to obtain the following equations.

$$X=X_0+\frac{S_{BF}}{HV}\int_0^t F_B dt \tag{10}$$

$$P=P_0+at+\frac{bS_{BF}}{HV}\int_0^t F_B dt \tag{11}$$

$$C=C_0-It-\frac{JS_{BF}}{HV}\int_0^t F_B dt \tag{12}$$

$$N_i=N_{i0}-\frac{S_{BF}}{HVY_{X/Ni}}\int_0^t F_B dt \tag{13}$$

In equations (10)-(13), the integral means the supplied volumetric amount of the pH-control solution by time t, which is easily measured by the volume change of the reserved pH-control solution. The concentrations of the residual biomass, PHB, and all nutrients in the batch culture at culture time t can be successfully estimated simultaneously by a simple real-time measurement of the supplied amount of the pH-control solution by time t.

Fed-Batch Culture

The stoichiometries describing a pH-stat fed-batch culture are

$$\frac{d(VX)}{dt}=\mu(VX) \tag{14}$$

$$\frac{d(VP)}{dt}=q(VX) \tag{15}$$

$$\frac{d(VC)}{dt}=C_F F_C-\left\{\frac{1}{Y_{X/C}}\frac{d(VX)}{dt}+\frac{1}{Y_{P/C}}\frac{d(VP)}{dt}\right\} \quad (16)$$

$$\frac{d(VN)}{dt}=S_{BF}F_B-\frac{1}{Y_{X/N}}\frac{d(VX)}{dt} \quad (17)$$

$$\frac{d(VM_i)}{dt}=M_{iF}F_C-\frac{1}{Y_{X/Mi}}\frac{d(VX)}{dt} \quad (18)$$

$$\frac{dV}{dt}=\alpha F_{total} \quad (19a)$$

where it is postulated that the sucrose solution and the ammonia solution are fed separately. Minerals are added to the sucrose solution.

In equation (19a), F_{total} is the total feed rate which is the sum of various nutrient solutions and the pH-control solution. The quantity α is a correction factor for the increment of the culture broth volume caused by feeding the total nutrient solution [l culture broth increament/l fed]. The value of α is usually less than unity when a highly concentrated nutrient solution is fed. In this study, the feeding of the nutrient solution composed of sucrose and minerals was in coupling with the ammonia solution feeding, so that Fc was proportional to F_B, i.e., $Fc=kF_B=(k+1)F_B$. Hence

$$F_{total}=F_C+F_B=(k+1)F_B \quad (20)$$

Therefore, equation (19a) is modified as follows

$$\frac{dV}{dt}=\alpha(k+1)F_B \quad (19b)$$

The integration of equation (19b) gives

$$V=V_0+\alpha(k+1)\int_0^t F_B dt \quad (19c)$$

After substituting equation (1) for μ (VX) of equation (14), the residual biomass concentration is obtained as

$$X=\left\{V_0X_0+\frac{S_{BF}}{H}\int_0^t F_B dt\right\}/V \quad (21)$$

The PHB concentration can be determined from equations (3) and (15) as

$$P=\left\{V_0P_0+at+\frac{bS_{BF}}{H}\int_0^t F_B dt\right\}/V \quad (22)$$

The integration of equation (16) combining with equations (1), (3), (8), and (9) yields the carbon source concentration as follows:

$$C=\left\{V_0C_0-I\int_0^t Vdt+\left(kC_F-\frac{JS_{BF}}{H}\right)\int_0^t F_B dt\right\}/V \quad (23)$$

The ammonium concentration can also be obtained from equation (17)

$$N=\left\{V_0N_0+S_{BF}\left(1-\frac{1}{HY_{X/N}}\right)\int_0^t F_B dt\right\}/V \quad (24)$$

The mineral concentrations can be estimated from equation (18) as

$$M_i=\left[V_0M_{i0}+\left\{(k-1)M_{iF}-\frac{S_{BF}}{HY_{X/Mi}}\right\}\int_0^t F_B dt\right]/V \quad (25)$$

Determination of Yields

The reliable determination of the residual biomass and PHB yield from each nutrient is essential to accurately estimate the concentrations. In order to estimate $Y_{X/C}$ and $Y_{P/C}$, it is important to know the amount of carbon transfer as carbon dioxide evolved in the residual biomass formation ($Y_{CO_2/X}$) and PHB biosynthetic pathway ($Y_{CO_2/P}$), respectively. To clarify the metabolic pathway of carbon source involved in microbial PHB production, refer to Fig. 1 [22]. The total amount of the carbon source consumed (C) is composed of two parts: the part converted to PHB (C_1) and the part converted to the non-PHB residual biomass (C_2). As carbon source is metabolized to both the residual biomass and PHB, a part of the carbon source is converted to carbon dioxide in each pathway.

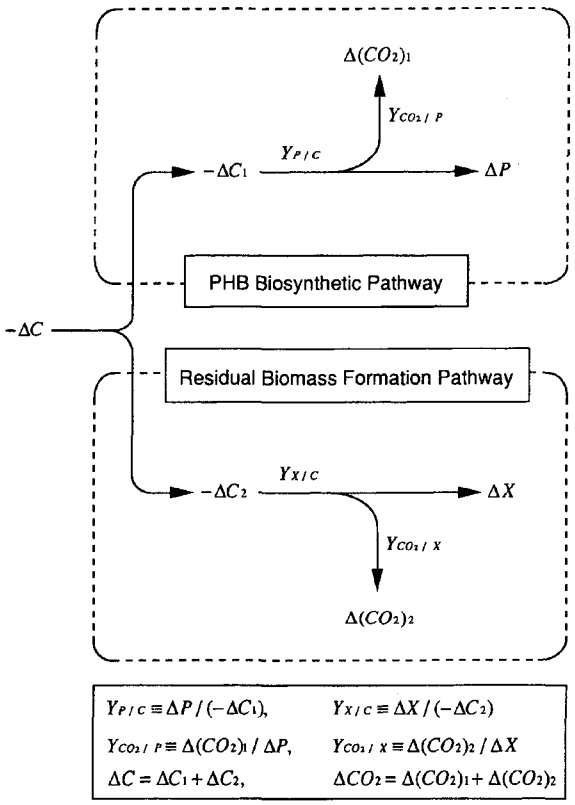

Fig. 1. Schematic diagram of the metabolic pathway of carbon source to residual biomass, PHB, and carbon dioxide.

The value of $Y_{CO_2/P}$ can be estimated from the PHB biosynthetic pathway. For PHB biosynthesis from sucrose, two moles of the PHB monomer and four moles of carbon dioxide per mole of sucrose are produced.

Sucrose $\rightarrow$ 4 CO_2+2/n PHB

Thus, $Y_{CO_2/P}$ and $Y_{P/C}$ for PHB biosynthesis are 1.02 (=2(44/86) and 0.5 (=2(86/342), respectively. The value of $Y_{P/C}$ is the same as that proposed by Yamane [22].

$Y_{X/C}$ can also be calculated from the following equation derived from the carbon balance involved in the residual biomass formation.

$$Y_{X/C}=\frac{\gamma_C}{\gamma_{CO_2}Y_{CO_2/X}+\gamma_X} \quad (26)$$

Where γ_C, γ_{CO_2}, and γ_X are the carbon contents in sucrose, carbon dioxide, and the residual biomass, which are 0.42 (=12×12/342), 0.27 (=12/44), and 0.50, respectively. The value of γ_X was determined from the elemental analysis of *A. latus* [23]. $Y_{CO_2/X}$ in equation (26) is calculated from total carbon balance as follows:

$$Y_{CO_2/X}=\frac{\gamma_{CO_2}(-\Delta C)-(\gamma_X\Delta X-(\gamma_P+\gamma_{CO_2}Y_{CO_2/P})\Delta P)}{\gamma_{CO_2}\Delta X} \quad (27)$$

Where Δ means the difference between the initial and final concentrations in batch culture.

Moreover, the yield of the residual biomass from each nutrient ($Y_{X/Ni}$) had already been determined by an elemental analysis [23].

MATERIALS AND METHODS

Microorganism and Medium

The strain used was *Alcaligenes latus* DSM 1123, which utilizes sucrose as a carbon source. The medium for the seed culture contained the following components per liter of distilled water [11]: 20 g of sucrose, 2 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 60 mg of $FeCl_2 \cdot 4H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, and 1 ml of trace elements solution. Each liter of trace elements solution contains 0.3 g of H_3BO_3, 0.2 g of $CoCl_3 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of $NaMoO_4 \cdot 2H_2O$, 28 mg of $NiSO_4 \cdot 7H_2O$, and 10 mg of $CuSO_4 \cdot 5H_2O$. The pH of the formulated medium was 7.0.

Cultivation

A loop of the organism from the stock culture kept on an agar plate was inoculated into three 100 ml seed cultures in three 500 ml round-bottom flasks and cultivated at 30°C for 24 h on a rotary shaker at 200 rpm. These precultures were centrifuged at 5,000 rpm for 15 min. The harvested cells were suspended in a 100 ml medium, a part of the main medium, and were used to inoculate into a 5 l jar fermenter (KMJ-5B, Mituwa, Japan) resulting in a 3 l medium. The culture temperature and air flow rate were 35°C and 1 vvm, respectively. The agitation speed was fixed at 500 rpm. The pH was maintained at 6.8 by a pH-controller (Able, Japan). The pH controller had a twin timer. Its on- and off-times were set at 6 sec and 6 sec, respectively. When the pH dropped below the low set point, the pH-control solution was fed in. When the pH increased above the low set point due to the addition of the pH-control solution, its feeding stopped. The supplied gravimetric amount of the pH-control solution which includes the ammonia solution (2.8%) or 1N-KOH solution was measured by a digital electronic balance (Libror EB-330H, Shimazu, Japan). Initially, 0.2 ml of adecanol LG-109 (Asahi Denka, Japan) per liter of medium was added as an antifoam.

pH-Stat Fed-Batch Culture

Two kinds of feed media were prepared, which were both a pH-control solution, *viz.*, an ammonia solution (2.8%, w/v) and a sucrose solution composed of sucrose (545 g/l), phosphate, and minerals. The compositions of the phosphate and minerals of *A. latus* cell mass for balanced growth were determined from an elemental analysis [10, 14, 18, 23]. These media were fed separately to the bioreactor to prevent precipitation.

The feed media were supplied simultaneously by the same type of pumps (Perista pump SJ-1211, Atto, Japan) which was controlled by a pH-controller, so that the sucrose and mineral media supplies were coupled with the ammonia solution feeding. The feed rate of the sucrose solution was 2.88 times as fast as that of the pH-control solution.

The initial medium volume in the bioreactor was 2 l. The initial concentration of sucrose and $(NH_4)_2SO_4$ were 10 g/l and 1 g/l, respectively. High-cell-density fed-batch cultures were also conducted as reported previously [20].

Analytical Procedure

The microbial growth was estimated by measuring the optical density of the culture broth at 660 nm after diluting with deionized water. The precise cell concentration was determined by weighing dried cells which were prepared after 10 ml of the culture broth was centrifuged at 15,000 rpm for 3 min, washed three times with distilled water, and dried at 105°C for 24 h.

The PHB concentration was measured using gas chromatography (GC-7AG, Shimazu, Japan) with benzoic acid as the internal standard [2]. The PHB content was estimated by dividing the analyzed amount of PHB with the total cell weight. The residual biomass concentration was determined as the difference between the total cell and PHB concentrations. That is, $X=X_{total}-P$.

The ammonium concentration was assayed using the Nesslerization method [6], and the sucrose concentration was analyzed by the anthrone-sulfuric acid method [17].

RESULTS

Relationship Between Cell Growth and the Fed Amount of pH-Control Solution

In order to study the relationship between the growth of the residual biomass and the fed amount of the pH-control solution, batch cultures of *A. latus* using a KOH or ammonia solution (2.8%) as a pH-control solution were carried out. The fed amount of the ammonia solution was linearly proportional to the growth of the residual biomass, yet that of the KOH solution increased logarithmically as the cells grew (Fig. 2a). Moreover, the ammonia solution, a weak alkali, was fed less than the KOH solution, a strong alkali, and yet produced the same residual biomass growth. The value of H for the ammonia solution was 14.07 (mmol ammonia/g residual biomass), which is equivalent to 0.197 (g nitrogen/g residual biomass). This is higher than 0.113, which is the value estimated by equation (2) using the nitrogen content of the total biomass [23]. This discrepancy may be due to both a partial loss of the fed NH_3 through evaporation accompanied with the exhaust gas and the accumulation of NH_3 in the culture liquid. The same proportional relationship was observed with the high-cell-density fed-batch culture (Fig. 2b).

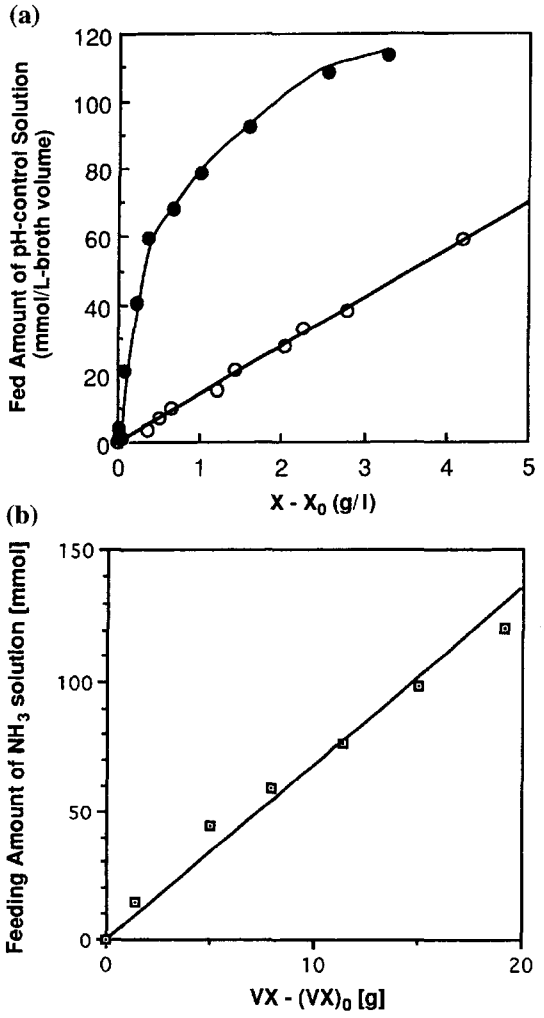

Fig. 2. Relationship between the residual biomass concentrations and the fed amount of pH-control solutions (a) in batch culture [KOH (●), NH_4OH (○)] and (b) in fed-batch culture.

Relation Between the Residual Biomass Growth Rate and the PHB Production Rate

As a characteristic of *A. latus*, the PHB production rate was in proportion to the residual biomass growth rate as shown in Fig. 3. Thus, equation (3) was also confirmed by the batch culture. The coefficient a in equation (3) was zero from the intercept, and b was 0.69 from the slope.

Determination of Yields

During the batch culture, 2.63 g/l of the residual biomass and 3.15 g/l of PHB were synthesized accompanying 16.1 g/l of sucrose consumption. From these results, $Y_{CO_2/X}$ was calculated as 3.97 (g/g) from equation (27), and $Y_{X/C}$ was 0.27 (g/g) from equation (31). Accordingly, the values of I and J of equation (8) and (9) were zero and 4.21, respectively.

Estimation of the Concentrations in the Fermentation of *A. latus*

In order to confirm the validity of this estimation method, the on-line concentrations of the residual cell mass, PHB, sucrose, and ammonium ion were on-line estimated using

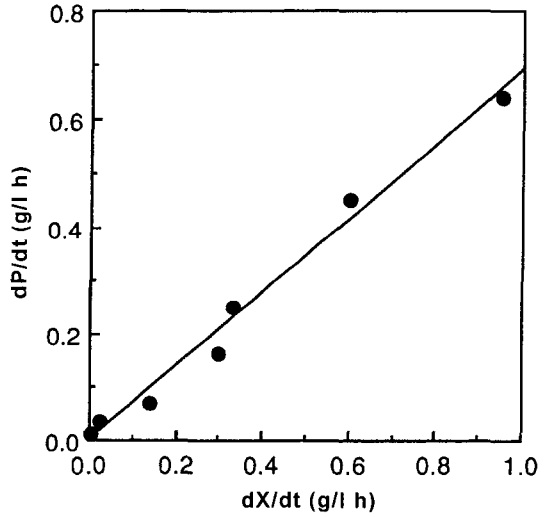

Fig. 3. Linear relationship between the residual biomass growth rate (X) and the PHB production rate (qX) in batch culture of *A. latus*.

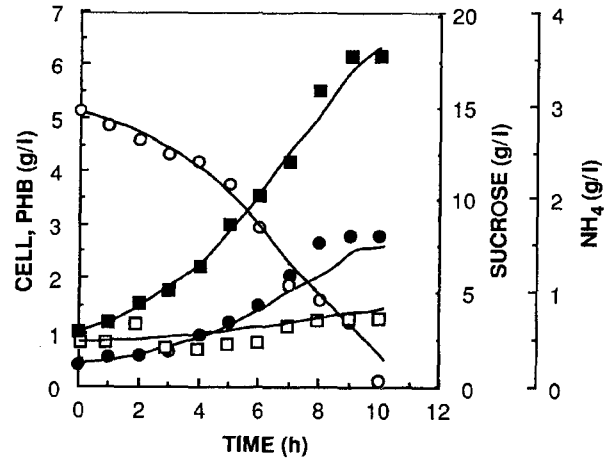

Fig. 4. Estimation of the concentrations of cell (■), PHB (●), sucrose (○), and ammonium (□) from the fed amount of pH-control solution (NH_4OH 2.8%, w/v) in batch culture of *A. latus*. The solid lines represent estimated values.

equations (10)-(13) for a batch culture, and using equations (21)-(24) for a fed-batch culture. An ammonia solution (2.8%) was used as a pH-control solution. The on-line concentrations were then compared with the experimental data.

In the batch culture of *A. latus*, despite a small discrepancy in the late exponential growth, this method successfully estimated the concentrations of the residual biomass and PHB as shown in Fig. 4. The sucrose concentration was also well estimated. The ammonium concentration was slightly overestimated. However, the estimation result showed the same increasing pattern as the experimental data with the culture time.

In the pH-stat low-cell-density fed-batch culture shown in Fig. 5, the cell concentration was under-estimated, yet the PHB concentration was well estimated. The ammonium concentration was slightly over-estimated as in the case of the batch culture. This method also estimated the high sucrose concentration over 100g/l successfully.

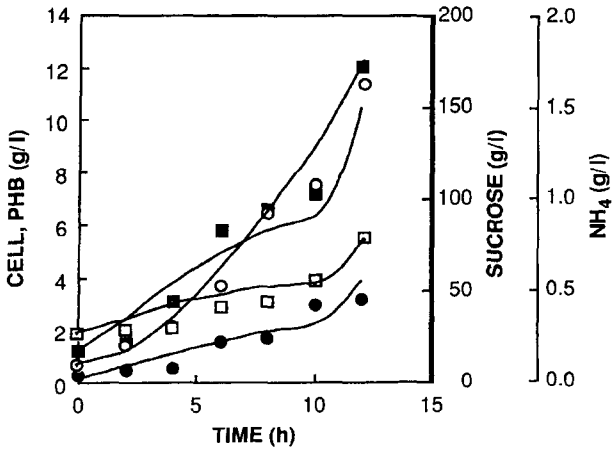

Fig. 5. Estimation of the concentrations of cell (■), PHB (●), sucrose (○), and ammonium (□) from the fed amount of pH-control solution (NH_4OH 2.8%, w/v) in fed-batch culture of *A. latus*. The solid lines represent estimated values.

DISCUSSION

When comparing the fed amounts of KOH and NH_4OH in the batch culture of *A. latus*, NH_4OH was fed less than KOH, and yet produced the same residual biomass growth as shown in Fig. 2. The reason seems that the ammonium ion (NH_4^+) of NH_4OH was taken up by the microbes as a nitrogen source, whereas most K^+ ion was accumulated unused.

As a result of estimating the culture of *A. latus*, a good coincidence with the experimental data was produced in the concentrations of residual biomass, PHB, sucrose and ammonium ion. From this result, it can easily be speculated that other nutrients including phosphate and minerals might also be precisely estimated because these concentrations vary in proportion to the ammonium concentration during fermentation.

The pumping speed of pH-control solutions was fixed during fermentation. Then, the supplied amount of the pH-control solution per unit time depends on the actual feeding time. That is, the pump automatically starts to operate when the pH decreases below the pH setpoint, and stops when the pH increases above this value. Hence, as the cell growth proceeds, the feeding times increase and the feeding period is also lengthened, so that the time interval between the feeding periods of the pH-control solution is shortened. Therefore, the integral of equations (10)-(13), (19c), (21), (22), (23), and (24) can be expressed as:

$$\int_0^t F_B dt = F_B \sum \Delta t_F \tag{28}$$

where $\sum \Delta t_F$ represents the sum of the operating time of the pH-control pump during the time interval from 0 to t. Equation (28) indicates that the supplied amount of the pH-control solution can be determined by only monitoring the operating time of the pump supplying the pH-control solution during fermentation.

Accordingly, a linear relationship is proposed between the residual biomass growth rate and the PHB production rate of *A. latus*. Generally, PHB-synthesizing bacteria, however, accumulate PHB in cells when cell growth is limited due to the depletion of some nutrients such as the nitrogen source, oxygen, phosphate, magnesium, or sulfur, etc. [12]. This means that the PHB production rate is no longer linear to the residual biomass growth rate. *A. latus*, however, synthesizes PHB during the exponential growth phase with no limitation of any nutrients.

In most fermentation processes, the pH is controlled automatically with feeding acids or alkali solutions.

Therefore, the proposed estimation method can also be applied to most fermentation systems. Moreover, if this estimation method is combined with a computer control scheme, it would be possible to estimate the concentrations of cells and nutrients on-line, and also to control nutrient concentrations optimally.

Acknowledgments

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NOMENCLATURE

a	constant in equation (3)
b	proportional constant in equation (3)
C	carbon source concentration (g/l)
F	feed rate (l/h)
H	required amount of pH-control solution to maintain initial pH (mol/g cell)
I	defined in equation (8)
J	defined in equation (9)
k	ratio of feed rates of F_C and F_B ($=F_C/F_B$)
M_i	concentration of mineral i (mol/l)
n	number of monomer in PHB
N	ammonium concentration (mol/l)
N_i	concentration of nutrient i except carbon source (g/l)
P	PHB concentration (g/l)
q	specific PHB production rate ((g PHB)/(g residual biomass)/h)
S_{BF}	concentration of pH-control solution (mol/l)
t	culture time (h)
V	volume of culture broth (l)
X	non-PHB residual biomass concentration (g/l)
$Y_{CO_2/P}$, $Y_{CO_2/X}$	ratio of amount of evolved carbon dioxide to PHB production and residual biomass synthesis, respectively (g/g)
$Y_{P/C}$	yield of PHB from carbon source (g/g)
$Y_{X/C}$, $Y_{X/Ni}$	yield of residual biomass from carbon and other nutrients, respectively (g/g)
$Y_{X/Mi}$	yield of residual biomass from nutrient i except carbon source and nitrogen source (g/g)

Greek Letters

α	correction factor of increment in culture broth volume (l culture broth increment/l fed)
γ_C, γ_{CO_2}, γ_R, γ_X	carbon contents of carbon source, carbon dioxide, PHB, and residual biomass, respectively
δ	nitrogen content of residual biomass
μ	specific growth rate of residual biomass (h^{-1})

Subscripts

F	feed
B	pH-control solution
C	carbon source
total	sum of feeding solution
0	initial

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