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Adaptive Estimation of Hairy Root Mass Using Conductometry

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Abstract An accurate and efficient method for measuring the mass of hairy roots using conductometry is established. A conductivity equation expressed in terms of the concentration of the ion species in the medium is suggested. By using this equation, the effect of the individual ions on the total conductivity can be quantitatively analyzed. An equation for the *in situ* estimation of the cell growth coefficient for determining the mass of hairy roots is established based on measurements of the nitrogen concentration and conductivity during cultivation. The proposed equation does not require preliminary experiments to determine the cell growth coefficient. Instead, the physiological characteristics of the plant species are reflected by introducing the cellular nitrogen content. Since the cell growth coefficient is determined by measuring the major ionic nutrient concentrations, it is more effective to express the dynamics of an actual culture system. This improved method for determining the mass of hairy roots was successfully utilized in a fed-batch culture system.

Key words: Plant hairy roots, conductivity, mass, cell growth coefficient, fed-batch

Plant cell/tissue cultures are one of the essential techniques for the production of economically valuable bioproducts, such as enzymes, flavonoids, pigments, pharmaceuticals, and the cell mass itself [2, 8, 9]. Thus, to optimize the bioprocess for the production of valuable products, fast and accurate measurements of the cell/tissue mass are very important. Generally, cell growth in suspension cultures can be evaluated by measuring one or more of parameters, such as the fresh or dry weight, optical density [16], protein and/or DNA contents, cell viability [4], and medium conductivity [7, 14]. However, the accurate measurement of cell growth in plant cell bioreactor is very difficult. Usually, plant cells are much bigger than microbes and grow as aggregates or embryo cells and organs. These facts make it difficult to obtain a homogeneous sample of a cell suspension, and therefore, more difficult to determine the cell mass in a plant cell culture system. In particular, in an organ culture using a bioreactor, it is impossible to obtain a homogeneous sample from the culture system. Therefore, research to obtain more efficient method for cell mass determination is essential for plant organ culturing.

Conductometry is an indirect method for cell mass determination, and is widely used in plant cell, hairy root, and myxobacterial culture systems [18]. A conductivity change in the medium is caused by the cellular uptake of ionic nutrients during cell growth. This cellular uptake of ionic nutrients during growth decreases the medium conductivity. The increase in the cell mass shows a linear correlation to the decrease in the conductivity of the medium [7, 12, 14]. The cell growth coefficient, β, can be defined as the increase in the cell mass per unit volume divided by the conductivity change due to a concentration change in the ionic nutrients consumed by the cells. Generally, the cell growth coefficient can be predetermined by the linear regression between changes in the dry weight and the medium conductivity under specific conditions, such as the cell line and culture medium. Since the cell growth coefficient is assumed to be a constant value, slight deviations between measurements and predictions have been observed in the lag and stationary growth phase [14]. If the environmental conditions change, the cell growth coefficient should be redetermined during the batch cultivation to apply the conductometry to further experiments. Yet, this is a time-consuming and labor-intensive process. Therefore, a new method for estimating the cell growth coefficient is a prerequisite for plant cell/organ culture systems.

Accordingly, the current study was undertaken to evaluate the effect of ionic nutrients on conductivity and suggests a conductivity equation expressed in terms of the concentrations of the ion species. Using this equation, the conductivity of various culture media can be predicted without measurement. In addition, a method for the *in situ*

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estimation of the cell growth coefficient was developed based on the medium conductivity change and nitrogen concentration during cultivation. This improved method for determining the mass of hairy roots was applied to batch and fed-batch cultivations.

MATERIALS AND METHODS

Culture Conditions

Carrot hairy roots were cultivated in a 250 ml Erlenmeyer flask (working volume: 50 ml) using Murashuge & Skoog's medium containing 30 g sucrose/l at 27°C and 120 rpm under dark conditions. The hairy roots were subcultured every 10 days in a 500 ml flask under the same conditions. The medium pH was adjusted to 5.8 with 2 N NaOH, followed by autoclaving at 121°C for 15 min.

Root Mass Measurement

The fresh hairy root weight was measured after being washed with distilled water and vacuum filtration on Whattman filter paper No. 2. The dry weight was measured after drying the roots at 80°C for 2 days.

Conductivity Measurement

The specific conductivity of the culture broth was measured using a conductivity meter (OM-1A, Toa Electronics, Co., Japan) with a conductivity cell (CG 201-PL) at 27°C.

Analysis

The elemental compositions of the roots were determined by GC (Yanaco MT-2, CHN corder). The sucrose concentration was measured by the DNS method [15] using a spectrophotometer (Pharmacia LKB., Ultrospec III), and the ammonium and nitrate concentrations were measured colorimetrically using the Berthlot [17] and Brucine [17] methods, respectively.

RESULTS AND DISCUSSION

Relationship Between Specific Conductivity and Ionic Components of Medium

First, the effect of various concentrations of the carbon source on the conductivity was investigated. Generally, sucrose, a major carbon source in plant cell cultures, is hydrolyzed into glucose and fructose by invertase located in the cell wall [3]. Thus, the effect of three types of carbon source on the conductivity was examined. As shown in Fig. 1, the conductivities exhibited very low values (below 0.05 mmho/cm) and the conductivity differences among the three types of carbon source were negligible. Therefore, the effects of the type of carbon source and concentration on the conductivity were considered to be insignificant.

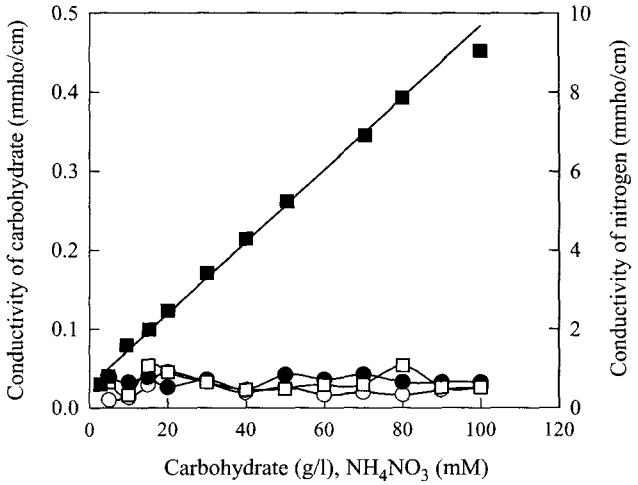

Fig. 1. Relationship between conductivity and nutrient concentration.
○: glucose, ●: fructose, □: sucrose, ■: NH_4NO_3.

The effect of the ammonium and nitrate concentrations on the conductivity was also investigated. The total concentration of nitrogen (ammonium and nitrate) in the MS medium was 60 mM, which accounted for over 60% of the total ionic nutrients in the MS medium. Ryu *et al.* [14] reported that the medium conductivity was directly proportional to the concentration of nitrate. As shown in Fig. 1, NH_4NO_3 showed a good linearity with the conductivity up to 80 mM. Since the initial concentration of the ammonium in the MS medium was 20.6 mM and that of nitrate was 39.4 mM, the conductivity could be successfully applied to a medium with a high concentration of nitrogen due to medium optimization or a fed-batch culture for controlling the nitrogen concentration.

The effect of the other important nutrients on the conductivity was also examined using various concentrations of KH_2PO_4, $MgSO_4$, $CaCl_2$, and NH_4Cl solution. The relationship between the nutrient concentrations and the conductivity exhibited a good linearity. In the case of KH_2PO_4 and $MgSO_4$, the conductivity measurements were performed at below 10 mM concentration, because the concentrations of these nutrients in the MS medium were about 1-3 mM. In all cases, the linearities were maintained up to 6 mmho/cm, and the experiments were performed at below the conductivity value of 6 mmho/cm to obtain reliable results. On the basis of these results, the specific conductivity of the individual ions in the MS medium is summarized in Table 1.

Generally, in a plant cell culture system, pH of the medium changes from 6 to 4 during cultivation. The profile of the medium pH normally shows an initial decrease during the lag phase and a slight increase during the exponential growth phase followed by a fairly constant value. Amino *et al.* [1] reported that this phenomenon was due to the

Table 1. Molar ionic conductivity of major inorganic components in MS medium.

Ion	Concentration (mM)	Conductivity (mmho/cm)	Molar conductivity (mmho/cm·mM)
K^+	20.10	0.979	0.0470
NH_4^+	20.60	1.070	0.0519
Ca^{2+}	2.09	0.132	0.0063
Mg^{2+}	1.50	0.132	0.0880
Cl^-	5.98	0.355	0.0594
NO_3^-	39.40	1.944	0.0493
$H_2PO_4^-$	1.25	0.060	0.0479
SO_4^{2-}	2.63	0.231	0.0880
Total	**93.55**	**4.903**	

physiological adaptation of the plant cells to enhance the invertase activity. Hence, the effect of the medium pH on the conductivity was examined.

As the pH in the MS medium changed, the association/dissociation reactions of $H_2PO_4^-$ and NH_4^+ were considered, respectively [10]. Using these equations, the value of the conductivity change due to a variation in the medium pH was determined. For a pH change of 3 (which is the maximum value in most plant cell culture systems), the variation in the concentration of ions was 0.0012 mM for NH_4^+ and 0.0078 mM for $H_2PO_4^-$. When the value of the conductivity change was very small (6.23×10^{-5} for NH_4^+, 3.74×10^{-4} for $H_2PO_4^-$), the effect of variation in the medium pH during cultivation on the conductivity measurement could be neglected. Therefore, based on the experiments, the equation for the specific conductivity related with the major ions in the MS medium would appear to be as follows.

$$K=0.0487[K^+]+0.0519[NH_4^+]+0.0631[Ca^{2+}]+0.0880[Mg^{2+}]+0.0594[Cl^-]+0.0493[NO_3^-]+0.0479[H_2PO_4^-]+0.0880[SO_4^{2-}]+\gamma \quad (1)$$

where γ is a constant that represents the sum of the contributions from the minor ions in the medium. The value of γ was found to be 0.18 in the MS medium. To evaluate this equation, the effect of the relative concentration of the MS medium on the conductivity was tested. When the medium was diluted, a proportionally decreasing conductivity was measured, and the measurements fit well with the values obtained when equation (1) was used (data not shown). The effect of various nitrogens in the MS media on conductivity was also tested. The conductivity measurements were found to be consistent with the results obtained from the calculation, as shown in Table 2. Yet, a slight deviation was observed at a high conductivity (above 6 mmho/cm).

Table 2. Comparison of conductivity between measured and calculated values in MS medium with various ion concentrations.

K^+ (mM)	NH_4^+ (mM)	NO_3^- (mM)	Cl^- (mM)	Measured (mmho/cm)	Calculated (mmho/cm)	Error* (%)
20.13	30	20	25.98	5.88	5.80	1.36
30.13	30	30	5.98	4.40	4.13	1.23
10.13	30	30	35.98	6.45	7.38	14.42
20.13	30	30	15.98	5.00	5.22	4.40
60.13	0	60	5.98	6.50	6.98	7.38
0.13	60	0	65.98	6.65	7.78	6.99

*Error (%)=|measured value-calculated value|/measured value×100.

Estimation of Cell Growth Coefficient Using Medium Conductivity and Nitrogen Concentration

The major ionic nutrients of an MS medium are ammonium and nitrate ions. These ions account for over 60% of the total ionic concentration. A change in the conductivity during cultivation is mainly caused by concentration changes in the nitrogen sources in a plant cell culture [7], and changes in the ammonium concentration in a myxobacteria culture system [18].

The growth of hairy roots was estimated using the following equation,

$$\Delta X=\beta\Delta K \quad (2)$$

where X: dry weight (g/l),
β: cell growth coefficient ($g\cdot l^{-1}/mmho\cdot cm^{-1}$),
K: conductivity (mmho/cm)

For an analytic estimation of the cell growth coefficient, β, the mass balance for nitrogen was derived from the following equation,

$$\frac{\beta C_N \Delta K}{14}=\Delta[NH_4^+]+\Delta[NH_3]+\Delta[NO_3^-] \quad (3)$$

where C_N: cellular nitrogen content (g nitrogen/g hairy root)

Figure 2 shows the batch profiles of the concentration of nitrogen, cellular nitrogen content, and β value. The cellular nitrogen content, C_N, represents the intracellular physiological state of the cell in the given environment, which is considered

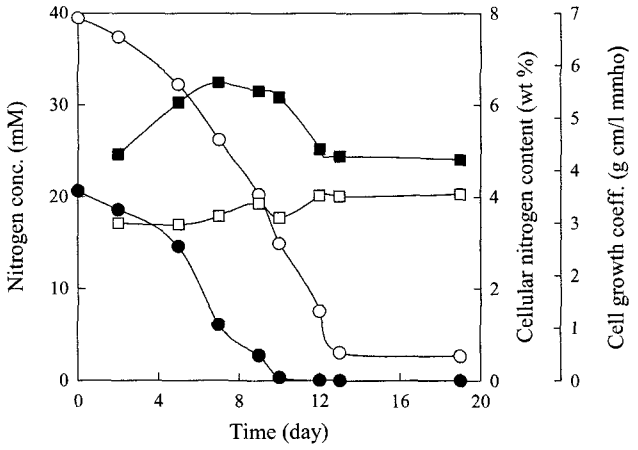

Fig. 2. Batch profiles of nitrogen concentration, cellular nitrogen content and cell growth coefficient.
○: NH_4, ●: NO_3, □: cellular nitrogen content, ■: cell growth coefficient.

as a constant [10]. It is assumed that the intracellular state is independent of the extracellular environmental conditions. However, after an increase in the cellular nitrogen content during the early growth phase, the cellular nitrogen content decreased as the nitrogen uptake rate of the hairy roots declined. Rose *et al.* [13] reported that a high rate of nitrogen uptake and metabolism relative to cell growth was followed by an increase in the cell mass relative to the cellular nitrogen during cultivation. The cellular nitrogen content has also been reported to be correlated with the concentration of ammonium in the medium [5]. Therefore, according to equation (3), the cell growth coefficient, β, is not a simple constant but a variable relative to the cell nitrogen content and concentration of nitrogen in the medium. Yet, it is difficult to determine the intracellular parameter, C_N, during cultivation, and it is preferable to express the cell growth coefficient by extracellular environmental parameters. As shown in Fig. 3, no significant relationship between C_N and β was detected, therefore, the average value of C_N from the experiment was used for the calculations in the other cases [5].

To develop an equation expressed using environmental parameters, the effect of the nitrogen concentration on the conductivity was quantitatively analyzed. Kino-Oka *et al.* [10] were the first to suggest an analytic expression of β, however, they estimated the nitrogen concentration an equation with a predetermined β. Gomez and Humphrey [6] suggested the parameter, $E_{E/N}$, that is the moles of nitrogen versus the total conductivity of the medium. They also kept the value constant during cultivation.

To estimate the cell growth coefficient, β, the conductivity equation (1) was simplified as follows:

$$K=0.05192[NH_4^+]+0.04934[NO_3^-]+\delta \quad (4)$$

To evaluate the effect of the concentration of nitrogen in the broth on the total conductivity during cultivation, a new dimensionless variable $K_{N/T}$ was introduced as follows,

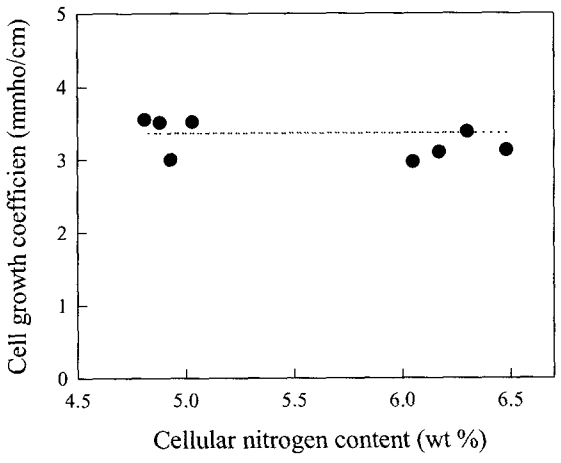

Fig. 3. Relationship between cell growth coefficient and cellular nitrogen content.

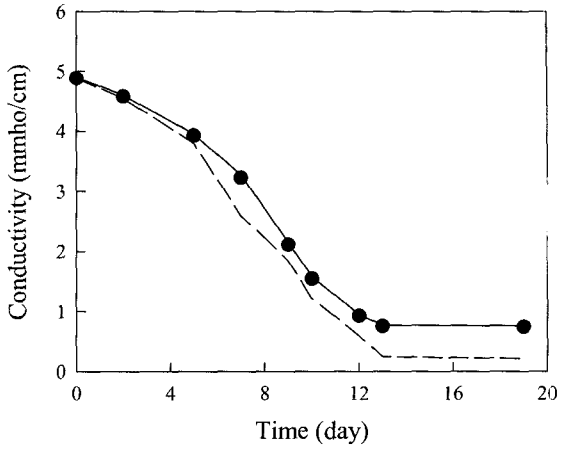

Fig. 4. Comparison of measured conductivity with calculated conductivity.
●: measurement, ——: variable $K_{N/T}$, -----: constant $K_{N/T}$

$K_{N/T}$=conductivity by nitrogen/total conductivity

$$(K_{N/T})_i=(K_{N/T})_o\frac{\{([NH_4^+]+[NO_3^-])/K\}_i}{\{([NH_4^+]+[NO_3^-])/K\}_o} \quad (5)$$

where $(K_{N/T})_o$=(1.07+1.944)/4.903=0.615

Equation (4) was modified using $(K_{N/T})_i$.

$$K_i=\frac{0.05192[NH_4^+]+0.04934[NO_3^-]}{(K_{N/T})_i} \quad (6)$$

Figure 4 shows that the conductivity measurement coincided with the value obtained from the equation (6), based on monitoring the nitrogen concentration during cultivation. Therefore, the cell growth coefficient, β, was *in situ* estimated using the following equation (7), while the mass of hairy roots was determined by using the equation (2).

$$\beta=\frac{14}{C_N}\frac{\Delta[NH_4^+]+\Delta[NO_3^-]}{\Delta K} \quad (7)$$

The time courses of the experimental β and calculated β are shown in Fig. 5(a) and Fig. 5(b), respectively. The cell growth coefficient, β, determined by experiment, was not a constant and exhibited an increasing trend during cultivation. As shown in Fig. 5(a), the time course of the calculated β value, determined using the variable C_N, showed a trend similar to the experimental value. After the cell growth coefficient decreased during the early growth phase, the value increased during the exponential growth phase. Although the experimental and calculated values showed the same profile during cultivation, the difference between the values was too wide. In addition, it is very difficult to determine the intracellular parameter, C_N, during cultivation. Using the constant $C_{N,ave.}$ system, as shown in Fig. 5(b), the profile of the calculated cell growth coefficient, determined by measuring the extracellular environmental concentration,

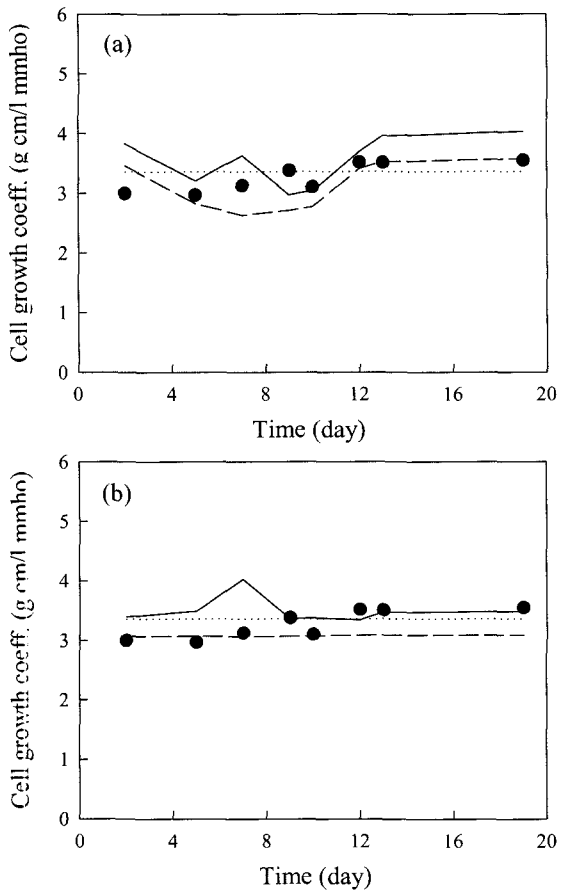

Fig. 5. Comparison of experimental values with calculated values for cell growth coefficient.
(a) variable C_N, (b) constant C_N. ●: measurement, ····: constant (3.36), —: variable $K_{N/T}$, ----: constant $K_{N/T}$.

was in agreement with the experimental profile, although a slight deviation was noticeable during the early growth phase. Furthermore, the mass of hairy roots obtained in the

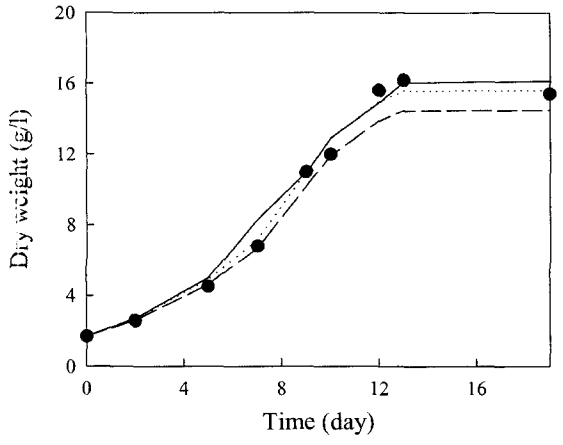

Fig. 6. Comparison of experimental values with calculated values for dry weight.
●: measurement, ····: constant (3.36), —: variable $K_{N/T}$, ----: constant $K_{N/T}$.

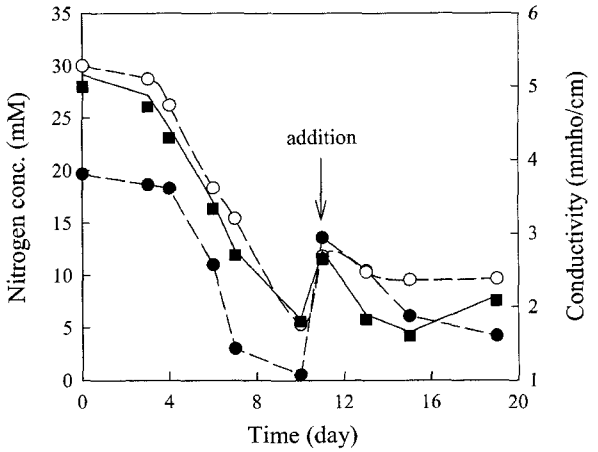

Fig. 7. Time cours of nitrogen concentration and conductivity in fed-batch culture.
○: NH_4, ●: NO_3, □: measured conductivity, —: calculated conductivity.

experiment displayed a good match with that obtained from the equation, as shown in Fig. 6.

Application of Conductivity to Fed-Batch Culture

To investigate the validity of the improved conductometry with environmental changes during cultivation, a fed-batch culture was performed. Ammonium and nitrate were added to the broth after cultivation for 11 days. As shown in Fig. 7, the concentrations of ammonium and nitrate in the broth increased to about 15 mM, and the profile of the conductivity showed the same trend as that for nitrogen. The conductivity measurement also exhibited a good agreement with the calculated conductivity, as shown in Fig. 7. Therefore, as environmental changes due to the feeding of nutrients affected a change in $(K_{N/T})_i$, an accurate estimation of the medium conductivity could still be performed.

To compare the previous reports using a constant β, the time courses of a predetermined β, empirical value β, and calculated value β are shown in Fig. 8. The empirically determined β showed an initial increase, then fairly constant value followed by an increase due to the feeding of nitrogen. The profile of the calculated value for the cell growth coefficient, β, showed the same trend as the empirical value, although a slight deviation was detected. Fig. 8 shows the time courses of the dry weight of carrot hairy roots actually measured by gravimetric determination and that estimated using conductometry. The results showed a good agreement, thus, the new method for the *in situ* estimation of the mass of hairy roots can successfully be applied to a fed-batch system.

The proposed equation has many advantages over a predetermined constant cell growth coefficient. i) The equation does not require preliminary experiments. Instead, introduction of the cellular nitrogen content reflects the physiological

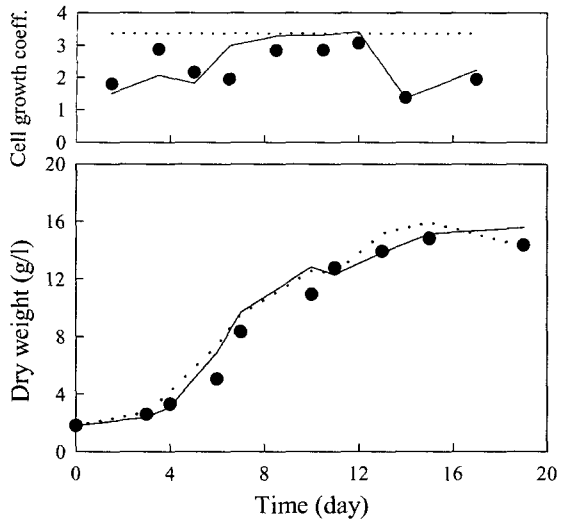

Fig. 8. Comparison of experimental values with calculated values for cell growth coefficient and dry weight in fed-batch culture.
●: measurement, ····: predetermined value (3.36), —: calculated value.

characteristics of the plant species. ii) Since the cell growth coefficient is determined by measuring the nitrogen concentration and conductivity, it is more effective in reflecting the dynamics of an actual culture system.

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