Kinetics and Modelling of Cell Growth and Substrate Uptake in *Centella asiatica* Cell Culture

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Abstract In this study, we have conducted kinetics and modelling studies of *Centella asiatica* cell growth and substrate uptake, in an attempt to evaluate cell growth for a better understanding and control of the process. In our bioreactor cultivation experiment, we observed a growth rate of 0.18/day, a value only 20% higher than was seen in the shake flask cultivation trial. However, the observed maximum cell dry weight in the shake flask, 10.5 g/L, was 14% higher than was achieved in the bioreactor. Ninety seven percentage confidence was achieved via the fitting of three unstructured growth models; the Monod, Logistic, and Gompertz equations, to the cell growth data. The Monod equation adequately described cell growth in both cultures. The specific growth rate, however, was not effectively predicted with the Logistic and Gompertz equations, which resulted in deviations of up to 73 and 393%, respectively. These deviations in the Logistic and Gompertz models may be attributable to the fact that these models were developed for substrate-independent growth and fungi growth, respectively.

Keywords: kinetics, modelling, *Centella asiatica*, Monod, Logistic, and Gompertz

INTRODUCTION

In recent years, interest in plant-derived pharmaceutical products and drugs has increased tremendously. In Malaysia, the value of the herbal market is estimated to be US$3.8 billion, and is growing by 10 to 20% annually [1,2]. The medicinal properties of *Centella asiatica* have been attributed to its secondary metabolites, the triterpenoids (TTP).

The study of kinetics provides insight into the dynamics of cell culture systems, as well as the interactions of organisms with their surrounding environments. Cell growth in a batch system is limited to the availability of essential nutrients and/or the accumulation of toxic products. This interaction can be assessed to construct a cell growth profile, which can then be subdivided into the lag, exponential, stationary, and death phases. Better understanding of these dynamics, as well as the determination of important kinetic parameter values, will facilitate both the optimization and scaling-up of bioprocesses.

Mathematical modelling generates quantitative knowledge which can then be utilized in the description and prediction of the behavior of a process under the influence of control variables. The kinetic parameters derived from the experimental data can then be fitted into mathematical models, such as the Monod, Logistic, and Gompertz equations, allowing for the description of metabolic reaction rates and their stoichiometry on the basis of present and past fermenter conditions. The description of actual processes and the mathematical prediction of probable bioreactor performance may prove useful with regard to advances in large-scale culture protocols. The growth of plant cells has been previously modelled using several unstructured models, including the Logistic, modified Monod, Gompertz, Contois, and Aiba equations, as well as modified Pazoutova's models [3]. Meanwhile, product synthesis is generally described via the Leudeking-Piret equation.

In this paper, we have conducted analyses of cell growth, sugar, ammonium, nitrate and phosphate uptake kinetics of *C. asiatica* cell suspension cultures in both shake flask (M) and bioreactor systems (B). The kinetic parameters were calculated via simple kinetic models. The experimental data has also been fitted into three unstructured models, namely the Monod, Logistic, and Gompertz equations.

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Materials and Methods

Cell Suspension Cultures

C. asiatica cell suspension cultures were initiated from callus culture obtained from the leaves of a CA-01 accession. The stock cultures were maintained as previously described [4]. Five mL batches of 7-day-old stock cultures were used as inocula. The cells were harvested daily to obtain the kinetic profiles for the shake flask cultures.

Bioreactor Inoculum Preparation

Cell suspension cultures for the bioreactor inocula were prepared via the inoculation of 80 mL of 7 or 8 day old stock cultures into 320 mL of M medium in 1,000 mL Erlenmeyer flasks with side-flow channels, to allow for bioreactor inoculation (Pyrex). The cultures were grown for 7 days prior to inoculation into the bioreactor.

Bioreactor System

The setup of the bioreactor system is shown in Plate 1. The stirred-tank bioreactor (Biostat A, B. Braun) consisted of a 2.5 L jacketed glass vessel (2 L working volume), equipped with 2-45°- inclined paddle-type impellers controlled at a constant rate of 100 rpm. The tip speed calculated from the impeller speed and geometry was 0.314 m/s. For better mixing, 4 equally spaced baffles were incorporated. Air was introduced via a stainless steel microsparger. Dissolved oxygen (DO) was detected using a sterilizable oxygen electrode (Ingold). Nonetheless, the oxygen electrode malfunctioned; therefore, we were unable to monitor dissolved oxygen throughout the entire culture period. Culture pH was determined with a pH electrode (Ingold). Culture temperature was controlled at 25 ± 1°C using a water jacket with a Frigomix®U unit (B. Braun). However, water loss due to evaporation by wet air exiting the bioreactor was not accounted for in this experiment. Foam was detected using an antifoam sensor, and the amount of antifoam addition was controlled by a PID controller. The cultures were illuminated by external fluorescent light at an intensity of 500 lux, generated by a table lamp. A stainless steel tube with an inner diameter of 0.25 in was used for the daily sampling of the culture. The tip of the tube was located at the bottom of the vessel, slightly below the impeller, to ensure that 10 mL of heterogeneous samples were sucked out.

Analytical Procedures and Statistical Analysis

Cell growth and triterpenoid contents were analyzed as previously described [4].

Statistical Analysis

All data are expressed as the mean values of two replicates, and were statistically analyzed via Duncan's Multiple Range Test using the SAS® System Release 6.12, Cary, USA.

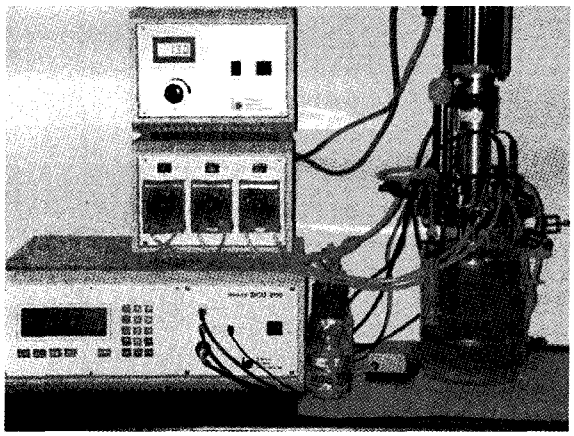

Plate 1. Biostat A assembly with stirrer-motor mounted to the top-plate of a 2.5 L glass vessel bioreactor.

RESULTS AND DISCUSSION

Kinetics of Cell Growth, Culture pH and Triterpenoids Production

The kinetics of cell growth and culture pH in the shake flask (M) and bioreactor (B) systems are depicted in Figs. 1A and B, respectively, and the kinetic parameter comparisons are shown in Table 1. Lag phase was not apparent in both cultures, but growth occurred slowly over the first 6 and 9 days in M and B, respectively. The exponential phase for M occurred from days 6 to 14 and days 9 to 21 in B. The maximum specific growth rates (μ_{max}) for M and B were 0.15 and 0.18/day, corresponding to a doubling time of 4.7 and 4.0 days, respectively. The doubling time of the cultures between the 4 and 5th days were comparable to the 5-day values reported for the *Morinda elliptica* [5] and *Nicotiana tabacum* [6] cell cultures. The maximum biomass attained in M was at 10.5 g/L, and lower in B, at 9.6 g/L. The maximum cell dried weight (CDW) is not necessarily indicative of the cell growth rate. Cell productivity was found to be higher in M at 0.86 g/L/d and B at 0.46 g/L/d. The cells entered the stationary and death phases on day 15 for M, and day 21 for B.

Foam formation, at the beginning of the culture, trapped the cells within the foam, thus decreasing initial inoculum density, and lengthening the culture period for B. It has been reported, in a study of an *Atropa belladonna* cell culture, that 55% of cells were detected in the foam layer after only 30 min of foaming [7]. The low dry cell weight observed in B was attributed to the relatively low pH observed throughout the culture period [8], and the loss of cells to foaming developed at an early culture stage [9]. As is shown in Fig. 1B, a drop in the pH of the culture, from 5.2 to 3.9, was observed on day 3 in M, and a drop to 2.7 was observed on day 9 in B. These drops are very consistent with the lag phase or the onset of the accelera-

Table 1. Comparison of kinetic parameters

Parameters	M	B
X_{max} (g/L)	10.5	9.6
μ_{max} (1/d)	0.147	0.175
t_d (d)	4.71	4.03
t (d)	15.0	21.0
P_r (g/L/d)	0.86	0.46
$q_{s\,max}$ (g sucrose/g cells/d)	1.65	3.75
$Y_{x/s}$ (g cell/g sucrose)	0.64	0.47
$Y_{mo/s}$ (mg MO/g sucrose)	0.25	ND
$Y_{ao/s}$ (mg AO/g sucrose)	0.21	ND
$Y_{ma/s}$ (mg MA/g sucrose)	0.29	ND
$Y_{aa/s}$ (mg AA/g sucrose)	0.02	ND

ND; not detected.

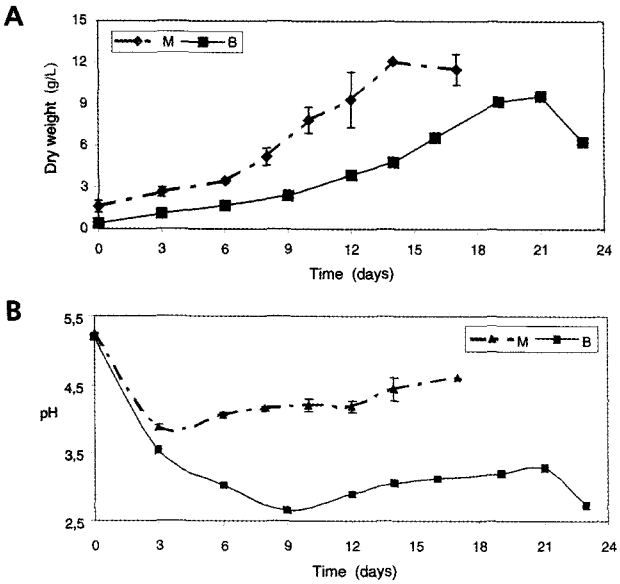

Fig. 1. Profile of cell dry weight and culture pH of *C. asiatica* cell cultures in shake flask (M) and bioreactor (B) experiments.

tion phase. The pH level of 2.7 in B may explain the slow cell growth culture rate. The pH value increased gradually to 4.5 in M from day 14 onwards. In B, however, the pH level remained below 3.5 from day 9 onwards. A similar pattern of changes in the culture pH of media have also been observed in several other suspended culture systems, including suspensions of *Catharanthus roseus* [10] and *M. elliptica* [5].

Madecassoside (MO) was not detected for the first 8 days in the M culture (Graph not shown). This corresponds well to the lag and acceleration or early exponential phases. MO production was detected at 3~4 mg/g CDW from day 10 onwards. Asiaticoside (AO) was not detected until day 10, at 1 mg/g CDW, and this increased gradually, to a maximum on day 17 of 3.34 mg/g CDW. Although Madecassic acid (MA) was detected at a level of 1.5 mg/g CDW on day 8 and reached a maximum on of 4.8 mg/g CDW on day 12, the detectable level of Asiatic

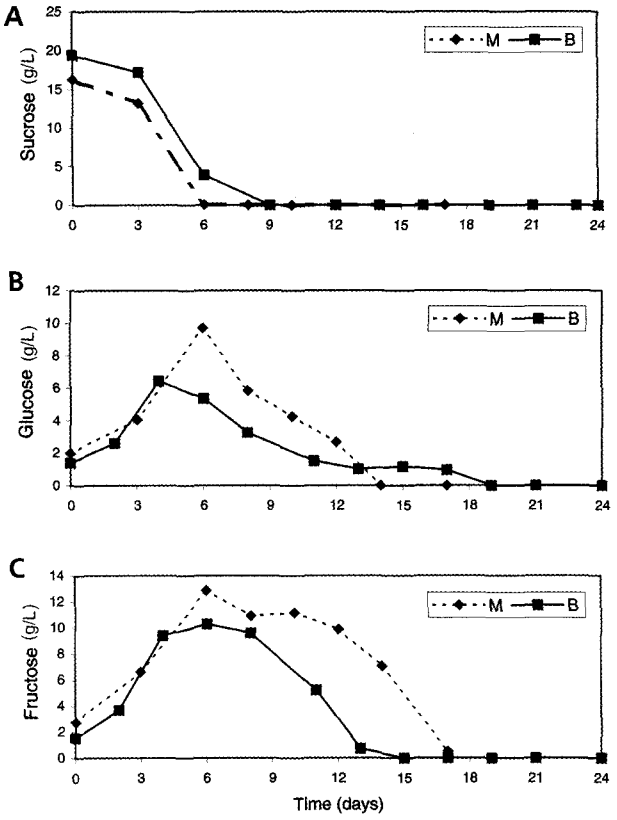

Fig. 2. Sucrose, glucose, and fructose uptakes in shake flask (M) and bioreactor (B).

acid (AA) remained below 1 mg/g CDW. TTP were detected in trace amounts in B. The product yield coefficients for the M culture are listed in Table 1. The yield coefficients were as follows: 0.25 mg MO, 0.21 mg AO, 0.29 mg MA, and 0.02 mg AA per gram of sucrose. The product formation categories were determined using the Leudeking-Piret equation. Each of the TTP categories were determined in the M culture (Data not shown). Plots of dP/dt against dX/dt and dP/dt against X did not indicate any straight-line relationship. Our failure to categorize TTP productions as growth, non-growth, or mixed growth-associated may be the result of the relatively low production yields with respect to cell growth.

Kinetics of Sugar Uptakes

Sugar uptake profiles in the M and B cultures are shown in Figs. 2A, B, and C. On the day of inoculation, the sucrose concentrations were below 30 g/L in cultures M and B, with the presence of glucose and fructose, which was attributed to autoclaving-induced hydrolysis. Sucrose was completely hydrolyzed into glucose and fructose after 6 days in culture M and 10 days in culture B. The concentrations of both glucose and fructose increased to a maximum coinciding with the day on which the sucrose had become completely hydrolyzed. Glucose was preferentially uptaken over fructose in both the M and B cultures. The concentrations of hexose sugars were

also observed to increase for the first 6 days in cultures M and B. Higher sugar consumption rates were observed during the exponential phase, on days 3 to 6 in cultures M and B, respectively. Glucose was completely used up in both the M and B cultures before the cultures achieved maximum cell concentration. Fructose was only completely used up during the stationary phase, during which the carbohydrate source may have been utilized primarily for cell maintenance.

As is shown in Table 1, the dry cell weight obtained per unit of sucrose consumed was higher in culture M, at 0.64 g cell/g sucrose, and lower in culture B, at 0.47 g cell/g sucrose. The wall-bound and excreted plant invertases responsible for sucrose hydrolysis are at their optimal levels at acidic pH values of around 4 [11]. In cultures M and B, during the lag phase and early exponential phase, pH values of around 4 were observed. This might explain, in part, the high sucrose hydrolysis rates observed in cultures M and B between days 3~6. Sugar uptake rates were predominant during the exponential phase, as the result of the relatively high metabolic activity. The accumulation of biomass has been closely associated with sugar concentrations in the medium, with the onset of dry biomass reduction coinciding with the complete removal of total sugars from the medium. This close association is suggestive of rapid metabolism, rather than storage, of the majority of the absorbed sugar [12]. *Vitis vinifera* cell cultures [13] and various *Papaver* species [14] do not accumulate and store carbohydrates in the form of starch granules, which induces cell death during sugar exhaustion. The high value of the total sucrose yield to biomass in M is comparable to that observed in a *C. roseus* culture at 0.78 g DW/g sucrose [15].

Nitrate, Ammonium, and Phosphate Uptake Kinetics

Nitrate, ammonium, and phosphate kinetics in the M and B cultures were shown in Figs. 3A, B, and C. Nitrate concentrations in M were depleted completely on day 14, coinciding with the maximum cell concentration. Ammonium uptake in M was relatively low over the initial 8 days, prior to the rapid uptake observed during the exponential phase. The nutrient consumption rate was slower in culture B than in culture M. The slow growth rate in the B cultures might be the result of the slow rate of ammonium, nitrate, and phosphate uptake. The slight increase in pH values after days 3 and 9 in M and B, respectively, was presumably attributable to the simultaneous uptake of ammonium and nitrate. Nitrogenous compounds are incorporated into the cells as amino acids, proteins, and nucleotides [16]. In rice [17] and *Panax notoginseng* [18] cultures, ammonia was completely depleted concomitantly with nitrate uptake. In strawberry cultures, ammonium and nitrate uptake occurred simultaneously [19]. In a *Taxus cuspidata* cell culture, nitrate uptake was shown to occur preferentially, supporting protein synthesis for growth and osmoregulation, as nitrate can be stored within the vacuoles [12]. The slower nutrient uptake rate registered in the B culture is thought to be indicative of slower metabolic activity within the

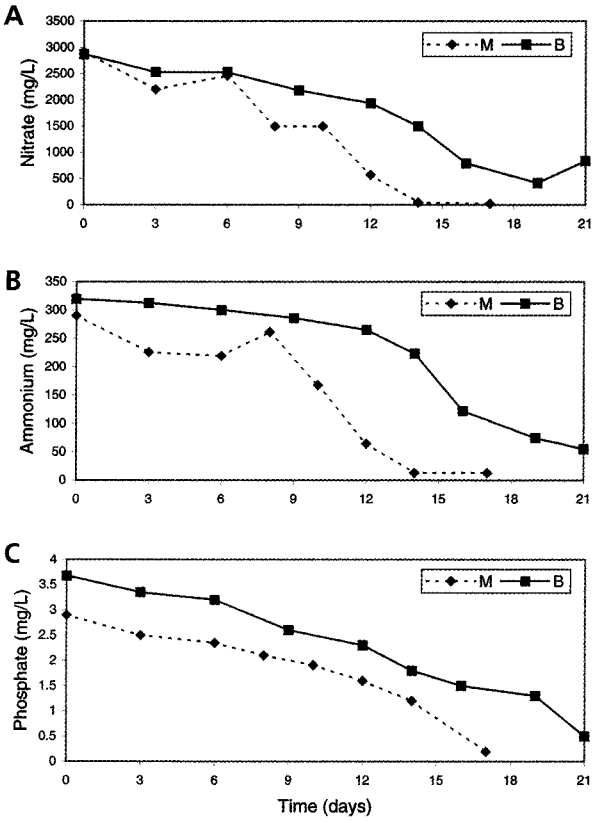

Fig. 3. Nitrate, ammonium, and phosphate uptake profiles in cultures M and B in shake flask (M) and bioreactor (B).

cells. The bioreactor operation in this study may also have been sub-optimal. The utilized phosphate is stored in the cells in some cultures, including cultures of *Panax quinquefolium* [20]. In this study, the cessation of cell growth occurred simultaneously with the complete exhaustion of ammonium, nitrate, and phosphate. Phosphate has been reported as a limiting nutrient in opium poppy cell cultures [21].

Modelling of Cell Growth and Substrate Uptake

Three unstructured models: the Monod (MD), Logistic (L), and Gompertz (GZ) models, were fitted to the cell growth data from the M and B experiments. The equations for these models are as follows:

$$\text{Monod: } \frac{dX}{dt} = \left[\left(\frac{\mu_{max} S}{K_s + S}\right)\right] X \quad (1)$$

$$\text{Logistic: } X(t) = \frac{X_o e^t}{1.0 - \left(X_o / X_m\right)(1.0 - e^t)} \quad (2)$$

$$\text{Gompertz: } \ln\left(X/X_o\right) = X_{max} \exp[1 - \exp(\mu_{max} / X_{ma})(\lambda - t)] \quad (3)$$

Each of the symbols is described in the Nomenclature

Table 2. Comparison of the performance and the kinetic parameter values of cell growth[a]

Parameters	M				B			
	E	MD	L	GZ	E	MD	L	GZ
X_{max} (g/L)	10.5	10.6	9.93	10.9	9.60	9.38	7.77	8.91
μ_{max} (1/d)	0.15	0.15	0.26	0.71	0.18	0.17	0.23	0.53
t_d (d)	4.71	4.62	2.67	0.98	4.03	4.01	2.96	1.31
λ (d)	3.0	3.0	2.0	3.0	9.0	10.5	9.0	12.0
P_r (g/L/d)	0.86	0.87	0.61	0.93	0.46	0.51	0.39	0.54
$Y_{x/s}$ (g cell/g sucrose)	0.64	0.65	0.61	0.65	0.47	0.48	0.39	0.57
K_s (g/L)	0.08	0.08	NA	NA	0.06	0.06	NA	NA
t-test (%)	NA	99.0	97.0	99.0	NA	100.0	100.0	100.0

[a]NA; not applicable, E; experimental.

list. Eq. (1) is iterative and the Berkeley-Madonna (Version 8.0.1, Macey, R. & Oster©, USA) simulation program was used for model fitting. For Eqs. (2) and (3), the MS-Excel spreadsheet program was used.

The kinetics of substrate uptake were evaluated using Eq. (4), as below:

$$-\frac{dS}{dt}=\frac{r_x}{Y_{x/s}} \tag{4}$$

The evaluation of the significance of deviation of the experimental and calculated data was conducted using statistical analysis (unpaired t-test) in the MS-Excel program.

Fig. 4A shows the growth curves of experimental and calculated data using the Monod (MD), Logistic (L), and Gompertz (GZ) models for the shake flask cultivation trials. Bioreactor cultivation evidenced a similar profile (Fig. 4B). All of the models were sufficient to describe the growth of the *C. asiatica* culture in both systems. These models fit well with the experimental data, with over 97% fitness (t-test). The μ_{max} value was selected to fit the experimental data to the model, such that a confidence level of 97% or higher (t-test) was achieved. The growth parameters (X_{max}, μ_{max}, t_d, λ, Pr, and $Y_{x/s}$) for the experimental (E) and calculated data using the three models are tabulated in Table 2. All growth parameters in the M and B cultures calculated using the Monod equations matched closely with the parameters calculated from the experimental data, better than those calculated using the Logistic model or the Gompertz equation. The deviation from the experimental data for the μ_{max} of the M and B cultures ranged from 28 to 73% in the Logistic model and 194 to 393% in the Gompertz equation.

The deviation from experimental data observed in conjunction with the Gompertz equation was far in excess of the deviation associated with the Logistic model, and this is probably attributable to the fact that the former was developed for the modelling of fungal growth. The deviation associated with the Logistic model was attributable to the fact that the model was developed to model substrate-independent growth. Cell growth is very much dependent on substrate availability, and most notably, sucrose availability. The inclusions of lag phase values (λ) in the Gompertz equation help to ameliorate this deviation. Another possible contribution to the observed deviations was the inability of the two models to predict expansion or lysis phase [22]. These models were developed for the evaluation of microbial cultures, and therefore do not consider the characteristics of plant cell cultures which are not shared with microbial systems, including the susceptibility of plant cells to shear [22], the existence of nonviable cells in much higher proportions in plant cell culture [23], the leaking of secondary metabolites into the medium [24], and the heterogeneity of plant cells [25]. All these factors may contribute to the deviations observed in simple, unstructured model equations.

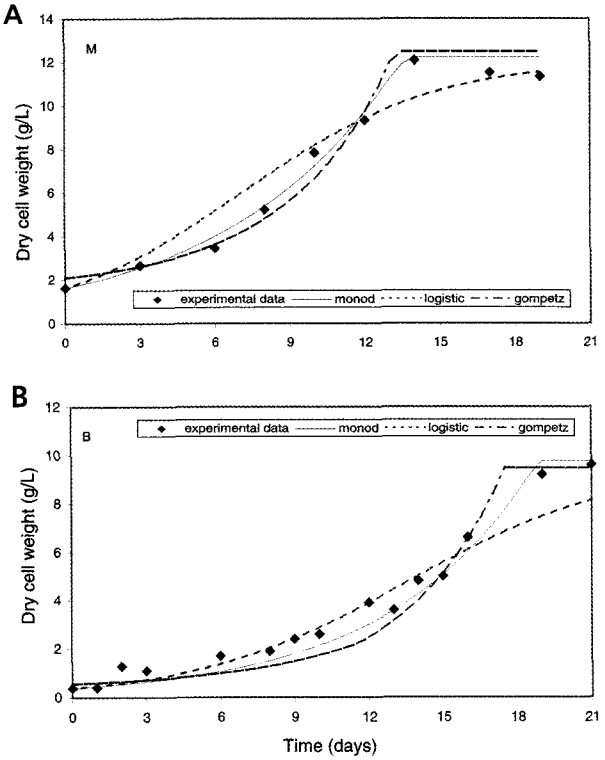

Fig. 4. Comparison of calculated and experimental data for cell growth.

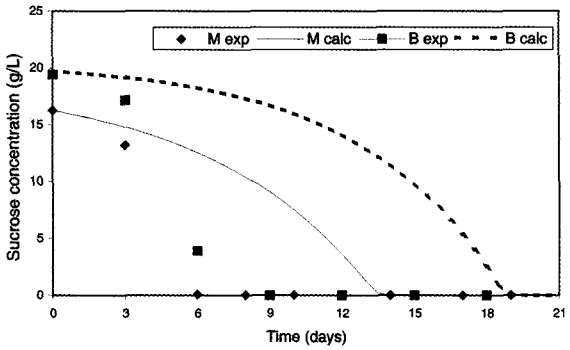

Fig. 5. Comparison of calculated and experimental data for sucrose uptake in batch culture.

The experimental and calculated sucrose uptake values recorded for the M and B cultures are listed in Fig. 5. From the graph, the model can be shown to deviate enormously from the experimental data. The model accurately describes the experimental data only during the early lag phase. Thereafter, sucrose depletion is predicted at a slower rate than was observed in the actual data. These deviations may be attributable to the fact that this model did not account for the hydrolysis of sucrose into glucose and fructose, but rather accounted only for substrate utilization by the cells. The model has been used primarily to describe glucose uptake in microbial cultures. However, plant cells may not directly utilize sucrose, but may take up the hydrolyzed sugar as glucose and fructose.

CONCLUSION

The kinetics of cell growth, culture pH, sugar, and macronutrient uptakes were studied in both shake flask (M) and stirred-tank bioreactor (B) systems, both of which employed a maintenance medium. The growth rate in culture B was approximately 20% higher than that observed in culture M, but the maximum cell concentration was 14% higher in culture M than in B. The biomass yield coefficient in M at 0.64 g/g sucrose was approximately 1.4 times higher than that of B. Sucrose, glucose, and fructose were utilized fully in both the M and B cultures. The Monod model satisfactorily described cell growth in all of the examined cultures. The substrate uptake model proved an accurate predictor only during the early lag phase of both cultures.

NOMENCLATURE

K_s saturation constant (g/L)
P product concentration (mg product/g DCW)
P_r Productivity (g/L/d)
r_x rate of cell growth (g/L/d)
S substrate concentration (g/L)
t cultivation time (d)
t_d doubling time (d)
X cell concentration (g/L)
X_m maximum cell concentration (g/L)
X_o initial cell concentration (g/L)
$Y_{x/s}$ growth yield coefficient (g CDW/g sucrose)
λ lag time (d)
μ specific growth rate (1/d)
μ_{max} maximum specific growth rate (1/d)

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