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The Culture of Chicken Embryo Fibroblast Cells on Microcarriers to Produce Infectious Bursal Disease Virus

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ABSTRACT

The cultures of chicken embryo fibroblast (CEF) cells in flasks, spinner bottles, and bioreactors were studied. The growth and metabolism characteristics of CEF cells and the feasibility of the CEF cell culture in bioreactor were investigated. The plating process of the CEF cells on GT-2 microcarriers in spinner bottles was studied, and a plating kinetic model was presented. The culture of CEF cells in 1.5 L CelliGen bioreactor to produce infectious bursal disease virus (IBDV) had met success. Whereas the additive microcarriers were fed during the culture, the cell density was increased 10 times as against seed cells adhering to microcarriers and the virus titer was as high as 7.5. All the aforementioned experimental results have laid the foundation for high density culture of CEF cells and process scale-up.

Index Entries: Bioreactor; chicken embryo fibroblast cell; cell culture; infectious bursal disease virus; microcarrier; plating kinetics.

INTRODUCTION

It is generally acknowledged that some cell lines can grow in suspension, and other lines and most primary cells are anchorage-dependent and require a substratum, on which they can adhere and spread for growth. Microcarrier culture is a culture system in which animal cells are grown as a monolayer on the surface of small solid particles and kept suspended in the culture medium by gently stirring. In this way, the characteristics of both suspension culture and monolayer culture are brought together and retaining all advantages of both culture systems. So it is possible to scale

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up the anchorage-dependent cell culture. By the large scale and high density animal cell culture, many valuable therapeutic biomedicines such as virus vaccines, monoclonal antibodies, interferon, and so on can be produced. With the development of society, the more the requirement of biomedicines is, the larger the cell culture scale is *(1)*.

The CEF cell is one of the commercially important primary cells, and can be used to produce many chicken vaccines, such as infectious bursal disease virus vaccines, Marck's disease virus vaccines, New City virus vaccines, and so on, and to express some genetic products *(2,3)*. In the early time, the culture was mainly performed in flasks or roller bottles on small scale. With the development and application of microcarrier technology, the culture scale and the virus yield have been greatly increased and a CEF cell culture in 140 L bioreactor was reported *(4)*. But in industrial practice, the CEF cells are cultivated still in roller bottles to produce chicken vaccines with the complicated operation, great labor, and frequent contamination. At the same time, SPF embryonated chicken eggs are expensive and in shortage in China, and the vaccine type and quantity are not satisfied. Recently, the propagation of IBDV in continuous cell lines was investigated *(5–7)*. In this work, we studied the culture technology of CEF cells to produce IBDV and laid the foundation for high density culture of CEF cells and process scale-up.

MATERIALS AND METHODS

Cell

Chicken embryo fibroblast cells were prepared from 10-day-old eggs *(8)*. The embryonated chicken eggs were kindly provided by Shanghai Songjiang Bio-Pharmaceutic Factory.

Virus

Infectious bursal disease virus (IBDV) was kindly provided by Shanghai Songjiang Bio-Pharmaceutic Factory.

Media

The cells were cultivated in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MD) or LH (Hanks' salt with 0.5% lactalbumin hydrolysate [Dxuid Limited, England]) with 10% new born calf serum (NBCS, ECUST, Shanghai, P.R. China).

Microcarrier

GT-2 microcarriers (ECUST, Shanghai, P.R. China) were used in the study *(8)*. They were made of denatured collagen by suspensive cross-linking technology. In phosphate-buffered-saline (PBS) solution, they were

transparent beads with a diameter of 120–200 μm, smooth surface, specific density of 1.02–1.04, and swelling factor (mL/g dry weight) of about 10. Microcarriers were equilibrated with PBS and autoclaved (121°C, 20 min) in batches of 1 g in 100 mL PBS. All glassware were presiliconized with silicon oil in order to prevent microcarriers from adhensing on the glass surface. The PBS was removed and fresh growth medium with appropriate serum was added. The microcarrier suspension was incubated at 37°C for at least one day, then the seed cells were inoculated *(8)*.

Cell Culture

The Culture in Flasks

Appropriate cells were inoculated in each of 25 mL-flasks, and fresh growth medium was added to 5 mL. Then cells were cultivated at 37°C in a 5% CO_2 incubator. Two or three flasks were sampled and analyzed daily.

The Culture in Spinner Bottles

Appropriate cells were inoculated in spinner bottles (500 mL, Wheaton, U.S.A), and fresh growth medium was added to 200ml. Then, cells were cultivated on biological stirrer (80rpm) in a 5% CO_2 incubator at 37°C. Samples were taken for analysis and 25–75% medium of the culture volume was changed during the culture.

The Culture in 1.5 L CelliGen Bioreactor

In a siliconized bottle with prepared microcarriers, one third of medium of last working volume was added and the cells were inoculated. Then, the bottle was incubated at 37°C. Each 45 min, the bottle was softly shaken to increase the attachment and adsorption of cells on microcarriers. After 6 h, the bottle content was transferred into bioreactor, fresh medium was added to the working volume (1200 mL), and temperature, dissolved oxygen (DO) and pH of the cell culture system were set and controlled automatically at 37°C, 50% air saturation and 7.10, respectively.

Virus Culture

When cells grew to a high density, IBD virus was inoculated. The inoculating virus was 4.7 mL virus liquid, which was virus titer of 3.0, per 10^7 cells. Then cells and virus were cultivated at 37°C for 3 or 4 d.

Analysis Methods

After cells on microcarriers were trypsinized for 10 min, the medium was added and softly blown. Cells were counted with a haemacytometer. The anchoraged cells were considered as viable cells *(8)*. Glucose and lactate concentrations were assayed with the GOD-POD method *(9,10)* and the

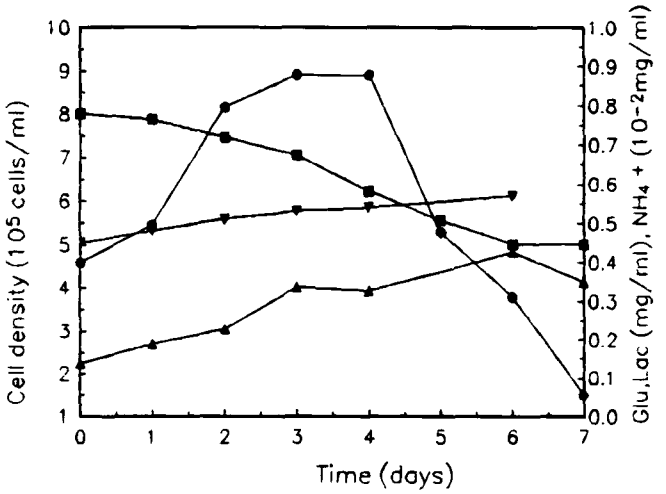

Fig. 1. The growth and metabolism of CEF cells cultured in LH medium. The inoculum density was 4.6×10^5 cells/mL. Glucose (■), Lactate (▲), Ammonia (▼), Cell density (●).

LDH method *(10)*, respectively. Ammonia was determined by urea nitrogen reagent kit *(11)*. Virus titers were assayed with the Behrens-Kärber method *(12)* and expressed with LD_{50}.

RESULTS AND DISCUSSION

Basic Growth Characteristics of CEF Cells

In the flask culture (25 mL, 5 mL medium), after inoculated with a density of 4.6×10^5 cells/mL the cells grew slowly in the first day because they were adapted to the new condition gradually and the essential factors needed by cell growth were synthesized continuously. In the second day, cells grew quickly and put into the exponential phase. Between the third and fourth days, cell densities attained maximum. Later, cells were into dead phase (Fig. 1). In correspondence with the cell density, glucose was depleted and lactate and ammonia were accumulated constantly with the increase of cell density. Later, when the cell viability declined, the rate of glucose consumption was decreased greatly. From the specific glucose consumption rate (q_{Glu}) and the specific lactate accumulation rate (q_{Lac}), the increase of lactate was consistent with the decrease of glucose. It implied that lactate mainly derived from glucose metabolism. From specific ammonia accumulation rate (q_{NH4}+), greater q_{NH4}+ in lag phase may be believed to be the vigorous synthetic metabolism of enzymes and chemical decomposition of glutamine. Greater q_{NH4}+ in the later stage could result from ammonia production by the lysis of died cells (Fig. 2).

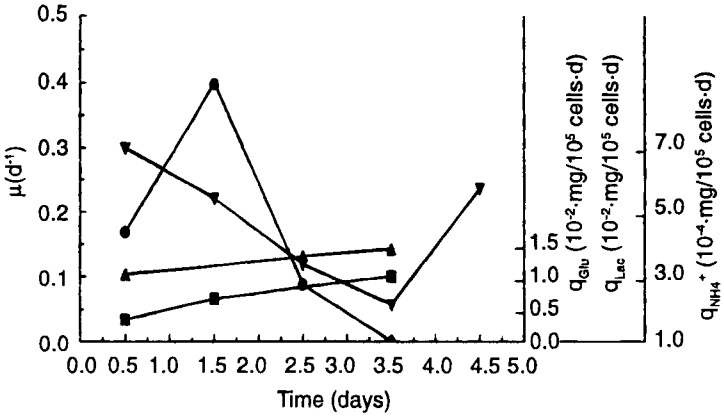

Fig. 2. The changes of the specific cell growth rate (μ) (●), the specific glucose consumption rate (q_{Glu}) (■), the specific accumulation rates of lactate (q_{Lac}) (▲), and ammonia (q_{NH4+}) (▼) during CEF cell culture in flasks.

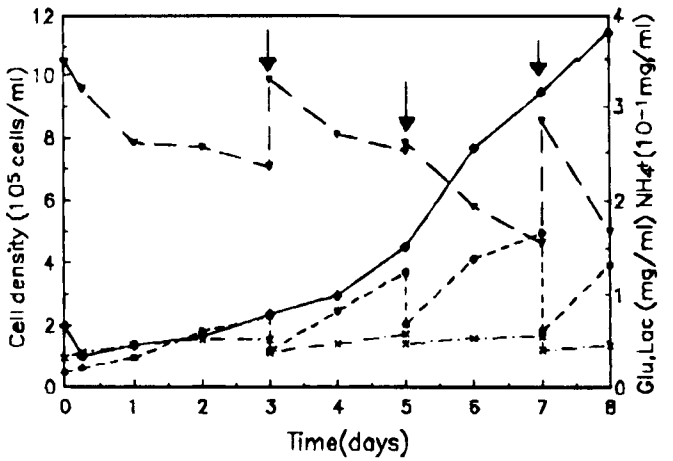

Fig. 3. The growth and metabolism of CEF cells cultured in a spinner bottle Cell density (◆), Glucose (▼), Lactate (●), Ammonia (X) (↓) changing medium.

Growth and Metabolism of CEF Cells in Spinner Bottles

2.0×10^5 cells/mL was inoculated in a spinner bottle with 200 mL medium in which 2g/L microcarriers were added. According to the cell growth, some medium was changed during the culture. The results were shown in Fig. 3.

In the cell growth process, the glucose consumption and the lactate accumulation were consistent with that in flask culture. The greatest specific cell growth rate was 0.5 d^{-1}, the greatest specific accumulation rates of lactate and ammonia were 0.19 and 0.001 mg/d/10^5 cells, respectively. On the eighth day, cell density attained to 1.15×10^6 cells/mL, and the micro-

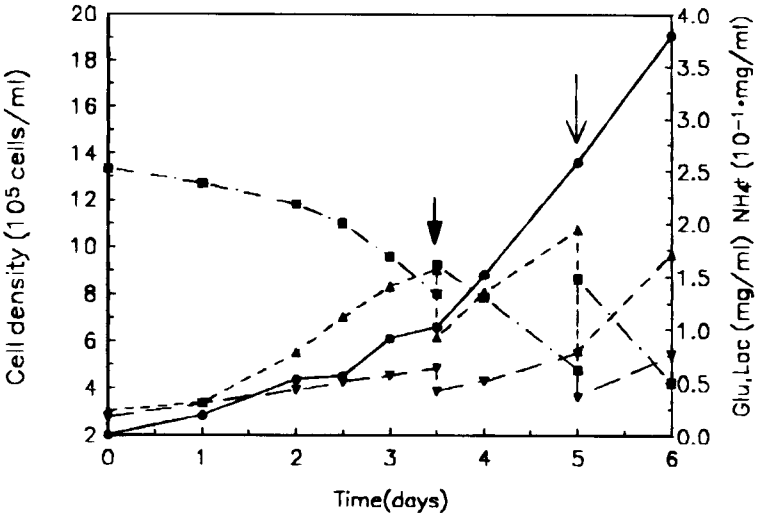

Figure 4. The growth and metabolism of CEF cells in 1.5 L CelliGen bioreactor Cell density (●), Glucose (■), Lactate (▲), Ammonia (▼) (↓) adding microcarriers and changing medium, (↓) changing medium.

carriers were covered with cells tightly. It was very difficult for cells to propagate continuously. At the time, IBDV was inoculated. After further culture of 3 d, the virus titer rose up to 6.25.

CEF Cell Culture in 1.5 L CelliGen Bioreactor

In 1.5 L CelliGen bioreactor, pH, dissolved oxygen (DO) and temperature were set and controlled at 7.10, 50% air saturation and 37°C, respectively. The pH was controlled at the set point by adding the 5.6% sodium bicarbonate or changing the CO_2 content in the gas phase. The agitation speed of the bioreactor was controlled at 30 rpm and 45 rpm before and after adding additional microcarriers.

At the beginning of culture, the cells grew quickly and specific cell growth rate was 0.3–0.35 d^{-1}, in comparison with the 0.19 d^{-1} in spinner bottles. The shorter lag phase derived from steady culture conditions in the bioreactor. When the cell grew to the density of 6.6×10^5 cells/mL after culture of 3.5 d, microcarriers were covered with cells and microcarriers of 2 g/L were added (the final microcarrier concentration was 5 g/L) with the medium change (Fig. 4). During 5 h after adding the microcarriers, agitation was stopped for the beads-to-beads transfer of cells and heating was switched off to avoid over-heat in parts. Every 30 or 45 min agitation and heating were run for 2 min to avoid the agglomeration of microcarriers and the lack of nutrients. The cells on old microcarriers had the tendency to transfer to new microcarriers when the old and new microcarriers contracted fully. "Bridges" between the old and new microcarriers appeared and the cells migrated to the new microcarriers. After 6 h, the new micro-

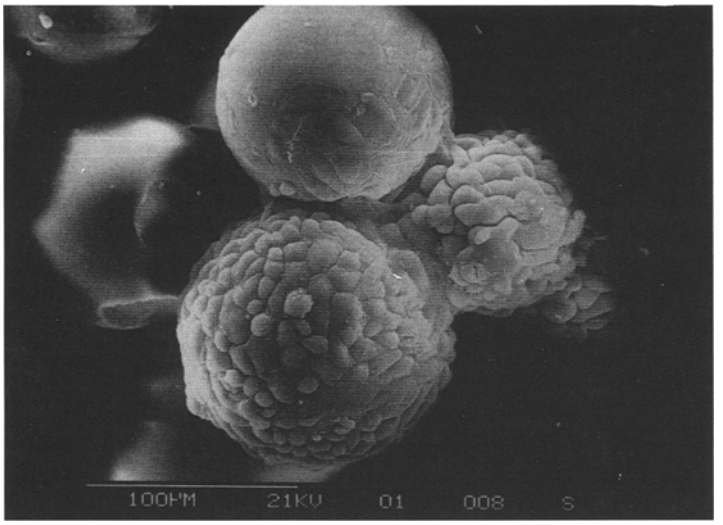

Fig. 5. The cell bridges between the old and new microcarriers.

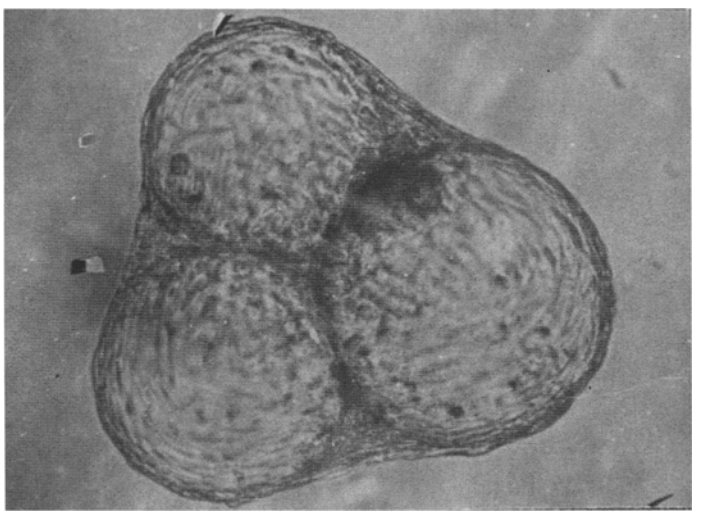

Fig. 6. CEF cells attached on GT-2 microcarriers after culturing for 6 d.

carriers were attached by cells (Fig. 5). Because of the increased attachment area, the enriched nutrient concentration and the diluted concentration of toxic by-products, the specific cell growth rate accelerated to about 0.4 d^{-1} and at the sixth day the cell density attained 1.91×10^6 cells/mL which was as 10 times as that at the beginning. Microcarriers were full of cells tightly (Fig. 6). New microcarriers would be added and fresh medium would be fed more frequently, otherwise it was difficult for cells to grow continuously. Considered the characteristics of primary CEF cells, IBDV was inoculated. After further 4 d culture, the virus titer was 7.5.

The Plating Kinetics of CEF Cells in Spinner Bottles

In the plating experiment, batch operation was taken. The cells inoculated in the spinner bottles were cultured in a 5% CO_2 incubator at 37°C. Every 1 h, the bottles were shaken to mix the cells and microcarriers. At the end of plating process, the bottles were put on biological stirrer and cells were cultivated regularly. It was defined that one period (1 h) was the time between bottle shaking and next. The plating rate of anchorage-dependent animal cells was previously considered to be the first order kinetics with respect to the concentration of the unattached viable cells *(13–15)*. It was expressed as:

$$\frac{dC}{dt} = k \cdot C$$

where, k is the rate constant (h^{-1}) and was influenced by the environment, cell quality, medium, and so on. C is the concentration of unattached cells with attachment ability. It was established only if the inoculated cell density was low, the positions that could be attached by cells were abundant and the chance which every cell attached to microcarrier was even. But in the experiment, the terms weren't met. Although the positions on microcarrier were much, the ones that can be used were limited because of the special effects of cells-cells and cells-microcarriers and the piled effects of microcarriers. At the same time, although some cells had the attachment ability, they hadn't the chance to attach in one period because of the far distance to the positions on microcarriers. So, the plating process could not be described as the first order kinetics simply. In the plating process, we defined two stages—contact stage and plating stage according to the batch operation. In the first stage—contract stage or mixture stage, cells contracted with microcarriers casually, and physical adsorption happened, but the effect was very small *(16,17)*. The contract chance was proportioned to the cells and positions on microcarriers. In the second stage—plating stage or stable stage, the cells adsorbed on microcarriers underwent some physiological reactions under the function of the plating factors. Thus, cells plated tightly and could not be separated from microcarriers in the regular conditions although the cells had not spread at that time. The plating kinetic model of CEF cells on microcarriers was expressed as:

contract stage:

$$C + M \xleftrightarrow{K} CM \tag{1}$$

plating stage:

$$CM \xrightarrow{k_2} CS \tag{2}$$

where

C: the unattached cells with attachment ability (cells/mL)
M: unattached position numbers on microcarriers which can be attached by cells in 1 mL
CM: cells that had been adsorbed to microcarriers but combined untightly (cells/mL)
K: the equilibrium constant in the contract stage
k_2: the rate constant in the plating stage
CS: cells adsorbed and plated on microcarriers tightly (cells/mL)

So,

$$CM = K \cdot C \cdot M$$
$$C = C_0 - CS_1 = a \cdot CT - CS_1$$
$$M = M_0 \cdot CMC - CS_1$$
$$CM_0 = K \cdot (C_0 - CS_1) \cdot (M_0 \cdot CMC - CS_1)$$
$$CM = CM_0 - (CS - CS_1)$$

where,

CT: the total inoculum cell density (cells/mL)
C_0: the density of cells which can be attached on microcarriers (cells/mL), and it was a fraction of the total inoculum cell density. $C_0 = a \cdot CT$. a ($0 < a < 1$) is the viability of inoculum cells, and is affected by the cell preparation process. Hence, a is different in different preparation batch
CS_1: the cells plated on microcarriers tightly at the end of the last period. At the beginning of plating process, CS_1 is zero
CS_2: the cells plated on microcarriers tightly at the end of this period
M_0: the position number which can be attached by cells on microcarriers in one milligram
CMC: microcarrier concentration (mg/mL)
CM_0: CM at the beginning of this plating period

Equation (2) can be considered the first order *(13–15)*, thus

$$-\frac{dCM}{dt} = k_2 \cdot CM \tag{3}$$

It is integrated as:

$$-k_2 \cdot \Delta t = \ln \frac{CM}{CM_0} = \ln \frac{CM_0 - (CS_2 - CS_1)}{CM_0} \tag{4}$$

$$K' = K \cdot (1 - e^{-k_2 \cdot \Delta t})$$
$$CS_2 = CS_1 + K' \cdot (a \cdot CT - CS_1) \cdot (M_0 \cdot CMC - CS_1) \tag{5}$$

Table 1
The Comparison of the Experimental and Calculating Values of the Plating Ratio of CEF Cells on GT-2 Microcarriers

Number	Inoculum density ($\times 10^5$ cells/ml)	Plating time (hs)	The experimental values	The calculating values
1	2	1	0.152	0.140
2	2	2	0.248	0.238
3	2	4	0.343	0.367
4	2	6	0.458	0.447
5	2	8	0.498	0.500
6	2	10	0.530	0.537
7	2	12	0.570	0.563
8	2	12	0.630	0.620
9	3	12	0.560	0.576
10	5	12	0.480	0.473

where Δt is the time of a period and 1 h in this experiment. When the same Δt is taken in the process, K′ is a constant.

The plating ratio is described as:

$$\phi = \frac{CS}{CT}$$

so,

$$\phi_2 = \phi_1 + K' \cdot (a - \phi_1) \cdot (M_0 \cdot CMC - \phi_1 \cdot CT)$$

ϕ_1: the plating ratio at the end of the last period
ϕ_2: the plating ratio at the end of this period

The parameters in the model can be estimated when CMC is 2 mg/mL and the spinner bottles were shaken one time every 1 h during the plating process. The cell viability is 0.65 according to the 1 to 7 date points and the cell viability is 0.72 according to the 8 to 10 date points because they were

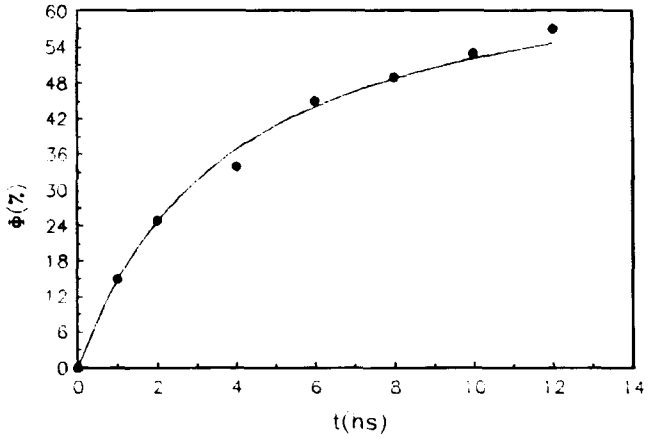

Fig. 7. The plating process of CEF cells on GT-2 microcarriers in spinning bottles.

not the same batch of cell preparation. K′ is 7.9×10^{-7}, and M_0 is 1.37×10^5. Table 1 and Fig. 7 compared the experimental values and the calculating values of the CEF cell plating ratio on GT-2 microcarriers.

In conclusion, growth and metabolism characteristics of CEF cells are similar to that of continuous cell lines. CEF cells can be cultivated on microcarriers in bioreactors. By the technology, cell density has been increased ten times as many as inoculating cell density and a higher virus titer has been attained. If the process is put into commercial production of IBDV, the benefit of the decreased requirement of SPF embryonated chicken eggs and enhanced virus yield will outweigh the increased cost because of the introduction of microcarriers and the prolongation of culture period. Its advantages will be especially obvious in large-scale production of IBDV or other virus vaccines.

NOMENCLATURE

μ	specific cell growth rate (d^{-1})
q_{Glu}	specific glucose consumption rate (mg/10^5cells/day)
q_{Lac}	specific lactate accumulation rate (mg/10^5 cells/day)
q_{NH4+}	specific ammonia accumulation rate (mg/10^5 cells/day)
CT	total inoculum cell density (cells/mL)
a	cell viability
CMC	microcarrier concentration (mg/mL)
CS	cells having plated on microcarriers tightly (cells/mL)
K	the equilibrium constant
k_2	the rate constant
ϕ	plating ratio

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