Modeling and Application of Controlled-fed Perfusion Culture of CHO Cells in a Bioreactor

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The controlled-fed perfusion culture, integrating the advantages of both the fed-batch and perfusion cultures, was applied in the culture of recombinant CHO cells expressing HBsAg. To control and optimize the process, a model was established to closely combine the feeding and perfusion operation on a basis of step-by-step strategy. The culture process was divided into many consecutive sections in the time dimension, and the feeding and perfusion rates in each section were calculated with the data in the former sections. The feeding operation was controlled by a feeding model to supplement with exact nutrients, while the perfusion operation was controlled by a perfusion model to keep byproduct concentration at low level. Linking by glucose, lactate and ammonia concentrations, the two separate models were integrated to globally control the cell culture process. By using this control strategy, the toxic byproducts were kept below their inhibitory concentrations, and a viable cell density of $13 \cdot 10^9$ cells L^{-1} and the HBsAg production of 1.65 mg L^{-1} were achieved in controlled-fed perfusion mode, representing an almost two-fold increase over a perfusion culture.

Key words:
Animal cell culture, bioreactor, CHO cell, controlled-fed perfusion, serum-free medium

Introduction

The market of recombinant proteins is now expanding for their use as diagnostic agents and therapeutic drugs. Most recombinant proteins under development are produced by mammalian cells because of their glycosylation ability, basically in a batch, fed-batch or perfusion culture mode. Each mode has its own merits and limitations with respect to the specific cell line and the expressed protein. To combine the advantages of both fed-batch and perfusion modes together, controlled-fed perfusion culture has been emerged and successfully applied in a case of hybridoma cells for monoclonal antibody production.[1] This culture mode is not only able to increase product concentration, but to reduce toxic metabolite accumulation and medium consumption. Principally, the medium was continuously perfused in and out a reactor to provide basic nutrients and discharge toxic byproducts, while the key nutrients were continuously fed to meet the demand of high-density cell growth and protein production. In such a controlled-fed perfusion mode, Feng *et al*.[2] reported an "oxygen uptake rate-amino acids (OUR-AA)" control strategy to maintain appropriate nutrient concentration and reduce dilution rate, in which the feeding of amino acids was controlled based on OUR variation. As a result, product concentration significantly increased. However, the decrease of dilution rate usually causes high byproduct accumulation such as ammonia and lactate because of the unbalance between the byproducts production and their elimination. Hence, the effects of byproducts accumulation on cell growth and protein production usually are the preparatory work to determine the threshold levels of these byproducts in the controlled-fed perfusion culture.[1,2]

As in a perfusion culture, the performance of a controlled-fed perfusion culture could be improved by dilution rate control and regulation to balance product concentration and byproduct accumulation. Feng *et al*.[2] attempted to control the perfusion rate according to CSPR (cell specific perfusion rate) in controlled-fed perfusion. However, the combination and modeling of feeding and perfusion controls of controlled-fed perfusion have not been reported and could have potential value for the control of byproduct and the improvement of productivity.

Three strategies have been reported to control nutrient supplement and perfusion rate in animal cell culture. The first one is based on a kinetic model dependent on bioreactor environmental parameters such as pH, OUR, specific consumption rates of amino acids and concentrations of glucose and glutamine.[3,4] In practice, the fed-batch culture or perfusion culture mode has been widely used to improve productivity. The fed-batch culture with serum-supplemented medium enhanced cell density

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by using a stoichiometric model for feeding strategy compared with non-kinetic model batch, fed-batch and perfusion culture.[5,6] Generally, these control models are cell line or interest protein specific, which restricts their applications, and lack of feedback control, which usually results in large deviations of culture parameters, unsatisfactory control accuracy and poor culture performance. The second strategy is on the basis of real-time automatic monitoring of some parameter such as glucose concentration, viable cell density, oxygen uptake rate (OUR), etc.[2,7,8] Real-time monitoring of OUR provides smooth, robust, and reliable signal and the monitoring scheme is applicable to mammalian cell bioprocesses of laboratory or industrial scale. It was found that the OUR indicating gradual reduction of cell respiration activity could provide an accurate control strategy for bioreactor. Nevertheless, a method like this must depend on the availability and quality of a highly automated bioreactor system. The third, a step-by-step strategy, is a combination of the two aforementioned strategies.[9,10] Hypothetically, the culture process is divided into many sections, and the operation in each section is controlled by kinetic model based on the metabolic characteristics of the cell line. The characteristics of cell metabolism were taken into account, and the feedback control was introduced into this strategy. Thus, the control accuracy can be improved in long-term culture, independent of complicated bioreactor system and frequent off-line analysis.

In this work, we established a combined control model for controlled-fed perfusion culture and applied it in the bioreactor culture of recombinant CHO cells for HBsAg production. The feeding operation was controlled by a feeding model to supplement with exact nutrients, while the perfusion operation was controlled by perfusion model to keep byproduct concentration at low level. These two models were linked by some variables to conduct the whole process. As such, the cell density and protein productivity were improved by sufficient nutrient supplementation and low byproduct level, and finally, an increased product concentration was achieved by lowering the perfusion rate.

Materials and methods

Cell line and medium

The recombinant CHO cell line expressing hepatitis B surface antigen (HBsAg) was provided by Wuhan Institute of Biological Products, China. Cells were cultured in DMEM with 10 % new-born calf serum, and were adapted into an in-house serum-free medium after about 10 passages. This serum-free medium is a 1:1 DMEM/F12 medium supplemented with amino acids, inorganic salts, vitamins, transferrin, and dextran sulfate, and trace elements.[11] All the reagents were purchased from Gibco or Sigma, USA. The osmotic pressure of serum-free medium was 320~330 mOsm kg^{-1}. In the bioreactor culture, 0.1 % Pluronic F-68 was added to protect cells from shear damage.[12] The composition of feeding medium in controlled-fed perfusion culture is given in Table 1. Perfusion medium is the aforementioned serum-free medium.

Table 1 – *Composition of feeding medium in controlled-fed perfusion culture of CHO cells*

Components	Concentration (mg L^{-1})	Components	Concentration (mg L^{-1})
L-Tryptophan	380	L-Phenylalanine	510
L-Tyrosine	225	L-Glutamic acid	676.5
L-Isoleucine	470	L-Asparagine	2600
L-Leucine	880	L-Proline	340
L-Valine	360	L-Serine	1500
L-Arginine	220	L-Threonline	600
L-Lysine · HCl	180	L-Glutamine	21900
L-Methionine	350	D-Glucose	90000

Bioreactor culture

Seed cells were inoculated in 1 L flasks with 0.3 L working volume and incubated on a shaker with 90~100 min^{-1}. The temperature and CO_2 partial pressure were controlled at 37 °C and 5 %, respectively. The cell inoculation density was $2 \cdot 10^8$ cells L^{-1}.

The batch and fed-batch cultures were conducted in a 1.5-L bioreactor with 1 L working volume. Cells were suspended in the bioreactor at an agitation speed of 50~60 min^{-1}. The inoculation cell density was $5 \cdot 10^8$ cells L^{-1}. Aeration was performed through a cylindrical sintered sparger at 0.02 L L^{-1} min^{-1}. The dissolved oxygen concentration (DO) was controlled by DO probe (InPro3030, METTLER TOLEDO, Germany) at 40 % air saturation by automatically adjusting the proportions of air, O_2 and N_2. The pH was controlled by regulating CO_2 partial pressure or feeding 5 % sodium carbonate. The pH and DO values in culture were detected by pH probe (InPro6800, METTLER TOLEDO) and DO probe (InPro3030), respectively. Samples of $2 \cdot 10^{-3}$ L were withdrawn at intervals of 8~12 h,

depending upon the growth status of the cells. The perfusion and controlled-fed perfusion cultures were conducted in a 5 L packed-bed bioreactor with 3.6 L working volume (CelliGen Plus, NBS, USA) with 200 g disk carriers (FibraCel Disks, NBS). After autoclave, the bioreactor was inoculated with $6 \cdot 10^8$ cells L^{-1}. Temperature, pH and DO were automatically controlled at 37 °C, 6.8, and 40 % of air saturation, respectively.

Assays

Cell number was counted with a hemacytometer. Cell viability was estimated by trypan blue dye exclusion method. Glucose and lactate were assayed with glucose oxidase (GOD-POD) and LDH methods, respectively, and ammonia concentration was determined by a reagent kit (Urea nitrogen type, Shanghai Institute of Biologicals, China).[13] HBsAg concentration was measured by an ELISA kit (Huamei Biological Co., Shanghai, China).[14] Osmolarity was measured using freezing-point depression method (Automatic cryoscopic osmometer FM-8P, Shanghai Medical University Instrument Factory, China).

Control strategies and models

In the preparatory experiments of the CHO cell culture, we investigated the relationship between the yield of lactate to glucose ($Y_{lac/glc}$) and the present glucose concentration. High glucose concentration corresponded to rapid lactate production, and $Y_{lac/glc}$ would be 0.2 mol mol^{-1} and 0.3 mol mol^{-1} if glucose concentration was kept at 7 mmol L^{-1} and 10 mmol L^{-1}, respectively (data not shown). Conventionally, $Y_{lac/glc}$ of 0.2 mol mol^{-1} was acceptable and favorable to avoid large amount of lactate accumulation. On the other hand, to prevent glucose exhaustion caused by deviation of control, glucose concentration could not be excessively low during a culture. Hence, in fed-batch, perfusion and controlled-fed perfusion cultures, 7 mmol L^{-1} glucose was set as a high limit in adjusting the feeding and perfusion rates.

The controlled-fed perfusion process is divided into many consecutive short sections to exactly control feeding and perfusion rates. The t_{n-1} is the starting time of the nth section. The feeding and perfusion rates of the (n+1)th section can be estimated by the data at the nth and the (n–1)th sections according to kinetic models. In the culture, feeding and perfusion media as well as base solution are flowed into bioreactor while the culture broth with negligible viable cells is continuously discharged. Their relationship can be described by

$$F_D = F_P + F_F + F_B \tag{1}$$

where F_D, F_P, F_F, and F_B are the discharging rate (L h^{-1}), perfusion rate (L h^{-1}), feeding rate (L h^{-1}), and base feeding rate (L h^{-1}), respectively. F_B is much smaller than F_D and F_P because the lactate production rate is at low level. Generally, F_F is also small as a result of high glucose concentration in feeding medium. Hence, F_D is supposed to equal F_P.

Feeding model

Glucose concentration in bioreactor is considered constant within a short time from t_{n-1} to t_n. Since the volume of additional base is negligible as compared to the culture volume, then,

$$\begin{aligned} C_{gP}\, F_{P(n+1)}\, (t_{n+1} - t_n) + C_{gF}\, F_{F(n+1)}\, (t_{n+1} - t_n) = \\ = C_{gn}\, F_{D(n+1)}\, (t_{n+1} - t_n) + S_{g(n+1)} \end{aligned} \tag{2}$$

where C_{gn}, glucose concentration at t_n, mmol L^{-1}; $S_{g(n+1)}$, glucose consumption in the (n+1)th section, mmol; C_{gP}, glucose concentration in perfusion medium, mmol L^{-1}; C_{gF}, glucose concentration in feeding medium, mmol L^{-1}.

Before calculating the $F_{F(n+1)}$ in the (n+1)th section, the $S_{g(n+1)}$ should be calculated. The $S_{g(n+1)}$ is relevant to X_{n+1} and μ_{n+1}, which denote viable cell density and specific growth rate in the (n+1)th section, respectively. So it changes with n. However, in a relatively short period of time, the average glucose consumption in the three continuous sections is assumed to be in a linear relationship, and the $S_{g(n+1)}$ can be estimated by the following equation if the sampling interval is short enough,

$$\frac{\dfrac{S_{g(n+1)}}{t_{n+1} - t_n}}{\dfrac{S_{gn}}{t_n - t_{n-1}}} = \frac{\dfrac{S_{gn}}{t_n - t_{n-1}}}{\dfrac{S_{g(n-1)}}{t_{n-1} - t_{n-2}}}$$

$$\begin{aligned} S_{g(n+1)} &= \frac{\dfrac{S_{gn}}{t_n - t_{n-1}}}{\dfrac{S_{g(n-1)}}{t_{n-1} - t_{n-2}}} \cdot \frac{S_{gn}}{t_n - t_{n-1}} \cdot (t_{n+1} - t_n) = \\ &= \frac{S_{gn}^2 (t_{n-1} - t_{n-2}) \cdot (t_{n+1} - t_n)}{S_{g(n-1)} (t_n - t_{n-1})^2} \end{aligned} \tag{3}$$

$$n \geq 2.$$

The deviation caused by estimated S is inevitable even though the interval is very short. This will result in the fluctuation of glucose concentration in bioreactor. To keep glucose concentration in bioreactor close to 7 mmol L^{-1}, the term $(7 - C_{g(n-1)})\, V$ is introduced to the eq. (2). Then,

$$C_{gP}\, F_{P(n+1)}\, (t_{n+1} - t_n) + C_{gF}\, F_{F(n+1)}\, (t_{n+1} - t_n) = \\ = C_{gn}\, F_{D(n+1)}\, (t_{n+1} - t_n) + S_{g(n+1)} + (7 - C_{gn})\, V \quad (4)$$

where V, working volume of bioreactor, L. $n \geq 2$.

$F_{F(n+1)}$ can be given by eq. (4) if $F_{D(n+1)}$ is known from perfusion model.

Perfusion model

In this work, the control of perfusion is mainly based on the balance between minimizing the influence of toxic byproducts on cell growth and increasing HBsAg concentration in broth. In controlled-fed perfusion culture, the byproducts produced in cell metabolism are removed by continuous medium discharge. The change of a byproduct between t_n and t_{n+1} can be described as

$$\Delta P_{n+1} = P_{p(n+1)} - P_{a(n+1)}\, F_{D(n+1)}\, (t_{n+1} - t_n) \quad (5)$$

where ΔP_{n+1}, change of byproduct between t_n and t_{n+1}, mmol; $P_{p(n+1)}$, byproduct production in the $(n+1)$th section, mmol; $P_{a(n+1)}$, average byproduct concentration in the $(n+1)$th, mmol L^{-1}; $F_{D(n+1)}$, discharging rate in the $(n+1)$th section, L h^{-1}. $n \geq 1$. $P_{a(n+1)}$ can be supposed to be equal to byproduct concentration at t_n if the duration from t_n to t_{n+1} is short enough.

The byproduct concentration at the $(n+1)$th section should be kept below its inhibitory value to avoid the inhibition of cell growth. It is expressed as

$$P_{n+1} = \frac{\Delta P_{n+1}}{V} + P_n < P_L \quad (6)$$

where P_{n+1}, byproduct concentration at t_{n+1}, mmol L^{-1}; P_L, inhibitory concentration of certain byproduct, mmol L^{-1}.

To obtain $F_{D(n+1)}$ in the $(n+1)$th section, $P_{p(n+1)}$ is estimated by the following equation when the sampling interval is short enough,

$$\frac{\dfrac{P_{p(n+1)}}{t_{n+1} - t_n}}{\dfrac{P_{p_n}}{t_n - t_{n-1}}} = \frac{\dfrac{P_{p_n}}{t_n - t_{n-1}}}{\dfrac{P_{p_{(n-1)}}}{t_{n-1} - t_{n-2}}}$$

$$P_{p(n+1)} = \frac{\dfrac{P_{p_n}}{t_n - t_{n-1}}}{\dfrac{P_{p_{(n-1)}}}{t_{n-1} - t_{n-2}}} \cdot \frac{P_{p_n}}{t_n - t_{n-1}} \cdot (t_{n+1} - t_n) = \\ = \frac{P_{p_n}^2 (t_{n-1} - t_{n-2}) \cdot (t_{n+1} - t_n)}{P_{p_{(n-1)}} (t_n - t_{n-1})^2} \quad (7)$$

Finally, $F_{D(n+1)}$ is calculated by the eq. (5). $n \geq 2$. $F_{F(n+1)}$ can be calculated by eq. (4) when $F_{D(n+1)}$ is gained from eqs. (5) and (6).

In the controlled-fed perfusion model, the variable linking the fed and perfusion models is C_g, which denotes glucose concentration in culture medium. During a culture, glucose is supplied mainly by feeding and partly by perfusion, which is determined by byproduct control model. In order to maintain appropriate glucose concentration in bioreactor, the feeding flow rate has to be adjusted with the change of perfusion flow rate. In this work, when glucose concentration in the nth section is lower than 7 mmol L^{-1}, the feeding begins and feeding volume in the $(n+1)$th section is calculated by eq. (4). When byproducts such as lactate and ammonia are close to the inhibitory concentration, the perfusion rate will be increased on the basis of eq. (5). If two or more byproducts need to be controlled simultaneously, the perfusion rate is dependent on the one close to its inhibitory concentration.

Results

Inhibitory effect and threshold of byproducts on cell growth

As shown in Fig. 1, the viable cell density in the fed-batch culture declined rapidly at 100 h while the total cell density still increased. This decline of cell viability could not be reversed by optimizing the feeding medium composition (data not

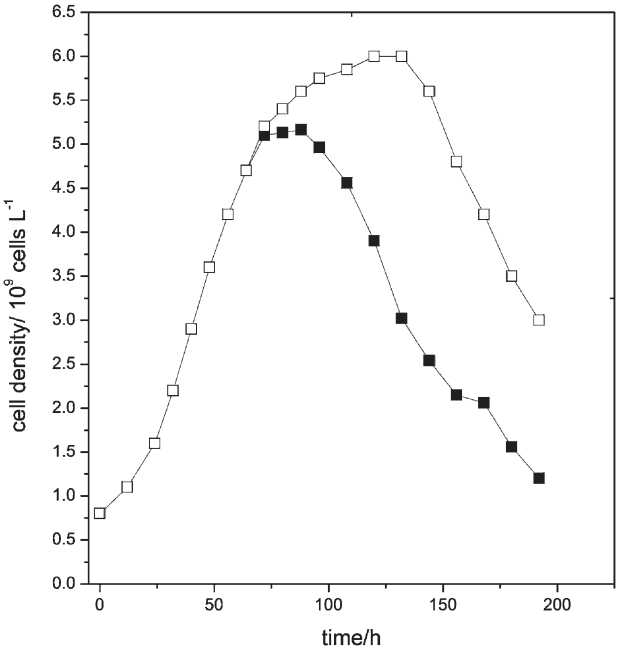

Fig. 1 – *Growth of CHO cells in fed-batch culture in a 1.5 L bioreactor. (□) Total cell density, (■) Viable cell density.*

shown). Generally, the decline of cell viability is mainly induced by ammonia or osmotic pressure in the culture of CHO cells. Therefore, we investigated the relationships between ammonia concentration/osmolarity and cell growth. The experiments were performed in duplicate in 0.1 L shake flasks. Firstly, different concentrations of NH_4Cl were added to the initial medium, and the ammonia concentration and viable cell density were measured at 72 h. Similarly, different concentrations of NaCl were added to evaluate the osmolarity effect on cell viability. Thus, the ammonia concentration of 5.4 mmol L^{-1} and the osmolarity of 460 mOsm kg^{-1} resulted in the decrease of viable cell density by 13 % and 10 % of maximum viable cell density, respectively (Fig. 2 and Fig. 3). If the reluctantly acceptable decrease of viable cell density were set at 10~15 %, we supposed that an ammonia concentration below 5.4 mmol L^{-1} and an osmolarity below 460 mOsm kg^{-1} would be the thresholds for cell growth inhibition. Also, the influence of lactate on CHO cells would be a result of high osmolarity.[15] In preparatory experiments, we found that the lactate accumulation of 50 mmol L^{-1} was coupled to osmolarity of 460 mOsm kg^{-1} in fed-batch culture. Addition of 50 mmol L^{-1} lactate just increased osmolarity by 60 mOsm kg^{-1} in medium. The reason why the osmolarity increased by almost 100 mOsm kg^{-1} in the culture was probably the feeding of sodium bicarbonate for pH adjustment and the accumulation of some low molecular weight metabolites. The regularity for change of osmolarity in the controlled-fed perfusion culture was similar with that in the fed-batch culture because of the low discharge rate. Hence, the inhibitory concentrations of ammonia and lactate were set at 5.4 mmol L^{-1} and 50 mmol L^{-1}, respectively.

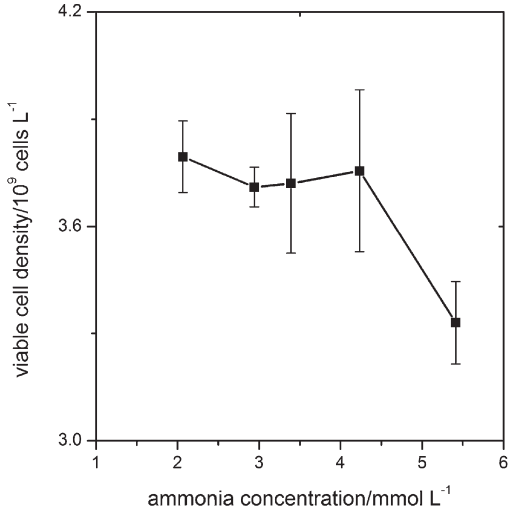

Fig. 2 – *Viable cell density at 72 h of CHO cell cultures in different concentrations of NH_4Cl. The error bar is standard deviation.*

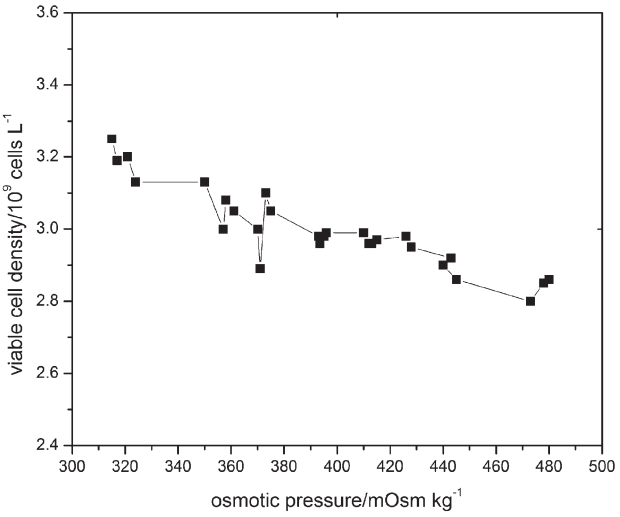

Fig. 3 – *Viable cell density at 72 h of CHO cell cultures at different osmotic pressures. Sodium chloride was added to provide a control for osmolarity effects.*

Comparison of different culture modes

Recombinant CHO cell growth and HBsAg production were evaluated in a 1.5 L bioreactor for batch and fed-batch cultures and a 5 L bioreactor for perfusion and controlled-fed perfusion cultures (Table 2).

In fed-batch culture, glucose concentration was controlled at 7 mmol L^{-1} with a feeding medium including glucose, glutamine and expendable amino

Table 2 – *Bioreactor performance with different culture modes at different scales*

Culture mode	In 1.5 L bioreactor		In 5 L bioreactor	
	Batch	Fed-batch	Perfusion	Controlled-fed perfusion
Maximum viable cell density (10^9 cells L^{-1})	3.4	5.1	10	13
Maximum specific growth rate (h^{-1})	0.055	0.054	0.062	0.058
Total glucose consumption (g L^{-1})	4.5	10.2	104.0	88.6
Maximum HBsAg concentration (mg L^{-1})	1.5	2.3	0.5	1.65
Maximun lactate concentration (mmol L^{-1})	35	45	32	50
Maximum ammonia concentration (mmol L^{-1})	2.4	5.3	2.2	5.8
Culture time (h)	120	192	480	384
HBsAg productivity (mg L^{-1} h^{-1})	0.0125	0.0125	0.029	0.028

acids (Table 1). A cell density of $5 \cdot 10^9$ cells L^{-1} and an HBsAg production of 2.3 mg L^{-1} were achieved after 190 h culture. HBsAg productivity in both batch and fed-batch cultures was 0.0125 mg L^{-1} h^{-1} (Table 2). The fed-batch culture did not give higher efficiency than batch culture in terms of HBsAg production. As shown in Fig. 1, the viable cell density in fed-batch culture declined rapidly at 100 h in spite of the continuous increase of total cell density.

In perfusion culture, glucose concentration was also controlled at about 7 mmol L^{-1}. The cell density had to be indirectly estimated by glucose consumption rate because of their concealment in packed disks bed which could not be taken out for cell counting. Based on the estimation, the maximum cell density reached $10 \cdot 10^9$ cells L^{-1}. As shown in Table 2, however, due to the dilution of perfusion, although the productivity of HBsAg in perfusion culture was significantly higher (0.029 mg L^{-1} h^{-1} in perfusion vs 0.0125 mg L^{-1} h^{-1} in batch and fed-batch culture), HBsAg concentration in perfusion culture was lower than that in batch and fed-batch cultures (0.5 mg L^{-1} vs 1.5 mg L^{-1} in batch and 2.3 mg L^{-1} in fed-batch). In contrast, controlled-fed perfusion culture was more efficient, and its HBsAg concentration was stably higher than 3-fold that in perfusion culture. As shown in Fig. 4, in controlled-fed perfusion culture of 400 h, the HBsAg concentration reached 1.65 mg L^{-1} at 240 h and kept at approximately the same level until the end of culture. The comparison between the two operation modes showed that low perfusion rate in controlled-fed perfusion culture brought about little impact on cell growth, but did increase HBsAg concentration.

In controlled-fed perfusion culture, glucose consumption rate began to decline at 200 h of culture, and it was stable from 220 h to 400 h. However, the cell density in supernatant (not including the cells in disc carriers) increased from $2 \cdot 10^8$ cells L^{-1} to $1 \cdot 10^9$ cells L^{-1} during the first 200 h culture. Thus, some suspension cells were continuously removed from the vessel with medium.

Assessment of the feeding and perfusion models

The exact estimations of glucose consumption and byproduct production are paramount for the proposed feeding and perfusion models. However, it is rather difficult to eliminate the error between the real concentration and model-estimated concentration. One way to reduce this error is to shorten the interval of serial sampling and off-line analysis. This would increase contamination risk and labor force. As shown in Fig. 5 and Table 3, for glucose consumption rate, ammonia production rate and lactate production rate, all the mean absolute percentage errors between actual values and estimated

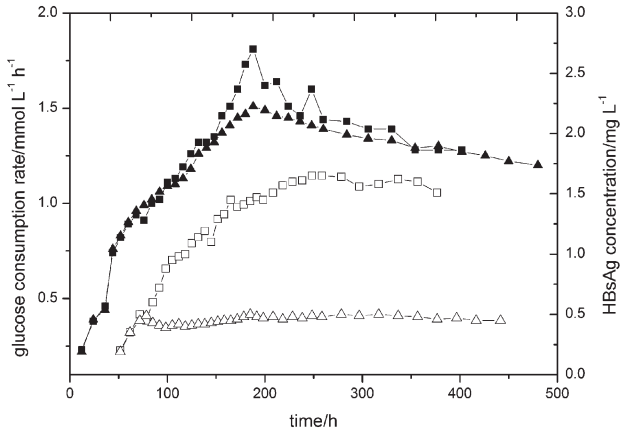

Fig. 4 – *Profiles of glucose consumption rate (■) and HBsAg (□) concentration in controlled-fed perfusion culture, glucose consumption rate (▲) and HBsAg (△) concentration in perfusion culture of CHO cells in a 5 L bioreactor*

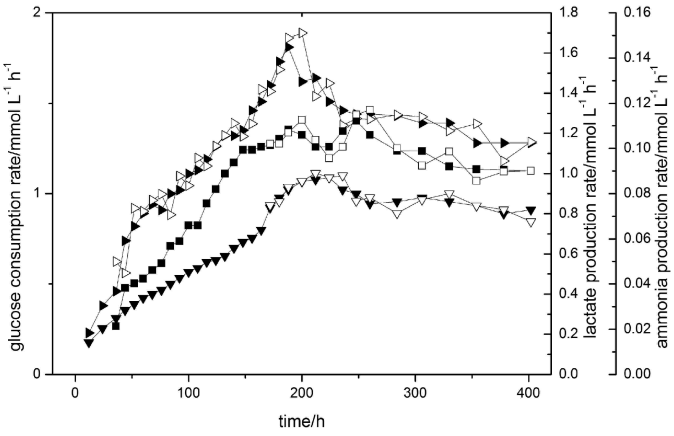

Fig. 5 – *Profiles of glucose consumption rate (▶), lactate production rate (▼), ammonia production rate (■), estimated glucose consumption rate (▷), estimated lactate production rate (▽), and estimated ammonia production rate (□) in controlled fed-perfusion culture of CHO cells in a 5 L bioreactor*

Table 3 – *Evaluation of deviation between actual value and estimated value by model*

	Average absolute error between actual value and estimated value by model (%)	Standard deviation of absolute error between actual value and estimated value by model (%)
Glucose consumption rate at 44~402 h	4.83	3.98
Ammonia production rate at 164~402 h	4.01	3.14
Lactate production rate at 172~402 h	2.87	2.56

ones by model were less than 5 %, and the standard deviations of absolute percentage errors were less than 4 %. This indicated that there were no dramatic deviations between real measured values and estimated ones. The estimation accuracy would decline with prolongation of the interval in the whole culture. In addition, a large disturbance of actual value might generate obvious deviation of the estimated one. For instance, the glucose consumption rate in the time period of 188~200 h was 1.62 mmol L^{-1} h^{-1}, less than that in the previous time period of 180~188 h by about 10 %, and this resulted in the deviations of actual glucose consumption rate within 188~200 h and 200~212 h from estimated ones by 14 % and –6 %. The main parameters of the models mentioned above are shown in Table 4.

Feeding and discharge control in controlled-fed perfusion culture

During the culture, glucose concentration was controlled below 7 mmol L^{-1} by adjusting feeding rate based on the feeding model a little higher than 7 mmol L^{-1} at the beginning of the short time of perfusion (Fig. 6). The feeding rate increased to maximum 0.0125 L h^{-1} at 180~188 h while the glucose consumption rate reached a maximum at 188 h (Fig. 4 and Fig. 6).

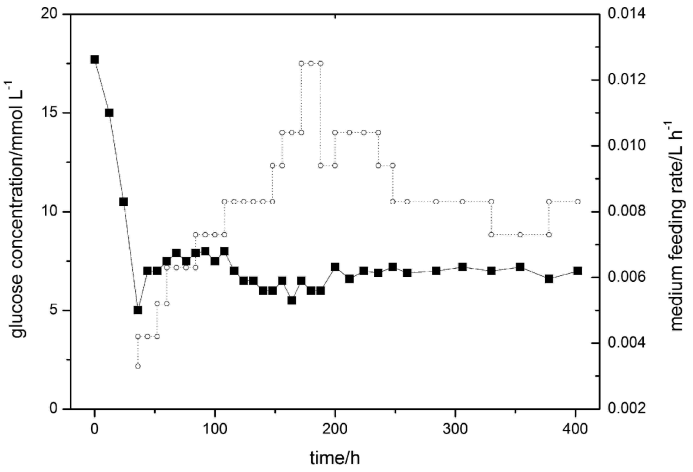

Fig. 6 – *Glucose concentration (■) and medium feeding rate (□) in controlled-fed perfusion culture of CHO cells in a 5 L bioreactor*

When the concentrations of ammonia and lactate increased to their inhibitory values, perfusion rate increased and was controlled according to the perfusion model. As shown in Fig. 7, the ammonia concentration reached 5.4 mmol L^{-1} at 220 h because of intensive metabolism at high cell density. Then, the perfusion control target shifted from glucose concentration to ammonia concentration. Until

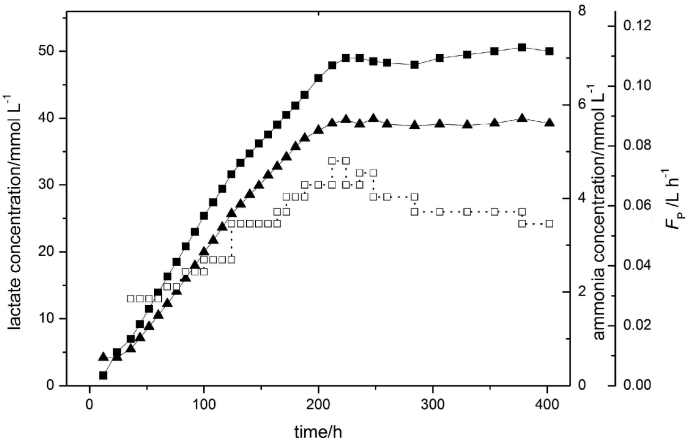

Fig. 7 – *Lactate (■) and ammonia (▲) concentrations and perfusion rate (□) in controlled-fed perfusion culture of CHO cells in a 5 L bioreactor*

the end of culture, ammonia and lactate concentrations and osmotic pressure stayed below their inhibitory values despite a low F_p, ranging from 0.029 to 0.075 L h^{-1} (Fig. 7 and Fig. 8), to minimize the dilution of HBsAg.

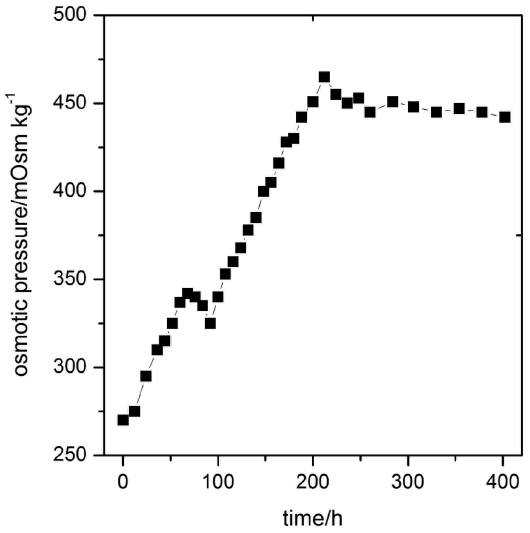

Fig. 8 – *Osmotic pressure in controlled-fed perfusion culture of CHO cells in a 5 L bioreactor*

Discussion

There are two reported ways to supplement nutrients in perfusion culture. One is to feed concentrated nutrient solution into the culture. Another is to fortify particular nutrients in perfusion medium.[1] However, it is still difficult to exactly supply nutrients to meet the dynamic demand of cells during a culture process. An alternative approach is to continuously adjust the supplementary components in responding to cell physiological state to enhance the feeding efficiency.[16] The stoichiometric rela-

Table 4 – *The main parameters of feeding and perfusion models in controlled-fed perfusion culture in a 5 L bioreactor*

Section number n	Time t (h)	Glucose consumption rate (mmol L^{-1} h^{-1})	Estimated glucose consumption rate (mmol L^{-1} h^{-1})	Ammonia production rate (mmol L^{-1} h^{-1})	Estimated ammonia production rate (mmol L^{-1} h^{-1})	Lactate production rate (mmol L^{-1} h^{-1})	Estimated lactate production rate (mmol L^{-1} h^{-1})	Feeding rate F_F (L h^{-1})	Perfusion rate F_P (L h^{-1})
1	0~12	0.23				0.167			
2	12~24	0.38				0.227			
3	24~36	0.46	0.63	0.021		0.285			
4	36~44	0.74	0.56	0.038		0.358		0.003	0.029
5	44~52	0.82	0.92	0.040		0.393		0.004	0.029
6	52~60	0.89	0.90	0.042		0.412		0.004	0.029
7	60~68	0.94	0.96	0.046		0.432		0.005	0.029
8	68~76	0.91	0.99	0.049		0.451		0.006	0.033
9	76~84	1.00	0.88	0.057		0.480		0.006	0.033
10	84~92	1.02	1.10	0.059		0.512		0.006	0.037
11	92~100	1.11	1.05	0.066		0.533		0.007	0.037
12	100~108	1.13	1.20	0.066		0.558		0.007	0.037
13	108~116	1.19	1.15	0.076		0.576		0.007	0.042
14	116~124	1.26	1.26	0.082		0.599		0.008	0.042
15	124~132	1.32	1.32	0.089		0.583		0.008	0.042
16	132~140	1.32	1.39	0.094		0.591		0.008	0.054
17	140~148	1.35	1.32	0.099		0.722		0.008	0.054
18	148~156	1.46	1.39	0.099		0.765		0.008	0.054
19	156~164	1.51	1.58	0.101		0.813		0.009	0.054
20	164~172	1.60	1.57	0.101	0.102	0.832		0.01	0.054
21	172~180	1.73	1.69	0.104	0.102	0.882	0.861	0.01	0.058
22	180~188	1.81	1.87	0.108	0.107	0.928	0.933	0.012	0.058
23	188~200	1.62	1.89	0.106	0.113	0.961	0.962	0.012	0.062
24	200~212	1.64	1.54	0.101	0.104	0.977	1.002	0.009	0.067
25	212~224	1.51	1.61	0.101	0.096	0.980	0.980	0.01	0.075
26	224~236	1.46	1.38	0.108	0.101	0.929	0.990	0.01	0.067
27	236~248	1.44	1.41	0.112	0.115	0.906	0.864	0.01	0.071
28	248~260	1.44	1.41	0.106	0.117	0.856	0.880	0.009	0.062
29	260~284	1.43	1.43	0.099	0.100	0.861	0.803	0.008	0.062
30	284~306	1.39	1.42	0.099	0.092	0.880	0.870	0.008	0.062
31	306~330	1.28	1.34	0.092	0.099	0.866	0.900	0.008	0.058
32	330~354	1.28	1.39	0.091	0.086	0.842	0.840	0.008	0.058
33	354~378	1.28	1.19	0.091	0.090	0.809	0.820	0.007	0.058
34	378~402	1.28	1.28	0.090	0.090	0.821	0.762	0.007	0.058

tionship between oxygen and amino acids consumption rate was applied in controlled-fed perfusion culture because the variation of OUR could reflect the physiology of cell to a certain extent.[2] However, until now, the physiology of a majority of cell lines is still complicated and hardly elucidated. The optimized controlled-fed perfusion mode with new feeding and perfusion strategies can provide a simple solution. In this study, glucose concentration was strictly controlled, while glutamine and other expendable amino acids were set at relatively high levels. Although the yield coefficients of ammonia and lactate were a little higher than those in perfusion culture, their concentrations were kept below inhibitory thresholds by the delicate adjustment of perfusion rate. The results showed that the lactate production was decreased by limiting glucose. However, the decrease of glucose concentration is limited by the cellular threshold of glucose dependence and the glucose fluctuation by control deviation. Anyway, byproduct production is inevitable even if the precise control is conducted. In this work, the feeding limitation and discharge control strategies in controlled-fed perfusion achieved both the high cell density in fed-batch culture and the low byproduct accumulation in perfusion culture.

There is successful implication of cell specific perfusion rate (CSPR) in perfusion or controlled-fed perfusion.[2] In this work, it is possible and simple to discharge according to CSPR. However, it depends on the data of exact cell density and the medium perfusion of a large amount and results in low product titer. Compared to CSPR, the strategy of perfusion control in the controlled-fed perfusion culture gives accurate control of nutrients and byproducts.

It is a novel application for CHO cell culture which retains cells with DISK microcarrier in perfusion serum-free culture. The cell can not adhere to DISK entirely without assistance of serum. However, our previous experiment showed that the growth, expression and metabolism of these cells were no different than that of single suspension culture cells, which indicated that the cells in DISK would be in a semi-suspension condition. On the other hand, there is a maximum retaining cell density of about $1.3 \cdot 10^{10}$ cells L^{-1} in this case which may be caused by lack of serum. Anyway, application of this culture mode on improving productivity of other cell lines remains questionable and it may be cell specific.

Long-term culture of more than one month could be achieved by perfusion culture or controlled-fed perfusion culture. However, as for controlled-fed perfusion culture, the cell growth was still influenced by toxic byproducts under low discharging rate even though the main known byproducts were controlled at low levels.[1] The production of these unknown toxic metabolites may be independent of cell growth rate and metabolism rate. This would limit the application of controlled-fed perfusion mode if the accumulation of toxic metabolites could result in serious effect on cell culture and could not be eliminated by low discharge rate. Besides, since the accumulation of toxic metabolites depends on cell line, the model parameters have to be determined beforehand when a new cell line is used.

For accurate control of culture process, estimated glucose consumption rate and estimated byproducts production rate were introduced to the control model. In this work, the culture process was theoretically divided into many sections, and the variables in every two neighboring sections have regularity in some respect based on the hypothesis that a line section formed by three neighboring points in curve is approximately straight. Hence, the variables in certain sections can be estimated by the variables in the former two sections. The short duration of every section kept deviation of the model so low that the control accuracy of the culture process was improved by circulating the estimating procedure.

The results indicated that the step-by-step strategy relying on the model guide made the controlled-fed perfusion more reasonable and achievable because it was able to avert the amplification of the fluctuation in the cell growth and metabolism caused by various unexpected reasons. Furthermore, the reliable control model of step-by-step progress has some characteristics of low cost implementation and high deviation tolerance, and is not dependent on complex and expensive online control instruments.

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Notations

HBsAg – hepatitis B surface antigen

F_D – discharging rate, L h^{-1}

F_P – perfusion rate, L h^{-1}

F_F – feeding rate, L h^{-1}

F_B – base feeding rate, L h^{-1}

C_{gP} – glucose concentration in perfusion medium, mmol L^{-1}

C_{gF} – glucose concentration in feeding medium, mmol L^{-1}

S_g – glucose consumption, mmol
P_a – average concentration of byproduct, mmol L^{-1}
P_L – limited concentration of byproduct, mmol L^{-1}
P_p – production of byproduct, mmol
HBsAg productivity = HBsAg production/ (culture volume · culture time)
Glucose consumption rate = Glucose consumption/ (culture volume · culture time)
Ammonia production rate = Ammonia production/ (culture volume · culture time)
Lactate production rate = Lactate production/ (culture volume · culture time)

References

1. *Yang, J. D., Angelillo, Y., Chaudhry, M., Goldenberg, C., Goldenberg, D. M.,* Biotechnol. Bioeng. **69** (2000) 74.
2. *Feng, Q., Mi, L., Liu, R., Xie, L., Tang, H., Chen, Z. N.,* J. Biotechnol. **122** (2006) 422.
3. *Jang, D. J., Barford, J. P.,* Biochem. Eng. J. **4** (2000) 153.
4. *Lin, J. Q., Takagi, M., Qu, Y. B., Yoshida, T.,* Biochem. Eng. J. **11** (2002) 205.
5. *Xie, L. Z., Wang, D. I. C.,* TIBTECH. **15** (1997) 109.
6. *Xie, L. Z., Wang, D. I. C.*, Biotechnol. Bioeng. **51** (1996) 725.
7. *Yoon, S., Konstantinov, K. B.,* Biotechnol. Bioeng. **44** (1994) 983.
8. *Frahm, B., Lane, P., Atzert, H., Munack, A., Hoffmann, M., Hass, V. C., Portner, R.,* Biotechnol. Prog. **18** (2002) 1095.
9. *Ozturk, S. S., Riley, M. R., Palsson, B. O.,* Biotechnol. Bioeng. **39** (1992) 418.
10. *Zhang, L., Shen, H., Zhang, Y. X.,* J. Chem. Technol. Biotechnol. **79** (2004) 171.
11. *Yi, X. P., Zhang, D. H., Sun, X. M., Zhang, Y. X.,* Chinese Patent (2009) No. 200910195505.7.
12. *Keane, J. T., Ryan, D., Gray, P. P.,* Biotechnol. Bioeng. **81** (2003) 211.
13. *Zhang, L., Shen, H., Zhang, Y. X.,* Chinese J. Biotechnol. **16** (2000) 373.
14. *Li, J. H., Sun, X. M., Zhang Y. X.,* Process Biochem. **41** (2006) 317.
15. *Zhang, Y. X.,* Adv. Biochem. Eng. Biotechnol. **113** (2009) 177.
16. *Li, L., Li, M., Feng, Q.,* Biotechnol. Appl. Biochem. **42** (2005) 73.