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High density culture of insect cells using rational medium design and feeding strategy

T.-W. Chiou, Y.-C. Hsieh, C. S. Ho

Abstract The objective of this study is to achieve high density cell culture by a rational medium design and feeding strategy. Insect cell/baculovirus expression system is one of the widely used methods for the production of heterologous proteins in the cell culture domain. Insect cell *Spodoptera frugiperda* Sf-21 and a recombinant baculovirus with encoded gene for human interleukin-5 were chosen as the model system in this study. A stoichiometric model was established to study the demand of nutrients, including glucose, 20 amino acids, and yeastolate, for the synthesis of cell mass. The coefficients for individual nutrients in the stoichiometric equation governing insect cell growth were determined from the information of cell mass and compositions. Based on the stoichiometric coefficients, the initial and supplemental media for fed-batch cell cultures were designed.

The experiments began with the inoculation of Sf-21 cells into a spinner flask with the initial medium, which provided a starting environment for achieving optimum cell growth. This was followed by the periodic feeding of supplemental medium designed by utilizing the stoichiometric equation that governs insect cell growth. With this strategy, it was demonstrated that the Sf-21 cell culture reached a cell density in excess of 1.9×10^7 cells/ml. During the cultivation process, the utilization of various nutrients and the production of metabolites were also monitored. Further experiments proved that high concentration of recombinant product (such as human interleukin-5) could be achieved by infecting the high density cells (resulting from the designed medium) with recombinant baculoviruses.

List of symbols

a	Amino acid
C_t	Total concentration of glucose and amino acids in the supplemental medium, mM
D_A, D_G, D_C, D_T	DNA base composition of adenine, guanine, cytosine, and thymine, mol %
i	Index of amino acids
M	Molecular weight, g/mole
M_D	Average molecular weight of DNA, g/mole
M_R	Average molecular weight of RNA
n	Total specie number of glucose and amino acid
N_n	Total cell number in the culture system
$\Delta N_{t,n}$	Net increase in total cells in the system between the time interval culture of the nth feeding and the $(n+1)$th feeding, cells
N_A, N_G, N_C, N_T, N_U	Total moles of adenine, guanine, cytosine, thymine and uracil, moles
N_{DNA}, N_{RNA}	Total moles of DNA and RNA per cell, mol/cell
P/O	Moles of ATP generated per mole NADH oxidized
R_A, R_G, R_C, R_U	RNA base composition of adenine, guanine, cytosine, and uracil, mol %
q_{ATP}	Specific ATP consumption rate, mmol/cell · h
ΔV_n^f	Feeding volume of the nth feeding, L
t	Culture time, h
W	Average dry cell weight, mg/cell
Z_C, Z_D, Z_L, Z_P, Z_R	Dry weight percentage of carbohydrate, DNA, lipid, protein, and RNA in cell mass, %

Greek symbols

μ	Specific growth rate, 1/h
β	Total stoichiometric coefficient of amino acids and glucose, mmol/cell
θ	Stoichiometric coefficient, mmol/cell
$\theta_{a,i}$	Stoichiometric coefficient for the ith amino acid, mmol/cell
$\theta_{a,i}^P$	Amino acid composition of cellular proteins, mmol of the ith amino acids/mg proteins
θ_{glc}	Stoichiometric coefficient for glucose, mmol/cell
θ_{ATP}	Stoichiometric coefficient for ATP, mmol/cell
θ_y	Stoichiometric coefficient for yeastolate, g/cell

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$\theta^{P}_{a,gln}$, $\theta^{P}_{a,gly}$, $\theta^{P}_{a,asp}$, $\theta^{P}_{a,glu}$	Composition of glutamine, glycine, aspartate, and glutamate in cellular proteins, respectively, mmol/mg protein
θ^{carb}_{glc}, θ^{lipid}_{glc}, θ^{DNA}_{glc}, θ^{RNA}_{glc}, θ^{en}_{glc}	Stoichiometric coefficient for glucose in the synthesis of carbohydrates, lipid, DNA, RNA, and energy production, mmol/cell
θ_{gln}, θ_{gly}, θ_{asp}, θ_{glu}	Stoichiometric coefficient for glutamine, glycine, aspartate, and glutamate, respectively, mole/cell

1 Introduction

Animal cell culture has become an important approach for the production of biologically functional proteins for human therapy. In recent years the insect cell/baculovirus expression systems have been widely employed for the synthesis of heterologous proteins [1–4]. Because they produce biologically active proteins more reliably than do bacterial or yeast expression systems, and yields generally exceed those from mammalian expression systems. *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is a baculovirus which hosts a number of insects such as *Spodoptera frugiperda*. The virus consists of a 128 kb double stranded, supercoiled, circular piece of DNA encased in the occlusion body. This occlusion body is primarily composed of polyhedrin, 29 kD structural protein. By utilizing the promoter for the polyhedrin gene and replacing the nonessential polyhedrin structural gene with the gene of interest, it is possible to produce large quantities of recombinant proteins [5–7].

Many in vitro animal cell culture techniques have been developed during the past several years, with most of them aiming at lowering the cost of animal cell products. The problem persists partly because of lower cell densities and lower product concentrations, incurring higher purification expenses. The conventional batch cell culture usually results in low cell density and short culture span as animal cells produce waste byproducts, such as ammonia and lactate, which are toxic to cell growth [8]. Perfusion/continuous cultivation requests extra medium, which costs, to maintain the endured growth of cells [9–11]. A stoichiometric feeding strategy was therefore designed to sustain the culture duration of the conventional batch cell culture [12–14].

Therefore an attempt of this study is to achieve high density of Sf-21 insect cell culture by a rational medium design and feeding strategy. Stoichiometric calculations can provide quantitative relationship between yields of biomass and product synthesis, maintenance, and energetic yields which are extremely important for process design [15, 16]. A rational approach for medium design is mainly based on mass composition of cells, yield coefficients, and the stoichiometry of growth and energy formation [17–19].

2 Theoretical analysis

2.1 Stoichiometric model

The required nutrients associated with the growth of insect cells are carbon, nitrogen, vitamins, inorganic salts and others such as serum and lactoalbumin hydrolysate, for instance (Fig. 1). The design of the minimal media did not focus on ions, vitamins and serum because ions were usually abundant in the commercial basal medium, vitamins were also added in the yeastolate, and 10% fetal bovine serum was assumed to provide sufficient growth factors and hormones for the insect cell growth.

Based on the supplement of glucose, amino acids and yeastolate, an overall stoichiometric equation for insect cell growth and energy formation, i.e. cell mass and ATP, can be represented as:

$$\theta_{glc}[\text{glucose}] + \sum_{i=1}^{20} \theta_{a,i}[\text{amino acid}]_i + \theta_y[\text{yeastolate}] = [\text{cell mass}] + \theta_{ATP}[\text{ATP}] \ , \quad (1)$$

where θ_{glc}[glucose] stands for stoichiometric coefficient of glucose, $\theta_{a,i}$ for stoichiometric coefficient of *i*th amino acid, θ_y for stoichiometric coefficient of yeastolate, and θ_{ATP} for stoichiometric coefficient of ATP synthesized in cell. The equation is similar to the one derived for mouse-mouse hybridoma cell line by Xie and Wang [12, 13].

There are in total 23 unknown stoichiometric coefficients for glucose, 20 amino acids, yeastolate and ATP in Eq. (1). In order to solve these stoichiometric coefficients, the formation of ATP and cell mass was simplified as follows:

(a) ATP production

Glucose can be oxidized for ATP production in the case that one glucose can generate 36 ATP's if one assumes a P/O ratio of 3:

$$C_6H_{12}O_6 \longrightarrow 36 \text{ ATP} \ . \quad (2)$$

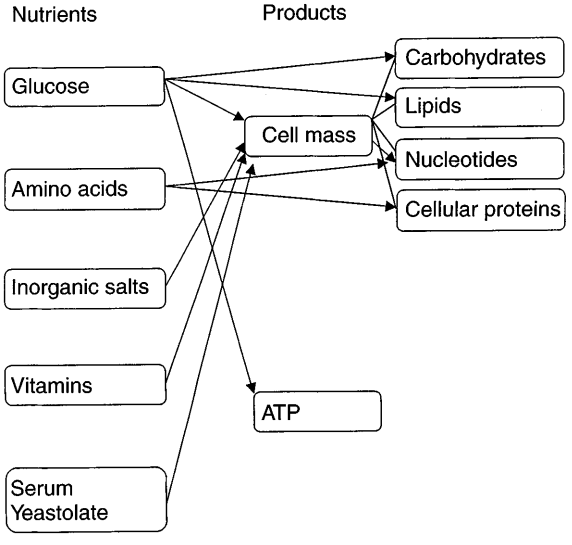

Fig. 1. Schematic pathway of synthesis of cell mass

(b) Carbohydrates synthesis
Cellular carbohydrates are optimally synthesized from glucose with the assumption that one glucose can provide 6 carbons for carbohydrate synthesis:

$$C_6H_{12}O_6 \longrightarrow 6\text{ -}(CH_2O)\text{-} \quad . \tag{3}$$

(c) Lipids synthesis
The most important carbon source for the synthesis of cellular lipids in animal cell cultivation is glucose. Cellular lipids are a mixture of fats, phospholipids and steroids. The formula of lipids $(CH_2O_xP_y)_m$ can be abbreviated as $(CH_2)_m$. One glucose can provide 6 carbons for lipid synthesis:

$$C_6H_{12}O_6 \longrightarrow 6\text{ -}(CH_2)\text{- lipids} \quad . \tag{4}$$

(d) Nucleotide synthesis
The deoxyribose in DNA and ribose in RNA are synthesized from glucose through pentose phosphate cycle. One glucose can provide 6 carbons for the synthesis of these pentose:

$$C_6H_{12}O_6 \longrightarrow 6/5\text{-}(X\text{-}C_5H_7O\text{-}PO_4)\text{-DNA} \quad , \tag{5}$$

$$C_6H_{12}O_6 \longrightarrow 6/5\text{-}(X\text{-}C_5H_7O_2\text{-}PO_4)\text{-RNA} \quad . \tag{6}$$

Purine derived to guanine and adenine, and pyrimidine derived to cytosine, uracil and thymine are synthesized from glutamine, aspartate and glycine:

$$2\text{Gln} + \text{Asp} + \text{Gly} \longrightarrow \text{guanine} + 3\text{Glu} \quad , \tag{7}$$

$$2\text{Gln} + \text{Asp} + 2\text{Gly} \longrightarrow \text{adenine} + 2\text{Glu} \quad , \tag{8}$$

$$2\text{Gln} + \text{Asp} \longrightarrow \text{cytosine} + 2\text{Glu} \quad , \tag{9}$$

$$2\text{Gln} + \text{Asp} \longrightarrow \text{uracil} + 2\text{Glu} + NH_4^+ \quad , \tag{10}$$

$$2\text{Gln} + \text{Asp} \longrightarrow \text{thymine} + 2\text{Glu} + NH_4^+ \quad . \tag{11}$$

(e) Protein synthesis
Cellular proteins are composed of all 20 amino acids. Therefore:

$$\sum_{i=1}^{20} \theta_{a,i}^{P}[\text{amino acid}]_i = \text{proteins} \quad , \tag{12}$$

where $\theta_{a,i}^{P}$ stands for the overall stoichiometric coefficient of all amino acids.

2.2 Calculation of stoichiometric coefficients

Since there was little information about the cell composition of insect cells, the stoichiometric coefficients were calculated according to either the data of insect cells obtained from our experimental results, or the data of animal cells obtained from literature. Cell compositions are assumed to be 38% proteins, 25% carbohydrates, 5% DNA, 13% RNA and 15% lipids [12, 13, 18, 20, 21]. Cell dry weight was measured as 6.1 mg/10^7 cells. The amino acid contents of cellular proteins were referred to those of Ferrance et al. [17]. The molar percentage of amino acids in Sf-21 insect cell was calculated and listed in Table 1.

Table 1. Molar percentage of individual amino acid in Sf-21 cell

Amino acid	Molar percentage (%)
Alanine	6.2
Arginine	7.0
Asparagine	5.2
Aspartate	5.2
Cysteine	2.8
Glutamate	6.2
Glutamine	6.2
Glycine	4.8
Histidine	2.7
Isoleucine	5.2
Leucine	7.7
Lysine	7.9
Methionine	3.3
Phenylalanine	4.8
Proline	4.7
Serine	4.1
Threonine	4.2
Tryptophan	2.3
Tyrosine	4.1
Valine	5.4

Therefore, the stoichiometric coefficients are represented as follows:

(a) ATP stoichiometric coefficient (θ_{ATP})
The stoichiometric coefficient for ATP can be represented as:

$$\theta_{ATP} = q_{ATP}/\mu \quad , \tag{13}$$

where q_{ATP} stands for the specific ATP consumption rate (mmol/cell · h); μ for specific growth rate (1/h). Assume that q_{ATP} equals to 10^{-9} mmol ATP/cell · h [12] and based on our measurement of μ equaling to 0.03 h^{-1} for Sf-21 cells, θ_{ATP} can be therefore obtained as:

$$\theta_{ATP} = 3.33 \times 10^{-11} \text{ mole/cell} \quad . \tag{14}$$

(b) Glucose stoichiometric coefficient (θ_{glc})
Based on the stoichiometric analysis, the stoichiometric coefficient for glucose is the sum of the five stoichiometric coefficients listed below for glucose in the synthesis of the cell components such as lipids, carbohydrates, pentoses in DNA and RNA, and energy ATP:

$$\theta_{glc} = \theta_{glc}^{lipid} + \theta_{glc}^{carb} + \theta_{glc}^{DNA} + \theta_{glc}^{RNA} + \theta_{glc}^{en} \quad . \tag{15}$$

By the definition of these five stoichiometric coefficients Eq. (15) can be expressed as:

$$\theta_{glc} = \frac{Z_L W}{M_{CH_2} \times 6} + \frac{Z_C W}{M_{CH_2O} \times 6} + \frac{Z_D W}{M_D \times 6/5} + \frac{Z_R W}{M_R \times 6/5} + \frac{\theta_{ATP}}{36} \quad . \tag{16}$$

Z_L, Z_C, Z_D, Z_R: the dry weight percentage of lipid, carbohydrate, DNA and RNA in cell mass (%); W: average total cell dry weight (mg/cell); M_{CH_2}: molecular weight of lipid (g/mole); M_{CH_2O}: molecular weight of carbohydrate

(g/mole); M_D: average molecular weight of DNA, (g/mole); M_R: average molecular weight of RNA.

The molecular weight of lipids and carbohydrates are known as:

$$M_{CH_2} = 14, \text{ and} \tag{17}$$

$$M_{CH_2O} = 30 \ . \tag{18}$$

Since the moles of pentoses in DNA and RNA can be calculated from the molar percentage of bases: adenine (D_A or R_A), guanine (D_G or R_G), cytosine (D_C or R_C), thymine (D_T) and uracil (R_U), the molecular weight of DNA and RNA are obtained as follows:

$$\begin{aligned} M_D &= M_{PO_4} + M_{C_5H_5O} + D_A M_{C_5H_4N_5} + D_G M_{C_5H_4ON_5} \\ &\quad + D_C M_{C_4H_4ON_3} + D_T M_{C_5H_5O_2N_2} \\ &= 307.8 \ , \end{aligned} \tag{19}$$

$$\begin{aligned} M_R &= M_{PO_4} + M_{C_5H_5O_2} + R_A M_{C_5H_4N_5} + R_G M_{C_5H_4ON_5} \\ &\quad + R_C M_{C_4H_4ON_3} + R_U M_{C_4H_3O_2N_2} \\ &= 320.3 \ . \end{aligned} \tag{20}$$

Consequently the value of θ_{glc} was calculated as 3.1×10^{-12} mole/cell.

(c) Amino acid stoichiometric coefficients ($\theta^P_{a,i}$)

All of the 20 amino acids are needed for the synthesis of cellular proteins. In order to calculate amino acid stoichiometric coefficients, the moles of adenine (N_A), guanine (N_G), cytosine (N_C), thymine (N_T), and uracil (N_U) should be obtained first:

$$\begin{aligned} N_A &= N_{DNA} \cdot D_A + N_{RNA} \cdot R_A \\ &= 8.5 \times 10^{-14} \, \text{mole/cell} \ , \end{aligned} \tag{21}$$

$$\begin{aligned} N_G &= N_{DNA} \cdot D_G + N_{RNA} \cdot R_G \\ &= 8.5 \times 10^{-14} \, \text{mole/cell} \ , \end{aligned} \tag{22}$$

$$\begin{aligned} N_C &= N_{DNA} \cdot D_C + N_{RNA} \cdot R_C \\ &= 8.5 \times 10^{-14} \, \text{mole/cell} \ , \end{aligned} \tag{23}$$

$$N_T = N_{DNA} \cdot D_T = 2.5 \times 10^{-14} \, \text{mole/cell} \ , \tag{24}$$

$$N_U = N_{RNA} \cdot R_U = 2.5 \times 10^{-14} \, \text{mole/cell} \ , \tag{25}$$

where the total moles of nucleotides in DNA (N_{DNA}) and RNA (N_{RNA}) are calculated from the cell composition and the average molecular weights of DNA (M_D) and RNA (M_R):

$$N_{DNA} = Z_D W / M_D = 8.5 \times 10^{-14} \text{ mole} \ , \tag{26}$$

$$N_{RNA} = Z_R W / M_R = 8.5 \times 10^{-14} \text{ mole} \ . \tag{27}$$

Then the stoichiometric coefficients of glutamine, glycine and aspartate, which contribute the syntheses of DNA and RNA, are calculated as follows:

$$\begin{aligned} \theta_{gln} &= [Zp\,W]\theta^P_{a,gln} + 3N_G + 2[N_A + N_C + N_T + N_U] \\ &= 8.65 \times 10^{-13} \text{ mole/cell} \ , \end{aligned} \tag{28}$$

$$\begin{aligned} \theta_{gly} &= [Zp\,W]\theta^P_{a,gly} + N_G + 2N_A \\ &= 3.9 \times 10^{-13} \text{ mole/cell} \ , \end{aligned} \tag{29}$$

$$\begin{aligned} \theta_{asp} &= [Zp\,W]\theta^P_{a,asp} + N_G + N_A + N_C + N_T + N_U \\ &= 4.3 \times 10^{-13} \text{ mole/cell} \ , \end{aligned} \tag{30}$$

$$\begin{aligned} \theta_{glu} &= [Zp\,W]\theta^P_{a,glu} - \{3N_G + 2[N_A + N_C + N_T + N_U]\} \\ &= -6.7 \times 10^{-13} \text{ mole/cell} \ , \end{aligned} \tag{31}$$

where N_A, N_G, N_C, N_T, and N_U represent moles of adenine, guanine, cytosine, thymine and uracil; Zp represent the dry weight percentage of protein in cell mass (%). The negative value of θ_{glu} was obtained due to the generation of glutamate from purine and pyrimidine synthesis. The stoichiometric coefficients for other amino acids are obtained as follows:

$$\begin{aligned} &\theta_{a,i} = [Zp\,W]\theta^P_{a,i} : \\ &\theta_{ala} = 1.6 \times 10^{-13} \text{ mole/cell}; \ \theta_{arg} = 9.2 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{asn} = 9.0 \times 10^{-14} \text{ mole/cell}; \ \theta_{cys} = 5.3 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{his} = 4.0 \times 10^{-14} \text{ mole/cell}; \ \theta_{lie} = 9.1 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{leu} = 1.3 \times 10^{-14} \text{ mole/cell}; \ \theta_{lys} = 1.3 \times 10^{-13} \text{ mole/cell}, \\ &\theta_{met} = 5.1 \times 10^{-14} \text{ mole/cell}; \ \theta_{phe} = 6.6 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{pro} = 9.3 \times 10^{-14} \text{ mole/cell}; \ \theta_{ser} = 8.9 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{thr} = 8.1 \times 10^{-14} \text{ mole/cell}; \ \theta_{try} = 2.6 \times 10^{-14} \text{ mole/cell}, \\ &\theta_{tyr} = 5.2 \times 10^{-14} \text{ mole/cell}; \ \theta_{val} = 1.1 \times 10^{-13} \text{ mole/cell} \ . \end{aligned} \tag{32}$$

Table 2 shows the comparison of all amino acid stoichiometric coefficients between our calculated values and the experimental values of Ferrance et al. [17] and Drews et al. [18]. Among them, the order of magnitude of calculated stoichiometric coefficients for all amino acids, except glutamate and alanine, is compatible to that of experimental values in the literature. The differences probably resulted from the assumptions of cell composition and the culture conditions. A stoichiometric coefficient of 8.7×10^{-13} mole/cell for glutamate, taken from our calculated value for glutamine, was used in the medium design because the calculated stoichiometric coefficient of glutamate was far from the experimental data [17, 18].

(d) Yeastolate stoichiometric coefficient (θ_y)

The stoichiometric coefficient for yeastolate was defined as:

$$\begin{aligned} \theta_y = &\text{ the amount of yeastolate supplied/} \\ &\text{maximal cell number formed} \ . \end{aligned} \tag{33}$$

In batch culture, 8 g/l yeastolate could provide cell to grow up to density of 1.8×10^7 cells/ml [17]. Therefore, θ_y was estimated as:

$$\begin{aligned} \theta_y &= (8 \times 10^{-3} \, \text{g/ml})/(1.8 \times 10^7 \, \text{cells/ml}) \\ &= 4.44 \times 10^{-10} \, \text{g/cell} \ . \end{aligned} \tag{34}$$

Table 2. Comparison of stoichiometric coefficients between our calculated values and experimental ones of Ferrance et al.'s and Drews et al.'s

Stoichiometric coefficients	Chiou et al.'s calculated stoichiometric coefficients	Ferrance et al.'s experimental stoichiometric coefficients	Drews et al.'s experimental stoichiometric coefficients
θ_{ala}	1.6×10^{-13}	-1.8×10^{-12}	-1.6×10^{-12}
θ_{arg}	9.2×10^{-14}	4.8×10^{-13}	1.0×10^{-13}
θ_{asn}	9.0×10^{-14}	1.1×10^{-13}	1.0×10^{-13}
θ_{asp}	4.3×10^{-13}	1.0×10^{-12}	1.0×10^{-13}
θ_{cys}	5.3×10^{-14}	8.3×10^{-12}	
θ_{glu}	-6.7×10^{-13}	1.6×10^{-12}	1.0×10^{-13}
θ_{gln}	8.7×10^{-13}	1.0×10^{-12}	1.0×10^{-13}
θ_{gly}	3.9×10^{-13}	2.0×10^{-13}	1.5×10^{-13}
θ_{his}	4.0×10^{-14}	4.7×10^{-14}	5.0×10^{-14}
θ_{lie}	9.1×10^{-14}	1.2×10^{-13}	9.0×10^{-14}
θ_{leu}	1.4×10^{-14}	1.7×10^{-14}	1.4×10^{-13}
θ_{lys}	1.3×10^{-13}	7.2×10^{-14}	1.2×10^{-13}
θ_{met}	5.4×10^{-14}	1.3×10^{-13}	5.0×10^{-14}
θ_{phe}	6.6×10^{-14}	1.8×10^{-13}	7.0×10^{-14}
θ_{pro}	9.3×10^{-14}	1.9×10^{-13}	1.3×10^{-13}
θ_{ser}	8.9×10^{-14}	5.0×10^{-13}	1.0×10^{-13}
θ_{thr}	8.1×10^{-14}	2.8×10^{-14}	9.0×10^{-14}
θ_{try}	2.6×10^{-14}	6.2×10^{-14}	3.0×10^{-14}
θ_{tyr}	5.2×10^{-14}	1.1×10^{-13}	5.0×10^{-14}
θ_{val}	1.1×10^{-13}	1.7×10^{-13}	1.1×10^{-13}

Table 3. Composition of initial medium

Components	Concentration (mg/l)
Inorganic salts	
$CaCl_2$	750
KCl	4100
$MgCl_2 \cdot 6H_2O$	2280
$MgSO_4 \cdot 7H_2O$	2780
$NaH_2PO_4 \cdot H_2O$	1013
$NaHCO_3$	350
Amino acids	
L-Alanine	7.5 (0.085 mM)
L-Arginine · HCl	10.5 (0.050 mM)
L-Asparagine	6.6 (0.050 mM)
L-Aspartate	33.0 (0.250 mM)
L-Cysteine	3.2 (0.027 mM)
L-Glutamate	75.0 (0.440 mM)
L-Glutamine	66.8 (0.440 mM)
Glycine	7.5 (0.100 mM)
L-Histidine · HCl · H_2O	5.2 (0.025 mM)
L-Isoleucine	5.9 (0.045 mM)
L-Leucine	9.2 (0.070 mM)
L-Lysine · HCl	10.9 (0.060 mM)
L-Methionine	3.7 (0.025 mM)
L-Phenylalanine	5.8 (0.035 mM)
L-Proline	7.5 (0.065 mM)
L-Threonine	5.4 (0.045 mM)
L-Tryptophan	3.1 (0.015 mM)
L-Tyrosine	4.5 (0.025 mM)
L-Valine	6.4 (0.055 mM)
Vitamins	
PAPA	0
Biotin	0
Choline · Cl	0.22
D-Ca Pantothenate	0.02
Folic acid	0.02
i-Inositol	0.04
Nicotinic acid	0.02
Pyridoxine · HCl	0.02
Riboflavin	0.002
Thiamine · HCl	0.02
Other components	
D-Glucose	279 (1.55 mM)
Fumaric acid	55
α-Ketoglutaric acid	370
Malic acid	670
Succinic acid	60
Sucrose	26680
Yeastolate	220
Lactoalbumin Hydrolysate	3330

2.3 Medium design

Two media were designed in the fed-batch experiment. An initial medium was employed to initiate the cultivation and followed by the feeding of a supplemental medium according to a stoichiometric feeding strategy. The initial medium was designed to provide basal nutrients for cells to grow up to 5×10^5 cells/ml. As shown in Table 3, it consisted of yeastolate, lactoalbumin hydrolysate, sodium bicarbonate, glucose, inorganic salts, amino acids, vitamins, and other components, with the addition of 10% fetal bovine serum. Inorganic salts were added to maintain the same osmolality of the Grace's medium.

The supplemental medium, which included glucose, yeastolate and amino acids, was designed for using 0.8 ml medium to supply the increase of 10^8 cells in 100 ml medium. Components of designed supplemental medium are listed in Table 4.

2.4 Feeding strategy

The amount of the supplemental medium needed to be added to the cell culture system can be estimated from the nutrient demand of cell growth according to our stoichiometric model. The amount of the nth feeding was determined based on the measured total cell density from the following equation:

$$\Delta V_n^f = \beta \Delta N_{t,n}/C_t - [\Delta V_{n-1}^f - \beta \Delta N_{t,n-1}/C_t] \ , \qquad (35)$$

where ΔV_n^f: feeding volume of the nth feeding (L); ΔV_{n-1}^f: feeding volume of the $(n-1)$th feeding (L); β: total stoichiometric coefficient of amino acids and glucose (mmol/cell); $\Delta N_{t,n}$: net increase in total cells in the culture system between the time interval of the nth feeding and the $(n+1)$th feeding (cells); $\Delta N_{t,n-1}$: net increase in total cells in the reactor between the time interval of the nth feeding and the $(n-1)$th feeding (cells); C_t: total concentration of glucose and amino acids in the supplemental medium (mM).

Table 4. Composition of supplemental medium

Components	Concentration (mg/40 ml)
Glucose	5600 (0.778 M)
Yeastolate	4400
Amino acids	
L-Alanine	0 (0.0 M)
L-Arginine · HCl	105 (0.0125 M)
L-Asparagine	66 (0.0125 M)
L-Aspartate	325 (0.0613 M)
L-Cysteine	32 (0.0066 M)
L-Glutamate·Na	735 (0.1088 M)
L-Glutamine	635 (0.1088 M)
Glycine	75 (0.0250 M)
L-Histidine · HCl · H_2O	52 (0.0063 M)
L-Isoleucine	59 (0.0113 M)
L-Leucine	92 (0.0175 M)
L-Lysine · HCl	109 (0.0150 M)
L-Methionine	37 (0.0063 M)
L-Phenylalanine	58 (0.0088 M)
L-Proline	75 (0.0163 M)
L-Serine	53 (0.0125 M)
L-Threonine	54 (0.0113 M)
L-Tryptophan	31 (0.0038 M)
L-Tyrosine	45 (0.0063 M)
L-Valine	64 (0.0138 M)

3 Materials and methods

3.1 Cell culture, medium, and experimental apparatus

Spodoptera frugiperda IPLB Sf-21 insect cells were used in the study. The cells are anchorage-independent. Cells were routinely maintained in T-flask in the medium of TNM-FH with 10% FBS (Gibco) at 27 °C in a water humidified incubator.

TNM-FH consisted of 46 g/l Grace's insect cell medium (Gibco), 3.3 g/l of yeastolate (Difco), and 3.3 g/l lactoalbumin hydrolysate (Gibco) (adjusting pH to 6.4 with NaOH).

Fed-batch experiments were carried out in a 250-ml spinner flask (Fig. 2). The 250-ml spinner flask was equipped with an impeller in the center. Oxygen supply was achieved by surface aeration as shown in Fig. 2. 4.3×10^5 cells/ml were initially inoculated into 50 ml of initial medium. Samples were taken every 24 h to measure the cell density. The supplemental medium was fed into medium when cells grow up to 5×10^5 cells/ml. The concentration of amino acids, glucose and lactate were monitored during the fed-batch culture.

As control, a batch culture of insect cells was performed, using 50 ml of medium TNM-FH with 10% FBS in a 250-ml spinner flask. An inoculum of 4.3×10^5 cells/ml was used to initiate the batch culture. Samples were taken to measure the cell density at 12 h intervals.

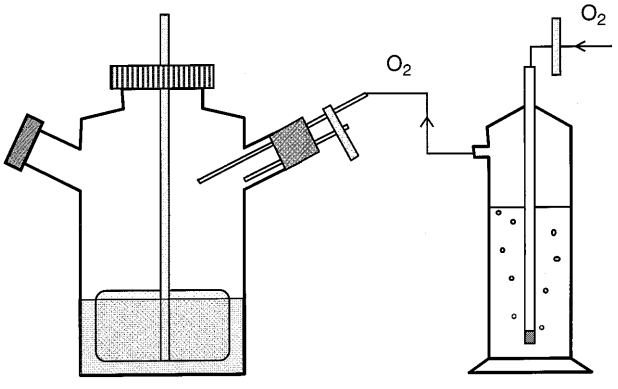

Fig. 2. Diagram of an agitated spinner flask with aeration apparatus

3.2 Determination of cell density and the concentration of amino acids, lactate, glucose and ammonia

Cell density was determined using a hemocytometer and the viability was assessed by 0.2% trypan blue (Gibco) exclusion. Amino acid concentration was determined by measuring derivatives of phenylisothiocyanate reacted with amino acids [22, 23], using HPLC (column: Phase Sep ODS2) which was equipped with UV monitor. Glucose, lactate and ammonium were enzymatically determined using Glucose [HK] assay kit (Sigma), Lactate assay kit (Sigma), and Ammonium assay kit (Sigma).

3.3 Recombinant virus, hIL-5 production and hIL-5 assay

A recombinant *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) with the encoded gene for human interleukin-5 (hIL-5) was used in this study. The recombinant virus was constructed and kindly provided by Dr J.J.-Y. Yen. For the production hIL-5, insect cells grown in TNM-FH with 10% FBS or the designed medium were harvested by centrifugation and resuspended in fresh TNM-FH with 10% FBS or the designed medium, respectively, and adjusted to the cell densities tested.

Infection was performed at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell. The titer of human interleukin-5 was determined by measuring ED_{50} of IL-5 dependent cell JYTF-1 [24]. JYTF-1 cells were maintained in the presence of small amount of hIL-5 using the medium of 90% PRMI-1640 (Gibco), 1% L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), 0.1% 2-mercaptoethanol (Gibco), and 10% FBS at 37 °C.

4 Results and discussion

4.1 High density cell culture by fed batch operation

Using the initial medium, supplemental medium and the feeding strategy based on the stoichiometric model, the fed batch culture experiments was performed. The experimental results are described as follows.

As shown in Fig. 3, Sf-21 cells reached a high density of 1.9×10^7 cells/ml by our stoichiometric feeding strategy. The cell density achieved was 3-fold to the density achieved in the batch culture (also shown in Fig. 3).

The concentration of glucose changed periodically due to the consumption by cells and fed-batch addition during

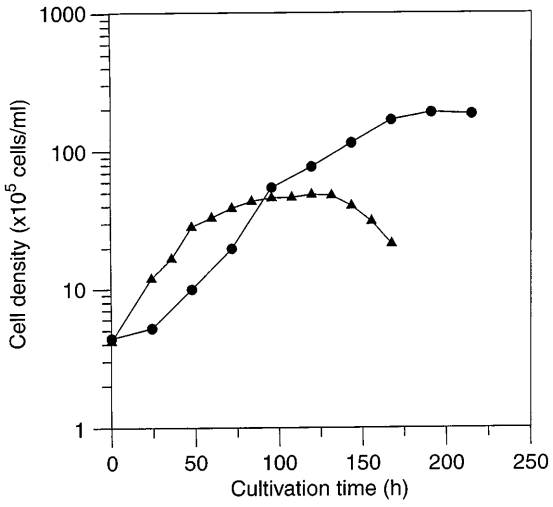

Fig. 3. The growth of Sf-21 insect cells in batch culture (▲) using TNM-FH with 10% FBS and fed-batch culture (●) using designed medium

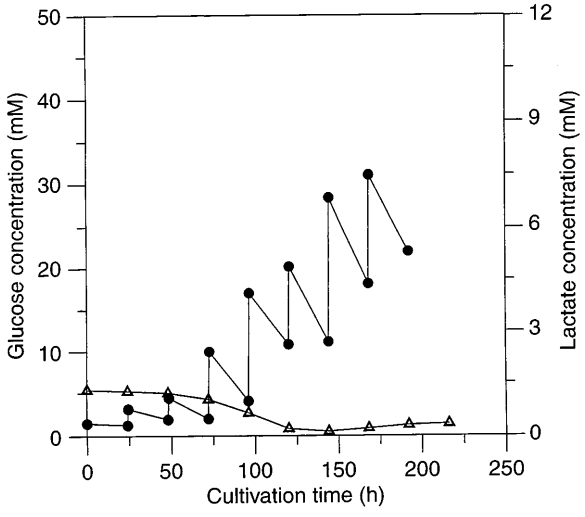

Fig. 4. Glucose (●) and lactate (△) concentration during stoichiometric fed-batch cultivation process

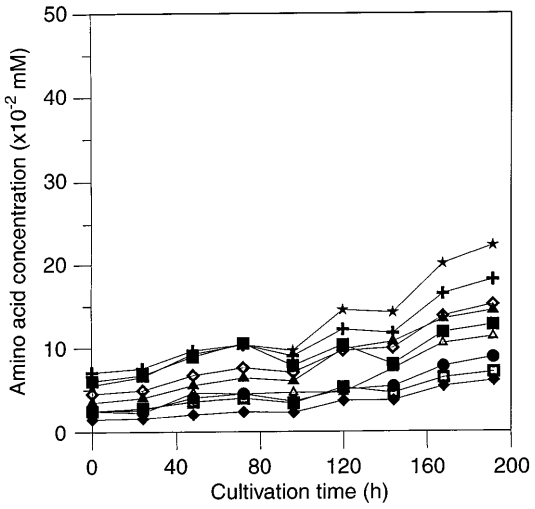

Fig. 5. Residual concentration of balanced amino acids before fed-batch supplement during stoichiometric fed-batch cultivation of SF-21. △, Histidine; ◇, Isoleucine; +, Leucine; ■, Lysine; □, Methionine; ▲, Phenylalanie; ◆, Tryptophan; ●, Tyrosine; ★, Valine

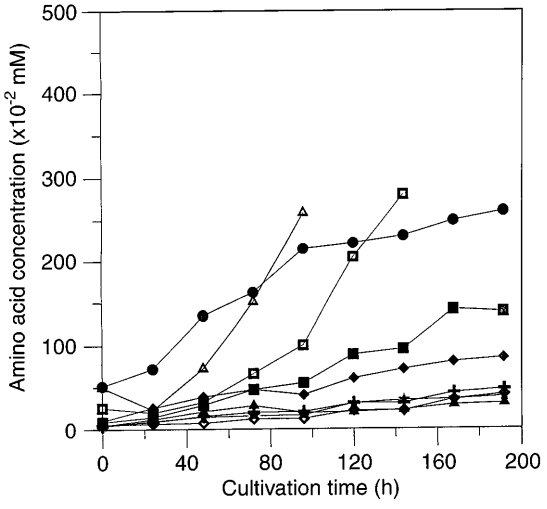

Fig. 6. Residual concentration of unbalanced amino acids before fed-batch supplement during stoichiometric fed-batch cultivation of SF-21. ■, Alanine; +, Arginine; ◇, Asparagine; □, Asparate; ●, Glutamate; △, Glutamine; ◆, Glycine; ▲, Serine; ★, Threonine

the cultivation process. The concentration of glucose remained above 25 mM after 200 h culture, suggesting that the supplement of glucose was sufficient (Fig. 4). The concentration of lactate remained below 1.5 mM during stoichiometric fed-batch cultivation of Sf-21 insect cells, suggesting that lactate was nearly completely oxidized and did not cause toxic effect for cells growth (Fig. 4). The concentration of ammonia was measured in a range of 1.3–3.7 mM during the cultivation process. It was well below the toxic level reported [25].

Based on the metabolic characteristics of amino acids, the amino acids were divided into two groups of balanced and unbalanced amino acids by Ferrence et al. [17]. Residual concentration of balanced amino acids and unbalanced amino acids were monitored respectively during the cultivation process. Figures 5 and 6 show the concentrations of balanced amino acids and unbalanced amino acids respectively during the cultivation. It is indicated that the culture was not limited by the availability of these amino acids during the cultivation process because their measured residual amounts were increasing.

The cell density reached a plateau after 156 h cultivation. Based on the observations, it was possibly due to the limitation of serum components or oxygen. It requires further investigations to elucidate the phenomenon.

4.2
The production of hIL-5 by the insect cell/ baculovirus expression system

Since TNM-FH supplemented with 10% FBS has been commonly used for insect cell/baculovirus expression system, the production hIL-5 at various cell densities was performed in TNM-FH with 10% FBS as control experiment. As shown in the Fig. 7, the medium could support

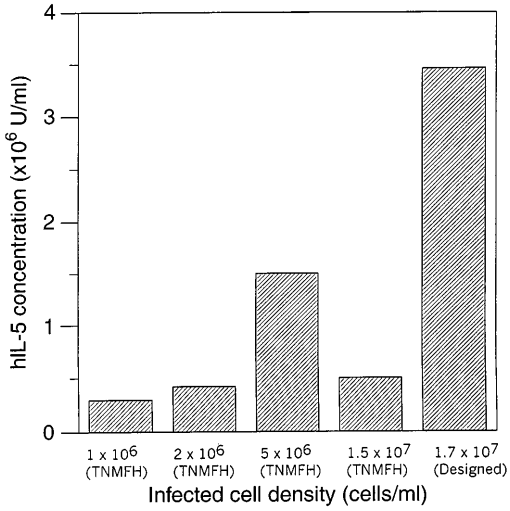

Fig. 7. Comparison of the total amount of hIL-5 produced per ml at various infected cell densities when Sf-21 cells were cultured in different media

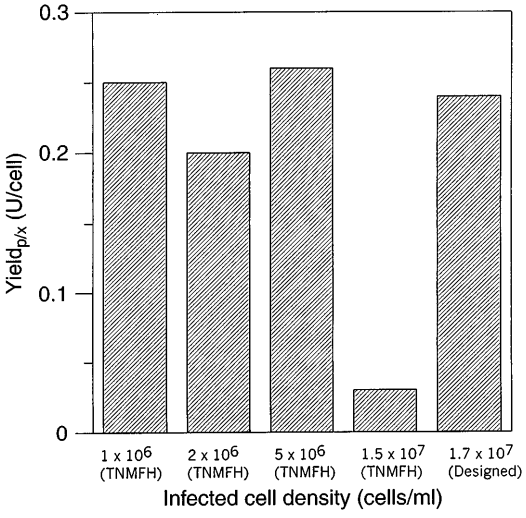

Fig. 8. Comparison of the specific amount of hIL-5 produced per cell at various infected cell densities when Sf-21 cells were cultured in different media

the production of hIL-5 by the expression system up to a cell density of 5×10^6 cells/ml. As the cell density increased from 1×10^6 to 5×10^6 cells/ml, hIL-5 production was also increased. The product yields ranged from 0.2 to 0.25 U/cell in these cell densities (Fig. 8). However, at a very high cell density such as 1.5×10^7 cells/ml, the hIL-5 concentration produced was much lower than that from a cell density of 5×10^6 cells/ml. The production yield dropped to 0.03 U/cell. It was probably due to the limitation of nutrient substrates provided by TNM-FH with 10% FBS.

Nevertheless, high concentration of hIL-5 could be achieved at very high cell density such as 1.7×10^7 cells/ml in our designed medium. 3.5×10^6 U/ml of hIL-5 was obtained and the production yield was 0.24 U/cell which was comparable to those obtained in TNM-FH with 10% FBS. The availability of nutrient substrates in our designed medium could support the expression of recombinant hIL-5 at high cell density.

5 Conclusions

A high cell density of 1.9×10^7 cells/ml was achieved using our designed medium and feeding strategy. Based on our analytical data, glucose and amino acids were sufficiently supplied and the cumulation of lactate and ammonia in the medium was very low during the fed-batch culture process. Using the commonly used insect-cell medium TNM-FH with 10% serum, very high cell density did not necessarily lead to very high product concentration due to substrate limitation. The resulted product yield was significantly decreased when the cell density exceeded 5×10^6 cells/ml. However, using our designed medium, the product yield was not sacrificed at high cell density (such as 1.7×10^7 cells/ml). This high cell density consequently led to the highest product concentration.

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