Fed-batch culture optimization of a growth-associated hybridoma cell line in chemically defined protein-free media

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Abstract An investigation was made to study the processes of fed-batch cultures of a hybridoma cell line in chemically defined protein-free media. First of all, a strong growth-associated pattern was correlated between the production of MAb and growth of cells through the kinetic studies of batch cultures, suggesting the potential effectiveness of extending the duration of exponential growth in the improvement of MAb titers. Second, compositions of amino acids in the feeding solution were balanced stepwisely according to their stoichiometrical correlations with glucose uptake in batch and fed-batch cultures. Moreover, a limiting factor screening revealed the constitutive nature of Ca^{2+} and Mg^{2+} for cell growth, and the importance of their feeding in fed-batch cultures. Finally, a fed-batch process was executed with a glucose uptake coupled feeding of balanced amino acids together with groups of nutrients and a feeding of CaCl_2 and MgCl_2 concentrate. The duration of exponential

cell growth was extended from 70 h in batch culture and 98 h in fed-batch culture without $\text{Ca}^{2+}/\text{Mg}^{2+}$ feeding to 117 h with $\text{Ca}^{2+}/\text{Mg}^{2+}$ feeding. As a result of the prolonged exponential cell growth, the viable and total cell densities reached 7.04×10^6 and 9.12×10^6 cells ml^{-1} , respectively. The maximal MAb concentration achieved was increased to approximately eight times of that in serum supplemented batch culture.

Keywords Calcium · Fed-batch culture · Growth-associated · Hybridoma · Magnesium · Monoclonal antibody (MAb) · Protein-free

Introduction

Among the approaches employed in mammalian cell culture processes, fed-batch culture has been one of the most widely used, due to its operational simplicity and higher yields of products on media as compared with those of batch, perfusion and continuous cultures (Xie and Wang 1994; Bibila and Robinson 1995; Zhou et al. 1997; de Zengotita et al. 2000; Dhir et al. 2000). The fundamental principle behind the success of fed-batch cultures lies in their capabilities to significantly increase the viable cell densities and to prolong the culture longevities at the same time (Luan et al. 1987; Miller et al. 1988; Bushell et al.

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1994; Zhou et al. 1995; Xie and Wang 1996; Xie et al. 1997). In practice, an effective feeding strategy should avoid the occurrences of nutrient limitations while minimizing the formation of metabolic byproducts such as lactate and ammonium (Wohlpert et al. 1990; Ozturk et al. 1992; Bushell et al. 1994; Xie and Wang 1994; Zhou et al. 1995; DiStefano et al. 1996). Previously, feeding strategies using stoichiometrically balanced amino acids in conjunction with measurable process parameters such as viable cell density, oxygen uptake rate (OUR) and glucose concentration were successfully employed in fed-batch cultures of mammalian cells (Ramirez and Mutharasan 1990; Duval et al. 1991; Xie and Wang 1994; Zhou et al. 1995; Stoll et al. 1996).

The optimization of cell culture media frequently necessitates the accurate information about medium components. Besides glucose and amino acids, the complex physiological requirements of mammalian cells may render other nutrient species limiting to cell growth, such as vitamins, lipids, phosphates and salts (Bibila and Robinson 1995; Xie and Wang 1996; de Zengotita et al. 2000). However, a thorough analysis of all the components in culture media is technically challenging, costly and time consuming. Therefore, the establishment of alternative methods which are able to efficiently determine limiting factors is critical to the successful optimization of culture media. Medium dilution or component deficient media have been used by several groups to determine limiting elements triggering apoptosis among amino acids and proteins (Mercille and Massie 1994; Franěk 1995; Franěk and Šrámková 1997; Fassnacht et al. 1998; Simpson et al. 1998). These approaches could also be properly adapted for the general purposes of limiting factor screenings among nutrients and toxicity assessments of metabolites such as ammonium.

Besides the physiological requirements for organic nutrients, the presence of a variety of minerals in culture media is essential to the growth of mammalian cells. Ca^{2+} , one of the most important metallic cations, plays important roles in many intracellular physiological activities, such as signal transductions regulating gene transcription, cell mitosis and apoptosis (Crabtree 2001). The other common cation, Mg^{2+} , is involved in

the ion transports across cell membranes and regulations of over 300 enzymes related to intracellular energy metabolism, fatty acids synthesis, ribosome stabilization and protein synthesis (Greenspan and Lowenstein 1968; Bais and Keech 1972; Guptas and Mildvan 1977). Therefore, Ca^{2+} and Mg^{2+} may be considered as constitutive building blocks for cells and therefore their availabilities in the media may contribute to the total numbers of cells obtainable in spite of the possibilities of re-uses after their releases from non-viable cells.

The major goal of this work was to investigate the fed-batch cultures of hybridoma JJ-1 cells in chemically defined protein-free media to produce monoclonal antibodies (MAbs). Batch studies were first carried out to identify the relationship between the formation of MAb and growth of cells. An amino acid balanced feeding solution was developed by studying the stoichiometrical relationships between the consumptions of amino acids and glucose uptake in batch and fed-batch cultures. Based on the observation made in limiting factor screening, a feeding of $\text{Ca}^{2+}/\text{Mg}^{2+}$ was implemented to the fed-batch culture in addition to the feeding of balanced amino acids with the other nutrient groups.

Materials and methods

Cell line and culture media

A murine hybridoma cell line, JJ-1, secreting MAbs against human tissue factor (hTF) was provided by Dr. Xintai Zhao, Shanghai Cancer Institute, China. Cells originally grown in DMEM supplemented with 10% FBS were adapted into and cultured routinely in a chemically defined protein-free medium (CD-HPFM). The CD-HPFM was a DMEM/F12 (1:1) based medium supplemented with non-essential amino acids (see Table 1), lipids (Tween 80 2.2 $\mu\text{l/l}$, water soluble linoleic acid 0.042, oleic acid 0.84, cholesterol 0.22 and V_E 0.085 mg/l), trace elements (TE) ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1×10^{-5} , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 3×10^{-3} , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 3×10^{-3} , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 1×10^{-3} , NaSeO_3 1×10^{-5} , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 1×10^{-6} , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1×10^{-6} , $\text{EDTA} \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$ 0.03

Table 1 Compositions of amino acids in the starting media, spent media, feeding media and calculated consumption ratios to glucose uptake

AA	Batch			Fed-batch C_0	FB01		FB02		FB03	
	C_0	C_r	R_i		C_f	R_i	C_f	R_i	C_f	R_i
Asp	0.150	0.070	−0.004	0.150	0.000	0.003	0.487	0.010	0.487	0.016
Asn	0.150	0.080	0.012	0.150	1.79	0.014	2.06	–	2.06	–
Arg	0.700	0.530	0.025	0.700	3.83	0.025	3.83	0.021	3.83	0.035
His	0.150	0.080	0.006	0.150	0.95	0.008	1.22	0.011	1.22	0.013
Ile	0.416	0.180	0.017	0.416	2.55	0.026	4.07	0.036	4.07	0.048
Leu	0.451	0.270	0.019	0.451	2.88	0.033	4.94	0.034	4.94	0.068
Lys	0.499	0.110	0.023	0.499	3.49	0.044	6.68	0.050	6.68	0.052
Met	0.116	0.000	0.013	0.116	1.93	0.020	3.05	0.020	3.05	0.021
Phe	0.215	0.120	0.009	0.215	1.36	0.015	2.24	0.019	2.24	0.020
Pro	0.250	0.260	0.001	0.250	0.186	0.001	0.186	0.004	0.186	0.006
Ser	0.350	0.210	0.012	0.350	1.78	0.010	1.54	0.016	1.54	0.038
Thr	0.449	0.240	0.010	0.449	1.54	0.023	3.36	0.023	3.36	0.034
Val	0.452	0.210	0.018	0.452	2.71	0.032	4.84	0.040	4.84	0.050
Cys	0.100	0.070	0.008	0.100	1.16	0.009	1.38	0.009	1.38	0.009
Trp	0.044	0.020	0.003	0.044	0.398	0.005	0.708	–	0.708	–
Tyr	0.452	0.090	0.008	0.452	1.16	0.016	2.38	0.013	2.38	0.012
Gln	4.00	0.650	0.355	0.300	53.2	0.375	56.3	–	56.3	–
Glu	0.150	0.180	−0.004	0.150	0	−0.026	0	−0.008	0	−0.003
Gly	0.350	0.380	−0.007	0.350	0	−0.018	0	−0.001	0	−0.020
Ala	0.150	1.43	−0.085	0.150	0	−0.084	0	−0.074	0	−0.067

C_0 , concentration in starting medium (mM); C_r , residual concentration at growth stalling time point (mM)

C_f , concentration in feeding medium (mM); R_i , calculated consumption ratio of amino acid_{*i*} to glucose uptake

and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 1×10^{-6} mM), ethanol-amine 3 $\mu\text{l/l}$, 2-mercaptoethanol 4 $\mu\text{l/l}$, and Pluronic F68 1.0 g/l. The concentration of glucose and glutamine was 17.5 and 4 mM, respectively, unless specified. All chemicals were purchased from Sigma Chemical Co. A 2 l Biostat M stirred-tank bioreactor (B. Braun Biotech, Inc.) was used in this study.

Culture conditions

Cells were maintained in 125 ml shaker flasks on a rotary shaker (90 rpm) in a 37°C 5% CO_2 incubator and passaged every 2 days at a seeding density of 2×10^5 cells ml^{-1} . Cells in the mid of exponential growth phase were harvested by centrifugation from a 1,000 ml shaker flask (250 ml working volume) and resuspended in fresh medium before the inoculation of the bioreactor. Operational parameters for the bioreactor were set as 37°C, pH 7.0, 50% of air saturation for the dissolved oxygen, and 70 rpm for impeller speed throughout the cultivation.

Analytical methods

Cell density and viability were estimated by a trypan blue dye exclusion method with a hemocytometer. The concentration of MAb was quantified by ELISA with a limit of detection of $50 \mu\text{g l}^{-1}$ with the confirmation of OD_{280} measurement after protein A purification (Harlow and Lane 1999). Concentrations of glucose, lactate and ammonium were measured with YSI 2300 STAT PLUS glucose/L-lactate analyzer and Model 95-12 ammonia electrode (Thermo Electron Co.), respectively. Amino acids in medium samples were analyzed by reversed-phase HPLC after precolumn AccQ.Tag (Waters Co.) derivatization. Samples of medium were diluted 5–20 times and analyzed against references of amino acids with a limit of detection of 50 pmoles.

Determination of MAb production pattern

The mathematical relationship between the concentrations of MAb (P) and viable cells (X) can be expressed by the following equations:

$$d\mathbf{P}/dt = k_1 d\mathbf{X}/dt + k_2 \mathbf{X} \quad (1)$$

$$\mu = d\mathbf{X}/\mathbf{X}dt \quad (2)$$

$$d\mathbf{P}/dt = (k_1\mu + k_2)\mathbf{X} \quad (3)$$

$$\mathbf{P}_t - \mathbf{P}_0 = (k_1\mu + k_2) \int_0^t \mathbf{X}dt \quad (4)$$

$$\mathbf{VI}_t = \int_0^t \mathbf{X}dt \quad (5)$$

$$\mathbf{q}_{\text{MAb}} = (k_1\mu + k_2) \quad (6)$$

where k_1 is the yield for growth-associated antibody production (pg cell^{-1}), k_2 is the specific productivity for non-growth-associated antibody production ($\text{pg cell}^{-1} \text{day}^{-1}$), μ is the specific growth rate of cells (day^{-1}), $\mathbf{q}_{\text{MAb}}$ is the general specific productivity ($\text{pg cell}^{-1} \text{day}^{-1}$), and $\mathbf{VI}$ is the viability index ($\text{cells ml}^{-1} \text{day}$) as the integration of viable cell density over a time period.

From these equations, three general types of product formation patterns can be referred in relation to cell growth, i.e. growth-associated, non-growth-associated and partially growth-associated. These patterns can be identified experimentally by the examinations of kinetic results of batch cultures and plotting of product concentrations against the viability indexes (Merten 1988; Shen et al. 1996; Dutton et al. 1998). For a predominantly growth-associated product, where $k_1\mu \gg k_2$, the accumulation of product is expected to largely happen during the active multiplication of cells and thus the prolonging of exponential cell growth is probably a more efficient measure for the improvement of product titer. For a cell line that has a characteristic of complete ($k_1 = 0$) or significantly partial non-growth-associated product formation, however, extending the longevity of a high cell density culture can more effectively help the accumulation of a product.

Stoichiometrical balances among amino acids in feeding solution

Based on our early experimental observation that, the consumptions of both amino acids and glucose were directly proportional to the yield of JJ-1 cells during the exponential growth phase in batch cultures, $\mathbf{A}_{i/\text{Glu}}$, the consumption ratios of

individual amino acids to glucose uptake were calculated with the following equations:

$$\mathbf{A}_{i/\text{Glu}} = \mathbf{q}_i/\mathbf{q}_{\text{Glu}} = d\mathbf{C}_i/d\mathbf{C}_{\text{Glu}} = (\mathbf{C}_{i,t_i} - \mathbf{C}_{i,t_{i-1}})/(\mathbf{C}_{\text{Glu},t_i} - \mathbf{C}_{\text{Glu},t_{i-1}}) \quad (7)$$

$$\mathbf{C}_{\text{feed},i} = \mathbf{A}_{i/\text{Glu}} \mathbf{C}_{\text{feed},\text{Glu}} \quad (8)$$

where $\mathbf{q}_i$ and $\mathbf{q}_{\text{Glu}}$ represent the specific uptake rate of amino acid i and glucose for the time period of $t_i - t_{i-1}$, $\mathbf{C}_{i,t_i}$ and $\mathbf{C}_{i,t_{i-1}}$ represent the concentration of amino acid i at time t_i and t_{i-1} , $\mathbf{C}_{\text{Glu},t_i}$ and $\mathbf{C}_{\text{Glu},t_{i-1}}$ represent the concentration of glucose at time t_i and t_{i-1} , $\mathbf{C}_{\text{feed},i}$ and $\mathbf{C}_{\text{feed},\text{Glu}}$ represent the concentration of amino acid i and glucose in the feeding solution, respectively.

Limiting factor screening

Five nutrient groups, including vitamins, TE, lipids, hypoxanthine and thymidine (HT), and Ca^{2+} and Mg^{2+} ($\text{Ca}^{2+}/\text{Mg}^{2+}$), were screened for their potentials to cause limitations on cell growth. The types and concentrations of vitamins included were the same as those listed in DMEM/F12 (1:1). The TE and lipid groups were the same mixtures of salts, fatty acids, cholesterol and V_E as described in the **Materials and methods**. The HT group consisted of Na hypoxanthine and thymidine at 2.39 and 0.365 mg/l, respectively. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ group was composed of CaCl_2 and MgCl_2 at 116.6 and 28.64 mg/l, respectively. Media of six different dilution levels of each group, 100, 87.5, 75, 50, 25 and 0% (complete CD-HPFM), were prepared by mixing the component-free CD-HPFM with the complete CD-HPFM at the ratios of 4:0, 3.5:0.5, 3:1, 2:2, 1:3, and 0:4. Aliquots of 3.5 ml were then added to each well in 6-well plates, followed by the inoculation at 2×10^5 cells ml^{-1} with 0.5 ml of cells in corresponding media before the incubation on a rotary shaker. Upon the completion of cultures, assessments were made to compare the maximal viable cell density under each condition to that in the complete CD-HPFM, $\mathbf{X}_{V_{\text{max,dilu}}}/\mathbf{X}_{V_{\text{max,com}}}$.

Fed-batch cultures

Fed-batch culture processes were studied with a feeding solution composed of four nutrient groups (vitamins, TE, lipids and HT) plus amino acids. The glucose and glutamine concentration was 1.1 and 0.3 mM in the starting medium, respectively. Cells in the mid of exponential growth phase were harvested to inoculate the 2 l bioreactor at the density of 2×10^5 cells ml⁻¹ in 1.0 l volume. The glucose concentration in the feeding solution was 150 mM while the other nutrients were tenfold of their concentrations in the starting medium. To maintain the concentration of glucose above 0.25 mM, medium feeding was started at about 12 h and then performed according to the viable cell density and glucose concentration measured at every 4–8 h. The feeding volume was calculated with the equations below:

$$q_{\text{Glu}} = -dC_{\text{Glu}}/Xdt \quad (9)$$

$$V_{\text{feed},i+1} = q_{\text{Glu}} X_i (t_{i+1} - t_i) V_i / C_{\text{feed,Glu}} \quad (10)$$

where $V_{\text{feed},i+1}$ represents the estimated feeding volume for the time period of $t_{i+1} - t_i$ at time t_i , X_i and V_i represent the viable cell density and total culture volume at time t_i , respectively.

On the basis of the implementations of amino acids and limiting factor screening, the feeding of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentrate was further implemented to the fed-batch culture. The concentration of CaCl_2 and MgCl_2 in the feeding solution was 21 and 14 mM, respectively, 20-folds of those in the starting medium. Shot feeding was initiated when viable cell density reached 1.0×10^6 cells ml⁻¹ (at about 50 h) and then performed at every 12 h.

Results

Batch cultures

When grown in DMEM with 10% FBS, as shown in Fig. 1, JJ-1 cells typically were able to reach a maximal viable cell density of about 1.8×10^6 cells ml⁻¹ with a final MAb concentration of 87 mg l⁻¹. After a weaning of more than 1,000 h, they were adapted to grow in the chemically defined protein-free medium CD-HPFM.

Fig. 1 Batch culture of JJ-1 cells in DMEM supplemented with 10% FBS

Results of a typical batch culture of JJ-1 cells in CD-HPFM were shown in Fig. 2a. The maximal density of viable cells reached 1.46×10^6 cells ml⁻¹ after 70 h. The production of MAb increased dramatically to approximately 116 mg l⁻¹ during the exponential growth phase of the first 58.5 h. In the stationary and decline phases of about 40 h, however, MAb formation slowed down dramatically and was accumulated to a final concentration of 136 mg l⁻¹. It should be noted that the levels of glucose, lactate and ammonium were probably not causing limitation or inhibitions on cell growth (Fig. 2b). The MAb produced during the exponential growth phase accounted for approximately 85% of the total. Although there existed differences in maximal viable cell densities and product yields between the serum supplemented and protein-free cultures, JJ-1 cells generally remained a similar pattern between the cell growth and MAb formation under both conditions. Figure 2c demonstrates the relationship between the MAb formation and viability index in five individual batches of JJ-1 cells in CD-HPFM. The entire course of batch culture was divided into two distinct regions, representing a period of active product formation during the exponential growth phase and that of less productiveness after the cells entered the stationary phase. The slopes calculated by statistical regression for both regions represented specific productivities of 125 and 6.2 pg cell⁻¹ day⁻¹ during and after the exponential growth phase. From the above

Fig. 2 Batch culture of JJ-1 cells in CD-HPFM. **(a)** Cell growth and MAb formation. **(b)** Concentration profiles of glucose, lactate and ammonium. **(c)** MAb formation

results, it is apparent that the production of MAb by JJ-1 cells was related to the exponential cell growth to a much higher extent than to the stationary and decline phases. That is to say, the MAb production pattern was strongly growth-associated for this particular cell line in spite of the continued product accumulation after the exponential growth phase. To effectively improve the yield of antibodies by JJ-1 cells, it is rational to focus on extending the time length of exponential growth phase rather than that of stationary and decline phases. Analyses of the residual amino acids in the medium at the stalling point of growth (58.5 h) revealed their different degrees of utilization by JJ-1 cells (Fig. 2d). Tryptophan was excluded in the analyses because of technical reasons. Among all of the amino acids analyzed, it

pattern. **(d)** Utilization profiles of amino acids at 58.5 h. Ala, Glu, Gly and Pro were omitted because they were accumulated during the cultures

was found that methionine was totally exhausted with more than 50% consumptions for several of the others. The consumption ratios of amino acids to glucose uptake were calculated and used to formulate the initial feeding solution for fed-batch culture (Table 1). Alanine, aspartic acid, glutamic acid and glycine were observed to accumulate by the cells during the culture and thus not given any further considerations in the design of feeding solution.

Stoichiometrical balance among amino acids in fed-batch cultures

With the use of the feeding solution based on the results of batch cultures, the fed-batch culture of JJ-1 cells was studied with an emphasis on the

Fig. 3 Fed-batch culture of JJ-1 cells in chemically defined protein-free medium (FB01). **(a)** Cell growth and MAb formation. **(b)** Concentrations of residual amino acids in the medium at 88 h

need of further adjustments of amino acid contents in the feeding solution (FB01). Compared with the results of batch culture in CD-HPFM, JJ-1 cells experienced a longer period of exponential cell growth of 88 h and reached a higher maximal viable cell density of $3.31 \times 10^6 \text{ cells ml}^{-1}$, resulting in a more than doubled yield of MAb, 304 mg l^{-1} (Fig. 3a). No further increase in MAb concentration was observed after the cell growth ceased. The analyses of residual amino acids at 88 h (Fig. 3b) and re-calculation of the consumption ratios of amino acids to glucose uptake (Table 1) apparently indicated the increased demands on some of the amino acids, especially isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine and tyrosine.

Based on these results, the feeding solution was modified to meet the changed requirements for these substrates (Table 1).

As the result of the above modifications to the feeding solution, improvements were subsequently observed in both cell growth and MAb production. In fed-batch process FB02, as a result of the further extended growth period of 97 h, the maximal viable cell density reached $3.89 \times 10^6 \text{ cells ml}^{-1}$ with a dramatically increased MAb yield of 559 mg l^{-1} , compared to $3.31 \times 10^6 \text{ cells ml}^{-1}$ and 304 mg l^{-1} in FB01 (Fig. 4a). Results also indicated that both glucose and glutamine were in abundance and were actually accumulated with the progression of the fed-batch culture, which were considered to be responsible

Fig. 4 Fed-batch culture of JJ-1 cells in chemically defined protein-free medium (FB02). **(a)** Cell growth and MAb formation. **(b)** Concentration profiles of glucose, lactate, glutamine and ammonium. **(c)** Concentrations of residual amino acids in the medium at 97 h

for the potentially inhibitory even toxic levels of lactate and ammonium accumulated, 48 and 9.4 mM, respectively (Fig. 4b). Analyses of residual amino acids at 97 h showed that their concentrations were neither lower than 50% of their initial values nor higher than 3 mM when the cells ceased to grow, suggesting that this feeding solution had probably achieved a stoichiometrical balance among amino acids at this stage (Fig. 4c).

Limiting factor screening

Besides amino acids, the full spectrum of nutrient requirements for cell growth also includes a variety of other small molecules in chemically defined protein-free medium. A series of screening experiments were carried out to help identify the group(s) of nutrients which most likely became limiting during the fed-batch cultures of JJ-1 cells, including (1) vitamins, (2) TE, (3) lipids, (4) HT and (5) $\text{Ca}^{2+}/\text{Mg}^{2+}$. Since the upper limits of these nutrients were commonly found in standard cell culture media, the growth of JJ-1 cells responded positively to all these nutrient groups (Fig. 5). However, the cells exhibited quite different sensitivities towards the concentration changes of these nutrient groups. When vitamins, TE, lipids and HT were diluted below 25% of their concentrations in the complete medium, the values of corresponding

Fig. 5 Normalized growth responses of JJ-1 cells to the dilution levels of different nutrient groups in chemically defined protein-free media for limiting factor screening

$\text{X}_{V_{\max,\text{dilu}}}/\text{X}_{V_{\max,\text{com}}}$ ($\text{X}_{V_{\max,\text{dilu}}}$ was the maximal viable cell density achieved under each dilution condition, and $\text{X}_{V_{\max,\text{com}}}$ was the maximal viable cell density achieved with the complete CD-HPFM) still remained above 80%, suggesting that these nutrients existed relatively abundantly in the media and were at lower risks to become limiting. In the $\text{Ca}^{2+}/\text{Mg}^{2+}$ limiting cultures, however, the highest viable cell density obtained was found to be basically in proportion to the levels of these ions available in the medium. Furthermore, the value of $\text{X}_{V_{\max,\text{dilu}}}/\text{X}_{V_{\max,\text{com}}}$ dropped sharply within the dilution range of 25–75%, and went down to zero when both cations were diluted to 25% of their concentrations in the complete medium. These results suggested that these two cations were probably of constitutive nature to the growth of JJ-1 cells and a continual supply of them to the medium could have important impacts on the viable cell densities in fed-batch cultures.

Fed-batch culture with $\text{Ca}^{2+}/\text{Mg}^{2+}$ feeding

On the basis of the FB02 run, a separate feeding of CaCl_2 and MgCl_2 concentrate was implemented to the fed-batch culture of JJ-1 cells. In the FB03 run, shot feeding of $\text{Ca}^{2+}/\text{Mg}^{2+}$ at a constant dose (except the first feeding) and a time interval of 12 h was started when the viable cell density reached 1.0×10^6 cells ml^{-1} (50 h). The exponential growth of JJ-1 cells was further prolonged to 117 h as the result of the dual feeding (Fig. 6a). The maximal densities of viable and total cells consequently reached 7.04×10^6 and 9.12×10^6 cells ml^{-1} respectively, significantly exceeding those of FB02 which received no $\text{Ca}^{2+}/\text{Mg}^{2+}$ feeding. The highest concentration of MAb reached 707 mg l^{-1} , which closely followed the active multiplication of cells and maximal viable cell density achieved. Similar to what was seen in FB01 and FB02, the MAb concentration slightly decreased during the decline phase, supporting our previous observation that extending the culture beyond the exponential growth had a less significant impact on the accumulation of MAb for JJ-1 cells if not at all.

Figure 6b illustrates that the concentration of glucose was generally maintained above 0.25 mM

Fig. 6 Fed-batch culture of JJ-1 cells in chemically defined protein-free medium with $\text{Ca}^{2+}/\text{Mg}^{2+}$ feeding (FB03). **(a)** Cell growth and MAb formation. **(b)** Concentration profiles of glucose feeding and lactate accumulation. **(c)** Concentration profiles of glutamine and ammonium

throughout FB03 with the help of manual feeding. The maximal concentrations of lactate, glutamine and ammonium at each feeding time point were depicted in Fig. 6b and c. As a result of the

gradually increased feeding volume required as the viable cell density increased, the cells experienced an increasing exposure to glucose and glutamine. Similar to those in FB02, both lactate

and ammonium experienced a gradual accumulation throughout the process and reached levels which might have significantly negative effects on the growth of cells. Analyses of residual amino acids in the medium at 117 h indicated further increased demands on arginine, leucine, isoleucine, serine, threonine and valine at this stage (Table 1).

Discussion

Because of the high specificities to their targets, antibody products have found many important applications in the diagnoses and therapeutic treatments of diseases (Goel and Batra 2001; Taylor 2003; Li et al. 2002). It should be noted that the success of antibody products has required a great deal of efforts made in order to improve the productivities and lower the costs for their manufacturing. One of the major obstacles to the improvement of process performance has been the low cell density in mammalian cell culture, compared with those in microbial processes. The complex cellular requirements for nutrients and high sensitivities to environmental factors have made the optimization of mammalian cell culture processes a challenging task (Xie and Wang 1994; Bibila and Robinson 1995; Zhou et al. 1995; Gong et al. 2005).

In this study, an investigation was made on hybridoma JJ-1 cells which secreted MAbs with potential applications in lung cancer treatments (Sawada et al. 1999; Salge et al. 2001; Chen et al. 2005; Minamiya et al. 2004). First of all, JJ-1 cells were found to characteristically exhibit a strong growth-associated pattern of product formation in both serum supplemented and chemically defined

protein-free media. In chemically defined protein-free batch cultures, the specific MAb productivity for the exponential growth period was found to be approximately 20 times of that for the stationary and decline phases (Table 2). This observation coincided with studies on several different hybridoma cell lines (Ramirez and Mutharasan 1990; Wohlpert et al. 1990), but was in contrast with the other results where the production of MAb predominantly happened after the exponential growth phase (Bloemkolk and Gray 1992; Sureshkumar and Mutharasan 1991; Suzuki and Ollis 1990). Although it is generally accepted that the improvements in MAb formation can be realized by promoting cell growth and extending the survival time of high cell density cultures (Luan et al. 1987; Miller et al. 1988; Bushell et al. 1994; Xie and Wang 1996; Zhou et al. 1997; de Zengotita et al. 2000), focusing on boosting the exponential cell growth has been proven to be more efficient for JJ-1 cells because of their much less product accumulation afterwards.

In order to promote the exponential cell growth, the stoichiometrical relationships between the consumptions of amino acids and glucose uptake were studied in both batch and fed-batch cultures to balance the compositions of amino acids in the feeding solution. A series of limiting factor screening experiments further led to the conclusions that cations Ca^{2+} and Mg^{2+} were constitutive for the cells and had the highest possibility to cause limitations on cell growth among the five nutrient groups tested. However, considering the possibilities to cause hazardous consequences by these two cations at elevated concentrations, such as cell aggregations, toxicities and incompatibilities with the other electrolytes in aqueous solutions (McKeehan et al. 1981;

Table 2 Process parameters estimated for different cultures

	Serum batch	Batch	FB01	FB02	FB03
Maximal viable cells (10^6 cells ml^{-1})	1.82	1.37	3.31	3.89	7.04
Maximal total cells (10^6 cells ml^{-1})	1.84	1.52	3.89	4.27	9.12
Duration of growth phase (h)	81.0	58.5	88.0	97.0	117
Maximal MAb (mg l^{-1})	87.0	136	304	559	707
μ (day^{-1})	0.912	0.816	0.816	0.792	0.864
k_1 (pg cell^{-1})	85.0	154	130	154	123
$k_1\mu$ ($\text{pg cell}^{-1} \text{ day}^{-1}$)	77.1	125	106	122	106
k_2 ($\text{pg cell}^{-1} \text{ day}^{-1}$)	–	6.2	–	–	–

Morton 1981), an independent feeding of a CaCl_2 and MgCl_2 concentrate was implemented to the fed-batch culture. With the dual feeding of an amino acid balanced medium together with the concentrates of four nutrient groups, and a concentrated CaCl_2 and MgCl_2 solution, the execution of fed-batch process was able to extend the duration of exponential growth to achieve 7.04×10^6 and 9.12×10^6 cells ml^{-1} for viable and total cell densities respectively, and to increase the MAb production to 707 mg l^{-1} . These results represented significant improvements in both cell growth and MAb production over those obtained in the initial serum supplemented batch culture (Fig. 7a and b). Table 2 summarizes the results and kinetic parameters obtained for JJ-1 cells in batch and fed-batch cultures in serum supplemented and chemically defined protein-free

media. Although there was a general decrease in the specific growth rate (μ) in the protein-free media, there is no doubt that the general increases in the yield for growth-associated antibody production (k_1) and specific productivity ($k_1\mu$) also contributed to the product formation in addition to the improved cell growth.

In this study, the level of glucose in the media was chosen as the process parameter to monitor and manually control the progression of fed-batch cultures. The subsequent consequences of this feeding strategy were the exposure of the cells to gradually increasing concentrations of glucose and glutamine as the cultures proceeded. Significant accumulations of lactate and ammonium were caused as the results of inefficient utilizations of these two substrates. The situation is expected to be improved by the use of automatic feeding to more precisely maintain the concentrations of nutrients in the media. Another important lesson learned from this study is that the cells may have changing requirements for nutrients in relation to glucose uptake as the optimization of media evolves. For example, the demands on amino acids arginine, leucine, isoleucine, serine, threonine and valine increased dramatically from FB01 to FB03. The situation would possibly become even more complicated when the utilizations of glucose and glutamine became less and less efficient as the fed-batch process went on. This may further emphasize the importance of more precise control of nutrient concentrations in fed-batch cultures.

Fig. 7 Performance comparisons of cell growth and MAb formation among different culture processes. **(a)** Cell growth. **(b)** MAb formation

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References

- Bais R, Keech B (1972) The magnesium ion (Mg^{2+}) activation of sheep kidney pyruvate carboxylase. *J Biol Chem* 247:3255–3261
- Bibila TA, Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. *Biotechnol Prog* 11:1–13
- Bloemkolk JW, Gray MR (1992) Effect of temperature on hybridoma cell cycle and MAb production. *Biotechnol Bioeng* 40:427–431

- Bushell ME, Bell SL, Scott MF, Spier RE, Wardell JN, Sanders PG (1994) Enhancement of monoclonal antibody yield by hybridoma fed-batch culture, resulting in extended maintenance of viable cell population. *Biotechnol Bioeng* 44:1099–1106
- Chen C, Yang Q, Patel S, Lei Y, McAleer L, Singleton J, Soltis D, Wang B (2005) Characterization of human tissue factor (TF)-specific monoclonal antibodies prepared using a rapid immunization protocol. *Hybridoma (Larchmt)* 24(2):78–85
- Crabtree GR (2001) Calcium, calcineurin, and the control of transcription. *J Biol Chem* 276:2313–2316
- de Zengotita VM, Miller WM, Aunins JG, Zhou W (2000) Phosphate feeding improves high-cell-concentration NS0 myeloma culture performance for monoclonal antibody production. *Biotechnol Bioeng* 69:566–576
- Dhir S, Morrow KJ, Rhinehart RR, Wiesner T (2000) Dynamic optimization of hybridoma growth in a fed-batch bioreactor. *Biotechnol Bioeng* 67:197–205
- DiStefano DJ, Mark GE, Robinson DK (1996) Feeding of nutrients delays apoptotic death in fed-batch cultures of NS0 myeloma cells. *Biotechnol Lett* 18:1067–1072
- Duval D, Demangel C, Munier-Jolain K, Miossec S, Geahel I (1991) Factors controlling cell proliferation and antibody production in mouse hybridoma cells: I. Influence of the amino acids supply. *Biotechnol Bioeng* 38:561–570
- Dutton RL, Scharer JM, Murray MY (1998) Descriptive parameter evaluation in mammalian cell culture. *Cytotechnology* 26:139–152
- Fassnacht D, Rössing S, Franěk F, Al-Rubeai M, Pörtner R (1998) Effect of Bcl2 expression on hybridoma cell growth in serum-supplemented, protein-free and diluted media. *Cytotechnology* 26:219–225
- Franěk K (1995) Starvation-induced programmed cell death of hybridoma cells: prevention by amino acid mixtures. *Biotechnol Bioeng* 45:86–90
- Franěk K, Šrámková K (1997) Cell suicide in starving hybridoma culture: survival-signal effect of some amino acids. *Cytotechnology* 23:231–239
- Goel A, Batra SK (2001) Antibody constructs for radio-immunodiagnosis and treatment of human pancreatic cancer. *Teratog Carcinog Mutagen* 21(1):45–57
- Gong X, Fang Q, Li X, Han X, Wu Y, Yang S, Shen BQ (2005) A high-throughput end-point assay for viable mammalian cell estimation. *Cytotechnology* 49:51–58
- Greenspan MD, Lowenstein JM (1968) Effects of magnesium ions, adenosine triphosphate, palmitoylcarnitine, and palmitoyl coenzyme A on acetyl coenzyme A carboxylase. *J Biol Chem* 243:6273–6280
- Guptas RK, Mildvan AS (1977) Structures of enzyme-bound metal nucleotide complexes in the phosphoryl transfer reaction of muscle pyruvate kinase. *J Biol Chem* 252:5961–5976
- Harlow E, Lane D (1999) Using antibodies a laboratory manual. Cold Spring, Harbor Press
- Li L, Das AM, Torphy TJ, Griswold DE (2002) What's in the pipeline? Prospects for monoclonal antibodies (mAbs) as therapies for lung diseases. *Pulm Pharmacol Ther* 15(5):409–416
- Luan YT, Mutharasan R, Magee WE (1987) Strategies to extend longevity of hybridomas in culture and promote yield of monoclonal antibodies. *Biotechnol Lett* 9:691–696
- McKeehan WL, McKeehaaaan KA, Ham RG (1981) The relationship between defined low-molecular-weight substances and undefined serum-derived factors in the multiplication of untransformed fibroblasts. In: Waymouth C, Ham RG, Chapple PJ (eds) *The growth requirements of vertebrate cells in vitro*. Cambridge University Press, New York
- Mercille S, Massie B (1994) Induction of apoptosis in nutrient-deprived cultures of hybridoma and myeloma cells. *Biotechnol Bioeng* 44:1140–1154
- Merten O-W (1988) Batch production and growth kinetics of hybridomas. *Cytotechnology* 1:113–121
- Miller WM, Blanch HW, Wilke CR (1988) A kinetics analysis of hybridoma growth and metabolism in batch and continuous suspension culture: effect of nutrient concentration, dilution rate, and pH. *Biotechnol Bioeng* 32:947–965
- Minamiya Y, Matsuzaki I, Sageshima M, Saito H, Taguchi K, Nakagawa T, Ogawa J (2004) Expression of tissue factor mRNA and invasion of blood vessels by tumor cells in non-small cell lung cancer. *Surg Today* 34(1):1–5
- Morton HJ (1981) Known cellular growth requirements and the composition of currently available defined media. In: Waymouth C, Ham RG, Chapple PJ (eds) *The growth requirements of vertebrate cells in vitro*. Cambridge University Press, New York
- Ozturk SS, Riley MR, Palsson BO (1992) Effects of ammonia and lactate on hybridoma growth, metabolism and antibody production. *Biotechnol Bioeng* 39:418–431
- Ramirez OT, Mutharasan R (1990) Cell cycle-and growth phase-dependent variations in size distribution, antibody productivity, and oxygen demand in hybridoma cultures. *Biotechnol Bioeng* 36:839–848
- Salge U, Seitz R, Wimmel A, Schuermann M, Daubner E, Heiden M (2001) Transition from suspension to adherent growth is accompanied by tissue factor expression and matrix metalloproteinase secretion in a small cell lung cancer cell line. *J Cancer Res Clin Oncol* 127:139–141
- Sawada M, Miyake S, Ohdama S, Matsubara O, Masuda S, Yakumaru K (1999) Expression of tissue factor in non-small-cell lung cancers and its relationship to metastasis. *Br J Cancer* 79:472–477
- Shen BQ, Clarke MF, Palsson BO (1996) Kinetics of retroviral production from the amphotrophic Ψ CRIP murine producer cell line. *Cytotechnology* 22:185–195
- Simpson NH, Singh RP, Perani A, Goldenzon C, Al-Rubeai M (1998) In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl-2 gene. *Biotechnol Bioeng* 59:90–98
- Stoll TS, Mühlethaler K, Stockar UV, Marison IW (1996) Systematic improvement of a chemically-defined protein-free medium for hybridoma growth and monoclonal antibody production. *J Biotechnol* 45:111–123

- Sureshkumar GK, Mutharasan R (1991) The influence on temperature on a mouse-mouse hybridoma growth and monoclonal antibody production. *Biotechnol Bioeng* 37:292–295
- Suzuki E, Ollis DF (1990) Enhanced antibody production at slowed growth rates: experimental demonstration and a simple structured model. *Biotechnol Prog* 6:231–236
- Taylor PC (2003) Antibody therapy for rheumatoid arthritis. *Curr Opin Pharmacol* 3(3):323–328
- Wohlpert D, Kiwan D, Gainer J (1990) Effects of cell density and glucose and glutamine levels on the respiration rates of hybridoma cells. *Biotechnol Bioeng* 36:630–635
- Xie L, Wang DIC (1994) Stoichiometric analysis of animal cell growth and its application of medium design. *Biotechnol. Bioeng.* 43:1164–1174
- Xie L, Wang DIC (1996) High cell density and high monoclonal antibody production through medium design and rational control in a bioreactor. *Biotechnol Bioeng* 51:725–729
- Xie L, Nyberg G, Gu X, Li H, Mollborn F, Wang DIC (1997) Gamma-interferon production and quality in stoichiometric fed-batch cultures of Chinese Hamster Ovary (CHO) Cells under serum-free conditions. *Biotechnol Bioeng* 56:577–582
- Zhou W, Rehm J, Hu W-S (1995) High viable cell concentration fed-batch cultures of hybridoma cells through on-line nutrient feeding. *Biotechnol Bioeng* 46:579–587
- Zhou W, Chen C-C, Buckland B, Aunins J (1997) Fed-batch culture of recombinant NS0 myeloma cells with high monoclonal antibody production. *Biotechnol Bioeng* 55:783–792