*Cytotechnology* **32:** 229–242, 2000.



# Effect of feed rate on growth rate and antibody production in the fed-batch culture of murine hybridoma cells

Jae Deog Jang[1] & John P. Barford[2*]
[1] *Department of Chemical Engineering, Sydney University, Sydney, NSW 2006, Australia;* [2] *Department of Chemical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong (SAR), China*
*(* Author for correspondence, E-mail: johnb@ust.hk)*

Received 20 April 1999; accepted 11 January 2000

*Key words:* fed batch, hybridoma, macromolecular composition, monoclonal antibody, substrate limitation, target specific growth rate

**Abstract**

Batch and fed-batch cultures of a murine hybridoma cell line (AFP-27) were performed in a stirred tank reactor to estimate the effect of feed rate on growth rate, macromolecular metabolism and antibody production. Macromolecular composition was found to change dynamically during batch culture of hybridoma cells possibly due to active production of DNA, RNA and protein during the exponential phase. Antibody synthesis is expected to compete with the production of cellular proteins from the amino acid pool. Therefore, it is necessary to examine the relationship between cell growth in terms of cellular macromolecules and antibody production. In this study, we searched for an optimum feeding strategy by changing the target specific growth rate in fed-batch culture to give higher antibody productivity while examining the macromolecular composition. Concentrated glucose (60 mM) and glutamine (20 mM) in DR medium (1:1 mixture of DMEM and RPMI) with additional amino acids were fed continuously to the culture and the feed rate was updated after every sampling to ensure exponential feeding (or approximately constant specific growth rate). Specific antibody production rate was found to be significantly increased in the fed-batch cultures at the near-zero specific growth rate in which the productions of cellular DNA, RNA, protein and polysaccharide were strictly limited by slow feeding of glucose, glutamine and other nutrients. Possible implications of these results are discussed.

## Nomenclature

| | | |
|---|---|---|
| $N_v$ | Viable cell concentration | cell $l^{-1}$ |
| $N_d$ | Dead cell concentration | cell $l^{-1}$ |
| $N_t$ | Total cell concentration | cell $l^{-1}$ |
| $V$ | Culture volume | l |
| $\mu_t$ | Target specific growth rate | $hr^{-1}$ |
| $\mu_{app}$ | Apparent specific growth rate | $hr^{-1}$ |
| $F_i$ | Feed rate | l $hr^{-1}$ |
| $F_o$ | Harvest rate | l $hr^{-1}$ |
| $Glc$ | Glucose concentration | mM |
| $Gln$ | Glutamine concentration | mM |
| $Lac$ | Lactate concentration | mM |
| $Amm$ | Ammonia concentration | mM |

| | | |
|---|---|---|
| $Glc_t$ | Glucose concentration in the feed | mM |
| $Gln_t$ | Glutamine concentration in the feed | mM |
| $Y_{glc}$ | Cell yield on glucose | cell $mmol^{-1}$ |
| $Y_{lac,glc}$ | Lactate yield on glucose | mmol $mmol^{-1}$ |
| $Y_{amm,gln}$ | Ammonia yield on glutamine | mmol $mmol^{-1}$ |
| $Ab$ | Antibody concentration in the medium | mg $l^{-1}$ |
| $X_m$ | Concentration of cellular macromolecules in the medium ($m$ = dna, rna, prt, lpd, psd) | mg $l^{-1}$ |
| $X$ | Cell mass (sum of cellular macromolecules) | mg $l^{-1}$ |
| k | Rate constant for the spontaneous decomposition of glutamine | $hr^{-1}$ |
| $Q_i$ | Specific metabolic rate of metabolites ($i$ = glc, gln, lac, amm) | mmol $cell^{-1}$ $hr^{-1}$ |
| $Q_{\text{ab}}$ | Specific antibody production rate | pg $(g\ cell)^{-1}$ $hr^{-1}$ |
| $Q_m$ | Specific production rate of macromolecules ($m$ = dna, rna, prt, lpd, psd) | g $(g\ cell)^{-1}$ $hr^{-1}$ |
| $\Delta(y)$ | Absolute error of a parameter | |
| $\text{Re}(y)$ | Relative error of a parameter | |

*Subscripts*

| | |
|---|---|
| glc | Glucose |
| gln | Glutamine |
| lac | Lactate |
| amm | Ammonia |
| ab | Monoclonal antibody |
| dna | Deoxyribonucleic acid (DNA) |
| rna | Ribonucleic acid (RNA) |
| prt | Protein |
| lpd | Lipid |
| psd | Polysaccharide |

## Introduction

Monoclonal antibodies (MAbs) have become one of the most important biological products gaining widespread usage in immunodiagnosis, tumor imaging, affinity chromatography, drug delivery system and cancer treatment. Since Köhler and Milstein first made hybridoma cells producing antibodies in 1975, hybridoma cell culture has been used widely to produce monoclonal antibodies. Therefore, attention has been focused on the mass production of monoclonal antibodies. However, to achieve higher productivity of monoclonal antibody, a better understanding on the relationship between animal cell metabolism and antibody production is needed.

Monoclonal antibody production has been reported to be non-growth associated in many cases (e.g. Miller et al., 1988; Ray et al., 1989) while it was reported to be growth associated in SP2/O myeloma cell culture by Robinson and Memmert (1991). The production rate of monoclonal antibodies was reported to be higher (Hayter et al., 1992; Miller et al., 1988; Ray et al., 1989) at lower growth rates or unchanged (Boraston et al., 1984; Takazawa et al., 1988) in response to lowering growth rates. The validity of growth and non-growth associated concepts for antibody production has been challenged (Phillips et al., 1991; Barford et al., 1996).

Antibody synthesis by hybridoma cells requires all the cellular machinery for protein synthesis including transcription, translation, posttranslational modification, and secretion because antibodies are proteins. On the other hand, antibody is not an essential product for hybridoma cell growth, thus its production might be expected to be less favored by fast-growing cell when compared to the production of cellular proteins. Therefore antibody productivity might be expected to increase by strictly limiting cell growth in fed-batch or continuous culture. Antibody productivity has been reported by several researchers to be higher in the G1 phase of the cell division cycle (Garatun-Tjeldstø et al., 1976; Golding et al., 1988; Ramírez and Mutharasan, 1990; Suzuki and Ollis, 1989). Linardos et al. (1992) suggested that the fraction of cells arrested in the G1 phase should be higher at lower dilution rates of continuous culture. Though cell death rate was also shown to be a function of the cell fraction arrested in the G1 phase of the cell cycle (Linardos et al., 1992), it is not yet clear whether this can be the major reason for the increase of antibody production at lower specific growth rate.

On the other hand, animal cells produce waste products such as ammonia and lactate which have been reported to be inhibitory to cell growth and/or antibody production. Excess supply of glucose and glutamine in the medium may cause accumulation of intracellular pyruvate which accumulates on both sides of the mitochondrial membrane. The excess pyruvate exceeding the mitochondrial capacity for oxidation is excreted as lactate and alanine while ammonia is produced in the initial steps of glutaminolysis (Ljunggren and Häggström, 1994). This substrate-concentration-dependent overflow metabolism can be reduced or prevented by using slow feeding of glucose and glutamine in fed-batch or continuous culture, since high specific growth rates favour this type of metabolism (Ljunggren and Häggström, 1994).

Fed-batch culture has been used extensively in animal cell culture to extend maintenance of viable cell population (Bushell et al., 1994) and to avoid depletion of glucose and/or glutamine (Glacken et al., 1986), as well as being able to operate at lower specific growth rates. Macromolecular composition of hybridoma cell has been studied previously by other researchers (Bonarius et al., 1996; Savinell and Palsson, 1992; Xie and Wang, 1994a; Zupke and Stephanopoulos, 1995). Biosynthetic rates for cellular macromolecules of hybridoma cells were calculated by Savinell and Palsson (1992) as part of metabolic networks to study metabolic flux. However, the effect of macromolecular metabolism on the production of antibody during hybridoma cell culture has not been extensively studied previously.

The present study investigated the relationship between the metabolic activity of cellular macromolecules and antibody production while examining specific metabolic rate of metabolites such as glucose, glutamine, lactate and ammonia as a function of the specific growth rate in the fed-batch cultures.

## Materials and methods

### *Cell line and medium*

The cell line used in this study was a murine hybridoma AFP-27, producing an IgG1 antibody which reacts with human $\alpha$-fetoprotein. The medium used in this study was based on DR medium (1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and RPMI) or eDR medium (DR with additional amino acids) with various concentrations of glucose and glutamine. 20 mM glucose and 4 mM glutamine in DR medium was used for batch culture on the 4 l scale. The inoculum for the fed-batch culture was prepared by serial subcultures, in which the culture volume was doubled daily from 30 ml up to 1 l with eDR medium of 5 mM glucose and 2 mM glutamine. The concentrations of amino acid in the eDR5/2 medium are as follows: 0.100 mM L-Ala, 1.550 mM L-Arg, 0.760 mM L-Asn, 0.300 mM L-Asp, 1.651 mM L-Cys, 0.068 mM L-Glu, 2.000 mM L-Gln, 0.266 mM Gly, 0.500 mM L-His, 1.769 mM L-Ile, 1.769 mM L-Leu, 1.528 mM L-Lys, 0.503 mM L-Met, 1.126 mM L-Phe, 0.087 mM L-Pro, 0.782 mM L-Ser, 0.794 mM L-Thr, 0.257 mM L-Trp, 0.784 mM L-Tyr, and 1.460 mM L-Val. Fetal calf serum (2%, v/v) was added to the main culture medium and feed medium.

Hybridoma growth was controlled during the fed-batch operation by setting a target specific growth rate at start of feeding. Target specific growth rate was used to calculate feed rate of feed medium with concentrated glucose and glutamine for exponential feeding in accordance with increased cell mass. Feed medium consists of concentrated glucose (60 mM) and glutamine (20 mM) as well as enhanced amino acids in 1:1 mixture of DMEM and RPMI. Glutamine and glucose concentration in the feed medium was designed to give a 1:3 ratio. This ratio was based on the profiles of typical batch growth of hybridoma cells in

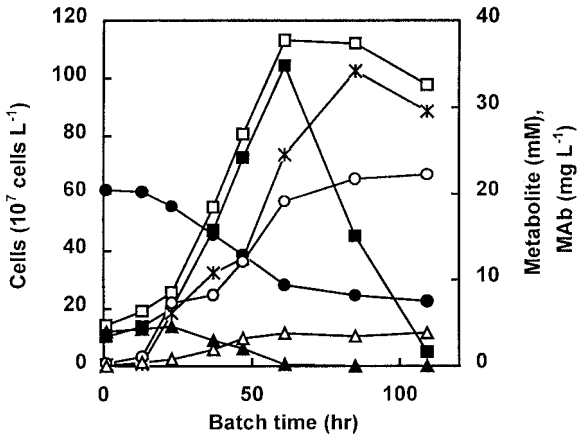


*Figure 1.* A typical batch culture profile of AFP-27 in 4 l medium of DR25/4 (1:1 mixture of DMEM and RPMI with 25 mM glucose and 4 mM glutamine). Total (empty square) and viable (filled square) cell concentration, monoclonal antibody (asterisk), and major metabolites including glucose (filled circle), glutamine (filled triangle), lactate (empty circle), and ammonia (empty triangle) are shown.

which 3–4 mM glutamine and 8–12 mM glucose were consumed during batch cultures of AFP-27 (Figure 1).

Though both glucose and glutamine are expected to be limited in fed-batch cultures in this strategy, feed rate was calculated using only glucose concentration in the culture to minimize the complexity of the operation.

*Cultivation conditions*

Working cell banks were made by freezing vials down in the presence of 20% fetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). Cells were rapidly thawed at 37 °C, washed once in medium and incubated at 37 °C, in a 25 $cm^2$ T-flask. Cells recovered from the frozen state were weaned from the high content of serum by a series of subculture with lower content of serum. All the subcultures were performed in the T-flasks with area of 25 $cm^2$ or 75 $cm^2$ and spinner flask with culture volume of 200–500 ml. In the subculture, temperature was maintained at 37 °C and agitation rate was set to 30 rpm.

Batch and fed-batch cultures in 2–4 l running volume were performed in a stirred tank reactor. The reactor consisted of a glass cylinder section (PS 300 Crown Corning, Sydney, Australia) sandwiched between two stainless steel plates with Teflon seals. Silicon tubing was wrapped around the glass cylinder as heating loop through which water was circulated using a thermocirculator (Grant LC10, Grant Instruments, Cambridge, UK) to control the culture temperature. The bottom plate incorporated a cooling loop through which tap water was circulated under control of a solenoid valve. A PT 130 resistance thermometer incorporated in the bottom plate was used to monitor the culture temperature. The top plate had a number of ports for feed, base/acid addition, sampling, and air inlet and outlet. An impeller shaft was incorporated with 2 marine type impellers in place. The impeller was driven by a removable variable speed motor (Baldor AP7427, Baldor, Fort Smith, AK, USA). The glass vessel was modified so that pH and oxygen probes could be side-mounted. The whole reactor set-up was mounted onto a movable trolley which could be moved to a services panel that supplied water, control gases and power.

Monitoring and control of the fermentation was performed using FC4 data logger and controller (Real Time Engineering, Sydney, Australia) interfaced with a 486 personal computer. Another computer unit was used to control feed rate using analogue output to peristaltic pumps (Watson-Marlow, MHRE2) by GENIE program (Advantech, MA, USA). pH was automatically controlled to 7.1 by adding 0.5 N NaOH and 0.5 N HCl and temperature was controlled to 37 °C automatically by the FC4 controller. DO was controlled automatically to be maintained between 10 and 50% by direct sparging of compressed air.

In fed-batch operation, an exponential feeding method was used to control the specific growth rate by feeding the concentrated feed medium previously described. In the calculation of the feed rate, cell yield from glucose was assumed to be constant and remaining glucose concentration in the reactor was assumed to be much lower than that of feed medium. Feed rate ($F_i$, l $hr^{-1}$) was calculated from target specific growth rate ($\mu_t$, $hr^{-1}$), viable cell concentration ($N_v$, cell $L^{-1}$), running volume ($V$, liter), cell yield from glucose consumed ($Y_{glc}$, cell $mmol^{-1}$), and glucose concentration in feed medium ($Glc_f$, mM) according to following equation (Pirt, 1957):

$$F_i = \frac{\mu_t N_v V}{Y_{glc} Glc_f} \tag{1}$$

In this equation, $Y_{glc}$ and $Glc_f$ were set to $2.11 \times 10^8$ cells $mmol^{-1}$ and 60 mM, respectively. Feed rate was recalculated after every sampling with updated cell numbers and running volume.

*Analytical methods*

Samples of more than 100 ml were withdrawn from

the culture about every 12 hrs for the assays of cell number, concentrations of nutrients and metabolites, and concentration of cellular macromolecules. Each culture sample was distributed into centrifuge tubes placed in cold ice (4 °C). Three of 10 ml culture samples were prepared for the assays of cellular DNA, RNA, and protein. Another 10–20 ml culture sample was prepared for the determination of dry cell weight. The remainder of the sample (50–60 ml) was prepared for the assay of cellular lipid. Cell pellets were harvested by centrifugation (1500 rpm, 10 min). The cell pellets were resuspended with a cold PBS (phosphate-buffered saline, 4 °C) solution then centrifuged again to wash the pellets for 10 min at 1500 rpm. The washed cell pellets were stored in – 40 °C freezer until assayed. This methodology reduces any subsequent macromolecular degradation in the samples.

Viable and dead cell number were counted on a hemocytometer (Neubauer, Germany) after diluting the sample 1:1 with 0.16% trypan blue in normal saline. Glucose, lactate, and ammonia were assayed using an enzymatic technique, in which the change in NADH concentration was monitored at 340 nm using a spectrophotometer (UNICAM SP1800 ultraviolet spectrophotometer). Total amount of antibody was measured using enzyme-linked immunosorbent assay (ELISA). Samples were diluted so that they fell into this approximate range. Normally, three dilutions of the sample were used in each plate. If only one reading was obtained for a particular sample (i.e. only one falling into the linear portion of the standard curve), the assay was repeated. Pre-column derivatization technique using FDNDEA (N,N-diethyl-2,4-dinitro-5-fluoro-aniline, Fluka, Buchs, Switzerland) was used to assay the concentration of amino acids in the cell culture supernatant (Fermo et al., 1988; Fermo and Vecchi, 1990).

DNA and RNA were purified from the cell lysate using the method of Halliburton and Thompson (1965). DNA concentration in the sample was determined by the diphenylamine method (Dische, 1955). RNA concentration was assayed by the orcinol method (Brown, 1946). Total cellular protein was assayed by using Coomasie Blue (Bradford, 1976) after extraction with sodium hydroxide. Total cellular carbohydrates were determined by the phenol-sulfuric method (Dubois et al., 1956) after lysis of cell pellets with lysis buffer (0.5% Triton X-100, 1 mM EDTA, 0.2 mM PMSF). The extraction of total cellular lipids was carried out using the methods of Folch and Sloane (1957). The final lower phase contained lipids was dried in a weighing pan at 40 °C in a vacuum desiccator until a constant weight. Dry cell weight was estimated by filtration of a known number of cell onto pre-weighed, 0.22 $\mu$m filter papers, followed by washing with PBS. A zero reading was recorded by washing duplicate filter papers with PBS alone. The filter papers were dried at 100 °C for more than 1 hr and subsequently weighed.

*Data analysis*

Experimental data from the batch and fed-batch cultures were analyzed by fitting to appropriate functions using the least-squares method. In the batch culture, time course data were divided into two phases – growth and death phase. In the fed-batch cultures, data were divided into three phases – batch, quasi-steady state, and death phase. Data from the growth phase of batch culture and the quasi-steady state of the fed-batch cultures were used to obtain specific rates for cell growth, toxic product formation, and monoclonal antibody production. A quasi-steady state was defined in this study as the state in which the rate of the net increase in glucose concentration in the culture is approximately zero, assuming that cell growth is limited by glucose during the period.

The equations used to calculate specific rates are described in Equations (2)–(12). Harvest rate ($F_o$) was included in the equations to include the changes in the total amount of cell mass and metabolites introduced by sampling. Specific rates best fitting the experimental data were searched for using the 'solver' function of Microsoft® Excel to minimise the difference between the actual and calculated data. Apparent specific growth rate ($\mu_{app}$) in this study was derived from the profile of viable cell concentration during the culture using Equation (2). At least 10 experimental points were used to estimate the average apparent specific growth rate.

$$\frac{\mathrm{d}(N_v V)}{\mathrm{d}t} = \mu_{app}(N_v V) - F_o N_v \tag{2}$$

$$\frac{\mathrm{d}(GlcV)}{\mathrm{d}t} = -Q_{glc}(N_v V) + F_i Glc_f - F_o Glc \tag{3}$$

$$\frac{\mathrm{d}(GlnV)}{\mathrm{d}t} =$$

$$-Q_{gln}(N_v V) - kGlnV + F_i Gln_f - F_o Gln \tag{4}$$

$$\frac{d(LacV)}{dt} = Q_{lac}(N_v V) - F_o Lac \qquad (5)$$

$$\frac{d(AmmV)}{dt} = Q_{amm}(N_v V) + kGlnV - F_o Amm \qquad (6)$$

$$\frac{d(X_{dna}V)}{dt} = Q_{dna}(XV) - F_o X_{dna} \qquad (7)$$

$$\frac{d(X_{rna}V)}{dt} = Q_{rna}(XV) - F_o X_{rna} \qquad (8)$$

$$\frac{d(X_{prt}V)}{dt} = Q_{prt}(XV) - F_o X_{prt} \qquad (9)$$

$$\frac{d(X_{lpd}V)}{dt} = Q_{lpd}(XV) - F_o X_{lpd} \qquad (10)$$

$$\frac{d(X_{psd}V)}{dt} = Q_{psd}(XV) - F_o X_{psd} \qquad (11)$$

$$\frac{d(AbV)}{dt} = Q_{ab}(XV) - F_o Ab \qquad (12)$$

Errors for measured and calculated parameters are shown in Table 1. Some of these are compared with those of Pörtner and Schäfer (1996) and are consistent with their findings. Absolute errors, $\Delta(y)$ for the parameters which were derived by addition or subtraction of measured ones were calculated using Equation (13). Relative errors, Re($y$) for the parameters which were derived by multiplication or division of measured ones were calculated using Equation (14) (Pentz and Shott, 1989).

$$\Delta(y) = \sqrt{\sum \Delta(y_i)^2} \qquad (13)$$

$$\mathrm{Re}(y) = \sqrt{\sum \left(\frac{\Delta y_i}{y_i}\right)^2}, \qquad (14)$$

where $i = 1, 2 \ldots$, n (number of parameters used in the equation).

## Results and discussion

### *Culture performance*

Hybridoma cell growth was successfully controlled during fed-batch operation by controlled feeding of feed medium (eDR60/20). Maximum viable and total cell density were increased about 3-fold in the fed-batch at a target specific growth rate of 0.025 $hr^{-1}$ compared to the batch level. Maximum antibody titer in the fed-batch at a target growth rate of 0.01 $hr^{-1}$ was 5-fold that of batch case (Table 2). As seen in Figure 2, the apparent specific growth rate in fed-batch cultures increased with target specific growth rate to some extent. The apparent specific growth rates have some deviation from the targeted ones both in higher and lower ranges, possibly because the yield value

*Table 1.* Relative errors, Re(y) for measured and calculated parameters in batch and fed-batch culture

| Measured parameter | Re(y) (%) | Calculated parameter | Re(y) (%) |
|---|---|---|---|
| $N_v$ | 6 [7][a] | $N_t$ | 8 [6] |
| $N_d$ | 6 [7] | $\mu$ | 11 [10] |
| $Glc$ | 3 [2] | $Q_{glc}$ | 7 [10] |
| $Gln$ | 7 [5] | $Q_{gln}$ | 12 [10] |
| $Lac$ | 4 [2] | $Q_{lac}$ | 9 [10] |
| $Amm$ | 6 [5] | $Q_{amm}$ | 11 [10] |
| $Ab$ | 12 [10] | $Q_{ab}$ | 21 [12] |
| $X_{dna}$ | 7 | $Q_{dna}$ | 16 |
| $X_{rna}$ | 3 | $Q_{rna}$ | 14 |
| $X_{prt}$ | 7 | $Q_{prt}$ | 16 |
| $X_{lpd}$ | 8 | $Q_{lpd}$ | 17 |
| $X_{psd}$ | 3 | $Q_{psd}$ | 14 |
| | | $X$ | 13 |
| | | $Y_{lac,glc}$ | 5 |
| | | $Y_{amm,gln}$ | 9 |

[a] Numbers in the brackets are errors reported by Pörtner and Schäfer (1996) for continuous culture.

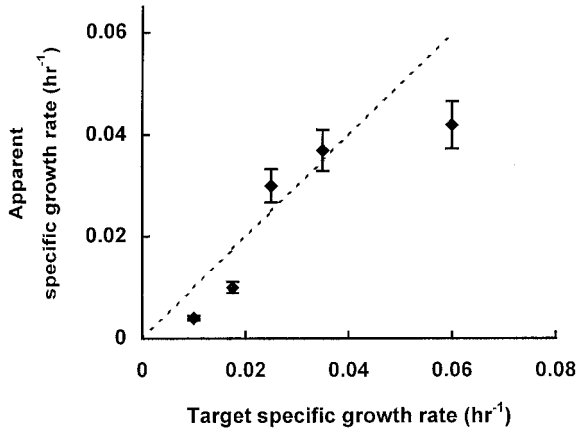


*Figure 2.* Relationship between target specific growth rate and apparent specific growth rate in the fed-batch cultures of AFP-27.

*Table 2.* Culture performance in batch and fed-batch cultures of AFP-27

| $\mu_t$ ($hr^{-1}$) | 0.01 | 0.0175 | 0.025 | 0.035 | 0.06 | Batch |
|---|---|---|---|---|---|---|
| Max. viable cells ($10^7$ cells $l^{-1}$) | 82.9±5.0 | 207.2±12.4 | *312.8*±18.8 | 244.8±14.7 | 97.8±5.9 | 104.4±6.3 |
| Max. total cells ($10^7$ cells $l^{-1}$) | 193.1±15.4 | 351.2±28.1 | *382.8*±30.6 | 280.2±22.4 | 118.5±9.5 | 113.2±9.1 |
| Max. MAb titre (mg $l^{-1}$) | *180.0*±21.6 | 56.5±6.8 | 73.3±8.8 | 84.7±10.2 | 43.9±5.3 | 34.2±4.1 |

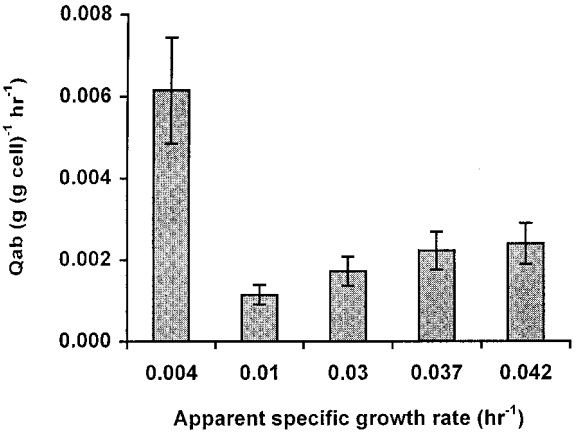


*Figure 3.* Specific antibody production rate as a function of the apparent specific growth rate in the fed-batch cultures of AFP-27.

($Y_{glc}$), used in the calculation of feed rate, was assumed to be constant, while actual yield could vary with different feed rates in the fed-batch cultures. The apparent specific growth rate could not be increased further above ca. 0.04 $hr^{-1}$ in this fed-batch cultures possibly due to the accumulation of ammonia and/or lactate (The apparent specific growth rate of the batch culture in the same reactor with 4 l running volume was 0.041±0.0045 $hr^{-1}$). Fed-batch culture which was fed by concentrated glucose and glutamine at a high rate (as the case with target specific growth rate of 0.06 $hr^{-1}$ in this study) was expected to be inefficient in terms of cell yield and antibody production, because high level of glucose and glutamine in medium would increase toxic product level without significant increase in cell growth or antibody production.

*Antibody production*

The specific production rate of antibody in this study showed significant increase in the near-zero apparent specific growth rate in the fed-batch cultures while showing lower specific production rate with little variation above 0.01 $hr^{-1}$ (Figure 3). This trend of sharp increase in the near-zero specific growth rate is consistent with result of Leno et al. (1992) reporting that the maximal antibody production rate occurred at the low dilution rate in continuous culture of hybridoma under adverse environmental conditions such as low nutrient supply. This also agrees with the findings that hybridoma cells might increase the production of antibody when subjected to stress such as nutrient limitation (Al-Rubeai et al., 1990). The issue of antibody productivity and its relationship with growth rate is one which remains contentious. The issues of experimental errors (Phillips et al., 1991) and the relationship between antibody productivity and cellular metabolism (Barford et al., 1996) still have not been fully resolved and require further study.

Hybridoma cells can be arrested in the G1 phase by means of isoleucine deprivation, serum depletion, stationary state reached after exponential phase, inhibition by drugs and oxygen supply limitation (Suzuki and Ollis, 1989). They proposed, from the study of their cell cycle model, that hybridoma cells in the G1 phase might often produce antibody at a rate considerably higher than that in the other cell cycle phases. And this was supported by the report of Ramírez and Mutharasan (1990). Therefore, the increase of the G1-arrested cell fraction could be another possibility for the increase of antibody productivity at the near-zero specific growth rate, though this assumption requires further study for verification.

*Metabolism of glucose, lactate, glutamine and ammonia*

Glucose has been limited during the quasi-steady state

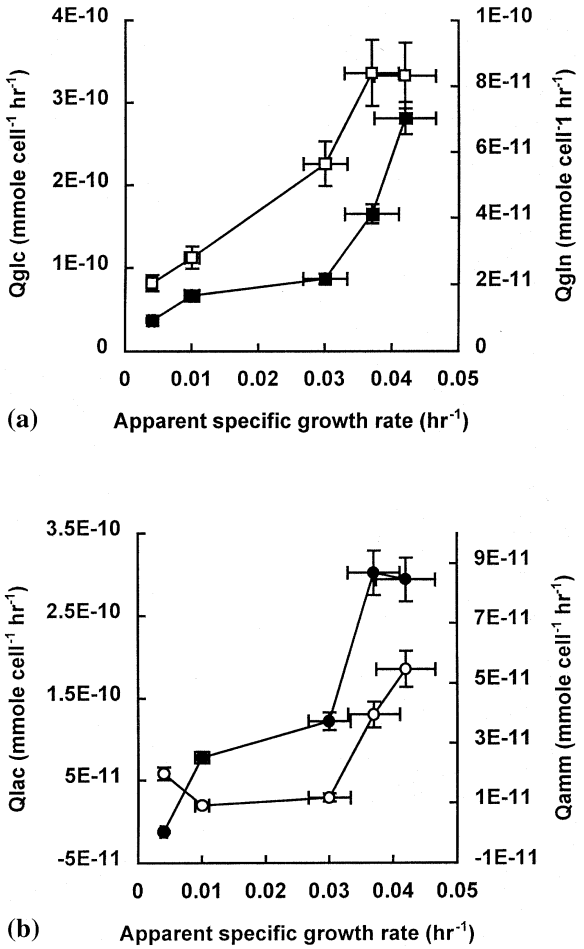


*Figure 4.* Specific consumption rate of (a) glucose (filled square) and glutamine (empty square), and specific production rate of (b) lactate (filled circle) and ammonia (empty circle) as a function of the apparent specific growth rate in the fed-batch cultures of AFP-27.

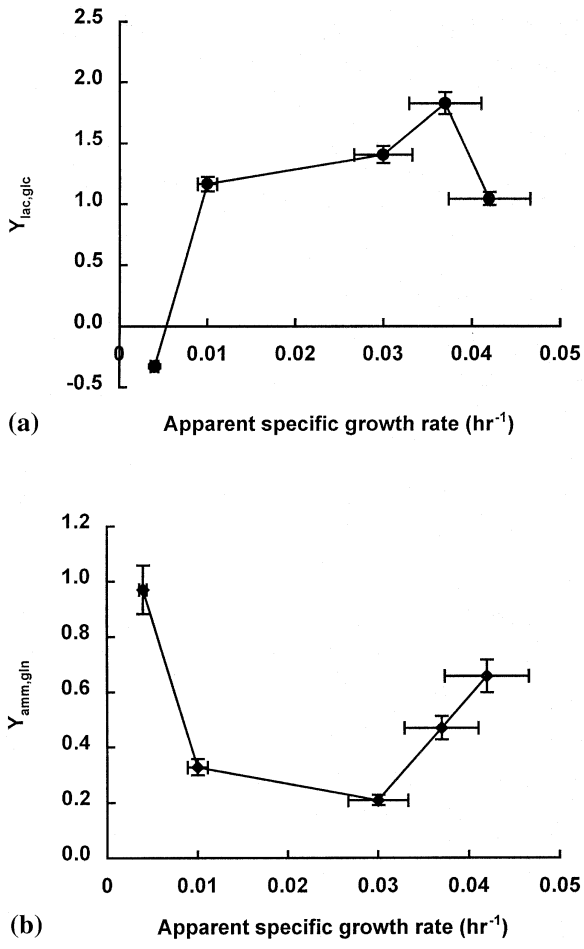


*Figure 5.* Profiles of (a) lactate yield from glucose (b) ammonia yield from glutamine as a function of the apparent specific growth rate in the fed-batch cultures of AFP-27.

of fed-batch culture by an exponential feeding strategy determined by a target specific growth rate and viable cell mass in real culture time. Specific glucose consumption rate decreased with decreasing apparent specific growth rate (Figure 4a) as reported by Frame and Hu (1991, from the continuous cultures of AFP-27-NP, non-producing murine hybridoma) and Robinson and Memmert (1991, from the continuous cultures of SP2/O myeloma cell line). The specific rate of glucose consumption increases with steeper slope in the higher specific growth rate range.

Glutamine has been maintained at low concentrations of less than 1.5 mM but was not exhausted during fed-batch operations. The specific rate of glutamine consumption was proportionally increased with increasing specific growth rate during fed-batch cultures as shown in Figure 3a. In the figure, the specific glutamine consumption rate was extrapolated to near zero at zero specific growth rate – different to that of glucose in which the specific rate was extrapolated to a value greater than zero. This implies that the requirement of glutamine for the maintenance activity might be negligible in calculating apparent specific consumption rate of glutamine in this hybridoma strain.

Lactate was accumulated through all the fed-batch cultures with all target specific growth rate except the lowest one of 0.01 $hr^{-1}$ in which lactate was not accumulated during quasi- steady state while glucose was consumed. Specific lactate production rate was decreased with decreasing target specific growth rate (Figure 4b). This implies that by limiting glucose

in the hybridoma cell culture, pyruvate in glycolysis pool has been more efficiently metabolized to secrete less lactate, which otherwise will be accumulated in the culture medium by overflow metabolism (Ljunggren and Häggström, 1994). The yield of lactate from glucose consumed in the batch and fed-batch culture of AFP-27 was plotted as a function of the specific growth rate (Figure 5a). In most ranges of the specific growth rate, the lactate yield from glucose was nearly constant but it was decreased significantly in the near-zero specific growth rate. This suggests that in near-zero specific growth rate the metabolism of glucose is more efficient.

Ammonia was accumulated during batch and fed-batch cultures with final concentration ranging from 1.5 mM to 4.5 mM. Ammonia was accumulated up to 4.5 mM in the fed-batch cultures with target specific growth rate of 0.025 and 0.035 $hr^{-1}$, which is higher than in the batch case in which the final ammonia level was 3.8 mM. The ability of the hybridoma cells to grow at higher ammonium concentrations could be explained by either or both of the slow increase of ammonia concentration and the adaptation of hybridoma cells to higher ammonia level in slow increase rate (Nielsen et al., 1992; Newland et al., 1994). The higher concentration of ammonia may also indicate a change in the metabolism of the of C/N sources. Specific ammonia production rate (Figure 4b) and the yield of ammonia from glutamine (Figure 5b), as a function of specific growth rate, showed the parabolic figures having minimum level around the specific growth rate of 0.02 $hr^{-1}$. At specific growth rates higher than 0.02 $hr^{-1}$, both specific ammonia production rate and ammonia yield from glutamine increased as the specific growth rate increased. This implies that, by slow feeding of glutamine, glutamine has been metabolized more efficiently to give less ammonia production with less spontaneous degradation because of the shorter residence time of glutamine in the culture medium (Schneider et al., 1996). The reasons for the increase of the specific production rate of ammonia and the increase of ammonia yield from glutamine at the near-zero specific growth rate of 0.01 $hr^{-1}$ are not so clear yet. More amino acids might have been used for carbon source instead of glucose which was extremely limiting in such culture condition. Thus more ammonium might have been liberated by the deamination of the amino acids.

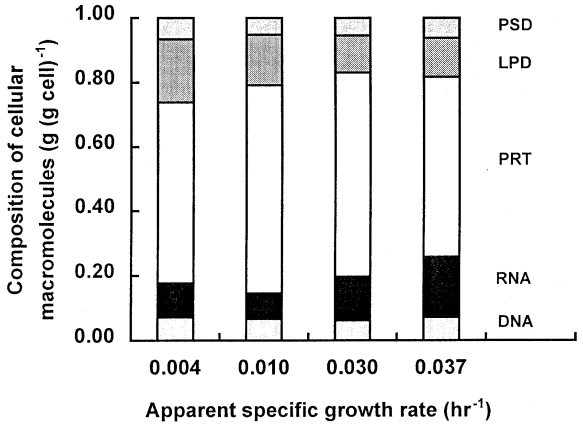


*Figure 6.* Macromolecular composition of the cells as a function of the apparent specific growth rate in the fed-batch cultures of AFP-27 (DNA, RNA, protein, lipid, polysaccharide from the bottom of each column).

*Macromolecular composition*

The composition of cellular macromolecules during batch and fed-batch cultures in this study was found to have similar range as others reported (Xie and Wang, 1994b; Bonarius et al., 1996), as shown in Table 3.

In the batch culture, cellular content of DNA, protein, and lipid per cell were observed to increase during the culture time, while RNA content exhibited a peak at the late exponential phase and then decreased possibly due to the extensive degradation (data not shown). The polysaccharide content in the batch culture was initially fairly high and decreased during the cell growth (data not shown). The initial high level of cellular polysaccharide content may be caused by high initial glucose concentration (25 mM) in the batch culture medium.

In the fed-batch cultures, cellular content of DNA, protein, and polysaccharide have been maintained nearly constant through all the fed-batch cultures, while RNA proportion was dynamically changed in response to the specific growth rate (Figure 6). RNA proportion was highest in the fed-batch with the apparent specific growth rate of 0.037 $hr^{-1}$. Lipid proportion was slightly increased with decreasing target specific growth rate. Two possibilities could be suspected for the increase of lipid content at lower specific growth rates. Firstly, the preferential breakdown of cellular macromolecules during sample preparation and assay might be suspected for the cause of the increase of lipid content at those specific growth rates. Secondly, the preferential leakage of macromolecules except lipid from non-viable cells during the culture could be

*Table 3.* Comparison of macromolecular composition (as percentage of dry cell mass)

| Macromolecule | Bonarius et al.[a] (1996) | Xie and Wang[b] (1994b) | This study[c] |
|---|---|---|---|
| DNA | 1.5 | 1.4 | 1.0–7.0 |
| RNA | 5.3 | 3.8 | 5.0–19.0 |
| Protein | 67.1 | 68.3 | 56.0–65.0 |
| Lipid | 10.0 | 13.5 | 12.0–24.0 |
| Total carbohydrate | 7.0 | 3.5 | 5.0–10.0 |

[a] Data for murine hybridoma cells grown in serum-free low-protein lipid-free medium with Primatone (animal tissue lysate).
[b] Data for animal cells, estimated for their model development.
[c] Data for murine hybridoma, AFP-27 in batch and fed-batch cultures at different specific growth rates.

suspected. Since cellular membrane of non-viable cell may be more unstable than that of viable one, cellular macromolecules such as DNA, RNA, polysaccharide and protein may leak from non-viable cell. Therefore higher fraction of non-viable cells in the lower specific growth rates may result in higher lipid contents.

*Macromolecular metabolism*

The specific production rate profiles of cellular macromolecules in the batch culture as a function of time are shown in Figure 7. In batch culture, the specific production rate of DNA, RNA and lipid has been maintained relatively constant, while the specific production rate of protein and polysaccharide decreased during the growth phase. The specific production rate of cellular polysaccharide in the batch culture is negative for much of the batch after recording an initial high specific production rate. This negative values after the initial high specific production rate in the batch culture, possibly implies that hybridoma cells, in high glucose level (20 mM in this study), may uptake and store the excess glucose in the form of polysaccharide within the cell and then utilize it later.

In the fed-batch culture, specific production rates of cellular macromolecules were also changed dynamically as the specific growth rate was changed. From the comparison of the specific production rate of cellular macromolecules in the fed-batch cultures with apparent specific growth rate of 0.037 $hr^{-1}$ and 0.004 $hr^{-1}$, it is apparent that the production rates of whole cellular macromolecules except lipid were lower in the lower specific growth rate (Figure 8). Since DNA synthesis is closely related to the cell growth which include DNA replication in a cell cycle, the specific production rate of DNA is expected to be proportional to the specific growth rate. In addition,

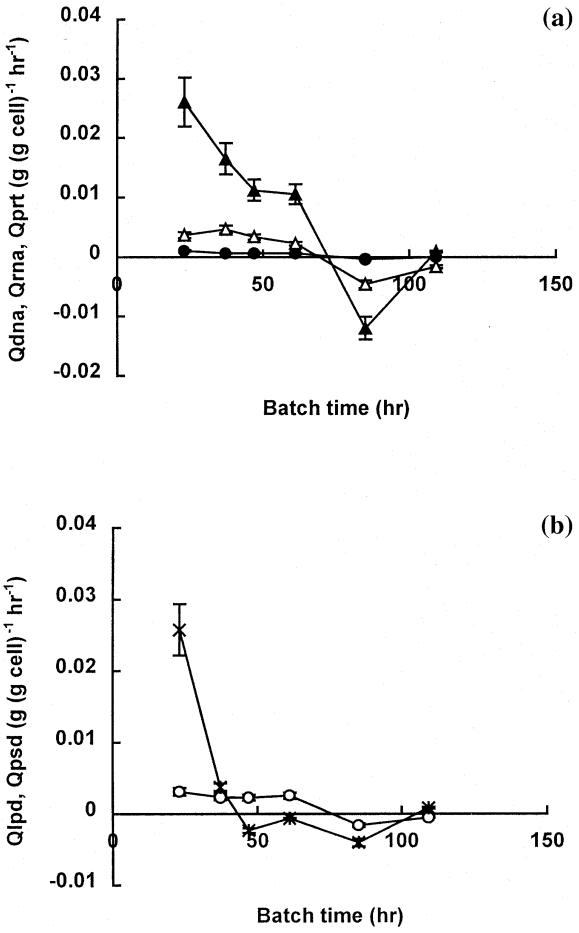


*Figure 7.* Specific production rate of cellular macromolecules during the time course of the batch culture of AFP-27 (DNA: filled circle, RNA: empty triangle, protein: filled triangle, lipid: empty circle, polysaccharide: asterisk).

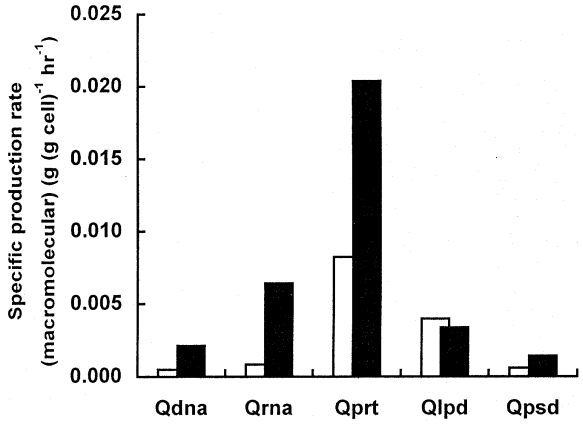


*Figure 8.* Specific production rate of cellular macromolecules in two fed-batch cultures of AFP-27 with different apparent specific growth rates of 0.037 $hr^{-1}$ (black bar) and 0.004 $hr^{-1}$ (white bar).

since RNA and protein are made from the transcription of template DNA and from the transcription of mRNA, respectively, the production rate of these macromolecules are expected to be interlinked. Specific RNA production rate (Qrna) is plotted against specific DNA production rate (Qdna) in Figure 9a, in which Qrna is nearly proportional to Qdna. However the specific protein production rate was shown to give a saturation curve as increasing the specific RNA production rate (Figure 9b).

The apparent specific growth rate in the fed-batch culture appeared to have positive correlation with the specific production rate of the cellular macromolecules of DNA, RNA, protein, and polysaccharide but not with lipid (Figure 10). When the specific production rates of cellular macromolecules were compared with the specific antibody production rate, it was found that the maximum specific antibody production rate occurred with low specific production rates of DNA, RNA, and protein at near-zero apparent specific cell growth rate (Figure 10). In addition, either high specific production rate or high cellular content of cellular lipid was observed to be positively related to the high antibody production rate in hybridoma cell culture (Figures 6 and 10).

Summarizing these results, the following implications could be drawn:

1) In fed-batch culture in conditions promoting high production rate of cellular macromolecules, the specific antibody production rate is low.
2) Low specific production rate of cellular protein may be required to divert resources of protein synthesis from the synthesis of cellular macromolecules to antibody synthesis.
3) Near-zero specific growth rate may be required to increase the proportion of cells with higher specific antibody production rate.

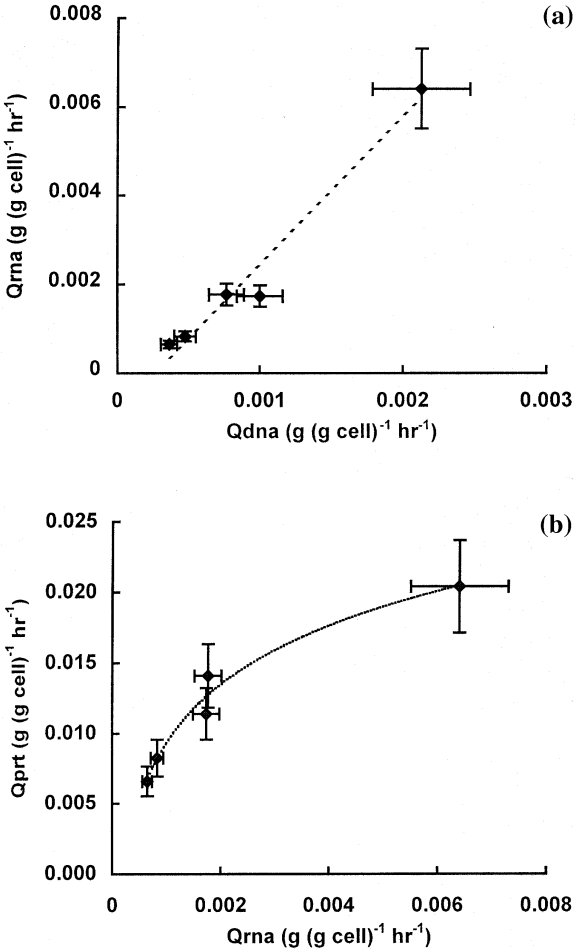


*Figure 9.* Relationships between the specific production rates of the cellular macromolecules in the fed-batch cultures of AFP-27. (a) Specific production rate of cellular RNA as a function of the specific production rate of cellular DNA, (b) specific production rate of cellular protein as a function of the specific production rate of cellular RNA.

## Conclusions

A set of fed-batch and batch cultures of murine hybridoma were performed to investigate how the cell growth in terms of macromolecular composition was associated with the antibody production as a function of the specific growth rate. From the average data of

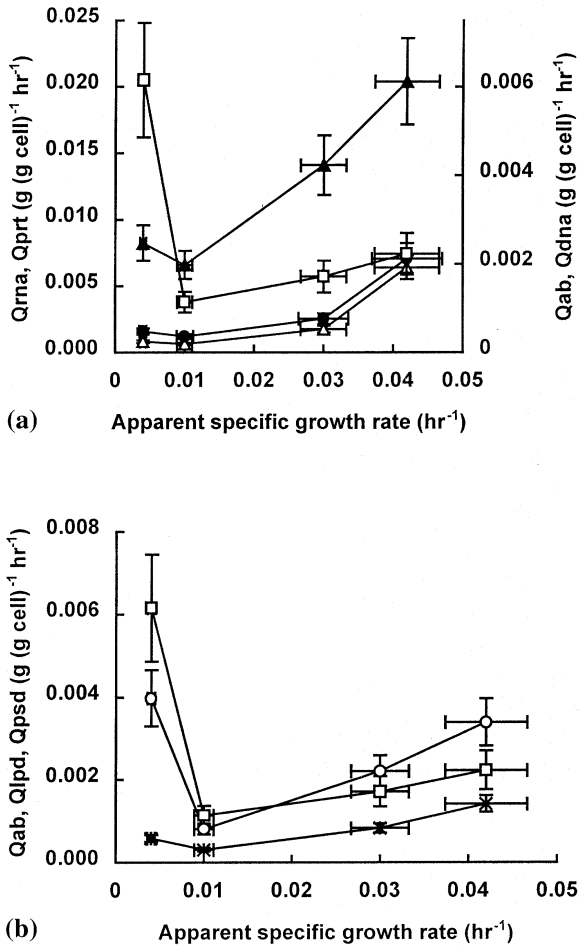


*Figure 10.* Specific production rate of (a) cellular DNA (filled circle), RNA (empty triangle), and protein (filled triangle), (b) lipid (empty circle) and polysaccharide (asterisk) together with specific antibody production rate (empty square) as a function of the apparent specific growth rate in the fed-batch cultures of AFP-27.

the quasi-steady state of the fed-batch cultures at different specific growth rates, some conclusions could be drawn:

1. The maximum viable and total cell concentration was obtained at the near-maximum apparent specific growth rate (0.037 $hr^{-1}$) in the fed-batch culture. The maximum cell concentration was decreased below this specific growth rate.
2. The efficiency of the metabolism of glucose and glutamine was greatly improved during fed-batch cultures which reduced lactate and ammonia significantly by feeding nutrient containing concentrated glucose and glutamine according to a feeding strategy designed to give lower specific growth rates. This agrees with others' reports (Bonarius et al., 1996; Glacken et al., 1986; Ljunggren and Häggström, 1994; Miller et al., 1988; Schneider et al., 1996).
3. The specific production rate of RNA was proportional to that of DNA over all the ranges obtained from the experiments, while the specific production rate of protein was proportional to that of RNA to a certain level beyond which it showed a saturated profile.
4. Antibody productivity was not strictly associated with cell growth in fed-batch cultures with concentrated feed medium. The maximum specific antibody production rate was obtained in the fed-batch culture at near-zero apparent specific growth rate.
5. The specific production rates of cellular macromolecules such as DNA, RNA, protein, and polysaccharides, were found to be proportional to the specific growth rate, while the maximum specific antibody production rate and specific production rate of lipids were obtained when the production of cellular protein was strictly limited at near-zero apparent specific growth rate. The increase of specific production rate of cellular lipid could be related to the preferential leakage of other macromolecules such as DNA, RNA, protein, and polysaccharides from non-viable cells.

In conclusion, this work suggests that the specific growth rates favoring high specific antibody production rate and high antibody concentration were different from those which favored high viable cell concentration. For the large-scale production of antibodies, fed-batch culture may provide some advantages such as operational simplicity, reliability, and flexibility for implementation in multipurpose facilities (Bibila and Robinson, 1995). Fed-batch culture is a compromise with high specific antibody production rate and high antibody concentration at high but not maximal viable cell concentration.

## References

Al-Rubeai M, Oh SKW, Musaheb R & Emery AN (1990) Modified cellular metabolism in hybridomas subjected to hydrodynamic and other stresses. Biotechnol Lett 12: 323–328.

Barford JP, Phillips PJ, Marquis CP & Harbour C (1996) Biosynthesis of protein products by animal cells. Are growth and non-growth associated concepts valid or useful? Cytotechnology 21: 133–148.

Bibila TA & Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotech Prog 11: 1–13.

Bonarius HPJ, Hatzumanikatis V, Meesters KPH, de Gooijer CD, Schmid G & Tramper J (1996) Metabolic flux analysis of hybridoma cells in different culture media using mass balances. Biotechnol Bioeng 50: 299–318.

Boraston R, Thompson PW, Garland S & Birch JR (1984) Growth and oxygen requirements of antibody-producing mouse hybridoma cells in suspension culture. Devel Biol Stand 55: 103–111.

Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254.

Brown AH (1946) Determination of pentose in the presence of large quantities of glucose. Arch Biochem 11: 269–278.

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.

Dische Z (1955) Color reactions of nucleic acid components. In: Chargaff E & Davison JN (eds) Nucleic Acids, Vol. 1 (p. 287) Academic Press, New York.

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.

Fermo I, Ruboni FM, Bolzacchini E, Arcelloni C & Bonini PA (1988) Pre-column derivatization of amino acids with N,N,-diethyl-2,4-dinitro-5-fluoroaniline and reversed-phase liquid chromatographic separation. J Chromatogr 433: 53–62.

Fermo I & Vecchi ED (1990) Serum amino acid analysis with pre-column derivatization: comparison of the o-phthaldialdehyde and N,N,-diethyl-2,4-dinitro-5-fluoroaniline method. J Chromatogr 534: 23–25.

Folch JL & Sloane SG (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 97–509.

Frame KK & Hu W-S (1991) Kinetic study of hybridoma cell growth in continuous culture. I. A model for non-producing cells. Biotechnol Bioeng 37: 55–64.

Garatun-Tjeldstø O, Pryme IF, Weltman JK & Dowben RM (1976) Synthesis and secretion of light-chain immunoglobulin in two successive cycles of synchronized plasmacytoma cells. J Cell Biol 68: 232–239.

Glacken MW, Fleischaker RJ & Sinsky AJ (1986) Reduction of waste product excretion via nutrient control: Possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol Bioeng 28: 1376–1389.

Golding B, Pillemer SR, Roussou P, Peter EA, Tsokos GC, Ballow JE & Hoffman T (1988) Inverse relationship between proliferation and differentiation in a Human TNP-specific B cell line. Cell cycle dependence of antibody secretion. J Immunol 131: 2564–2568.

Halliburton IW & Thompson RY (1965) Chemical aspects of compensatory renal hypertrophy. Cancer Res 25: 1882–1887.

Hayter PM, Kirkby NF & Spier RE (1992) Relationship between hybridoma growth and monoclonal antibody production. Enzyme Microb Technol 14: 454–461.

King KL & Cidlowski JA (1998) Cell cycle regulation and apoptosis. Ann Rev Physiol 60: 601–617.

Köhler G & Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495–497.

Leno M, Merten O-W & Hache J (1992) Kinetic analysis of hybridoma growth and monoclonal antibody production in semi-continuous culture. Biotechnol Bioeng 39: 596–606.

Linardos TI, Kalogerakis N & Behie LA (1992) Cell cycle model for growth rate and death rate in continuous suspension hybridoma cultures. Biotechnol Bioeng 40: 359–368.

Ljunggren J & Häggström L (1994) Catabolic control of hybridoma cells by glucose and glutamine limited fed batch culture. Biotechnol Bioeng 44: 808–818.

Merlo GR, Fiore L, Basolo F, Woods-Cook K & Hynes NE (1997) In mammary epithelial cells p53-mediated apoptosis in response to DNA damage is dependent on the agent and can be influenced by growth factors. Endoc Rel Cancer 4: 55–66.

Miller WM, Blanch HW & Wilke CR (1988) A kinetic 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.

Newland M, Kamal MN, Greenfield PF & Nielsen LK (1994) Ammonia inhibition of hybridomas propagated in batch, fed-batch, and continuous culture. Biotechnol Bioeng 43: 434–438.

Nielsen LK, Niloperbowo W, Reid S & Greenfield PF (1992) Avoiding rapid growth at high cell densities: A potentially important optimization criterion for hybridoma cultures. Cytotechnology 9: 21–27.

Pentz M & Shott M (1989) Handling Experimental Data. Open University Press, Milton Keynes, U.K.

Phillips PJ, Marquis CP, Barford JP & Harbour C (1991) An analysis of some batch and continuous kinetic data of specific monoclonal antibody production from hybridomas. Cytotechnology 6: 189–195.

Pirt SJ (1957) Principles of microbe and cell cultivation. John Wiley & Sons, New York.

Pörtner R & Schäfer T (1996) Modeling hybridoma cell growth and metabolism – a comparison of selected models and data. J Biotechnol 49: 119–135.

Ramírez 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.

Ray NG, Karkare SB & Runstadler Jr, PW (1989) Cultivation of hybridoma cells in continuous cultures: Kinetics of growth and product formation. Biotechnol Bioeng 33: 724–730.

Robinson DK & Memmert KW (1991) Kinetics of recombinant immunoglobulin production by mammalian cells in continuous culture. Biotechnol Bioeng 38: 972–976.

Savinell JM & Palsson BO (1992) Network analysis of intermediary metabolism using linear optimization. I. Development of metabolism formulation. J Theor Biol 154: 421–454.

Savonnière S, Zeghari N, Miccoli L, Muller S, Maugras M & Donner M (1996) Effect of lipid supplementation of culture media on cell growth, antibody production, membrane structure and dynamics in two hybridomas. J Biotechnol 48: 161–173.

Schneider M, Marison IW & Stocker U (1996) The importance of ammonia in mammalian cell culture. J Biotechnol 46: 161–185.

Suzuki E & Ollis DF (1989) Cell cycle model for antibody production kinetics. Biotechnol Bioeng 34: 1398–1402

Takazawa Y, Tokashiki M, Hamamoto K & Murakami H (1988) High cell density perfusion culture of hybridoma cells recycling high molecular weight compounds. Cytotechnology 1: 171–178.

Xie L & Wang DI (1994a) Applications of improved stoichiometric model in medium design and fed-batch cultivation of animal cells in bioreactor. Cytotechnology 15(1–3): 17–29.

Xie L & Wang DI (1994b) Fed-batch cultivation of animal cells using different medium design concepts and feeding strategies. Biotechnol Bioeng 43: 1175–1189.

Zupke C & Stephanopoulos G (1995) Intracellular flux analysis in hybridomas using mass balances and *in vitro* $^{13}$C NMR. Biotechnol Bioeng 45: 292–303.