Descriptive parameter evaluation in mammalian cell culture

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Abstract

Several methods exist for assessing population growth and protein productivity in mammalian cell culture. These methods were critically examined here, based on experiments with two hybridoma cell lines. It is shown that mammalian cell culture parameters must be evaluated on the same basis. In batch culture mode most data is obtained on a cumulative basis (protein product titre, substrate concentration, metabolic byproduct concentration). A simple numerical integration technique can be employed to convert cell concentration data to a cumulative basis (cell-hours). The hybridoma lines used in this study included a nutritionally non-fastidious line producing low levels of MAb and a nutritionally fastidious hybridoma with high productivity. In both cases the cell-hour approach was the most appropriate means of expressing the relationship between protein productivity and cell population dynamics. The cell-hour approach could be used as the basis for all metabolic population parameter evaluations. This method has the potential to be used successfully for both prediction and optimization purposes.

Abbreviations: CH_{vol}, cumulative volumetric cell-hours (million cell-hours per milliliter); VI, Viability Index ((Million cells per milliliter) (X hours))

Introduction

A need exists to develop robust techniques for evaluating protein production by mammalian cells in culture. In particular, understanding protein production would benefit greatly from widely applicable evaluation techniques for describing growth and productivity. Since batch culture is the operating mode typically employed for biopharmaceutical production, evaluation techniques should be applicable primarily to batch culture, although applicability to other modes (fed-batch, cyclic batch, continuous, and perfusion) is certainly acceptable as well. Currently, the techniques used for describing production in batch culture suffer in two key areas: 1). There is a lack of uniformity between researchers in methods used to evaluate population parameters, making comparisons between results difficult. 2). Many of the techniques currently in use for evaluating population parameters are based on correlations with specific growth rates and viable cell concentrations. These correlations are not adequate for

mammalian cells, primarily because, while the production of macromolecules such as proteins occurs within a time frame of hours, the mammalian genetic time domain is on the order of days (Lloyd *et al.*, 1982).

Parameters commonly used in describing batch mammalian cell culture are summarized in Table 1. Population growth (cell proliferation) is conventionally assessed in terms of the concentration of cell per unit volume of medium (X), frequently termed the cell density (Xie and Wang, 1996; Merten, 1988; Takahashi *et al.*, 1994; Suzuki and Ollis, 1990). In addition, the maximum viable cell concentration attained (X_{vmax}), the specific growth rate (μ), the percent viability (%V), and the average viable cell concentration (X_{vavg}) are all employed as measures of the state of the population (Xie and Wang, 1996; Merten, 1988; Takahashi *et al.*, 1994; Suzuki and Ollis, 1990).

Production of secreted (extracellular) proteins is commonly measured as the concentration of protein in the medium (Xie and Wang, 1996; Merten, 1988). Hence, production is the end point of synthesis, post-

Table 1. Some parameters used in describing batch hybridoma growth and monoclonal antibody production

Parameter	Symbol	Formula	Reference
viable cell density	X_v	$\frac{\text{viable cell number}}{\text{volume of medium}}$	
maximum cell density	$X_{v_{\max}}$		
average cell density	$X_{v_{\text{avg}}}$	$\frac{\sum_{j=i}^f X_{v_j}}{n}$	
specific population growth rate	μ_{avg}	$\frac{\sum_{j=i}^f \frac{\ln(X_{v_{j+1}}/X_{v_j})}{(t_{j+1}-t_j)}}{n}$	Dutton (1992)
	μ	$\mu_{\max} \frac{GLC}{K_{GLC} + GLC} \frac{GLN}{K_{GLN} + GLN}$	Miller <i>et al.</i> (1988), de Tremblay <i>et al.</i> (1992)
	μ	$\mu_{\min} + \frac{(\mu_{\max} - \mu_{\min})(GLC - GLC_{th})}{K_{GLC} + (GLC - GLC_{th})}$	Frame & Hu (1991a)
percent viability	%V	$(X_v X t^{-1})100\%$	
protein product accumulation rate	dP/dt	$\mu_a X_v + b X_v$	Xie & Wang (1996), Shirai <i>et al.</i> (1994)
or			
volumetric productivity	P_{VOL}	$\frac{(c_2 - c_1)}{(t_2 - t_1)}$	Merten (1988)
product titre	M_{Ab}	$\frac{\text{mass or moles } M_{\text{Ab}}}{\text{volume of medium}}$	
maximum product titre	$P_{\max}$		
viability index	VI	<i>integral of X_v</i>	Luan <i>et al.</i> (1987)
cumulative volumetric cell-hours	CH_{VOL}	$\sum_{j=i}^f \frac{(X_{v_{j+1}} - X_{v_j})}{\ln(X_{v_{j+1}}/X_{v_j})} (t_{j+1} - t_j)$	<i>this paper</i>
specific productivity	$q_{M_{\text{Ab}}-I}$	$\frac{P_{\max}}{(X_{v_{\text{avg}}})(t_f)}$	
	$q_{M_{\text{Ab}}-II}$	$\left(\sum_{j=g}^h \left(\frac{(P_{j+1} - P_j)}{(X_{v_{j+1}} - X_{v_j})} \right) \right) \left(\frac{\mu_{\max}}{n} \right)$	Merten (1988)
	$q_{M_{\text{Ab}}-II}$	$\left(\sum_{j=i}^f \left(\frac{(P_{j+1} - P_j)}{(X_{v_{j+1}} - X_{v_j})} \right) \right) \left(\frac{\mu_v}{n} \right)$	Hayter <i>et al.</i> (1992), Goergen <i>et al.</i> (1992)
	$q_{M_{\text{Ab}}-III}$	$\frac{M_{\text{Ab}}}{VI}$	Luan <i>et al.</i> (1987)
	$q_{M_{\text{Ab}}-III}$	$\frac{M_{\text{Ab}}}{CH_{\text{vol}}}$	<i>this paper</i>
yield of cells on substrate	$Y_{X, S}$	$\frac{X t_{\max}}{(S_f - S_i)}$	
specific consumption of substrate	q_s or q_{CON} eg: q_{GLC}	$\frac{GLC}{CH_{\text{vol}}}$	Xie & Wang (1996), <i>this paper</i>
	q_s	$\frac{\mu}{Y_{X, S}} + \frac{aS}{b + S}$	de Tremblay <i>et al.</i> (1992)
	q_s	$\frac{\mu}{Y_{X, S}} + m$	Miller <i>et al.</i> (1988)
	q_s	$\mu \left(\frac{1}{Y_{X, S}} - \frac{\beta}{Y_{P, S}} \right) + \frac{\alpha}{Y_{P, S}} - \frac{\mu_{\min}}{Y_{X, S}}$	Frame & Hu (1991b)
specific production of metabolites	q_{PROD} eg: q_{LAC}	$\frac{LAC}{CH_{\text{vol}}}$	Xie & Wang (1996), <i>this paper</i>
yield of metabolite on substrate	eg: $Y_{\text{LAC, GLC}}$	$\frac{q_{\text{LAC}}}{q_{\text{GLC}}}$	<i>this paper</i>

X : total cell concentrations; a, b, α, β ; constants; c, P : Product concentrations; S : substrate concentration; f : final; i : initial; g : start exponential growth phase; h : end exponential growth phase; n : number of observations; GLC: glucose concentration; LAC: lactate concentration; GLN: glutamine concentration; m : maintenance coefficient; t : culture time; μ_v : variable specific growth rate.

translational modifications, intracellular transport, and secretion (Suzuki and Ollis, 1990). Productivity may be represented by one or more of three parameters: 1). the specific productivity (q_P) (Xie and Wang, 1996; Merten, 1988, Suzuki and Ollis, 1990; Luan *et al.*, 1987) which is the amount of protein product produced per cell per unit time; 2). The protein product accumulation rate (dP/dt) (Xie and Wang, 1996; Merten, 1988), also termed the volumetric productivity (P_{vol}); and 3). the maximum protein product concentration (P_{max}) (Xie and Wang, 1996; Merten, 1988). The latter two are often expressed as functions of the specific growth rate (Takahashi *et al.*, 1994; Geartner and Dhurjati, 1993a, b) or the viable cell concentration (Xie and Wang, 1996; Takahashi *et al.*, 1994; Suzuki and Ollis, 1990), respectively.

The specific productivity, q_P , has been calculated in at least three different ways. For example, a method for evaluating the specific productivity was developed by Luan and co-workers (1987). In their technique, the volumetric cumulative product concentration measurements over the course of a batch culture were linearly regressed against the integral of the cell density measurements (which the authors called the "Viability Index") over the same time interval. In this study, the applicability of these population parameters for describing and assessing the growth and productivity of two hybridoma cell lines is tested. The Viability Index, while mathematically relevant, has not been clarified as to its biological significance. We present the theory of the cell-hour to resolve this matter. We propose the cell-hour as an independent parameter for describing and assessing population growth in batch culture mode, as well as for use in the evaluation of the specific productivity.

Materials and methods

Cell lines inoculum preparation, and culture propagation

Two different murine (mouse-mouse) hybridoma cell lines were used in this study. The first line, M4-1 hybridoma, is nutritionally non-fastidious, but a low producer of an IgG₁ type monoclonal antibody against bovine light chain IgG. This cell line was obtained courtesy of Center for Infectious Diseases (Ottawa, Canada). The second line, B72.3 hybridoma (ATCC # HB 8108), is nutritionally fastidious, but a high producer of an IgG₁ type monoclonal antibody against

human colon carcinoma.

Seed cultures were stored frozen in serum containing 10% v/v DMSO in the vapour phase of a liquid nitrogen dewar. Cell populations were maintained for a maximum of 2 months (a maximum of 85 generations) in 175 cm² tissue culture flasks as cyclic batch static cultures. These cell populations were routinely fed every third day by removing 75% of the culture volume (whole broth) and replacing with an equal volume of fresh medium. The cultures were propagated in a controlled environment incubator (Forma Scientific) at 37 °C in a 95% relative humidity, 5% CO₂ atmosphere. Inocula were taken on the third day following a feeding.

Experimental runs consisted of M4-1 and B72.3 hybridomas propagated in 175 cm² T-flasks, housed in a controlled environment incubator, as static batch cultures. A 10 mL inoculum (75% to 85% viability) was added to 90 mL of fresh environment-equilibrated medium for a total working volume of 100 mL, to yield an inoculum concentration of approximately 0.1×10^6 viable cells/mL. Experimental runs were allowed to proceed until viability of the cell population dropped below 10%.

Medium formulation

The M4-1 cell line was cultured in Minimum Essential Modified Eagle's (MEM) medium (ICN/Flow Laboratories Inc., Costa Mesa, CA) as the basal medium, supplemented with 1.5 g/L glucose to yield an initial glucose concentration of 2.5 g/L; 4.0 g/L sodium bicarbonate; and 50 mg/L sodium pyruvate. All supplements were obtained from Sigma Chemical Co. (St. Louis, MO).

The nutritionally fastidious B72.3 hybridoma was cultured in Dulbecco's Modified Eagle's (DMEM) Medium (ICN/Flow Laboratories Inc., Costa Mesa, CA) as the basal medium, containing 4.0 g/L glucose, and supplemented with 4.0 g/L sodium bicarbonate and 50 mg/L sodium pyruvate.

Powdered supplemented basal MEM or DMEM was dissolved in pyrogen free 18 MOhm-cm water and filter sterilized through a 0.22 µm membrane filter. Prepared basal medium was stored at 4 °C for a maximum of 3 weeks prior to use. Complete medium was prepared immediately prior to use by fortifying the MEM basal medium with 50 IU/mL penicillin and 50 mg/mL streptomycin; 4 mmol/L glutamine; and 10% v/v horse serum. The DMEM basal medium was completed for use by the addition of 50 IU/mL penicillin

and 50 mg/mL streptomycin; 4.0 mmol/mL glutamine; and 20% v/v foetal bovine serum. The above chemicals were all obtained from ICN/Flow Laboratories Inc. (Costa Mesa, CA). Complete medium was pH equilibrated to 7.2 for use in a 5% CO₂ atmosphere.

Analytical methods

Total cell concentration was determined on an electronic particle counter (Electrozone/Celloscope, Particle Data Inc.) having a 100 μ m aperture. A whole broth sample was diluted twenty fold in an isotonic aqueous solution. The average of five counts was dilution-corrected to yield a total cell concentration (cells/mL) in the culture broth.

Cell viability was determined with a haemocytometer using the Trypan Blue Exclusion Test (Phillips, 1973). At least 100 cells were counted within one minute of dye addition, and within 30 minutes of sampling. The percent viability was determined as a ratio of the number of non-stained (viable) cells counted to the total number of cells counted. Viability counts were repeated at least three times for each sample and then averaged.

The pH was measured within one minute of sampling, using an Orion research digital pH/millivolt meter 611 (Orion Research Co., Boston, MA) with a combination pH electrode.

Monoclonal antibody concentration

Monoclonal antibody concentration was determined using a modified version of the competitive Enzyme Linked ImmunoSorbent Assay (ELISA) originally described by Bosworth *et al.* (1983). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO. The 96 wells of a first microassay plate (plate 1) (Falcon 3912 MicroTest III flexible assay plate; Becton Dickinson, Oxnard, California) were coated with 50 μ L of mouse IgG₁ at a concentration of 1 μ g/mL NaHCO₃, and incubated at 4 °C for 6 to 10 hours. The 96 wells of a second microassay plate (plate 2) (Corning ELISA plates, 25805-96; Corning Glass Works, New York) were prewashed with 100 μ L of PBS (0.14 mol/L sodium chloride, 0.003 mol/L potassium chloride, 0.0015 mol/L potassium phosphate monobasic, and 0.008 mol/L sodium phosphate dibasic in pure water) containing 0.05% (v/v) Tween-20 (PBS-Tween) for a minimum of 10 minutes. The PBS-Tween solution was then discarded and four 65 μ L replicates of each dilution of each sample (diluted from 1 to 400 fold)

or IgG₁ type antibody standards (ranging from 0.01 to 10 μ g/mL) were introduced into the wells. An equal volume (65 μ L) of sheep anti-mouse IgG₁ at a 400 fold dilution in PBS was then mixed with the samples and standards in each well, and the plate was incubated at 4 °C for one hour.

Following incubation, 50 μ L aliquots were transferred from plate 2 to plate 1, which had been washed at least four times with 200 μ L/well of PBS-Tween just prior to the transfer to remove unbound mouse IgG₁. Plate 2 was then discarded and plate 1 was incubated at 4 °C for 30 minute incubation, plate 1 was washed at least four times with 200 μ L/well of PBS-Tween and 50 μ L of a 500 fold dilution of avidin-alkaline-phosphatase was added to each well. Following a one hour incubation at 4 °C, the plate was washed at least four times with 200 μ L/well of PBS-Tween and then rinsed twice with PBS and once with glycine buffer (pH 6.4 – 7.5). Enzymatic reaction was then initiated with 50 μ L of *p*-nitrophenol phosphate substrate solution at a concentration of 1 μ g/mL of glycine buffer at pH 10.4, and incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 25 μ L of 2 mol/L NaOH and the solution was left in the dark at room temperature for 30 minutes.

Absorbance was determined immediately following this final incubation at 405 nm using a Microplate Reader Model EL308 (Bio-TEK Instruments). Absorbance was calibrated to IgG₁ concentration (w/v) using a multivariable linear least squares procedure (Hayward *et al.*, 1991). The MAb concentration of each sample was calculated as the arithmetic average of the replicates from all of the dilutions which fell within the linear region of the absorbance to concentration calibration curve.

Results and discussion

The conventional representation of batch mammalian cell culture is shown in Figure 1. This methodology has inherent conceptual problems. For example, the production of monoclonal antibody (MAb) is an obvious function of the concentration of viable cells, apparently varying with the cell population growth phase. Although this seems to suggest that productivity is at least partially growth phase associated, the exact relationship of difficult to assess quantitatively.

A second frequently used technique for assessing population growth is the maximum viable cell concentration ($X_{v_{max}}$) attained, which is thought to indicate

Figure 1. Conventional representation of batch culture viable population curve and monoclonal antibody titre curve. Cells were propagated as free suspension static batch cultures in 100 mL working volume T-flasks. Clearly, the production of monoclonal antibody is a function of the viable cell concentration, but the exact relationship is difficult to assess from this representation of the data. Monoclonal antibody production appears to vary with the population growth phase. squares: Xv; circles: MAb; a) M4-1 hybridomas; b) B72/3 hybridomas.

Table 2. Population growth and productivity batch culture parameters of free suspension M4-1 and B723.3 hybridoma cells

	$t(X_{V(\max)})$ (h)	$X_{V(\max)}$ (c mL ⁻¹)	$X_{V(\text{avg})}$ (c mL ⁻¹)	$t(P_{\max})$ (h)	$P_{\max}$ ($\mu\text{g mL}^{-1}$)	$P_{\max} X_{V^{-1}}$ (pg c ⁻¹)	$\mu_{\max}$ (h ⁻¹)	$q_{\text{MAb-I}}$ (pg c ⁻¹ h ⁻¹)	$q_{\text{MAb-II}}$ (pg c ⁻¹ h ⁻¹)	$q_{\text{MAb-III}}$ (pg c-h ⁻¹)	$q_{\text{MAb-IIId}}$ (pg c-h ⁻¹)
M4-1	124	0.980	0.328	310	0.902	2.75	0.026	0.009	0.014	0.008	0.008
B723.3	141	0.994	0.395	310	141	357	0.018	1.146	1.700	0.935	2.926

c: cell; c-h: cell-hour

the success of population growth (Xie and Wang, 1996) and, by extension, the biological capacity for production of the system (Table 2). While a large number of cells will invariably produce a larger amount of protein product than will a small number of cells in the same time span, the single measurement of the instantaneous viable cell concentration has not been found to correlate with the maximum product titre ($P_{\max}$) achieved in a batch culture (Bushell *et al.*, 1993; Suzuki and Ollis, 1990; Xie and Wang 1994a; Merten, 1988). The average number of viable cells over the course of the batch culture ($X_{V\text{avg}}$) (Table 2) is probably a better indicator of both the biological capacity for production and the success of population growth or maintenance. The maximum product concentration can be assessed as a function of $X_{V\text{avg}}$ (Table 2).

Apparent population growth rate

The specific growth rate, μ , is derived from the instantaneous viable cell concentration, as the number of cells produced per cell per hour (usually expressed as h⁻¹). The specific growth rate changes continuously over the course of the batch process, as shown in Figure 1. In spite of this obvious variability, μ is generally utilized as a constant, frequently with $\mu_{\max}$ taken to be the value of this parameter (Table 2) (Hayter *et al.*, 1992; Goergen *et al.*, 1992; Xie and Wang, 1994a).

In recent years, the effect of μ on the rate of productivity and on the maximum product titre has become a topic of intense investigation (Hayter *et al.*, 1992; Bushell *et al.*, 1993; Goergen *et al.*, 1992; Takahashi *et al.*, 1994; Suzuki and Ollis, 1990). In particular, MAb has frequently been shown to be at least partially growth phase associated, and is therefore sensitive to changes in the apparent population growth rate. While an obvious indicator of population growth, μ is only superficially connected to protein productivity.

The cell cycle in batch culture

Underlying population growth are the life and proliferative cycles of the individual cells comprising the population. The proliferative cycle of the individual cells is known as the cell cycle. The cell cycle has been partitioned into four distinct phases: G₁, S, G₂ and M. Currently, much attention is focused on the relationship between protein productivity and cell cycle phases (Suzuki and Ollis, 1990; Kromenaker and Srienc, 1994; Mastrangelo and Betenbaugh, 1995; Cartwright, 1994; Mercille and Massie, 1994; Franek, 1995; Park and Ryu, 1994). While the length of S phase, G₂ phase, and M phase, are relatively constant, the length of the G₁ phase, particularly early G₁ phase, is variable (Baserga, 1985). The length of the early G₁ phase can actually extend indefinitely, and cells in such a quiescent state are said to have left the cell cycle and entered a resting state, G₀ or G_Q. Cells in this state are 'resting' only with respect to proliferative activity. This variability introduces a distribution in the specific growth rate: subpopulations divide at variable rates. The apparent specific population growth rate is equal to the true specific population growth rate only if the entire population is viable, synchronized, and proliferating at the maximum possible rate. Clearly, this can rarely be the case.

While much of the investigative effort continues to be placed on the proliferative events of the cell cycle phases, many workers are centering on RNA and protein synthetic activity, as this is pertinent to protein productivity of a cell population (Suzuki and Ollis, 1990; Cartwright, 1994; Kromenaker and Srienc, 1994). For example, it has been reported that many 'secondary metabolites', such as MAbs, are preferentially synthesized during the G₁ cell cycle phase (Hayter *et al.*, 1992; Kromenaker and Srienc, 1994; Tziampazis and Sambanis, 1994; Martens *et al.*, 1993; Singh *et al.*, 1994), while there is an absence of translation during G₂ and M cell cycle phases (Suzuki and Ollis, 1990). Theoretically, cell cycle phase-specific productivity can be ascertained from non-segregated popula-

tion data. For example, if the MAb is preferentially produced during the G_1 cell cycle phase, then specific productivity should increase as population growth rate decreases, and *vice versa*. This productivity pattern is termed negatively growth-associated, while in growth-associated productivity, the protein production rate increases with the specific growth rate (Tziampazis and Sambanis, 1994).

Life span of individual cells in culture varies both from cell line to cell line and within cell populations, but is on the order of 2 to 2.5 days (de la Broise *et al.*, 1991).

The life span of a non-transformed, or normal, mammalian cell *in vivo* is a maximum of about 50 doublings (Baserga, 1985), after which it drops out of the cell cycle permanently and enters senescence. This type of senescence is termed apoptosis, or programmed cell death. It is likely that environmental conditions may affect the life span of individual cells. Numerous stimuli, including viral infection and deprivation of cell growth factors, can induce apoptosis of cells grown in culture (Mastrangelo and Betenbaugh, 1995). This natural process of dying is distinct, both in time span and in physiological state, from the general degenerative, traumatic cell death, termed necrosis (Cartwright, 1994).

Apoptotic and necrotic cells have a number of defining characteristics pertinent to protein production from a cell population. In contrast to necrotic cells which exhibit a marked decrease in RNA and protein syntheses (Mercille and Massie, 1994; Franek, 1995; Singh *et al.*, 1994), apoptotic cells exhibit increases in some RNA and protein syntheses. Increases in the specific productivity during the decline phase of batch cultures could be explained if the protein product is preferentially synthesized by the increasing number of apoptotic cells. It has also been suggested that apoptotic cells exhibit increased secretion of intracellularly accumulated proteins (Al-Rubeai *et al.*, 1992). In contrast, where protein productivity is associated with proliferation, it has been assumed that retarding the transition of a cell population into apoptosis will increase protein titre (Mercille and Massie, 1994; Mastrangelo and Betenbaugh, 1995; Franek, 1995).

Specific productivity

A descriptor of the specific productivity, q_{MAb} ($\mu\text{g}/\text{cell}/\text{hour}$), can be calculated from the maximum product concentration per culture hour and normalized with respect to the average cell concentration. Such a

way of expressing q_{MAb} , here labelled q_{MAb-I} (Table 2), is based on an assumption that all cells are in an identical physiological state throughout the batch culture (i.e. a homegeneous, time-invariant cell population), although this is biologically unreasonable. For example, growing cells are obviously physiologically distinct from dying cells. This parameter (q_{MAb-I}) can serve as good estimation of productivity, but is not useful in formulating fine optimization strategies, as it has been found to vary from batch run to batch run (run dependent) (Bushell *et al.*, 1993), suggesting that it changes as the physiological make-up of the cell population changes.

In batch culture, a second form of the specific productivity (q_{MAb-II}), which is the protein product (MAb) concentration divided by the viable cell concentration (Hayter *et al.*, 1992), indicates that productivity is increasing throughout the decline phase when the viable cell number is very low and decreasing (Figure 1). While cells undergoing apoptotic senescence have been shown to increase the production of some proteins (Mercille and Massie, 1994; Singh *et al.*, 1994), and while all senescent cells may also be increasing the rate of secretion or release of accumulated intracellular MAb (Leno *et al.*, 1992; Flickinger *et al.*, 1992; Al-Rubeai *et al.*, 1992), there is no biological justification for this apparently very high rate of production from so few cells which are in a dying state. Rather, this apparent phenomenon is an artifact which arises from comparing cumulative MAb production (which the MAb concentration in the medium is a measure of) with the instantaneous measure of the biological capacity for production (i.e. the cell number). This is effectively comparing an integral function to a derivative function. In part, this contradiction has been recognized by constraining the use of q_{MAb-II} to the exponential growth phase (Goergen *et al.*, 1992; Gaertner and Dhurjati, 1993a, b), where the specific growth rate is approximately constant, and, therefore, the trace of the cumulative MAb production approximately parallels the trace of the cell number (Figure 1). When the slopes of these two traces are compared, q_{MAb-II} is presented as a specific monoclonal antibody rate with respect to culture time. Merten's (1988) method of calculating q_{MAb-II} in this way is shown in Table 2. Ideally, such a parameter should be applicable over the entire course of the culture, therefore, the validity of q_{MAb-II} , calculated in this way, is questionable. Attempts to apply this method of calculating the specific productivity over the entire batch run by utilizing a variable μ , results in complex curves (Table 1) which

are difficult to interpret (Hayter *et al.*, 1992; Goergen *et al.*, 1992; Al-Rubeai *et al.*, 1992).

A third representation of this parameter, which is gaining acceptance, expresses the protein concentration as a function of the so-called Viability Index (VI) (Luan *et al.*, 1987). This parameter is also called the specific productivity, and has been given the same symbol (q_{MAb}), leading to confusion. Here, this form of the specific productivity is labelled $q_{\text{MAb-III}}$. Luan and co-workers defined the Viability Index as "the number of viable cells at each count integrated over the duration of the experiment". Their data illustrated "a very good direct correlation between the Viability Index and the cumulative MAb concentration over the entire course of a batch hybridoma culture", suggestive of a physical basis which would substantiate the validity of this parameter, VI, and of calculating q_{MAb} as a function of VI. The methods of calculation for VI and $q_{\text{MAb-III}}$ are shown in Table 1.

Cell hours, cumulative production and specific productivity

The calculation of the Viability Index transposes the instantaneous measurement of the biological capacity into a cumulative measurement of the biological capacity for production on a unit volume of medium. Since protein synthesis and secretion is a spatio-temporal property of living cells, expressing production as the integral of viable cells (i.e. the cumulative length of time that live cells have been present in the batch run) per unit volume is a useful approach. However, Viability Index is a vague term, which we therefore propose be replaced by the term "cumulative volumetric cell hours", CH_{vol} , defined as the cumulative number of hours spent in the medium by all viable cells. Our method of calculating CH_{vol} is presented in Table 1. With respect to productivity, CH_{vol} represents the cumulative productive (eg. MAb producing) life span of cells in the medium (bioreactor). It is crucial to recognize that one cell has the potential to produce as much MAb in ten hours as ten cells can produce in one hour. In both cases, the total number of production hours is identical. The use of the CH_{vol} parameter allows for the assessment of productivity and of the biological capacity for production on the same (cumulative) basis (Figure 2).

Alternatively, cumulative antibody concentration data can be manipulated to yield the instantaneous antibody production, for direct comparison with the measured viable cell concentration. Mathematically, this would involve finding the derivative of the func-

tion representing the cumulative antibody concentration data over the entire course of the culture. However, this may prove problematic, in that the specific productivity does not necessarily remain constant over the entire course of the culture. That is, the cumulative antibody data trace may be composed of the combination of more than one function, as the number of cells in various subpopulations (physiological states, such as different cell cycle phases) changes over time.

The volumetric productivity (P_{vol} or dP/dt), which is frequently calculated as a function of the specific growth rate and the viable cell concentration (Table 1) (Xie and Wang, 1994a; Shirai *et al.*, 1994), is an estimated measure of the instantaneous antibody production. The deviation of dP/dt from direct proportionality to μ has been accommodated by the inclusion of a second, non- μ dependent, term. This term is referred to as the non-growth associated term.

The maximum volumetric productivity has been found to correlate directly with μ_{max} (Hayter *et al.*, 1992; Goergen *et al.*, 1992). In contrast, μ_{max} , which is often cited as a measure of the success of the propagation system, does not correlate with the maximum titre. The average volumetric productivity has no obvious connection to the viable cell concentration curve. This is not surprising when the length of the proliferative cycle of animal cells is compared with the length of the epigenetic time domain. In contrast to microorganisms, where genetic and epigenetic activities are contained within approximately the same time domain (measured in hours), the length of the animal cell cycle is measured on the order of days, while protein production proceeds on the order of hours (Lloyd *et al.*, 1982). Apparently nongrowing cells may produce significant quantities of protein. hence, protein productivity from animal cells is more closely correlated to the length of time spent in the reactor by all viable cells, than it is correlated to the frequency of division (growth rate) of that viable cell population. It should be noted that the cell-hour parameter, CH_{vol} , is inclusive of all non-growth associated metabolic activity. In this context, it is apparent that the yield of cells on a particular substrate ($Y_{X/S}$) has limited interpretive value with respect to optimization of productivity.

The method of evaluating the specific productivity as a function of cumulative volumetric cell hours, can and should be extended to the evaluation of metabolic parameters. Equations on this basis for specific production of lactate and specific consumption of glucose are presented in Table 1. The metabolic time domain (for ATP synthesis) which is measured on the

Figure 2. Free suspension static batch cultures of hybridomas producing monoclonal antibodies, represented as the cumulative biological capacity for production (cell-hours) and associated cumulative product titre. Recalculated from the data in Figure 1. Clearly, protein productivity is closely correlated to cell-hours. Here, it is possible to discern that MAb productivity by M4-1 hybridomas does not appear to vary with growth rate, while MAb productivity by B72.3 hybridomas may increase during the decline growth phase. triangles: CHvol; circles: MAb; a) M4-1 hybridomas; b) B72.3 hybridomas.

order of minutes, is even further removed from the time frame of cell division than is protein productivity. Specific glucose consumption rate, specific glutamine consumption rate, specific lactate production rate, specific ammonia production rate, etc., expressed in this way are inclusive of growth-, maintenance-, and protein-product-associated metabolic activities. Yields of metabolic byproducts on substrates, such as the yield of lactate on glucose ($Y_{LAC/GLC}$), can then be calculated from the specific rates, as shown in Table 1.

Association of productivity to population growth phase and to specific growth rate

CH_{vol} is a direct quantification of the "load" on the medium, while the maximum volumetric cell hours attained is a quantification of the success both of population growth and/or maintenance and of medium utilization for the support of the population. By simply re-plotting cell population and product data on the same (cumulative) basis (Figure 2), growth-phase-associated, non-growth-phase-associated, or partially-growth-phase-associated productivity is qualitatively obvious graphically, and quantitatively simple to analyze mathematically. When cumulative volumetric product concentration is linearly regressed against cumulative volumetric cell hours, as proposed by Luan *et al.* (1987), the nature of the association between these two parameters is immediately, and quantifiably apparent. The dependence of the cumulative MAb concentration on the volumetric cell hours is shown in Figure 3. The applicability of this approach for both B72.3 and M4-1 hybridomas is evident.

As is the case for the M4-1 hybridomas (Figure 3a and Table 2), a single line of constant slope (constant $q_{MAb-III}$) over the entire course of the batch culture, indicates non-growth-phase-associated productivity, suggesting that the protein is produced at approximately the same rate during all phases of the cell cycle as well as during periods of quiescence and during senescence. The slight hint of increase in the specific productivity during the late decline population growth phase suggests that apoptotic cells may exhibit a marginal increase in the production of this MAb.

In contrast, the B72.3 hybridoma cell line exhibits partial growth phase association of productivity (Figure 3b and Table 2) as indicated by two distinct linear lines. These lines both have non-zero positive slopes, indicating that MAb production occurs in all population growth phases of the batch. For the B72.3 hybridoma cell line, $q_{MAb-III}$ is greater during the decline phase

of the culture than during the other population phases of the culture. This productivity pattern can be explained by one or more of the following four theories: 1). The protein is produced preferentially and/or at a faster rate during the quiescent (G_1/G_0) states. 2). The protein is produced preferentially during apoptosis. 3). Accumulated intracellular protein is secreted from quiescent and/or senescent cells. 4). The protein is released *on masse* as cells lyse. This data also suggests that MAb may not be produced by the B72.3 cell line during the S, M, and G_2 , phases of the cell cycle. That is, as the specific growth rate decreases, productivity increases.

The two hybridoma cell lines examined in this study represent two different productivity patterns: non-growth-phase-associated and partially-growth-phase-associated. A third type of productivity pattern is possible. Were the MAb productivity growth-phase-associated, it would be restricted to some population growth phases, and excluded from others. Such exclusion would be indicated by one or more lines with a slope of zero.

In addition to productivity patterns based on correlations to population growth phases, it is possible to distinguish productivity patterns based on correlations to specific growth rates of the population. The two cell lines examined in this study represent two of the four possible relationships between the specific productivity and the specific growth rate. In the B72.3 cell line, specific productivity is inversely related to the specific growth rate (negatively growth-associated). In the M4-1 cell line, the specific productivity remains constant as the specific growth rate changes (non-growth-associated), as is the case with all cell lines exhibiting non-growth-phase-associated productivity.

A third type of cell line, which exhibits increasing specific productivity with increasing specific growth rate (growth-associated), suggests that product is produced preferentially or exclusively during the S, M, and G_2 , phases of the cell cycle. Conditions which extend exponential growth (perhaps including continuous culture mode) should therefore optimize productivity of this type of cell line.

In the fourth type of cell line, the relationship of productivity to growth rate is less clear, and may exhibit several different regions of association-types (negatively growth-associated, growth-associated, and non-growth associated regions). This type of association is termed partially growth-associated. When the regions of association-type vary rapidly, a plot of the specific productivity against the specific growth rate results in complex curves. When the specific pro-

Figure 3. The specific productivity of monoclonal antibody by hybridoma cells is easily determined from the linear relationship between cumulative product titre and cumulative cell-hours. a) M4-1 hybridomas: non-growth-phase-associated; non-growth-associated. b) B72.3 hybridomas: partially-growth-phase-associated; negatively growth-associated.

ductivity changes with the physiological state of the population (growth phase), these complex curves may loop back and forth as the batch run proceeds. As discussed above, the relationship of productivity to specif-

ic growth rate is, at best, loose, so that many cell lines fall into this category. Optimization strategies based on the specific growth rate for such cell lines are difficult to formulate.

Figure 4. Theoretical examples of the basic types of protein production kinetics. a) Protein titre as a function of the biological capacity for production (volumetric cell-hours) during the different population growth phases. The slopes of the lines are the specific productivities. 1 non-growth-phase-associated (constant slope); 2 growth-phase-associated (at least one zero slope); 3 partially-growth-phase-associated (variable slopes, no zero slopes). b) Specific productivity as a function of the specific growth rate. This correlation's biological basis is ambiguous. 1 non-growth-associated (constant zero slope); 2 growth-associated, negatively-growth-associated (constant positive, or negative, slope); 3 partially-growth-associated (at least partially non-linear).

Concluding remarks

Analyses of the parameters associated with the evaluation of batch cultures of mammalian cells must be performed on the same basis. Measured concentrations of substrates, metabolic byproducts, and protein products represent cumulative values. We have shown that a simple numerical integration technique can be employed to convert the instantaneous viable cell number to cumulative cell-hours. Specific productivity and consumption parameters can then be calculated on the basis of volumetric cell-hours. Using this approach, three basic patterns of protein production as a function

of the non-segregated cell population emerge. Theoretical examples of these patterns are illustrated in Figure 4a.

For comparison, the possible correlations of the specific productivity to the specific growth rate are presented in Figure 4b. Partial growth association results in complex patterns, suggesting that correlations of specific productivity to specific growth rate may not have a clearcut biological basis. Particularly, the non-linear patterns shown in Figure 4b can be more readily interpreted by analysis of the epigenetic time domain (Figure 4a).

Simplicity, both in measurement techniques and in

data analysis, is a highly desirable attribute of evaluation techniques. Evaluation of the physiological makeup of the population, or detailed cell cycle analysis, may not be necessary to the development of a successful optimization strategy. Here, for example, conditions which maintain a high population concentration, but slow specific population growth rate should maximize the productivity of the B72.3 cell line. The prolongation of the population decline phase should serve to enhance the product titre. Conversely, the rate of transit through the population growth phases should have no impact on the productivity of the M4-1 cell line, and therefore, for a given number of cell hours, the faster the growth rate, the faster product will accumulate. Hence, evaluation techniques at the whole population (macro or non-segregated) level may be sufficient for optimization purposes; and it may be unnecessary to evaluate the system at the individual cell (micro or segregated) level.

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