Verhulst and stochastic models for comparing mechanisms of MAb productivity in six CHO cell lines

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Abstract The present study validates previously published methodologies—stochastic and Verhulst—for modelling the growth and MAb productivity of six CHO cell lines grown in batch cultures. Cytometric and biochemical data were used to model growth and productivity. The stochastic explanatory models were developed to improve our understanding of the underlying mechanisms of growth and productivity, whereas the Verhulst mechanistic models were developed for their predictability. The parameters of the two sets of models were compared for their biological significance. The stochastic models, based on the cytometric data, indicated that the productivity mechanism is cell specific. However, as shown before, the modelling results indicated that G2 + ER indicate high productivity, while G1 + ER indicate low productivity, where G1 and G2 are the cell cycle phases and ER is Endoplasmic Reticulum. In all cell lines, growth proved to be inversely proportional to the cumulative G1 time (CG1T) for the G1 phase, whereas productivity was directly proportional to ER. Verhulst's rule, “the lower the intrinsic growth factor (r), the higher the growth (K),” did not hold for growth across all cell lines but held good for the cell lines with the same growth mechanism—i.e., r is cell specific. However,

the Verhulst productivity rule, that productivity is inversely proportional to the intrinsic productivity factor (r_x), held well across all cell lines in spite of differences in their mechanisms for productivity—that is, r_x is not cell specific. The productivity profile, as described by Verhulst's logistic model, is very similar to the Michaelis–Menten enzyme kinetic equation, suggesting that productivity is more likely enzymatic in nature. Comparison of the stochastic and Verhulst models indicated that CG1T in the cytometric data has the same significance as r , the intrinsic growth factor in the Verhulst models. The stochastic explanatory and the Verhulst logistic models can explain the differences in the productivity of the six clones.

Keywords CHO cell lines · Batch cultures · MAb productivity · Stochastic models · Verhulst logistic models

Introduction

Bioindustry is expanding at an exponential rate to meet the increasing demands for industrial chemicals and pharmaceutical products, which until recently were still the prerogative of the chemical industry. Industrial biotechnology, also called “red biotechnology,” is based on enzymes and cells (prokaryotic and eukaryotic) and is dominated by producers of biopharmaceuticals (KET 2011; Whelan and Keogh 2012; ABB review 1/12). The World Economic Forum

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(WEF) forecasts that the overall worldwide potential of the bioindustry is upwards of \$230 billion (with added benefits of being cleaner and preserving a greener environment) to the global economy by 2020. Thus, the biopharmaceutical industry is a formidable component of the bioindustry.

Mammalian cells, unlike their bacterial counterparts, have the ability to posttranslate these high-value products to make them biologically active. However, an urgent commercial need exists to improve the efficiency and productivities of the bioprocesses for two reasons: to reduce the spiralling experimental costs and, more importantly, to counter the emerging competition from non-cell culture technologies, such as in vitro ribosome display and transgenic animals (Duncan 2002). These improvements can be either *biological* or *culture* based (Sidoli et al. 2004). Modelling of the bioprocess enables the identification of key state variables, which have a direct effect on increasing efficiency by increasing purity, quality, and productivity (Sidoli et al. 2004). The present work studied the biological approach for improving productivity via cloning.

Initially, prokaryotic cells, like *Escherichia coli*, were used to produce therapeutic proteins. However, it was soon realised that recombinant therapeutics are more complex, requiring the posttranslational for their biological effectiveness. Only eukaryotic cells have necessary metabolic machinery for the post-translation (Butler 2005). Genetic manipulation of mammalian cells involves recombinant technologies. More recently, the enhanced interest in mammalian cell cultures is associated with the recombinant protein technology developed in the 1970s and 1980s (Butler 2005). Biopharmaceuticals produced from mammalian cell bioprocesses are defined as recombinant proteins, monoclonal antibodies (MAb), and nucleic-acid-based products (Butler 2005). From 2006 to 2011 on average, 15 novel recombinant protein therapeutics have been approved by the US Food and Drug Administration (FDA) annually (Lai et al. 2013). Presently, most pharmaceutical companies use the methotrexate (MTX) amplification technology or the glutamine synthetase (GS) system for developing new mammalian cell lines (Lai et al. 2013).

Several criteria are applied in the selection of cell lines (Li et al. 2010). In the present study the yield and viability were considered critical in the selection of producers cell lines.

The kinetics of growth and productivity were followed by flow cytometry and biochemical assay. The flow cytometry data of the six CHO cell lines consisted of the viable cell density (VCD), the cell cycle phases (G1, S, and G2), the mitochondrial membrane potential/mitochondrial mass (MMP/MM) ratio i.e., mitochondrial activity, the endoplasmic reticulum (ER), and the Golgi apparatus (GA). The biochemical assays are determination of monoclonal antibody (MAb), glucose and lactate. The productivity data for the six cell lines were computed by Simpson's rule to determine specific productivity (MAb mg/10⁶ cells), and the cytometric data were modelled by stepwise multivariate regression method to ascertain which of the state variables had causative effects on the productivity (Shirsat et al. 2013). The specific productivity and cell viability results were reviewed in the context of the respective explanatory models. Verhulst models were developed for growth and productivity of the cell lines to determine the biologically significant model constants: the carrying capacity (K_x) and the intrinsic factor (r_x) for productivity (Shirsat et al. 2014). The parameters in stochastic models were compared with the parameters in Verhulst models so as to get further insight into the growth and productivity mechanism in the CHO cell lines.

Materials and Methods

Batch cultures of CHO cell lines

Six CHO cell lines (38, 47, 76, 150, 160 and 164) producing cB72.3 IgG4 monoclonal antibody with varying levels of productivity. The cell lines were kindly provided by Lonza Biologics (Slough, UK). The cell lines were generated by transfection of the suspension-variant derivatives CHO-K1 (CHOK1SV) with the glutamine synthetase (GS) expression vector pcB72.3. The initial culture seeding density was 3×10^5 cells/ml. All the cultures were grown in triplicate and incubated at 37 °C in a shaker incubator at an agitation rate of 140 rpm. Samples were taken at a 24-h interval and analysed by flow cytometry for viable cell number, cell cycle (G1, S, and G2), MMP/MM, ER and GA (Shirsat et al. 2013). Cell-free supernatant was used to determine monoclonal antibody by ELISA (Carroll et al. 2007), glucose (Blood Glucose Meter, Bayer Healthcare, Newark, New

Jersey, USA) and lactate (Accutrend Lactate Meter, Roche Diagnostics, Mannheim, Germany).

Verhulst population balanced growth and productivity models

Shirsat et al. (2014) developed Verhulst logistic models for cell populations in the exponential and decline (death) phases:

$$\frac{dN}{dt} = rN \left(1 - \frac{N}{K} \right) \quad (1)$$

where N = viable cell density (VCD/ml), K = carrying capacity or equilibrium density (VCD/ml), and r = intrinsic growth factor (h^{-1}). Integration of Eq. (1) yields: (for the exponential phase):

$$N(t) = \frac{K}{1 + \left(\frac{K-N_0}{N_0} \right) e^{-rt}} \quad (2)$$

where N_0 = initial cell density (VCD/ml) at $t = 0$ and $N(t)$ = cell density at $t = t$. For the decline (death) phase, change the intrinsic growth factor r from $+$ to $-$ the initial cell number from N_0 to N_{max} , and t_0 to t_{max} . Thus,

$$N(t) = \frac{K}{1 + \left(\frac{K-N_{max}}{N_{max}} \right) e^{r(t-t_{max})}} \quad (3)$$

where N_{max} = maximum cell density (VCD/ml) at time, $t = t_{max}$. Model constants K and r are determined by plotting cell density $N(t)$ ($\log N(t)$) versus the specific rate of growth $(1/N)dN/dt$. The y-intercept, r (h^{-1}), is intrinsic growth factor and x-intercept, K (VCD/ml) is carrying capacity or growth potential (Shirsat et al. 2014).

Shirsat et al. (2014) extended the Verhulst population balanced model (2) to productivity of monoclonal antibody (MAB):

$$\frac{dX}{dt} = r_x X \left(1 - \frac{X}{K_x} \right) \quad (4)$$

which on integration yields:

$$X(t) = \frac{K_x}{1 + \left(\frac{K_x-X_1}{X_1} \right) e^{-r_x t}} \quad (5)$$

where X_t = MAb concentration, day 1, $X(t)$ = MAb concentration at time t days, K_x = potential for maximum or saturation concentration of MAb ($\mu\text{g/ml}$) and r_x = intrinsic productivity factor (h^{-1}). Model constants K_x and r_x were determined by plotting the experimental data $\ln X(t)$ versus $(1/X) dX/dt$; the intersect at the y-axis gives the value of r_x (h^{-1}), and the one at x-axis represents the value of K_x (MAb $\mu\text{g/ml}$) (Table 1; Shirsat et al. 2014). The values of the productivity saturation (potential) constant, K_x , and the intrinsic productivity factor, r_x , were used to rank the cell lines by their magnitude for growth, productivity, and specific productivity (Table 1).

Statistical modelling of cytometric data

Most biological systems are too complex to allow the relationship between the independent and dependent variables to be ascertained by experimental methods alone. Furthermore, the task is compounded where there is interdependency between the so-called independent variables themselves. To address this, a statistical methodology was developed to identify quantitative aspects of the regulatory mechanisms underlying proliferation and recombinant protein production in cell culture (Shirsat et al. 2013). The regression analysis is best suited for uncovering the true functional relationship between the categorical variables and a response variable by developing explanatory models (Gold 1977).

Therefore, statistical techniques as described by Shirsat et al. (2013) were employed to develop explanatory models for the six cell lines, using cytometric data of growth (VCD), productivity (Mab), cell cycle phases (G1, S and G2), and organelles (MMP/MM, ER and GA). Since several competing independent variables were involved regarding a given dependent variable, stepwise multivariate regression was carried out using Regression_Forecasting software (supplied by Micromail Ltd., Cork, Ireland). The Stochastic explanatory productivity models for the six cell lines are given in Table 4 and selected stochastic explanatory models for comparison of the six cell lines are given in Table 5.

Table 1 Comparison of key Verhulst model parameters of six cell lines

Cell line	K , VCD $\times 10^6$ /ml	r , h^{-1}	K_x , MAb, $\mu\text{g}/\text{ml}$	r_x , h^{-1}
38	7.121 (1)	0.349 (4)	391 (3)	0.688 (4)
47	7.099 (2)	0.332 (2)	575 (1)	0.531 (5)
76	6.922 (4)	0.347 (5)	550 (2)	0.516 (6)
150	6.965 (5)	0.340 (3)	340 (4)	0.702 (3)
160	7.266 (6)	0.446 (6)	89 (6)	0.952 (1)
164	7.022 (3)	0.327 (1)	235 (5)	0.736 (2)

The numbers in parenthesis represent the “rankings”

Verhulst model constants: K carrying capacity for growth, K_x carrying capacity or potential for productivity, VCD viable cell count 10^6 cells/ml, MAb monoclonal antibody $\mu\text{g}/\text{ml}$, r intrinsic growth factor, r_x intrinsic productivity factor

Simpson’s rule

The cumulative variable time (CVT) is a good indicator of relative performance of a state variable over a given time period. CVT for the state variables (VCD, MAb, G1, S, G2, MMP/MM ratio, ER, and GA) were computed by integrating time-course observations over 8 days (Shirsat et al. 2013).

$$y_{st} = \int_0^8 (at^2 + bt + c) dt \quad (6)$$

where y_{st} = state variable, t = time, days, a , b , and c are equation constants.

The results of CVT ($y_{\text{state-variable}} \times \text{time}$) for VCD and MAb are given in Table 2, cell cycle phases (G1, S, and G2), MMP/MM ratio, ER, and GA, in Table 5 and glucose and lactate, in Table 7.

Selection criteria based on productivity (max), specific productivity (yield), viability, and cumulative cell time

Li et al. (2010) described several criteria to apply in the most critical decision of selecting a cell line

suitable for a large-scale bioprocess. The present work considered maximum productivity, specific productivity (yield), cumulative cell time, and cell viability in the selection of the best of the six cell lines. Robust cell growth with high viability and low lactate synthesis is usually desirable. High-lactate-producing cell lines should not be chosen in order to avoid the dramatic osmolality increase that accompanies the addition of base needed to maintain pH (Li et al. 2010).

Productivity (max)

The batch processes were harvested for MAb productivity after 8 days (arbitrarily chosen), and the productivity on the 8 days was taken as the maximum.

Yield

Since the antibody expression rate in mammalian cells is usually not associated with growth, the final titre is equal to the specific productivity q_p multiplied by the integral of viable cell density over culture duration (Xie and Wang 1995; Li et al. 2010) i.e.,

Table 2 Comparison of cumulative growth and productivity of six cell lines

Cell line	VCD $\times 10^6$ /ml	MAb, $\mu\text{g}/\text{ml}$	MAb, $\mu\text{g}/\text{VCD} \times 10^6/\text{day}$
38	35.22 (2)	1179.4 (3)	4.19 (4)
47	30.55 (3)	1462.7 (1)	5.99 (1)
76	28.30 (4)	1312.5 (2)	5.78 (2)
150	26.42 (5)	1021.6 (4)	4.84 (3)
160	39.17 (1)	302.2 (6)	0.96 (6)
164	25.97 (6)	755.2 (5)	3.64 (5)

The numbers in parenthesis represent the “rankings”

VCD viable cell count, 10^6 cells/ml for growth, MAb monoclonal antibody $\mu\text{g}/\text{ml}$ for productivity

$$\text{Titre} = q_p f N dt \quad (7)$$

where q_p is the specific productivity (amount of protein product produced per unit cell per unit time), N is cell density, and t is time. The specific productivity of individual cells was compared for efficacy of protein secretion. To date, typical production-cell-line-specific productivities range from 20 pg/cell/day to 70 pg/cell-day and peak cell densities (from 5 to 30×10^6 cells/ml) in a 10–14 days fed-batch process (Li et al. 2010).

Viability

The viability of the cell lines was measured by Trypan Blue staining and presented as a ratio of viable cells to total cell numbers at 24-h intervals.

Cumulative time for growth, glucose consumption, and lactate production

Protein productivity is directly proportional to viable cell mass, viability, and culture longevity (Kumar et al. 2007). Therefore, the cumulative cell time (CCT) for all cell lines is computed by Simpson's rule, Eq. (6), using the growth data. Cumulative times for glucose consumption and lactate production were also computed because of their link to growth and viability.

Results and discussion

Verhulst models for growth and productivity

The growth and productivity data, as modelled by Verhulst equation, are presented in Fig. 1. The

Verhulst models were developed to determine the growth and MAb productivity potential (K) and the intrinsic factor (r) of the six CHO cell lines (Shirsat et al. 2014). The results are given in Table 1.

The figures in the brackets rank the K s and r s according to their relative magnitude.

Evaluation of constants in the Verhulst growth models

In the Verhulst model, the previous study (Shirsat et al. 2014) indicated a relationship between r and K —“the lower the intrinsic factor (r), the higher the potential (K) for growth or productivity”. However, the growth results indicate that the intrinsic growth factor r is not inversely proportional to growth, implying that r is cell specific (genetic makeup), reflecting the cell machinery (i.e., physiology) of the cell line (Krebs 1996; Thomas 1990). Nevertheless, the Verhulst rule for growth holds for the same cell line with different cultivation modes (e.g., batch and fed-batch) or medium composition (different levels of limiting substrate), as found by Shirsat et al. (2014). Thus, the intrinsic factor r in the Verhulst model is of biological significance, reflecting the genetic makeup of the cell line.

Mammalian cells do not achieve their full growth potential K (carrying capacity) because of the inhibitory effects of the end metabolites, such as lactate and ammonia, insufficient availability of nutrients, and general adverse conditions for growth. Therefore, as an alternate to growth potential, K , the concept of cumulative cell time (CCT), i.e., cell viability and longevity, was computed (Simpson's Rule) for the six cell lines over 8 days. Also, cumulative productivity times (CPTs) were computed

Fig. 1 Verhulst models for the data of the six CHO cell lines: **a** Growth and **b** productivity

for the cell lines to compare their specific productivity. The cumulative times of growth and productivity are presented in Table 2.

Table 2 makes it clear that no direct relationship exists between the viable cell density and productivity for different cell lines. In fact, the results (Table 2) indicate that cell line 160, with the highest CCT ($39.17 \text{ VCD} \times 10^6/\text{ml}$), had the lowest productivity ($302.2 \text{ Mab, } \mu\text{g}/\text{ml}$). Therefore, the six cell lines were ranked according to their specific productivities ($\text{Mab, } \mu\text{g}/\text{VCD} \times 10^6/\text{day}$), as shown in Table 2.

Evaluation of constants in the Verhulst productivity models

In contrast to growth, the productivity, except for cell lines 47 and 76, indicates that K_x and r_x have a perfect inverse relationship, i.e., the lower the r_x , the higher the K_x (Table 1). In cell lines 47 and 76, the differences in their K_x and r_x are marginal. This point is discussed again later.

Unlike K , the maximum growth potential, the six cell lines achieved K_x , their maximum productivity potential. The marginal shortfall in productivity of cell lines 47 and 76 occurred because the process was terminated prematurely.

Growth and productivity

The main focus of work was to determine the relative efficiencies of the cell lines on the basis of their specific productivities. Higher growth does not necessarily mean higher potential for productivity because, in some cases, protein production is not associated with growth (Dutton 1998). Antibody production appears to be nongrowth associated kinetics, i.e., production continues even in the decline (death) phase (Hayter 1989; Agrawal et al. 1989; Goudar et al. 2005; Shirsat et al. 2014), negatively

“growth associated” kinetics, i.e., increase in productivity at reduced growth rate (Korke et al. 2004; Kumar et al. 2007), or a combination of the two (Frame and Hu 1991; Linardos et al. 1991; Miller et al. 1986a, b).

The CHO cell lines were ranked according to the magnitude of their respective cumulative times for viability, productivity, and specific productivity ($\text{Mab, } \mu\text{g}/\text{VCD} \times 10^6$) (Table 3). Cell line 47, ranked first, had the highest productivity ($1462.7 \text{ Mab, } \mu\text{g}/\text{ml}$) with a modest CCT ($30.45 \text{ VCD} \times 10^6/\text{ml}$), whereas cell line 160, ranked the last for productivity, had the highest CCT ($39.17 \text{ VCD} \times 10^6/\text{ml}$) but the lowest productivity ($302.2 \text{ Mab, } \mu\text{g}/\text{ml}$), thus confirming that the productivity is not always directly proportional to viability. The growth results also indicate that the cell lines reached only 55 % of their full potential, K_s (VCDs/ml), for growth because of the inhibitory effects of lactate and other adverse environmental factors.

The comparison of growth and productivity clearly indicates that higher CCT does not necessarily mean higher productivity, nor does highest cumulative time for productivity mean the best specific productivity ($\mu\text{g}/10^6\text{cells}/\text{day}$). Thus, cell line 47 was the best, followed by cell lines 76, 150, 38, 164, and 160 (Table 3).

Verhulst model and productivity

Describing the population of CHO cells in the exponential and decline (death) phases required two separate Verhulst models, whereas a single Verhulst exponential model can describe the productivity in batch and fed-batch cultures because productivity is asymptotic (Shirsat et al. 2014). The productivity profiles of all six cell lines indicated that they plateau once the full potential of productivity is reached i.e., K_x , the carrying capacity (Fig. 1b). Data published by

Table 3 Ranking of six cell lines according to growth and productivity

VCD viable cell count ($\text{VCD} \times 10^6/\text{ml}$), MAb monoclonal antibody ($\text{Mab, } \mu\text{g}/\text{ml}$), MAb/VCD ($\mu\text{g}/10^6\text{cells}$) ranking

Cell line	VCD-ranking	Mab-ranking	Mab/VCD/day-ranking
38	2	3	4
47	3	1	1
76	4	2	2
150	5	4	3
160	1	6	6
164	6	5	5

Goudar et al. (2005) show that, in the case of hybridoma cells, productivity increases exponentially (up to day 8), plateauing thereafter (days 8–11; cf. Figure 4[b], Goudar et al. 2005). On the other hand, the hybridoma population grows exponentially, peaks on the fifth day, and starts declining after that (cf. Figure 4[a], Goudar et al. 2005). Thus, the results (Goudar et al. 2005) show that productivity continues in the late exponential and early decline (death) phases, as found by Hayter (1989) and Agrawal et al. (1989). Therefore, proliferation control strategies are typically implemented in the middle or late phases of exponential growth for improving productivity and longevity (Kumar et al. 2007). However, uncontrolled proliferation beyond a certain desired cell density is also undesirable because depletion of nutrients and oxygen, as well as accumulation of toxic metabolites and cell death could lead to product degradation (Al-Rubeai and Singh 1998; Zeng et al. 1998; Zeng and Deckwer 1999). Hence, prior knowledge of the productivity potential as predicted by the Verhulst productivity model (Shirsat et al. 2014) would help to terminate the bioprocess once the potential is reached; thus, avoiding the late decline (death) phase. Incidentally, the specific productivity (q_p) has a profile almost identical to the growth curve (Figure 4[b], Goudar et al. 2005).

Mechanisms of protein (antibody) production, the Michaelis–Menten enzymatic equation, and the Verhulst productivity model

The ER is responsible for many homeostatic responses, including folding and maturation of newly synthesised secretory and transmembrane proteins (Kleizen and Braakman 2004). In addition, the ER is also the site for membrane and secreted protein biosynthesis (transcription; Malhotra and Kaufman 2011). Proteins are translocated into the ER lumen (the space inside the tubular structure) in an unfolded state and require protein chaperons and catalysts (of protein folding) to assist in proper folding (translation), which takes place in the GA (Hua and Graham 2009; Malhotra and Kaufman 2011). Thus, the formation of antibodies is an enzymatic reaction which can be described by the Michaelis–Menten equation. The productivity profile as described by the Verhulst model (Fig. 2) resembles the profile of Michaelis–Menten enzyme kinetics, i.e., they are both

asymptotic—after the initial exponential reaction rate, the curve plateaus as the substrate concentration nears zero. Therefore, the Verhulst logistic equation describing productivity is similar to the Michaelis–Menten enzyme kinetic equation.

Statistically significant models for productivity

The observed differences in productivity (Mab, $\mu\text{g/ml}$) and in the productivity rate ($\text{MAB}\mu\text{g/VCD} \times 10^6$) in the six cell lines (Table 2) could be due to the different underlying mechanisms in the protein secretion. Therefore, cytometric data for the six cell lines were modelled by stepwise multivariate regression analysis to develop statistically significant (explanatory) models for productivity, as described by Shirsat et al. (2013) (Tables 4 and 5).

The cell cycle phases have been linked with the proliferation rate (Al-Rubeai and Emery 1990; Cooper 2001; Jorgensen and Tyers 2004; Müller and Lasche 2004; Arakaki et al. 2006; Lee et al. 2007; Mitra et al. 2009) and the content of the GA (Wang and Seeman 2011) and ER (Rapoport et al. 2004; Plemper and Wolf 1999) and are considered connected to MAb productivity (Uchiyama et al. 1997; Uchiyama and Shioya 1999; Frykman and Srienc 2001). A detailed review is in Shirsat et al. (2013).

Kumar et al. (2007) have described several approaches for optimising cell cycle parameters. One of these approaches has involved cell cycle arrest in the G1 phase by lowering the temperature (Kumar et al. 2007), because several genes, such as those involved in ribosome biogenesis and translation, are expressed highly in the G1 phase (Al-Rubeai and

Fig. 2 Comparison of productivity potential K_x (max) predicted and actual

Table 4 Stochastic explanatory productivity models for the six cell lines

Cell line		R^2
38	$y = 3.70 - 0.020G1 - 0.037ER$	0.775
38	$y = 0.249 - 0.072G2 - 0.013ER$	0.923
38	$y = 1.305 + 0.087G2 - 0.010ER - 0.015Mito$	0.955
47	$y = 3.246 + 0.005G1 - 0.024ER$	0.761
47	$y = 4.979 - 0.054G2 - 0.024ER$	0.923
47	$y = 4.929 - 0.054G2 - 0.024ER + 0.0000Mito$	0.923
76*	$y = 6.752 - 0.043G1 - 0.047ER$	0.882
76	$y = 3.150 + 0.030G2 - 0.035ER$	0.863
76	$y = 1.362 - 0.024G2 - 0.026ER + 0.024Mito$	0.959
150**	$y = 1.617 + 0.007G1 - 0.028ER$	0.742
150	$y = 3.988 - 0.045G2 - 0.023ER$	0.923
150	$y = 2.755 + 0.043G2 - 0.026ER + 0.014Mito$	0.935
160	$y = 1.617 + 0.007G1 - 0.028ER$	0.742
160	$y = 2.696 - 0.028G2 - 0.031ER$	0.763
160***	$y = 2.160 - 0.028G2 - 0.031ER + 0.006Mito$	0.763
164	$y = 5.112 - 0.038G1 - 0.038ER$	0.924
164	$y = 1.570 + 0.046G2 - 0.033ER$	0.898
164	$y = 6.049 - 0.044G2 - 0.039ER - 0.005Mito$	0.925

These models are all statistically significant models based on the level of significance of the variable

* Not valid because $G1 \approx ER = 0.94$

** Not valid because $G1 \approx ER = 0.90$

Colinearity; *** Model is not statistically significant

G1, *S*, *G2* cell cycle phases, *ER* endoplasmic reticulum, *Mito* mitochondrial membrane; and potential/mitochondrial mass (MMP/MM ratio), R^2 goodness of fit

Table 5 Comparison of selected stochastic explanatory models for the six cell lines

Cell line	Model	R^2	Mab $\mu\text{g/ml}$
38	$y = 1.305 + 0.087G2 - 0.010ER - 0.015Mito$	0.955	1179.4 (3)
47	$y = 4.979 - 0.054G2 - 0.024ER$	0.923	1462.7 (1)
76	$y = 1.362 - 0.024G2 - 0.026ER + 0.024Mito$	0.959	1312.5 (2)
150	$y = 3.988 - 0.045G2 - 0.023ER$	0.923	1021.6 (4)
160	$y = 2.696 - 0.028G1 - 0.031ER$	0.742	302.2 (6)
164	$y = 6.049 - 0.044G2 - 0.039ER - 0.005Mito$	0.925	755.2 (5)

G1, *S*, *G2* cell cycle phases, *ER* endoplasmic reticulum, *Mito* mitochondrial membrane; potential/mitochondrial mass (MMP/MM ratio), R^2 goodness of fit

Emery 1990; Al-Rubeai et al. 1992; Fussengger et al. 2000; Kaufmann et al. 2001; Ibarra et al. 2003; Bi et al. 2004; Trummer et al. 2006).

A cell, in reality, is a “biological machine” equipped with all the necessary machinery (enzymes, transporters, pumps, etc.) to perform the necessary tasks of making large quantities of complex glycosylated proteins to produce a desired product, like MAb (Thomas 1990). However, it has been argued that introducing powerful expression vectors into mammalian cells and amplifying gene copies is of little use unless the cells possess the necessary internal machinery to process the message and make large quantities of the desired product (Thomas 1990).

The data for cell cycle phases and organelles are characterised by different cellular activities, morphologies, and even mechanical properties (Mitchison 1971; Needham et al. 1990; Ramirez and Mutharasan 1990; Henderson et al. 1992). Thus, cytometric data are ideally suited for developing a segregated model for mammalian cells because it is a true representation of cellular physiology. The previous study (Shirsat et al. 2013) has shown that the measurable state variables, like cell cycle phases, and organelles are the best indicators of growth flux and productivity, embodying the performance of cell physiology and, by implication, cell machinery. Therefore, flow cytometry, which describes cell machinery, is ideally

suites for the optimisation of cell culture for productivity.

Cytometric data for the six cell lines were analysed by a stepwise multivariate regression method (Shirsat et al. 2013) to determine which of the cycle phases and organelles were causatively related to the MAb productivity (Table 4). The models with the minimum number of state variables with the best “goodness of fit” (R^2) with minimum state variables (Chatterjee and Price 1991) were chosen for comparative study.

The modelling results indicate that the underlying mechanism of productivity is specific to the cell line, even though all six cell lines were cloned from the same parent CHO cells (Table 4). The similarities and differences in their underlying mechanisms of productivity are discussed below.

Biological significance of the explanatory models

The cytometric data from the six cell lines were modelled using regression methods (Shirsat et al. 2013) to develop the explanatory models presented in Table 4.

Multivariate analysis of cytometric data indicated that G2 and ER were causatively linked to MAb productivity in cell lines 47 and 150 ($R^2 \approx 0.92$), because inclusion of mitochondria made no or slight difference in R^2 ; G2, ER, and mitochondria were involved in MAb productivity in cell lines 38, 76, and 164 ($R^2 \approx 0.96$); in cell line 160 ($R^2 \approx 0.76$), no clear link between MAb productivity and (G1 + ER) or (G2 + ER) was found, and there was no involvement of mitochondria. Therefore, the explanatory models selected for each cell line in Table 4 were used to compare their performances with reference to the cell cycle phases and organelles (Table 5).

The CVT (cumulative variable time) of the independent state variables for the six cell lines over 8 days were computed using Simpson’s Rule (Table 6). Since G2 and ER were causatively linked to MAb productivity in cell lines 47 and 150, the CVTs of the two cell lines were compared. The differences in the cell times for G2 of 47 and 150 were marginal (228.8 and 215.4, respectively) and not statistically significant. However, the differences in their cumulative times for ER, which were substantially different (472.4 and 335.7, respectively) and statistically significant led to higher productivity in cell line 47 (1462.7 Mab $\mu\text{g/ml}$) than in cell line 150 (1021.6 Mab $\mu\text{g/ml}$). In fact, their productivity ratio was the same as

their cumulative ER times i.e., approximately 1.4. Similarly, the comparison of cell lines 76 and 164, which had the same model parameter (G2 + ER + MMP/MM) and comparable cumulative MMP/MM times, indicated that their differences in productivity (411.8 and 264.4, respectively) could have been entirely due to the differences in their ER (1312.2 and 755.2, respectively)—i.e., their productivity ratio (1.73) is of a similar order as their ER ratio (about 1.58). Thus, their productivity is directly proportional to their ER content i.e., ER content is a good index for MAb productivity in CHO cell lines.

However, cell lines 38 and 76 had the same underlying mechanism, yet they had considerably different cumulative times for ER [208.2/411.8 (approximately 1:2), respectively], which do not reflect differences in their MAb productivity (1179.4 and 1312.5 Mab $\mu\text{g/ml}$, respectively, i.e., 0.90:1). Therefore, other factors must be at work, which could explain the efficient conversion of ER into productivity in cell line 38. One explanation could be longevity, which was found to directly relate to productivity—i.e., increased longevity led to increased productivity (Kumar et al. 2007). Cell line 38 had a CCT value of 35.22, considerably higher than the CCT of 28.3 for cell line 76 (i.e., approximately 24 %). Secondly, as mentioned before, the ER is the site for synthesising proteins (antibody) (Malhotra and Kaufman 2011), and MMP/MM is needed to provide energy via ATP for protein synthesis (Thomas 1990). In the stochastic productivity models for cell line 38 and 76, mitochondria were a statistically significant component. The results indicated that the productivity ratio (about 0.9) of the two cell lines—76 and 38—had the same proportion as the ratio of their cumulative times for MMP/MM (879.3 and 820.6, respectively, i.e., about 0.9). Thus, the cumulative times for mitochondria can explain the productivity differences between the two cell lines. Thirdly, the previous study (Shirsat et al. 2013) showed that the cell arrest in the G1 phase is detrimental to both growth and productivity. Differences in the percentage of G1 cell time between cell lines 38 and 76 (338 and 449, respectively) were considerable, resulting in lower CCT (longevity) and inefficient conversion of ER into productivity in cell line 76. The same explanations apply for the considerable productivity differences between cell lines 38 (1179.4) and 160 (755.2), even though cell line 38 had much lower ER content than 160 (208.2 and 264.4, respectively).

Table 6 Comparison of cumulative *variable* times (CVT) for six cell lines

Cell line	G1 phase	S phase	G2 phase	ER	MMP/MM	GA	Mab $\mu\text{g/ml}$
38	338.0 (6)	238.7	222.6 (3)	208.2 (5)	820.6 (4)	111.7	1179.4 (3)
47	369.7 (5)	341.3	228.8 (1)	472.4 (1)	902.0 (1)	134.8	1462.7 (1)
76	449.4 (2)	131.1	226.0 (2)	411.8 (2)	879.3 (2)	124.1	1312.5 (2)
150	416.5 (3)	168.3	215.4 (5)	335.7 (3)	779.5 (5)	86.9	1021.6 (4)
160	401.5 (4)	212.4	186.0 (6)	180.4 (6)	676.0 (6)	72.5	302.2 (6)
164	454.5 (1)	130.0	215.9 (4)	264.4 (4)	836.4 (3)	114.1	755.2 (5)

V variable, G1 S, G2 phases, ER endoplasmic reticulum, MMP/MM mitochondrial membrane potential/mitochondrial mass, GA Golgi apparatus, MAb monoclonal antibody

Incidentally, in all three lines (38, 76, and 164), the cumulative times for the G1 phase proved to be good indicators of cumulative cell time (CCT)—i.e., the higher the G1 contents (338.0, 449.4, and 454.5, respectively), the lower the CCT (35.217, 28.2, and 25.97, respectively). Similarly, cell lines 47 and 150 also indicated that the higher the cumulative G1 times (370.0 and 417.5, respectively), the lower the CCT (30.55 and 26.42, respectively). Thus, the cumulative G1 phase time is a growth indicator, the same as r , the intrinsic growth factor in the Verhulst logistic growth model, and CCT is equal to K, the carrying capacity.

The foregoing discussion makes it evident that the cumulative ER time (in conjunction with G2) is the main indicator of MAb productivity in CHO cell lines. Cumulative ER time alone would not correlate to result in productivity unless the cells possessed the necessary internal machinery, such as enzymes (Thomas 1990) for converting ER into MAb. Productivity did not appear to be directly proportional to biomass, but viability (i.e., CCT) played a vital role in productivity (Kumar et al. 2007), because productivity continued in the decline (death) phase (Hayter 1989; Agrawal et al. 1989). The G1 cell phase was a good indicator of CCT. These deductions from the stochastic models and CVT can shed light on the underlying mechanism of MAb productivity in CHO cell lines and, as such, could provide useful guidance to geneticists engaged in developing recombinant mammalian cells for producing antibody.

Glucose consumption and lactate production

Since glucose consumption and lactate production directly impacted growth and indirectly affected

productivity, cumulative times for them were computed by Simpson's Rule (Eq. 6), as shown in Table 7.

Cell line 47, with the best productivity (1462.7), had the lowest cumulative consumption of glucose (20.41) and the least cumulative production of lactate (97.75). In contrast, cell line 160, with the worst productivity (302.2), had the highest cumulative production of lactate (121.16) as well as the highest cumulative cell time (CCT = 39.17). These findings suggest that the dominance of glycolysis (i.e., glucose consumption), associated with proliferation and lactate production, adversely affected productivity. A reverse phenomenon occurs during metabolic shift, when cells start consuming lactate instead of glucose (Shirsat et al. 2014)—i.e., from proliferation to protein synthesis (Korke et al. 2004; Kumar et al. 2007).

Comparison of productivity performance (key parameters of the six CHO cell lines)

Several key parameters were considered in selecting the cell line for large-scale production (Li et al. 2010). Conventionally, the cell lines were ranked according to their specific productivity, based on the cell

Table 7 Cumulative variable times (CVT) where V, variable = glucose (mM/ml-8 days) and lactate (mM/ml-8 days)

Cell line	Glucose consumption	Lactate production
38	20.93	109.15
47	20.41	97.75
76	23.25	115.36
150	21.36	113.68
160	21.37	121.16
164	22.34	108.67

Table 8 Summary of results

Cell line	38	47	76	150	160	164
Viability	35.22 (2)	30.55 (3)	28.30 (4)	26.42 (5)	39.17 (1)	25.97 (6)
Productivity (total)	1179.4 (3)	1462.7 (1)	1312.5 (2)	1021.6 (4)	302.2 (6)	755.2 (5)
Productivity (max)	386 (3)	478 (1)	438 (2)	344 (4)	78 (6)	247 (5)
Specific productivity	4.19 (4)	5.99 (1)	5.78 (2)	4.84 (3)	0.96 (6)	3.64 (5)
Volume. productivity	147.4 (3)	182.8 (1)	164.7 (2)	127.7 (4)	37.8 (6)	94.4 (5)
Productivity efficiency	33.49 (4)	47.88 (1)	46.37 (2)	38.67 (3)	7.72 (6)	29.08 (5)
Glucose	20.93 (2)	20.41 (1)	23.25 (6)	21.36 (3)	21.37 (4)	22.34 (5)
Lactate	109.1 (3)	97.3 (1)	115.4 (5)	113.7 (4)	121.2 (6)	108.8 (2)

Cell line—Recombinant CHO-cell line, Viability—VCD $\times 10^6$ /ml-8 days, Productivity (total)—Mab, $\mu\text{g}/\text{ml}$ -8 days, Productivity (max)—Mab, $\mu\text{g}/\text{ml}$, Specific productivity—Mab $\mu\text{g}/\text{cells} \times 10^6$ -day, volumetric productivity (Mab $\mu\text{g}/\text{ml}/\text{day}$), Productivity efficiency, CPT/CCT—Mab $\mu\text{g}/\text{cells} \times 10^6$, glucose (mM/ml-8 days), and lactate (mM/ml-8 days)

population, i.e., μg or $\text{pg}/\text{cell}/\text{day}$. However, MAb productivity in the CHO-lines was not directly proportional to the cell mass, and it also continued in the decline (death) phase. Therefore, the CHO cell lines were ranked according to their volumetric specific productivity (μg or $\text{pg}/\text{ml}/\text{day}$), as well as by their cumulative productivity time/cumulative cell (viable) time (i.e., μg or $\text{pg}/\text{cells} \times 10^6$ -day), as shown in Table 8. The productivity performance of mammalian cells is adversely affected by lactic acid, a metabolic endpoint of glycolysis, because of osmolality (Li et al. 2010).

Table 8 sets out the cumulative times over 8 days for the key parameters: viability (VCD/ml), productivity (Mab $\mu\text{g}/\text{ml}$), productivity (max) (Mab $\mu\text{g}/\text{ml}$), specific productivity ($\mu\text{g}/10^6$ VCD/day), volumetric productivity (Mab $\mu\text{g}/\text{ml}/\text{day}$), glucose (mM/ml), and lactate (mM/ml).

The results indicate that cell line 47 was the best producer of MAb because it had the highest total productivity, maximum productivity, specific productivity, and volumetric productivity with the least amount of glucose consumption and lactate production. In contrast, cell line 160 had the highest lactate content and the worst productivity. The specific productivity ranking of cell line 38 changed (4) to (3) when volumetric productivity criterion was applied.

Conclusions

The underlying mechanisms of productivity are specific to the cell line, even if they are cloned from the same

parent cells. Therefore, the criteria for productivity performance should be reviewed in conjunction with the findings of the Verhulst and stochastic explanatory models for growth and productivity.

The Verhulst law for growth—“the intrinsic growth factor, r , is inversely proportional to growth”—applies only where the growth mechanism is the same. Thus, the intrinsic growth factor is cell specific. The results of the stochastic model for growth indicated that the cumulative G1 times of cell lines, similar to the Verhulst intrinsic growth factor, are “inversely proportional to growth” where the underlying mechanism for growth is the same—i.e., G1-cumulative times are cell specific. Thus, both r and G1 reflect the genetic makeup of the cell line.

However, the intrinsic productivity factor, rx , in the Verhulst productivity model is a good indicator of Kx , the productivity potential, across all the cell lines. Thus, rx is not cell specific. The Verhulst asymptotic models, describing the productivity of the CHO cell lines, are very similar to Michaelis–Menten enzymatic equation. This should be further investigated to establish if the conversion of unfolded proteins in the ER into folded proteins is purely enzymatic, requiring viable cells, but independent of other cellular activities related to growth. The statistically significant (i.e., explanatory) models can explain the productivity. However, in addition to the explanatory model parameters, the influence of other state variables on productivity should be considered in interpreting the relative productivity performance of the cell lines. Specific productivity is not directly proportional to total productivity.

If the selected cell line is destined for large fed-batch cultures, then the explanatory models should be based on the data derived from the bioprocesses, replicating the medium composition and feeding strategy to be employed in large-scale production. A previous study (Shirsat et al. 2013) has shown that the batch and fed-batch modes differ in their underlying mechanisms of growth and productivity. Thus, the enhancement of productivity is achieved not only by selecting highly productive cell lines but also by optimising culture medium composition and bioreactor operation conditions (Li et al. 2010). The present study shows that flow cytometry could be of immense value in optimising cell cultures with respect to the extracellular variables (media composition and growth environment) but also in evaluating the performance of recombinant mammalian cells.

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