ORIGINAL RESEARCH

Enhanced recombinant M-CSF production in CHO cells by glycerol addition: model and validation

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Abstract Addition of stimulatory chemical such as glycerol was found to increase recombinant protein production in Chinese hamster ovary (CHO) cells. However, glycerol influenced cell mitosis and reduced cell growth rate. We developed a controlled proliferation strategy to utilize the stimulation of glycerol on recombinant protein production and mitigate the problem of growth inhibition. The approach is to apply a two-stage process, where cells are cultured without glycerol for a period of time in order to obtain enough cell density and then glycerol is added to achieve high specific productivity. In addition, a model for predicting the profiles of cell proliferation and recombinant protein production was developed and validated. A two-stage process, addition of 1% glycerol after 1 day of growth, could increase the final production of macrophage-colony stimulating factor (M-CSF) by 38% compared with the value obtained without addition of glycerol.

Keywords CHO cells · Controlled proliferation · Glycerol · M-CSF · Model · Optimization

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Introduction

Chinese hamster ovary (CHO) cells are used extensively for the production of recombinant proteins. However, the productivity of mammalian cells is low as compared with that of prokaryotic hosts, which increases the cost of recombinant protein production and purification. Strategies have been developed in order to increase recombinant protein production in CHO cells, such as the regulation of culture temperature (Fox et al. 2004), the addition of chemicals (Kim et al. 2005; Rodriguez et al. 2005), bioreactor engineering (Nienow 2006), and inhibition of cell apoptosis (Arden and Betenbaugh 2006). For example, Fox et al. (2004) used a biphasic temperature process to maximize the recombinant protein, i.e. cells are grown at high temperature for a certain period of time to obtain a suitable cell density, followed by shifting the culture to a reduced temperature, where the specific productivity is higher, for the remaining period. Besides, these authors also developed a simple and useful framework by using the Monod model to predict the cell proliferation and recombinant protein production under different temperature conditions.

Glycerol promotes cystic fibrosis trans-membrane conductance regulator protein folding and enhances its synthesis in NIH 3T3 cells (Brown et al. 1996). Additionally, glycerol aids the formation of a solvent shell around a protein molecule as a protein stabilizer, and increases the viscosity of a solution for

prevention of protein association (Fagain 1997; Mishra et al. 2005). Rodriguez et al. (2005) also reported that glycerol could stabilize the secreted β-interferon by CHO cells and result in reduced aggregation. Despite the recognized value of glycerol on recombinant proteins stability and production described above, there are no attempts to optimize the time point at which glycerol is added. The aim of this study was to construct and validate a model for the prediction of cell proliferation and recombinant protein production after glycerol addition. Effects of glycerol on the specific protein productivity and cell proliferation were evaluated using a recombinant CHO cell line, which could secret recombinant macrophage colonystimulating factor (M-CSF). Additionally, the Monod-model-based framework was developed to predict the trends of cell proliferation and M-CSF production for batch cultures with glycerol addition at day 0, 1, 2, 3, or 4 following inoculation. From these predicted results, the optimal time point at which to add glycerol was decided by comparing the maximal M-CSF production. Moreover, M-CSF is a multiple-function cytokine not only for hemopoietic system, but also for non-hemopoietic systems, such as the breast function, pregnancy and osteoclastogenesis. M-CSF also acts as the principal regulator of the survival, proliferation, and differentiation of macrophages and their precursors (Li et al. 2002).

Materials and methods

Cell line and cell culture

Genetically engineered CHO cells, obtained from ATCC (CRL-10154), were used to express M-CSF under the pressure of 1 μM methotrexate. The cells were initially cultivated in DMEM/F12 supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), and adapted to grow in a serum-free HyQ SFM4 medium (Hyclone) in 120 mL Erlenmeyer flasks with 100 rpm rotation. For every tested condition, two separate experiments were conducted and each experiment was repeated in triplicate in 12-well tissue culture plates (BD Biosciences, Rockville, MD, USA). Therefore, total six repeats were performed for every condition. The standard deviation and coefficient of variance are calculated using

the six repeats. The culture was inoculated at a concentration of 5×10^5 cells mL^{-1} and harvested after 120 h of cultivation. The viability was determined by the trypan blue exclusion method and the cell concentration was determined by Multisizer 3 (Beckman Counter, Beds, England, UK). Aliquots of spent media were taken and kept frozen at $-20\,°C$ for further ELISA analysis.

A set of experiments were performed with or without the glycerol addition at the beginning of the culture and were used to generate data for the model development (Model construction). Following this first set of cultures, the glycerol addition experiments at specified times were performed for validating the model (Model validation). For these experiments, the cells were supplied with 1% glycerol at the modeled times, which were 1, 2, 3, or 4 days following inoculation. The stock solution of glycerol is 50% (v/v in water) and is sterilized by autoclave. Error bars on figures are one standard deviation (SD).

The concentration of M-CSF in media samples after appropriate dilution was detected by use of ELISA kit (DuoSet DY216; R&D systems, Minneapolis, MN, USA). A solution of $3,3',5,5'$- tetramethyl-benzidine and H_2O_2 (Kirkeguard & Perry Laboratories Inc., Gaithersburg, MD, USA) was added as the peroxidase substrate. After 20-min incubation, 2N H_2SO_4 solution was added to stop the reaction and the plates were measured at a wavelength of 450 nm. Each sample was tested in duplicate to reduce the uncertainty of ELISA measurement. For the assay of M-CSF aggregation in culture media, some harvested media samples (100 μL) were denatured by boiling with 1 μL of 10% SDS and 1 μL of 2-mercaptoethanol for 3 min (Rodriguez et al. 2005). The M-CSF content was then determined by ELISA assay as described above.

Modeling

The goal of this model is to predict time profiles of cell proliferation, glucose consumption, and M-CSF production after glycerol addition at day 0, 1, 2, 3, or 4 following inoculation. From these predicted results, the optimal time point at which to add glycerol was decided by comparing the maximal M-CSF production. Experiments of cultures without glycerol (as a control) and with glycerol supplement at day 0 were conducted to obtain the kinetic parameters needed for

the model, namely, specific growth rate (μ), specific glucose consumption rate (qs), and specific M-CSF production rate ($q_{M\text{-}CSF}$). The model consists of batch balance equations on cell proliferation, glucose concentration, and M-CSF production (Eqs. 1–3). In addition, the Monod model was adopted to predict specific growth rate (Eq. 4) and specific glucose consumption rate (Eq. 5):

$$\frac{dX}{dt} = \mu.X \tag{1}$$

$$\frac{dS}{dt} = -q_s.X \tag{2}$$

$$\frac{dP}{dt} = q_{M-CSF}.X \tag{3}$$

$$\mu = \frac{\mu_{MAX}.S}{K + S} \tag{4}$$

$$qs = \frac{q_{MAX}.S}{Ks + S} \tag{5}$$

Parameters in Eqs. (4–5) were determined by the hyperbola-mode regression of data from control and glycerol supplement cultures using the Sigmaplot package (Systat Co., Point Richmond, USA). The assumptions used in this model include that glucose (S) is the rate-limiting substrate in the batch culture of CHO cells and the Monod equations is valid for predicting specific growth rate and specific glucose consumption rate. The model also assumes that model parameters (μmax, K, qmax, Ks, and $q_{M\text{-}CSF}$) changed instantaneously from the time of glycerol addition. This instantaneous change is assumed for the reason of simplicity of modeling. The specific M-CSF productivity ($q_{M\text{-}CSF}$) was assumed to be a time–invariant constant and could be determined by the slope of cumulative M-CSF concentration vs. the integral of viable cell density (IVCD) (Fox et al. 2004). IVCD is the result of integration of Eq. 1. These values of model parameters were simultaneously changed from values of control culture to those of glycerol-supplement culture after the addition of glycerol. Glycerol addition at day 1, 2, 3, or 4

post-inoculation was performed and evaluated. The optimal time for glycerol addition is determined by comparing peak profiles of M-CSF production at the specified time of glycerol addition. The ordinary differential equations (Eqs. 1–3) combined with the above assumptions, were solved by a built-in function "NDSolve" in the *Mathematica* package (Wolfram Research Inc. Champaign, IL, USA) to obtain time profiles of cell, glucose, and M-CSF. NDSolve can find numerical solutions to a wide range of ordinary and partial differential equations using the given initial and boundary conditions. This software iteratively solves these differential equations by applying a multi-step method, which starting at a particular value of independent variable and using an adaptive procedure to determine the size of these steps. If the solution appears to be varying rapidly in a particular region, the package reduces the step size so as to be able to track the solution better. Eventually, the solutions covering the whole range of independent variable are found.

Results and discussion

Effects of glycerol

The influence of different concentrations of glycerol on M-CSF production and CHO cell proliferation is demonstrated in Fig. 1. Glycerol inhibited cell proliferation as it increased from 1.0 to 2.0%.
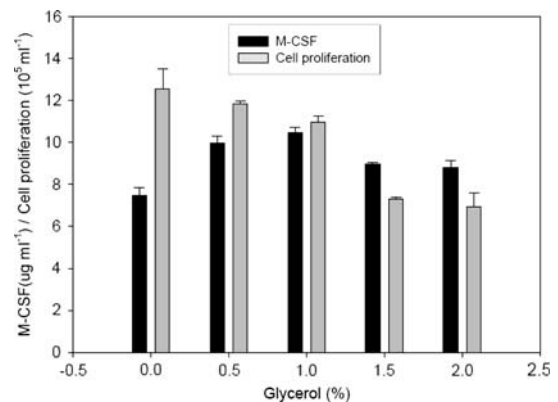

Fig. 1 Effects of glycerol concentration on CHO cell proliferation and M-CSF production. The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates). Error bars in the figure stand for one standard deviation (SD)

Glycerol at the range of 0.5 to 2.0% could stimulate M-CSF production as compared with that of control culture. However, more than 1.0% glycerol addition significantly repressed CHO cell proliferation. Brown et al. firstly observed that glycerol acts as a chemical chaperone and promotes recombinant protein synthesis in NIH 3T3 cells (Brown et al. 1996). Glycerol was also known to stabilize proteins against chemical and thermal denaturation (Timasheff 2002). Additionally, glycerol has been used to increase the refolding yield of proteins such as citrate synthase, thiosulfate sulfurtransferase, pancreatic elastase, and malate dehydrogenases in the in-vitro studies (Gorovits et al. 1998; Jaspard 2000; Mishra et al. 2005; Tieman et al. 2001). Glycerol also prevents human β-interferon aggregation during synthesis but does not dissociate pre-formed aggregates in CHO cells (Rodriguez et al. 2005). However, M-CSF produced by CHO cells did not have the aggregation problem as proved by no increase in M-CSF titer after the treatment of samples with reducing chemicals such as SDS and 2-mercaptoethanol (data not shown). Cell proliferation was not significantly different between the control culture and 1% glycerol-supplement culture in their research (Rodriguez et al. 2005). The inhibition threshold of 1.5% glycerol on the interferon-β producing CHO cells was different from the threshold of 1.0% glycerol on the M-CSF producing CHO cells in this study (Fig. 1). Therefore, the inhibition threshold of glycerol on cell proliferation seemed to be strain-dependent. The causes of proliferation inhibition by glycerol were studied by using nuclei stains. We found the binucleated cells in our glycerol-supplement cells after 24-h culture but not at any time in the control culture (photos not shown). Additionally, the growth arrest and an increase in specific recombinant protein productivity have some connection. The productivity is likely enhanced because growth-arrested cells do not need to devote cellular resources to biomass production (Fussenegger and Bailey 1999). As demonstrated in Fig. 1, the addition of 1% glycerol had the highest stimulatory effect on M-CSF production. Therefore, the concentration of glycerol was decided to be 1% for the following experiments. Additionally, a two-stage process was developed in the next sections to maximize the production of M-CSF by CHO cells. The strategy is to culture the cells without glycerol for a period of time in order to obtain enough cell density and then glycerol is added to achieve high specific productivity.

Model construction

Control culture (no glycerol addition) and culture with 1% glycerol supplement at day 0 were cultivated to obtain the cell proliferation, M-CSF production, and glucose consumption data. As demonstrated in Fig. 2, the addition of glycerol could increase the M-CSF production but inhibit the cell proliferation. Additionally, the glucose consumption of glycerol supplement culture was slower than that of control culture. The batch data of cell proliferation and glucose consumption for the culture with or without glycerol addition were further adopted to get the Monod kinetic parameters of specific growth rate (μ) and specific glucose consumption rate (qs) using the following equations, where x_1 represents the cell concentration at time t_1, and g_1 represents the glucose concentration at time t_1.

$$\mu = (x_2 - x_1)/(t_2 - t_1)/((x_1 + x_2)/2)$$

$$qs = (g_2 - g_1)/(t_2 - t_1)/((x_1 + x_2)/2)$$

The Monod equation is the most commonly applied unstructured, non-segregated model of microbial growth. This equation can describe substrate-limited growth when growth is slow and cell
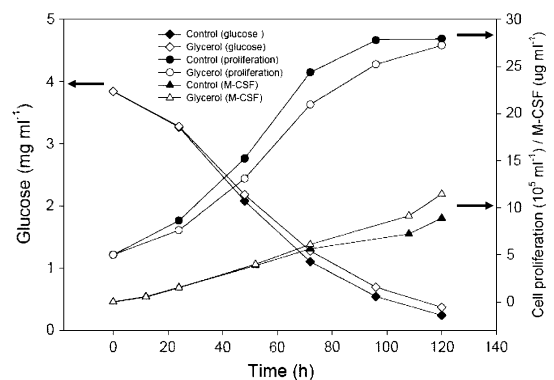

Fig. 2 Time course of cell proliferation, M-CSF, and glucose consumption for CHO cells. Control culture and culture with 1% glycerol supplement at day 0 were cultivated to obtain these data. The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates) and the coefficient of variance is less than 10%

concentration is low. Under these conditions, the release of toxic waste is slow. The growth rate can be related simply to the limiting substrate (Shuler and Kargi 2002). The experimental results and predictions by Eqs. (4–5) are shown in Fig. 3. As demonstrated in Fig. 3(a, b), the Monod equation (Eq. 4) could fit the experimental data of glucose concentration and specific growth rate. The r^2 values of specific growth rate regression for control and glycerol-supplement culture are 0.955 and 0.946, respectively. Whereas, the specific glucose consumption rate was found to follow the proposed Monod equation (Eq. 5) under both culture conditions (Fig. 3c, d). The regression error of the specific glucose consumption rate was relatively small as compared with that of the specific growth rate (Fig. 3c, d). The r^2 values for the regression of the specific glucose consumption rate in control and glycerol-supplement culture are 0.987 and 0.978, respectively. However, the estimation deviation in specific growth rate and specific glucose consumption rate would cause the prediction errors of cell proliferation and M-CSF production.

The specific M-CSF productivity (q_{M-CSF}) was determined by the slope of cumulative M-CSF concentration vs. the integral of viable cell density as described in the paper (Fox et al. 2004) The CHO cell line exhibits an 1.4-fold increase in specific M-CSF productivity under the glycerol-supplement condition (1.512 pg M-CSF cell^{-1} day^{-1}) vs. control culture (1.080 pg M-CSF cell^{-1} day^{-1}) as shown in Fig. 4. Nevertheless, the linear correlation between cumulative M-CSF concentration and the integral of viable cell density is not very well as indicated in the values of r^2 for control culture (0.775) and for glycerol-supplement culture (0.918). The kinetic parameters regressed from experimental data of control and glycerol addition cultures are summarized in Table 1. The inhibitory effect on cell proliferation by addition of glycerol was obvious as demonstrated in the values of maximum specific growth rate (μ_{max}). The μ_{max} of control culture is 1.30-fold of that of glycerol-supplement culture (0.922 vs. 0.710 day^{-1}). Besides, the maximum specific glucose consumption rate (q_{max}) of control culture is 1.57-fold of that of glycerol-supplement
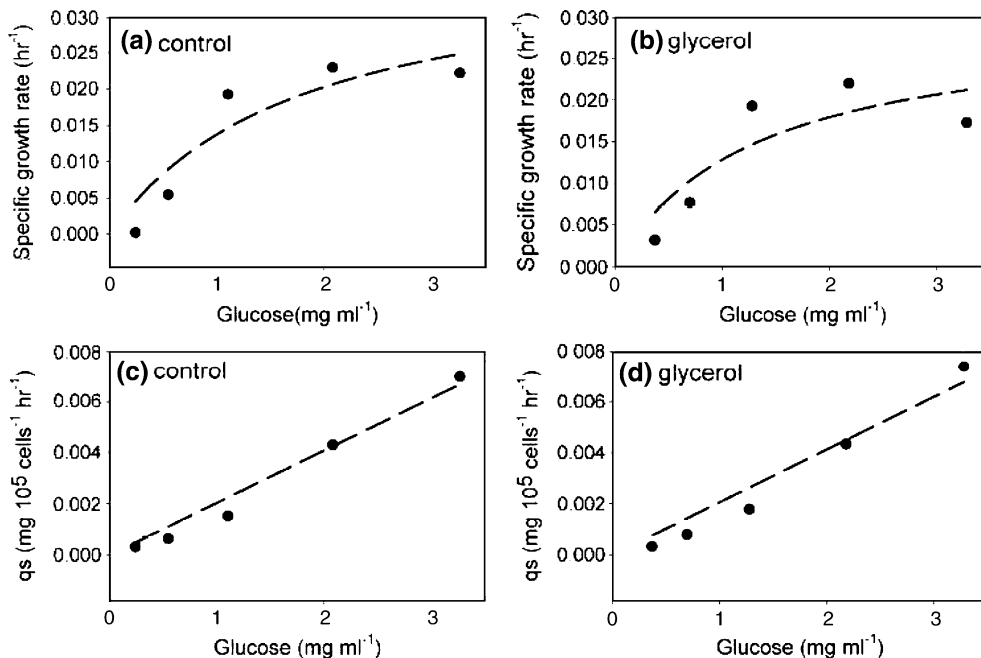

Fig. 3 Specific growth rate (a, b) and specific glucose consumption rate (c, d) as a function of glucose concentration. The specific growth rate and glucose consumption rate were obtained from data of Fig. 2. The glycerol was added at the concentration of 1%. The dashed lines were determined by using the hyperbola regression in the Sigmaplot package. The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates) and the coefficient of variance is less than 10%
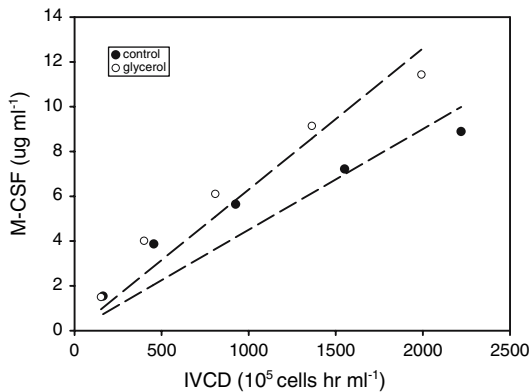
Fig. 4 M-CSF production as a function of the integral of viable cell density (IVCD). Average specific production rate, $q_{M\text{-}CSF}$, is the slope of the least–squared linear fit for experimental data from cultures without glycerol (control) and with glycerol supplement. The glycerol was added at the concentration of 1%. The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates) and the coefficient of variance is less than 10%

Table 1 Kinetic parameters for the proposed model

Parameters	Control culture	Glycerol-added culture
μmax (1 h^{-1})	0.0384	0.0296
K (mg mL^{-1})	1.7871	1.2965
$q$max (mg cell^{-1} h^{-1})	1792	1138
Ks (mg mL^{-1})	875925	550810
qM-CSF (μg cell^{-1} h^{-1})	4.5×10^{-8}	6.3×10^{-8}

culture (0.430 vs. 0.273 mg cell^{-1} day^{-1}). From these kinetic results, the effects of glycerol on stimulation of recombinant M-CSF and reduction of growth rate were confirmed again. Furthermore, the ''NDSolve'' in the *Mathematica* package in conjunction with the kinetic parameters in Table 1 was used to solve the ordinary differential equations (Eqs. 1–3) and to predict the trends of M-CSF production, glucose concentration, and cell proliferation in the following section. In contrast to the Microsoft Excel program used by Fox et al. (2004), this study adopted the *Mathematica* package to find the solutions to the ordinary different equations (Eqs. 1–3).

Model validation

The model was validated by comparing the experimental data and prediction profiles for cell

proliferation and M-CSF production. The experimental and predicted profiles of cell proliferation and M-CSF production for cultures with glycerol supplemented at the specified time were shown in Fig. 5. The difference between the values of model prediction and experimental data was quite small when glycerol added at day 1, 2, 3, and 4. The agreement on prediction and the experimental results suggests that the assumptions used in this model are reasonable. Additionally, this model can predict the general trends of cell proliferation and M-CSF production in CHO cells. From Fig. 5, the maximal M-CSF production (12.25 μg mL^{-1}) is obtained by adding glycerol at day 1.

In a batch culture, the cell population includes individual cells that may differ from each other. A cell can be further divided into different components that account for respiration, proliferation, and protein synthesis. Therefore, a structured and segregated model can realistically represent the growth kinetics of cells (Shuler and Kargi 2002). However, the computation of structured and segregated model is very complex. In this study, we describe the CHO cell proliferation by using the Monod model, which is a unstructured and non-segregated model often used to describe the growth kinetics for different micro-organisms. Similar assumptions were used by Fox et al. (2004) to simplify the calculation, i.e. the kinetic parameters were assumed to be constant and independent of the time of temperature shift or glycerol addition. However, the Monod model could well predict the growth kinetics of CHO cells in Fox's study and our case.

The model could be further validated by comparing its ability to predict final cell concentration and M-CSF production at the end of a 5-day batch culture. Table 2 shows the predicted vs. experimental values for maximal cell density and M-CSF production. The prediction by the model matches the experimental M-CSF production and cell proliferation. The comparison of peak profiles of M-CSF prediction pointed out that glycerol addition at day 1 resulted in a maximum M-CSF production for a 5-day batch culture (Table 2). The optimal glycerol addition day, namely, day 1, was demonstrated in Fig. 5 and Table 2. The final production titer of M-CSF with glycerol addition at day 1 is increased by 38% over the value obtained in culture without the addition of glycerol (12.25 vs. 8.89 μg mL^{-1}). Although glycerol
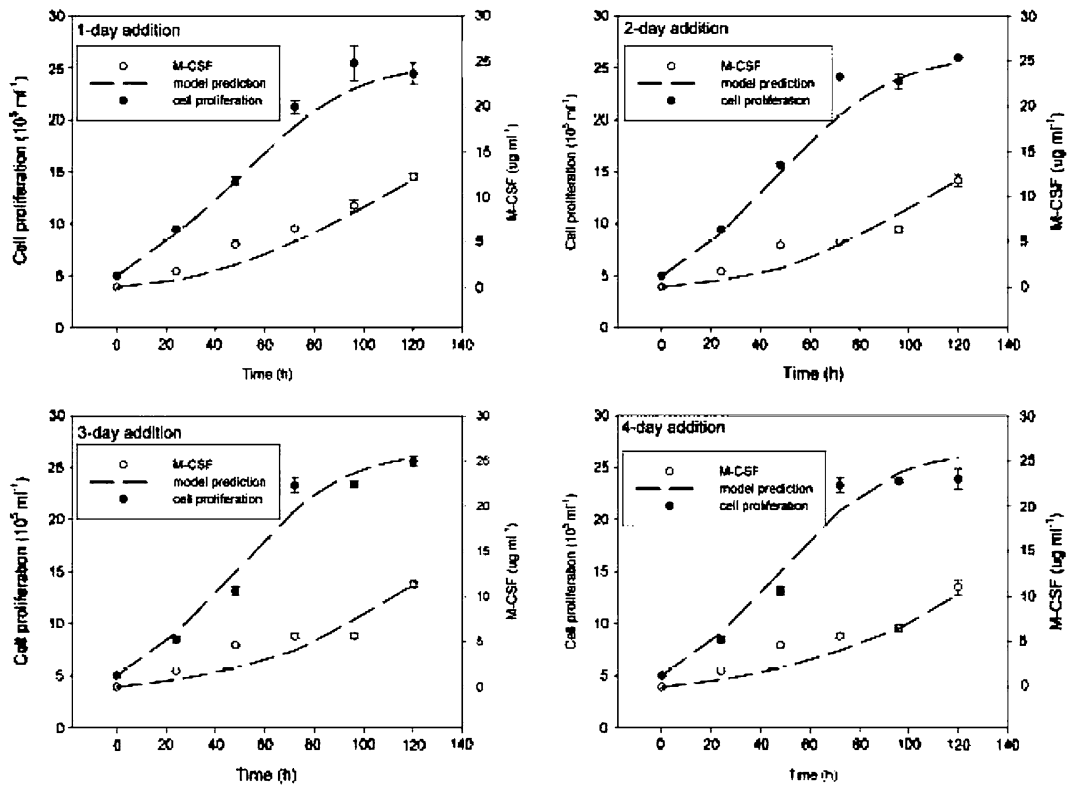
Fig. 5 Profiles of cell proliferation and M-CSF production for cultures with 1% glycerol added at day 1, 2, 3, and 4. The model (Eqs. 1–3) in conjunction with kinetic parameters in Table 1 was used to predict the profiles of M-CSF production and cell proliferation with the aid of the built-in function ''NDSolve'' in the *Mathematica* package. Model prediction (dash line) compared with actual cell proliferation (closed circle) and actual M-CSF (open circle). The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates). Error bars in the figure stand for one standard deviation (SD)

Table 2 CHO cell proliferation and M-CSF production at the end of 5-day batch culture[a]

Addition day of glycerol	M-CSF (μg mL^{-1})		Viable cells (10^5 mL^{-1})	
	Model prediction[b]	Experimental results	Model prediction[b]	Experimental results
Control	9.18	8.89 (0.39)	25.92	27.94 (1.39)
0	11.47	11.43 (0.49)	23.77	27.24 (0.41)
1	11.89	12.25 (0.33)	24.59	24.41 (1.04)
2	11.88	11.77 (0.69)	25.45	25.97 (0.02)
3	11.28	11.32 (0.37)	25.91	25.56 (0.49)
4	10.27	11.00 (0.86)	25.93	23.09 (1.00)

[a] The glycerol was added at the concentration of 1% at the specified day. Figures in parentheses stand for standard deviation. The results are an average of two separate experiments (each in triplicate in 12-well tissue culture plates).

[b] The model (Eqs. 1–3) in conjunction with kinetic parameters in Table 1 was used to predict the values of M-CSF production and cell proliferation with the aid of the built-in function ''NDSolve'' in the *Mathematica* package.

addition could improve final M-CSF titer, the strategy could be improved in some aspects. For example, the specific M-CSF productivity is assumed to be a constant and is influenced by the glycerol only. These simplified assumptions need to be validated when the model is applied to other controlled proliferation of

mammalian cells. Finally, the effects of glycerol addition on the quality and the purification of recombinant proteins merit more investigations.

In summary, we found that glycerol added in the culture medium can reduce the growth rate and increase the specific M-CSF productivity in CHO cells. A mathematical model to predict M-CSF production and cell proliferation was developed. As predicted by Fox et al. (2004), this modeling framework developed for temperature shift experiment is also applicable for optimizing controlled proliferation by glycerol. The agreement between the prediction and experimental data for the glycerol addition is quite satisfactory. Here glycerol addition successfully increase the final M-CSF production. By utilizing this strategy, final production titer of M-CSF is increased by 38% over the value obtained in culture without the addition of glycerol. This model has the potential to be applied to other controlled proliferation of mammalian cells using environmental, chemical or genetic means.

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