Published in final edited form as:

Biotechnol Prog. 2009 ; 25(6): 1650–1659. doi:10.1002/btpr.240.

Kinetic Simulation of a Centrifugal Bioreactor for High Population Density Hybridoma Culture

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Abstract

Demand for increasingly complex post-translationally modified proteins, such as monoclonal antibodies (mAbs), necessitates the use of mammalian hosts for production. The focus of this paper is a continuous centrifugal bioreactor (CCBR) capable of increasing volumetric productivity for mAb production through high density hybridoma culture, exceeding 10^8 cells/mL. At these extreme densities environmental conditions such as substrate and inhibitor concentrations rapidly change, dramatically affecting growth rate. The development of a kinetic model predicting glucose, mAb, lactate, and ammonium concentrations based on dilution rate and cell density is shown in this paper. Additionally, it is found that pH affects both growth rate and viability, and a range of 6.9 to 7.4 is needed to maintain growth rate above 90% of the maximum. Modeling shows that operating an 11.4 mL CCBR inoculated with 2.0×10^7 cells/mL at a dilution rate of 1.3 h^{-1} , results in a predicted growth rate 82% of the maximum value. At the same dilution rate increasing density to 6.0×10^7 cells/mL decreases the predicted growth rate to 60% of the maximum; however, by increasing dilution rate to 6.1 h^{-1} the growth rate can be increased to 86% of the maximum. Using the kinetic model developed in this research the concentration of glucose, mAb, lactate, and ammonium are all predicted within 13% of experimental results. This model and an understanding of how RPM impacts cell retention serve as valuable tools for maintaining high density CCBR cultures, ensuring maximum growth associated mAb production rates.

Keywords

High Population Density Bioreactor; Hybridoma; Kinetic Simulation; pH; Inhibition

Introduction

Increasing demand for recombinant proteins, particularly monoclonal antibodies (mAbs), for therapeutic and diagnostic needs has fueled research on novel bioreactors capable of increasing production without sacrificing quality [1-3]. Proteins requiring post-translational modifications such as glycosylation, phosphorylation, and folding demand the use of mammalian hosts, while production requirements exceeding grams per patient per year necessitate increased production capabilities [4-7]. Consequently, bioreactor systems including fed-batch and various perfusion systems designed to increase volumetric productivity may be used to meet these production needs [1,8,9].

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Many different techniques exist for protein production by mammalian cells, each with varying advantages and limitations. Batch cultures of hybridoma cells are used for *in vitro* production of mAbs, but low cell densities on the order of 10^6 cells/mL, and decreased viabilities limit productivity [10-12]. Continuous feed stirred tank reactor (CSTR) cultures can be used to maintain high viabilities but process densities are further limited to $2-3 \times 10^6$ cells/mL because of wash-out as cells are entrained in passing medium. Thus, cell retention devices must be employed to maintain high viability cultures as densities and medium flow rates proportionally increase. Densities can be increased as high as 20×10^6 cells/mL in CSTR culture if cells are separated from spent medium and returned to the culture through techniques such as centrifugation, membrane separation, or a vertical sedimentation column [13,14].

Cell immobilization can also be employed to prevent wash-out and is accomplished using hollow fiber bioreactors (HFBs) and packed bed bioreactors (PBRs). Both HFBs and PBRs are capable of supporting cell densities up to 10^8 cells/mL while maintaining high cell viability [2,7,15-17]. Though volumetric productivity is dramatically increased over batch or continuous suspension cultures, membrane fouling, diffusional limitations, and non-uniform gradients commonly plague HFBs and PBRs leading to heterogeneous product formation and inactive portions of the biomass [16-20].

In addition to these high density bioreactors a continuous centrifugal bioreactor (CCBR) has been developed; capable of sustaining highly viable cultures at densities above 10^8 cells/mL [21,22]. The CCBR is similar to the Beckman Elutriation System and a system patented by Kinetic Biosystems, Inc. that is seeing practical application for use in waste stream remediation by flowing through an immobilized culture of bacterial cells [23,24]. The typical problems associated with HFBs and PBRs are not observed within the CCBR due to the unique method of cell immobilization through the balance of centrifugal forces with opposing drag and buoyant forces. To maintain a homogeneous fluidized bed of cells fluid must flow around individual cells such that drag forces can suspend each cell against the centrifugal field, consequently providing convective transport of nutrients and wastes to and from cells. However, needed for the CCBR are models that adequately predict nutrient and metabolite concentrations within the reactor ensuring maximal growth rate is maintained for the duration of the culture. Kinetic modeling provides a tool to estimate growth rate dependence on the concentration of substrates such as glucose or glutamine, and metabolites including lactate and ammonia, each of which are directly affected by dilution rate and cell concentration. Increasing lactate and ammonium ion concentrations not only impact growth rate but also result in lower medium pH, which further contributes to growth rate depression [12,25-29].

In this paper we present a kinetic growth model derived from low density batch studies and verify that the developed model accurately predicts the CCBR culture environment. We have chosen glucose as the substrate, as well as the metabolites ammonium and lactate as the primary parameters of interest and demonstrate their role in growth rate inhibition. We go on to demonstrate model reliability for the extremely dense culture environment within the CCBR, and discuss the utility of this model for understanding high density cultures and their rapidly changing growth environment. Furthermore, we have performed studies that tie culture medium pH to growth rate inhibition and reduced viability; emphasizing the need to maintain pH at an optimal value. This paper will demonstrate how the CCBR is an excellent analytical tool for studying high population density systems, though industrial development shows its potential as a process production system [23,24].

Theory

Kinetic Modeling

For this work a generalized model proposed by Han and Levenspeil [30] was specified and applied to the CCBR culture. This model was chosen as it simultaneously accounts for multiple product and substrate inhibition to determine cellular growth rate. Ammonium and lactate were chosen as inhibitory parameters given that they are both recognized as major byproducts of metabolism which inhibit growth, while glucose is a major energy source required by cells. Additionally, each model parameter can be sensed with either enzyme or ion selective electrodes making online detection easily available for process control to further optimize the CCBR.

Concentrations of cells, glucose (substrate), mAb, ammonium ion and lactate are represented by C_{Cell} , C_G , C_{mAb} , C_A , and C_L respectively, the observed growth rate is determined with Eq. 1 while μ_{max} is the maximum specific growth rate. Critical inhibitor concentrations where the observed growth rate is equal to zero are represented by C_{A_max} and C_{L_max} , respectively. The Monod constant, C_M , is defined as the concentration of the limiting substrate resulting in half of the maximum growth rate, while n , m , p , and q are orders of inhibition and vary depending on the type of observed inhibition, D is dilution rate, and C_{Cell_out} represents the concentration of cells leaving the reactor when it has filled to capacity. Additionally, cell growth rate is coupled to concentrations of glucose, mAb, ammonium, and lactate through yield coefficients represented by Y_{GC} , Y_{mAbC} , Y_{AC} , and Y_{LC} in Eq. 2-5 where C_{i0} is the feed concentration of any of the aforementioned species.

Dilution rate is defined as F/V , the inverse of mean residence time, where F is the flow rate of fresh medium into the reactor and V is the reactor volume (11.4 mL); since volume remains constant, dilution rate changes are analogous to changing the amount of fresh medium fed to the CCBR. Therefore, a fresh feed flow rate of 1 mL/min results in a dilution rate of 0.09 min^{-1} and a residence time of 11.4 min. Consequently, the effect of changing dilution rate is apparent almost immediately when compared to the time scale of an entire culture. The Runge-Kutta method was used to approximate the numerical solution for this set of equations where initial conditions correspond to concentrations of each species in fresh culture medium, e.g., 400 mg/dL for glucose whereas all other species have an initial condition of zero concentration.

$$\frac{\partial C_{Cell}}{\partial t} = \mu_{max} \cdot \frac{\left(1 - \frac{C_L}{C_{L_max}}\right)^n \cdot \left(1 - \frac{C_A}{C_{A_max}}\right)^m \cdot C_G \cdot C_{Cell}}{C_G + C_M \cdot \left(1 - \frac{C_L}{C_{L_max}}\right)^q \cdot \left(1 - \frac{C_A}{C_{A_max}}\right)^p} - (D \cdot C_{Cell_out}) \quad (1)$$

$$\frac{\partial C_G}{\partial t} = D(C_{G0} - C_G) - Y_{GC} \cdot \left(\frac{\partial C_{Cell}}{\partial t}\right) \quad (2)$$

$$\frac{\partial C_{mAb}}{\partial t} = D(C_{mAb0} - C_{mAb}) + Y_{mAbC} \cdot \left(\frac{\partial C_{Cell}}{\partial t}\right) \quad (3)$$

$$\frac{\partial C_A}{\partial t} = D(C_{A0} - C_A) + Y_{AC} \cdot \left(\frac{\partial C_{Cell}}{\partial t} \right) \quad (4)$$

$$\frac{\partial C_L}{\partial t} = D(C_{L0} - C_L) + Y_{LC} \cdot \left(\frac{\partial C_{Cell}}{\partial t} \right) \quad (5)$$

Centrifugal Bioreactor

The CCBR retains cells within the reactor through increasing settling velocities, as a result of centrifugal force, which offset opposing buoyant and drag forces associated with high medium throughput required to sustain dense long term cell cultures [21,22]. Each of the aforementioned forces and their relative directions within the bioreactor are shown in Figure 1. The settling force on a cell is due to the difference between the buoyancy of the particle in the suspending medium and the gravitational force, enhanced due to centrifugation. The settling force at any point within the reactor is the difference between the enhanced gravitational force and buoyancy described by Eq. 6; where F_s is the force of sedimentation, R is the radius of the particle or cell, ρ_p and ρ_f represent the density of the particle and suspending fluid respectively, ω is the angular velocity and r is the distance from the center of rotation to the point of interest.

$$F_s = \frac{4}{3}\pi R^3 (\rho_p - \rho_f) \omega^2 r \quad (6)$$

Counteracting the settling force in the bioreactor is a drag force imposed on any particle contained within a moving stream. Determination of the Reynolds number for the particle (Re_p) in a fluidized bed shows that at the reactor inlet and outlet, regions with highest superficial velocity, the Re_p is at a maximum of 0.36, less than the requirement of unity for flow to be considered laminar. The equation for the drag on a particle in a laminar stream, Stokes Drag, is shown in Eq. 7; where F_d is the drag force on the particle, μ_f is the viscosity of the fluid, R is the radius of the particle, and V_0 is the velocity of the stream.

$$F_d = 6\pi\mu_f R V_0 \quad (7)$$

Briefly, the reactor chamber is designed such that centrifugal and drag forces diminish proportionally as the distance from the center of rotation is decreased. This is achieved through the conical shape of the bioreactor and results in retention of cells within the chamber at a homogeneous density. Rigorous evaluation and development of the design equations for a counter-flow centrifugation system are presented by Sanderson and Bird [31]. In addition to the settling and drag forces present in the CCBR the Coriolis force which results from the system rotation can affect the fluid dynamics within the CCBR system. Operation of the CCBR without cells present results in a fluid flow profile significantly impacted by Coriolis forces [32], however this flow pattern is disrupted when cells are present (data not yet published). Consequently, Coriolis forces have little impact on the distribution of nutrients throughout the CCBR chamber and thus have an insignificant influence on growth kinetics.

For the work examined in this paper the forces on a particle are summed, and hindered settling or particle interaction effects as a function of bed porosity, ε , to the 2.7 power are included [33]. To account for the density difference between a single particle and the suspending fluid a term $(\rho_p - \rho_{\text{susp}})$ replaces the typical $(\rho_p - \rho_f)$ term, however, $(\rho_p - \rho_{\text{susp}})$ equals $(\rho_p - \rho_f) \times \varepsilon$. Then the velocity of the particle relative to the container is set to zero by setting the fluid velocity equal to the absolute value of the particle settling velocity. One then solves for velocity by dividing by the culture porosity ε to arrive at Eq. 8.

$$v = \frac{2}{9} R^2 (\rho_p - \rho_f) \omega^2 r \frac{\varepsilon^{4.7}}{\mu_f} \quad (8)$$

In this paper values of the fluid velocity are taken at the centroid of the volume of the cone containing cells and the porosity is assumed to be uniform throughout this volume. Calculations of inlet fluid velocities or porosity using Eq. 8 for comparison with experimental values are sensitive to cell diameter, cell density, and particularly sensitive to the exact reactor volume fraction containing cells. Consequently, comparison of predicted porosity and fluid velocity with experimental conditions results in poor agreement without precise knowledge of this volume. However, predictions of the effect of changing CCBP angular velocity, RPM, on porosity can be reliably made if the superficial velocity, v , remains constant. This results in a simple relationship between porosity, ε , and angular velocity as indicated in Eq. 9.

$$\varepsilon_2 = \varepsilon_1 \cdot \left(\frac{\omega_1}{\omega_2} \right)^{\frac{2}{4.7}} \quad (9)$$

Materials and Methods

Cell Culture

The cell line used for this research was the mouse hybridoma MM1A [34]. This cell line secretes an IgG1 monoclonal anti-body specific to the epsilon chain of the CD3 T-cell receptor complex. Medium used in all cultures was Dulbecco's Modified Eagle Medium (DMEM) which was supplemented to achieve the following final concentrations; 10% Calf Bovine Serum (CBS) (Hyclone, Logan, UT), an additional 2 mM L-Glutamine for an initial concentration of 6 mM, 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 10 mM HEPES Buffer, and 3×10^{-5} M 2-mercapto-ethanol. High glucose DMEM was used for all experiments except batch cultures for determining kinetic constants, in which case glucose free DMEM was supplemented to varying concentrations of glucose. All medium ingredients other than CBS were purchased from Invitrogen (Carlsbad, CA). Cell counts and viability assays for all cultures were performed using a Trypan Blue Exclusion Assay and a Hemocytometer.

Batch Culture

Initial rate experiments were conducted to determine kinetic model parameters according to the approach outlined by Glacken *et al.* [35]. The approach results in pseudo-steady state relationships as the measurements are taken over a short time at low cell concentrations to ensure cell growth rate is solely a function of known constant concentrations of inhibitor and or substrate.

Initial rate experiments were carried out in 12-well tissue culture plates containing 4 mL of medium. Varying concentrations of filter sterilized glucose (Sigma, St. Louis, MO), ammonium chloride (J.T. Baker, Phillipsburg, PA), or sodium lactate (Sigma, St. Louis, MO) were added to glucose free DMEM in individual wells. Each well was inoculated with 2.0×10^5 total cells from a larger stock being maintained in exponential growth phase. Cells were counted every 14 hours to determine growth rate at each concentration. The lowest concentration of each inhibitor resulting in a growth rate of zero was used as the maximum concentrations in the kinetic model, while orders of inhibition were determined from Lineweaver-Burke plots [30].

Batch culture was also used to determine the effect of pH on growth rate and viability. The pH of the culture medium in a 12-well plate was changed with the addition of 1 M hydrochloric acid (Sigma, St. Louis, MO) or 1 M sodium hydroxide (Sigma, St. Louis, MO). Cells were seeded at a lower concentration of 5×10^4 total cells per well as it was found that more consistent results were obtained using a single count at the end of 48 hours for calculating the growth rate. Counting cells every 14 hours as described above for the nutrient and metabolite studies led to periods of irregular pH as the medium equilibrated between normal laboratory atmosphere and the increased CO₂ incubator atmosphere. Following the 48 hour culture period pH was immediately measured ensuring the value reflected equilibrium with incubator CO₂ concentrations. Finally cells were counted to assess viability and growth rate as a function of pH.

Yield coefficients for glucose, ammonium ion, lactate, and mAb were determined using a 25 mL batch culture with an initial concentration of 2×10^5 cells/mL. Concentrations of cells and every species of interest were determined every 14 hours. By plotting species concentration versus cell concentration the yield coefficient is determined from the linear region of the resultant curve.

Bioreactor Culture

The centrifugal apparatus used for this research study is a COBE® Spectra™ Apheresis System (Gambro BCT Inc., Lakewood, CO) manufactured for use as a blood separation device. The bioreactor chamber itself consists of a single conical chamber (11.4 mL) which is part of the Cell Purging tubing set (Gambro BCT Inc.) made for attachment to the COBE® Spectra™. Cell Purging tubing sets are supplied sterile from the manufacturer, and intended for single use applications. To conserve cost, tubing sets in our laboratory are used for multiple individual experiments and sanitized by pumping 70% ethanol through the entire system for at least 24 hours between experiments. Prior to reactor start-up tubing sets were modified to reflect the flow profile shown in Figure 2, where medium is routed to and from the reactor through tubes using an anti-twister mechanism similar to that shown by Van Wie *et al.* [22].

After sanitization the tubes and reactor were emptied of ethanol, then rinsed and filled with sterile DMEM. All air was removed from the system prior to start-up as the presence of bubbles results in a disruption of the cell bed during reactor operation, and ultimately loss of CCBR culture. Reactor experiments were begun by spinning the Spectra™ rotor at a given RPM, e.g. 650 RPM, corresponding to 60 G's at the reactor inlet and 35 G's at the reactor exit. Next the feed pump, which combines flow from the fresh feed and recycle streams, was adjusted to supply 5.2 mL/min of medium to the reactor, corresponding to a fluid speed of 0.37 cm/s at the reactor entrance, 0.035 cm/s at the cell bed center of mass, and 0.016 cm/s at the reactor's widest point. Typically $2.0 - 2.5 \times 10^8$ total MM1A cells, being maintained in the exponential growth phase in a batch culture process, were supplied to the system through a valve connected to the inoculation line. Initially the product pump was set to 0.25 mL/min; this automatically sets the fresh feed to the same flow rate based on a mass balance

and in this case corresponds to a dilution rate of 1.3 h^{-1} . Product flow rate was increased as needed to maintain adequate nutrient supply and pH.

Sampling the reactor to assess cell viability and density was accomplished by stopping the centrifugal rotor and allowing the feed pump to remain running at an increased rate of 16.5 mL/min for 20 min. This operation resulted in the even distribution of cells throughout the recycle loop resulting in a single continuously stirred tank reactor (CSTR) with a total volume of 31.5 mL. This process was repeated at least once per day and most often twice per day to assess cell density and viability while medium collected from the product stream was stored for later determination of glucose, ammonium, lactate and MAb concentrations.

MAb Quantification

MAb concentration was determined using the Mouse IgG LL NANORID Kit (The Binding Site Limited, Birmingham, UK). Briefly, the NANORID Kit quantifies MAb concentration by diffusion through an agarose gel containing a mono-specific antibody. Samples are added to a cylindrical well and over time allowed to reach equilibrium between complex formation and breakdown with the mAb contained in the gel; consequently, ring diameter is proportional to sample concentration. Ring diameter was determined with a Transidyne General Calibrating Viewer (Transidyne General Corporation distributed by Kellestad Laboratories Inc., Chaska, MN).

Glucose, Ammonium Ion, and Lactate Quantification

Glucose was quantified using Glucose Color Reagent (Raichem, San Diego, CA). The method contained within the package insert was adapted for use with a 96-well plate. Briefly, 150 μL of color reagent was added to each well followed by 10 μL of either standard or sample diluted 1:10 added to specific wells. The reaction was allowed to go to completion by incubating for 10 min at 37°C . Then the plate was read at a 500 nm wavelength using a Spectra Rainbow plate reader (SLT Instruments, Austria). Sample concentration was determined from a standard curve.

Ammonium ion was quantified using Ammonia Reagent (Raichem, San Diego, CA). The method contained within the package insert was again adapted for use with a 96-well plate. Briefly, 110 μL of Reagent 1, containing 2-Oxoglutarate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) is added to 20 μL of sample. After an initial absorbance measurement at 340 nm using a Victor3 plate reader (Perkin Elmer, Waltham, MA), 5 μL of Reagent 2 containing glutamate dehydrogenase is added to the mixture catalyzing the formation of L-glutamate and NADP^+ . The amount of NADPH oxidized is equal, on a molar basis, to the amount of ammonium ion contained in the sample. A second absorbance reading at 340 nm is made following 5 minutes of incubation at room temperature. The absorbance from Reading 2 is subtracted from the absorbance from Reading 1 and the resultant change in absorbance was used for concentration determination using a standard curve.

Lactate was quantified using a lactate reagent from Sigma Diagnostics (Sigma Diagnostics, St. Louis, MO). The procedure was again adapted for use in a 96-well plate; specifically, 10 μL of either standard or sample diluted 1:10 was added to appropriate wells, and then 100 μL of reagent, containing lactate oxidase which reacts with lactic acid and catalyzes the oxidative condensation of chromogen precursors to produce a colored dye was added to each well. Concentration was determined from a standard curve after reading the plate at 540 nm with the Spectra Rainbow plate reader.

Results and Discussion

Impact of pH

Decreased culture pH will occur when the buffering capacity of the medium is overcome; this can happen due to accumulation of lactic acid, as most of the glucose during mammalian cell culture is metabolized to lactate [15,26,36], or the formation of carbonic acid through the release of CO₂ during cellular respiration [15]. The individual impact of extracellular pH on growth rate and viability was studied in low density batch cultures (inoculation density of 1.25×10^4 cells/mL) to ensure buildup of metabolites did not further effect pH or cellular growth rate. Results are shown in Figure 3. Due to error associated with the pH measurement the average of the growth rate and viability for replicate experiments is reported for measurements within 0.05 pH units. Results reveal that viability and growth rates are both reduced as cultures deviate from an optimal pH of 7.2. Figure 3 indicates for maintenance of 80% of the maximum growth rate pH must be maintained between 6.8 and 7.6, and for 90% of the maximum growth rate to be observed pH must be maintained between 6.9 and 7.4.

Model Parameters

As outlined in the Methods section initial rate batch culture experiments were conducted to determine the growth model constants in Eq. 1-5, the values for which appear in Table 1. The initial rate approach for determining constants was used as it most resembles the conditions within the CCBP. Using the initial rate approach cells are subjected to a constant concentration of substrate or inhibitor and the growth rate response for the condition is observed, much the same during CCBP operation. At steady state in the CCBP, when the reactor is full of cells, the growth rate and rate at which cells are removed from the system by the product stream are equal; consequently a constant cellular concentration is maintained, resulting in constant concentrations of substrate and inhibitors at a single dilution rate.

In this paper steady state is modeled by setting both the cellular growth rate and rate at which cells leave the system in Eq. 1 equal to zero. Although this is not physically correct, conceptually it serves the same purpose as setting the rate at which cells are leaving the reactor equal the cellular growth rate, because both result in zero change in CCBP cell concentration. Additionally, because the concentration of cells is unchanged with respect to time during steady state Eq. 2-5 will also equal zero, resulting in constant concentrations of all modeled parameters.

Types of cellular growth rate inhibition and orders of inhibition were determined through construction of Lineweaver-Burk plots, $1/\mu$ vs. $1/C_{\text{Glucose}}$ for both ammonium ion and lactate [30]. The Lineweaver-Burk plot for lactate suggests uncompetitive inhibition as varying inhibitor concentrations provide lines with the same slope and as inhibitor concentrations increase y-intercept values also increase. A similar double reciprocal plot for ammonium ion shows that as inhibitor concentrations are increased the resultant slopes also increase. However, because of the lack of intersection at a single point and because the resultant orders of inhibition m and p are greater and less than zero, respectively, ammonium exhibits a general type of inhibition as proposed by Han and Levenspiel [30].

Glucose was chosen as the lone substrate in this model although it is well known that both glucose and glutamine are necessary substrates providing the major energy and carbon sources for mammalian cell growth. A multiple substrate model can be developed from one of three forms; the interactive or multiplicative form, additive form, or non-interactive form to predict growth rate dependence from both glucose and glutamine [37]. However, development of such a model would require twice as many fitted parameters and it is best if

a simpler model can be selected that will adequately predict cell growth. Additionally, it will be shown that glutamine does not reach inhibitory levels due to spontaneous degradation or metabolic depletion, based on observed CCBR ammonium ion concentrations and literature values of the ratio of consumed glutamine to ammonia production. Therefore, glutamine can be omitted as a modeled parameter eliminating the need for a multiple substrate model for practical system simulation.

Combined Impact of pH and Predicted Inhibition

The impact of pH along with substrate and metabolite concentrations on growth rate is further illustrated for a CCBR seeded with 2.5×10^8 cells (2.2×10^7 cells/mL for the 11.4 mL CCBR) and operated at a constant dilution rate of 1.3 h^{-1} and 650 RPM. As shown in Figure 4 there is an initial lag phase of 45 h after which exponential growth is observed until cells fill the reactor at a density of 5.8×10^7 cells/mL. At this point pH drops to a minimum of 6.75 where Figure 3 shows growth rates will be limited to 77% of the maximum value from pH alone.

Normalizing Eq. 1 to the maximum specific growth rate of 0.065 h^{-1} will provide a fraction of the maximum specific growth rate which will be observed at a particular cell concentration and dilution rate. Doing so results in a model predicted growth rate 61% of the maximum. Individual contributions for each metabolite ion can be determined by evaluating the single exponential term in the numerator containing the inhibitor of interest; in the same fashion substrate inhibition can be determined if C_G is divided by the denominator of Eq. 1. Individual contributions of lactate, ammonium, and substrate are 0.92, 0.77, and 0.86, respectively, where a value of 1 would indicate no inhibition. Therefore, the combined inhibition from pH, metabolite accumulation, and substrate depletion results in a predicted growth rate that is 47% of the maximum ultimately contributing to a decline in CCBR culture density to 4.6×10^7 cells/mL. The lower concentration of cells results in decreased glucose consumption and metabolite production and an observed rise in pH to 6.85, consequently the overall inhibition is reduced and a growth rate which is nearly 85% of the maximum results.

Inhibiting pH levels can be eliminated through increasing dilution rate to provide adequate buffering capacity. Additionally, increasing dilution rates minimize inhibition through lowering lactate and ammonium ion concentrations while raising substrate levels. When pH, substrate and inhibitor concentrations are regulated by stepwise increases in dilution rate from an initial value of 1.3 h^{-1} up to 5.2 h^{-1} , as shown in Figure 5, cells experience a lag phase followed by an exponential growth phase until cells fill the reactor at a steady state concentration of 8.5×10^7 cells/mL. During the initial steady state, from 52 to 143 h when the dilution rate varies between 3.5 and 4.4 h^{-1} , the collective set of inhibition terms in Eq. 1 predict a growth rate multiplier between 0.72 and 0.78 for the maximum growth rate. Lactate concentrations provide little inhibition with a term value between 0.94-0.96, glucose has slightly lower values between 0.91-0.93, and the ammonium term has the largest predicted impact between 0.84 and 0.88. The pH inhibition is minimal as pH was maintained between 6.90 and 7.35 with an average pH of 7.12 corresponding to an inhibition multiplier of 0.96 of the maximum growth rate. Collectively the overall inhibition during the initial steady state results in a growth rate between 69-75% of the maximum.

If the centrifugal force is increased without a subsequent increase in drag force (i.e. at constant inlet flow rate) a higher population density can be maintained within the reactor. To illustrate this point, Figure 5 shows results for an RPM increase from 650 RPM at 143 h to a final RPM of 1200 at 181 h, which corresponds to a total increase in centrifugal force by a factor of 3.4. The RPM was increased through a series of steps as follows; 750 RPM at 143h, 850 RPM at 166 h and finally 1200 RPM at 181 h. An initial increase in RPM from

650 to 750 will increase the centrifugal force by a factor of 1.3 and using Eq. 9 result in a 38% increase in cell number. The second increase in RPM at 166 h to 850 increases the centrifugal force by a factor of 1.7, corresponding to a predicted 70% increase in cell density to 1.4×10^8 cells/mL which is the density determined experimentally at 1200 RPM. Based on Eq. 9 cell density should have reached 2×10^8 cells/mL at 1200 RPM. Predictions of cell density using Eq. 9 are within 30% of experimentally determined values reinforcing the need for extremely accurate cell bed volume determinations and a better understanding of all the fluid dynamics affecting cell density before more accurate predictions can be made.

During the final steady state the ammonium ion multiplier is 0.84, while lactate and glucose multipliers are 0.94, 0.91, respectively; a pH multiplier of 0.95 is due to an average pH of 7.06 from 170-210 h. Consequently, an overall growth rate which is 68% of the maximum would likely necessitate an increase in dilution rate to maintain the culture for weeks or months. To maintain a growth rate which is 90% of the maximum and ensure the culture is sustained the dilution rate would have to increase to 22 h^{-1} – an explanation and thorough analysis of this will be provided in the model utility section to follow.

As has been consistently shown through predictions with the kinetic model the ammonium ion term has the largest deleterious impact on cellular growth rate for the MM1A cell line. Work by Hassell *et al.* [26] and Doyle and Butler [25] shows the effect of ammonium ion on cellular growth rate supporting its significant impact on MM1A growth rate. First of all, the work shows growth rate sensitivity to ammonium ion is cell line dependant. Secondly, it is the small quantity of dissolved NH_3 instead of the measured NH_4^+ concentration that has the greatest effect on growth rate. This is supported by the fact that IC_{50} values of ammonium chloride increase as the ratio of $\text{NH}_3/\text{NH}_4^+$ is decreased with decreasing pH [25,26]. Because the pK_a of ammonium ion is 9.25, at the optimal pH for cell growth of 7.2, 99% of dissolved ammonia will be the less toxic protonated species, making the ammonium ion phenomenon more important at pHs above the optimal value for cell growth.

Model Efficacy

Figure 6 is used to illustrate the accuracy of the model in predicting actual concentrations for a CCBR experiment seeded with 1.6×10^7 cells/mL, and run for 275 h at 700 RPM with a constant inlet flow rate of 5.2 mL/min with an initial dilution rate of 1.3 h^{-1} . During operation pH was checked every 12 hours and as pH declined toward 7.0 dilution rate was adjusted by incremental increases of $\sim 1 \text{ h}^{-1}$ ensuring pH remained between 7.0 and 7.2 with an average of 7.1 over the entire culture. According to Figure 3 the pH range for this experiment corresponds to conditions with little inhibition from pH and observed growth rates greater than 96% of the maximum specific growth rate.

Three dilution rate increases are observed following the lag phase as the initial dilution rate of 1.3 h^{-1} is increased to 5.0 h^{-1} over a 48 h period. Consequently, simultaneous step changes in monitored species concentrations are seen depending on whether species are being consumed or produced in the CCBR. Results shown in Figures 6A and 6B indicate that following an initial 35 h lag phase exponential growth is observed resulting in a density of 3.7×10^7 cells/mL that remains relatively constant over the period covering 55-135 h. At the beginning of this period the overall inhibition multiplier is 0.80 and through a series of dilution rate increases is raised to 0.88 at 95 h.

Although there was no apparent change in process conditions a new steady state with a density to 4.5×10^7 cells/mL is observed at 140 h. The increase in cell concentration can be explained by considering that the new less inhibitory conditions provides an environment more supportive of cell growth. After a lag phase of about 45 h from the last dilution rate change, which is similar in duration to the lag phase experienced at the start of culture, an

increased cellular growth rate now exceeding the rate of removal from the reactor results allowing the cone to fill to a slightly larger extent. Of significance is the comparison of CCBR species concentrations to model predicted values derived from Eq. 2-5, shown in Figure 6. Both modeled and actual concentrations are normalized before finding relative standard deviations; cells are normalized to 1.0×10^9 total cells, mAb and glucose to 70 $\mu\text{g}/\text{mL}$ and 400 mg/dL respectively, while ammonium and lactate are normalized to maximum inhibitor concentrations of 5.8 mM and 930 mg/dL , respectively. As shown in Figure 6, we see excellent agreement over the entire 260 h run. Relative standard deviations between model predicted and experimentally determined concentrations for the various species are 8.1% for glucose, 13% for mAb, 9.3% for ammonium ion, 3.8% for lactate, and 7.2% for the cell concentration (when considering the model is set to a steady population density at a given RPM).

Although glutamine is not predicted with this model it can be shown it does not contribute significantly to growth rate inhibition based on literature values of kinetic parameters and measured ammonium ion concentrations. It has been shown for various hybridoma cell lines that ammonia is produced at a ratio of 0.4 - 0.8 mols per mol of glutamine consumed [10,36,38,39]. Medium used in the experiments for this paper is supplemented with glutamine prior to culture, resulting in an initial concentration of 6 mM. If one uses the lower limit yield coefficient of 0.4 mols ammonia per mol glutamine consumed, the largest theoretical amount of glutamine consumed will result. Using the average ammonium ion concentration over the final 100 h of experimental culture in Figure 6, 0.75 mM, a minimum glutamine concentration of 4.1 mM will result. Additionally, Acosta et al. reported a Monod constant for glutamine of 0.08 mM [38]. Therefore, for glutamine depletion to result in a 10% reduction in growth rate concentrations would have to fall below 0.72 mM, which is significantly lower than the worst case scenario calculated above. Therefore, we conclude that inhibition due to glutamine is insignificant in the current study.

In addition to cellular metabolism glutamine is spontaneously degraded to ammonia and pyrrolidone carboxylic acid and the rate of degradation is both medium type and pH dependant [40]. Over the course of 400 h Ozturk and Palsson [40] found up to 40% of medium glutamine could decompose, and not accounting for this decomposition raised the yield coefficient of mols of ammonia produced per mol of glutamine consumed from the actual value of 0.52 to an apparent value of 0.68. Not accounting for glutamine decomposition can produce up to a 300% error when determining cellular consumption of glutamine and production rates of ammonia [40]. However, because growth rate inhibition depends on total ammonia concentration, knowing whether ammonia is produced by cells or as a result of decomposition is of little importance for calculating total growth rate inhibition, as in this paper. Furthermore, the low concentration of ammonium ion measured in the culture medium indicates that at worst only 30% of the initial glutamine is consumed. According to the kinetic considerations already stated, the initial concentration would have to be reduced by nearly 90% before significant inhibition due to glutamine depletion is observed.

Implications for Centrifugal Bioreactor Scale-Up

Using this kinetic model to optimize the conditions within the CCBR will result in a maximum cellular growth rate. Although, the volume of the CCBR is small, 11.4 mL, the productivity of the system in terms of mAb production rate can be quite significant. Using the rate of mAb production in the final 100 h of Figure 6, 40 $\mu\text{g}/\text{mL}$, and the dilution rate of 4.3 h^{-1} it is possible to produce 48 mg/day of mAb. To increase productivity it is possible to increase reactor volume; for example tubing sets are available from Gambro which use a 40 mL reactor. Furthermore, our lab has made modifications to the CCBR rotor allowing two reactor chambers to be cultured simultaneously. The rotor could easily be modified to hold 4

reactors and if each were of the 40 mL type the total system volume would be increased 15-fold over the current apparatus discussed in this paper, thus making it possible to produce over 600 mg/day of mAb. Production could easily be increased beyond this to over a gram per day if culture population density is increased through changes in rotor RPM.

Traditional scale-up of the CCBP process where reactor size is increased to meet production needs would likely result in complications such as extreme amounts of power required for system rotation in addition to mechanical instability during the rotation of large reactor volumes. Traditional scale-up also limits the applicability of small volume kinetic analysis due to the increased mechanical stresses on cells from mixing requirements and nutrient gradients within the bioreactor which lead to non-uniform growth rates. However, it appears more likely that CCBP scale-up would include many small volume reactors operating in parallel, similar to that which has been done commercially by Kinetic Biosystems, Inc. for use in waste water processing [23,24]. As each small volume reactor in a scaled-up process would provide the same uniform environment the kinetic analysis developed in this manuscript would directly apply, providing accurate environmental predictions during cell culture.

Bioreactor process optimization is not solely achieved through increasing cellular growth rates at various densities, as in this study, but through jointly maximizing cellular productivity and density. Cellular protein productivity is often not directly related to growth rate; commonly protein production rates increase when cells are stressed or in a growth arrested state [12,17,41-43]. Unfortunately, increased protein productivity observed during these conditions is countered by apoptosis which results from culture stresses that may include nutrient limitation, metabolite accumulation, and mechanical stresses [44-47], thereby limiting the overall productivity of the bioreactor. Apoptosis, or programmed cell death, is a physiological process characterized by the suicidal response of a cell to a non-lethal physiological or environmental stimulus [41,44].

Within the current study the method of cellular death, be it necrosis or apoptosis, was not differentiated as understanding the collective impact of process conditions on cellular growth rate during CCBP operation was the primary objective. However, as has been presented in this manuscript manipulation of the dilution rate influences inhibition of both substrate and metabolite concentrations. Identification of substrate and metabolite concentrations which result in apoptosis versus necrosis and the relation to protein productivity is of interest for future studies to further optimize the system. Additionally, due to the unique cell immobilization method within the CCBP mechanical stresses and their possible role in activation of apoptosis pathways should be studied to further understand cell culture optimization for the CCBP technology.

Model Utility

Given our model can accurately predicts cell, glucose, mAb, lactate and ammonium ion concentrations, we can learn valuable information about what is required to maintain optimal growth conditions at extreme culture densities. This can be done for a variety of cell concentrations achievable through increases in CCBP RPM up a maximum density of 3×10^8 cells/mL.

To assess the impact of dilution rate we can rearrange the model in Eq. 1 for the normalized growth rate $\mu/\mu_{\max}$ to determine the fraction of maximum growth rate that will be achieved for various cell densities as presented in Figure 7. For maintaining higher population densities Figure 7 clearly shows the necessity of increasing dilution rate which has the impact of reducing the concentration of toxic metabolites and increasing the concentration of substrate. For example, to maintain a cell concentration of 1×10^7 cells/mL above 90% of

the maximum growth rate requires a dilution rate of 1.66 h^{-1} whereas μ will be a mere 31% of the maximum value at the same dilution rate when the population density increases to 2×10^8 cells/mL. This type of analysis provides a valuable tool for finding the most effective dilution rate to maintain optimum growth rate conditions.

In order to maintain a density of 2×10^8 cells/mL at 90% of the maximum growth rate our model indicates a dilution rate of 33 h^{-1} and a corresponding fresh feed flow rate of 6.3 mL/min would be required. Because the required fresh feed flow rate is higher than the 5.2 mL/min inlet feed rate we've used thus far the inlet feed rate would need to increase in order to accommodate the necessary dilution rate. From this discussion it is apparent that to maintain the desired density within the reactor while increasing inlet feed rate the RPM would also have to be increased to ensure cells are retained within the reactor.

Conclusions

The CCBP system used for this work immobilizes cells for continuous high density mammalian cell culture for protein production. High density culture increases volumetric protein productivity, but lends itself to rapid changes in environmental conditions leading to growth rate inhibition through substrate depletion, metabolite accumulation, or suboptimal culture pH. A kinetic model describing mAb concentrations and the dependence of cellular growth rate on glucose, ammonium ion, and lactate concentrations has been developed using low density small volume batch studies. Additional batch studies show an optimum pH of 7.2 for the MM1A hybridoma; furthermore, viabilities and growth rates are maintained within 90% of maximum values when pH values are between 6.9 and 7.4.

An example is presented for a culture operated at an initial population density of 2.2×10^7 cell/mL and dilution rate of 1.3 h^{-1} at which growth rate is predicted to be 80% of the theoretical maximum. As biomass increases to 4.6×10^7 cells/mL an unchanged dilution rate reduces the predicted growth rate to 67% of the maximum through accumulation of ammonium ion and lactate in addition to glucose depletion, the individual contributions of each to overall inhibition are 0.81, 0.93, and 0.89, respectively. However, kinetic modeling predicts, and experimental results confirm, that by increasing the dilution rate from 1.3 to 5.2 h^{-1} growth rate inhibition in the CCBP will be significantly reduced.

Furthermore, due to the unique cellular immobilization technique of the CCBP every cell is individually bathed in culture medium which enhances the likelihood each cell is subjected to the same steady-state environmental conditions. Consequently, the proposed kinetic model may be extended to predict dilution rate requirements for much higher density cultures of 2×10^8 cells/mL and above. Finally, the design of the CCBP which provides continuous medium provision and removal of toxic metabolites is an ideal system for the study of high density culture, providing a tool to identify characteristics inherent to any reactor designed to enhance productivity through increased cell density.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the Biotechnology Training Grant (NIH 5T32-GM008336-16) and the Gene & Linda Voiland School of Chemical Engineering and Bioengineering for graduate student support and supplies. Equipment in the form of the COBE® Spectra Apheresis System and Cell Purging tubing sets in addition to corporate technical research staff consultation are a result of a donation by Gambro BCT Inc., Lakewood, CO. Mr. R. Hutchinson of the Washington State University College of Engineering and Architecture Machine Shop was instrumental in design and fabrication of the CCBP chambers used in these experiments.

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Figure 1. Indicates the forces acting on individual cells within the CCB and their relative direction of action on that cell. Cell immobilization in this manner prevents wash-out allowing for increased perfusion of medium throughout the fluidized bed of cells, maintaining both high density and viability during culture.

Figure 2.

Process flow diagram for CCBR used in the culture of hybridoma cells. The 11.4 mL reactor was inoculated with 175-250 million cells which were subsequently cultured for 14-28 days. A 1L feed tank (FF tank) provides fresh DMEM and spent medium is removed by the product pump. Because the CCBR behaves as a continuously stirred tank reactor, a sample taken of the product stream is representative of concentrations of substrates and metabolites in the reactor.

Figure 3.

Cell growth rate and viability dependence on medium pH. As pH deviates from the optimum of 7.2 both growth rate and viability are affected to an increased degree. To maintain viability and growth rate above 90% pH must be maintained within a range of 6.9 to 7.4 throughout a culture.

Figure 4.

Impact of pH on CCBR culture at a 1.3 h^{-1} dilution rate. As cell population increases to $5.8 \times 10^7 \text{ cells/mL}$ inhibition increases as substrate concentration and pH both decrease while inhibitory metabolites increase resulting in an overall growth rate predicted to be 47% of the maximum. Growth rate is calculated based on a combined term including a fit to the pH growth rate curve and the lactate, ammonium and glucose inhibition factors multiplying the maximum specific growth rate in Eq. 1. Due to significant inhibition cell concentration decreases to $4.6 \times 10^7 \text{ cells/mL}$.

Figure 5.

The impact of dilution rate and CCB-RPM on inhibition and cell culture density. To diminish inhibition effects dilution rate can be used to increase substrate concentration while decreasing lactate, and ammonium ion levels, and maintaining pH buffering capacity. A steady state concentration of 8.5×10^7 cells/mL is maintained with a dilution rate between 3.5 and 4.4 h^{-1} corresponding to an overall normalized model-predicted growth rate between 0.72 and 0.78. Limited inhibition ensures that as centrifugal force is increased a second steady state at increased cell concentration will result.

Figure 6.

Comparison of model predicted and actual concentration data collected every 12 h during 261 h CCBP run. Comparison of model predicted values and actual normalized measured concentrations show all relative standard deviations are below 13%: (A) Cell concentrations were normalized to 1×10^9 total cells; glucose to 400 mg/dL; (B) Concentrations of lactate were normalized to 929 mg/dL; ammonium ion to 5.7 mM; and mAb to 70 μ g/mL. The calculated inhibition value consists of all terms multiplying $\mu_{\max}$ in Eq. 1.

Figure 7.

Maximum growth rate as a function of varying dilution rates and cell concentration maintained within the CCBR. As the cell population density is increased the dilution rate must also be increased to prevent inhibition due to lactate and ammonium ion accumulation as well as glucose depletion.

Table 1

Values of the constants for the proposed kinetic model in Eq. 1-5.

Model Parameter	Value
$\mu_{\max}$	0.065 h^{-1}
$C_{L_{\max}}$	929 mg/dL
$C_{A_{\max}}$	5.78 mM
C_M	16 mg/dL
n (lactate)	0.22
m (ammonium ion)	0.52
p (ammonium ion)	-1.16
q (lactate)	0.55
Y_{GC}	$15.0 \times 10^{-5} \text{ (mg/dL)/(cell/mL)}$
Y_{mAbC}	$5.6 \times 10^{-5} \text{ (}\mu\text{g/mL)/(cell/mL)}$
Y_{AC}	$0.13 \times 10^{-5} \text{ (mM)/(cell/mL)}$
Y_{LC}	$17.1 \times 10^{-5} \text{ (mg/dL)/(cell/mL)}$