

INCREASING SERUM CONCENTRATIONS DECREASE CELL DEATH AND ALLOW GROWTH OF HYBRIDOMA CELLS AT HIGHER AGITATION RATES

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Summary Hybridoma cells CRL-8018 were grown in a well controlled 1.2 l bioreactor at high agitation rates (220 rpm) with fetal bovine serum (FBS) concentrations varying between 1% and 10%. No net growth was observed at FBS concentrations below 5%, while higher FBS concentrations allowed faster growth. The inferred death rate shows a continuous decrease with FBS concentrations. 10% FBS allows cell growth even at 280 rpm in our bioreactor. A simple model is proposed for the quantitative description of the FBS effect on cell growth and death.

Introduction

Growth of anchorage-dependent and freely suspended animal cells in mixed bioreactors offers a variety of advantages such as: scaleability, ease of controlling and programming important bioreactor parameters (pH, DO, nutrient concentrations), relatively uniform bioreactor conditions, process monitoring, and use of existing industrial capacity from other biological processes. Mixing is desirable in bioreactors in order to increase oxygen transfer and provide a homogeneous environment for growth. However, compared to microbial cells, animal cells, which lack cell walls, are more sensitive to and can be damaged by hydrodynamic forces. The hydrodynamic sensitivity is often referred to as "shear" sensitivity. Hydrodynamic cell damage in microcarrier cultures has been recently reported in the literature (Cherry and Papoutsakis, 1988; 1989; Croughan et al., 1987; 1989). "Shear" sensitivity of freely suspended cells is also a problem of practical and fundamental importance. Freely suspended cells (such as hybridoma cells for production of monoclonal antibodies, insect cells and various transformed cells for production of recombinant proteins) can be grown in a variety of bioreactor configurations, such as microencapsulation reactors, hollow-fiber reactors, air-lift reactors, and stirred-tank reactors. Cells more resistant to hydrodynamic damage (or media components and additives that render cells more resistant to such damage) would simplify the design, operation, and productivity of most bioreactors and also would allow more efficient membrane processing of the cells for cell recycle and harvesting.

Shear effects on various suspended cells (blood and tumor cells) in laminar flows have been widely reported in the biomedical engineering literature (Chittur et al., 1988; McIntire et al., 1987; Petersen et al., 1988). Reports on "shear" effects on cells for biotechnological applications are less numerous. Shear sensitivity of insect cells in agitated and aerated suspensions and in a viscometer has been reported (Tramper et al., 1986). Handa-Corrigan and co-workers (1989) have studied the damage mechanisms of suspended cells due to gas sparging in bioreactors. Shear sensitivity of hybridoma cells has been examined in viscometric studies by us (Petersen et al., 1988) and others (Smith et al., 1987). Flow effects in various channel flows on mouse myeloma cells, Hela cells and mouse L929 cells have also been reported (Augenstein et al., 1971; McQueen et al., 1987).

We have reported recently (Kunas and Papoutsakis, 1989a) on the hydrodynamic damage of hybridoma cells in agitated bioreactors with surface aeration. We shall shortly show (manuscript in preparation) that in such bioreactors, cell damage is solely the result of bubble entrainment and break-up in the vortex area around the impeller shaft. In this paper we discuss the protective effect of serum against such agitation damage in terms of apparent growth rates and death rates at high agitation levels.

Materials and Methods

Cell Culture and Media

CRL-8018 hybridoma cells (ATCC), which produce an IgM antibody directed against hepatitis B surface antigen, were used in all experiments. The cells were adapted to grow with 1% Nutridoma medium additive and 1% fetal bovine serum (FBS). After adaptation, the cells were routinely cultured in Dulbecco's modified Eagle's medium (DME; Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 1% v/v FBS (Hyclone Laboratories, Logan, UT, U.S.A.), 1% v/v Nutridoma-NS (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.), 2 mM glutamine (Sigma), and 50 units/ml penicillin, 0.05 mg/ml streptomycin, and 0.1 mg/ml neomycin (PSN; Sigma) before filtering. The serum was denatured at 56° C for 30 min. before use.

Bioreactor Cultures

Large scale experiments utilized two Setric Genie 2C bioreactors (Setric Genie Industriel, Toulouse, France) operated with a 1.2 l working volume. Temperature was controlled at 37° C, and the dissolved oxygen concentration was maintained above 65% saturation with air by addition of O₂ into the reactor headspace. The pH was maintained at 7.2 by addition of either CO₂ into the headspace or 0.5 N NaOH to the culture medium. The impeller used for agitation was 7 cm in diameter with four blades pitched at 57° to the horizontal, each blade being 7 cm by 1.8 cm in dimension. Spinner flask cultures were used as inocula for the bioreactors as well as for non-agitated (T-flask) control cultures. The bioreactor agitation speed was kept at 60 rpm, a non-damaging speed for the cells, until the cells had entered exponential growth. At a cellular concentration of 4-5 x 10⁵ cells/ml, the agitation rate was increased to the desired rate, generally 220 rpm. Variations in the cells' ability to withstand hydrodynamic forces due to different inoculum histories, length of lag phase, or culture age (Petersen et al., 1988) were largely overcome by allowing each batch culture to reach the same point in exponential growth before the desired agitation was imposed.

Assays

Total cell concentrations were determined using a coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.). Culture viability of random samples was checked using trypan blue dye exclusion in a hemacytometer. The viability was consistently found to be above 95% during exponential growth.

Growth and Death Rate Calculations

Samples were taken every 3-5 hours until at least 5 samples within the exponential growth phase had been obtained. The apparent growth rate, μ_{app} , is determined by linear regression of the exponential phase of the growth curve, which is a plot of the natural log of the cell concentration, C, versus time, t. Unless stated otherwise, the growth rates reported are the apparent growth rates. The death rate, k, was determined by subtracting the apparent growth rate from the average growth rate of the T-flask control cultures (0.055 hr⁻¹). Error bars for the growth and death rates represent the 95% confidence level for a regression parameter.

Results

We assessed the protective effect of FBS against agitation cell damage in a well controlled bioreactor using the protocol discussed above in paired experiments in order to avoid the difficulties associated with the varying "shear" sensitivity of the cells (Petersen et al., 1988). Characteristic pairs of experimental results are shown in Fig. 1. Effective cell growth

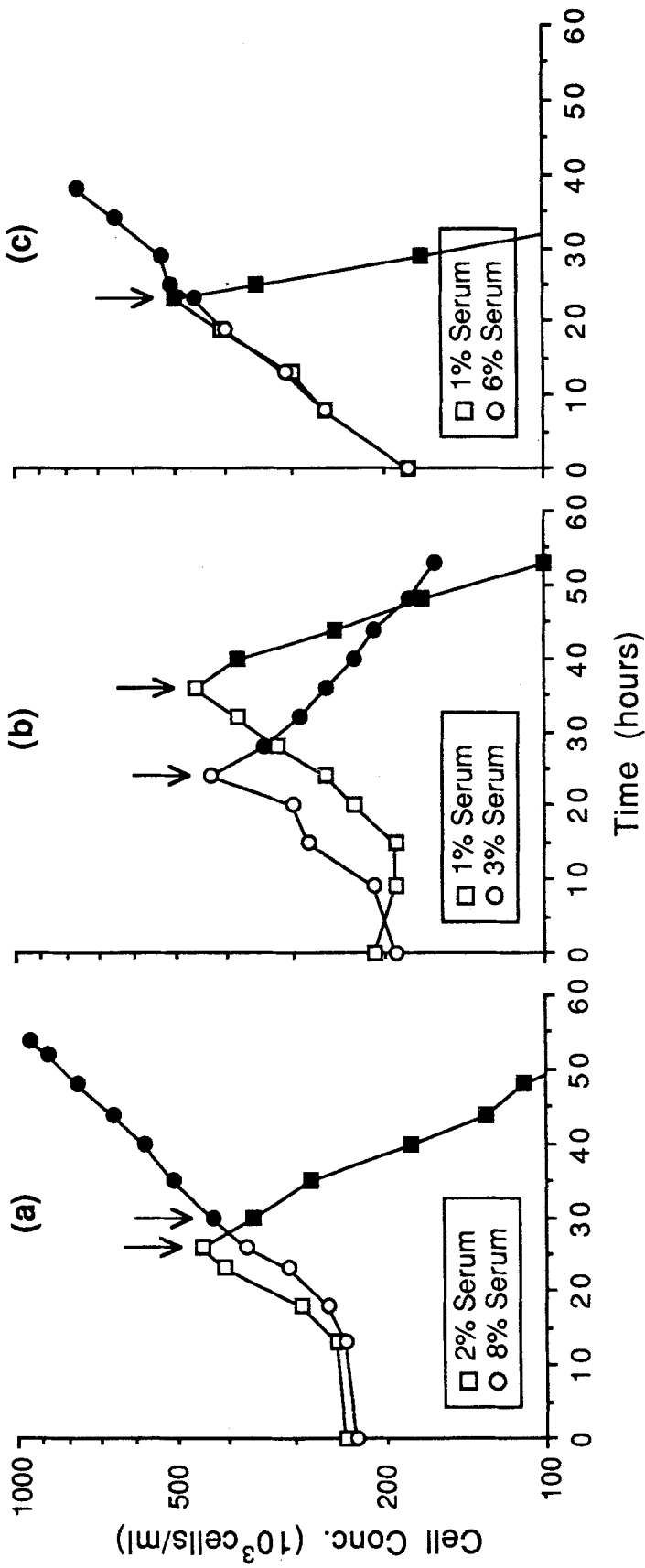


Fig. 1. Typical growth curves for cells grown in bioreactors at various serum concentrations. Curves on the same graph (a, b, or c) represent cultures inoculated with the same inoculum source and grown simultaneously in two bioreactors. The agitation rate was increased from 60 to 220 rpm during batch growth at the point indicated by the arrows. Apparent growth rates from the exponential phases were computed using the linear portions of the curves indicated by the filled symbols.

(positive μ_{app}) at 220 rpm does not occur until FBS concentration is above 4-5%. At progressively lower FBS concentrations, a larger negative μ_{app} reflects an increasingly greater death rate. The μ_{app} results are summarized in Fig. 2, which also shows the effect of FBS concentration on a *calculated* death rate, k . We write that

$$\mu_{app} = \mu_o^* - k \quad (1)$$

where μ_o^* is the intrinsic rate of cell growth found to be 0.055 hr^{-1} from control T-flask experiments. If we assume that μ_o^* does not vary with agitation intensity, and since control T-flask experiment show that μ_o^* is not affected by FBS concentration in the range of 1-10% (Kunas and Papoutsakis, 1989b), we use Eq. (1) to calculate k . The death rate calculations shown in Fig. 2 indicate an appreciable cell death even at 10% FBS.

The μ_{app} and death rate data can be fitted with saturation type equations as follows:

$$k = k_{max} - \frac{k^* S}{S + a_1} \quad (2)$$

$$\mu_{app} = \mu_o^* - k = \frac{a_2 + a_3 S}{S + a_1} \quad (3)$$

where

$$a_2 = (\mu_o^* - k_{max})a_1 \quad (4)$$

$$a_3 = \mu_o^* - k_{max} + k^* \quad (5)$$

$$\mu_o^* = \begin{cases} \mu_o & S > 1 \\ 0 & S < 1 \end{cases} \quad (6)$$

S is the %FBS concentration (dimensionless), a_2 is also dimensionless, and k_{max} , k^* , a_2 , and a_3 have units of hr^{-1} . Eq. (6) accounts for the fact that the cells do not grow at 0% FBS, but grow at a constant μ_o rate at FBS concentrations above 1%. k_{max} is the maximum death rate at a given rpm (for $S = 0$). The model is essentially valid for S between 1 and 10% and, with the proper constraints, displays logical asymptotic behavior, namely that for $S = 0$, $\mu_o = 0$ and $k = k_{max}$; and for $S \rightarrow \infty$, $k = k_{max} - k^* \geq 0$ and $\mu_{app} = a_3$. Since the constraint $k_{max} - k^* \geq 0$ is applied, a_1 is also greater than 0. From our data and using nonlinear regression analysis with the aforementioned constraint (SYSTAT software package, Evanston, IL 60201), it is found that

$$k_{max} = k^* \quad (7)$$

Then, the model reduces to:

$$k = \frac{k_{max} a_1}{S + a_1} \quad (8)$$

$$\mu_{app} = \frac{a_2 + \mu_o S}{S + a_1} \quad (9)$$

Fitting the data to Eqs. (8) and (9) using nonlinear analysis gives parameter values of $k_{max} = 1.27 \text{ hr}^{-1}$, $a_1 = 0.187$, and $a_2 = -0.228 \text{ hr}^{-1}$, which are consistent with Eq. (4). The model results are plotted on Fig. 2.

One would expect a given FBS concentration to have a quantitatively different effect on μ_{app} or k depending on the agitation intensity (rpm). This is demonstrated in Fig. 3, which

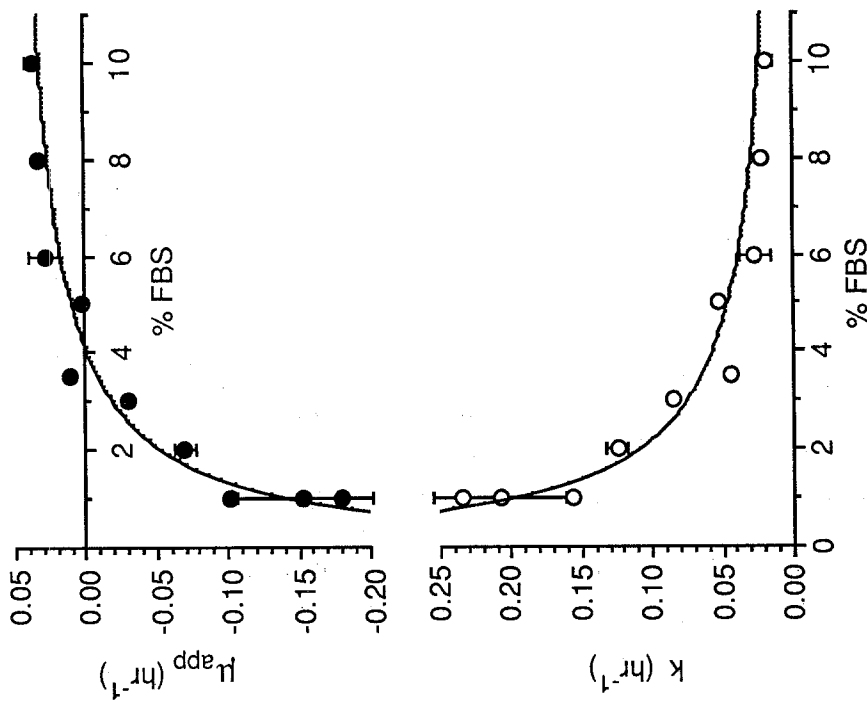


Fig. 2. Apparent growth rate (filled symbols) and death rate (open symbols) versus serum concentration for cells grown in a 1.2 L bioreactor at 220 rpm. Error bars represent the 95% confidence limit for a regression parameter. Solid lines are model predictions.

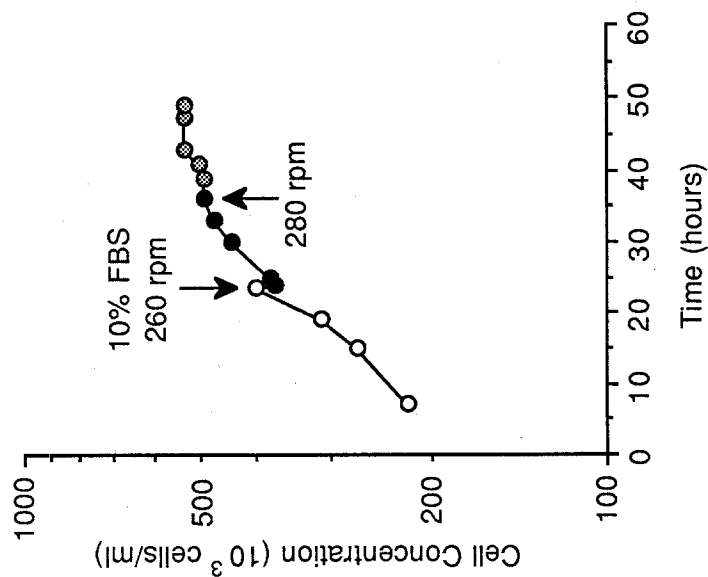


Fig. 3. Growth curve for cells in a bioreactor containing 1% serum. The serum concentration was increased from 1% to 10% at the point indicated by the first arrow followed 5 min. later by an increase in agitation from 60 to 260 rpm. The agitation rate was increased to 280 rpm at the point demarcated by the second arrow. Using the points represented by the black symbols (260 rpm) and the gray symbols (280 rpm), the growth rates are calculated to be 0.0243 ± 0.0045 and 0.0079 ± 0.0071 , respectively (95% confidence limit for a regression parameter).

shows that 10% FBS allows the cells to grow with different μ_{app} and k values at 260 and 280 rpm. At 220, 260, and 280 rpm, the corresponding pairs of $[\mu_{app}, k]$ are (units of hr^{-1}) [0.0362, 0.0188], [0.0243, 0.0307], and [0.0079, 0.0471], respectively. These findings are supported by additional experiments (results not shown).

Discussion

Fetal bovine serum, although it does not affect the cell growth in static cultures (T-flasks), allows the cells to grow at higher agitation levels at progressively higher concentrations. For example, while the cells will not grow at 220 rpm at FBS levels below 4-5%, in the presence of 10% FBS they can grow even at 280 rpm (Figs. 2 and 3). We show elsewhere (Kunas and Papoutsakis, 1989b) that the protective effect of FBS is mostly physical, but not through its small effect on the medium viscosity. 10% FBS increases the medium viscosity by 13% (results not shown). On the other hand 50 and 100% increases in viscosity using high MW dextran do not increase the apparent growth rate at 220 rpm and actually result in lowered apparent growth rates (Kunas and Papoutsakis, 1989a).

The simple model of Eq. (1) is consistent with patterns of glucose and glutamine utilization and cell yields (Kunas and Papoutsakis, 1989b). However, it may still be possible that μ_0 is affected by the agitation intensity. The parameters of Eqs. (2) and (3) would vary with the level of agitation as Fig. 3 demonstrates, and may also vary with the cell type since different cells have different sensitivities to hydrodynamic forces.

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