Analysis of the use of fortified medium in continuous culture of mammalian cells

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Abstract

Continuous culture is frequently used in the cultivation of mammalian cells for the manufacturing of recombinant protein pharmaceuticals. In such operations a large volume of medium is turned over each day, especially in the case where cell recycle, or perfusion cultivation, is practiced. In principle, the volumetric throughput of medium can be reduced by using a more concentrated feed while maintaining the same nutrient provision rate. Overall, the medium components are divided into two categories: 'consumable nutrients' and 'unconsumable inorganic bulk salts'. In such fortified medium, the concentrations of consumable nutrients, but not bulk salts, are increased. With a stoichiometrically-balanced medium, the large amount of nutrients fed into the culture is largely consumed by cells to give rise to residual concentrations of these nutrients in their optimal range. However, unless care is taken to initiate the continuous culture, overshoot of nutrients may occur during the transient period. The high nutrient concentration during overshoot may be inhibitory by itself, or the resulting high osmolality may retard the growth. Using a mathematical model that incorporates the growth inhibitory effect of high osmolality we demonstrate such a potentially catastrophic effect of nutrient and osmolality overshoot by simulation. To avoid overshoot a controlled nutrient feeding scheme should be devised at the initiation of continuous culture.

Introduction

Mammalian cells are widely used for the production of therapeutic and diagnostic biologicals. Some of these products, especially viral vaccines, are produced using anchorage dependent cells. However, in the last decade, an increasing number of products, notably monoclonal antibodies and proteins derived from gene technology, have been produced by cells that grow in suspension. The predominant method of cultivating suspension cells is to employ stirred tank bioreactors. Traditional batch or simple continuous cultures in stirred tank bioreactors can only achieve a relatively low cell concentration and low volumetric productivity. Therefore, considerable efforts have been devoted to developing production systems with high cell concentration and long production periods.

Fed-batch cultures, wherein nutrient feeding is employed to sustain cell growth and production, have been used to increase the cell and product concentrations (Bibila, Ranucci et al., 1994; Bushell, Bell et al., 1994; Xie and Wang, 1994; Zhou, Rehm et al., 1995; Xie and Wang, 1997). However, the accumulation of metabolites, such as lactate and ammonia, eventually inhibits cell growth and limits product concentration and culture time. To sustain long production periods, continuous flow is necessary for culture fluid renewal. Simple continuous stirred tank bioreactors have been shown to sustain steady operations over extended time periods (Birch, Lambert et al., 1987; Miller, Wilke et al., 1988; Frame and Hu, 1991; Kurokawa, Tatsuya et al., 1993). However, the relatively low cell and product concentration attainable at steady state makes it unattractive for manufacturing purposes.

Continuous cultures with cell retention or recy-

cle are increasingly being used to increase the cell concentration and the productivity for a given reactor volume (Kitano, Shintani et al., 1986; Takazawa and Tokashiki, 1989; Batt, Davis et al., 1990; Comer, Kearns et al., 1990; Seamans and Hu, 1990; Tokashiki, Arai et al., 1990; de la Broise, Noiseux et al., 1991; Hu and Peshwa, 1991; Hu and Piret, 1992; Kyung, Peshwa et al., 1994; Laporte, Shevitz et al., 1995; Takamatsu, Hamamoto et al., 1996). These cultures, often referred to as perfusion cultures, can lead to 5-10 fold increase in cell concentration and product throughput over conventional batch and continuous cultures. However, the higher productivity is offset by the greatly increased medium throughput needed to purge the bioreactor of inhibitory metabolites. To overcome these drawbacks of perfusion cultures, it would be desirable to achieve similar cell concentrations with a lower medium throughput while supplying a sufficient amount of nutrients. This can be done by increasing the nutrient concentration in the feed. The potential peril is that the optimal concentration of nutrients for mammalian cell growth is in a relatively narrow range compared to those for microbial or plants cells. Furthermore, the culture environment must be well balanced in osmolality. A high osmolality is known to be growth inhibitory (Ozturk and Palsson, 1991; Oyaas, Ellingsen et al., 1994; Oh, Chua et al., 1995). Increasing the nutrient concentrations in the feed will inevitably increase its osmolality, and may lead to an increased osmolality in the culture.

Cell culture medium has two major categories of constituents: the nutrients consumed by cells, and other 'unconsumable' components, primarily inorganic components which balance the osmolality, provide pH buffer and correct ionic balances. These inorganic salts, in a strict sense, are also 'consumable'. Except for sodium bicarbonate, cells also take them up to provide essential constituents of cells. However, under typical cultivations, the amount consumed by cells is only a very small fraction of that present in the culture medium. In a typical cell culture medium the consumable organic nutrients have a smaller contribution to the total osmolality than the inorganic species. The consumption of organic nutrients causes osmolality to decrease. In many cases, such a decrease in osmolality is partially offset by metabolites produced during cultivation. In a typical batch culture, the change in osmolality due to nutrient consumption and metabolite production is relatively small, as the inorganics are the major contributors of the osmolality. However, in certain cases, a fortified medium, in

Figure 1. Schematic of a perfusion bioreactor with cell recycle.

which the concentrations of consumable nutrients are sometimes increased by ten-fold, is used (Zhou, Rehm et al., 1997). In such situations, the consumption of these nutrients does cause a change in osmolality.

In a continuous culture, it is possible to formulate a proper feed medium so that the supply and consumption of nutrients is balanced. At a high cell concentration at steady state where the nutrient consumption rate is high, a feed with fortified nutrient concentration can be used. The osmolality of the feed is higher than the optimal level for cell growth. Since these nutrients are consumed, they do not contribute to the osmolality in the culture except through the metabolites produced. In other words, despite the use of fortified medium with a high osmolality in the feed, the composition and osmolality of the culture fluid can be maintained at optimal levels by feeding stoichiometric amounts of nutrients.

However, the above stoichiometric considerations do not account for any inhibition caused by overshoot during the transient stage of the culture. Upon switching from batch mode to a continuous culture, cell growth rate and nutrient consumption gradually change and finally settle to the steady state values. A step change from batch culture to continuous process, with the fortified medium as the feed, may result in an overshoot of nutrient concentration and osmolality, and result in growth inhibition. In such cases, the cells might settle to a totally different and much lower steady state concentration. The above considerations call for a more detailed examination of the transient behavior with respect to potential inhibitory effects, which is a focus of this paper.

The model

For general consideration a continuous culture system with cell recycle will be discussed (Figure 1). The dilution rate, D , is defined as the total flow rate through the system (Q) divided by the working volume (V) of bioreactor and the retention ratio (Vits and Hu, 1992), α , as:

$$\alpha = 1 - \frac{X_1}{X}$$

where X is the cell concentration in the reactor and X_1 , the cell concentration in the effluent stream. Referring to the bioreactor in Figure 1, the balance equation for cell mass can then be written as:

$$\frac{dX}{dt} = \mu X - D(1 - \alpha)X \quad (1)$$

where μ is the specific growth rate of the cells. The first term refers to the cell growth while the second term refers to the washout of the cells. The decrease in cell concentration due to cell death is neglected.

The feed constituents have been divided into two categories: those consumed by the cells (nutrients, S_i) and those not consumed by the cells (unconsumables or salts, U_j). The balance equations for these can be written as:

$$\frac{dS_i}{dt} = D(S_{fi} - S_i) - q_{s,i}X \quad (2)$$

$$\frac{dU_j}{dt} = D(U_{fj} - U_j) \quad (3)$$

where $q_{s,i}$ is the specific uptake rate of nutrient i ; S_{fi} , the concentration of nutrient i in the feed; and U_{fj} , the concentration of salt j in the feed. The equations consist of input, output and consumption terms.

Another important variable for the system is the production of potentially inhibitory metabolites (I_k), notably lactate and ammonia. Assuming their concentration in the feed is negligible, the balance equation would consist of the production by cells and washout in the effluent:

$$\frac{dI_k}{dt} = q_{I,k}X - DI_k \quad (4)$$

where $q_{I,k}$ is the specific production rate of metabolite k . A similar equation can be written for the product.

As discussed in the introduction, high osmolality is known to have a profound effect on cell growth. The initial osmolality in the continuous culture is considered to be optimal for cell growth. This is generally

true because the change in the osmolality during the batch culture preceding the continuous culture is usually low. The osmolality in a batch culture varies from 280 to 320 at the end of a batch culture that is within the range for optimal cell growth. It is further assumed that the osmolality is only affected by the change in the concentrations of all the dissolved species and the corresponding dissociable ionic species. The use of a serum free or almost protein-free medium (Zhou and Hu, 1994) is also assumed and therefore the contribution of the proteins to the overall osmolality can be neglected. Thus, the major contributors to osmolality are only the components in the basal medium (namely nutrients and unconsumables) and the metabolites. Additionally, carbon dioxide partial pressure in the gas phase and the mass transfer characteristics in the reactor affect the dissolved CO_2 concentration, and hence osmolality. Base addition for pH control also perturbs osmolality. The balance equation for osmolality can thus be written as:

$$\frac{dOs}{dt} = a \left[\sum \zeta_{s,i} \frac{dS_i}{dt} + \sum \zeta_{u,j} \frac{dU_j}{dt} + \zeta_{I,k} \frac{dI_k}{dt} + \zeta_b \frac{dB}{dt} + 2H \frac{dCO_2}{dt} \right] \quad (5)$$

where Os denotes osmolality; a , the activity coefficient accounting for the deviation from ideality; and B , base added to the culture per unit reactor volume. We further assume the total species derived from dissolved CO_2 (HCO_3^- , CO_3^{2-} and CO_2) to be in equilibrium with CO_2 in the gas phase. Their concentrations are thus related to the CO_2 partial pressure in the gas phase by Henry's law constant (H) for CO_2 . $\zeta_{s,i}$, $\zeta_{u,j}$, $\zeta_{I,k}$ and ζ_b are the number of dissociable ionic species for nutrient S_i , salt U_j , metabolite I_k and base respectively.

The specific growth rate is assumed to follow Monod type kinetics with a limiting substrate (Miller, Wilke et al., 1988; Frame and Hu, 1991). The inhibitory effects of high osmolality and metabolite concentration on specific growth rate is assumed to be multiplicative (Bree, Dhurjati et al., 1988; Glacken, Adema et al., 1988). An expression that fits the existing data in literature is used to describe the inhibition due to increased osmolality (Figure 2). An osmolality of 300 mOsm is assumed to be optimal for cell growth; any value greater than this causes growth inhibition as described by the third term in Equation 6. The expression for metabolite is that reported by Glacken et al.

Figure 2. Comparison of proposed expression (—) for osmolality inhibition with the literature data. Data from Ozturk et al. (●), Oh et al. (▲), Oyaas et al., (■).

(Glacken, Adema et al., 1988).

$$\mu = \mu_{\max} \frac{S}{K_s + S} \frac{K_I}{K_I + I^2} \frac{O_s}{O_s + K_{os}(O_s - 300)^2} \quad (6)$$

where K_s , K_{os} and K_I are constants.

Kinetic parameters and calculations

Glucose is chosen as the growth limiting substrate and lactate as the potential growth inhibitory metabolite. Reported values of the kinetic parameters for glucose and lactate are used (Frame, 1988; Glacken, Adema et al., 1988). The kinetic parameters for the osmolality inhibition term are determined by curve fitting of literature data (Ozturk and Palsson, 1991; Oyaas, Ellingsen et al., 1994; Oh, Chua et al., 1995), as shown in Figure 2.

The composition of medium, i.e., the concentrations of nutrient and unconsumable species used in our studies with hybridoma cells (Zhou and Hu, 1994; Zhou and Hu, 1995; Zhou, Rehm et al., 1995), is used for the calculations. The specific rates for nutrient consumption from those studies are used and are summarized in Table 1.

The CO_2 partial pressure in the gas phase is set to be constant. Thus, the contribution of CO_2 to the change in osmolality can be ignored. We further assume the change of NH_3 concentration to be relatively small. This is a valid assumption because in a typical cell culture, NH_3 accumulates to a maximum value of 3–6 mM (Wang, Zhang et al., 1993; Zhang, A. et al., 1993; Bibila, Ranucci et al., 1994) contributing little to the overall osmolality. Therefore, lactate is the only metabolite that contributes significantly to osmolality. Moreover, we assume that the molar amount of base added to maintain a neutral pH equals that of the lactic acid produced. This has been shown experimentally before (Zhou and Hu, 1994). Therefore, $\text{d}B/\text{d}t = \text{d}L/\text{d}t$, where L is the Lactate concentration in the reactor. We also assume that the medium behaves like an ideal solution, i.e., a is 1.0. Equation 5 thus simplifies to

$$\frac{\text{d}O_s}{\text{d}t} = \left[\sum \zeta_{s,i} \frac{\text{d}S_i}{\text{d}t} + \sum \zeta_{u,j} \frac{\text{d}U_j}{\text{d}t} + 2 \frac{\text{d}L}{\text{d}t} \right] \quad (7)$$

In the following simulations, we only consider the case where no cell recycle is employed. Though the simulation is conducted for a simple continuous culture, the results are certainly applicable to cases where

Table 1. List of used kinetic parameters and constants

Parameter	Value
$\mu_{\max}$	0.055 hr ⁻¹
K_s	0.05 mM
K_{os}	0.01 mOsm ⁻¹
K_L	12,000 mM ²
qGlucose	2x10 ⁻¹⁰ mmol cell ⁻¹ hr ⁻¹
qL	3x10 ⁻¹⁰ mmol cell ⁻¹ hr ⁻¹
qGlutamine	0.8x10 ⁻¹¹ mmol cell ⁻¹ hr ⁻¹
qHistidine	1.8x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qThreonine	1.8x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qArginine	1.1x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qTyrosine	3.7x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qMethionine	1.3x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qValine	4.1x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qPhenyl-alanine	1.3x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qIsoleucine	4.3x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qLeucine	5.1x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qLysine	2.5x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qAsparagine	3.0x10 ⁻¹³ mmol cell ⁻¹ hr ⁻¹
qGlycine	1.4x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qGlutamate	2.0x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qAlanine	-3.3x10 ⁻¹¹ mmol cell ⁻¹ hr ⁻¹
qSerine	4.3x10 ⁻¹² mmol cell ⁻¹ hr ⁻¹
qAspartate	-3.0x10 ⁻¹³ mmol cell ⁻¹ hr ⁻¹

cell recycle is employed. The values of the kinetic parameters and constants used for the simulations are summarized in Table 1.

Results

To achieve high steady state cell concentration in a continuous culture, the concentration of consumable components in the feed should be increased. However, during the transient period following the initiation of continuous flow, the residual nutrient concentration often deviates substantially from the steady state values. Such a deviation may inhibit cell growth. In particular, the use of concentrated feed may result in an overshoot of residual nutrient levels. The high concentration of nutrient or the associated high osmolality may inhibit cell growth. To investigate this, the dynamics of a continuous culture using a fortified feed are illustrated by using the model equations and kinetic parameters listed above. These equations were solved by the Adams Predictor-Convactor method, using Mathematica® 3.0 (Wolfram Research, Inc.,

Champaign, Illinois). To illustrate the effect of nutrient overshoot more clearly, only the inhibitory effect of osmolality is considered. The metabolite inhibition term is dropped from the growth rate equation (Equation 6). The results would be similar if the metabolite inhibition term is included.

For comparison, a control continuous culture using the batch medium (referred to as 1x) as the feed is also shown. The dilution rate is set to 85% of the maximum growth rate. The initial cell condition is set to be 5% of the expected steady state in the control culture. The cell, metabolite and nutrient concentrations are then normalized: reference for cell and metabolite concentrations are the steady state concentrations obtained in the control, while the reference for rate-limiting nutrient is that of the control feed. The osmolality is also normalized with respect to its optimum value of 300 mOsm.

Overshoot due to fortified feed

Three different feed concentrations are used in simulation. The concentration corresponding to the batch medium, as used in the control culture, is denoted as 1x. The other two concentrations, two fold and three fold concentrated, are denoted as 2x and 3x, respectively. In the 2x and 3x medium, only the concentrations of the consumable components are increased to higher levels. Concentration of the unconsumables or salts is maintained at levels of the batch medium. The relative osmolalities of the 2x and 3x feed are 380 and 415 respectively. The dilution rate and initial conditions used for all simulations are identical.

The results of these simulations are shown in Figure 3. With 1x medium as the feed, the cell concentration initially increases and after approximately 400 time units, reaches a steady value of 1 (Figure 3a). The nutrient concentration increases initially and overshoots to a value of 0.9 before decreasing to a steady state value of 0.02 at the same time when the cell concentration reaches the steady state value. This is shown in Figure 3b. The metabolite concentration, shown in Figure 3c, increases proportional to the cell concentration. The osmolality increases slowly upon switching to continuous operations and finally reaches a steady state value of 1.07.

In the case where 2x medium is used as the feed, the cell concentration again increases rapidly after switching to continuous culture. However, after approximately 150 time units, it starts to lag behind that of the control (Figure 3a). The rate-limiting substrate

Figure 3. Effect of concentrated feed in a continuous culture (—): 1x feed (control); (•••••): 2x feed; and (----): 3x feed. (a) shows the cell concentration; (b), the nutrient concentration; (c), the metabolite concentration and (d) the osmolality.

concentration rises to 1.9 at 100 time unit as shown in Figure 3b. This overshoot of nutrient concentration results in a corresponding increase of osmolality (Figure 3d), which in turn inhibits cell growth rate and causes the increase in cell concentration to lag behind that of 1x feed. As the cell concentration increases, the concentration subsides to a steady state value of 0.09. The metabolite concentration is proportional to cell concentration (Figure 3c). Due to a higher metabolite concentration, the osmolality does not decrease when the nutrient accumulation subsides. The steady state osmolality is 1.23.

In the third case, where 3x medium is used as feed, the growth is retarded from the onset of continuous culture (Figure 3a). The nutrient concentration (Figure 3b) overshoots to 2.9 and remains at a high value of 1.9 at steady state. The metabolite concentration, which is proportional to cell concentration, is

also lower than the case of 2x feed (Figure 3c). The osmolality is much higher than the other two cultures in the transient phase and eventually reaches a steady state value of 1.28 (Figure 3d).

In the absence of any inhibition, the steady state cell concentration in cultures is proportional to the concentration of feed medium. However, with 3x feed, the steady state cell concentration is substantially lower than the culture with 2x feed. As in the case of 2x feed, the reduced growth rate and steady state cell concentration is caused by the nutrient overshoot in the transient phase. The nutrient overshoot, though not inhibitory per se, results in a high osmolality that inhibits cell growth. As the cell concentration increases, the nutrient accumulation subsides due to an increase in nutrient consumption. In the culture with 2x feed, the nutrient accumulation is completely alleviated. However, the osmolality remains higher than the control

due to a higher metabolite concentration. In the culture with 3x feed, the growth inhibition caused by nutrient overshoot persists and prevents the cells from reaching a high concentration.

The simulations above demonstrate the potentially detrimental effect of nutrient overshoot during the transient period following continuous culture initiation. In these simulations, the potential inhibitory effect of a high nutrient concentration is not considered, nor is the metabolite inhibition. The effect of overshoot on the time delay in reaching steady state and on the maximum cell concentration will be even more profound if such growth inhibition is considered.

Effect of Initial Cell Concentration

The analysis above illustrates the growth inhibition on use of fortified medium due to nutrient accumulation and high osmolality during the transient period. Such overshoot is largely caused by the supply rate of nutrients being greater than the rate of consumption by cells. Increasing the consumption rate by increasing the cell concentration can eliminate the nutrient overshoot encountered during initial stages. A simple way to do so is to initiate the continuous flow at a cell concentration close to the estimated steady state level. The following simulation illustrates the case where a 3x feed is used for two cultures at the same dilution rate but different initial cell concentrations of 0.05 and 2.5. The first value, 0.05, is one used in the previous section while the latter (2.5) is close to the expected steady state value of 3.0 if growth rate is not inhibited.

The results of these simulations are shown in Figure 4. With an initial cell concentration of 0.05, an overshoot in nutrient concentration and the corresponding high osmolality results in growth inhibition and settles in a relatively low steady state cell concentration. For an initial cell concentration of 2.5, there is no overshoot of nutrient concentration and the cell concentration settles to a steady state value of 2.3 rather quickly (Figure 4a). The metabolite concentration (Figure 4c) also reaches a high steady state value of 2.2 – much higher than the culture with low initial cell concentration. This in turn results in a high osmolality (Figure 4d).

Depending on the initial cell concentration, the dynamic behavior in the transient period differs. These simulation results demonstrate that using a sufficiently high initial cell concentration, the overshoot of nutrients can be minimized. This, in turn, would increase the steady state cell concentration. However,

the steady state cell concentration achieved with a high inoculum is still lower than the maximum the fortified nutrient could support. This is due to a high residual nutrient and metabolite concentration, which increases the osmolality. The high residual limiting nutrient concentration is to compensate the negative effect of high osmolality, as described in equation 6. In this particular example of simulation both cultures have a high steady state osmolality, one (with a low initial cell concentration) caused by a high nutrient concentration, the other (with a high initial cell concentration) caused by a high metabolite concentration.

Effect of dynamic feed

As demonstrated above, the nutrient overshoot during the transient phases can be overcome by starting the continuous culture with a higher cell concentration. This increases the initial nutrient consumption to balance the supply rate. Similar effect can be obtained by controlling the nutrient supply rate to balance the consumption. This can be accomplished by dynamically feeding the nutrient at a rate proportional to the increase in cellular demand. The expression for this dynamic feed is derived from Equation 2.

$$\frac{dS_i}{dt} = D(S_{fi}(t) - S_i(t)) - q_{s,i}X(t) \quad (2)$$

One possible strategy of dynamic feeding is to maintain the nutrient concentration at a constant level that is sufficient to sustain a desired growth rate. This sets dS_i/dt to zero. Furthermore, if no growth inhibition occurs, S_i can be assumed to be small in comparison with S_{fi} . Thus, substituting these values in equation 2 and then taking derivatives over time gives:

$$\frac{dS_{fi}(t)}{dt} = \frac{q_{s,i}}{D} \frac{dX(t)}{dt} \quad (8)$$

This equation can now be solved along with the other model equations discussed earlier to simulate cultures with dynamic feed.

Two continuous cultures initiated at identical cell conditions and dilution rate, but with different feeding strategies are shown in Figure 5. In the first case, the feed is 3x; in the other, the nutrient is fed dynamically as described by Equation 8. The initial concentration for the dynamic feed is 0.13 (calculated to meet the initial nutrient demand of the cells). The first culture has already been described in the previous two sections and the graphs are reproduced in Figure 5 for

Figure 4. Effect of initial cell concentration in a continuous culture (----): low initial cell concentration; (—): high initial cell concentration. (a) shows the cell concentration; (b), the nutrient concentration; (c), the metabolite concentration and (d) the osmolality.

comparison. With the dynamic feed, the cell concentration increases to a relative value of 1.9 – almost two times higher than the first culture (Figure 5a). The nutrient level decreases slightly and then stays constant at a low level of 0.07 (Figure 5b). The metabolite concentration, shown in Figure 5c, increases to 1.9 – again, almost two times higher than the first culture. The osmolality (Figure 5d) too decreases initially, due to a decrease in residual nutrient concentration, and then rises as the metabolite increases. The steady state value of osmolality is 1.22 – only slightly lower than the first culture.

In the above cultures, the steady state cell concentration in the culture with dynamic feed is higher than the culture to which a step change to the 3x feed is applied. This is because the residual osmolality for the first 600 time units is much lower than the culture

with constant 3x medium as feed. This results in a higher growth rate during the transient phase and thus higher steady state cell concentration. As the metabolite concentration increases, the osmolality increases to an inhibitory level, causing the growth rate to decrease till the steady state value is reached. The culture with constant 3x feed, on the other hand, maintains a low growth rate right from the beginning of continuous flow, and therefore the cell concentration is seen to lag behind the other culture.

Discussion

The cell and product concentrations, in animal cell cultures, can be increased by the use of concentrated media. However, the range in which the state vari-

Figure 5. Effect of dynamic feeding in a continuous culture (----): 3x feed; (—): with dynamic feeding. (a) shows the cell concentration; (b), the nutrient concentration; (c), the metabolite concentration and (d) the osmolality.

ables can be maintained in order to maintain optimal growth conditions is narrow. For example, the nutrient concentrations as well as osmolality need to be kept in a narrow range to avoid inhibition of growth rate. This prevents the use of concentrated medium in a batch culture. However, concentrated medium has been widely used in fed-batch cultures, to increase the cell and product concentrations by a factor of nearly 10 over batch cultures (Zhou, Rehm et al., 1995; Bibila, Ranucci et al., 1994; Bushell, Bell et al., 1994; Xie and Wang, 1996). In these cultures, the feed consists of only essential nutrients. Thus, although the feed osmolality is high, most of the nutrients are consumed, contributing little to residual osmolality. A similar strategy can be applied to a continuous culture. By balancing the nutrient concentration in the feed to the demand of the cells, the accumulation of nutrients can be avoided.

The above principle can be applied to a steady state continuous culture with relative ease. However, as demonstrated in this paper, any transient overshoot before the onset of steady state can cause a significant increase in the osmolality due to the accumulation of nutrients in the culture. It is also possible that such high nutrient concentrations may cause growth inhibition. A high nutrient level in the feed may cause an increase in metabolite concentration which can also cause growth inhibition. Since the metabolite accumulation occurs after a large amount of nutrient is consumed, the osmolality inhibition is likely to precede the metabolite inhibition during the transient period. Therefore, in this study we concentrated on the effect of overshoot of osmolality to emphasize its detrimental effect. In all cases, the overshoot is caused by a nutrient supply rate exceeding the cellular demand. In some cases, when the inhibition is not severe,

the time to attain steady state is delayed, but eventually the inhibition is overcome because of the increased nutrient consumption caused by an increase in the cell concentration. However, in other cases, a severe transient osmolality inhibition causes the cells to settle in a different steady state with a lower cell concentration and the inhibition effect is never alleviated. This inhibitory effect can be avoided by balancing the nutrient supply and cellular consumption rates during the transient period. There are two strategies to achieve this balance: increasing the cellular demand when the culture is switched from a batch to a continuous mode (as shown in Figure 4) or, feeding the nutrient at the consumption rate (Figure 5). However, the former is not very practical, as starting a culture at a high cell concentration often requires concentrating the cells from the inoculum seed culture. The latter strategy of increasing the nutrient feed concentration as the cell concentration increases, seems more practical.

A dynamic feeding strategy to adjust the feed concentration in the transient phase, depending on the changing nutritional demand, can be implemented in many ways. If there is sufficient data available, one can evolve a time profile of the feed concentration from the previous nutrient consumption profiles of all the nutrients involved. However, in most cases, the cell growth and nutrient consumption profiles are not entirely reproducible and the use of a predetermined nutrient feeding strategy is likely to lead to a significant deviation from the set point of the residual nutrient concentration. It is therefore necessary to have an on-line estimation of the cell concentration, the residual nutrient concentration, the nutrient demand and a well designed medium delivery system. This can also be done using off-line measurements and increasing the feed nutrient concentration stepwise. However, a more desirable way of implementing this strategy is to adopt an on-line instrumentation and estimation of the metabolic rate of the culture. Such a strategy is commonly used in microbial fermentations (Konstantinov, Nishio et al., 1991; Zhong, Konstantinov et al., 1994; Konstantinov, 1996).

The past few years have witnessed significant developments in on-line instrumentation and control for mammalian cell cultures. Several reports have focused on real time monitoring of biomass concentration (Konstantinov, Chuppa et al., 1994). These include the use of turbidity probes: with a high sensitivity laser sensor (Konstantinov, Pambayun et al., 1992) or with an infrared optical sensor (Merten, Palfi et al., 1987). These methods, however, could measure

only the total cell concentration and could not distinguish between dead and viable cells. Moreover, the nutritional requirement of the cells is not always proportional to the cell concentration. In many cases, the specific consumption rate changes during the course of cultivation (Zhou, Rehm et al., 1997). Therefore, others have adopted on-line measurement of parameters that reflect cellular metabolism. These include the use of OUR (Fleischaker and Sinskey, 1981; Fleischaker, 1982; Nicholson, Hampson et al., 1991; Kyung, Peshwa et al., 1994; Zhou and Hu, 1994), estimation of the ATP production rate (Fleischacker, 1986; Glacken, 1991) and measurement of lactic acid production (Fleischacker, 1986; Glacken, Fleischaker et al., 1986; Hu and Oberg, 1990; Glacken, 1991; Matanguihan, Pambayun et al., 1993). More recently, direct measurements of glucose using enzymatic and non-enzymatic techniques have been applied (Konstantinov, Tsai et al., 1996). The non-enzymatic glucose monitoring techniques include HPLC (Kurokawa, Park et al., 1994) and near-IR spectrophotometry (Riley, M. et al., 1997). Enzymatic instruments applied include *in situ* glucose probes, flow injection analyzers (FIA) and non-FIA analyzers. As an example, Konstantinov et al. (Konstantinov, Tsai et al., 1996) applied the YSI 2700 glucose/lactate analyzer along with an external aseptic sampling module to monitor glucose and lactate on-line. Some or all of these on-line measurement systems can be adapted to control the transient phase feed concentrations as desired.

The simulations shown in Figures 4 and 5 indicate that in cases where the residual concentrations are controlled at low levels, the metabolite concentration eventually reaches a high value. High metabolite concentrations increase the osmolality, which in turn inhibits cell growth. Thus, with fortified medium, as long as metabolite concentration is high, steady state cell concentration will be lower than that without metabolite accumulation. In the above simulations, the metabolite inhibition term was dropped from Equation 6 to emphasize the effect of osmolality. When this term is included, the steady state cell concentration is reduced further (data not shown), as expected. It thus becomes obvious that by using fortified medium alone, one will have only limited success in increasing cell concentration. However, if the metabolism of the cells can be altered to reduce or eliminate the metabolite production, a much higher cell concentration can be achieved. Previous reports in literature (Kurokawa, Park et al., 1994; Zhou, Rehm et al.,

1997) have indicated the possibility of changing the metabolism of mammalian cells by culturing them in low glucose and glutamine environment in fed-batch cultures. Recently it has been demonstrated that this altered metabolism of cells can be maintained in continuous cultures (Hu, Zhou et al., 1998). In this report, the cells were grown in a fed-batch mode with glucose maintained at low levels and the cells were allowed to change metabolism. When the cells were still in the exponential growth phase, the culture was switched to a continuous operation. The dilution rate and feed nutrient concentrations were based on the cell growth rate and their nutrient demand at the time of switching to continuous mode. The cells maintained the altered metabolism in the continuous phase and the metabolite production was near zero.

The above considerations indicate the possibility of using concentrated media in continuous cultures of mammalian cells to increase the cell and product concentrations proportionally. The implementation of this strategy, however, requires careful manipulation due to potential inhibitory effects during the transient phase. The metabolite inhibition further limits the cell concentration achievable. Therefore, a careful implementation of on-line estimation and control is required to maintain the culture conditions at optimal levels. The manipulation of cell metabolism to reduce or eliminate metabolite production also assumes an important role if the cell and product concentrations are to be increased significantly.

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