OPTIMIZATION TECHNIQUES FOR THE PRODUCTION OF MONOCLONAL ANTIBODIES UTILIZING HOLLOW FIBER TECHNOLOGY

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I. Introduction

Hollow fiber technology has many advantages for large-scale mammalian cell culture:
1) High cell densities (> 10^8 cells/ml);
2) Efficient distribution of nutrients and removal of metabolic waste products;
3) Secreted proteins can be harvested in a concentrated form, because they cannot pass through 6-10,000 MW cut-off fibers;
4) Cells are immobilized in the fiber bundle allowing cell-free harvests;
5) Secreted proteins can be harvested very pure (60-95%);
6) Hollow fiber technology is well suited for long-term continuous culture.

Even though these advantages have been well documented for some time,4-8, hollow fiber technology has not been utilized commercially on a large scale until recently. This is because of the technical problems associated with the scale-up of this technology. These limitations to the scale-up of hollow fiber technology are:
1) The need for sophisticated control of the culture environment surrounding densely packed cells.
2) The formation of metabolite and oxygen gradients in the hollow fiber bioreactor.
3) The lack of sufficient nutrient diffusability through a dense cell mass.

A discussion of these limitations and the solutions which we have employed are explained in the following sections.

II. ACUSYST-P

The Acusyst-P cell culture system is designed to automate the production of mammalian cell-secreted products. The system provides controlled conditions for scale-up cell culture through incorporation of advanced hollow fiber technology, continual media feed, and state of art process control strategies. The Acusyst-P allows monitoring and feedback control of vital parameters to maintain cell growth, viability, and secretion.

The Acusyst-P consists of two independently controlled flowpaths, which contain 6 hollow fiber cartridges each, or a total of 12 cartridges per instrument. Each hollow fiber cartridge in the Acusyst-P flowpath has a total fiber surface area of 1.4 cm². Cultures within these cartridges routinely reach densities of 5 x 10^6 to 10^8 cell/ml. Hence, each instrument can support 10^11 to 10^12 viable cells over an extended period of time (months).

Process control in the Acusyst-P is specifically designed to meet the requirements of a dense culture. The software uses computer controlled algorithms to balance nutrient feed, waste removal, and to maintain pH at specified setpoints. Critical parameters such as pH, oxygen, glucose, lactic acid and
serum levels fluctuate rapidly in a dense culture, thus a process control technique that responds rapidly is essential. This control strategy relies on continued media feed, online pH and dissolved oxygen monitoring, and offline glucose and lactic acid monitoring. This strategy combined with data entry provides specific control over culture conditions.

In addition to control parameters, the Acusyst-P is capable of continual harvest product. Which allows the user to predict future product concentrations. This is especially relevant with cells that are sensitive to product feedback inhibition. In addition to continual product harvest, each flow path can be gradually weaned from serum, such that hence culture become essentially serum free. Hollow fiber cartridge technology provides a demonstrated advantage in this regard, which significantly reduces downstream product purification costs.

III. control of the Culture Environment

The need for an advanced system of process control for the extracellular environment is an inherent difficulty with all dense culture scale-up technologies. Systems operating at high cell densities (5x 10^6 cells/ml) experience rates of change in culture conditions that are more rapid than low density culture (5x 10^6 cells/ml)^9,10. The principal reason for this rate of change is that the densely packed cells are exposed proportionally to a smaller volume of media at any one time, thus the buffering and nutrient capacity of the culture media is extremely limited. This causes the loss of sufficient pH control and the rapid accumulation or depletion of metabolites. To overcome these difficulties in hollow fiber technology, we have developed sophisticated process control software for pH, glucose, and lactate to maintain user determined setpoints. pH is controlled primarily by gassing, with base addition and medium dilution providing secondary and tertiary control systems. Glucose is controlled by glucose factor addition, with medium addition available as a secondary glucose control capability. Lactate is controlled by base addition and medium dilution.

A. pH CONTROL

The software utilizes a hierarchy of three different algorithms to control pH within ± 0.03 pH units. The first algorithm controls the CO2 mixture. Bicarbonate buffered culture medium allows pH to be controlled at a setpoint by automatically increasing or decreasing the amount of CO2 that is delivered to the gas exchange cartridge (Figure 1). The second algorithm is base (NaOH) addition to the culture medium. The addition of base rejuvenates the buffering capacity of the medium and counters the increased lactic acid production rate of a growing culture. The third control algorithm is medium dilution, as this is the most expensive method. Increased medium delivery to the culture effectively increases pH by removing waste products, i.e. lactic acid, more rapidly.

B. GLUCOSE and LACTIC ACID CONTROL STRATEGIES

The metabolic data is measured offline and is then entered into the computer. The software then calculates medium and glucose pump rate corrections from the entered data based on desired setpoints 24 hours in the future. The software does this by determining the time in hours between samples, the total volumes pumped by each pump, and the average flow rates of each substance over the sample interval. This information, combined with known glucose concentrations in medium, glucose factor, and serum (data supplied by the user), provides all information necessary for calculation of glucose consumption and lactate production values, and for control of pump speeds to maintain the user desired setpoints.

The following definitions will be used in the uptake and production formulas which follow:
Figure 1a

Acusyst-P Flowpath Recirculation Circuit

Figure 1b

Flow Path, Extracapillary Space Expansion Circuit
Note: CV 1-3 are Checkvalves.
Fm = flowrate of medium
Fg = flowrate of glucose factor
Fs = flowrate of serum factor
Fb = flowrate of base add factor
Gm = conc. of glucose in medium
Gg = conc. of glucose in glucose factor
Gs = conc. of glucose in serum factor
Ft = total flowrate = Fm + Fg + Fs + Fb
E = \( \exp(-\frac{Ft}{V}) \)
T = time in hours since last sample
V = system volume in ml = 1146 + (190 \times (# of cartridges))

1. GLUCOSE UPTAKE

Glucose concentration is controlled at a user-defined setpoint by combining medium with a supplemental glucose factor feed. The software first calculates the lactate production rate (L.P.R.) and then the glucose uptake rate (G.U.R.), based on actual sample concentration. The amount of glucose required to maintain a specific level is determined and compared to the glucose contributed through continued media feed. If a need for additional glucose exists, a flow rate is calculated for a supplemental glucose factor feed. Glucose factor feed is accomplished by attaching a separate media source supplemented with 3000 mg % glucose.

Glucose uptake for the most recent sample period is based on the following formula:

\[
\text{GUR} = \frac{Gx - FtG - E(Gx - FtGo)}{1 - E}
\]

where:
GUR = Glucose uptake Rate
G = current glucose reading
Go = previous glucose reading
Gx = \((FmGm) + (FgGg) + (EsGs)\)

If no fluids have been pumped into the flowpath since the last sample, the uptake rate is defined to be:

\[
\text{GUR} = \frac{(V)(Go-G)}{T}
\]

2. LACTATE PRODUCTION RATE

Similar to glucose, lactate concentrations are measured offline and date subsequently entered into the computer. The software then calculates the production rate and determines a medium pump rate that controls lactic acid concentration around a predetermined setpoint.

The calculation of lactate production is:

\[
\text{LPR} = \frac{(Ft)(L-LoE)}{1 - E}
\]

where:
LPR = Lactate Production Rate
Lo = lactate concentration or previous sample
L = lactate concentration of current sample

If no fluids have been pumped into the flowpath since the last sample, the lactate production rate is defined to be:

\[
\text{LPR} = \frac{(V)(Lo-L)}{T}
\]
3. **GLUCOSE AND LACTIC ACID PROJECTION FORMULAS**

Based on glucose uptake and lactic acid production rates, the software attempts to maintain glucose concentration at the glucose setpoint in each flowpath, and the lactate concentration at the lactate setpoint. The projection is attempted for the 24 hours immediately succeeding the sample; the metabolic calculation algorithm is executed every time sample data is entered, so pump speed estimates are assumed to be constant over the following 24 hour period.

The glucose and lactate levels for any sample period may be determined by rearranging the formulas in the preceding sections.

At time \( t \) (defined to be 24 hours after the current sample), the glucose concentration \( (G_t) \) and lactate concentration \( (L_t) \) will be:

\[
G_t = \frac{Gx - GUR + (E)(GUR + FtG + Gx)}{Ft}
\]

where

\( G_t = \) glucose concentration at time \( t \)
\( G = \) current glucose concentration;

and

\[
L_t = \frac{(E)(FtL - LPR) + LPR}{Ft}
\]

where

\( L_t = \) lactate concentration at time \( t \)
\( L = \) current lactate concentration

Optimally, \( G_t \) and \( L_t \) correspond closely with the user’s glucose and lactate setpoints. Direct solution of the projection equation is not possible. The technique used to solve for the necessary pump rate that will give a glucose or lactate concentration which is acceptably close to the setpoint is a binary search with all variables held constant except flowrate.

The medium pump rate required to control lactate concentration is calculated first, followed by glucose factor rate (using the updated medium pump rate previously calculated), followed by another medium pump adjustment to supplement glucose if the glucose factor pump is unable to satisfy demand.

IV. **Control of Metabolite and Oxygen Gradients**

An additional limitation to scale-up utilizing hollow fibers for the growth of mammalian cells has been the formation of oxygen and metabolite gradients caused by what is referred to as the Starling effect\(^7\). This is a convective flow through the extracapillary space (ECS) of the cartridge caused by pressure differences from the front to the back in response to luminal media flow\(^11\) for review. These gradients permit only part of the cell mass within the bioreactor to be at the optimal conditions for growth, while the cells at the outlet of the bioreactor are subjected to depleted media conditions. To resolve this problem, a secondary flow through the ECS of the cartridge is created by controlling the ultrafiltrative flow from the lumen of the fibers past the cell mass. This media is then automatically transferred into and out of an expansion chamber attached to the ECS of the bioreactor (Figure 1). By this method the fluid dynamics of the ECS environment are greatly improved, causing the cells to be bathed with an even distribution of nutrients and improving the removal of waste and inhibitory products\(^13\).
Control of the dissolved oxygen concentration has been found to be extremely important for cell growth and monoclonal antibody production for a variety of hybridomas. The control of dissolved oxygen and the associated oxygen gradients within the cell mass has been considered one of the missing factors in the use of hollow fiber technology. The extent of the oxygen gradients in hollow fiber bioreactors has recently been thoroughly characterized, and was found to be controlled in this system by variable media delivery rates through the lumen of the hollow fibers. Removing the requirement of adding pure oxygen to the gassing mixture eliminates the possibility of oxygen becoming a cytotoxic factor. The next area of interest for the optimization of hollow fiber type systems, along with any other scale-up system is the effect of nutrient and waste metabolite concentrations on the ability of the cells to grow and, more importantly, to produce.

V. MONOCLONAL ANTIBODY PRODUCTION

A major problem in the large-scale production of monoclonal antibodies in a ACUSYST-P has been to determine the most favorable environment for the cells. Our initial approach was to analyze kinetic data from a 5-7 day batch culture. Between 0.5 – 1.0 x 10^6 cells/ml were inoculated into T 25 flasks. Aliquots were removed daily and assayed for glucose, lactate, cell number, pH and product concentration. A computer program was written which generated curves to fit the kinetic culture. These curves were used to determine the growth rates and production rates per cell number for each hour of the study. A typical hybridoma cell “finger print” from this computer model is shown in Figure 2. Cells grown in this manner follow a predictable pattern of an initial lag phase, exponential growth phase, slowing to a preliminary stationary phase, stationary phase and a subsequent death phase. Glucose consumption follows an initial lag phase, followed by an exponential phase concomitant with the cell growth phase. Lactate is produced in approximately a 1:1 ratio to glucose consumption. PH begins in the range of 7.35 - 7.45 and steadily decreases with time. Late in the death phase pH may show a slight increase. Product accumulates proportionally to cell growth, but continues to increase even during the death phase.

The increase in product concentration during the death phase is associated with increased production per cell, not with intracellular product released from lysed cells (unpublished data). The environmental conditions which cause the shift from stationary phase to death phase is commonly attributed to one or a combination of the following: Low nutrient levels, high metabolic waste products, low pH levels, or cellular produced feedback inhibition. The exact mechanism causing cell population to shift into the death phase is probably an intricate combination of all these factors, and perhaps other as yet undefined factors. The essential amino acid concentrations were also tested to determine if their uptake rates followed the trend of the glucose uptake rate. The amino acid uptake rates appeared to follow the glucose uptake rate and surprisingly, tyrosine, methionine, valine and
lysine were actually utilized very quickly along with L-glutamine for the AFP-27 murine hybridomas tested here (Table 1).  

Our initial process control strategy for hybridoma cells (S.O.P.-standard operating procedure) was to control the cellular environment such that conditions were not allowed to deteriorate past the environmental conditions associated with the stationary phase. Setpoints for pH, glucose and lactate were selected at the apex of the growth curve (Figure 3). This process control strategy resulted in very unpredictable production. Table 2 shows production data from four different hybridoma cell lines in an ACUSYST-P. Total production ranged from 0.40 to 2.10 grams/day. No correlation existed between production in a T-flask and production in an ACUSYST-P. Another problem was that the production data was not always repeatable from each production run. With the same process control setpoints, production from the same cell line could vary up to 400%. To determine the cause of this variability, we investigated the metabolic kinetic data from two production runs from the same murine hybridoma which resulted in significantly different production.

In the first production run, glucose was consumed and lactate produced until the levels reached the set control points, 150 mg% and 100 mg% respectively. From that point, glucose and lactate were kept in control. pH was maintained throughout at 7.15. Total production over a 12 day period was 2.61 grams of IgG.

In the second production run, glucose was kept at relatively high levels initially (200 mg%) then allowed to fall to relatively low levels (125 mg%). Lactate was kept low initially (100 mg%) then gradually reached very high levels (200 mg%). PH gradually fell in time, then rose slightly toward the end of the run. Total production over a 12 day period was 18.55 grams of IgG.

**Table 1.** Amino Acid data for AFP-27 cells grown in McCoy’s SA media. Cell/ml is (x 10⁵), amino acid data is in micromolar.

<table>
<thead>
<tr>
<th>Days</th>
<th>Cell/ml</th>
<th>ARG</th>
<th>LYS</th>
<th>GLN</th>
<th>THR</th>
<th>ALA</th>
<th>VAL</th>
<th>ILE</th>
<th>MET</th>
<th>HIS</th>
<th>GLU</th>
<th>TYR</th>
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<tr>
<td>0</td>
<td>1.17</td>
<td>91</td>
<td>43</td>
<td>905</td>
<td>28</td>
<td>76</td>
<td>37</td>
<td>56</td>
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<td>69</td>
<td>18</td>
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<td>1</td>
<td>2.33</td>
<td>86</td>
<td>30</td>
<td>766</td>
<td>11</td>
<td>95</td>
<td>32</td>
<td>47</td>
<td>14</td>
<td>33</td>
<td>66</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>9.11</td>
<td>76</td>
<td>19</td>
<td>438</td>
<td>11</td>
<td>101</td>
<td>16</td>
<td>25</td>
<td>6</td>
<td>34</td>
<td>67</td>
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<td>48</td>
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<td>6</td>
<td>163</td>
<td>9</td>
<td>22</td>
<td>4</td>
<td>31</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
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<td>3.78</td>
<td>46</td>
<td>6</td>
<td>262</td>
<td>6</td>
<td>214</td>
<td>10</td>
<td>20</td>
<td>3</td>
<td>29</td>
<td>29</td>
<td>0</td>
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**Table 2.** Production data from an Acusyst-P.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Product</th>
<th>Harvest Concentration(ug/ml)</th>
<th>Total(g/day)</th>
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</thead>
<tbody>
<tr>
<td>mouse x mouse IgG</td>
<td>5800</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>human x mouse IgG1</td>
<td>420</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>mouse x mouse IgG2</td>
<td>1000</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>human x mouse IgG2a</td>
<td>1109</td>
<td>2.10</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 3.** Common "Finger Print" of Kinetic Data for Batch Culture with S.O.P. (Standard Operating Procedure) control parameters selected.
These results indicated that the metabolic environment around the cells can have a significant influence on the cellular behavior. Conditions that more closely simulated the dynamic environment found in static culture resulted in higher production than the more well-controlled environment in the first production run.

This led us to investigate whether we could create conditions which would favor cell production. The metabolic “finger print” for these cells was reexamined to determine growth setpoints. The point at which the rate of cell number increase was greatest was determined. Glucose and lactate setpoints were determined at that point (see Figure 4). PH at this point was very low (about 6.8). This was considered to be too low to initiate cell growth. Therefore, the pH setpoint was selected at the point where the rate of cell growth was greatest (see Figure 4).

Production setpoints were selected at the point where production per cell was greatest (see Figure 5). Greatest production per cell occurred well into the death phase. Nutrient uptake on a per cell basis, usually characterized by glucose and glutamine utilization, is generally greatest during the exponential growth phase, when the cells are producing the greatest biomass and increasing cell size. This uptake rate is commonly between 1.6 and 2.0 x 10^{-5} mg/10^6 cell hour, and then slows to nearly zero. An uptake rate of 0.7 to 1.9 x 10^{-4} mg/10^6 cell hour is commonly seen for glucose by the hybridomas in our laboratory and remains fairly steady during the stationary and death phases. This implies that if the environmental conditions surrounding these phases of the growth curve could be maintained, without cell death, then the efficiency of media utilization to antibody production would be optimal. We tested the effect of high levels of lactate on cells in static culture (see Figure 6). Lactate inhibited the growth of cells, but did not affect viability. However, high levels of lactate also caused an increase in the production per cell (see Figure 7). These results were ideal for continuous hollow fiber culture, as they implied that a confluent culture could be inhibited from growing by high lactate levels allowing the culture to last a longer time. Additionally, the culture would potentially produce more per cell. Therefore, the lactate and glucose production setpoints were selected at the point of highest production per cell. The pH setpoint was selected at the apex of the growth curve. It has been noted in our laboratory that if the
lactate levels in the bioreactors are allowed to reach concentrations above 400 mg%, and pH is not controlled above 6.5, the cells die almost immediately. However, under the same conditions if the pH is controlled at 6.8 or above the cells show no detrimental effects (unpublished data).

**Figure 6**
Growth curves of AFP-27 hybridoma cells grown in varying lactate concentrations.

**Figure 7**
Production of IgG per cell number for AFP-27 hybridoma cells grown in varying lactate concentrations.
FIGURE 8
Comparison of glucose uptake rate (GUR) and lactate production rate (LPR) for growth phase and product phase setpoints.

The comparison of growth phase parameters to production phase parameters gave the results expected. The metabolic growth parameters, G.U.R. and L.P.R., showed that the flowpaths controlled at the growth phase conditions increased their metabolic rates exponentially (Figure 8). Since these metabolic rates are primarily dependent on the viable cell number, one may assume that the cell population is increasing at a similar rate. The calculated doubling time of the cells in the system, utilizing this metabolic data, was about 22 hours, compared to 18 hours in batch culture systems.

The systems controlled at the production phase conditions showed the opposite response compared to the growth phase systems. When the flowpaths were controlled at the production phase parameters the cells repeatedly did not show any substantial increase in their metabolic rates (Figure 8), implying a near constant viable cell population of $2 \times 10^9$ cells, based on metabolic rates. One may speculate that metabolic rates do not increase in this system because the death rate is equal to the growth rate within the cell population. If this were true, then the visible cell density should have increased as the concentration of dead cells increased. However, there was no substantial increase in the visible cell mass during these experimental runs. The differences between the cells grown at the growth phase and production phase parameters can easily be seen in the metabolic rates shown in Figure 8.

With this data on growth and production phase parameters, it was possible to grow the hybridomas to high densities quickly and then shift to new control parameters that would keep the cells at or near a stable population. This approach in contrast to conventional production systems would now allow for product to be removed sooner and at a higher concentration. The metabolic rates for this new
FIGURE 9
Comparison of glucose uptake rate (GUR) and lactate production rate (LPR) for the standard operating procedure (S.O.P.) and the enhanced process control.

FIGURE 10
Comparison of production per day of one flowpath in an Acusyst-P between S.O.P. and the enhanced process control.
control strategy compared to the rates at the standard control conditions are shown in Figure 9. These show that during the growth phase there is a substantial increase in the cellular rate of metabolism, and that after the cells had grown it was possible to maintain a highly viable culture. Figure 10 shows the difference in production rates for these two culture strategies. In the culture that was grown quickly and then maintained, there was an increase in production of about 100% over the standard setpoints.

VI. CONCLUSIONS AND DISCUSSIONS

There is sufficient data to speculate that for the optimization of monoclonal antibody production from hybridomas, a controlled extracellular environment is necessary. Utilizing this control in hollow fiber systems allows optimal cell growth "in-vitro" with the ability for subsequent inhibition of growth and maintenance of a viable cell population for production. The preliminary data shown here implies that selecting the environmental parameters associated with the cellular response desired will allow that response to be maintained when controlled in a hollow fiber cartridge. There is also the possibility that for optimal production of monoclonal antibodies from hybridomas utilizing hollow fiber technology, it is necessary to use two sets of control parameters, one for cell growth and another for cell production. Utilizing this control allows for the achievement of high cell viability in dense culture for maximum production. Furthermore, this strategy could become a standardized method to determine process control strategies for any cell line.

REFERENCES

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15. Tyo, M. (manuscript in progress).
