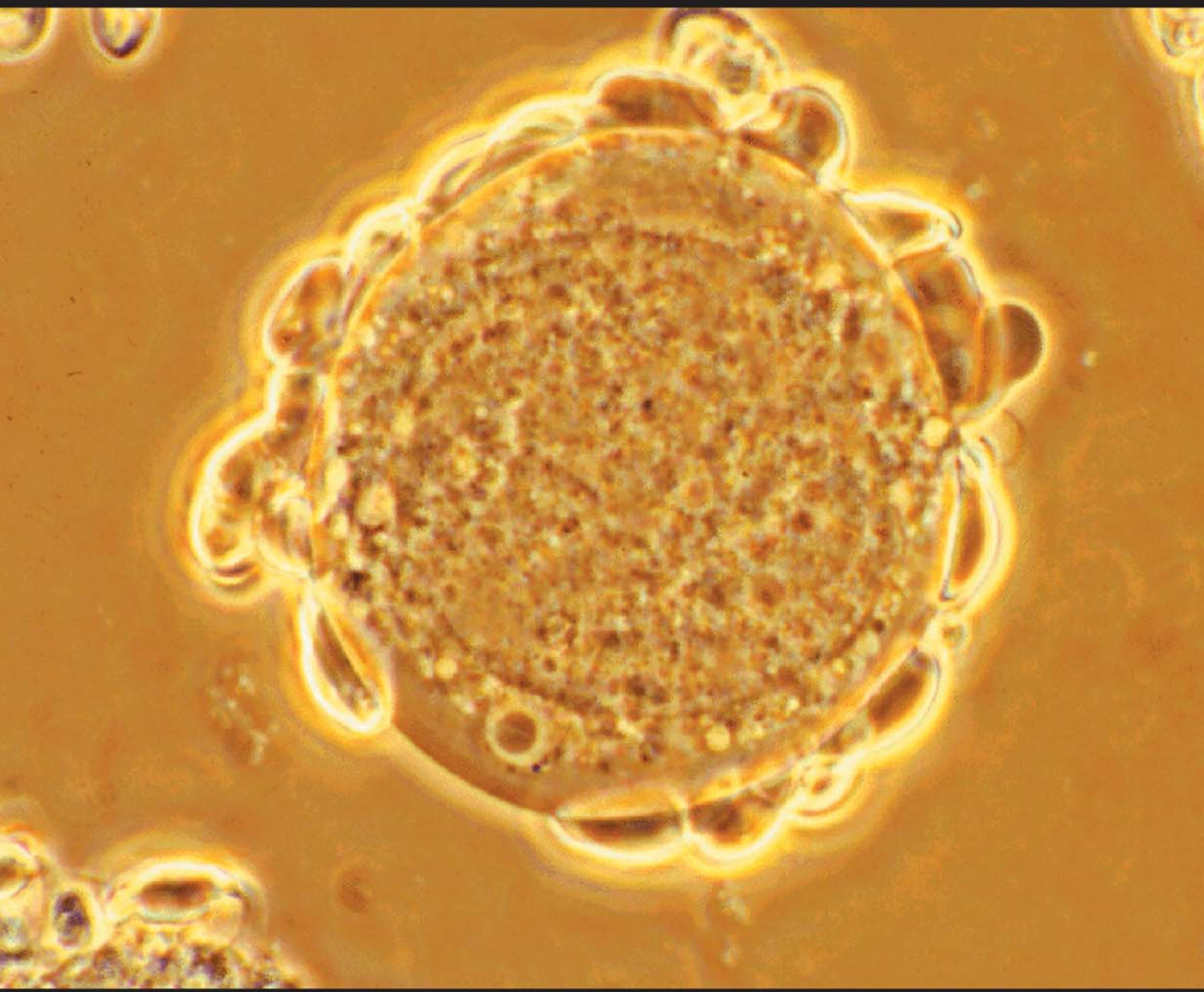
A Publication of
The Williamsburg BioProcessing Foundation

January/February 2003
ISSN 1538-8786

BioProcessing JOURNAL

Advances & Trends In Biological Product Development



Vol. 2/No. 1

www.bioprocessingjournal.com

Advances in High Cell Density Culture Technology Using the Sf-9 Insect Cell / Baculovirus Expression System — The Fed-Batch Approach

BY CYNTHIA B. ELIAS, ARNO ZEISER AND AMINE KAMEN

Table 1: Recombinant proteins produced on a large scale (3-100L) at the Biotechnology Research Institute using the insect cell-BEV system.

Reporter Proteins	β-galactosidase: Secreted alkaline phosphatase (SEAP) Green fluorescent protein (GFP)
Intracellular proteins	Bovine rotavirus nucleocapsid protein (rBRVP6) Human protein tyrosine phospatase (hPTP1C) Herpes simplex virus ribonucleotide reductase (HSVRR) Viral PyLT nuclear protein Human ribonucleotide reductase subunits (hRR_1, hRR_2) GATA differentiation factor protein Human cytosolic phospholipase (cPLA2) Human lipoxygenase (h5LO) Human cyloxygenase ($hCOX_1$, $hCOX_2$) Human phosphodiesterase (PDE IV family) Cysteine proteases Cytochrome P450s Oxido reductase Cathepsin
Secreted proteins	Papain Yeast serine proteases Calnexin Epidermal growth factor receptor exracellular domain (EGFR-ED) Type II transforming growth factor receptor extracellular domain (TGFβRII-ED) Human interleukin (rhIL-5) Human transcobalamin II protein (rTCII) Human glycosyltransferase I (rGnTI) Human nerve growth factor receptor kinase extracellular domain (trkA-ED) Bone morhogenetic protein receptors Extracellular domain (BRK proteins) rat UDP-glucosyl transferase (RUG-T)
Membrane proteins	G protein subunits Human muscarinic receptor (HM1) Dopamine receptor (DOP1) Serotonin receptor (5HT1α) Yeast G proteins (STE4, STE5, STE18)

The Sf-9 insect cell / baculovirus expression system is one of the most commonly used protein expression systems. It is the preferred system for generating large amounts of protein in a short period of time, and it has been successfully used to express several hundreds of different proteins. A representative list of the different proteins made in our laboratory over the past decade with the Sf-9 insect cell / BEVS system is given in Table 1. These proteins are often used in drug screening studies and structure function analysis. Proteins intended for therapeutic purposes are not normally produced using this technology, although a few examples do exist. There is also an unexplored potential for the cells to be used for the production of recombinant viral vectors. Recent reports demonstrating the ability of baculoviruses to express proteins in mammalian cells, with mammalian promoters, indicate that BEVS technology might soon have a major role to play in the field of gene delivery. There

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have also been reports on the use of the system to generate recombinant viral vectors, such as adeno-associated viral vectors (AAV), which are difficult to produce in large amounts using traditional transfection techniques in mammalian cells. Additionally, there have been reports on the generation of stable insect cell lines that are able to express recombinant proteins continuously. These developments have led to a resurgence of interest in the use of insect cells as protein expression systems.

For most of these applications, it is necessary to develop processes that can operate on a large scale, and in an efficient, reproducible and robust manner. It is also desirable to maximize the productivity of the process with respect to the volumetric and cell-specific yields. Some of the developmental work conducted on insect cell technology at the Biotechnology Research Institute, with reference mainly to the *Spodoptera frugiperda Sf-9* insect cell line, is presented here. The focus of this work is real-time monitoring of the bioprocess to gain information on the physiological status of the cells, and then extending this analysis to develop rational feeding strategies for high cell density conditions. The resulting fed-batch strategy leads to an increase in the volumetric productivity without a loss of cell-specific yield, and thus truly intensifies the process at a given scale.

Table 2: Comparison of cell growth and β-galactosidase production in fed-batch culture of Sf-9 cells using different feeding strategies.

Feeding strategy	Number of Feeds	Max cell density reached	Cell density at infection cells/mL	β-gal activity U/mL	Specific β-gal activity U/10^6 cells	Control[a] U/10^6 cells
Pulse	5	38 x 10^6	-	-	-	-
Pulse	4		13 x 10^6	19.51	12.5	31.0
Semi continuous	4	52 x 10^6	-	-	-	-
Semi continuous	3		13 x 10^6	372.16	28.62	31.0

[a]Control: β-galactosidase production with cells infected at 2.5x10^6 cells/mL in batch culture in shake flask with complete medium replacement.

Background

The *Spodoptera frugiperda Sf-9* baculovirus expression vector system (BEVS) is one of the most commonly

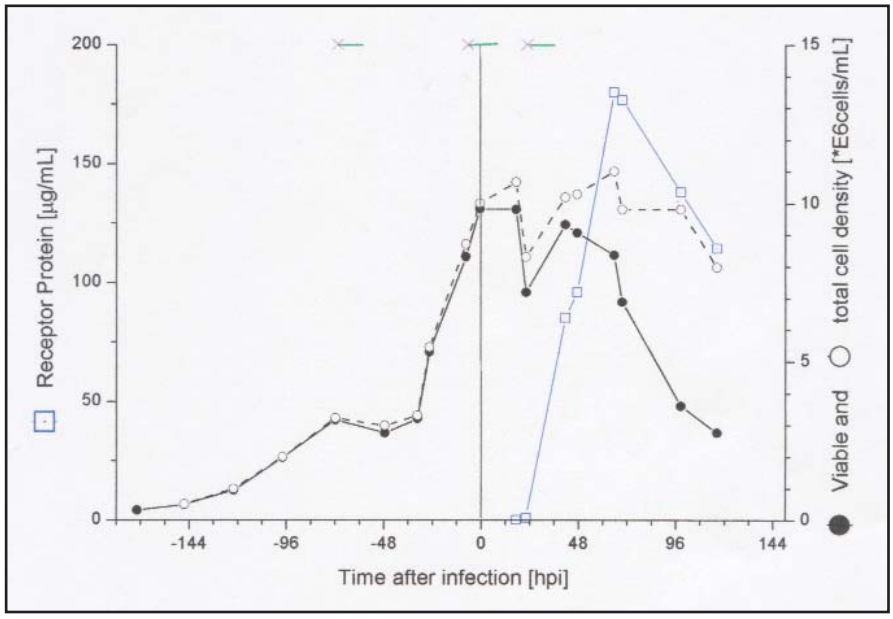

Figure 1. The time course of viable and total cell densities, and the production of a receptor protein in a fed-batch culture of Sf-9 cells growing in Excel-420 medium (JRH Biosciences, Lenexa, KS). The x marks the time of addition, and the horizontal line indicates the time period over which the addition was made.

used insect cell systems for the production of recombinant proteins.[11,15,17]

A tremendous amount of research has been directed at improving and optimizing this process to obtain higher yields of recombinant protein by independent research groups.[3,8,16,18] The protein yield of a system can be increased by achieving higher cell densities and producing at these densities.[4,9] Other reports describe work done on increasing the maximum cell density with improved medium in both fed-batch and perfusion culture.[22,21,16,23,1,10] To a greater or lesser extent, all of these approaches were successful in increasing the cell densities. Maximum cell densities of 10^7 cells/mL can be routinely achieved in some commercial serum-free media.[20] Nevertheless, higher per cell productivity is not always the result.

In synchronously infected cultures in serum-containing medium, the volumetric productivity of baculovirus expressed proteins increases with an increase in the cell density only up to a critical density of 1-3x10^6 cells/mL.[3,13,14] It has been reported that this density varies between 5x10^6 to 7x10^6 for serum-free medium.[18,19,5] It has been consistently found that the critical cell density at infection is about 3-4x10^6 cells/mL for serum-free, and between 1-2x10^6 cells/mL in serum-containing media. However, going beyond these densities results in a loss in cell-specific productivity. In general terms, the limitations to higher productivity may be due primarily to nutrient depletion or to the accumulation of toxic metabolites. The latter is not a major problem with Sf-9 cells, since there are several published reports which show that these cells do not accumulate waste metabolites such as lactate and ammonia. In addition, the accumulation of alanine by these cells seems to have no effect on their growth.[23,1,6] This effect would indicate that the limitations to growth and production are essentially a result of nutrient depletion. The fed-batch mode was therefore adopted as a

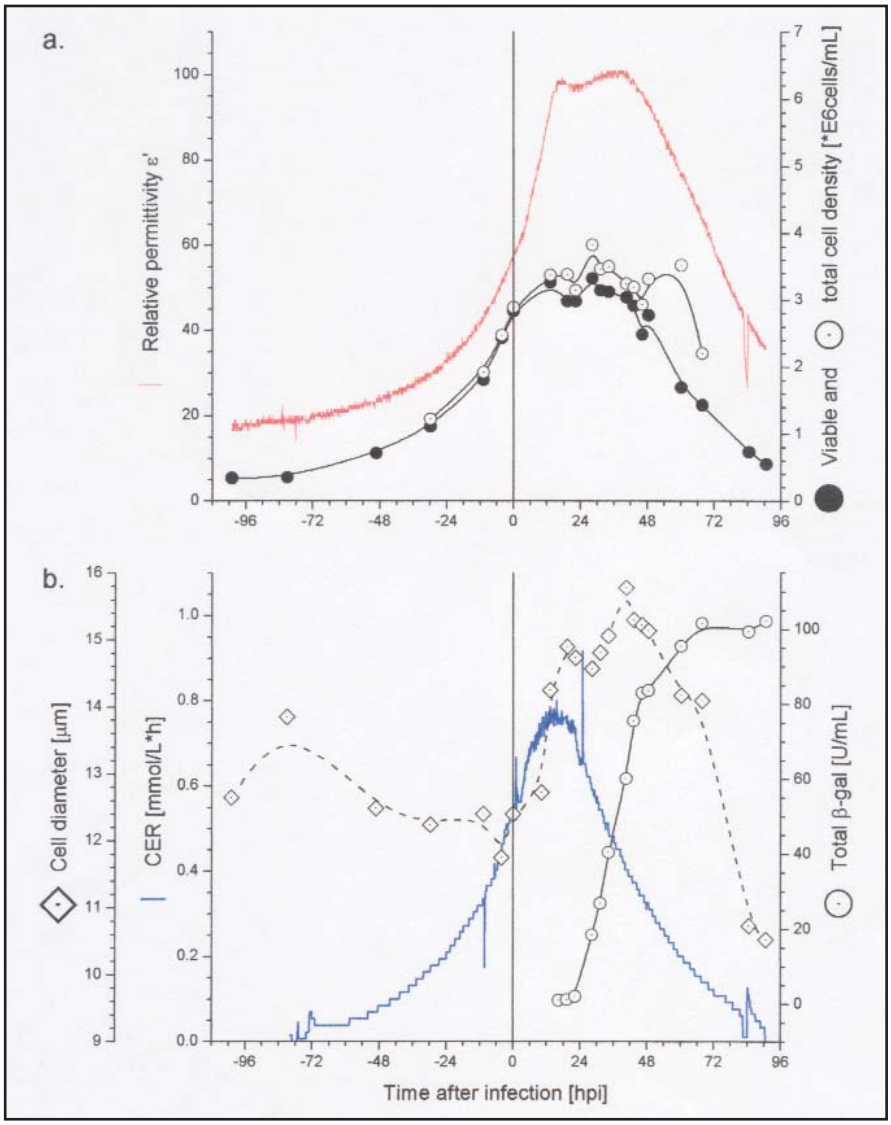

Figure 2: The time course of relative permittivity, viable and total cell densities (Fig. 2a), CO_2 evolution rate (CER), cell diameter, and total β-galactosidase (Fig. 2b) concentration in a batch culture of Sf-9 cells infected at a MOI=10.

method to overcome these limitations. This method offers several advantages over the traditional perfusion methods to replace the depleted culture medium. Noteworthy among the advantages is the elimination of special devices for cell retention, as well as the avoidance of handling large volumes of media.

The fed-batch method also avoids the use of enriching medium which initially causes deleterious imbalances in the osmolarity. A significant amount of work had been done earlier in our laboratory that identified the key nutrients for the growth and production process. The results led to the development of nutrient cocktails, comprising multiple components, which were added as step additions in the fed-batch culture. This development allowed the growth of cells to a maximum density of $38x10^6$ cells/mL.[2] However, the problem of production at higher cell densities was still largely unresolved. A seminal breakthrough was achieved by using the feed cocktails and combining them with a feeding regimen more attuned to the nutrient consumption of the cells. The results of these experiments, and the on-line monitoring and control systems used to achieve this higher production, are described below.

METHODS

Cells, Media, Virus, Bioreactor and Accessories

Sf-9 insect cells were maintained in SF900-II medium (GIBCO-Invitrogen in Carlsbad, CA) at 27 °C in shaker

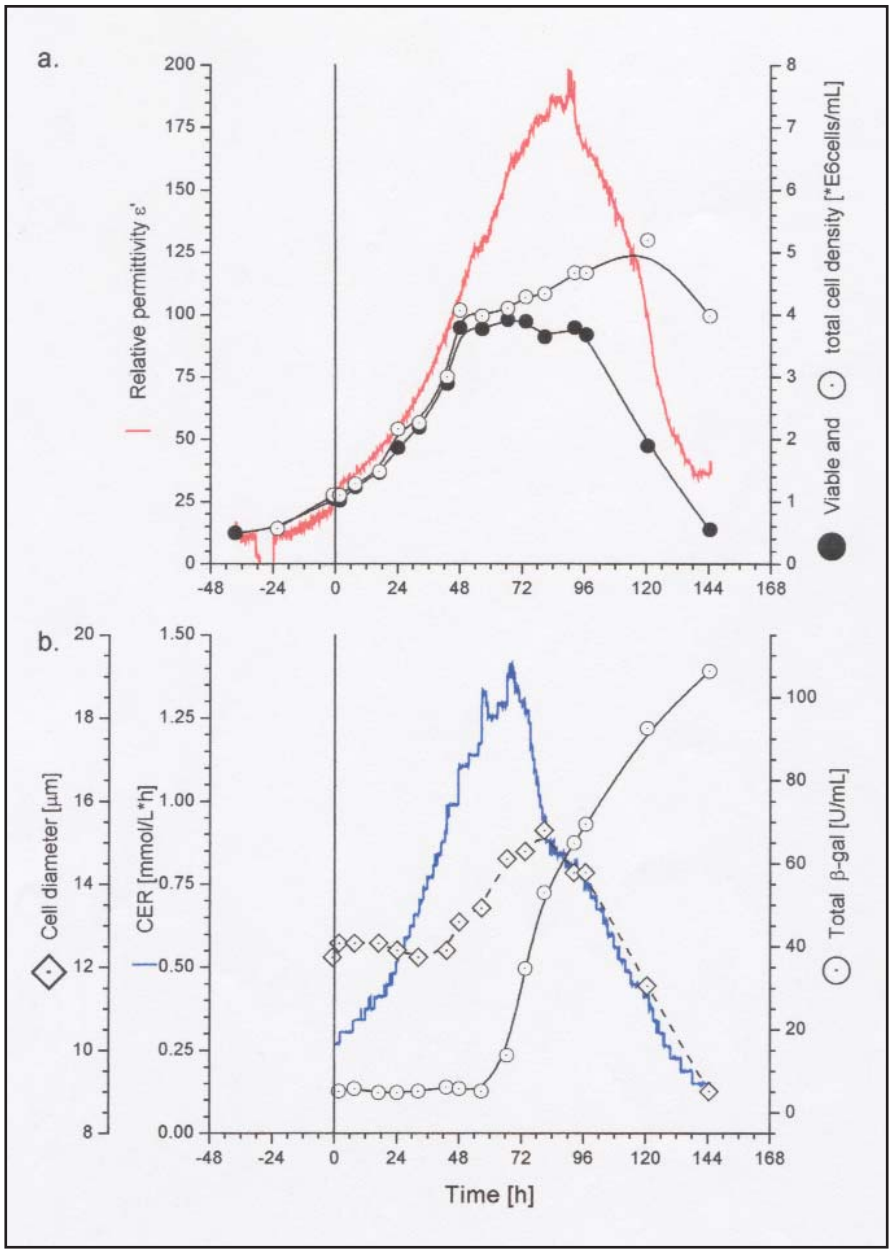

Figure 3: The time course of relative permittivity, viable and total cell densities (Fig. 3a), CO_2 evolution rate (CER), cell diameter, and total β-galactosidase (Fig. 3b) concentration in a batch culture of Sf-9 cells infected at a MOI=0.001

flasks that were agitated at 110-120 rpm. A recombinant baculovirus expressing β-galactosidase was amplified in the Sf-9 cells.

Bioreactor and Associated Instrumentation

The experiments were carried out in a Chemap 3.5 L, Type SG, bioreactor (Männedorf, Switzerland). A Chemap FZ-2000 control unit was used to control the temperature and agitation speed at 27 °C and 120 rpm respectively. Dissolved oxygen (DO) was monitored using a polarographic electrode, and pH was measured with a gel electrode (Ingold, Andover, MA). The DO was maintained at 40% of air saturation using a monitoring and control system as described in Kamen *et al.*[12] Capacitance (pF) and conductance (mS) were measured on-line using the Biomass Monitor (BM), model 214M (Aber Instruments Ltd., Aberystwyth, U.K.), and the relative permittivity (ε') was calculated as described in Zeiser *et al.*[24] The CO_2 generated by the system was measured in the outlet gas with an infrared analyzer (Servomex, 1400B4, Norwood, MA), and this data was used to calculate the carbon dioxide evolution rate (CER). Using an initial culture volume of 2.5 L, exponentially dividing cells were seeded at initial densities ranging from $3x10^5$ to $5x10^5$ per mL. Details of the nutrients and feeding regimen used in the fed-batch experiments have been described in Elias *et al.*[7] Culture samples were removed regularly and stored at -80 °C for off-line analysis.

Off-line Analysis

Culture sampling, sample preparation, β-galactosidase quantitation, and both viable and total cell counting methods are described elsewhere.[12] The osmolality of thawed supernatant samples was measured on an Osmometer (Advanced Instruments, Inc. Needham Heights, MA). The residual glucose and lactate concentrations in the culture supernatants were measured using the IBI Biolyzer Rapid Analysis System (Kodak, New Haven, CT).

Results and Discussion

The results from using different feeding strategies in the fed-batch experiments are summarized in Table 2. The results show that a semicontinuous feeding mode resulted in higher cell densities. A maximum cell density of about $52x10^6$ cells/mL could be reached using this feeding method, and there were fewer total feeds as compared to the pulse feeding method. The pulse feeding method yielded about $38x10^6$ cells/mL. The results of these two fed-batch methods were also compared for production of recombinant protein at high cell densities, where the cells were infected at a density of $14x10^6$ cells/mL.[7] It is evident from these results that by using the semi-continuous feeding method, cell-specific productivity could be maintained at cell densities as high as $14x10^6$ cells/mL. The volumetric productivity could be increased four-fold and thus resulting in a high yield process. Producing a commercially useful recombinant receptor protein (Fig. 1) then validated this process.

In addition to the feeding strategy, rigorous on-line monitoring and process control has been key to achieving success with high cell density production. The monitoring and process control was implemented on two levels. The first level involves the physical parameters such as pH, temperature, and dissolved oxygen (DO). In the case of the Sf-9 cells, pH control is not essential as there is no significant change during the culture period. Metabolic studies done in our laboratory, and independently by other workers, have shown that these cells do not accumulate large amounts of lactate or ammonia in batch cultures. The DO was controlled using a monitoring and control system with a supplementation of the oxygen fraction from an oxygen/nitrogen gas mixture. This system allows a direct correlation of the oxygen fraction used to the oxygen uptake rate of the cells, and can be combined with the carbon dioxide evolution rate (CER) to gain information on the physiological status of the cells. The CER can be calculated from the on-line measurement of the CO_2 in the exhaust gas. In our studies, we combined these results with the viable biomass, as measured on-line with the Biomass Monitor (BM). The details of the measurement method have been described in Zeiser *et al.*[24] Briefly, the probe measures the capacitance of the culture that results from the presence of viable cells. It simultaneously measures the conductance of the culture medium. This measurement is then used to calculate the relative permittivity (ε'), which takes into account the constant for the probe and the probe geometry. For a Sf-9 cell culture in serum-free medium, that was infected at an MOI of ten and resulted in a synchronous infection, the typical CER profiles and relative permittivity (ε'), along with the cell densities, cell diameter, and protein production, are shown in Figure 2. The cell density at infection was $3x10^6$ cells/mL. The profile of ε' closely follows the viable cell density in the growth and post infection period. It can be correlated to the increase in cell diameter as measured with a Coulter Counter®. In a synchronously infected culture, the peak in the CER is generally observed between 18-24 hpi (Kamen et al 1996), and corresponds to a plateau in the ε' profile. Toward the end of the culture, the downward trend in the ε' profile corresponds to the decrease in cell viability in the post infection period. In cases where a low MOI (0.001 pfu/cell) was used and resulted in asynchronous infection, the CER peak and the ε' profile plateau were found to shift to a later time (between 24-48 hpi) (Fig. 3). Therefore, these parameters can be used to gauge the physiological status of the cells and are also very good indications of the synchronicity of infection.[25] The synchronicity of infection is an important factor in deciding when to feed and harvest a fed batch culture. It is very important that the feeding regimen be designed to maintain exponential growth to achieve high cell density and to maintain the cells in the exponential phase up to the time of infection. The typical CER profiles and ε' during a fed-batch process are shown in Figure 4.

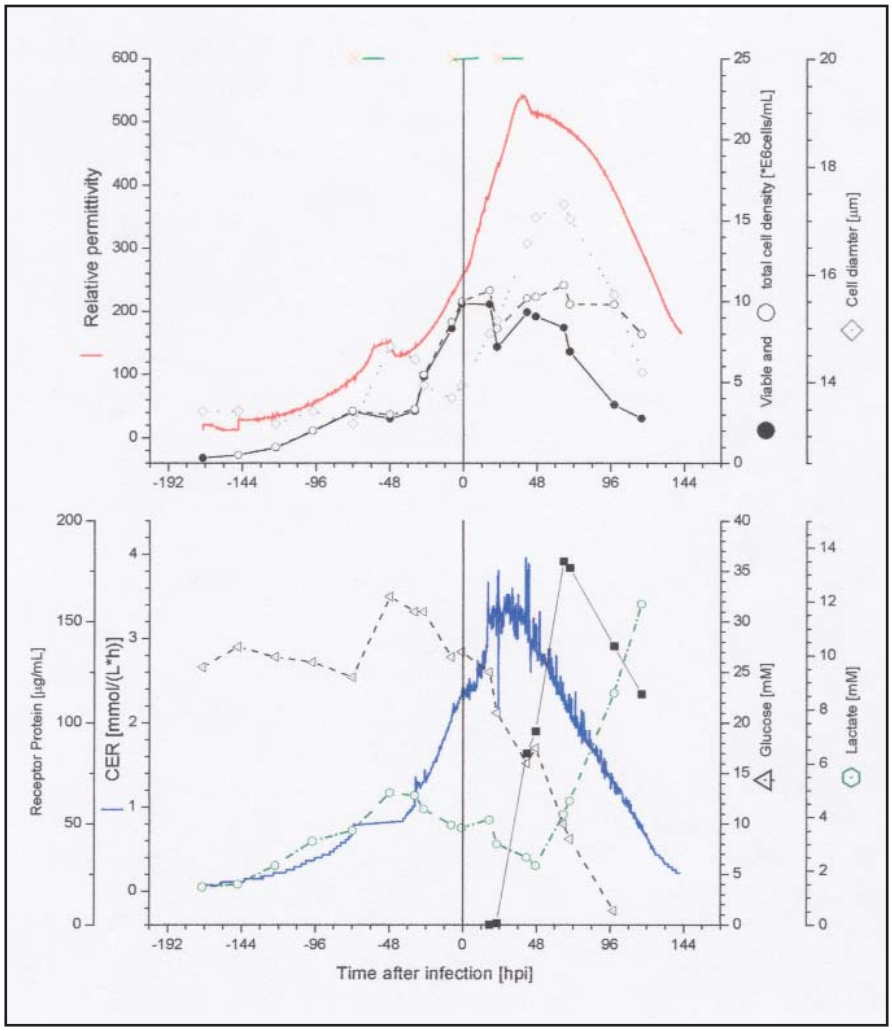

Figure 4: The time course of relative permittivity, cell diameter, and total and viable cell density (Fig. 4a.); CO_2 evolution rate (CER), glucose and lactate concentration, and the amount of receptor protein (Fig. 4b) produced in fed-batch culture of Sf-9 cells with semi-continuous nutrient feed. The x marks the time of addition, and the horizontal line indicates the time period over which the addition was made.

In the present experiment, these profiles were used as indicators for starting feeds, and for predicting the infection and harvest times. In cases where the infection is not synchronous and cells continue to grow post infection, additional feeds will be required to supply sufficient nutrients for the additional cell growth and protein production. The profiles in this case can also be used to determine the harvest time for optimal protein production.

Combining a feeding strategy with an on-line monitoring process has been successfully used to design fed-batch processes for a number of recombinant proteins with more than one insect cell line and medium. The process is being currently scaled up to 20 L. The results of the scale-up and its adaptation to other cell lines will soon be published separately.

CONCLUSIONS

The fed-batch approach has been shown to be an extremely useful and feasible method for achieving high cell density production of recombinant proteins with the insect cell / baculovirus system. The system is also being studied for applications involving coinfection with two or more baculoviruses for production of active proteins.

Recent developments have resulted in insect cells that have been transfected and cloned to express proteins stably. These developments offer a new arena for exploiting the potential of this system for high cell densities, and thus maximizing the volumetric productivity and facilitating further scale-up.

An on-line monitoring system is crucial to the success of the process, and its utility in monitoring fed-batch and perfusion systems are being further validated. A strategy for using this monitoring system to automate and control a fed-batch is also under study. The system is robust and has the potential for use with different cell

lines and different media. It clearly offers a powerful tool for use with more recent applications of insect cell-based processes.

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