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Optimisation of protein expression and establishment of the Wave Bioreactor for Baculovirus/insect cell culture

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Abstract

As the interest of research is beginning to shift from genomics to proteomics the number of proteins to be expressed is rapidly increasing. To do so, well-established, high-level expression systems and rapid, cost-effective production means are needed. For addressing the latter, a novel cultivation system for recombinant cells, the Wave Bioreactor™ has recently become available. We describe the set-up and the optimisation of parameters essential for successful operation and growth of insect cells to high cell densities in the Wave Bioreactor. According to our experience, the Cellbag™ system compares very favorably to conventional cultivation vessels such as bioreactors and roller cultures with respect to simplicity of operation and cost. Additionally, we developed a rapid and simple protocol for assessing expression and production conditions for the Baculovirus/insect cell system applicable to many different genes/proteins. Important parameters like MOI, TOI, peak cell density (PCD) and expression levels are determined in pre-experiments on small scale to achieve optimal expression of a given protein. These conditions are subsequently transformed and applied to large scale cultures grown in nutrient-supplemented medium in the Wave Bioreactor.

Introduction

Over the past two decades the Baculovirus/insect cell system has emerged as one of the most successful expression systems with multiple applications (for review see Kost and Condreay, 1999). Large-scale recombinant protein production using this system is commonly done in roller bottles, spinner cultures or bioreactors and a large amount of literature is available on optimisation of insect cell cultivation conditions, media and feeding strategies as well as virus production and infection kinetics (Elias et al., 2000; Chan et al., 1998; Zeiser et al., 2000; Petricevich et al., 2001; Savary et al., 1999).

A very recent development for large-scale cultivation of cell lines is the Wave Bioreactor™ System, which was first described by V. Singh (1999) and is now commercially available from Wave Biotech AG (Tagelswangen, Switzerland) and Wave Biotech, LLC (Bedminster, NJ, USA). The Wave Bioreactor consists of an air inflated flexible plastic bag (CellBag™) partially filled with cell suspension and placed on a rocking thermoplate. Aeration is performed via the headspace of the bag. The rocking motion induces undulation ensuring mixing, off-bottom suspension, and an enlarged and constantly renewed surface to increase oxygen transfer. The presterilised cellbags of different size are discarded after use, thus eliminating the need for cleaning and sterilisation.

It is claimed that the Wave Bioreactor is suitable for cultivation of many different cell types (e.g. hybridomas, mammalian cells, plant cells; see Wave Biotech AG at www.wavebiotech.ch), but due to its novelty, very few detailed protocols on essential operational parameters and cultivation conditions are as yet available or published.

Thus, the aim of this work was to establish suitable conditions for high cell density Sf-9 insect cell culture in the Wave Bioreactor and, based on optimisation studies on kinetic and nutritional parameters, to develop a generic protocol for rapid production of recombinant proteins in the Baculovirus/insect cell expression system.

Materials and methods

Cell lines and cell culture media

Spodoptera frugiperda Sf-21 (Vaughn et al., 1977) were maintained in T-flasks in ExCell401 (J.R.H. Biosciences, Lenexa, KS, USA) fortified with 10% FCS (Amimed, Allschwil, Switzerland). *Spodoptera frugiperda* Sf-9 insect cells, a clonal isolate of Sf21 cells, were maintained in 250 ml cultures in serum free SF-900II medium (Life Technologies, Basel, Switzerland) in 850 cm^2 roller bottles (Corning, NY). Growth temperature for all cells was 28 °C. All growth and expression studies were performed with Sf-9 cells; for virus titre determination by plaque assay Sf-21 cells were used.

The SF-900II medium (powder formulation) was prepared in-house and stirred for 5 h before filtration to ensure that all components were well dissolved (Wong et al., 1996). 0.1% Pluronic F-68 was routinely added to the medium. Yeastolate Ultrafiltrate (Gibco/Life Technologies, 50 × concentrate), and Lipid concentrate (100 × Gibco/Life Technologies) were added as described in Table 2. All media preparations were sterilised by filtration through a 0.1 μm filter (Meissner, Camarillo, CA, USA).

Virus production and titre determination

As a model protein for our studies served the extracellular domain of the adhesion molecule ICAM-1 fused to the constant region of an immunoglobulin light chain (c_κ) as tag (Bleijs et al., 2001). Recombinant AcNPV virus was generated using the BacPAK6 kit (Clontech, Palo Alto, CA, USA). Following plaque purification and master stock production, working virus stocks were produced in Sf-9 cells: 500 ml roller cultures were grown to 1.5×10^6 cells ml^{-1} density and infected with virus at a MOI of 0.1. After 4–5 days the virus containing supernatant was harvested at 5000 g for 15 min at 4 °C. Plaque assays for virus titration were performed according to King and Possee (1992).

Cell-culture systems

Roller bottles (850 cm^2, Corning, NY, USA) were used for small scale cultures with a maximum working volume of 500 ml. For volumes exceeding 250 ml a vented cap was used to enhance oxygen supply. Turning speed of the roller bottle device was 8 rpm.

The Wave Bioreactors (Wave Biotech AG, Tagelswangen, Switzerland) were operated with 20-l cellbags equipped with a modified sampling port. The bags were connected to media and inoculum tanks under a laminar flow and then installed on the rocking unit (Figure 1). For aeration of the head space an in-house gas mixing station was used, which allowed manual adjustment of oxygen concentrations in the aeration gas from 21 to 100%. Aeration gas was humidified by sparging through water to reduce medium evaporation.

Analytical and downstream processing

Off-line analytics were performed using a BioProfile 200 Analysator (Nova Biomedical Corp., Waltham, MA, USA), which simultaneously determines pH, osmolarity, DO (dissolved oxygen) concentration, CO_2 partial pressure and the concentration of Na^+, K^+, Ca^{2+}, NH_4^+, glucose, glutamine, glutamate and lactate within 4–5 min. Cell counts were performed using an improved Neubauer hemacytometer. Viability of uninfected cultures was measured by trypan blue (0.2%) exclusion. In infected cells the fraction of trypan blue stained cells does not correspond to the viability, as also living cells are stained due to the impaired membrane integrity through infection (Kamen et al., 1996). Therefore, infected cells were counted after approx. 3 min incubation with trypan blue, which allows a more precise differentiation between stained and unstained cells. Recombinant protein production was quantified by sandwich ELISA by means of a monoclonal rat-α-mouse-cκ coating antibody produced in-house, and a biotinylated rat-α-mouse-κ light chain antibody for detection (Zymed, San Franscisco, USA). At harvest the cells were pelleted at 3000 g for 15 min at 4 °C. The cell supernatant was stored at –80 °C for further downstream processing (protein purification).

Results and discussion

Establishment of the Wave Bioreactor system

The Wave Bioreactor represents a novel cell culture

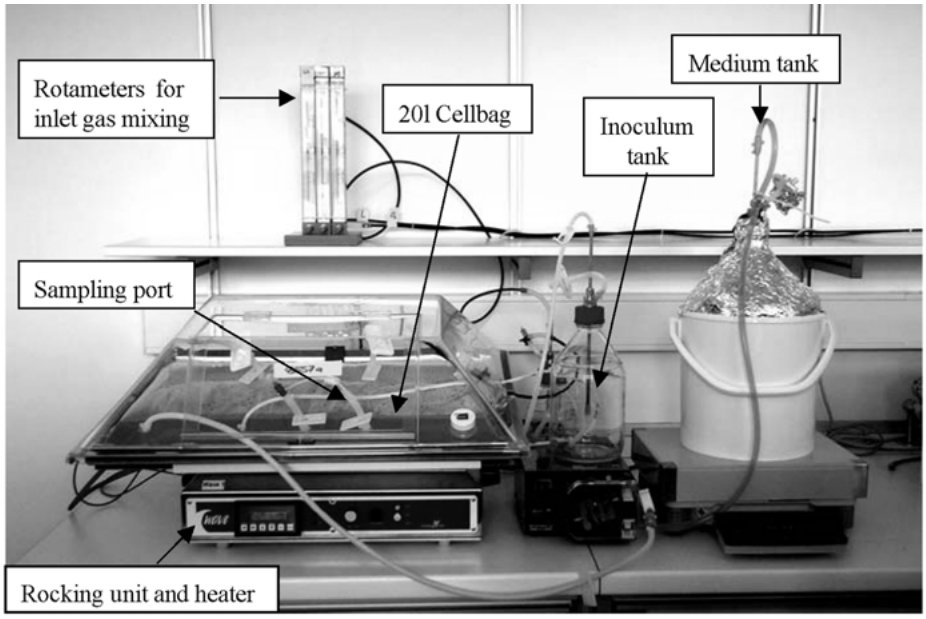

Figure 1. Wave Bioreactor with 20-l cellbag fitted with medium and inoculum tank, sampling port and gas supply.

system for which currently only little information is available for application with insect cell cultures. Parameters which can be modulated and adjusted in this culture system comprise the agitation strategy (rocking rate, rocking angle), the aeration strategy and the culture volume.

The influence of different agitation intensities (rocking rate and angle) on the growth rate of Sf-9 cells in 20-l cellbags is shown in Figure 2. The starting volume was 1 l (as recommended in the Wave Bioreactor instruction manual) at a density of 5×10^5 cells ml^{-1}. When cell densities reached 2×10^6 ml^{-1} the cultures were stepwise re-diluted to 1×10^6 ml^{-1} by medium addition until the final culture volume of 10 l was reached.

The highest growth rate of 0.028 h^{-1} was observed for the intermediate agitation intensity, which correlates well with maximum reported growth rates of 0.028 to 0.035 h^{-1} (Power et al., 1994; Bailey, 1991). During the first 40 h post inoculation, however, all three cultures showed the same low specific growth rate of approx. 0.014 h^{-1}. After this lag-phase the two cultures with the lower agitation intensities resumed faster growth, whereas the culture with 24 rocks per minute remained at its low growth rate. As also visible in Figure 2 the CO_2 partial pressure was as low as 2 kPa in the first 40 h post inoculation. It was speculated that the initial lag phase of all three cultures was caused by enhanced CO_2 stripping from the small starting volume of 1 l, rather than by cell damage from turbulent flow at that small volumes.

In a further experiment both the influence of agitation and the influence of aeration on cell growth were tested (Table 1). Two batches were operated at constant agitation with rocking rate 20 min^{-1} and angle 7°; for the other two batches a variable agitation strategy was applied starting with rocking rate 14 min^{-1} and angle 5° followed by stepwise increase at the timepoints of medium addition to the final values of 20 min^{-1} and 7°, respectively. Alternately, these 4 batches got either normal, constant head space aeration from the beginning, or the aeration was switched off for the first 36 h. Starting volume for all four batches was 2 l at a density 5×10^5 cells ml^{-1}. The specific growth rates obtained in these runs are listed in Table 1.

No lag phase was observed in all four cultures. Highest specific growth rates were obtained in cultures with the aeration switched off in the beginning, independently of the agitation strategy applied. When aeration was applied from the beginning, however, the specific growth rate was significantly better in combination with a variable agitation strategy.

This procedure proved to be optimal for fed-batch cultivation of insect cells in 20-l Wave cellbags, since it requires only small inoculum volumes and allows

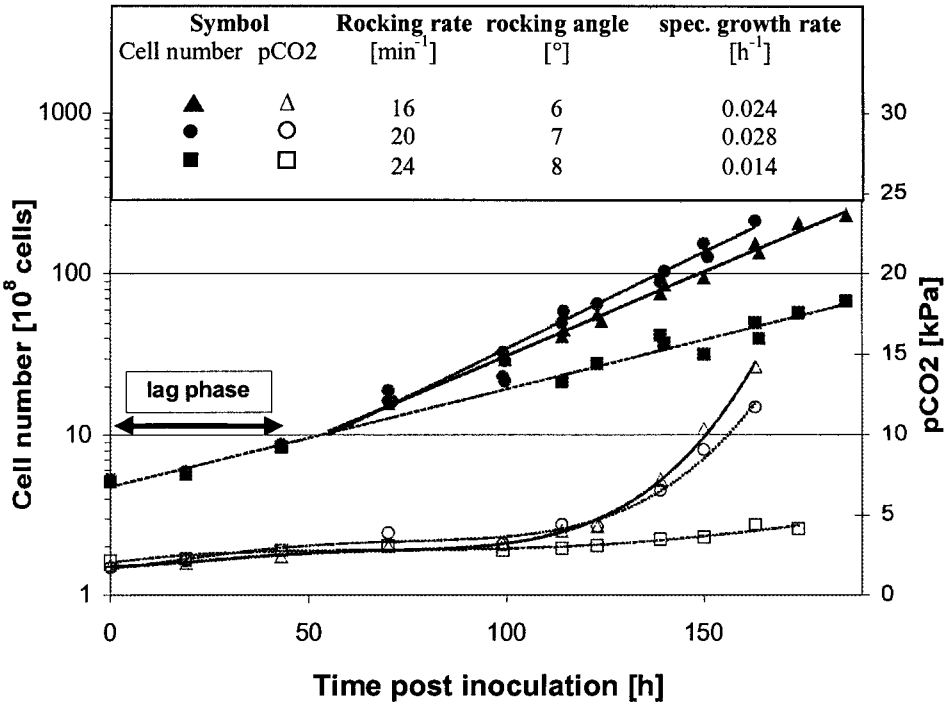

Figure 2. Growth curves of Sf-9 cells and CO_2 partial pressure in 20-l cellbags under different agitation parameters.

Table 1. Specific growth rates for different agitation intensities and aeration strategies in 20-l cellbags

Run	Agitation strategy	Aeration strategy	Specific growth rate (h^{-1})
1	Variable	From beginning	0.027
2	Variable	No aeration for t < 36 h	0.028
3	Constant	From beginning	0.020
4	Constant	No aeration for t < 36 h	0.029

cell growth at high specific growth rates without initial lag-phase. A typical example of Sf-9 cell growth in 20-l cellbag with variable agitation strategy and without aeration for the first 36 h post inoculation is shown in Figure 3. Starting volume was 2.5 l with a cell density of 5×10^5 ml^{-1}. When cell densities reached 2×10^6 ml^{-1} the cultures were stepwise re-diluted to 1×10^6 ml^{-1} by medium addition, until the final culture volume of 10 l was reached. Rocking rate and angle were increased with the volume as follows: 14 min^{-1} 5° for 2.5 l, 17 min^{-1} 6° for 5 l, and 20 min^{-1} 7° for 10 l culture volume. Aeration rate was increased from 0.2 to 0.4 l min^{-1}, and the oxygen concentration in the inlet gas was adjusted to maintain the DO above 50% of saturation. Cells grew without lag phase at a specific growth rate of 0.030 h^{-1}.

With the aim to develop a protocol as generic as possible, a simpler but just as effective approach was envisaged. The incremental filling (fed-batch) of the cellbag was abandoned and the culture was started with the final volume of 10 l. This requires a larger inoculum for an initial cell density of 3×10^5 ml^{-1}, but allows aeration from the beginning of the cultivation process and less interventions during the process. By this approach the cells grew also without lag phase and reached specific growth rates of up to 0.034 h^{-1} (Figure 4). Thus all further batches of Sf-9 insect cells in 20-l cellbags were done according to the following

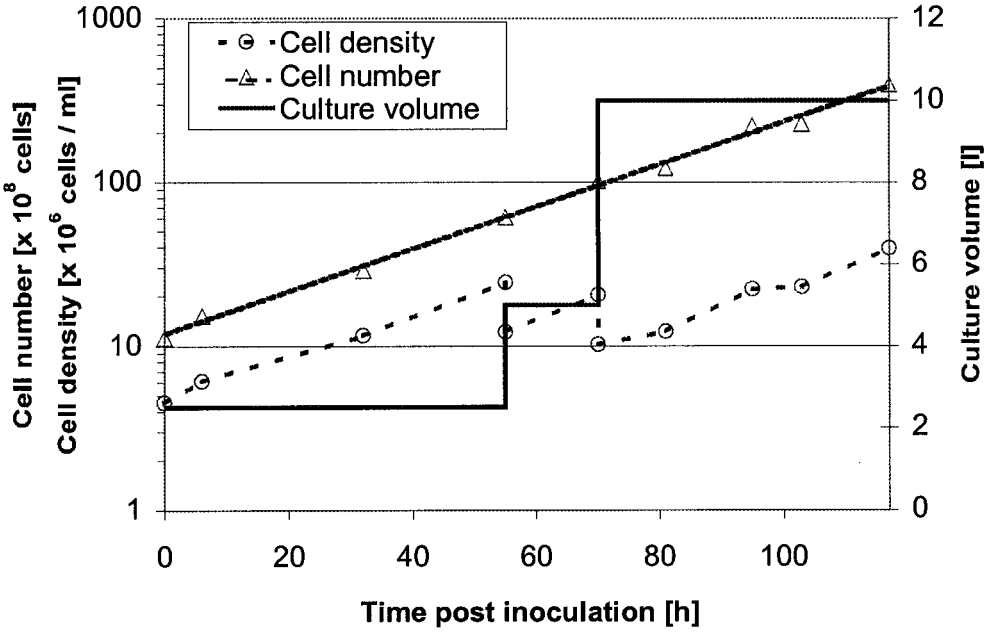

Figure 3. Typical growth curve in 20-l cellbag with variable agitation strategy and without aeration for the first 36 h post inoculation.

cultivation protocol:

- Starting conditions:
  - volume 10 l;
  - cell density 3×10^5 ml^{-1};
  - aeration 3 l h^{-1} air;
  - agitation: rocking rate 20 min^{-1} and rocking angle 7.5°.
- During the course of cultivation the agitation intensity is adjusted to increasing cell density; the oxygen concentration in the inlet gas is increased at higher cell densities to maintain the DO above 50% of saturation.

Expression studies

In general, the titre of a recombinant protein expressed in Baculovirus-infected insect cell cultures is a function of kinetic and nutritional parameters. Supplying additional nutrients, e.g. by fed-batch processes, has been shown to be very effective in producing high levels of recombinant protein (Taticek and Shuler, 1997; Chan et al., 1998; Bédard et al., 1994; Elias et al., 2000).

Just as important as sufficient nutrients supply is the adjustment of the kinetic parameters in such a way that the cells can transform as much nutrients as possible to recombinant protein. The two key parameters are the multiplicity of infection (MOI) and the time of infection (TOI), which is expressed as cell density at infection time. Licari and Bailey (1992) and Power et al. (1994) developed mathematical models for simulating the influence of TOI and MOI on cell growth, virus formation and recombinant protein expression. The main conclusions of these models are that an adequate combination of TOI and MOI is needed to obtain maximum protein expression: too early expression and cell lysis results in unspent medium; too late expression results in low expression levels due to nutrient limitation.

Both, nutritional and kinetic parameters were investigated on the example of recombinant ICAM-cκ expression.

Nutritional parameters

In a Plackett-Burman fractional factorial design experiment in 200 ml roller bottle cultures 6 different feeding strategies using media supplements were investigated, of which only the addition of yeastolate and a lipid mix showed an effect on recombinant protein expression (data not shown). The influence of these two supplements on peak cell density, i.e. the maximum obtainable cell density in non-infected insect cell cultures, was further analysed in a 2^2 factorial

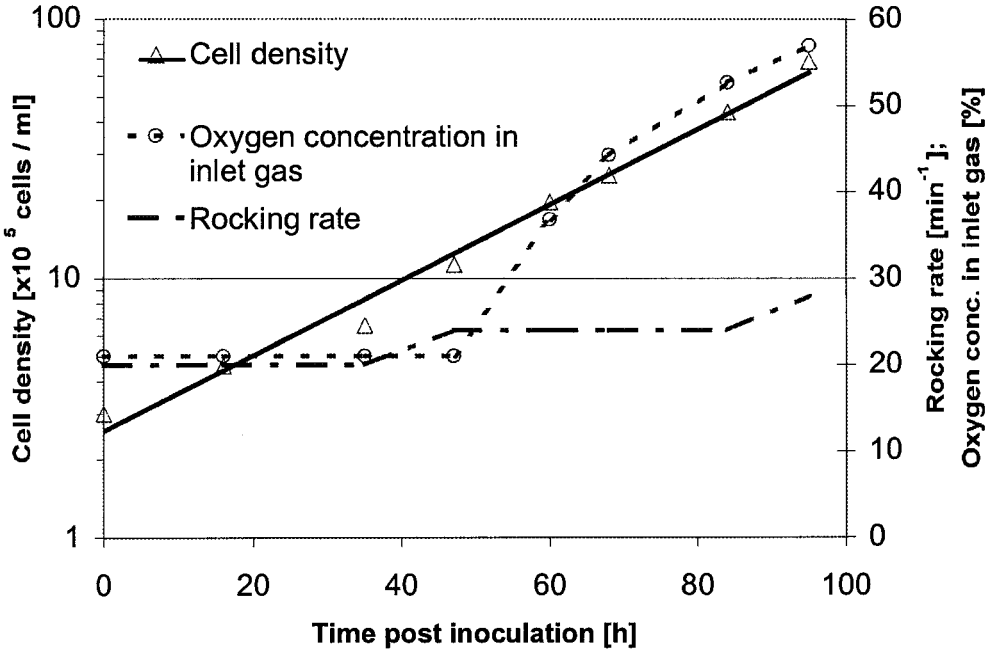

Figure 4. ypical growth curve for Sf-9 cells in 20-l cellbags with 10 l starting volume.

design experiment. Yeastolate and lipid mix were added directly after inoculation of the cultures at a cell density of 5×10^5 Sf-9 cells ml^{-1}. Experiments were performed in duplicate. When the DO dropped below 70% of saturation, the roller bottles were flushed with 300 ml sterile oxygen. As shown in Table 2, only yeastolate addition had a significant positive effect on peak cell density whereas lipid mix addition caused rather a reduction of peak cell density. The growth curves of the Sf-9 cultures with and without yeastolate addition are shown in Figure 5. Data points are averages of each duplicates, and the end points of the 'error bars' show the underlying real data. Until day 6 growth curves run in parallel for cultures with and without yeastolate addition. After day 6, however, the cell density of the cultures without yeastolate declines after having reached a peak cell density of 5.6×10^6 ml^{-1}. In contrast, the cultures with yeastolate addition continued growing for another day and finally reached an average peak cell density of 9.2×10^6 ml^{-1} which is ~60% higher than the peak cell density of the cultures without yeastolate addition.

These results are in accordance with recently published data on the beneficial effects of protein hydrolysate addition, in particular yeastolate addition, to insect cell culture media, while the benefit of lipid mixture addition proves to be controversial (Ikonomou et al., 2001; Bedard et al., 1994; Chan et al., 1998).

Table 2. Effects of yeastolate and lipid mix addition on peak cell density

Run	Yeastolate	Lipid mix	Peak cell density
1	0	0	5.6×10^6 ml^{-1}
2	4 g l^{-1}	0	9.2×10^6 ml^{-1}
3	0	1 ×	4.9×10^6 ml^{-1}
4	4 g l^{-1}	1 ×	8.5×10^6 ml^{-1}

Kinetic parameters

To determine the optimum TOI (at given MOI) with respect to the peak cell densities (PCD) a series of experiments were performed using SF-900II medium without yeastolate addition. Sf-9 cells were grown in parallel roller bottle cultures with volumes of 350 ml each. At different cell densities ranging from 0.4×10^6 ml^{-1} to 5.3×10^6 ml^{-1} (= 7 to 98% of PCD) one culture each was split into three 100 ml cultures (in 2-l roller bottles) and infected with 0.5 MOI ICAM-cκ virus. In parallel, a non-infected culture was grown under the same conditions which reached

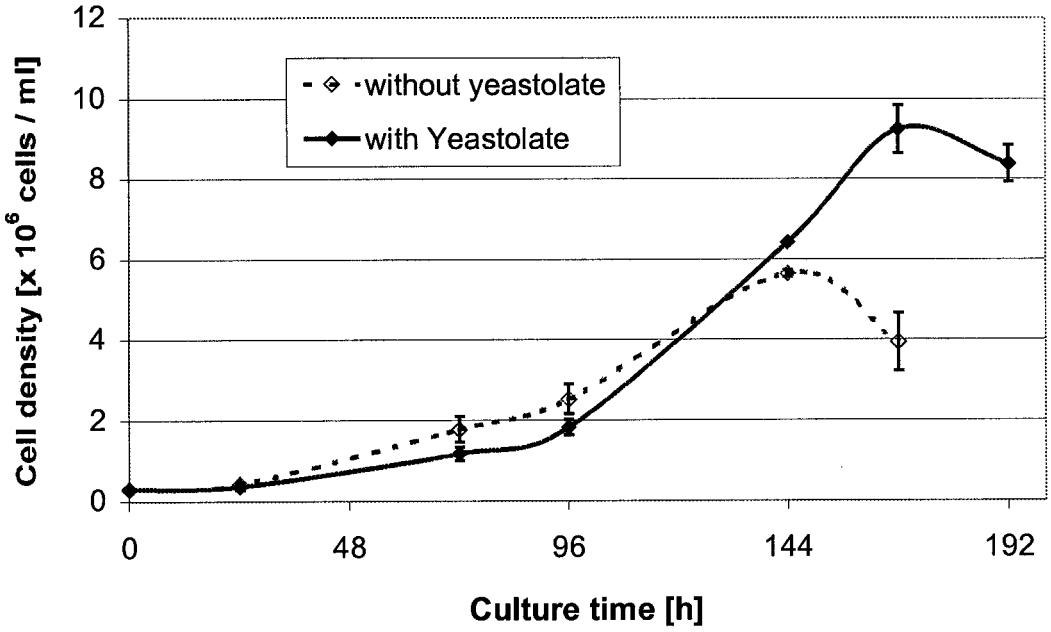

Figure 5. Growth curves for Sf-9 cells grown in roller bottles with and without yeastolate supplementation. Data points are averages of duplicates; the end points of the 'error bars' show the underlying real data.

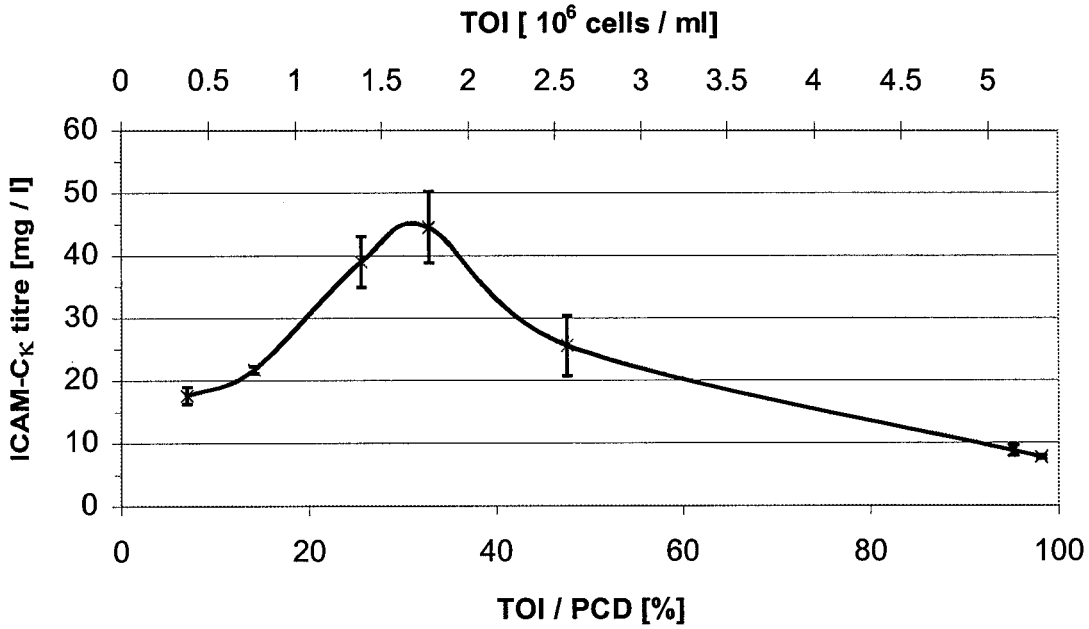

Figure 6. Recombinant ICAM-cκ titre as function of TOI and TOI/PCD. Error bars indicate standard deviation from triplicate experiments. Peak cell density without infection (PCD) was 5.4×10^6 ml^{-1}.

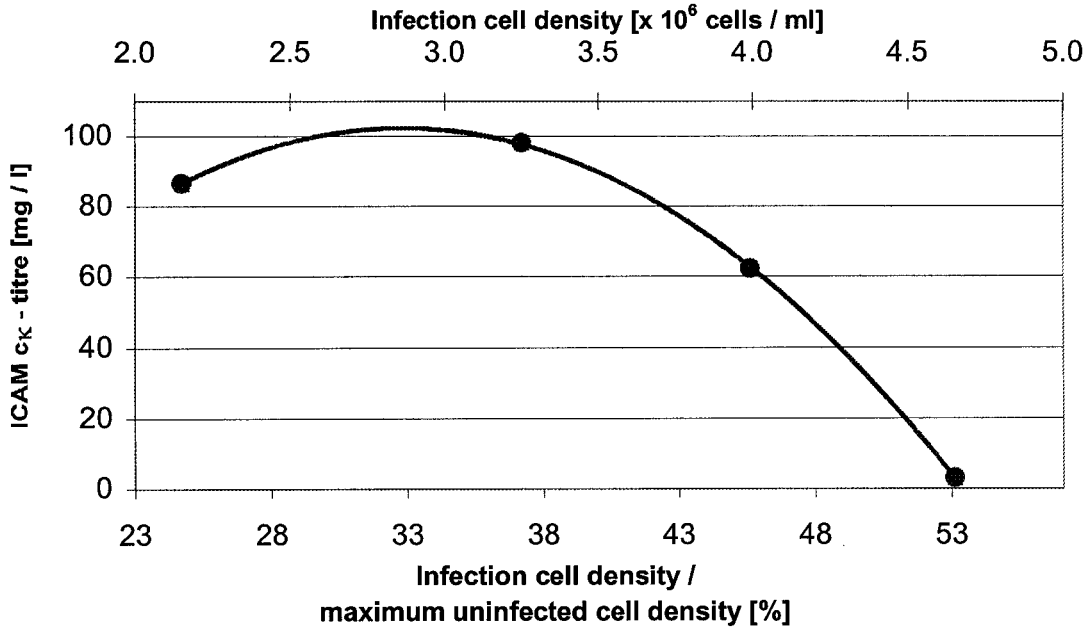

Figure 7. ICAM-cκ titre as function of TOI and TOI/PCD. PCD was taken as 8.8×10^6 cells ml^{-1}. The value at TOI = 3.25×10^6 cells ml^{-1} is the mean of three cultures.

a PCD of 5.4×10^6 cells ml^{-1}. Cell free samples were taken daily for ICAM-cκ quantification. The maximum titres as function of TOI (10^6 ml^{-1}) and TOI/PCD (%) are shown in Figure 6. Mean values and standard deviations for triple batches are shown. Highest ICAM-cκ titres were observed when the infection cell density was between 25 and 40% of PCD. The maximum titre of 45 mg l^{-1} was observed for TOI = 1.77×10^6 ml^{-1} (= 33% of PCD) after 5 days post infection.

A similar experiment was performed for large-scale Sf-9 cultures grown in 20-l cellbags and supplemented with yeastolate. These cultivations were performed following the fed batch approach as outlined and shown in Figure 3. At cell densities of $3.5–4.5 \times 10^6$ ml^{-1} 4 g l^{-1} yeastolate ultrafiltrate was added to the cultures. Cells were infected with 0.5 MOI ICAM-cκ virus at different TOI, as shown in Figure 7. A non-infected culture grown in parallel under the same conditions reached a PCD of 8.8×10^6 cells ml^{-1}. Under these conditions maximum ICAM-cκ titres were in the range of 90–100 mg l^{-1}, which is approx. twice the titre as obtained in the roller bottle cultures without yeastolate addition. Again, the highest titres were observed for TOI ranging between 25 and 40% of PCD, leading to the suggestion that TOI and PCD under different nutrient levels show the following relationship:

$$\frac{\mathrm{TOI}_{\text{low nutrients}}}{\mathrm{PCD}_{\text{low nutrients}}} = \frac{\mathrm{TOI}_{\text{high nutrients}}}{\mathrm{PCD}_{\text{high nutrients}}} . \qquad (1)$$

This relationship facilitates the estimation of the optimal TOI at a given MOI in nutrient-supplemented Wave cultures, as the TOI/PCD ratio can easily be determined in pre-experiments on small scale without nutrient supplementation.

Generic protocol

As the relationship of Equation (1) proved to be applicable for a large variety of different recombinant viruses the following generic expression protocol was introduced:

1. PCDs of non-infected Sf-9 cells grown (a) at non-supplemented standard conditions in roller bottles, and (b) under optimal conditions in Wave Bioreactors are once determined and used as reference for determination of TOI/PCD for individual Baculovirus clones.
2. MOI is adapted to individual protein characteristics (e.g. secreted vs intracellularly expressed proteins).

3. TOI (at chosen MOI) is determined by small scale kinetic experiments without nutrient addition, and set in relation to respective PCD; steps 2 and 3 can be performed in parallel.
4. Scale-up to Wave Bioreactors is performed by translating the optimum TOI/PCD ratio from small scale kinetic experiments to the PCD obtained in Wave Bioreactors with nutrient supplementation according to Equation (1).
5. As criterion for the determination of the time point of harvest, the fraction of trypan blue stained cells is taken, resembling the infection rate of the cells: in the small-scale experiments for determination of MOI and TOI (steps 2 and 3) a relation between recombinant protein titre and the fraction of trypan blue stained cell is determined. The large-scale culture is harvested when the infection rate correponds to the value obtained in correlation to highest recombinant protein titres in the pre-experiments.

Conclusions

In summary, this report describes a comprehensive protocol for the rapid generation of proteins using the Baculovirus/insect cell expression system in conjunction with cultivation and production using the novel Wave Bioreactor. We assessed and defined the most critical parameters involved in bioreactor operation as well as in protein expression using the secreted ICAM-1-c_κ fusionprotein as model. We have meanwhile validated this protocol by successful expression of a large number of other proteins, also belonging to different protein classes.

Acknowledgements

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