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High-density cultivation of insect cells and production of recombinant baculovirus using a novel oscillating bioreactor

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Abstract

A novel two-compartment bioreactor, BelloCell®, was used to cultivate insect cells and a maximum yield of 4.6×10^9 cells was attained. The cells were immobilized in a packed bed fixed in the upper chamber, and the bellow in the lower chamber was compressed and released in an alternating fashion. The motion resulted in gentle, cyclic movement of the medium that was contained in the lower chamber and consequently exposed the cells to air in an oscillatory manner, thus rendering adequate aeration and uniform cell distribution in the bed. The baculovirus yield produced in BelloCell® could amount up to 3.3×10^{17} pfu using as little as 1.1 l medium in the production run. Besides, BelloCell® was extremely easy to handle and operate. These benefits underline the potential of BelloCell® for simple, economical and high-density cell culture and protein/virus production.

Introduction

Cell culture technologies play essential roles in the production of recombinant proteins and viral vectors. Higher product yield and subsequent lower cost often hinge on whether high cell density is achieved. Currently, roller bottles and stirred tank bioreactors are systems of choice for large-scale applications owing to the well-understood principles of scaling parameters and the ease of process control in homogeneous systems. For anchorage-dependent cells, microcarriers can be introduced for cell attachment. The maximum cell densities achieved in these reactors, however, are usually lower than desired due to the mass transfer limitation, toxic by-product accumulation, sensitivity to shear stress and contact inhibition. To alleviate these problems, a number of different reactors have been developed, such as hollow fiber (Alauotila et al. 1994) and packed-bed bioreactors (Hu et al. 2000). The hollow fiber bioreactor generates low shear stress, but requires exterior oxygenation system and high circulation rate to provide sufficient oxygen supply. Packed bed bioreactor such as Celligen Plus™ (New Brunswick Scientific, Edison, NJ), on the other hand, has been developed to promote gentle liquid circulation through annular packed bed with low shear stress but good oxygen transfer. This reactor, however, requires sophisticated skills to operate, and the capital cost associated with the control system is high. Recently, a disposable Wave Bioreactor™ was proposed for convenient suspended cell culture, for which rocking provides good mixing and oxygen transfer to support cell growth, but the maximum cell density attained is limited to 4–5 $\times$ 10^6 cells ml^{-1} (Singh 1999). Other systems such as CellCube® (Corning Life Sciences, Corning, NY) and Cell Factories™ (Nalge Nunc International, Roskilde, Denmark) have also been available for immobilized cell culture, yet the cells are cultured on stacked surfaces

and oxygen transfer might impose a potential problem.

To overcome the aforementioned drawbacks in the existing reactors, a novel disposable bioreactor, BelloCell®, was developed by Cesco Bioengineering Co. (Hsinchu, Taiwan) for simple, economical and high-density cell culture in the laboratory scale. The reactor consists of two compartments: upper and lower chambers. The cells are immobilized in a packed bed fixed in the upper chamber, and the bellow in the lower chamber is compressed and released in an alternating fashion. The motion results in cyclic upward and downward movements of the medium and the fluid flow is so gentle that it results in low shear stress. Moreover, massive oxygenating surface area is created with simple medium movement relative to the immobilized cells, without any agitation or air sparging.

Despite so many advantages, the feasibility of using BelloCell® for animal cell culture has not been documented in the literature. In this study, we developed a simple process for the cultivation of insect cells as well as for the production of baculovirus using BelloCell®. We report for the first time that BelloCell® is a suitable bioreactor for insect cell culture.

Materials and methods

Cell, media and virus

Insect cell Sf-9 was propagated in spinner flasks as described previously (Hu et al. 2002). The medium, TNM-FH (Gibco BRL, Gaithersburg, MD), was supplemented with 10% fetal bovine serum (FBS, Sigma). The recombinant baculovirus, Bac-CEH, was constructed previously to encode *egfp* gene under the polyhedrin and the cytomegalovirus immediate-early (CMV-IE) promoters so that enhanced green fluorescence protein (EGFP) could be expressed in infected insect and mammalian cells, and allowed for facile detection of the expression in the cells (Hu et al. 2003). Besides, the virus was also designed to overexpress histidine-tagged gp64 on the virus envelope which would facilitate the virus binding to insect cells. The virus titers were determined following standard end-point dilution methods as described previously (Hu et al. 2003; O'Reilly et al. 1992).

Carriers for cell immobilization

The porous carrier for cell immobilization, BioNOC™ (Cesco Bioengineering Co.), was non-woven fabric made by polyester sheathed with high-density polyethylene. No other binder materials were involved in the manufacturing process. The non-woven fabrics were cut into strips of 5 mm in width and 10 mm in length. The distribution of pore size ranged between 50 and 200 μm, with the average being 125 μm. One gram of BioNOC™, when packed in BelloCell®, occupied $\approx$15 cm^3 space and had a void volume of $\approx$4 cm^3, but provided total surface area of $\approx$2000 cm^2 g^{-1} according to the manufacturer. The carriers were pre-packed in the bioreactor and pre-sterilized by gamma irradiation (intensity = 25 kGY).

Bioreactor system and operation

The bioreactor, BelloCell®, was provided by Cesco Bioengineering Co. (Hsinchu, Taiwan). It consisted of an upper chamber holding a carrier bed (by carrier support plate) that provided surface for cell attachment, and a compressible lower chamber (bellow) which contained the medium as shown in Figure 1(a). The upper chamber was made of polyethylene terephthalate and the bellow was made of low-density polyethylene. Two versions were available: BelloCell® 300 and BelloCell® 500, with working volumes of 300–350 ml and 500 ml, respectively. The reactor used in this study (BelloCell® 300) was pre-packed with 6.5–7 g BioNOC™ carrier which constituted a bed volume of $\approx$100 cm^3 (void volume $\approx$28 cm^3). The top lid was equipped with a 0.22 μm PTFE filter for ventilation.

For inoculation, the cells grown to mid-exponential phase ($\approx 1 \times 10^6$ cells ml^{-1}) were centrifuged at 1000 g and resuspended in 30 ml fresh medium as the inoculum. The top lid was simply opened, 300 ml medium was added and the bellow was compressed and released several times to wet the carrier bed. After 2 h, 30 ml cell inoculum was added and the reactor bottle was swirled gently to dispense the inoculum. The bellow was compressed and released intermittently to aid the uniform distribution of the cells, then the reactor was locked up to the bottom cap and sit in the incubator at 27 °C. After 3 h of attachment, the

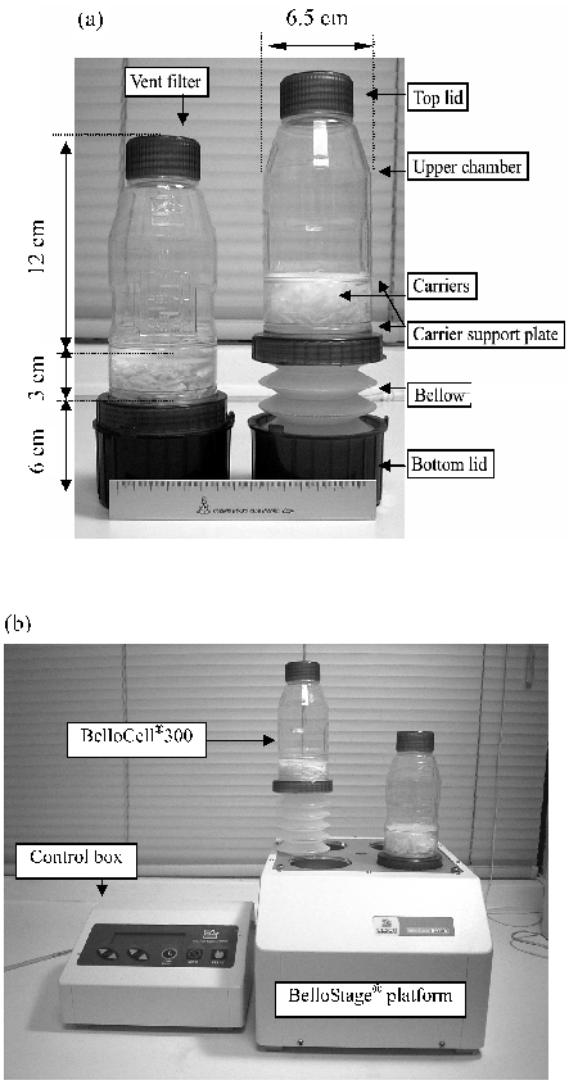

Figure 1. Schematic illustration of the bioreactor. (a) The reactor on the right shows that the bellow was released. On the left is the reactor with the bellow compressed and locked up by the bottom lid. The compartments and dimensions of the disposable reactor are illustrated in the figure. (b) The reactors were mounted onto the BelloStage® platform which was connected to the control box.

whole reactor was mounted onto BelloStage® platform (Figure 1(b)) which contained a 4-position stepping-motor able to move the plates upward and downward (the 4 plates were located inside the BelloStage® and not visible in the figure). The upper chamber of the reactor was fixed in position while the ascending movement of the plate lifted the bellow so as to raise the medium level to submerge the carriers. This phase enabled the nutrient supplementation. After a period of delay time (1–15 s), the plate descended and the medium flowed back to the lower bellow and the carriers were exposed to air for oxygen transfer. The moving rate of the plate (and hence the bellow) and the delay time at two ends could be adjusted from the control box. The whole reactor setup except the control box was put into the incubator at 27 °C.

During the culture, the glucose and glutamine concentrations were monitored twice daily in order to determine the timing for medium exchange. The medium exchange was performed by removing the reactor from BelloStage® and titillating so the medium could be removed by aspiration, new medium containing additional glutamine (400 mg l^{-1}) and glucose (3500 mg l^{-1}) was then fed to the reactor. For virus infection, the used medium was decanted and 300 ml fresh serum-free TNM-FH containing a pre-determined amount of virus was added to the reactor. The reactor was returned to the BelloStage®. After 2 h of incubation, FBS was supplemented to make the 10% solution. During the virus production phase, 2 ml medium was taken to assess the viral titer and cell damage, but no medium exchange was carried out.

Analytical assays

The concentrations of glucose, glutamine and lactate were measured using YSI 2700 biochemistry analyzer (Yellow Spring Instruments, Yellow Springs, OH). The dissolved oxygen (DO) and pH of the medium during the culture were occasionally measured using DO (Mettler-Toledo, Greifensee, Switzerland) and pH probes (Suntex, Taipei, Taiwan). The activity of lactate dehydrogenase (LDH) was measured using a commercial kit (Sigma No.DG1340-K). The percentage of cell damage in the virus production phase was quantified by a recently developed method (Ma et al. 2002), and is represented by:

$$\%\text{cell damage} = \frac{\text{LDH activity change in each time interval}}{\text{Total LDH contained within the cells}} \times 100\%$$

The total LDH contained within the cells was determined by measuring the difference in LDH

activity prior to infection and 7 days after infection (after the cells totally lysed and the LDH was completely released).

Cell count

The density of cells in the spinner flasks was counted by a hemacytometer and the viability was determined by trypan blue dye exclusion. For the direct measurement of final cell yield in the reactor, a DNA assay (Kim et al. 1988) was conducted after the culture was completed. The cell number was determined by measuring the amount of DNA released from BioNOC^{TM} using Hoechst 33258 dye (Fluka, Basel, Switzerland). DNA released from a known amount of Sf-9 cells was used to generate the standard curve. The cell number during the reactor operation was estimated indirectly by measuring the glucose consumption rate as described previously (Kaufman et al. 2000). A specific glucose consumption rate of 0.2 mg per 10^6 cells per day, which was determined previously (Taticek and Shuler 1997) and confirmed in our laboratory in spinner flask studies (not shown), was used to calculate the cell number.

Scanning electron microscopy (SEM)

At the end of the culture, the carriers were removed from the bed according to their locations in the bed (top, middle, bottom). Carriers were fixed with 2% glutaraldehyde and then postfixed in 1% osmium tetroxide. Subsequently, the samples were dehydrated in a graded series of ethanol, dried with supercritical carbon dioxide, and spattered with gold film. The carriers were then visualized by SEM (Hitachi, Japan).

Results and discussion

Cell growth in BelloCell®

To investigate the feasibility of high-density cell cultivation in BelloCell®, Sf-9 cells (12×10^7 cells) were inoculated into the reactor with total medium volume of 330 ml. Three hours after inoculation, the measurement of residual cell density in the medium revealed that over 99% of the cells were immobilized (not shown), demonstrating that the cell immobilization was quick and efficient

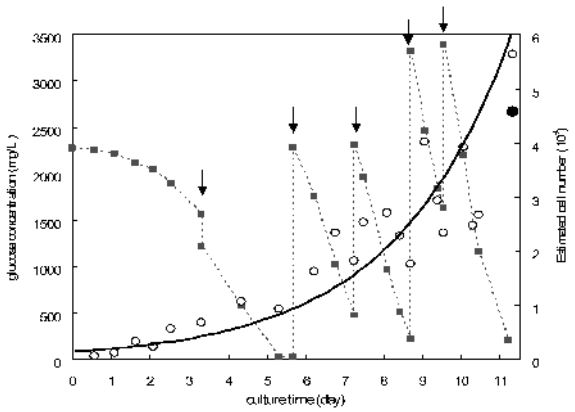

Figure 2. Time-course profiles of glucose concentration (■), cell number estimated via the specific glucose consumption (○) and the fitted cell growth curve (solid line). The cell number determined by the DNA assay at the end of culture is also shown (●). The medium (200–330 ml) was exchanged at the time points indicated by the vertical arrows.

under the specified condition. The linear rate of the plate (of the BelloStage®) was then set at 1 mm s^{-1} with the delay time being 10 s and the culture commenced. The culture was terminated after day 11 because our preliminary experiments suggested that the glucose consumption rate culminated at day 11–12 and then gradually declined under the same conditions. Figure 2 shows the time-course profiles of glucose concentration, estimated cell number and the fitted cell growth curve. The cell number determined by the DNA assay at the end of culture is also shown.

After a lag phase of ≈2 days, the glucose concentration dropped precipitously while the cell number rose considerably thereafter, marking the onset of exponential phase. During the exponential phase, 200–300 ml medium was replaced at the time points indicated by the vertical arrows to keep the pH above 5.5 and to prevent the glucose from depletion. As shown in Figure 2, the cell yield was measured to attain 4.6×10^9 after 11 days, which was close to the estimated yield (5.6×10^9) and suggested that the specific glucose consumption rate provided a reasonable estimation for the cell number. The final volumetric cell density achieved by the repeated-batch operation using BelloCell® thus corresponded to 1.4×10^7 cells ml^{-1} medium and the density based on bed volume corresponded to 6.4×10^7 cells cm^{-3} (total effective bed volume was 72 cm^3).

Sf-9 cells have been cultivated in suspension in various types of bioreactors such as stirred tank, airlift, spin-filter perfusion, and helical-ribbon impeller stirred tank (for review, see (Taticek et al. 1995)). Besides, insect cells have been immobilized on various substrata such as microcarriers (Wickham and Nemerow 1993), Fibra-Cel disks (Ikonomou et al. 2002) and glass beads (Chung et al. 1993), and were maintained in suspension by fluid flow. The maximum cell densities achieved in these studies lie in the range of 3–14.5 $\times$ 10^6 cells ml^{-1}. To enhance the cell density, packed bed reactors have been frequently utilized for high-density mammalian cell cultivation (Hu et al. 2000; Kaufman et al. 2000), but are rarely utilized for insect cell cultivation. In one study, Sf-21 cells were immobilized on Fibra-Cel disks and grown in a 50 ml packed bed reactor in perfusion mode (Kompier et al. 1991), but the maximum cell density achieved was only 6 $\times$ 10^6 cells cm^{-3} bed and the baculovirus titer obtained was in the range of 10^8–10^9 pfu ml^{-1}. In another packed bed reactor (Chiou et al. 1998), the maximum density of Sf-21 was elevated to 5.2 $\times$ 10^7 cells cm^{-3} cellulose foam, but the experiment was conducted in a tiny reactor composed of several cellulose foams and was not adapted to production scale. Comparing to the aforementioned bioreactors, BelloCell®, also a packed bed reactor, could be used to grow Sf-9 to very high cell yield and density in the laboratory scale, demonstrating its advantage and potential for insect cell cultivation. Furthermore, the total medium volume used throughout the culture was only 2 l. The high cell yield on such low medium consumption suggests that this process is also economical.

Also note that the average doubling time from day 3 to day 11 was $\approx$35 h, which was longer than typical doubling time of 18–34 h in suspended cultures (Wu et al. 1989). This is often encountered because the processes associated with immobilized cells are often characterized by low specific growth rates (Reiter et al. 1992). The repeated-batch operation in this experiment was adequate to support high cell yield, but might be sub-optimal for fast cell growth. Adaptation of the system to perfusion with precisely controlled nutrient feeding and culture conditions may further raise the growth rate. A modified reactor equipped with additional ports for perfusion operation is being developed (personal communication) and further optimization with perfusion is underway.

Distribution of cells in the bed

As the process was terminated after day 11, it was important to investigate whether the surface for cell growth was already completely occupied. The carriers at three different layers of the bed (top, middle, bottom) were collected, processed and examined by SEM and are shown in Figure 3(b–d). Comparing to the blank carrier without attached cells (Figure 3(a)), it is evident that the cells completely occupied all the space outside the matrix fiber regardless of the locations of the cells in the bed, and continued cultivation would not result in better yield. Moreover, the cells appeared to be distributed uniformly, suggesting that the reactor generated a uniform flow field to facilitate the even distribution of cells, although the detailed analysis of flow pattern has not been available. If the cells are not distributed evenly, some carriers might have populations near confluency (where a high MOI would be necessary) and others with a low density (where a lower MOI would be optimal). Therefore, the uniformity ensures that further infection would not be complicated by the heterogeneity.

Oxygenation

Oxygen transfer limitation constitutes a major barrier to achieve high density in cell culture. High air sparging and agitation rates in the stirred tank reactors are required to maintain sufficient oxygen transfer but also generate high shear stress and foaming problems detrimental to animal cells. One prominent advantage of the BelloCell® is the cyclic exposure of the cells to ambient air, which is likely to greatly facilitate the oxygen transfer. To confirm this, the concentrations of DO and lactate in the medium were measured at various time points. As shown in Figure 4, DO varied between 20 and 70% air saturation depending on the timing of measurement and medium exchange. These values remained within the optimal range for insect cell culture (Taticek and Shuler 1997), thus oxygen was not a limiting factor, particularly when the cells were exposed in the gas phase for 'breathing'. This hypothesis can be further realized by the

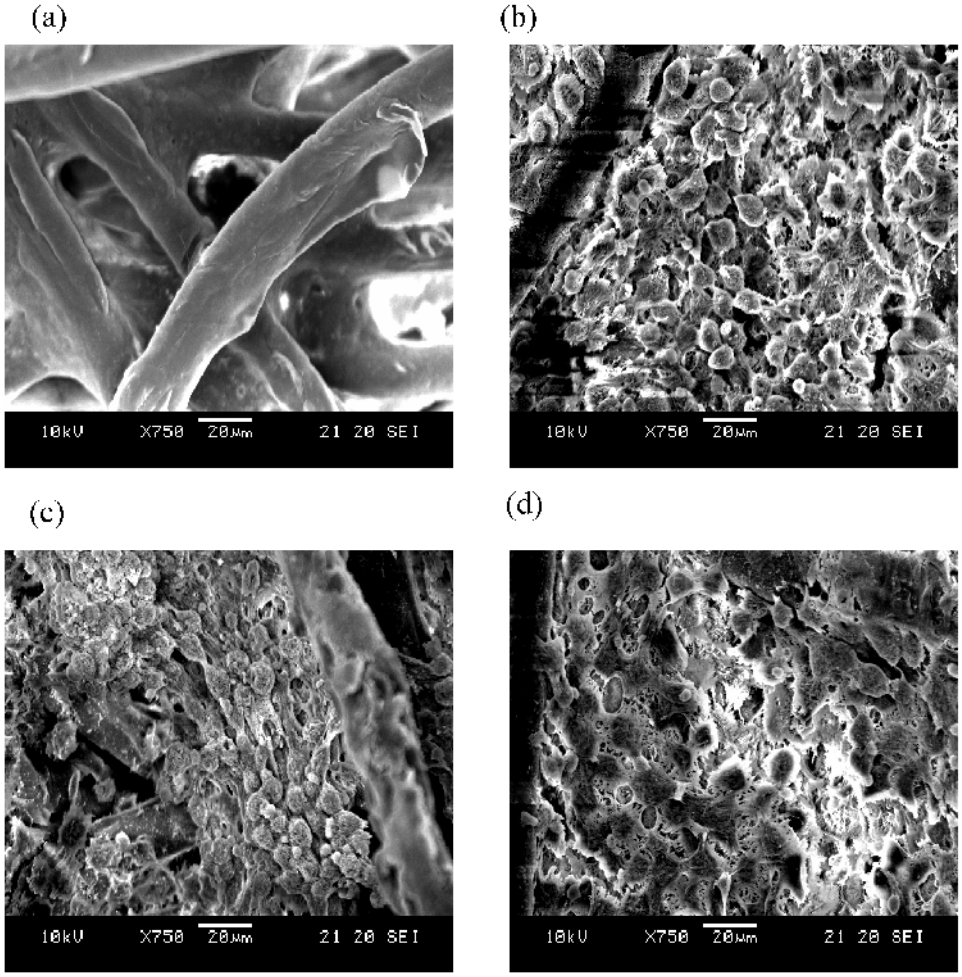

Figure 3. The scanning electron micrographs of cells and carriers: (a) the blank carrier included for comparison; (b–d) the carriers taken out at the top, middle and bottom of the bed, respectively. Magnification = 750×. Bar = 20 μm.

profile of the molar ratio of lactate production to glucose utilization (L/G, solid squares in Figure 4). A value of L/G equal or greater than 2 implies a switch from aerobic to anaerobic metabolism (Obradovic et al. 1999). As shown in Figure 4, the values of L/G were fairly high before day 1, which probably resulted from the adaptation of cells to the new environment in the packed bed. But L/G dropped below 2 quickly after the adaptation period and remained below 2 until day 10 and 11. In the last two days, the high density might have contributed to the increase of L/G but the values were on the borderline and were acceptable. Nonetheless, the profile suggests that aerobic metabolism was carried out without oxygen deficiency during the early- to mid-exponential phase. Both the DO and lactate measurements support our hypothesis that BelloCell® provides sufficient oxygenation for aerobic metabolism.

Virus production using BelloCell®

Now that high cell yield could be attained in BelloCell®, we further tested the capability of BelloCell® for viral vector production. The low linear flow rate (1 mm s^{-1}) ensuring the low shear environment in this system will be particularly suitable for the production of enveloped virus which would otherwise lose its infectivity under high shear stress. In this study, a recombinant enveloped baculovirus (Bac-CEH) was used as the model product. The cells were grown similarly as described

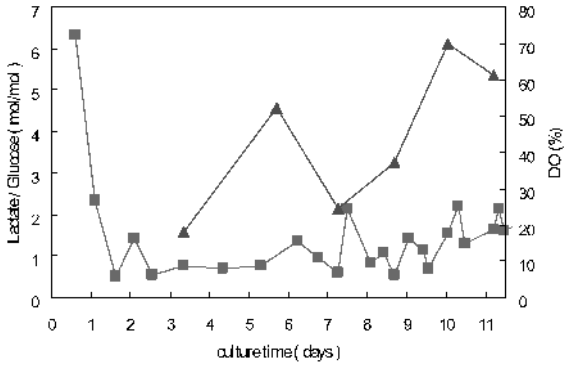

Figure 4. Time course profiles of DO (▲) and the molar ratio of lactate production to glucose utilization (■).

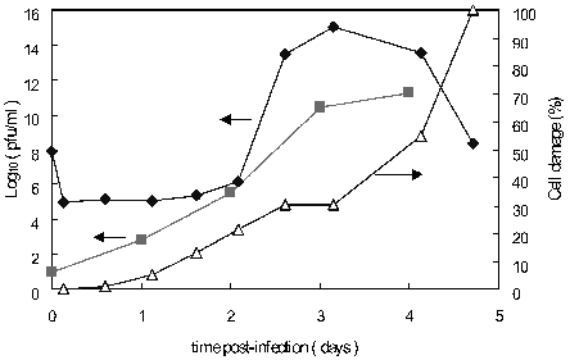

Figure 5. Time course profiles of the virus titers produced in BelloCell® (◆) as well as in spinner flasks (■), and percentage of cell damage in the BelloCell® system (△) assessed by LDH activity variation.

above to 2.6×10^9 cells and infected by Bac-CEH at MOI 10. As shown in Figure 5, the initial virus titer dropped sharply from 8.7×10^7 to 9×10^4 pfu ml^{-1} within 3 h, suggesting that >99% of the viruses successfully entered the immobilized cells with the gentle fluid motion. The virus titer remained low at the first two days post-infection (dpi) and then increased dramatically from day 2 to day 3, culminating at 1.1×10^{15} pfu ml^{-1}. As a result, the total virus yield that could be recovered from 300 ml medium corresponded to 3.3×10^{17} pfu. In addition to the high titer, the total medium used in this production run was only 1.1 l (800 ml for growth phase and 300 ml for infection phase). The high yield on low medium consumption demonstrated that this reactor was suitable for baculovirus production.

For comparison, the virus production was also performed in the spinner flask (200 ml working volume) as described previously (Hu and Bentley 2000). The maximum titer was only 1.8×10^{11} pfu ml^{-1} at day 4 (Figure 5). Thus, the virus concentration produced in BelloCell® (1.1×10^{15} pfu ml^{-1}) was significantly higher in comparison with that produced in the spinner flask. This enormous advantage over spinner flasks could be explained by: (1) BelloCell® provided better oxygen and nutrient transfer so that the cells produced more virus particles, in particular, oxygen demand would become more intense after infection and thus the good oxygen transfer could enhance the production of virus particle; (2) the low shear environment resulted in less damage to the virus envelope and hence reduced the number of defective particles. The combined effect might have accounted for the significant increase in the virus titer produced in BelloCell®.

Also note that Bac-CEH was designed to overexpress histidine-tagged gp64. It was hypothesized that the high titer resulted not only from the highly efficient production of virus particles, but also from the significantly high affinity of Bac-CEH to cells or more efficient endosomal escape inside the cell which are required for viral DNA transport to the nucleus. It has been shown that extra copies of gp64 on baculovirus envelope can enhance the virus infectivity and protein expression for up to 100-fold (Tani et al. 2001). The production of Bac-CEH in the spinner flask (1.8×10^{11} pfu ml^{-1}) might yield comparable amounts of virus particles comparing to other normal recombinant baculoviruses produced in the spinner flask ($\approx 5 \times 10^8$ pfu ml^{-1}), but Bac-CEH probably had considerably higher infectivity and thus resulted in higher titer. Taken together, these factors might have contributed to the high titer produced in BelloCell®, but the contribution of each factor remains unclear at this stage.

Figure 5 also depicts the cell damage quantified by LDH assay as an indicator of the cell health. As shown, the percentage of cell damage rose gradually in the first three days and then exhibited a sharp increase from 3 dpi. In contrast, the virus titer increased dramatically at 2 dpi and reached the peak at 3 dpi when the cell damage corresponded to ≈30%, then declined. The drop in virus titer might be due to the cell lysis and release of

cellular proteases to the medium, which subsequently degraded the virus. The coincidence of maximum titer and sharp increase in cell damage was also observed previously (Hu and Bentley 2000), hence the cell damage determined by LDH assay might be able to serve as an indicator for optimal harvest time. In this case, 3 dpi was optimal for virus harvest.

Conclusion

Using BelloCell® reactor, a high cell yield of 4.6×10^9 cells was attained using 2 l medium by repeated-batch operation. This final yield corresponded to a 38-fold growth and was comparable or superior to those in other reactor systems. The cell distribution was uniform in the bed as visualized by SEM. The total baculovirus yield achieved using BelloCell® could be up to 3.3×10^{17} pfu, demonstrating that this system is particularly suitable for baculoviral vector production. Moreover, the total volume of medium consumed for the whole production was only 1.1 l (because the cells were grown to only 2.6×10^9 cells), which translates into a highly cost-effective process. In particular, BelloCell® requires no air sparging, no pumping and no agitation and thus provides a low shear environment. Owing to the simple design, BelloCell® was extremely easy to handle and operate. Moreover, up to 4 BelloCell® reactors could be operated on one BelloStage® platform simultaneously. These benefits enable BelloCell® to become a simple and economical system for high-density cell culture and protein/virus production.

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