Biotechnology Letters (2005) 27: 1059–1065
DOI 10.1007/s10529-005-8450-3

A novel oscillating bioreactor BelloCell: implications for insect cell culture and recombinant protein production

Jen-Te Lu, Yao-Chi Chung, Zun-Ren Chan & Yu-Chen Hu*
Department of Chemical Engineering, National Tsing Hua University, 300, Hsinchu, Taiwan
**Author for correspondence (Fax: +886-3-571-5408; E-mail: yuchen@che.nthu.edu.tw)*

Received 3 March 2005; Revisions requested 22 March 2005 and 10 May 2005; Revisions received 3 May 2005 and 13 May 2005; Accepted 20 May 2005

Key words: baculovirus, BelloCell, immobilization, packed bed reactor, protein production

Abstract

BelloCell is a novel packed bed bioreactor that allows alternating nutrient and gas transfer to a culture. *Spodoptera frugiperda* Sf-9 grown in the BelloCell (300 ml culture) reached $1.3–1.5\times10^7$ cells ml^{-1} in 7–8 days and the total baculovirus-expressed protein yield was 2.3-times that in a stirred tank bioreactor (600 ml culture). The superior cell and protein yields underline the potential of BelloCell for cell culture and recombinant protein production.

Introduction

To date, roller bottles and stirred tank reactors are widely used for animal cell cultures, but increasing the maximum cell density and protein yield is difficult due to mass transfer limitation and toxic by-product accumulation. Alternatively, cells can be immobilized in hollow fiber, fluidized bed or packed bed reactors. Hollow fiber reactors generate low shear stress but require an exterior oxygenation system and a high circulation rate to provide adequate O_2 (Alauotila *et al.* 1994). Fluidized bed and packed bed reactors are designed to maximize the cell density, but sophisticated skills are required for operation and the capital costs are high (Hu *et al.* 2000). Although disposable reactors, such as CellCube (Corning Life Sciences, Corning, NY) and Cell Factories (Nalge Nunc International, Roskilde, Denmark), have been developed to save the capital costs, the maximum cell densities are limited by contact inhibition.

Recently, a novel disposable bioreactor, BelloCell, composed of a packed bed in the upper chamber and "bellows" in the lower chamber was developed for animal cell culture (Cesco Bioengineering Co.). The oscillating compression and relaxation of the bellows enables the immobilized cells to be sequentially submerged into medium for nutrient supply and exposed to the ambient air for gas exchange. We have demonstrated that BelloCell is capable of supporting high-density insect cell culture and baculovirus production but the process time is lengthy (Hu *et al.* 2003a). Furthermore, the effects of various process parameters on the cell growth rate, cell yield, duration of lag phase and cell distribution were not evaluated in the previous study (Hu *et al.* 2003a). The present study is therefore aimed at investigating the effects of these parameters so as to further improve the process. In addition, the performance of this system for recombinant protein production was compared with that of the conventional stirred tank bioreactor.

Materials and methods

Cell, media and baculovirus

Insect cell *Spodoptera frugiperda* Sf-9 (American Type Culture Collection, Manassas, VA) was

cultivated using TNM-FH medium (Gibco BRL) containing 10% (v/v) fetal calf serum (FCS, Sigma). The recombinant baculovirus, Bac-CE, was constructed previously to express enhanced green fluorescent protein (EGFP) as the reporter protein (Hu *et al.* 2003b). The virus was amplified and titered as described elsewhere (Hu *et al.* 2003b).

Carriers for cell immobilization

Three different carriers were used for cell immobilization in this study. Fibra-Cel disk was made of polyester non-woven fabric laminated to a polypropylene screen and was commercially available (Bibby Sterilin). BioNOC (Cesco Bioengineering Co.) was made of polyester sheathed with high-density polyethylene. BioNOC II (Cesco Bioengineering Co.) was similar to BioNOC but it was non-woven fabric made of 100% polyester. The carriers were pre-packed in BelloCell and pre-sterilized by gamma irradiation.

BelloCell system setup and operation

BelloCell is a disposable plastic bottle (working volume 300 ml) that comprises two compartments: upper and lower chambers. The upper chamber contains two carrier support screens to accommodate the carriers that constitute the packed bed. The compressible lower chamber (designated as "bellows" thereafter) containing the medium could be compressed and released by BelloStage (Cesco Bioengineering Co., Hsinchu, Taiwan) in an alternating manner (Figure 1a and b). The linear moving rate of the holding plate was set at 1 mm s^{-1} unless otherwise noted and the culture started.

Cell count and viability measurement

The cell densities in the suspension cultures were measured by a hemacytometer and the viabilities were determined by trypan blue dye exclusion (Hu *et al.* 2003a). The number of immobilized cells during the BelloCell operation

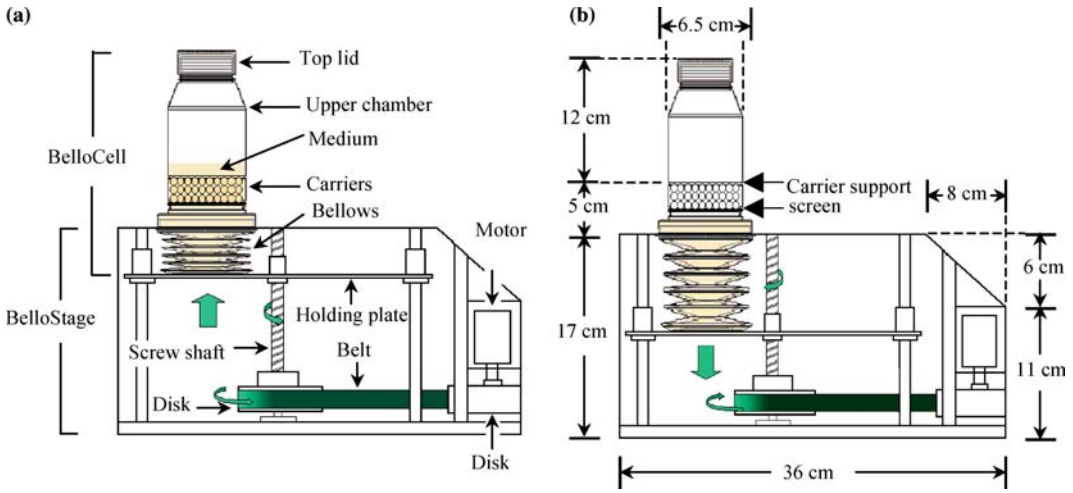

Fig. 1. Schematic illustration of BelloCell and BelloStage. (a) The BelloStage platform contains a motor whose power was transmitted through a belt drive and a screw shaft. The ascending movement of the holding plate lifted the bellows and raised the medium level to submerge the carriers, thus allowing for nutrient transfer. (b) After a delay time (10 s), the descending movement of the holding plate dropped the medium to the lower bellows, thus exposing the carriers to air for oxygen transfer. The cyclic change in the rotating direction of the screw shaft moved the holding plate upward and downward. Prior to cultivation, the packed carriers (bed volume = 100 cm^3) were immersed in PBS for 24 h. Sf-9 cells were centrifuged and resuspended in 30 ml fresh medium. Fresh medium (270 ml) was added from the top and the bellows was compressed and released. After 1 h, the cell inoculum was added and the reactor was mounted onto the BelloStage in the 27 °C incubator. The timing and strategy of medium replenishment, and when the maximum glucose uptake rate (GUR) occurred, were determined based on the glucose concentration and pH (Hu *et al.* 2003a). After the maximum GUR occurred, the GUR fluctuated around the maximum value for 1–2 days and then dropped rapidly. Concomitant with the decrease in GUR, pH swiftly dropped below 6 and the culture was terminated. The carriers were removed for cell count and viability measurement.

was estimated by converting the calculated glucose uptake rate (GUR) to the estimated cell number based on the assumption of a constant specific GUR (0.2 mg per 10^6 cells per day). Such indirect estimation of cell number in immobilized cell cultures has been commonly adopted (Racher & Griffiths 1993, Kaufman *et al.* 2000).

At the end of the culture, 60 pieces of carriers were taken from the top, middle and bottom layers in the bed, respectively, to determine the final cell yield in BelloCell. Every 30 pieces was mixed with 3 ml lysis solution (0.1% Crystal Violet in 0.1 M citric acid) and incubated for 1 h at 37 °C. The nuclei were released by vortexing and counted by a hemacytometer. The viable cell count was evaluated by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay (Kompier *et al.* 1991). The viability was calculated as the ratio of viable to the total cell number (determined by nuclei counting).

Virus infection and protein production

For protein production in the BelloCell, 9×10^7 cells were inoculated and the reactors were operated as described above. The spent medium was decanted prior to infection, and then 270 ml fresh medium containing Bac-CE was added for infection. After 1 h of operation, 30 ml FCS was supplemented and the production phase continued for 5.5 days without medium exchange. For protein production in the stirred tank bioreactor, Biostat B (B. Braun Biotech International) was used for culturing 600 ml Sf-9 cells (agitation = 80 rpm, dissolved O_2 = 50%). The cells (1.5×10^6 cells ml^{-1}) were withdrawn, centrifuged aseptically and resuspended in 100 ml virus-containing TNM-FH. The infected cells were shaken mildly for 1 h and then inoculated back into the reactor with 500 ml fresh medium (600 ml working volume). The production phase continued for 5.5 days without medium exchange.

Fluorescence intensity (FI) measurement

During the production phase, 2 ml medium was taken daily to measure the extracellular fluorescence intensity (FI) spectrophotometrically ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 520 nm). For intracellular FI in BelloCell, the carriers were taken as described above, soaked in phosphate buffered-saline (PBS, five carriers per ml) in 15 ml tubes, sonicated for 10 min and centrifuged. The FI in the supernatant was measured and converted to the overall intracellular FI. For intracellular FI in Biostat B, the cells were sonicated in PBS, and the FI in the supernatant was measured similarly and converted to the overall intracellular FI. The total FI was the sum of the intracellular and extracellular FI and served as the indicator of the EGFP yield in BelloCell and Biostat B.

Results and discussion

Effects of carriers

To evaluate the performance of BelloCell for culturing insect cells in different carriers, BioNOC, BioNOC II and Fibra-Cel were employed for cell growth. Figure 2 shows that the cells immobilized on different matrices had different growth kinetics. Table 1 indicates that the measured final yields on BioNOC and BioNOC II were only 18% and 7% less than the estimated maximum yields. Considering that the final cell numbers were measured 1–2 days after the estimated maximum cell numbers were reached, this small margin was negligible, thus validating the use of GUR to track the growth kinetics of insect cells on these two carriers. However, the

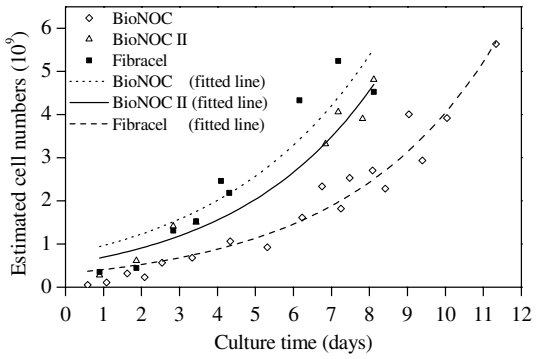

Fig. 2. Time-course profiles of estimated cell numbers in three different carriers. Approximately 1.2×10^8 cells were inoculated then the reactors were operated as described in Figure 1. The cell numbers were estimated by GUR (symbols) and the lines fitted using the Monod equation are shown. For simplicity, the data are shown over the culture time until the maximum GUR was attained.

Table 1. The growth characteristics using different carriers.

	Carriers		
	BioNOC	BioNOC II	Fibra-Cel
Max. cell number (10^9 cells)[a]	5.6 ± 0.2	4.1 ± 0.2	5.3 ± 0.2
Final cell number (10^9 cells)[b]	4.6 ± 0.1	3.8 ± 0.4	2.8 ± 0.2
Final cell yield (fold)[c]	47 (38)	34 (32)	44 (22)
Duration of lag phase (day)	2.5	<1[d]	<1[d]
Specific growth rate (h^{-1})	0.014	0.019	0.022
Viability (%)	N/D	85	78

[a]Maximum cell number was estimated by GUR (glucose uptake rate) and is denoted as mean ± SD.
[b]Final cell number was measured by nuclei counting and is denoted as mean ± SD.
[c]Fold increase in final cell yield was calculated based on the cell numbers estimated by GUR and measured by nuclei counting (quoted in parenthesis) compared to the inoculated cell number.
[d]Duration of the lag phases was estimated to be < 1 day because the first glucose samples were taken at 24 h post-inoculation, and the exact starting time of log phase was unknown.

final number of cells on Fibra-Cel was only 53% of the estimated number, probably due to serious cell detachment from Fibra-Cel over culture time because increasing concentration of cells suspended in the medium was observed after 4 days (up to 6.7×10^5 cells ml^{-1} at day 7, not shown). Consequently, Fibra-Cel was not suitable for insect cell immobilization in BelloCell.

The measured cell densities using either BioNOC or BioNOC II corresponded to ≈1.5- and 1.3×10^7 cells per ml in the 300 ml culture, which far exceeded $1–4\times10^6$ cells ml^{-1} normally achieved in suspension cultures using TNM-FH medium (Bedard *et al.* 1993, Akhnoukh *et al.* 1996). Overall, the cells grew 38- and 32-fold on BioNOC and BioNOC II, respectively (Table 1) and the cell yields were high compared to those using other immobilizing carriers (Kompier *et al.* 1991, Archambault *et al.* 1994). All these data further validated their use in BelloCell. However, although BioNOC possessed higher capacity for cell growth (19% more) compared to BioNOC II, the lag phase was significantly longer and the specific growth rate (0.014 h^{-1}) was lower (Table 1), which are not desired from a bioprocess perspective. Only BioNOC II resulted in reasonably short lag phases and high growth rates; moreover, the cell viability on BioNOC II remained 85%. Taken together, BioNOC II was more suitable for use in BelloCell in terms of final cell yield and growth rate, and was utilized in all subsequent experiments.

Effects of inoculum size

To determine the dependence of growth kinetics on inoculum size, BelloCell reactors (working volume 300 ml) were operated as described above but seeded at five different inoculum sizes: 3, 6, 9, 12 and 24×10^7 cells (corresponding to 1, 2, 3, 4 and 8×10^5 cells per ml culture). Figure 3 depicts that the growth profoundly depended on the inoculum size and the effect was more striking at low inoculum size. Table 2 reveals that the duration of exponential phases and specific growth rates in all five cultures were fairly close, while a longer lag phase (e.g. 2.8 days) was observed for lower inoculum size

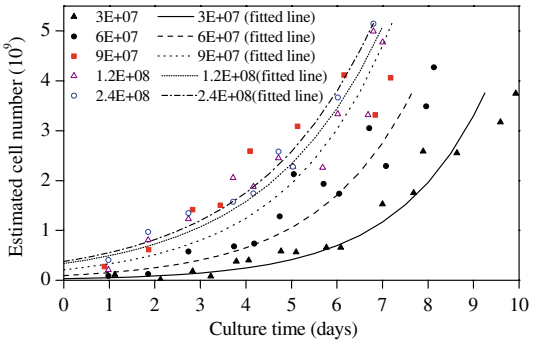

Fig. 3. Time-course profiles of estimated cell numbers in cultures with different inoculum sizes (3, 6, 9, 12 and 24×10^7 cells). The cell numbers were estimated by GUR (symbols) and the lines fitted using the Monod equation are shown. For simplicity, the data are shown over the culture time until the maximum GUR was attained.

Table 2. The growth characteristics using different inoculum sizes.

	Inoculum size ($\times 10^7$ cells)				
	3	6	9	12	24
Max. cell number (10^9 cells)[a]	3.7 ± 0.2	4.3 ± 0.2	5.2 ± 0.2	4.1 ± 0.2	5.2 ± 0.2
Final cell number (10^9 cells)[b]	4.3 ± 0.2	N/D	4.6 ± 0.2	3.5 ± 0.4	N/D
Final cell yield (fold)[c]	125 (142)	71	57 (51)	34 (32)	21
Duration of lag phase (day)[d]	2.8	2.3	<1[d]	<1[d]	<1[d]

[a]Maximum cell number was estimated by GUR and is denoted as mean ± SD.
[b]Final cell number was measured by nuclei counting and is denoted as mean ± SD.
[c]Fold increase in final cell yield was calculated based on the cell numbers estimated by GUR and measured by nuclei counting (quoted in parenthesis) compared to the inoculated cell number.
[d]Duration of the lag phases was estimated to be <1 day because the first glucose samples were taken at 24 h post-inoculation, and the exact starting time of log phase was unknown.

(e.g. 3×10^7 cells), suggesting that the longer culture time needed at lower inoculum size mainly stemmed from the requirement for adaptation. Once the cells resumed growing, the cells exhibited similar behavior in all cultures. Among these five inoculum sizes, 3×10^7 cells resulted in 142-fold increase in the final cell yield, which was higher than those achieved in other carrier types and reactors (Reiter *et al.* 1991, Archambault *et al.* 1994). The drawback was that the lag phase was lengthy. On the contrary, an inoculum of 24×10^7 cells resulted in a short lag phase (<1 day), but the cell number increased only 21-fold and the inoculum had to be prepared from 250 ml spinner flasks. In contrast, inoculum sizes between 9- and 12×10^7 cells resulted in short lag phases (<1 day) and achieved 51- and 32-fold cell growth in about 7–8 days. Moreover, the inoculum could be expanded readily in 100 ml spinner flasks. Therefore, inoculum sizes of 9- and 12×10^7 cells (corresponding to $3–4\times10^5$ cells per ml suspension culture) are suggested.

Effects of linear moving rate

In all previous experiments, the linear moving rates of BelloStage holding plate were set constant at 1 mm s^{-1} for both upward and downward movements. Since the linear moving rate of the plate is the major variable influencing the growth and distribution of the cells in the bed, the effects were studied at three different rates: 0.5, 1, and 2 mm s^{-1}. As shown in Figure 4, the binding efficiency varied slightly with the linear rates in the first 30 min. Nonetheless, virtually all cells (>97%) attached to BioNOC II after 1 h in all three cases, confirming that BelloCell reactor generated sufficient fluid flow to impart efficient binding, even at low linear rate. The ensuing growth characteristics (Table 3) show that the lengths of the lag phase were less than 1 day in all three cases, suggesting that the cells adapted readily in the BelloCell reactor regardless of the linear rate.

Whether the linear rates influenced the cell distribution at the end of the culture was also investigated by measuring the distribution of cells in different layers of the bed (not shown). We found that the cell distribution was nearly

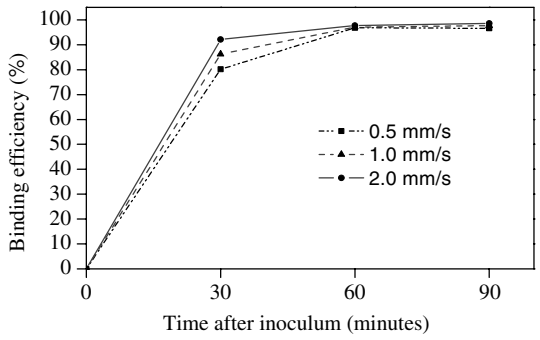

Fig. 4. Variation of cell binding efficiency to BioNOC II over time at different linear moving rates. Approximately 9×10^7 cells were inoculated and the reactors were operated as described above. The residual cell concentrations in the media were measured to calculate the binding efficiencies of cells. The binding efficiency was calculated as 1-(cell number remaining in the medium/initial cell number). No cell settling was observed at the bottom of BelloCell during the 90 min span.

Table 3. The growth characteristics using different linear moving rates.

	Linear moving rate[a] (mm s^{-1})		
	0.5	1	2
Final cell number (10^9 cells)[b]	5.6 ± 0.4	4.6 ± 0.2	4.9 ± 0.2
Final cell yield (fold)[c]	66 (62)	57 (51)	50 (54)
Duration of lag phase (day)	<1[d]	<1[d]	<1[d]
Specific growth rate (h^{-1})	0.014	0.019	0.022

[a]The cells were grown using varying linear moving rates of the holding plate.
[b]Final cell number was measured by nuclei counting and is denoted as mean ± SD.
[c]Fold increase in final cell yield was calculated based on the cell numbers estimated by GUR and measured by nuclei counting (quoted in parenthesis) compared to the inoculated cell number.
[d]Duration of the lag phases was estimated to be <1 day because the first glucose samples were taken at 24 h post-inoculation, and the exact starting time of log phase was unknown.

uniform in all three layers and seemed independent of the linear rates. This finding confirmed that these three linear rates generated gentle and uniform flow fields. The uniformity was achieved as soon as the cells attached to the carriers and ultimately resulted in uniform cell growth in the reactor.

Recombinant protein production using BelloCell

The capability of BelloCell and Biostat (a commercial stirred tank bioreactor) for protein production was compared using EGFP as the model product. Table 4 reveals that the total and volumetric yields of EGFP obtained in BelloCell (300 ml culture) were 2.3- and 4.5-times of those in Biostat B (600 ml culture), respectively, thus demonstrating the advantage of BelloCell over conventional stirred-tank bioreactor for protein production.

Table 4. The comparison of product yields obtained in BelloCell and Biostat B[a].

	Density at infection (cells per ml culture)	Total EGFP yield (units)	Volumetric EGFP yield (units per ml culture)
BelloCell	1.3×10^7	1.4×10^4	46.3
Biostat B	1.5×10^6	6.2×10^3	10.3

[a]The total volumes of BelloCell and Biostat B were 300 and 600 ml, respectively. The cells in BelloCell and Biostat B were infected at MOI (multiplicity of infection) 10. The EGFP yield was measured as described in Materials and methods.

Conclusion

Our data show that the BelloCell is suitable for insect cell culture and recombinant protein production. Considering process time and final cell yield, inoculating 9- and 12×10^7 cells onto Bio-NOC II and setting the linear rate at 1 mm s^{-1} is suggested for repeated-batch operations, in which final yields of ≈3.8–4.6×10^9 cells can be achieved and the process time was significantly decreased from 11–12 days (Hu *et al.* 2003a) to 7–8 days. Furthermore, the whole operation required only 1.5–2 l of medium and needed no sparging or mixing, thus reducing the mechanical stress. Thanks to the simple design, BelloCell was extremely easy to handle and operate. Segregation of the cells and medium also permits either fed-batch, semi-continuous or continuous operation without cell washout. All these benefits, in conjunction with the high yield and low medium consumption, underline the potential of BelloCell as an efficient and economical system for cell culture and protein production.

Acknowledgement

The authors gratefully acknowledge the support of the National Science Council (grant NSC 92-2622-E-007-024-CC3), Taiwan, and of the Ministry of Economic Affairs (grant MOEA 92-EC-17-A-17-S1-0009). We also wish to thank Mr. King-Ming Chang, Dr. Yu-Chi Wang and Ms. Fen-Lang Lin of Cesco Bioengineering, Co. (Taiwan) for valuable assistance and comments.

References

Akhnoukh R, Kretzmer G, Schugerl K (1996) On-line monitoring and control of the cultivation of *Spodoptera frugiperda* Sf9 insect cells and beta-galactosidase production by Autographa californica virus vector. *Enzyme Microb. Technol.* **18**: 220–228.

Alauotila S, Marjamaki A, Matikainen MT, Jalkanen M (1994) Use of a hollow-fiber bioreactor for large-scale production of alpha 2-adrenoceptors in mammalian cells. *J. Biotechnol.* **37**: 179–184.

Archambault J, Robert J, Tom L (1994) Culture of immobilized insect cells. *Bioprocess Eng.* **11**: 189–197.

Bedard C, Tom R, Kamen A (1993) Growth, nutrient consumption, and end-product accumulation in Sf-9 and BTI-EAA insect cell cultures: Insights into growth limitation and metabolism. *Biotechnol. Prog.* **9**: 615–624.

Hu Y-C, Kaufman J, Cho MW, Golding H, Shiloach J (2000) Production of HIV-1 gp120 in packed-bed bioreactor using the vaccinia virus/T7 expression system. *Biotechnol. Prog.* **16**: 744–750.

Hu Y-C, Lu J-T, Chung Y-C (2003a) High-density cultivation of insect cells and production of recombinant baculovirus using a novel oscillating bioreactor. *Cytotechnology* **42**: 145–153.

Hu Y-C, Tsai C-T, Chang Y-J, Huang J-H (2003b) Enhancement and prolongation of baculovirus-mediated expression in mammalian cells: Focuses on strategic infection and feeding. *Biotechnol. Prog.* **19**: 373–379.

Kaufman JB, Wang G, Zhang W, Valle MA, Shiloach J (2000) Continuous production and recovery of recombinant Ca^{2+} binding receptor from HEK 293 cells using perfusion through a packed bed bioreactor. *Cytotechnology* **33**: 3–11.

Kompier R, Kislev N, Segal I, Kadouri A (1991) Use of a stationary bed reactor and serum-free medium for the production of recombinant proteins in insect cells. *Enzyme Microb. Technol.* **13**: 822–827.

Racher AJ, Griffiths JB (1993) Investigation of parameters affecting a fixed bed bioreactor process for recombinant cell lines. *Cytotechnology* **13**: 125–131.

Reiter M, Bluml G, Gaida T, Zach N, Unterluggauer F, Doblhoffdier O, Noe M, Plail R, Huss S, Katinger H (1991) Modular integrated fluidized-bed bioreactor technology. *BioTechnology* **9**: 1100–1102.