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Baculovirus production for gene therapy: the role of cell density, multiplicity of infection and medium exchange

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Abstract One of the major concerns regarding the use of insect cells and baculovirus expression vectors for the production of recombinant proteins is the drop in production observed when infecting cultures at high cell densities; this work attempts to understand this so-called cell density effect in the scope of baculovirus production for gene therapy purposes. A *Spodoptera frugiperda* insect cell line (Sf-9) was cultured and infected in serum-free medium, and the patterns of production of a recombinant baculovirus expressing the green fluorescent protein (GFP) were analyzed at different cell concentrations at infection (CCIs) and multiplicities of infection (MOIs). The results confirm that a cell density effect on productivity occurs which is dependent on the MOI used, with a high MOI "delaying" the drop in production to higher cell densities. Medium replacement at the time of infection using a high MOI considerably improved baculovirus production, with the different production indicators, namely the titer, specific yield, amplification factor, and time of harvesting, increasing with cell concentration for the CCI range tested. Virus titers as high as 2.6×10^{10} IP.mL^{-1} were obtained in cultures infected at 3.5×10^{6} cells.mL^{-1}, while the amplification factor was roughly 19 times higher than the highest value obtained without medium exchange.

Keywords Baculovirus · Gene therapy · Cell density effect · Sf-9 cells · Multiplicity of infection · Medium exchange

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Introduction

The infection of Sf-9 cells by baculoviruses (BVs) has proven to be an efficient and versatile system for the production of a wide repertoire of biological products, from single proteins to multi-subunit complexes such as virus-like particles and adeno-associated viruses (Maranga et al. 2002; Patterson et al. 1995; Urabe et al. 2002). More recently, an increasing number of investigators have tackled the use of BVs for gene therapy applications (Kost and Condreay 2002). The manufacturing of modified BV vectors able to direct gene expression in mammalian cells represents a safer alternative over classical mammalian viruses. The validation of this approach implies generating knowledge on the mechanisms governing tissue targeting, gene delivery, and host immunological responses to the engineered BVs. Furthermore, in order to facilitate the use of BVs in gene therapy, the development of efficient production processes to amplify the engineered vectors is needed.

Most of the studies reported on bioprocesses using the BV/insect cell system are focused on the production of recombinant proteins rather than the vector itself (Ikonomou et al. 2003). In terms of process productivity, it is desirable to infect a highly productive insect cell culture at later stages of

growth, when higher cell densities can lead to higher titers of product. However, early on investigators have come to the conclusion that recombinant protein yields decrease in batch cultures infected during the later stages of growth (Caron et al. 1990; Reuveny et al. 1993). To cope with this phenomenon, a number of strategies ranging from improving medium composition, total or partial replenishing of medium at infection, nutrient supplementation schemes, fed-batch processes and perfusion cultures have been used to improve production (Ikonomou et al. 2003). Although the literature related to the production of BV vectors is very scarce, some early works demonstrated similar effects of cell density on the yields of wild-type viruses in a *Trichoplusia ni* cell line (Stockdale and Gardiner 1977; Wood et al. 1982). Accordingly, Klöppinger et al. (1990) found that the production of *Autographa californica* nuclear polyhedrosis virus using a *S. frugiperda* cell line was inhibited at a cell density of 1.5×10^6 cells.mL^{-1}.

Another significant factor influencing protein production is the multiplicity of infection (MOI), i.e., the number of infectious particles (IP) per cell added to a culture. More specifically, the interplay between MOI and cell concentrations at infection (CCI) is of utmost importance for the design of efficient infection strategies and to better comprehend the BV–insect cell system. Reports addressing this issue agree that, in batch culture, recombinant protein titers are relatively insensitive to MOI when the cells are infected during the early exponential phase, whereas from middle to late exponential phase a strong positive correlation exists between the two factors (Licari and Bailey 1992; Power et al. 1994).

The present work focuses on assessing the effect of cell density on the production of recombinant BVs assuming a comparative perspective with knowledge related with protein production. The questions addressed in this work are: Is there a cell density effect on the production of BVs by infection of Sf-9 cells in serum free medium? If so, is this effect dependent on the MOI used? Finally, can this effect be repressed by restoring initial medium conditions at the time for infection? For this purpose, several indicators of system productivity were evaluated for infection experiments where different CCIs at both low and high MOI were tested. Then, the combinations of CCIs and MOIs that yielded better production results were subject to medium replacement at the time of infection.

Materials and methods

Cell line, culture medium, and maintenance

The host insect cell line Sf-9, ECACC 89070101, originally isolated from the pupal ovaries of *Spodoptera frugiperda*, was provided by Dr. Otto W. Merten (Genethon, France). Cultivation was performed in 500 mL Erlenmeyer flasks (Corning, USA) with 50 mL working volume after thawing cells from an initial passage number of 17. Sf900II serum-free medium (Gibco, Invitrogen, USA), specifically designed for Sf-9 cells, was used throughout this work. Cells for maintenance were re-inoculated every 3 days at a cell density of $4–5\times10^5$ cells.mL^{-1} and kept in a humidified incubator operated at 27°C and 90 rpm. Cellular concentration was determined by cell count in a Fuchs–Rosenthal chamber and the viability assessed by the Trypan Blue exclusion method. To assess cellular lysis, the activity of lactate dehydrogenase (LDH; EC 1.1.1.27) released to the medium was measured by following the rate of pyruvate reduction to lactate. This reaction is coupled with the oxidation of NADH to NAD^+, which can be measured spectrophotometrically at 340 nm (Racher et al. 1990).

Virus and viral stock

The recombinant baculovirus Ac-hisGFP, provenient from the parental *A. californica* nuclear polyhedrosis virus, was provided by Dr. Monique M. van Oers (Laboratory of Virology, Wageningen University, The Netherlands), containing a GFP gene under the control of the polyhedrin promoter. Recombinant BVs were amplified by infecting Sf-9 cells at 1×10^6 cells.mL^{-1} with a MOI of 0.1 IP.cell^{-1}, in a 250-mL spinner flask (Wheaton, USA), and culture bulk was harvested after 5 days incubation at 27°C and 150 rpm. After harvesting, cells were seeded by centrifuging at 1,700×*g* for 10 min, and the supernatant containing viruses was stored at 4°C and protected from light for several months.

Experimental cultures and sampling

Sf-9 cells, after several times passage to ensure consistent high viabilities above 95%, were infected with recombinant BVs for evaluation of virus production and culture behavior. Experimental cultures were at a passage number not higher than 31. Cultures were performed in 125 mL spinner flasks (Wheaton, USA) at 27°C and with an agitation of 150 rpm. A total of six independent experiments were performed varying the CCI (1×10^6 cells.mL^{-1} (CCI 1), 2×10^6 cells.mL^{-1} (CCI 2), and $3–4\times10^6$ cells.mL^{-1} (CCI 3), corresponding to 24, 48, and 72 h of culture, respectively) for two different MOIs (0.6 IP.cell^{-1}—low MOI and 30 IP.cell^{-1}—high MOI). Three additional infections with a high MOI of 6 IP.cell^{-1} were carried with medium exchange at the time of infection, and the same set of CCIs was used. In these cultures, cells were centrifuged and re-suspended to the desired concentration in 10 mL of spent medium. Thereafter, recombinant BVs were inoculat-

ed, the volume was completed to 50 mL with fresh medium, and the cultures were maintained under continuous stirring to promote virus adsorption during 1 h. Finally, the volume was completed to 125 mL with fresh medium. Every 24 h, culture samples were taken and centrifuged at 1,700×*g* at room temperature for 10 min, in order to separate cells from the supernatant. Aliquots of the supernatant were then maintained at 4°C for virus titration and at −20°C to measure glucose, lactate, ammonia, and concentrations of free amino acids.

Analytical methods

Glucose and lactate concentrations were determined with automated enzymatic assays (YSI 7100 Multiparameter Bioanalytical System; Dayton, OH, USA). Ammonia was quantified enzymatically using a UV assay (No 1112732035; Boehringer Manheim, R-Biopharm AG, Darmstadt, Germany).

Amino acids were quantified by high performance liquid chromatography analysis using a reverse phase 3.9×150 mm column (AccQ.Tag, Waters, USA). A pre-column derivatization technique was used based on the Waters AccQ.Tag Amino Acid Analysis method. Briefly, both primary and secondary amino acid derivatives were produced by mixing with 6-aminoquinolyl *N*-hydroxysuccinimidyl-carbamate, allowing their separation and quantification by fluorescence detection at 395 nm. Prior to derivatization, sample proteins were precipitated by adding an equal volume of acetonitrile and removed by centrifugation at 12,400×*g* for 15 min, at room temperature. An internal standard (α-aminobutyric acid) was added to ensure consistent measurements between runs.

Virus titration

Virus titers were determined by an end-point dilution assay. In this method, 96-well plates containing 100 μL of cellular suspension at a density of 0.5×10^6 cells.mL^{-1} were incubated for 1 h at 27°C. Dilutions of the virus samples were performed serially in SF900II medium, from $1{:}10^4$ to $1{:}10^{11}$, and 100 μL were used to infect Sf-9 cells monolayers after removing the medium from plate wells. Ten replicates for each dilution were performed in the same plate, and two independent plates were infected for each viral sample. A dilution of $1{:}10^2$ was used as positive control. SF900II medium was used as negative control. Plates were screened after 7 days for GFP signal under an inverted fluorescence microscope, using an excitation wavelength range between 450 and 490 nm. The 50% tissue-culture infectious dose ($TCID_{50}$), i.e., the dilution which is sufficient to infect half of the cells, was calculated using equations described elsewhere (King and Possee 1992). This value was then converted to plaque-forming units (or number of infectious particles) by the relationship: $PFU=0.69\times TCID_{50}$ (King and Possee 1992).

Estimation of specific metabolic rates and production indicators

Specific consumption and production rates of important molecules of cell metabolism such as glucose, lactate, glutamine, serine, and alanine were estimated dividing the slopes of concentration profiles by the average of cell density along defined time intervals (48–144, 72–168, and 96–192 h). For the infection experiments, the average of cell density was calculated for the entire post-infection time, since linear concentration profiles after infection were observed for these compounds. The specific yield values presented were estimated by dividing the maximum titer (infectious particles per milliliter) by the CCI used in each infection experiment. The amplification factor was calculated as the ratio between maximum titer and the number of IPs per milliliter at the time of infection. Time of harvesting (TOH) corresponds to the culture time when the maximum titer was obtained.

Results

The effect of cell density on the production of recombinant BVs was assessed by infecting Sf-9 cultures at CCIs of 1, 2, and $3–4\times10^6$ cells.mL^{-1}, both at a MOI of 0.6 and 30 IP.mL^{-1}, in SF900II serum-free medium. The production indicators calculated for each condition tested were the maximum titer, specific yield, amplification factor and TOH. Additionally, specific metabolic rates of glucose, lactate, glutamine, serine and alanine were assayed, and the metabolic profiles of glucose and lactate were analyzed. Three additional experiments were carried out by exchanging the medium at the time of infection with a high MOI.

Infections at low MOI

When Sf-9 cells were infected with a recombinant BV (Ac-hisGFP) at a low MOI, a marked decrease in the growth rates upon infection was obtained, followed by a drop in viable cell density (Fig. 1a). Cellular lysis was also monitored throughout culture time, with an increase in extracellular LDH activity consistent with loss in viability (data not shown). Figure 2 shows typical profiles of glucose and lactate in uninfected and infected Sf-9 cultures. It is clear from the lower slopes in the glucose concentration profiles when compared to the uninfected culture that glucose uptake was down-regulated after infection (Fig. 2a). Lactate accumulated in the uninfected culture up

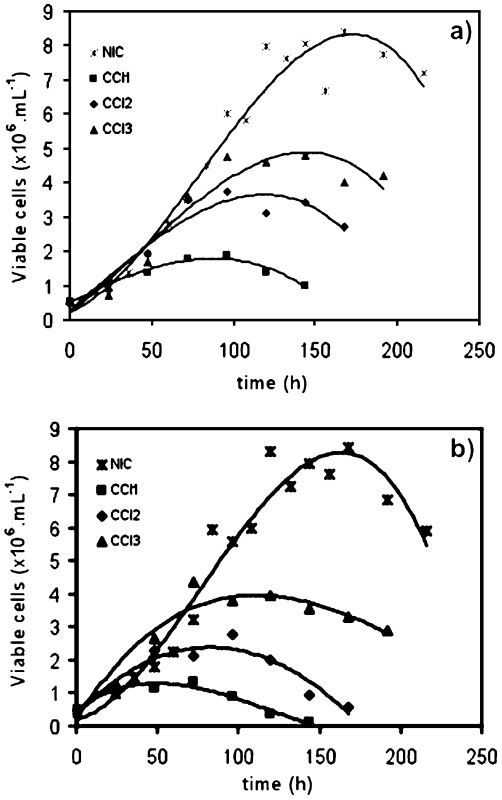

Fig. 1 Uninfected and infected Sf-9 cultures growth in SF900II serum-free medium. Infections with a recombinant BV (Ac-hisGFP) were carried either with a low MOI (**a**) and a high MOI (**b**), for different CCIs, without medium exchange. *NIC* uninfected cells

to approximately 12 mM (Fig. 2b), with a mean specific production rate of 10.57±0.75 nmol.(10^6 cells)$^{-1}$.h^{-1}, its concentration decreasing then at the end of the culture. In the infected cultures, lactate accumulation only occurred at CCI 3, with a specific production rate of 9.59±0.57 nmol.(10^6 cells)$^{-1}$.h^{-1}. In contrast, ammonia did not accumulate significantly in the medium at any stage of cultivation for all experiments performed, remaining approximately constant in the range of 4 to 6 mM (Fig. 3).

Figures 4 and 5 show the specific glucose and amino acid uptake rates for uninfected and infected cultures without medium exchange. It can be seen that the consumption of these nutrients clearly decreased after viral infection at low MOI. Interestingly, glucose uptake decreased both during the later stage of growth and after infection at a high CCI. The same pattern was observed for the consumption rates of serine and, more prominently, in the case of glutamine. The rates of alanine production are also represented. This was the only amino acid accumulating in the culture medium. None of the amino acids analyzed was depleted in the infected cultures, although serine was completely exhausted at the end of the uninfected culture (data not shown).

Regarding recombinant BV production, it can be seen in Table 1 that all the production indicators declined by one order of magnitude when cells were infected at progressively higher CCIs. The titer decreased from 0.18 to 0.007×10^9 IP.mL^{-1} and the specific yield decreased from 172 to 2 IP.cell^{-1}. Moreover, the amplification factor dropped from 287 to 3, and the TOH was 120 h for all CCIs tested.

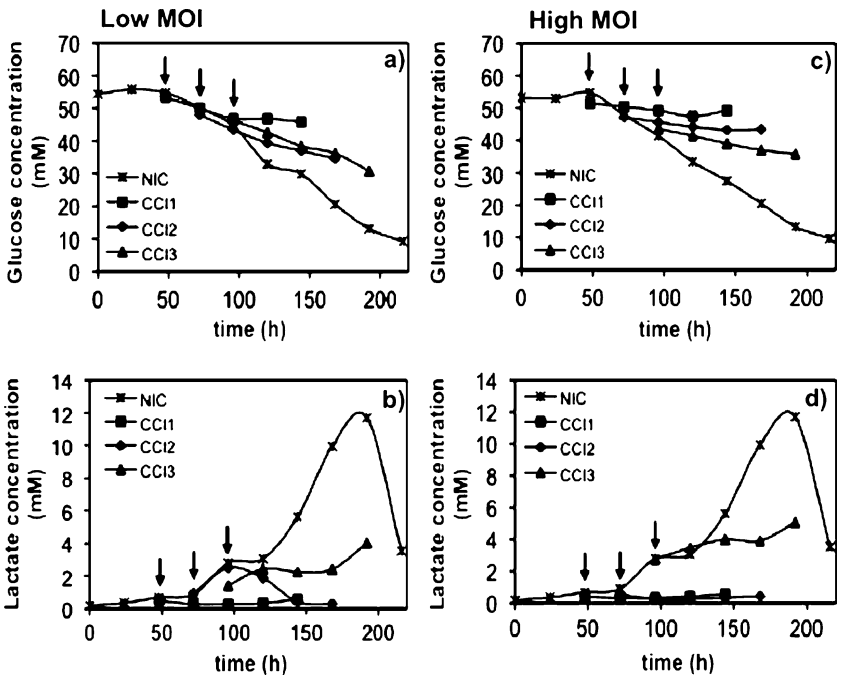

Fig. 2 Metabolic profiles of glucose (**a**, **c**) and lactate (**b**, **d**) for uninfected and infected Sf-9 cultures with a low and a high MOI, without medium exchange. *Arrows* indicate the time of infection for the three CCIs tested

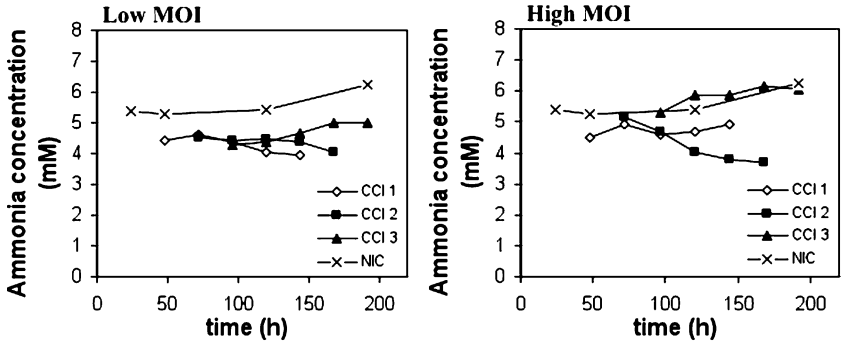

Fig. 3 Concentration profiles of ammonia in culture medium after infection with recombinant Ac-hisGFP are shown. Infections were carried out either with a low or high MOI, both for three different CCIs, without medium exchange at the time of infection. *NIC* uninfected cultures

Infections at high MOI

For the infections performed at high MOI, it is clear from the growth curves (Fig. 1b) that cell growth was strongly inhibited after virus infection, independently of the CCI. Glucose profiles were qualitatively similar to those of the low MOI infections (Fig. 2c), although evidencing a further down-regulation of glucose uptake which is probably associated with the synchronous infection of the cells. As in the case of the low MOI infections, lactate accumulated only at CCI 3 (Fig. 2d) with a specific production rate of 5.90±0.28 nmol.$(10^6$ cells$)^{-1}.h^{-1}$. The ammonia concentration remained in the same interval of 4 to 6 mM (Fig. 3). After calculating the specific consumption fluxes of glucose (Fig. 5), it was possible to confirm that these were lower than the uninfected culture and lower than the low MOI infections for CCIs 2 and 3. A decreasing trend of glucose consumption fluxes with increasing CCI was evident in these experiments. The uptake rates of serine and glutamine were lower than the uninfected and low MOI infected cultures for all CCIs. None of the amino acids analyzed was exhausted as for the previous infections.

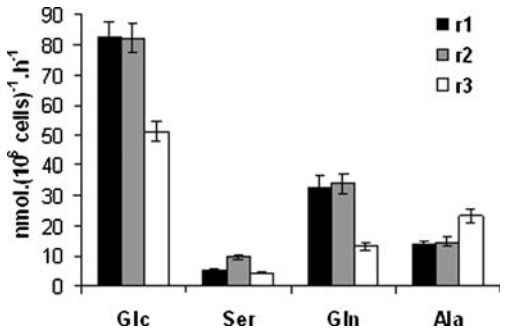

Fig. 4 Specific consumption and production rates of glucose and selected amino acids calculated for uninfected Sf-9 cultures. The values presented for alanine (*Ala*) represent rates of accumulation in the culture medium. *r1*, *r2*, and *r3* refer to the rates calculated for the following time spans: 48–144, 72–168, and 96–192 h, respectively. The *error bars* presented correspond to the linear regression error of the corresponding slope. The errors involved in the measurements of the primary variables were 5% (Glc) and 10% (Ser, Gln, Ala and cell number). *Glc* glucose, *Ser* serine, *Gln* glutamine, *Ala* alanine

The results for virus production at high MOI were quite contrasting with what was observed for infections at low MOI (Table 1). The titer increased with CCI from 0.55×10^9 to 9×10^9 IP.mL^{-1}, while the maximum specific yield was obtained at CCI 2, corresponding to a value of 2,475 IP.$cell^{-1}$. In the same way, the amplification factor for this set of infections was higher for CCI 2 (83), but lower than the highest amplification factor obtained at low MOI (287 for CCI 1).

Infections at high MOI with medium exchange

In order to discriminate the influence of medium condition on the cell density effect, experiments with medium exchange at the time of infection were performed using a high MOI. As explained in the "Materials and methods" section, cells were centrifuged and re-suspended in fresh medium to the three CCIs tested. The main observations related to the metabolic activity of Sf-9 cells after infection are depicted in Fig. 6. Glucose uptake rate was lower in these experiments when compared to what was observed for uninfected and the other infected cultures (Fig. 6). Besides, the characteristic decrease of glucose and amino acids consumption with increasing CCI appears to be reversed.

Higher amounts of recombinant BV were obtained for this set of infections (Table 2). It can be seen that all the production parameters increased with CCI. At CCI 3, a specific yield of 9,306 IP.$cell^{-1}$ was obtained, corresponding approximately to a threefold increase with respect to the higher value obtained for high MOI without medium exchange (2,475 IP.$cell^{-1}$, CCI 2). Additionally, the highest amplification factor was roughly 19 times higher than that obtained without medium exchange at CCI 2. In terms of bioprocess time, a significant decrease in TOH was observed in relation to the previous infections.

Discussion

The inhibition of cell division observed after virus inoculation demonstrates that viral entry into the cells has

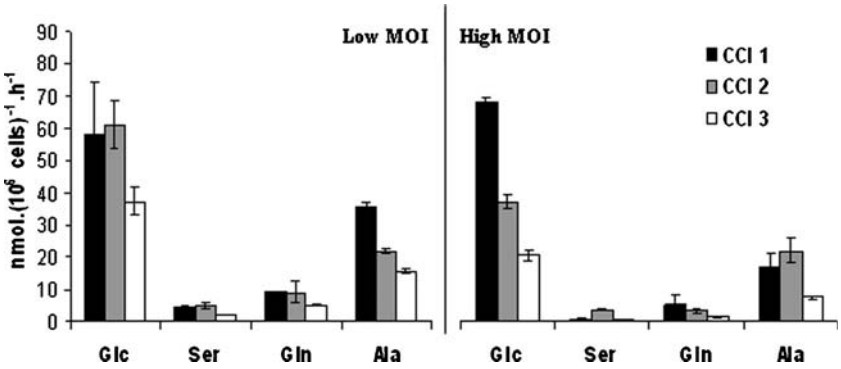

Fig. 5 Specific consumption and production rates of glucose and selected amino acids calculated for infected Sf-9 cultures at two MOIs and different CCIs, without medium exchange. The values presented for alanine represent rates of accumulation in the culture medium

a profound impact on their physiology and metabolic state (Fig. 1). This drop, which has been reported before in other works (Elias et al. 2000), was more pronounced for the infections at high MOI and reflects the synchronous infection of the cells.

Several authors have shown that lactate does not usually accumulate in Sf-9 cultures. Therefore, the significant build up of lactate in the uninfected cultures during later stages of growth (Fig. 2b,d) indicates a limitation in oxygen availability in spinner flasks (Rhiel and Murhammer 1995). On the other hand, the final drop at the end of cultivation time suggests an uptake of lactate possibly as a result of the lower glucose concentrations (Mendonça et al. 1999). During the infection experiments, lactate accumulation was reduced, probably due to the lower consumption of glucose. Final levels around 4–5 mM were obtained in the infections at CCI 3, thus further suggesting a limitation in oxygen supply at higher cell densities. The low levels of ammonia production along all uninfected and infected cultures (Fig. 3) have already been described to be related with its detoxification through the production of alanine, which is normally released by Sf-9 cells (Drews et al. 2000). Besides, the concentration values measured were between 4 and 6 mM, being lower than the 20 mM previously reported to be inhibitory for Sf-9 cells (Öhman et al. 1996).

Comparing the metabolic rates presented in Figs. 4 and 5, it can be seen that glucose uptake appears to be lower after viral infection, hence directly correlating with its utilization for cell growth. An interesting observation was that this rate also decreased at later stages of growth in the uninfected cultures and after infection at high CCI. This could be related to possible limitations of a given compound not yet identified, or the accumulation of inhibitory/regulatory factors present in spent medium down regulating cellular metabolism (Doverskog et al. 1997). Oxygen limitation could also play a role, as indicated by the accumulation of lactate both at later stages of growth and after high cell density infection. However, ongoing experiments in fully controlled bioreactors have shown similar metabolic patterns in terms of nutrient uptake rates (data not shown), thus supporting that the effects assessed were mainly due to regulatory effects rather than to oxygen limitation. Besides glucose, glutamine, and serine were consistently consumed during all experiments. This was expected since glutamine has been demonstrated to be the major nitrogen source for Sf-9 cells biosynthetic metabolism, being also used as a carbon and energy source (Drews et al. 2000), while serine can be directly incorporated in the central metabolism by conversion to pyruvate. Overall, lower specific consumption rates, as described for glucose, can be observed for these two amino acids after infection,

Table 1 Summary of Ac-hisGFP baculovirus production indicators for different cell concentrations at infection (CCIs) and multiplicities of infection (MOIs)

	Low MOI				High MOI			
	Titer[a]	Specific yield[b]	Amplification factor[c]	TOH[d]	Volumetric titer[a]	Specific yield[b]	Amplification factor[c]	TOH[d]
CCI 1	0.18	172	287	120	0.55	486	17	120
CCI 2	0.07	36	61	120	5.6	2,475	83	120
CCI 3	0.007	2	3	120	9	2,060	69	96

[a] Infectious particles per milliliter (10^9 IP.mL^{-1})
[b] Infectious particles per cell (IP.cell^{-1})
[c] Ratio between volumetric productivity and number of viruses per milliliter at the time of infection (volumetric productivity/(CCI×MOI))
[d] Time of harvest (hours)

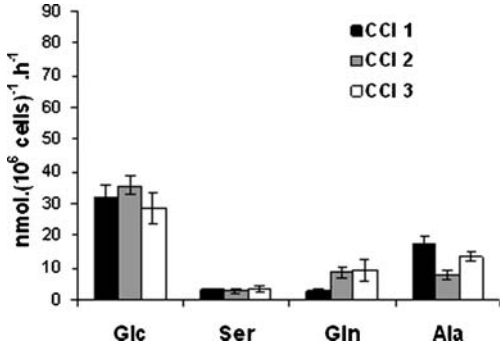

Fig. 6 Specific consumption and production rates of glucose and selected amino acids calculated for Sf-9 cultures infected at high MOI and different CCIs, with medium exchange at the time of infection. The values presented for alanine represent rates of accumulation in the culture medium

especially at high MOI when infections tend to be synchronous. The decrease in serine consumption rates at higher CCI is less evident when compared with glucose and glutamine uptake rates; however, one should take into consideration the proportionality between rate values and associated errors that, even with low deviations, may blur the interpretation of the results. The only amino acid to build up in the culture medium was alanine, which has been described as the major metabolic by-product in Sf-9 cells (Drews et al. 2000). It can be estimated from the specific fluxes presented in Figs. 4 and 5 that the amount of glucose diverted to alanine production ranged from 8% to 23% in uninfected cultures and from 12% to 30% in infected cultures without medium exchange, considering glucose the sole carbon source. Although concentrations up to 100 mM have been not shown to impact productivity (Bédard et al. 1993; Öhman et al. 1995), the channeling of glucose to by-product formation implies a decrease in the efficiency of its oxidation for energy production and biosynthesis.

The metabolism of Sf-9 cells after exchanging the medium at infection with a high MOI yielded relevant results (Fig. 6). The lower consumption rates of glucose in comparison to the values of uninfected and the other infected cultures (Figs. 4 and 5) show a marked shift in cell physiology. The literature is scarce in information regarding the metabolic behavior of cells after medium restoration at infection. We suggest that exposing the cells to fresh medium will induce an adaptation process somewhat resembling the lag phase present during the beginning of a new culture. In Sf-9 cells, this is characterized by extremely low consumption rates of glucose (see Fig. 1). Thereafter, viral infection takes over hindering biomass formation and substrate consumption. Besides, the characteristic decrease of the consumption rates at high CCI is not so evident for glucose and serine in this set of experiments and even appearing to be reversed in the case of glutamine. This is probably related with the requirements for higher BV productivities seen at higher CCI.

Considering the production of BV in the infections performed at low MOI, it is possible to see that Sf-9 cells grown in serum-free medium exhibit the same behavior previously described for the production of recombinant proteins, clearly demonstrating a cell density effect in the system (Table 1). When infections were carried out at high MOI, higher productivities were obtained in cultures infected at higher CCI. Studies that have addressed the combined effects of CCI and MOI on recombinant protein titers demonstrate that for each CCI there is an optimum MOI for maximum protein production (Licari and Bailey 1992; Power et al. 1994). More specifically, Power et al. (1994) showed that for a high MOI, the protein titer increases exponentially in proportion to the number of cells at the time of infection until a certain point, beyond which production starts to decrease. In their particular case, this happened for a cell density of 2.4×10^6 cells.mL^{-1}. The titers that we obtained increased until CCI 3 (9×10^9 IP.mL^{-1}), although the highest value is in the same order of magnitude than that obtained at CCI 2 (5.6×10^9 IP.mL^{-1}).

The cell density effect in high MOI infections can be more markedly observed when considering specific yields, where the highest value was obtained at CCI 2 (2,475 IP.cell^{-1}), decreasing thereafter at CCI 3 (2,060 IP.cell^{-1}). In this work, the values presented are based on the initial infection cell density (Wang et al. 1993). We also estimated specific yields by two other methods: (1) dividing by the highest cell density obtained after infection and (2) considering the average number of cells from infection until the maximum titer was obtained. Overall, the values show the same pattern along the CCI range for both MOIs. However, considering the average number of cells is not accurate since it accounts for the decrease in cell number following viral infection, leading to an overestimation of specific BV yields. On the other hand, dividing the maximum titer by the highest cell number after infection is more biologically relevant, especially in the case of low MOI infections. Nevertheless, the choice of using CCI as

Table 2 Summary of Ac-hisGFP baculovirus production indicators for different cell concentrations at infection (CCIs) and a high multiplicity of infection (MOI) with medium exchange

	Titer (10^9 IP.mL^{-1})	Specific yield (IP.cell^{-1})	Amplification factor[c]	TOH (h)
CCI 1	0.3	177	30	96
CCI 2	13	5,652	942	72
CCI 3	26	9,306	1,550	72

[a] Volumetric productivity/(CCI×MOI))

the leveling parameter avoids the variability inherent to virus infection and cellular response in terms of growth inhibition, yielding coherent, comparable results between experiments.

There is a scarcity of data in the literature regarding the factors that affect BV production by insect cells. One of the few studies that has addressed this issue also shows the same pattern of decreased productivity with increasing CCIs along a wide range of cell densities at infection, obtaining a maximum specific yield of 657 plaque-forming units (PFU).cell^{-1} for a cell concentration of 4.5×10^{6} cells.mL^{-1} (Radford et al. 1997). In our work, the maximum specific yield for high MOI infections (Table 1) was obtained at 2×10^{6} cells.mL^{-1}, corresponding to a value of 2,475 IP.cell^{-1} which is approximately four times higher than the 657 PFU.cell^{-1} reported previously. Differences may be attributed to the different MOIs and recombinant viruses used in each case: Radford's BV had been constructed for the high expression of secreted β-galactosidase and infections were performed at a MOI between 1 and 2 IP.cell^{-1}, whereas the virus used in this work expresses a GFP protein and infections were carried out at a MOI of 30 IP.cell^{-1}.

The viral amplification factor was higher for CCI 2, decreasing then at CCI 3; the fact that this higher value of 83 was still lower than the highest amplification factor obtained at low MOI (287 for CCI 1) suggests that, in terms of bioprocess performance, working at low CCI and low MOI is the best option for virus propagation if no improvements in the culture conditions are attempted.

After exchanging the medium at the time of infection, the cell density effect was completely overcome for the range of CCIs tested, since all the production indicators were maximized at CCI 3, with maximum titer and specific yield of 2.6×10^{10} IP.mL^{-1} and 9,306 IP.cell^{-1}, respectively (Table 2). Furthermore, a marked decrease in the culture time for the titers to reach maximum values (TOH=72 h) was observed, which contributes to the efficiency of the bioprocess. According to the literature, the replenishment of fresh medium along with the inoculation of viruses has proved to be an efficient way of restoring the yields of different proteins in high density Sf-9 cultures (Caron et al. 1990; Lindsay and Betenbaugh 1992; Tom et al. 1995). From a metabolic perspective, this increase in protein yield after medium replacement has been related with nutrient depletion rather than the accumulation of inhibitory by-products (Bédard et al. 1994; Weiss et al. 1992). This is consistent with the fact that the common by-products excreted by mammalian cell lines, ammonia and lactate, do not usually accumulate to toxic levels in Sf-9 cells (Drews et al. 1995). However, most of these studies lacked a detailed description of cellular metabolism, failing to identify the specific components responsible for improved productivities. Exceptionally, Radford et al. (1997) have reported a complete depletion of cystine in infected cultures at high cell density, which was correlated with the decrease in recombinant protein production. In our study, we could not observe a depletion of any of the carbon sources and amino acids analyzed. This fact might indicate that the depletion of other components of the medium (eventually vitamins or trace elements) might be limiting BV production. However, different other factors have been pointed as possible explanations for limited productivities in older cultures without medium exchange. Some authors have shown previously the linking between oxygen uptake rate, infection, and protein production in the BV–insect cell expression system (Palomares et al. 2004; Lecina et al. 2006), while others even stated that control of oxygen supply post-infection is critical and determined that to obtain good productivities oxygen concentration should be above 20% of air saturation, which corresponds to spinner flasks at a cell density of 3.0×10^{6} cells.mL^{-1} and lower (Taticek and Shuler 1997). From another perspective, the decreasing degree of synchronization taking place during the course of a growing culture has been suggested to negatively impact protein production (Calles et al. 2006), to which the accumulation of autocrine factors down-regulating metabolism and proliferation (Doverskog et al. 1997) would enhance this negative effect. Therefore, resuspending cells in fresh medium would be effective in overcoming both these issues. Finally, product degradation through virus- or cell-encoded proteases cannot be excluded, as some authors have identified proteolytic activity in the insect cell–BV system (Cruz et al. 1999; Naggie and Bentley 1998).

However, total medium exchange, though feasible for small scale cultures, is not suitable for higher production scales, increasing the cost of the bioprocess due to the need for separation devices and extra medium costs. On the other hand, the addition of complex nutrient mixtures such as different kinds of serum substitutes and hydrolysates may be beneficial for increasing recombinant BV yields (Ikonomou et al. 2003), but hinder the identification of the individual components that may be limiting production. The use of such complex mixtures would involve higher costs for operation and variability in process performance. Considering this, the authors sustain that future improvement of the insect cell–BV system at production scale is highly dependent on the detailed characterization of medium composition and insect cells metabolism along culture time and after infection. Moreover, the development of modeling structures combining the information from infected and uninfected cellular metabolism with infection kinetics would be extremely useful for bioprocess optimization. The growing application of recombinant BVs for gene therapy represents an opportunity to deepen the knowledge around this system.

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