Effect of partial medium replacement on cell growth and protein production for the High-FiveTM insect cell line

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Abstract

The potential of spent medium to support the growth and recombinant protein production of High-FiveTM cells was investigated. Growth in medium consisting of three parts fresh and one part spent medium was comparable to that in fresh medium (maximal specific growth rates of 0.028 and 0.029 h⁻¹, and maximal cell densities of 4 and 4.5 × 10⁶ cells ml⁻¹, respectively). Glucose exhaustion coincided with an abrupt decrease of viability. Of 15 amino acids analyzed, not a single one was completely exhausted at the end of the growth phase. Growth in medium consisting of equal parts spent and fresh medium led to lower maximal cell concentration (2.9 × 10⁶ cells ml⁻¹) with a smoother death phase. Glucose supplementation at the beginning of the culture or at the end of the growth phase did not lead to an increase of either the maximal cell density or the specific growth rate. Infection of High-FiveTM cells at three different densities (1.4, 2.5 and 4.2 × 10⁶ cells ml⁻¹) without medium change led to monotonically decreased specific productions for β -galactosidase. Partial (75%) or total medium replacement at the higher infection density restored the specific production at the levels of the intermediate density infection (321, 292 and 389 U.(10⁶ cells)⁻¹, respectively).

Introduction

The baculovirus expression vector system (BEVS) is one of the most powerful systems for recombinant protein production, combining high expression levels with acceptable post-translational modifications (Jarvis 2003; Luckow 1991). These and other favorable features have made the BEVS a subject of industrial-scale development (Agathos 1996; Ikononou et al. 2003). One of the major considerations in the large-scale culture of insect cells is the need for medium replenishment before baculovirus infection. The protein production in the BEVS takes

place within a rather narrow time window, the BEVS being a lytic, transient expression system. It has been clearly demonstrated that the ‘cell density effect’, i.e. the dramatic decrease of specific productivity with increasing infection density is rather due to nutrient limitation than to waste accumulation (Chico and Jäger 2000; Radford et al. 1997). Partial or total replacement before or immediately after infection (Hensler et al. 1994; Lindsay and Betenbaugh 1992; Reuveny et al. 1993; Tom et al. 1995), glucose and glutamine addition (Wang et al. 1993), one-step addition of nutrient mixture (Bédard et al. 1994a, b) and fed-batch culture

(Bédard et al. 1997; Elias et al. 2000; Nguyen et al. 1993) have all been proven efficient strategies for restoration of specific production in the BEVS system.

Re-use of the spent medium can be highly desirable in the context of mass production of recombinant proteins. Medium re-cycling coupled with perfusion culture of a hybridoma cell line (Kempken et al. 1991), led to cost reduction (37% savings) and medium volume reduction (38% savings). The same approach resulted in increased monoclonal antibody (mAb) production in a repeated batch process, performed in a 100-l pilot scale bioreactor (Riese et al. 1994). In both cases, a concentrate of amino acids and glucose was used to supplement the spent medium, instead of fresh complete medium. However, accumulation of waste products did not permit the re-use of spent medium for more than one cycle in the case of perfusion and more than four repeated batches in the case of the pilot process. Insect cells can tolerate higher levels of by-products, such as ammonia, lactate or alanine and higher medium osmolality (Agathos et al. 1990) than mammalian cells and extended re-use of spent medium could be envisaged in their large-scale processing.

A similar approach was recently proven quite efficient in the BEVS system (Wu et al. 1998). Spent medium from stationary phase cultures could support growth and protein production of Sf-9 and Tn5 cells when supplemented with glucose, glutamine, yeastolate and 20% of fresh medium. Volumetric and specific productivities were similar to those in fresh medium (Wu et al. 1998). Thus, spent medium from batch processes appears to contain substantial quantities of essential nutrients and can be efficiently re-used. The exhausted nutrients can be relatively cheap ingredients such as glucose and glutamine or components of both the basal medium and the serum fraction in the case of serum-supplemented media (Roberts 1984).

Re-cycle of conditioned medium can offer other advantages apart from the reduction of waste volume. Cultured animal cells may need autocrine factors for their growth and this is probably why occasionally the inoculum size can directly affect the growth and final densities reached (Lauffenburger and Cozens 1989; Lee et al. 1992). Invertebrate cells *in vitro* have been shown to constitutively secrete growth factors (Doverskog et al. 1997, 1999; Ferkovich and Oberlander 1991). Sf-9 cells in

serum-free medium express insulin-like growth factor I (Doverskog et al. 1997) as well as a soluble protein with a MW of 27 kDa with high affinity for insulin (Doverskog et al. 1999), both of which are directly implicated in the regulation of cell proliferation. Growth factors currently used in mammalian cell culture have no positive effects in insect cell culture (Nishino and Mitsunashi 1995). At the same time invertebrate growth factors are difficult to isolate and purify (Ferkovich and Oberlander 1991). Thus, re-use of spent medium enriched in autocrine growth factors can be especially advantageous in the context of serum-free insect cell culture where expensive supplements are generally needed to substitute the growth promoting fractions of serum.

We present here results from experiments with spent medium of High-Five serum-free culture. Evidence is shown as to the beneficial effect of conditioned medium on cell growth and recombinant protein production, namely β -galactosidase (β -gal) production. About 25% spent medium appears to be the best proportion for both the growth and infection phases of the BEVS system, using the High-Five cell line.

Materials and methods

Cell lines and media

Details on adaptation of cells in suspension, serum-free culture, routine cell culture and media can be found elsewhere (Ikonomou et al. 2001). The Ex-CellTM 405 medium, specially designed for the Tn5 cell line, was purchased from JRH Biosciences (Lenexa, KS).

Baculovirus

A recombinant AcMNPV baculovirus carrying the *Escherichia coli* β -gal gene under the control of the polyhedrin promoter was purchased from Invitrogen (San Diego, CA). Amplification of the virus was performed in either 100 mm Petri dishes or shake-flasks by infecting Sf-9 cells with a multiplicity of infection (MOI) of 0.1 plaque-forming units per cell (pfu cell⁻¹), which is the ratio of baculovirus titer (pfu ml⁻¹) to the viable cell concentration at the time of infection (cells ml⁻¹). Virus titer was determined by the end-point dilution assay in 60-well

(Terasaki) plates (Nalge Nunc International, Rochester, NY). The baculovirus employed for the infection studies had a titer of 9×10^7 pfu ml⁻¹.

Effect of conditioned medium on cell growth and protein production

All experiments were performed on an orbital shaker (model C24, New Brunswick Scientific, Edison, NJ) at 27°C and 150 rpm. The medium was Ex-Cell™ 405.

For the experiments concerning the effect of conditioned (spent) medium on cell growth, High-Five cells growing exponentially (viable cell concentration of 2.4×10^6 cells ml⁻¹) were employed. The spent medium of this culture was recovered after centrifugation at $100 \times g$ for 10 min and filtration with a 0.22- μ m filter. Duplicate 125-ml shake-flasks (Corning, Acton, MA) with 25 ml liquid volume were inoculated at a density of 0.3×10^6 cells ml⁻¹. Three ratios of spent medium were tested, namely 0% (fresh medium), 25% and 50%. To avoid cell aggregation, 10 U/ml heparin were added to each flask. Cell count was performed daily using a Neubauer improved hemocytometer and cell viability was determined by the trypan blue exclusion method (0.2% final dye concentration). Glucose concentration was determined using an enzymatic kit (Cat. No:10716251035, Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany). Amino acid analysis was performed by HPLC (Fekkes et al. 1995).

For the infection studies, a 500-ml shake-flask with 100 ml liquid volume was employed. When viable cell concentration reached 1.4×10^6 cells ml⁻¹, 20 ml were transferred each to duplicate 125-ml shake-flasks without medium change and were infected at a MOI of 5 pfu cell⁻¹ (1.5 ml virus inoculum), with a baculovirus coding for the β -gal gene. The same procedure was followed for the infection at 2.5×10^6 cells ml⁻¹ (2.8 ml virus inoculum). Finally, when cell density reached 4.2×10^6 cells ml⁻¹, three 20 ml samples were centrifuged, cells were re-suspended in the same volume but with different conditioned medium proportions, namely 0% (total medium replacement) 25% and 100% (no medium replacement), and infected at a MOI of 5 pfu cell⁻¹ (4.7 ml virus inoculum). Sampling was performed daily. About 400 μ l sample were centrifuged at $500 \times g$ for 3 min. Pellets and supernatants

were separated and kept at -20°C until the β -gal assay (intracellular and extracellular, respectively). The latter was a modification of Miller's method (Miller 1972). Briefly, 10 μ l of appropriately diluted sample were mixed with 1000 μ l Z-buffer (pH 7.0) in a cuvette at 37°C. Afterwards, 200 μ l of an *o*-nitrophenyl β -D-galactoside (ONPG) solution (4 mg ml⁻¹ in a 0.1 M phosphate buffer, pH 7.0) were added. When sufficient yellow color was developed, the reaction was stopped with addition of 500 μ l Na₂CO₃ (1 M). The absorbance was measured at 420 nm and the β -gal concentration was calculated by using the following formula:

$$\beta\text{-gal concentration} = \frac{(A/t)}{\text{slope}} \times \text{dilution factor}$$

where the β -gal concentration is expressed in U ml⁻¹, t is the incubation time (min), A is the absorbance and the slope [0.0119 absorbance min⁻¹ (U ml⁻¹)⁻¹] was calculated from the calibration curve of a β -gal standard (X grade, G 2513, Sigma, St Louis, MO). One unit is defined as the β -gal amount that will hydrolyze 1.0 μ mol of ONPG to *o*-nitrophenol and D-galactose per minute at pH 7.3 and 37°C. For both growth and infection studies, no re-adjustment of pH and osmolality was attempted.

The effect of glucose supplementation on the growth of High-Five cells was evaluated by adding glucose in duplicate 125-ml shake-flasks with 25 ml liquid volume. High-Five cells were inoculated at 0.2×10^6 cells ml⁻¹. About 250 μ l of 1 M glucose solution were added either at the time of inoculation (0 h) or at the end of the growth phase (120 h), to ensure a 10 mM increase in glucose concentration. Sampling for cell counting was performed daily.

Results and discussion

High-Five cell growth

The effect of seeding density was decoupled from the effect of conditioned medium on cell growth as cells had been centrifuged before inoculation and the seeding density was the same for all the experimental conditions. Figure 1 shows the effect of different medium proportions on the growth of High-Five cells. Maximal viable cell concentration was the

Figure 1. Effect of conditioned medium on growth of High-Five cells. Duplicate 125-ml flasks with 25-ml liquid volume were agitated at 150 rpm. The inoculation density was $0.3 \times 10^6 \text{ cells ml}^{-1}$ and the medium was Ex-Cell 405. The viable cell concentration (A), viability (B) and glucose concentration (C) are shown for flasks in fresh medium (0%, open triangles), in three parts fresh and one part spent medium (25%, open circles) and in equal parts of fresh and spent medium (50%, open squares). Vertical bars at each point in (A) indicate half of the difference between viable cell concentrations from duplicate flasks for this point.

same for flasks with 0% and 25% conditioned medium but decreased at 50% spent medium (Table 1).

Specific growth rates were essentially the same for all three conditions (0.028, 0.029 and 0.026 h^{-1}) (Table 1). Therefore, increase in spent medium proportion did not confer any significant improvement in maximal specific growth rate. The effect of conditioned medium on cell growth has been mainly studied for the Sf-9 cell line (Hensler et al. 1994; Kioukia et al. 1995a, b). Hensler et al. (1994) found that Sf-9 cells in Ex-Cell 401 medium reached the same final densities when growth took place in either highly conditioned (50%) or fresh medium.

On the contrary, Kioukia et al. (1995a, b) observed a reduction in specific growth rate and final cell density with increasing carry-over of spent medium, which they attributed to the presence of cytotoxic, growth inhibiting products. High-Five cells can reach high densities ($>8 \times 10^6 \text{ cells ml}^{-1}$) even in the presence of significant by-product amounts (Rhiel et al. 1997). Ammonia accumulation has been found to negatively affect protein production by *Trichoplusia ni* cells (Donaldson et al. 1999; Yang et al. 1996). Nevertheless, the possible inhibitory effects of this and other metabolites on cell growth for High-Five cells are not yet known and their study was beyond the scope of this work. The similar growth rates but the reduced maximal cell concentration for 50% spent medium found in our study imply that either autocrine growth factors exert minimal biological activity or that the beneficial effect of conditioned medium factors is outbalanced by nutrient limitation. On the other hand, cultures containing a certain proportion of spent medium (25% and 50%) had a stationary phase of about 50 h with high viability ($>80\%$), which was reduced for the fresh medium culture (Figure 1A and B). In addition, the culture in 50% spent medium was characterized by a smoother death phase, whereas the culture in 0% spent medium showed an abrupt decrease of viability once the maximal cell concentration was reached (Figure 1A and B). We have previously shown that insect cells had a remarkably prolonged stationary phase in a hydrolysate-containing medium as long as glucose was available (Ikonomou et al. 2001). This phase was attributed to the antiapoptotic properties of Primatone RL hydrolysate, which have been at first observed with hybridoma cell culture (Schlaeger 1996). The similar cell behavior observed

Table 1. Kinetic and metabolic parameters for High-Five cells in different proportions of conditioned medium^a.

Proportion of conditioned medium	Specific growth rate (h ⁻¹)	Population doubling time (h)	Maximal viable cell concentration (10 ⁶ cells ml ⁻¹)	Glucose specific consumption rate (10 ⁻¹⁷ mol cell ⁻¹ s ⁻¹)
0% (fresh medium)	0.028	24.7	4.5	6.5
25%	0.029	23.7	4.0	5.5
50%	0.026	26.1	2.9	4.8

^a Data are from duplicate 125-ml shake flasks with 25 ml liquid volume agitated at 150 rpm. The kinetic parameters were calculated for the exponential part of the growth phase.

in the present work may imply that conditioned medium of insect cell culture could contain factors with analogous properties. Recent articles support the hypothesis that conditioned medium from cultured cells contains autocrine or paracrine anti-apoptotic activity. Quiescent (non-growing) mouse embryonic 10T 1/2 cells export proteins, members of the secreted apoptosis-related protein family in their growth medium that inhibit the onset of programmed cell death (Melkonyan et al. 1997). Furthermore, microglial cells have been shown to secrete neuroprotective factors *in vitro* that prevent neuronal apoptotic death (Polazzi et al. 2001; Toku et al. 1998). At present, no data exist as to an analogous activity in cultured insect cells. Thus, the hypothesis of antiapoptotic activity of spent medium, appealing as it may be, needs substantial investigation.

Glucose level was about the same at the beginning for all three conditions and was exhausted by 137 h (Figure 1C). Spent medium was from the mid-exponential phase of a High-Five culture (see 'Materials and Methods' section). Consumed glucose at that point can be as low as 10 mM (Rhie et al. 1997). This fact and the expected inaccuracy in measuring high glucose concentrations may account for the same observed glucose levels despite the different spent media proportions, hence the initial glucose values must be viewed with some caution. The specific consumption rate of glucose (q_{glc}) decreased with increased proportion of spent medium during the exponential growth phase but the differences between the q_{glc} values were not significant (Table 1). Again, the different post-growth profiles at the three conditions suggest that either the major limiting factor is other than glucose or that concentration of autocrine factors in the 50% spent medium culture is adequate to prevent the dramatic decrease in viable

Figure 2. Amino acid concentration in Ex-Cell 405 medium. Culture conditions are the same as in Figure 1. The amino acid concentration is shown for fresh medium (Ex-Cell 405), for flasks inoculated in fresh medium after 166 h (0%) and for flasks in three part fresh and one part spent medium after 166 h (25%).

cell concentration and viability. The hypothesis of amino acid(s) limitation was tested by measurement of the amino acid content of culture supernatants at 166 h, i.e. after the dramatic drop in viability seen in the 0% and 25% spent medium cultures (Figure 2). Of the 15 amino acids measured, none was exhausted at that time point. Alanine accumulated to about 40 mM, which is quite ordinary for insect cell culture. Glucose has been shown to be the main source for alanine synthesis in Sf-9 cell culture, in several instances (Bédard et al. 1993; Mendonça et al. 1999; Öhman et al. 1995). Initial glucose and glutamine concentrations are respectively, 52 and 32 mM for the Ex-Cell 405 medium (JRH Biosciences 2000). Thus, an excessive alanine production is to be

expected in batch culture of High-Five cells in this medium all the more because this amino acid also serves to remove excessive ammonia from the medium (Öhman et al. 1995). Although such an alanine concentration is not considered toxic for insect cells (Bédard et al. 1994a, b) it may affect negatively their energy metabolism (Öhman et al. 1995).

Glucose supplementation

To determine whether glucose supplementation could retard cell death, 10 mM glucose was added either at inoculation (0 h) or at the end of the growth phase (120 h) (Figure 3). As expected, specific growth rate and maximal viable cell concentration were not affected by the different initial glucose content (Table 2). A similar independence had been observed previously with Sf-9 cells in different media (Drews et al. 1995; Ferrance et al. 1993) when glucose was added at various time-points. Therefore, neither glucose nor one of the 15 amino acids shown in Figure 2 appear to be the limiting nutrients in the serum-free culture of High-Five cells. Nevertheless, addition of glucose at the end of the growth phase seems to render somewhat smoother the death phase of High-Five cells (Figure 3).

Infection studies

To investigate the effect of the infection cell density on protein productivity when no medium change takes place, cells from the same culture were infected at different time-points of growth (See 'Materials and Methods' section). In that way, any variations due to different passage number were eliminated by using cells of the same exponentially growing population. Infections were performed when cells in the inoculum flask reached 1.4×10^6 cells ml^{-1} (low density), 2.5×10^6 (mid-density) and 4.2×10^6 (high density), without medium replacement. To see if medium replenishment can restore $q_{\beta\text{-gal}}$, additional infections of High-Five at the high density were performed with total and partial (75%) medium replacement. All infections were synchronous, as no cell growth took place post infection. The results are shown in Table 3.

Volumetric production of $\beta\text{-gal}$ ($Q_{\beta\text{-gal}}$) increased from 640 to 970 U ml^{-1} when the infection cell density increased from 1.4 to 2.5×10^6 cells ml^{-1}

Figure 3. Effect of glucose supplementation on growth of High-Five cells. Duplicate 125-ml flasks with 25 ml liquid volume were agitated at 150 rpm. The inoculation density was 0.2×10^6 cells ml^{-1} and the medium was Ex-Cell 405. The viable cell concentration (A) and viability (B) are shown for flasks without glucose supplementation (control, open triangles), with addition of 10 mM glucose during inoculation (0 h, open circles) and with addition of 10 mM glucose at the end of the exponential growth phase (120 h, open squares). Vertical bars at each point in (A) indicate half of the difference between viable cell concentrations from duplicate flasks for this point.

(Table 3). On the other hand, this increase was accompanied by a decrease in the specific production ($q_{\beta\text{-gal}}$). The decrease of per cell production indicates that no medium replacement can result in paucity or exhaustion of essential factors for protein production. This was particularly evident at the

Table 2. Kinetic parameters for the growth of High-Five in Ex-Cell 405 with glucose supplementation^a.

	Specific growth rate (h ⁻¹)	Population doubling time (h)
Control (no glucose supplementation)	0.031	22.3
Glucose addition at <i>t</i> = 0 h	0.032	21.6
Glucose addition at <i>t</i> = 120 h	0.031	22.3

^a Data are from duplicate (except for the control) 125-ml shake-flasks agitated at 150 rpm with 25 ml liquid volume. The kinetic parameters were calculated for the exponential part of the growth phase. An increase of 10 mM in glucose concentration was achieved by adding 250 μ l of 1 M glucose solution.

Table 3. Volumetric and specific β -gal productions for High-Five cells infected at different cell concentrations^a.

Infection density (10 ⁶ cells ml ⁻¹)	Volumetric β -gal production (U ml ⁻¹)	Specific β -gal production [U · (10 ⁶ cells) ⁻¹]
1.4 (100% spent medium)	640	457
2.5 (100% spent medium)	970	389
4.2 (0% spent medium)	1228	292
4.2 (25% spent medium)	1348	321
4.2 (100% spent medium)	551	131

^a 125-ml shake-flasks with 25 ml liquid volume inoculated at 0.2×10^6 cells ml⁻¹ and agitated at 150 rpm were infected at different viable cell densities with a MOI of 5. The volumetric production is the maximal total concentration of β -gal (extracellular + extracellular) for each infection density whereas the specific production is the volumetric production divided by the viable cell density at the time of infection (0 h p.i.).

high density infection at 4.2×10^6 cells ml⁻¹ with no medium change when both $Q_{\beta\text{-gal}}$ and $q_{\beta\text{-gal}}$ declined significantly to 551 U ml⁻¹ and to 131 U $\times 10^6$ cells⁻¹, respectively. Partial (three parts of medium) or total medium replenishment restored the $q_{\beta\text{-gal}}$ to levels close to that of the mid-density infection ($q_{\beta\text{-gal}}$ at high density was about 70% of $q_{\beta\text{-gal}}$ at low density). The change of medium was not sufficient *per se* to fully restore $q_{\beta\text{-gal}}$; other factors, such as oxygen limitation (Taticek and Shuler 1997) or the difference in growth phases (early and late exponential) (Doverskog et al. 2000; Kioukia et al. 1996) may account for this phenomenon.

Taticek and Shuler (1997) found that an atmosphere rich in oxygen (80%) could maintain the per cell protein activity of Tn5 cells, infected at high densities (6×10^6 cells ml⁻¹). As for the cell cycle effect, work with Sf-9 cells showed that cells from the exponential phase produced more β -gal compared to cells obtained from the stationary phase (Kioukia et al. 1995a, b). Furthermore, it was recently suggested that the decrease in productivity depends on autoregulatory events for the Sf-9 cell line and that medium renewal can only partially restore the specific production (Doverskog et al. 2000). The restoration of $q_{\beta\text{-gal}}$ level resulted, in turn, in doubling of the $Q_{\beta\text{-gal}}$ value compared to that of the low density infection (1348 versus 640 U ml⁻¹), an outcome of considerable practical importance in bioprocessing. The fact that infection with partial change of medium showed better results than that with complete medium change shows that probable nutrient dilution did not affect negatively the protein production. Taticek and Shuler (1997) have shown that neither glucose nor glutamine (nutrients that were most probably exhausted in the spent medium at 4.2×10^6 cells ml⁻¹) control protein expression at high cell density infections of Tn5 cells. Furthermore, spent medium may contain secreted growth-promoting factors with a positive effect on protein production (Jesionowski and Ataai 1997). These factors are absent after a total medium change and will only be produced after some time in the case of a non-synchronous infection or not produced at all in the case of a synchronous infection. It was previously shown that spent medium from insect cell culture can be re-used for both growth and β -gal production when supplemented with glucose, glutamine, yeastolate and 20% of fresh medium (instead of 75% in our case) (Wu et al. 1998). However, the medium in that study was IPL-41 supplemented with 10% fetal bovine serum, which may be unsuitable if serum-free culture is required and if the presence of autocrine factors is to be exploited.

When compared with values reported in the literature (Table 4), our β -gal titers ($Q_{\beta\text{-gal}}$) are among the highest reported. The $q_{\beta\text{-gal}}$ corresponding to the peak $Q_{\beta\text{-gal}}$ compares favorably with the $q_{\beta\text{-gal}}$ of other *T. ni* cell lines in suspension systems.

Overall, our results indicate that growth and protein production of High-Five cells in a high proportion of spent medium (25%) compares well with that

Table 4. Production of β -gal by *T. ni* cell lines in agitated systems and serum-free media^a.

Cell line	Medium	Infection density (10^6 cells ml^{-1})	MOI (pfu cell $^{-1}$)	Culture system	$Q_{\beta\text{-gal}}$ (U ml^{-1})	$q_{\beta\text{-gal}}$ [U.(10^6 cells) $^{-1}$]	Source
TN5B1-4 DS 5000	Ex-Cell 405	2	20	Spinner flasks (80% O ₂)	705	352 ^b	Dee et al. (1997)
High-Five	IS BAC	2	9	Shake-flasks	1100	550 ^b	Gilbert et al. (1996)
Tn-5B1-4	Ex-Cell 401	1.1–1.5	10	Spinner flasks	575	n.d.	Taticek et al. (2001)
High-Five	YPR	7.2	2	Cultispher-S supports in shake-flasks	1728	240	Ikonomou et al. (2002)
High-Five	Ex-Cell 405	4.2	5	Shake-flasks	1348	321	This work

^a $Q_{\beta\text{-gal}}$ is the peak volumetric production for total β -gal reported in each publication. $q_{\beta\text{-gal}}$ corresponds to the $Q_{\beta\text{-gal}}$ reported here and it was calculated by dividing the $Q_{\beta\text{-gal}}$ by the infection density.

^b Calculated.

in fresh serum-free medium. This finding can be integrated in the design of feeding strategies for perfusion or repeated batch systems. Although the presence of autocrine growth factors is not explicitly demonstrated, it is suggested that such factors exist and have a beneficial effect on growth and protein expression.

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