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# Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells

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*Key words:* Adenovirus, human 293S cells, recombinant protein, scale-up, metabolism

## Abstract

Human 293S cells, a cell line adapted to suspension culture, were grown to $5\times10^6$ cells/mL in batch with calcium-free DMEM. These cells, infected with new constructions of adenovirus vectors, yielded as much as 10 to 20% recombinant protein with respect to the total cellular protein content. Until recently, high specific productivity of recombinant protein was limited to low cell density infected cultures of no more than $5\times10^5$ cells/mL. In this paper, we show with a model protein, Protein Tyrosine Phosphatase 1C, how high product yield can be maintained at high cell densities of $2\times10^6$ cells/mL by a medium replacement strategy. This allows the production of as much as 90 mg/L of active recombinant protein per culture volume. Analysis of key limiting/inhibiting medium components showed that glucose addition along with pH control can yield the same productivity as a medium replacement strategy at high cell density in calcium-free DMEM. Finally, the above results were reproduced in 3L bioreactor suspension culture thereby establishing the scalability of this expression system. The process we developed is used routinely with the same success for the production of various recombinant proteins and viruses.

*Abbreviations:* CFDMEM – calcium-free DMEM; CS – bovine calf serum; hpi – hours post-infection; J+ – enriched Joklik medium; MLP – major late promoter; MOI – multiplicity of infection (# of infectious viral particle/cell); q – specific consumption rate (mole/cell.h); pfu – plaque forming unit (# of infectious viral particle); Y – yield ($\mu$g/E6 cells or mole/cell)

## Introduction

The technological potential of adenovirus vectors (AV) in various applications such as 1) recombinant protein production, 2) live viral sub-unit vaccines production and 3) gene transfer for establishing stable cell lines or for gene therapy (reviewed in Berkner, 1988, 1992; Gerard and Meidell, 1993; Graham and Prevec, 1992) currently gives rise to growing interest from biotechnologists. All of these applications will require the production of large quantities of either recombinant proteins or AV stocks. However, so far, no significant research efforts have been directed towards the scale-up of the AV expression system.

Helper-independent AVs were developed in the early 80's for high-level expression of recombinant proteins in human cells. By deleting the E1 and E3 regions of the adenovirus genome, transcription cassettes up to 7.0Kbp could be inserted in AV (Gluzman *et al.*, 1982). While the deletion of the E3 region only affects the ability of the AV to efficiently propagate in whole animals (Berkner, 1988), the deletion of the E1 region prevents it's replication in all mammalian cells (either in vivo or in vitro). However, AV lacking the E1 and E3 regions can be propagated in the human 293 cell line which constitutively expresses the adenovirus E1 polypeptides (Graham *et al.*, 1977, Berkner, 1988). Thus, the AV/293 expression system has a double lock security feature built-in that restricts the propagation of replication defective recombinant viruses to the complementing 293 cell line. Typically, the construction of replication defective AV for recombinant protein production is accomplished by inserting Major Late promoter-based (MLP) expression cassettes in place of

the deleted adenovirus El region. The adenovirus MLP is one of the strongest mammalian promoters and its transcriptional activity is responsible for the accumulation of the abundant adenovirus late proteins which represent collectively as much as 30–40% of total cellular proteins in adenovirus-infected cells (Ginsberg, 1984). However, due to the complexity in the regulation of gene expression in adenovirus, the recombinant protein production, using the first generation of expression vectors, has never exceeded 4% of the total proteins (estimated from data in Berkner, 1992). Consequently, the development of the full potential of AV as a high-level expression system has lagged behind other similar expression vectors such as the baculovirus/insect cells system (reviewed in O'Reilly *et al.*, 1992).

Recently, we have reported the construction of a new adenovirus expression vector (pAdBM5) that allows for the production of unprecedented levels of recombinant protein in AV-infected 293 cells (Massie *et al.*, 1994). In 293 cells infected with AV derived from the pAdBM5 transfer vector (AdBM5), the recombinant protein can accumulate at levels up to 10-20% of total cellular proteins (equivalent to 30–60 $\mu g/10^6$ cells), which makes it the most abundant protein in the infected cells. This yield compares advantageously to established expression systems, such as baculovirus/ insect cell (Bac), for the production of non-secreted protein. Massie *et al.* (1994) compared the production of herpes simplex virus ribonucleotide reductase R1 and R2 subunits in both optimized culture of AV/293 and Bac systems. While the R2 subunit was about 5 fold more abundant and active in AV/293 than in Bac infected Sf9 cells, the R1 subunit was produced at roughly similar level in both systems. However, the amount of active soluble R1 obtained from AV/293 was at least 5 times higher than in Bac/Sf9 presumably due to better folding of the R1 protein in 293 cells. In terms of scale-up, the fact that, contrarily to Bac, AV virions remain concentrated within the cell long after yields have reached maximum levels, facilitating virus collection and concentration (Graham and Prevec, 1992) is also an advantage over Bac.

Another scale-up issue was the difficulty to design a large scale unit for the culture of adherent cells. The adaptation of the original 293A (anchorage-dependant) cells to suspension culture was a pre-requisite for the scale-up of the AV/293 system. The 293N3S subline developed by Graham (1987) by passage of the 293A cells through nude mice, was the first subclone of 293 cells successfully adapted to suspension culture. In our hands however, the 293N3S cells had a relatively long initial lag phase in suspension, a low growth rate, and a strong tendancy to clump, even in calcium-free medium. We then tested another subline, the 293S cells (Cold Spring Harbor Laboratories), obtained by gradual adaptation to suspension growth. The 293S cells grew more readily in suspension with no initial lag phase, a doubling time of 24 h and minimal clumping in calcium-free medium. Furthermore, 293S cells produced equivalent level of recombinant proteins compared to 293A (Massie *et al.*, 1994). The 293S cell line was therefore chosen for further process development.

Medium limitation and/or by-product inhibition is an important scale-up problem in animal cell culture in general and the AV/293 system is not an exception. Although 293S cells could reach plateau density of 2–5 $\times 10^6$ cell/mL depending on the culture medium, productive infection with AV was restricted to cell density lower than $5 \times 10^5$ cells/mL in batch culture without medium replacement. In this paper, we present a two-step approach, undertaken to improve the volumetric yield of the AdBM5/293S recombinant protein production system with the model protein Protein Tyrosine Phosphatase 1C (PTP1C). This 68kDa enzyme is a highly phosphorylated intracellular phosphatase that plays a crucial role in signal transduction and is a potential target for cancer therapy (Shen *et al.*, 1991). In the first step a medium replacement strategy has been applied, in order to rapidly overcome any medium-related limitation/inhibition problems. We will show the success and the limitations of this strategy. In a second step, an extensive metabolic analysis has been undertaken in order to identify more precisely what was limiting or inhibitory in the medium. This knowledge was then applied to a specific addition and control strategy of the infected culture. This longer procedure has lead to higher volumetric productivity with lower medium expenses.

## Materials and methods

### *Cells, medium and virus*

The 293A cells were used for plaque assay. The cells are derived from human kidney fibroblast transformed with Ad5 DNA and express the E1A and E1B proteins constitutively (Graham *et al.*, 1977). 293A were obtained from ATCC and sub-cultured twice weekly in DMEM with 10% fetal bovine serum in 25 $cm^2$ T-flasks. The 293S were obtained from Dr. Michael

Matthew (Cold Spring Harbor Laboratories). 293S were kept frozen in liquid nitrogen until used. A fresh cell aliquot was thawed every two months and maintained in 100 mL spinner flask at 37 °C, 5% $CO_2$ by diluting twice a week to cell densities 1–$3\times10^5$ cells/mL with complete Joklik + medium (J +) described below.

J+ medium was inspired from Chillakuru *et al.* (1991) who used enriched DMEM for cultivation of vaccinia virus in HeLa cells. It was made of Joklik medium (calcium-free modification of MEM, Sigma) supplemented with 2.5 g/L glucose (total 4.5 g/L, 25mM, Sigma), 1X MEM essential amino acids (Gibco), IX MEM non-essential amino acids (Gibco), 1X MEM vitamin solution (Gibco), 0.11 g/L Na.pyruvate(Gibco), 5.7 g/L $NaHCO_3$ (Sigma) and 2.5 g/L HEPES buffer (Sigma). The mixture was then adjusted to pH=6.75 and filter-sterilized. Calcium-free DMEM (CFDMEM, custom made, Gibco) was also tested. This medium was supplemented to yield a final concentration of 4.5 g/L glucose and 0.11 Na.pyruvate, equivalent to J+. However, CFDMEM was different from J+ as it did not contain any other non-essential amino acids except serine (0.1mM in J+ vs 0.4mM in CFDMEM) and it's buffering capacity consisted in 3.7 g/L of $NaHCO_3$. Both media were always completed with 5% iron supplemented bovine calf serum (CS)(Hyclone) and 0.1% (w/v) pluronic F-68 (Gibco) unless otherwise stated.

The replication defective AV, AdBM5-PT, have been constructed in Dr Shen's laboratories, to produce protein tyrosine phosphatase (PTP1C)(Zhao *et al.*, 1993). A stock of the virus has been constituted and used throughout all of the experiments: $6\times10^9$ cells were infected at a multiplicity of infection (number of viruses/number of cells or MOI) of 1 and harvested 72 hours post-infection (hpi). The cell pellet was then diluted to $10^7$ cells/mL with J+ and then freezed-thawed three times to liberate the virus. The stock has then been titrated to $1.2\times10^9$ pfu/mL by standard plaque assay method.

*Culture and infection in spinner flasks*

Unless otherwise stated, culture and production runs were done in 100 mL siliconized spinner flasks (Bellco) with 50 mL of cell suspension in a 37 °C, 5% $CO_2$, humidified incubator. Samples were taken on a daily basis for viable and total cell count and were kept at –80 °C for further analyses. Aliquots of $1\times10^6$ cells were centrifuged (13,000 g), the cell pellet was washed twice in PBS and then frozen at –80 °C.

Growth of 293S cells in batch cultures were initiated by inoculating fresh J + medium with 1–$2\times10^5$ cell/mL in exponential growth phase.

Production runs were prepared by first centrifuging (600g, 15 min) aliquots of a cell culture in the exponential phase or in the very beginning of the plateau phase. To insure that the viral adsorption phase was identical for each assay, uniform conditions were imposed for the initial incubation of the cell/virus mixture. The cell pellets were then resuspended with the AdBM5-PT virus in either spent or fresh medium at a cell density of $10^7$ cells/mL and a MOI of 10 to insure synchronous infection. These concentrated cell/virus suspensions were incubated 2 hours and then diluted at various cell densities with spent or fresh medium. The infected cultures were incubated 3–5 days while samples were taken once or twice daily. For medium replacement experiments, infected cultures were centrifuged at 600g for 15 min and the spent medium discarded and replaced with the same volume of fresh medium. In a few cases, pH was periodically adjusted (2–3 times/day) in spinner flasks by addition of 7.5% $NaHCO_3$ until the color of the culture returned to 7.1. In spinner flasks, pH was estimated by the medium color compared to standard flasks (red-orange at pH $\approx$ 7.1, yellow at pH $\leq$ 6.5).

*Bioreactor description and operation*

A 3.5 L bioreactor (Chemap CF-3000 with a CBC-10 control unit) was used with 2.7 L of culture volume in order to scale-up the spinner productions. The tank was equipped with 3 surface baffles to break the liquid surface and increase mass transfer. Mixing was performed with a marine impeller rotating at 100 RPM. The temperature was maintained at 37 °C with a water jacket. D.O. and pH probes (Ingold) were mounted for monitoring and control purposes. The pH was controlled at 7.0 by intermittent addition of 7.5% $NaHCO_3$ solution. Feed gas composition was regulated by the sequential opening of electro-valves: $CO_2$ was kept at 7% and $O_2$ set-point was under the control of dissolved oxygen (DO) in order to maintain DO above 20%. Operation parameters were sent to a Compaq Deskpro PC for data acquisition.

The bioreactor infection protocol was identical to the one used for spinner cultures except that transfer of fluid to and from the bioreactor was achieved through sterile connections instead of under a biological hood.

*Analytical methods*

Viable and total cells were counted on a haemacytometer. Viability was assessed by dye exclusion using erythrosine B. The 293S cells having the tendancy to agglomerate, special care was taken to separate the clumps without affecting viability.

Medium composition was analyzed via HPLC. The various amino acid concentrations were measured by a reversed phase method as described previously by Kamen *et al.* (1991). The glucose and organic acid concentrations were obtained using an Interaction Ion-300 organic acid column (Chemicals Inc) with 0.0033N sulphuric acid as a mobile phase and two detectors: a refractive index detector (model 410, Millipore) and a spectrophotometer detector (model 490, Millipore) at 210 nm.

The oxygen uptake rate was measured using a YSI model 53 biological oxygen monitor, following the protocol provided with the system.

SDS-PAGE electrophoresis of cellular proteins was performed as follows. Frozen cell samples were thawed and diluted to $10^7$ cell/mL in extraction buffer (80 mM Tris-HCI pH 6.8, 2% (w/v) SDS and 10% (v/v) glycerol) and then sonicated (Heat Systems-Ultrasonics Inc, model W-375) 5s, 90W. Cell extracts (10 $\mu$L) were diluted with 10$\mu$L of NOVEX (San Diego, CA) sample buffer, containing 0,5% (v/v) $\beta$-mercaptoethanol. The diluted samples were heated at 85 °C for 5 min. and centrifuged 15s in an Eppendorf centrifuge before being loaded on a 8% acrylamide NOVEX precasted gel ($10^5$ cells per lane). The SDS-PAGE was run for 90 min. at 125 V following the NOVEX procedures.

*Protein Tyrosine Phosphatase activity*

PTP1C activity was measured according to the method described by Pot *et al.* (1991) with the following modifications. Batch analysis was performed by doing multiple dilutions in 96-well plates where samples were quantified against a PTP1C standard (kindly provided by Dr S.Shen). However, it was found that when diluted in the extraction buffer alone, the activity of the purified enzyme was drastically reduced. In order to stabilize the enzymatic activity, the purified PTP1C was diluted in a cell lysate obtained by adding 400 $\mu$L of the extraction buffer (described below) per $1 \times 10^6$ non-infected cell pellet. Samples, stored at –80 °C, were thawed on ice and resuspended at $2.5 \times 10^6$ cells/mL in extraction buffer: 25mM Tris-HC1, pH = 7.5, 10 mM $\beta$-mercaptoethanol, 2mM EDTA and 0.5% (v/v) Triton X-100. Aliquot volumes of 1 to 6 $\mu$L were transferred in the 96 well plate followed by 95$\mu$L of pNPP reagent: 25mM pNPP, 1.6mM DTT, 40 mM MES, pH=5. The plate was incubated at room temperature for 10 min and then 100 $\mu$L/well of 0.2N NaOH solution was added to stop the reaction. The plate was read at 405 nm using a Titertek Multiskan MCC microplate reader. A calibration curve was obtained from dilutions of the standard and the PTP1C content of the samples was calculated from that curve.

The specific PTP1C activity has been verified to be equivalent for cell samples and purified standard. For dilutions of identical activities, the PTP1C band obtained on PAGE for the purified standard was always equal or less than the PTP1C band for a cell sample. The stability of the frozen PTP1C standard was also assessed by series to series reproducibility of the activity calibration curve.

*Yield (Y) and specific consumption rate (q) calculations*

During growth experiments, the limits of the exponential growth phase were identified by first determining the zone of linear relationship on the plot of the natural log of cell concentration ln(X) vs time (t). The specific growth rate ($\mu$) was then estimated as the slope of that ln(X) vs t plot and the doubling time ($t_d$) was computed: $t_d = \ln(2)/\mu$. The cellular yield per mole of consumed substrate ($Y_s$) was calculated by dividing the quantity of cells produced by the quantity of substrate consumed during the exponential growth phase, while the product per cell yield was the mole produced divided by the quantity of cell produced during the exponential growth phase. The specific substrate consumption ($q_s$) rate was obtained by using: $q_s = \mu/Y_s$.

During infection, since cells do not grow significantly, Y and q were calculated differently. Specific substrate consumption rates were estimated during the period of initial linear consumption by dividing the quantity consumed by the time interval and the mean cell concentration during that period. Product yield was obtained by dividing the maximum quantity produced by the total cell concentration at that time.

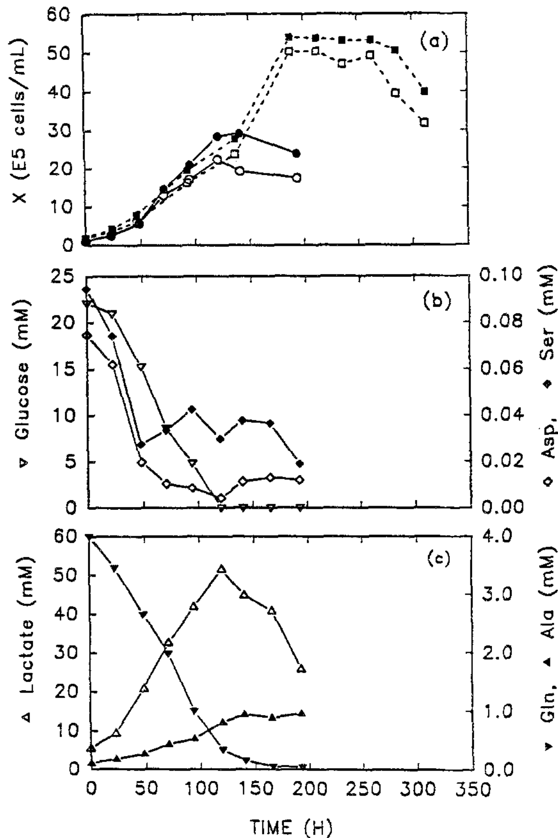


*Fig. 1.* Typical growth curves of 293S cells in Joklik+ and CFD-MEM. (a) o, • viable and total cells in J+, □, ■ viable and total cells in CFDMEM. Key metabolites profiles for cultures grown in Joklik+ medium: (b) ▽ glucose, ◇ aspartate, ◆ serine, and (c) △ lactate, ▼ glutamine, ▲ alanine.

## Results and discussion

### *Growth kinetic of 293S cells in suspension culture*

Typical growth curves for the 293S cells in J+ and CFDMEM, together with the variation of key medium components in J+ is shown in Fig. 1(a) through (c). Viable and total cell densities are shown in Fig.1(a). 293S cells were inoculated at $0.15\text{x}10^6$ cells/ml in complete J+ medium. Exponential growth started soon after cell inoculation and was maintained for a period of 3 days with a doubling time of 24 h. The growth was then linear for the next two days followed, at day 5, by a plateau of $2–3\times10^6$ cells/mL. At that point, the viability of the culture did not decrease sharply but rather stayed at the plateau for a few days before declining.

As can be seen in Fig.1(b) and (c), the growth of 293S cells in J+ was characterized by a substantial consumption of: glucose ($Y_{glucose}10^{11}$ cell/mole or $q_{glucose} = 29\times10^{-14}$ mole.(cell.h)$^{-1}$ in the exponential phase), aspartate ($Y_{asp} = 7.1\times10^{12}$ cell/mole or $q_{asp} = 0.4\times10^{-14}$ mole.(cell.h)$^{-1}$), serine ($Y_{ser} = 6.7\times10^{12}$ cell/mole or $q_{ser} = 0.43\times10^{-14}$ mole.(cell.h)$^{-1}$) and glutamine ($Y_{gln} = 5\times10^{11}$ cell/mole or $q_{gln} = 5.8\times10^{-14}$ mole.(cell.h)$^{-1}$). Although glucose and glutamine were consumed at a higher specific rate, aspartate and serine were depleted first since they are at a much lower concentration in the medium (0.1 mM compared to 25mM for glucose and 4mM for glutamine). Ammonia never exceeded 2mM at which concentration it was tested to be non-inhibiting for 293S growth (data not shown). On the other hand, lactate was the main by-product of the culture ($Y_{lact} = 2\times10^{-11}$ mole produced/cell) while a marginal amount of alanine was produced (1mM at day 6). Indeed, 293S cells did not oxidize significant amounts of glucose; most of it was used through glycolysis. Lactate has been found to impede 293S cell growth at concentrations of 20mM or more (data not shown). Consequently, lactate accumulation might have caused the shift from exponential to linear growth around day 3, although depletion of aspartate and serine may also be involved. Lactate accumulated to 50 mM by day 5, at which point glucose was depleted and the cells stopped dividing. Glucose and/or glutamine depletion around day 5 was most probably the cause of the culture entering the plateau phase, after which the cells started consuming lactate.

The 293S cells were also grown in complete CFD-MEM (Fig.1(a)). As can be seen, while the growth kinetics were equivalent in both media up to day 5, beyond that point, cells in CFDMEM kept growing for another 3 days reaching a cell density of $5\times10^6$ cells/mL. In contrast, cells in J+ entered the plateau phase at this time. A 3 day plateau then occurred before the cell density started decreasing. As described in the previous section, the only difference between CFD-MEM and J+ is the lower buffering capacity and the absence of non-essential amino acids with the exception of serine which is four times more concentrated. The need for higher levels of serine and/or the effect of the buffers (especially HEPES) on cell/substrate yields (glucose or glutamine) could explain the better performance of CFDMEM for 293S cell growth.

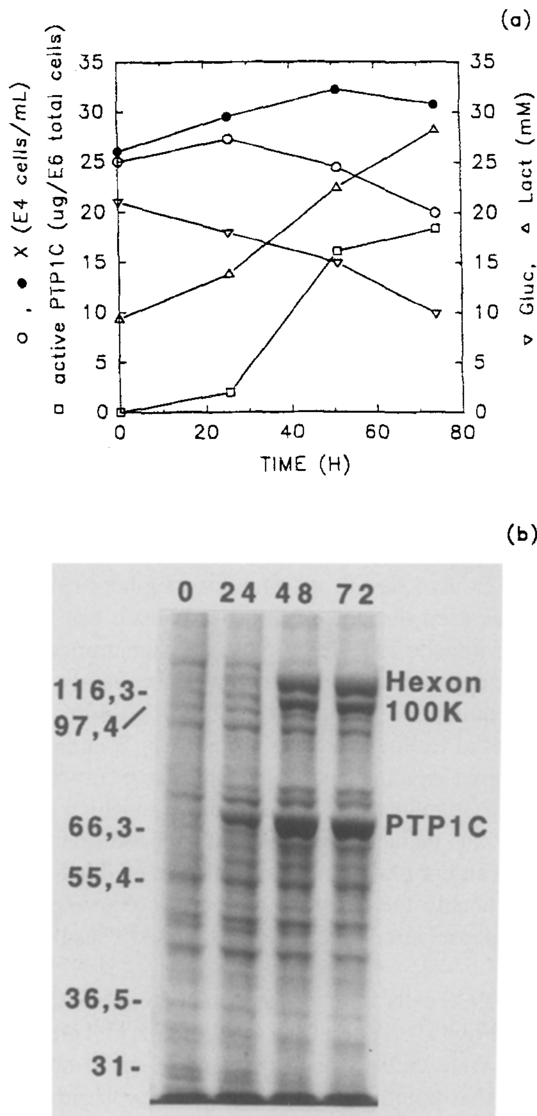


*Fig. 2.* Typical batch production of PTP1C at low cell density ($0.25\times10^6$ cells/mL) in Joklik+ o, • viable and total cells, □ PTP1C, ∇ glucose, △ lactate; (b) SDS-PAGE of cell extracts sampled at different time the infection, $10^5$ cells per lane: 0, 24, 48 and 72 hpi.

### *Production of PTP1C in AdBM5-PT infected 293S at low cell density*

Fig. 2(a) shows the increase in cell density with time for infecfed 293S cells seeded at $0.25\times10^6$ cells/mL. The active PTP1C cell content, glucose consumption as well as the lactate accumulation are also shown. The growth rate for infected cells was close to zero and cell viability fell gradually during the 3 day production phase. This is typical of a viral infection where the virus utilizes the host's cellular machinery towards the production of it's own DNA, RNA and proteins, thus impeding cellular growth and eventually causing cell death. Active PTP1C was produced at a constant rate and reached a level of 18 $\mu$g/$10^6$ cells 3 days post-infection. Furthermore, as can be seen by SDS-PAGE of the cell extract (Fig.2b) for samples at 0, 24, 48 and 72 hpi, the PTP1C band at 68kDa followed the same accumulation kinetics as the activity assay. It can also be seen in Fig.2(b) that PTP1C constituted the most abundant cellular protein, overtaking the hexon and 100K viral proteins.

As for the metabolites' evolution, glucose consumption as well as lactate accumulation were significant but not limiting the culture (Fig.2a); the other components in the medium were also not limiting (data not shown). This was expected since at low cell density infections, conditions are optimal for production because no limitations and/or inhibitions take place. However, specific consumption rates for glucose, $46\times10^{-14}$ mole.(cell.h)$^{-1}$ and glutamine, $9.4\times10^{-14}$ mole.(cell.h)$^{-1}$ were 60% higher than during growth while the rates for aspartate and serine, q= $0.25\times10^{-14}$ mole.(cell.h)$^{-1}$ decreased by 40% with respect to growth. The significant increase in glucose and glutamine consumption rate, both primary sources of energy, indicate a general acceleration of the cell metabolism during infection. Once again, lactate accumulated at a specific rate twice that of glucose consumption ($1\times10^{-12}$ mole.(cell.h)$^{-1}$) implying complete glycolysis.

### *Production of PTP1C at higher cell densities: effect of medium replacement*

A first series of experiments was undertaken in order to precisely establish the potential of the production medium in terms of the maximum cell density at infection that would not impair product yield. 293S cells were infected at different days in a culture and therefore at different densities (day 2, $0.6\times10^6$, day 3, $1.3\times10^6$ and day 4, $1.7\times10^6$ cells/mL) have been infected and resuspended at their initial cell densities in either (a) their spent medium, (b) fresh medium or (c) fresh medium followed with a medium replacement at 24 hpi. The resulting PTP1C yields at various time points are presented in Fig. 3 (a) through (c) and compared to a culture control infected a low cell density ($0.25\times10^6$ cells/mL). It can be seen in Fig. 3(a) that at $0.6\times10^6$

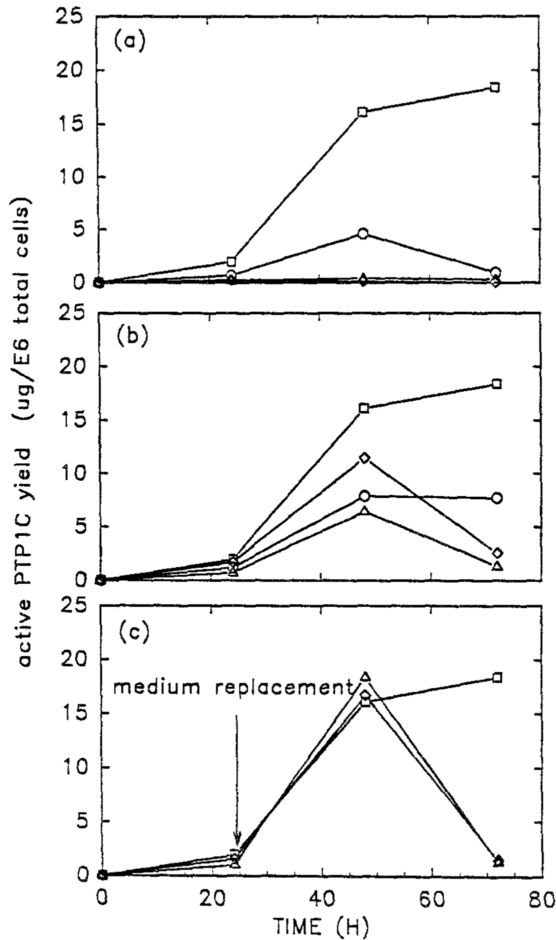


*Fig. 3.* PTP1C production in J+ at different cell densities. Infected cells resuspended in: (a) spent medium, (b) fresh medium, and (c) fresh medium with medium replacement at 24 hpi. Cell densities at infection: ○ $0.6\times10^6$, ◇ $1.3\times10^6$ and △ $1.7\times10^6$ cells/mL. □ duplicate controls at $0.25\times10^6$ cells/mL resuspended in spent or fresh medium.

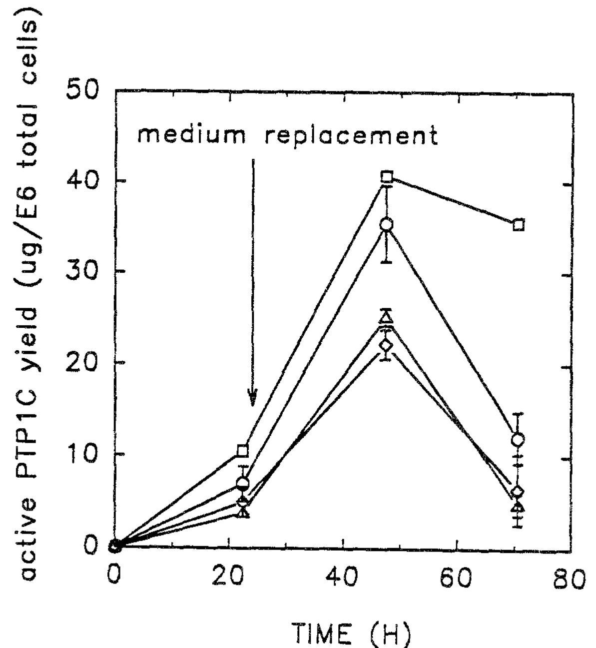


*Fig. 4.* PTP1C production in fresh J+ at different cell densities with one medium replacement at 24 hpi. ○ $2\times10^6$, ◇ $3\times10^6$, △ $4\times10^6$ cells/mL. □ control at $3\times10^5$ (without medium replacement). Means of duplicates are represented (± S.D.).

cells/mL or above, no significant amount of PTP1C was produced when the infected cells were resuspended in their spent medium. However, when resuspended in fresh medium (Fig.3b) production regained 50% of the maximum productivity with respect to the control at all cell densities. Furthermore, a second medium replacement at 24 hpi (Fig.3c) allowed for a sustained maximum specific productivity, even at the highest cell density (initial $1.7\times10^6$ led to a final $2.2\times10^6$ cells/mL).

These results clearly establish the existence of a substrate limitation and/or a by-product inhibition at high cell densities, a problem which can be partially remediated by an initial cell resuspension in fresh medium (Fig.3b) and completely restored with a medium change at 24 hpi (Fig.3c), resulting in a maximum productivity comparable to optimal low cell density infection. It also establishes that the cell culture growth stage (from early exponential at day 1 to beginning of plateau phase at day 4) do not influence protein production since cells infected in their 4th day of culture produced as much PTP1C as one day old infected culture, as long as the medium is not limiting and/or inhibiting. However, this medium replacement strategy was apparently only effective for a period of 24 to 48 h since the PTP1C activity decreased abruptly at 72 hpi. Analysis of total cell protein by SDS-PAGE showed that the loss in activity at 72 hpi was not concurrent with an equivalent loss in total PTP1C content (data not shown). The decrease in PTP1C activity ohserved at 72 hpi was therefore not due to protein degradation, but rather to an unknown mechanism such as protein aggregation, as previously observed for the HSV R1 subunit expressed with a similar AV (Massie *et al.*, 1994) or a major change in the phosphorylation state of the protein. Resolving this issue will require further investigation.

In order to evaluate the limit of this initial and 24 hpi medium replacement strategy, PTP1C yield was tested following infection of 293S cells at 2, 3 and

$4\times10^6$ cells/mL. As presented in Fig. 4, at 48 hpi, the yield of PTP1C for infection at $2\times10^6$ cells/mL ($35\pm4.3$ $\mu$g/$10^6$ cells) was not significantly different from the control (40$\mu$g/$10^6$ cells), while for higher cell densities yields were 40% inferior to the control. Past 48 hpi, the active product yield fell to zero for all the experiments at high cell densities while the low density control remained constant. These results show that $2\times10^6$ cells/mL is the maximum cell density at which daily medium replacement with J+ medium allows for the maintenance of maximum specific productivity. At cell densities higher than $2\times10^6$ cells/mL, volumetric productivity as well as specific product yield per cell decreases thereby increasing production and purification costs.

*Production of PTP1C at high cell density in a 3L bioreactor*

Since CFDMEM was more efficient for 293S growth than J+, its performance during an infection was tested. However, a preliminary experiment comparing both media for an infection at high cell density showed that CFDMEM acidified more rapidly than J+, which was not the case during cellular growth. At 72 hpi, in cell cultures infected at $1.3\times10^6$ cells/mL in fresh medium with a medium replacement at 24 hpi, the pH dropped below 6.5 (yellow medium) in CFDMEM while pH was roughly equal to 6.8 (orange medium) in J+ (data not shown). As a result, the PTP1C activity was also lower in CFDMEM (20 $\mu$g/$10^6$ cells) than in J+ (32 $\mu$g/$10^6$ cells) at 48 hpi.

In order to assess the correlation between active PTP1C production and pH as well as the scalability of the process, an infection experiment was performed in a pH-controlled 3L Chemap bioreactor. The results are shown in Figure 5 for a culture infected at $2\times10^6$ cells/mL (MOI=10) in CFDMEM. The bioreactor was compared to infected cell controls: two 50mL spinner flasks initially taken from the bioreactor, one with and the other without periodical pH adjustment.

The PTP1C specific productivity was equivalent in the bioreactor and in the pH controlled spinner with a peak of 45 $\mu$g active PTP1C/$10^6$ cells at 48 hpi, followed by a slight decrease in the active PTP1C concentration. By contrast, in the spinner flask without pH control, the accumulation of active PTP1C stopped at 30 hpi with a peak of 30 $\mu$g active PTP1C/$10^6$ cells, followed by a rapid reduction in activity, falling close to zero by 52 hpi. A correlation between the PTP1C activity loss and pH decrease was observed. While at

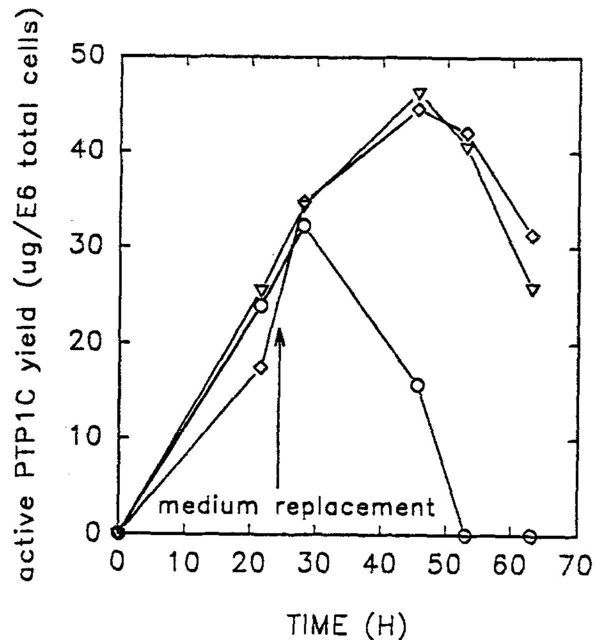


*Fig. 5.* Production of PTP1C in bioreactor at $2\times10^6$ cells/mL with fresh CFDMEM and a medium replacement at 24 hpi. ◇ bioreactor, ∇ spinner control with periodical pH adjustments, o spinner control without pH adjustments.

48 hpi the medium was already yellow in the non-controlled spinner (pH $\leq$ 6.5), the medium was still red-orange in the bioreactor and the pH adjusted spinner (pH $\approx$ 7). However, the decrease in pH does not fully explain the PTP1C activity loss since the PTP1C activity also decreased slowly in a pH controlled environment past 48 hpi. The pH control only delays the lytic process that inevitably takes place during adenoviral infection, while permitting the maximum product yield to be attained.

These results show that it is possible to maintain maximum specific production rate at high cell densities, by medium replacement at 0 and at 24 hpi, in a pH controlled culture at the 50 mL spinner flask scale as well as 3L bioreactor scale. Although peak product yield varied among the experimental runs, one can expect to obtain 40 to 45 $\mu$g PTP1C/$10^6$ cells, equivalent to 15% of the total cellular protein content (based on 300$\mu$g total protein/$10^6$ cells) or 90 mg PTP1C per Litre of culture (at $2\times10^6$ cells/mL) compared to 13.5 mg/l for $0.3\times10^6$ cells/mL. These figures are comparable to productions of Herpes Simplex Virus ribonucleotide reductase subunits R1 and R2 obtained previously in our lab with other AdBM5 AV's (Massie *et al*, 1994). Although this process is very effective with respect to cell yield and protein purification for

an intracellular product, it is not optimal in terms of medium expenses. In fact, with two medium changes, the yield of product per spent medium is equivalent to 30 mg PTP1C per L of medium (at $2\times10^6$ cells/mL) which is not much higher than 15 mg/L of medium for productions at $3\times10^5$ cells/mL without medium replacement. We then turned our attention to the analysis of key metabolites in order to improve yield based on spent medium.

*Analysis of key metabolites during the infection phase*

Samples from the infection of $1.3\times10^6$ 293S cells/mL in fresh J+ medium without medium replacement at 24 hpi (for which PTP1C yield has been presented in Fig.3b) have been analyzed for their content in glucose, organic acids, and amino acids. In this experiment, PTP1C was only 60% the level of it's maximum specific activity due to limitation of nutrients and/or inhibition of by-products. The results are presented in Fig. 6.

The total cell density increased from $1.3\times10^6$ to a mean value of $1.9\times10^6$ cells/mL during the first 24 h and remained constant thereafter. This slight initial increase of about 20% in cell count is routinely observed for infected culture. However, the maintenance of viability up to 96 hpi is peculiar to infection in limiting and/or inhibiting environment. This could be explained by the fact that under sub-optimal conditions of infection, the overall cycle of virus reproduction would occur at a lower rate, thereby reducing the infection stress on the cell which in turn would result in a prolonged viability.

Glucose was completely depleted before 48 hpi. Based on the first 24 hour period, the specific glucose consumption rate, was $38\times10^{-14}$ mole glucose.(cell.h)$^{-1}$ which is a 30% increase compared to $q_{glucose}$ during cell growth. However, glucose consumption rate was reduced by 17% compared to infection at low cell density. Furthermore, while glucose was totally transformed into lactate during growth and infection at low cell density, only 45% of it was metabolized through glycolysis during the infection phase at high cell density; 22mM of glucose yielding only 20mM of lactate instead of 44mM for a complete glycolysis. It appears that while the general metabolic activity was higher compared to growth phase, in this case there was a glucose limitation, thereby reducing glucose consumption rate as well as lactate production rate. This in turn should increase the cellular oxygen requirement. The specific oxygen consumption rate

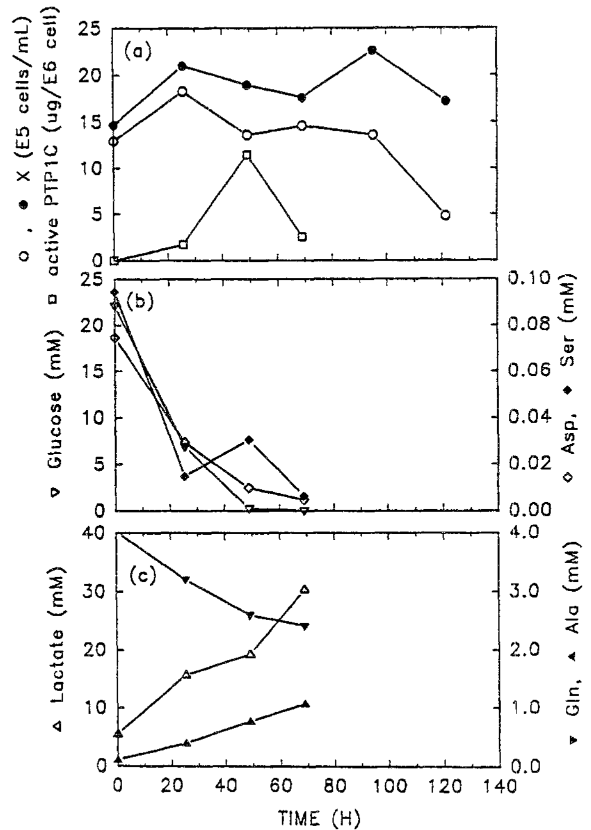


*Fig. 6.* Key metabolites evolution in a batch production of PTP1C in fresh J+ at initial cell density of $1.3\times10^6$ cells/mL: a)o, • viable and total cells in J+, □ PTP1C yield; (b) ▽ glucose, ◇ aspartate, ♦ serine, and (c) △ lactate, ▼ glutamine, ▲ alanine.

($q_{O2}$) has been measured in growth phase as well as during infection. Indeed, the average value obtained during infection, $q_{O2} = 16\times10^{-14}$ mole $O_2$.(cell.h)$^{-1}$ was twice as high as for exponential growth, $q_{O2} = 8\times10^{-14}$ mole $O_2$.(cell.h)$^{-1}$. This is consistent with a significant increase in metabolic rate during the infection phase compared to the growth phase and a reduced glycolysis rate with respect to nonlimiting medium conditions.

During infection, aspartate and serine specific consumption rates were low ($\approx 0.2\times10^{-14}$ mole.(cell.h)$^{-1}$ for both), but, given the high cell density, were rapidly depleted by 48 hpi. Glutamine was consumed ($2.2\times10^{-14}$ mole.(cell.h)$^{-1}$) but not depleted and alanine was produced ($1\times10^{-14}$ mole.(cell.h)$^{-1}$). These specific consumption rates were smaller than those obtained for infection at low cell density and exponential growth. This might be due to glucose limitation. However, aspartate and serine, although depleted, were not limiting. Indeed, compar-

isons of PTP1C production in J+ and CFDMEM at high cell densities were not found to be significantly different, even though CFDMEM did not contain aspartate but four times the serine concentration of J+. In other production runs, 6mM glutamine (instead of 4mM) as well as 10% serum addition (instead of 5%) have also been tested, but did not give higher productivities (results not shown). However, a 50% drop in PTP1C production was observed in absence of serum compared to the usual 5% CS.

In summary, in serum supplemented culture, the production of PTP1C with the AdBM5/293S system in CFDMEM was mainly limited by glucose depletion and inhibited by the pH drop caused by lactate accumulation.

### Effect of specific additions during a production run

In order to apply and verify the above results, PTP1C production was compared in a glucose addition vs a medium replacement experiment. A culture at $1.5 \times 10^6$ cells/mL was infected, resuspended in fresh CFDMEM with 2.5 g/L HEPES and aliquoted into three 50mL spinner flasks. In the first spinner the culture was centrifuged and the medium replaced with fresh CFDMEM + HEPES at 24 hpi. In the other two, 0.5 mL of a 200 g/L glucose solution was added to the culture at 24 hpi (+2 g/L, or 11 mM glucose addition). In one of these pH was periodically adjusted.

Figure 7 shows that, PTP1C production followed a similar profile in both the medium replacement and the glucose addition experiment where pH was controlled. The two feeding strategies yielded a maximum active PTP1C content of 25 $\mu$g/$10^6$ cells at 36–48 hpi. Since it has been shown that at high cell density without medium replacement or glucose addition, active PTP1C yield declined after 24 hpi, it is clear that glucose addition is responsible for sustained PTP1C production, equivalent to production with medium replacement. It therefore confirms that glucose is most probably the major limiting substrate of PTP1C production.

In the third spinner (glucose addition without pH control) the PTP1C activity decreased linearly after 24 hpi and was absent by 48 hpi. This severe drop in activity is again correlated with a decrease in pH; indeed, the medium was already yellow at 36 hpi or earlier (pH $\leq$ 6.5) in the glucose addition spinner without pH control while it was maintained around red-orange (pH $\approx$ 7) in the other spinner with glucose addition. The exclusive relation between pH and PTP1C activity

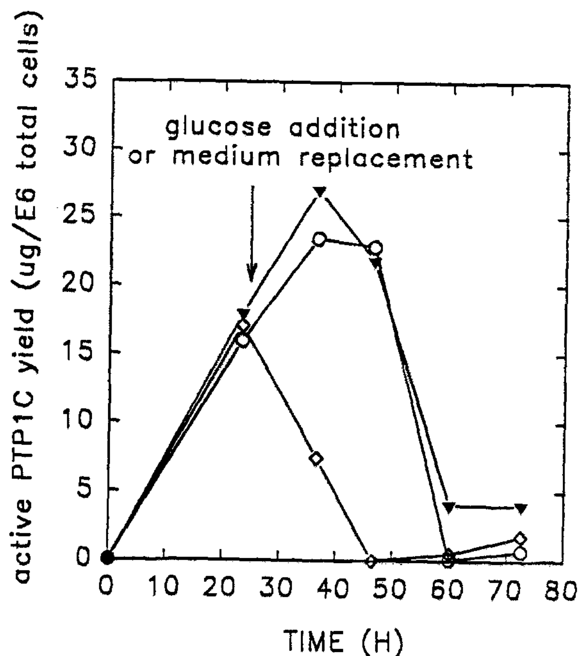


*Fig. 7.* Production of PTP1C in fresh CFDMEM at $1.6 \times 10^6$ cells/mL. ○ medium replacement at 24 hpi, ▼ glucose addition with periodical pH adjustments, ◇ glucose addition without pH control.

was confirmed by lactate analysis since lactate concentrations attained equivalent level (40–50 mM) in both glucose-supplemented spinners (data not shown). It is therefore the pH decrease as such and not lactate production, that has a negative effect on active PTP1C yield. This is encouraging since pH is easier to control than lactate production.

### Conclusion

In this paper we have presented the first results concerning the scale-up of a high-level recombinant protein production AV/293 system. The 293S cells have been shown to be able to grow to plateau cell densities of $5 \times 10^6$ cells/mL in calcium-free DMEM. With an initial and a 24 hpi medium replacement, the specific PTP1C yield could be maintained at it's maximum level up to infected cell densities of $2 \times 10^6$ cells/mL. Under these conditions, volumetric productivities of 90 mg/L could be attained. At an infected cell density of $1.6 \times 10^6$ cells/mL, the replacement of the 24 hpi medium change by a 2 g/L glucose addition, together with periodical pH adjustments, allowed the same specific productivities, but at lower medium expenses.

It is expected that glucose fed-batch in pH-controlled bioreactor will further improve these performances.

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