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Comparison of manufacturing techniques for adenovirus production

Paddy Iyer, Jeffrey M. Ostrove* & Dominick Vacante
BioReliance Corporation, 9900 Blackwell Road, Rockville, Maryland 20850 USA

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Abstract

We have compared three different production methods, which may be suitable for the large scale production of adenovirus vectors for human clinical trials. The procedures compared 293 cells adapted to suspension growth in serum-free medium in a stirred tank bioreactor, 293 cells on microcarriers in serum-containing medium in a stirred tank bioreactor, and 293 cells grown in standard tissue culture plasticware. With a given virus, yields varied between 2000 and 10,000 infectious units/cell. The stirred tank bioreactor routinely produced between 4000 and 7000 infectious units/cell when 293 cells were grown on microcarriers. The 293 cells adapted to suspension growth in serum-free medium in the same stirred tank bioreactor yielded between 2000 and 7000 infectious units/cell. Yields obtained from standard tissue culture plasticware were up to 10,000 infectious units/cell. Cell culture conditions were monitored for glucose consumption, lactate production, and ammonia accumulation. Glucose consumption and lactate accumulation correlated well with the cell growth parameters. Ammonia production does not appear to be significant. Based on virus yields, ease of operation and linear scalability, large-scale adenovirus production seems feasible using 293 cells (adapted to suspension/serum free medium or on microcarriers in serum containing medium) in a stirred tank bioreactor.

Introduction

Adenovirus vectors have various applications in the field of gene therapy and as viral oncolytics (Crystal, 1995). Methods of adenovirus production for phase 1 clinical trials have relied on standard tissue culture plasticware for the most part. Apart from being labor intensive, these methods have limited potential for scale-up. We have compared three different production methods for the generation of adenovirus vectors. Adenovirus production in a stirred tank bioreactor using both anchorage dependent 293 cells and 293 cells adapted to growth in suspension using serum-free medium were compared to cultures grown on tissue culture plasticware. Bioreactor cultures containing 293 cells were infected at a multiplicity of infection (MOI) of 5–10 when the cell density reached 1.5 – 2.5 $\times 10^6$ cells/ml. At 48 to 72 hours post inoculation, viral infected cultures were harvested and virus quantitation was performed using standard 50% tissue culture infectious dose ($TCID_{50}$) assay (Schmidt, 1989). Glucose consumption, lactate accumulation and ammonia production from the cultures were monitored using a Kodak metabolite analyzer.

* Presently with NeuroVir, Vancouver BC, Canada

Methods

Human embryonic kidney (HEK) 293 cells obtained from American Type Culture Collection (ATCC) and grown in fetal bovine serum (Hyclone) containing medium (DMEM, 10% FBS) in 162 cm^2 tissue culture flasks were used to seed Cytodex 3 microcarrier (Pharmacia Biotech) cultures. HEK 293 cells adapted to growth in suspension using serum-free medium in 100 ml shaker flasks were used to seed suspension bioreactor cultures in serum-free medium. The serum-free medium used was supplied by Life Technologies, Inc. A benchtop stirred tank bioreactor (Artisan, Inc., Waltham, MA) with a 4L working volume and height/diameter ratio of 2:1 was used. Oxygen, air and

CO_2 were supplied by headspace aeration and agitation provided by a marine impeller. In the bioreactor, the temperature was controlled at 37 °C, Dissolved Oxygen (D.O.) as a percent of air saturation was controlled at 50% and the pH was controlled at 7.3 using CO_2 or 7.5% sodium bicarbonate. Total cell densities for microcarrier cultures were determined using nuclei staining method (Nahapetian, 1986), while viable cell densities for suspension cultures were determined by the Trypan Blue dye-exclusion. Cell densities were determined on days 1, 3, and 6 post-seeding and glucose consumption, lactate and ammonia production were monitored using a metabolite analyzer (Kodak). When the cell densities reached 1.5 – 2.5 $\times$ 10^6 c/ml, a one volume fed-batch exchange was performed. After the fed-batch exchange, an adenovirus, Ad-dl312 (Jones, 1979) with Ad-5 backbone and E1A region deleted was introduced into the culture at a MOI of 5-10. After infection, conditions were maintained as above for cell growth. At 48 and 72 hours post infection, samples were collected for virus titer assays and glucose, lactate and ammonia analysis. Virus quantitation was performed using the $TCID_{50}$ assay (Schmidt, 1989) on the clarified infected cell lysate.

For experiments using plasticware, HEK 293 cells grown in DMEM with 10% FBS in 162 cm^2 tissue culture flasks were used to seed tissue culture plasticware (500 cm^2 Nunc™ trays). Day 1 post seeding, Ad-dl312 was introduced into the culture at a MOI of 5-10. Samples were collected 48 hours post-infection and virus quantitation was performed.

Results and Discussion

HEK 293 cells in serum-containing medium were grown in 2L and 4L microcarrier bioreactor cultures. The cells seeded at 1.5 $\times$ 10^5 c/ml reached 2 $\times$ 10^6 c/ml typically by day 6 or 7 of the culture. During this time, increased glucose consumption and lactate production was noticed (Figure 1). No trend toward increased ammonia production in the same time period was detected. This seems to indicate that glutamine at levels present in the medium may not be significant as an energy source. A fed-batch, one volume exchange on day 6 or 7 of the culture (Figure 1). These conditions were considered ideal for the infection phase and yielded between 4000 and 7000 infectious units per cell (Table 1). Specific consumption and production rates prior to infection are shown in Table 2.

Table 1. Adenovirus production using HEK 293 cells grown on microcarriers with serum-containing medium in a bioreactor system

Culture	Volume (Liters)	Cell Density[1] (cells/ml)	Virus Titer ($TCID_{50}$/cell)	Total yield (Total $TCID_{50}$)
Bioreactor	2	1.9 x 10^6	4500	1.7 x 10^{13}
Bioreactor	2	2.0 x 10^6	7300	2.9 x 10^{13}
Bioreactor	4	2.2 x 10^6	4500	4.0 x 10^{13}
Bioreactor	4	1.9 x 10^6	3600	2.7 x 10^{13}
Dishes[2]	10	5.0 x 10^5	10000	5.0 x 10^{13}

[1] At the time of infection
[2] Nunc™ (500 cm^2 – surface area)

Cells adapted to suspension growth in serum-free medium as opposed to microcarrier cultures in serum containing medium are easier to scale-up in large scale production systems such as 100 to 1000 liter stirred tank bioreactors. Other investigators (Garnier, 1994 and Nadeau, 1996) have demonstrated that HEK 293 cells adapted to suspension growth can be used for adenovirus production in stirred tank bioreactors. There is no need to develop complicated procedures as in the case with microcarrier cultures where cells may have to be harvested from seed microcarrier cultures prior to inoculating large scale systems. Serum-free culture medium apart from providing a less complex feedstream for downstream processing may also eliminate the need for animal-derived protein components, which may impact cost and minimize regulatory compliance issues.

HEK 293 cells were adapted to suspension growth by weaning cells off serum-containing medium. Once adapted, these cells have been maintained for more than 60 days and passaged nine times in 100 ml shaker cultures with consistent cell growth parameters and cell viabilities (Figure 2). The 293 cells adapted to suspension growth were used to seed 2L bioreactor suspension cultures. The cells seeded at 5 $\times$ 10^5 c/ml reached 2 $\times$ 10^6 c/ml by day 6 or 7 of the culture. Adenovirus that was added after a one volume fed-batch exchange led to virus yields between 2000 and 7000 infectious units per cell (Table 2).

As with microcarrier cultures in serum-containing medium, increased glucose consumption and lactate production was noted in the cell growth phase with the effect becoming more pronounced in the virus production phase (Figure 3). An optimized culture infection procedure using standard tissue culture plastic ware (500 cm^2 Nunc trays) provided the highest adenovirus

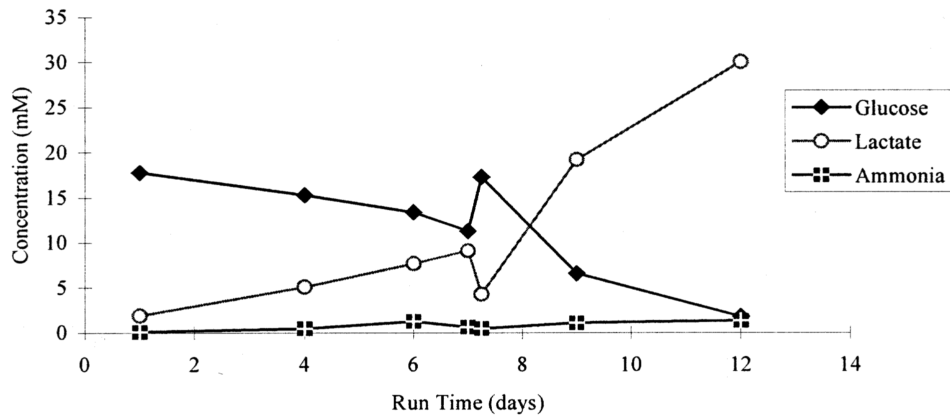

Figure 1. Glucose, lactate and ammonia profiles in a 2L bioreactor microcarrier culture. A one volume fed–batch exchange was performed on Day 7.

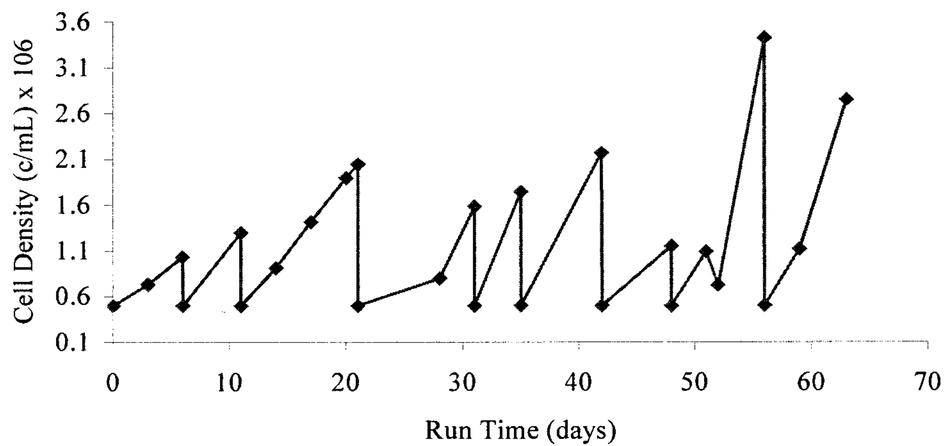

Figure 2. 293 cells adapted to suspension growth in serum free medium in 100 ml shaker cultures.

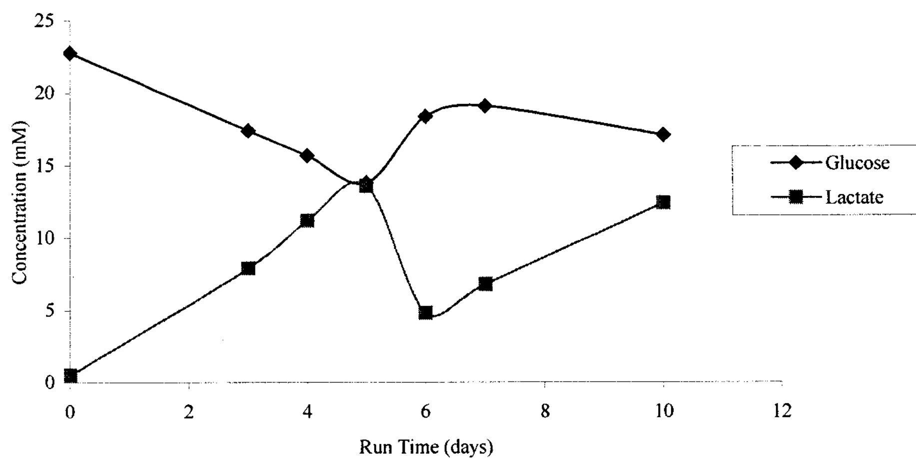

Figure 3. Glucose, lactate profiles in a 2L bioreactor serum-free suspension HEK 293 cell culture during growth phase. In this instance, the culture was split on day 5 by adding fresh medium.

Table 2. Specific consumption rates obtained in a 2L microcarrier bioreactor culture prior to infection

Culture time (Days)	Glucose consumption rate (mM/10^9 c/day)	Lactate production rate (mM/10^9 c/day)
1	2.78	3.56
4	2.79	3.82
6	1.66	1.11

Table 3. Adenovirus production using 293 cells adapted to suspension cultures in serum-free medium

Culture	Volume (Liters)	Cell Density[1] (cells/ml)	Virus Titer ($TCID_{50}$/cell)	Total yield (Total $TCID_{50}$)
Bioreactor	2	1.7 x 10^6	1900	6.5 x 10^{12}
Bioreactor	2	1.7 x 10^6	4000	1.4 x 10^{13}
Bioreactor	2	2.3 x 10^6	6400	2.9 x 10^{13}
Bioreactor	2	2.3 x 10^6	2100	9.7 x 10^{12}

[1] At the time of infection.

yields. Virus yields from such an optimized system were up to 10,000 infectious units per cell (based on $TCID_{50}$ assay).

Nunc™ dishes were seeded one day prior to infection at 1×10^5 c/cm^2 (5×10^5 c/ml) and harvested two days after infection. This procedure probably allowed less time for metabolic wastes to accumulate and made more nutrients available per cell as opposed to bioreactor cultures which were typically infected at 2×10^6 c/ml, 6–7 days post-seeding. Also, shear forces on the cells are virtually absent in the static culture of the dishes. We believe these factors may have contributed to providing the highest adenovirus yield per cell. Virus yields in Nunc™ dishes from other adenovirus constructs (some of which were proprietary client material) were compared to the Ad-dl312 construct (used in this study). Depending on the type of adenoviral construct, viral yields varied from 1700 to 12,000 infectious units per cell (based on $TCID_{50}$ assay).

Conclusion

Although standard tissue culture plasticware provided the highest adenovirus yields, the methods are labor intensive and scale limited. We have shown that with stirred tank bioreactor and anchorage dependent HEK 293 cells, it is possible to produce adenovirus yields that are linearly scaleable. Further, we have successfully adapted 293 cells to grow in suspension culture in serum-free medium in shaker and stirred tank bioreactor cultures. Virus yields from suspension 293 cells in serum-free medium, show promise for large scale adenovirus production given the potential ease of suspension cell scale-up operation. A serum free medium formulation will provide a less complex feedstream for downstream processing. Efforts are ongoing to optimize virus yields for such a system.

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Address for correspondence: Dominick Vacante, BioReliance Corporation, 14920 Broschart Road, Rockville, Maryland 20850, U.S.A.
Phone: 301-610-2543; Fax: 301-251–0437;
E-mail: dvacante@bioreliance.com