Aujeszky's disease virus production in disposable bioreactor

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A novel, disposable-bag bioreactor system that uses wave action for mixing and transferring oxygen was evaluated for BHK 21 C13 cell line growth and Aujeszky's disease virus (ADV) production. Growth kinetics of BHK 21 C13 cells in the wave bioreactor during 3-day period were determined. At the end of the 3-day culture period and cell density of 1.82 x 10^6 cells ml^{-1}, the reactor was inoculated with 9 ml of gE^- Bartha K-61 strain ADV suspension ($10^{5.9}$ $TCID_{50}$) with multiplicity of infection (MOI) of 0.01. After a 144 h incubation period, 400 ml of ADV harvest was obtained with titre of $10^{7.0}$ $TCID_{50}$ ml^{-1}, which corresponds to 40,000 doses of vaccine against AD. In conclusion, the results obtained with the wave bioreactor using BHK 21 C13 cells showed that this system can be considered as suitable for ADV or BHK 21 C13 cell biomass production.

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1. Introduction

Demand for vaccine production requires high-yield, stable bioproduction systems and implementation of new technologies. A cell culture device that uses a presterile, single-use plastic bag for cell cultivation has been introduced. The *Wave Bioreactor 20SPS™* (Wave Biotech AG, Tagelswangen, Switzerland) is a cultivation system developed for semi-industrial cultivation of many different cell types (e.g. hybridomas, mammalian cells, plant cells) (Weber *et al* 2002). Due to its novelty, very small number of protocols about cultivation parameters for different cell lines or virus production are available. The wave bioreactor (figure 1) consists of three components: (i) fixed rocker base unit with thermoplate, *Cellbase 20SPS*; (ii) device for data connection, *Instrument Rack SPS19''*; and (iii) disposable, presterile, plastic bag, *Cellbag 2L*.

The cell suspension is cultivated inside the *Cellbag2L*, placed on the thermoplate of the rocker base unit, *Cellbase 20SPS*, which rocks at specified frequency and angle. The gentle rocking motion of the bioreactor creates waves that promote mixing of the cell culture fluids (figure 2). As a result, cells are not exposed to large variations in shear forces and thus are able to grow in a more stable physical environment. Fresh atmosphere is passaged through the headspace of the cellbag, keeping it rigidly inflated. The wave motion of culture liquid generates turbulent surface, which results in much greater volumetric oxygen transfer coefficient (k_La) than in any static culture or spinner flasks (Singh 1999). This effectively eliminates the need for a complex dissolved oxygen control system. The presterilized cellbags are disposable after use, which eliminates the need for repeated washing and sterilization.

The Aujeszky's disease virus (ADV), belonging to the *Alphaherpesvirinae* family, is the cause of a natural infection in swine population. To reduce its incidence and financial losses, the vaccination of pigs with attenuated live or inactivated vaccines is widely performed. A laboratory-attenuated ADV replicates well in BHK 21 cells (Puentes

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Abbreviations used: ADV, Aujeszky's disease virus; $TCID_{50}$, tissue culture infectious dose 50.

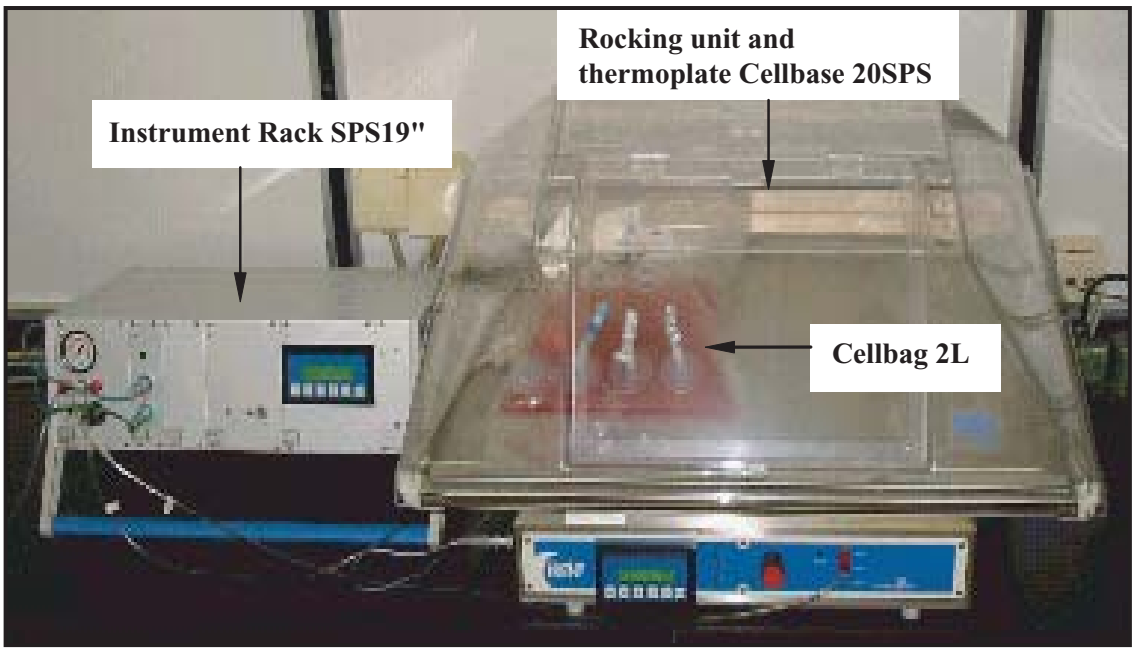

Figure 1. The *Wave Bioreactor 20SPS*™ system with fixed rocker base unit Cellbase 20SPS, Cellbag2L and Instrument Rack SPS.

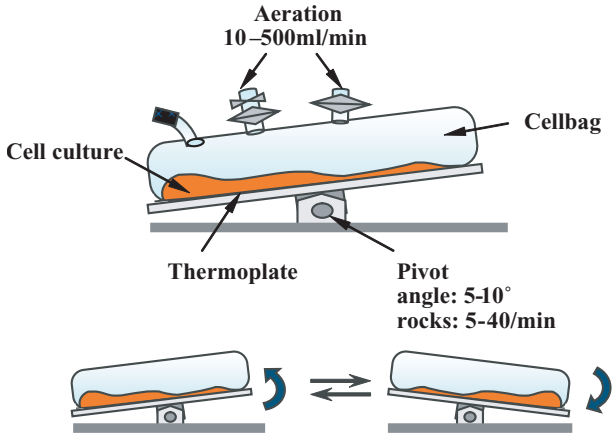

Figure 2. The wave bioreactor is a novel system for cell culture cultivation. The cell cultivation is performed in a disposable plastic bag (cellbag) placed on an incubation platform (thermoplate) that rocks at a specified frequency and angle. This rocking motion promotes mixing of the culture fluid inside the cellbag and efficient oxygen transfer without shear or bubbles.

et al 1993), which can be cultivated in different cell culture systems like T-flasks, roller bottles or spinner cultures in order to produce ADV.

The objective of this study was to investigate utility of the wave bioreactor system for cultivation of BHK 21 C13 cell line growing in suspension, and ADV production.

2. Materials and methods

2.1 *Cell line, medium and cell culture conditions*

The BHK 21 C13 cells (ATTC-CCL 10) were cultivated in Glasgow BHK 21 medium (GIBCO, Paisley, Scotland) supplemented with 5% (v/v) fetal bovine serum (FBS) (GIBCO, Paisley, Scotland). Antibiotics were not used.

Before starting the propagation in the wave bioreactor, the initial biomass production was performed in the cellspin bioreactor (IBS Integra Biosciences, Switzerland) in humidified atmosphere containing 5% of CO_2 at 37°C with initial cell concentration of 5 x 10^5 cells ml^{-1} and initial volume of 100 ml of culture medium. Cultivation in spinner flask was performed during 3 days with addition of 120 ml of fresh media on the second day. On the third day the cell suspension was diluted to final volume of 450 ml and cell density of 5.5 x 10^5 cells ml^{-1}. The cell suspension was then transferred directly into the inflated *Cellbag 2L*™ of the wave bioreactor system. All transfer activities were performed inside a laminar flow cabinet. The cell cultivation in the wave bioreactor was carried out after placing the cellbag on the rocking unit of the system heated at 37°C and at rocking speed of 10 rpm with tilt angle of 6°. Before the cell inoculation, the bag was rigidly inflated with atmosphere consisted of 95% of air + 5% of CO_2 using gas mixing module *Gasmix 20SPS*™. This atmosphere was regularly changed and refreshed within each 24 h during 9 days of maintained

cultivation and ADV production. The inlet and outlet filters of the cellbag were opened for aeration at flow rate of 0.2 l min^{-1} for several hours daily to avoid extensive medium evaporation. The medium exchanges were accomplished by stopping the rocking motion of the bioreactor, letting the suspended cells to sediment to the bottom of the bag, and then pumping out supernatant liquid. About 65% of the spent medium was removed from the cellbag. The fresh warmed medium was added to the remaining contents of the cellbag and then the initial rocking setting of the bioreactor was resumed.

2.2 *Cell counting*

Cells were counted using a Fuchs-Rosenthal hemacytometer. Viable and non-viable cells were determined by trypan-blue exclusion method. Viable cells are impermeable to trypan-blue and therefore the cells are transparent while non-viable cells are blue-dyed. The specific growth rate μ (h^{-1}) was estimated by the following equation: $\mu=(\mathrm{Ln}X_n-\mathrm{Ln}X_{n-1})/(t_n-t_{n-1})$, where X represents the viable cell density per ml, t represents the time-points of sampling expressed in hours and the subscripts n and $n-1$ stand for two successive sampling points.

2.3 *Glucose and lactate analysis*

Glucose and lactate were quantified enzimatically. The glucose was quantified using Glucose-PAP colorenzymatic assay kit (Herbos, Sisak, Croatia) while the lactate was quantified by the UV-test (R-Biopharm AG, Darmstadt, Germany).

2.4 *Virus strain, virus production and titration*

The gE^- ADV (Bartha K-61 strain), multiplied in PK-15 cell culture provided by the Veterina Ltd. (Kalinovica, Croatia), was used throughout this study.

The BHK 21 C13 cells were infected by the gE^- Bartha K-61 strain of AD virus at cell concentration of 1.82 x 0^6 cells ml^{-1} and inoculation volume of 2% (v/v) of the culture volume. The virus production was performed at 37 ^{0}C in the wave bioreactor containing 450 ml of culture medium during 6 days. The samples were taken daily to determine cell concentration as well as concentration of glucose and lactate. The virus titre was determined by the Spearman-Kärber method and expressed in tissue culture infectious dose ($TCID_{50}$ ml^{-1}) on the second, the fourth and the sixth day post-infection using secondary cell culture of chicken fibroblasts (KF/1) by means of the microtitration method (Lojkić *et al* 1992). All assays were performed in duplicate.

3. Results and discussion

3.1 *Cell culture growth in the wave bioreactor*

The growth profile of BHK 21 C13 cells in the wave bioreactor was established and typical growth curve is presented in figure 3. The cells were propagated in 200 ml of media in spinner flask, prior to cultivation in the bioreactor. During this propagation, the cells started their exponential growth phase. After their transfer into the *cellbag* of the bioreactor and addition of 300 ml of fresh media, this phase continued for the next 3 days. The initial cell density in the bioreactor was 5.2 x 10^5 cells ml^{-1} and it reached its maximum (19 x 10^5 cells ml^{-1}) at the fourth day of the cultivation period by an apparent beginning of plateau phase. The maximum growth rate was 0.0285 h^{-1}.

The cell culture in the bioreactor was maintained in batch mode at rocking rate of 10 rpm, tilt angle 6° and aeration conditions as previously described.

3.2 *Virus production*

Once it has been determined that the wave bioreactor system could support the high cell density of BHK 21 C13 cells, the next step was to evaluate the ADV production capability. The cells were seeded at initial cell density of 5.5 x 10^5 cell ml^{-1} in culture volume of 450 ml (figure 4). The cells were cultivated at a rocking rate of 10 rpm, tilt angle 6° and aeration conditions as described previously. During the second day of cultivation and having obtained cell concentration of 13.3 x 10^5 cells ml^{-1}, about 65% of the culture medium was replaced with the same volume of fresh medium in order to avoid nutrient limitation and to increase cell density. At the third day the cell density reached 1.82 x 10^5 cells ml^{-1} and the cell viability was equal to 99%. The specific growth rate was ranged from 0.008 to 0.028 h^{-1} (mean 0.016 h^{-1}). The obtained specific growth rate was comparable to growth rates achieved when HEK-293 and CHO cell lines were cultivated in the wave bioreactor system (Namdev and Lio 2000). The glucose, as one of the major carbon and energy sources in cell culture media and its by-product lactate, were measured during BHK 21 C13 cell cultivation. The glucose and lactate concentrations during the cultivation process are shown in figure 5. At the end of the cultivation period, the glucose concentration was 15.4 mM while the lactate concentration was 5.9 mM. The inhibitory level of lactate is 22 mM, as reported for stirred BHK 21A culture (Cruz *et al* 2000), and the results of our study have shown that lactate level were significantly under the growth limit.

When the BHK 21 C13 cells reached concentration of 1.82 x 10^5 cells ml^{-1}, the cells were infected with 9 ml of

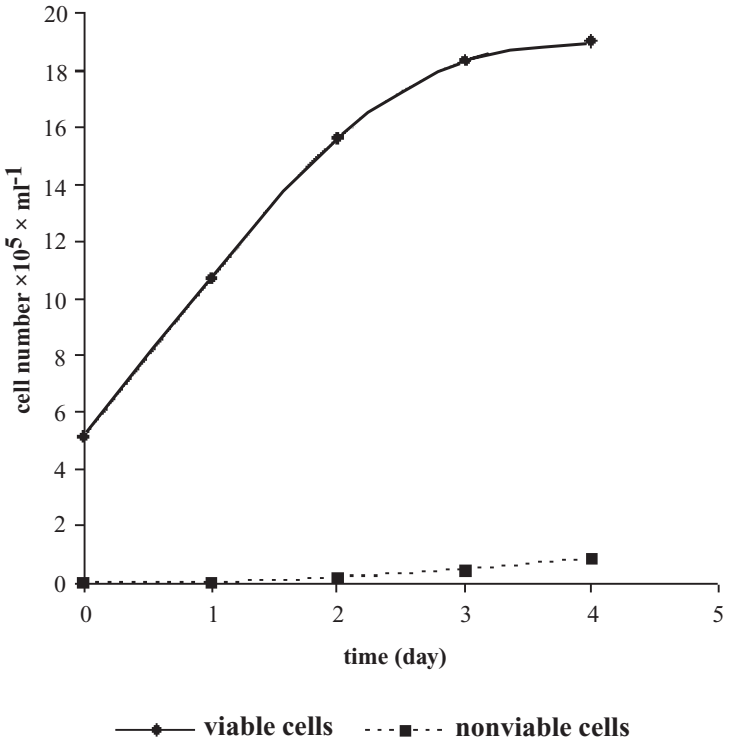

Figure 3. Typical growth curve of BHK 21 C13 cells cultivated in batch mode in the wave bioreactor.

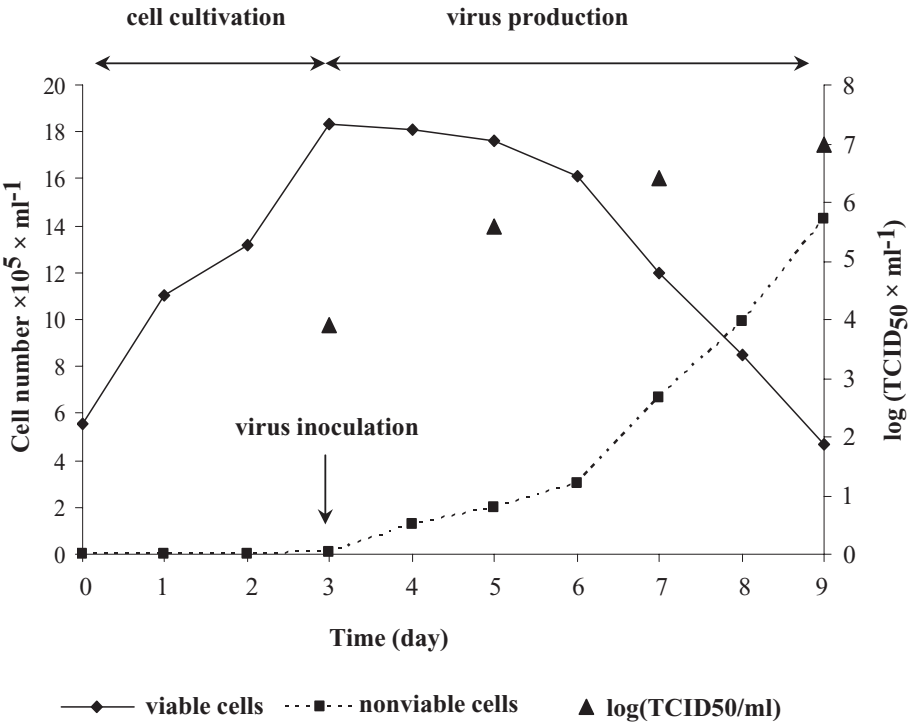

Figure 4. Growth of BHK 21 C13 cells in the wave bioreactor and evolution of viable/nonviable BHK 21 C13 cells during AD virus production.

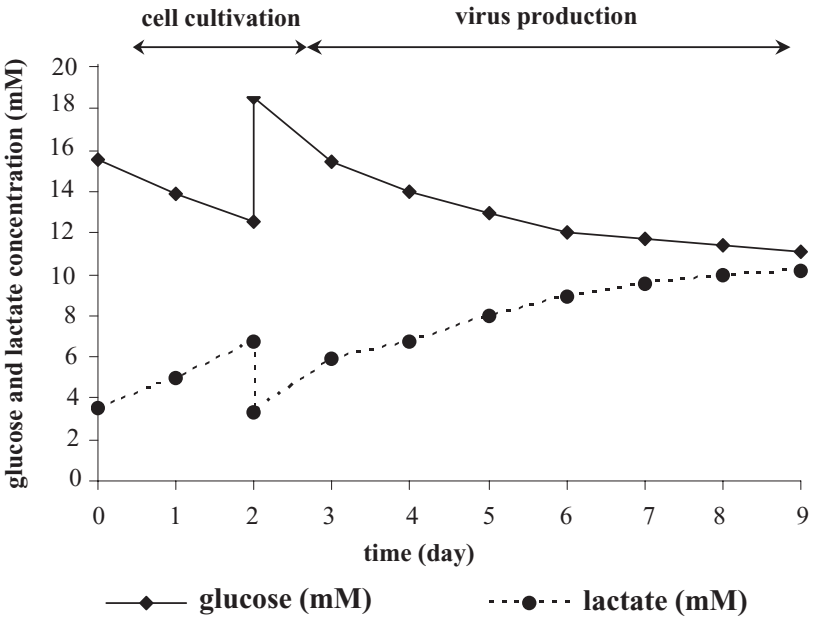

Figure 5. Glucose consumption and lactate production during BHK 21 C13 cell growth and ADV production in the wave bioreactor.

gE$^-$ Bartha K-61 strain virus suspension ($10^{5.9}$ $TCID_{50}$) with multiplicity of infection (MOI) of 0.01 and continued to incubate in the wave bioreactor system (figure 4). This titre of the seed virus was chosen according to our previous experiments of AD virus production using multiple harvest process (Gaurina Srček *et al* 2004) when the main intention was to keep the cells alive for longer period in order to achieve higher virus production. On the first day post-infection there was 99% of viable cells. During next 6 days the cell number decreased slowly from 90% on the second day post-infection to 62% on the fourth day post-infection and to 25% at the end of the process. The virus titre expressed in $TCID_{50}$ ml^{-1} on the second day post-infection was $10^{5.6}$ and on the fourth day post infection was $10^{6.4}$ (figure 4). The virus harvest obtained on the sixth day post-infection yielded 400 ml and virus titre calculated using the Spearman-Kärber formula was $10^{7.0}$ $TCID_{50}$ ml^{-1}. Since one-AD vaccine dose requires $10^{5\pm0.5}$ $TCID_{50}$ (Veterina Ltd, Kalinovica, Croatia, *Technical Documentation*), 40,000 vaccine doses could be prepared from the crude harvest during this experiment.

The Aujeszky vaccine is routinely produced in 150 l fermentors by Smith Kline-RIT (Baijot *et al* 1987) and 2x better results in the antigen units were obtained when microcarrier cultures were performed in comparison to stationary cultures. This difference is explained by the difference in cell number, better regulation of pH and better oxygen supply,. The new technology of mixing, and therefore better oxygen supply, could also be a reason for higher virus titre obtained in our experiment. Comparison of oxygen-transfer data obtained in spinner flasks and wave bioreactor system is reported by Singh (1999). According to this data, optimization of rocking mechanism provided k_La for oxygen transfer of 2–4 h^{-1}, which is sufficient to grow up to 7 x 10^6 cells ml^{-1}. Also, the adenovirus production by human embryonic kidney cell line 293 showed that the system is capable to maintain adequate dissolved oxygen levels at achieved cell densities of 2.7 x 10^6 cells ml^{-1}. At the end of the ADV production (figure 5) the glucose concentration was 11.1 mM and the lactate concentration was 10.2 mM, which was still under inhibitory levels reported for stirred BHK 21A culture (Cruz *et al* 2000).

The results of this study showed that BHK 21 C13 cells could be cultivated successfully in the wave bioreactor in order to produce ADV with obtained virus titre of $10^{7.0}$ $TCID_{50}$ ml^{-1} and 40,000 doses of AD vaccine. The ADV production was carried out with relatively low titre of the seed virus which was the reason for 6 days infection cycle and 25% of viable cells at the end of process. Our further experiments with ADV production are oriented to obtaining a higher virus titre and cell concentrations. Therefore, this will reduce the time and costs of production, which will lead to further improvements of the whole process.

In conclusion, BHK 21 C13 cells could be cultivated successfully in the wave bioreactor to cell density of 1.82 x 10^5 cells ml^{-1} and viability of 99%. The ADV titre obtained during this process was higher than in previously published

data with simple harvest process using spinner technology. Therefore, this type of bioreactor can be recommended for routinely AD and other virus productions, particularly considering the fact that its scalability of 1:10 makes it a favourable bioreactor with several advantages.

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