

# Primjena životinjskih stanica u proizvodnji cjepiva protiv bolesti Aujeszokoga

Igor Slivac<sup>1</sup>, Višnja Gaurina Srček<sup>1</sup>, Kristina Radošević<sup>1</sup>, Ivana Kmetić<sup>2</sup>, Zlatko Kniewald<sup>1,3,\*</sup>

<sup>1</sup> Laboratory for Cell Culture Technology and Biotransformations

<sup>2</sup> Laboratory for Toxicology, Faculty of Food Technology and Biotechnology, University of Zagreb, 6 Pierotti St., HR-10000 Zagreb, Croatia

<sup>3</sup> član emeritus HATZ-a u Odjelu bioprocenog inženjerstva

\* Corresponding author (Fax, 385-1-46 05 065; Email, [zlatko.kniewald@pbf.hr](mailto:zlatko.kniewald@pbf.hr))

Za Godišnjak HATZ 2018 INOVACIJA, a na temu „Inovacije i patenti članova Akademije tehničkih znanosti Hrvatske“, prilog u nastavku pri uređivanju negdje je zagubljen.

Sada predlažem Uredništvu HATZ da ga sada uključi u Godišnjak 2020. zajedno sa znanstvenim radom našeg tima koji je objavljen 2006. godine i rezultat je navedenoga istraživanja. Baza podataka ResearchGate (RG) prati korištenje znanstvenih radova u proteklom razdoblju. Posebno su zanimljivi podaci našega rada od početka COVID 19 krize i neposredno prije toga. Baza podataka je otvorena i javno dostupna svim zainteresiranima iz cijelog svijeta, a cjelovit rad je dostupan u bazi podataka bez naknade. Rad je u proteklom razdoblju, a posebice od 2018 pregledan 1393 puta i to 230 od članova RG, a 1163 su nečlanovi RG. Citiranost rada 21 puta. Stanje 12.2.2021.

## Razlozi:

1. Predloženi tekst sadrži izvješće o rezultatima dugogodišnjeg istraživačkog projekta u suradnji s PLIVA d.d. i postignutim rezultatima. (Tekst u priritku).

U popisu objavljenih radova iz tog projekta nalazi se i rad:

Slivac I., Gaurina – Srček V., Radošević K., Kmetić I., Kniewald Z. **Aujeszky's disease virus production in disposable bioreaktor. :J. Biosci.(2006) 31 (3) 363-368,**

2. Uvidom u ResearchGate (podaci u privitku od 2004 godine do 2020.) uočen je visoki znanstveni interes za ovaj naš rad do kojega dolazi otvaranjem pandemije COVID 19 i žurne potrebe proizvodnje cjepiva.

**Sažetak:** *Ispitan je novi sustav bioreaktora s reaktorskom posudom za jednokratnu upotrebu (Wave bioreactor). Bioreaktor, koji koristi valovito djelovanje za miješanje i prijenos kisika ispitan je kod umnožavanja mase stanica stanične linije BHK 21 C13 pri proizvodnji virusa Aujeszkijeve bolesti (ADV). Praćena je kinetika rasta BHK 21 C13 stanica u bioreaktoru tijekom trodnevnog razdoblja. Na kraju trodnevnog uzgoja BHK 21 C13 stanica i pri gustoći stanica od  $1,82 \times 10^6$ /stanice  $\text{ml}^{-1}$ , reaktor je inokuliran s 9 ml  $gE^-$  Bartha K-61 soj ADV suspenzija ( $10^{5.9}$  TCID<sub>50</sub>) s indeksom infekcije (MOI) od 0,01. Poslije inkubacije od 144 h, dobiveno je 400 ml ADV biomase s titrom od  $10^{7.0}$  TCID<sub>50</sub>  $\text{ml}^{-1}$ , što odgovara 40.000 doza cjepiva protiv ADV. Posebna pogodnost ovog postupka je, što se proizvedena biomasa može u istom spremniku uskladištiti do trenutka daljnjeg tehnološkog postupka, i s novim spremnikom nastaviti proizvodnja iste ili druge biomase bez posebnih dugotrajnih postupaka čišćenja i sterilizacije. Ovisno o potrebi, volumen spremnika se može povećavati bez zahtjevnog scale-up postupka.*

**Zaključno:** *Rezultati dobiveni ovim bioreaktorom s BHK 21 C13 stanicama pokazali su da se ovaj sustav može smatrati uspješnim za proizvodnja ADV ili samo proizvodnju BHK 21 C13 biomase stanica.*

*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 Aujeszký'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  $1.82 \times 10^6$  cells  $\text{ml}^{-1}$ , the reactor was inoculated with 9 ml of  $gE^-$  Bartha K-61 strain ADV suspension ( $10^{5.9}$  TCID<sub>50</sub>) 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<sub>50</sub>  $\text{ml}^{-1}$ , 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.*

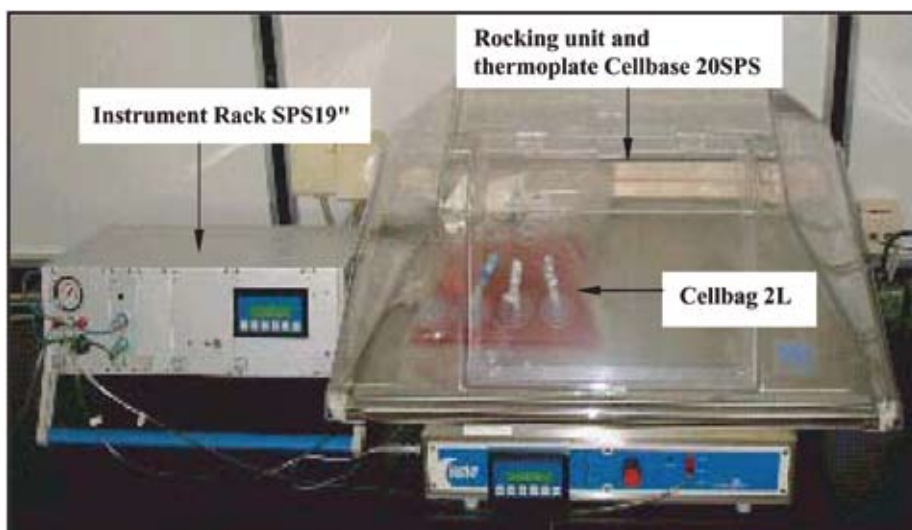
**Keywords:** *Aujeszký's disease virus, baby hamster kidney cells, cell culture, disposable bioreactor, virus titre*

## 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,

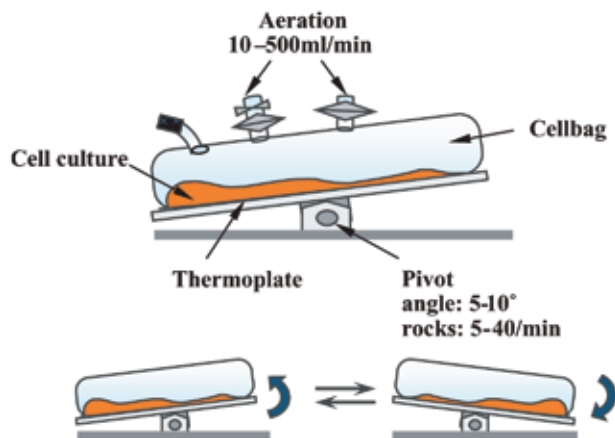
single-use plastic bag for cell cultivation has been introduced. The *Wave Bioreactor 20SPS™* (Wave Biotech AG, Tagelswangen, Switzerland) is a cultivation system developed for semi-industrial cultivation of many different cell types (e.g. hybridomas, mammalian cells, plant cells) (Weber *et al* 2002). 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.



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

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 passed 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 ( $k_L a$ ) 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.



**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.

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 *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<sub>2</sub> at 37°C with initial cell concentration of 5 x 10<sup>5</sup> cells ml<sup>-1</sup> 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  $5.5 \times 10^5$  cells  $\text{ml}^{-1}$ . The cell suspension was then transferred directly into the inflated *Cellbag 2L<sup>TM</sup>* 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  $37^\circ\text{C}$  and at rocking speed of 10 rpm with tilt angle of  $6^\circ$ . Before the cell inoculation, the bag was rigidly inflated with atmosphere consisted of 95% of air + 5% of  $\text{CO}_2$  using gas mixing module *Gasmix 20SPS<sup>TM</sup>*. 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  $0.2 \text{ l min}^{-1}$  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 trypanblue 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  $\mu$  ( $\text{h}^{-1}$ ) was estimated by the following equation:  $\mu = (\ln X_n - \ln X_{n-1}) / (t_n - t_{n-1})$ , 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 enzymatically. 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<sup>-</sup> 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<sup>-</sup> Bartha K-61 strain of AD virus at cell concentration of  $1.82 \times 10^6$  cells ml<sup>-1</sup> and inoculation volume of 2% (v/v) of the culture volume. The virus production was performed at 37°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<sub>50</sub> ml<sup>-1</sup>) 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  $5.2 \times 10^5$  cells ml<sup>-1</sup> and it reached its maximum ( $19 \times 10^5$  cells ml<sup>-1</sup>) at the fourth day of the cultivation period by an apparent beginning of plateau phase. The maximum growth rate was 0.0285 h<sup>-1</sup>. 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  $5.5 \times 10^5$  cell ml<sup>-1</sup> 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  $13.3 \times 10^5$  cells ml<sup>-1</sup>, 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  $1.82 \times 10^5$  cells ml<sup>-1</sup> and the cell viability was equal to 99%. The specific growth rate was ranged from 0.008 to 0.028 h<sup>-1</sup> (mean 0.016 h<sup>-1</sup>). 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.

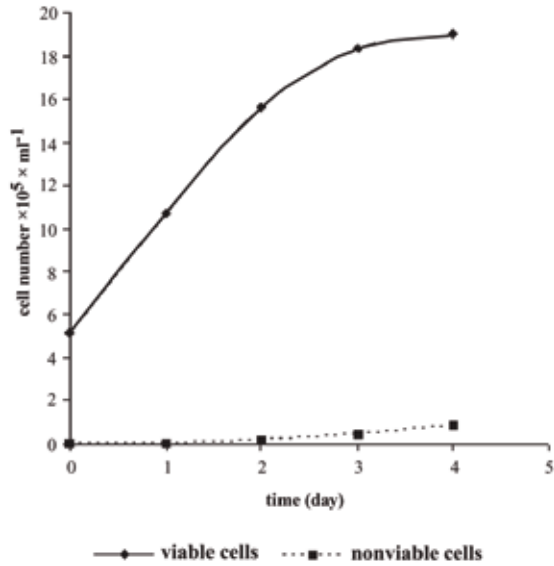


Figure 3. Typical growth curve of BHK 21 C13 cells cultivated in batch mode in the wave bioreactor.

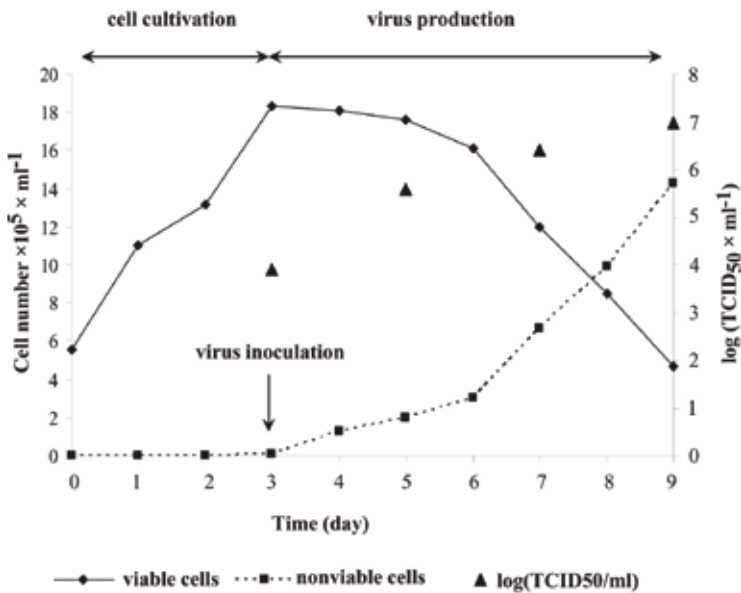
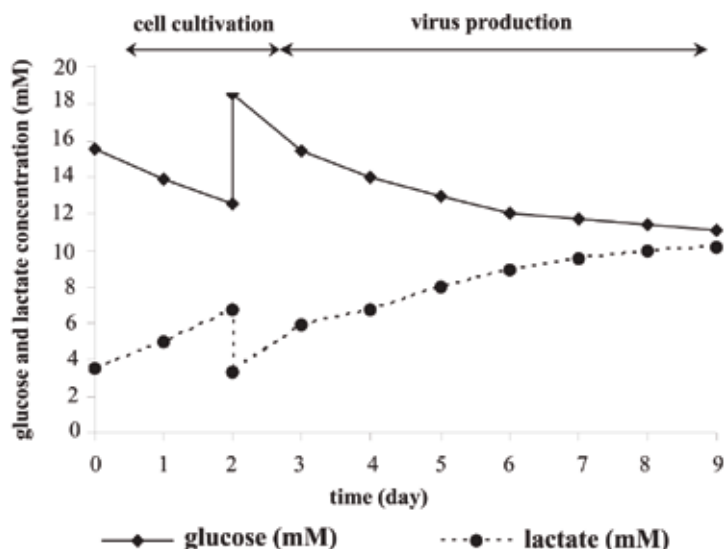


Figure 4. Growth of BHK 21 C13 cells in the wave bioreactor and evolution of viable/nonviable BHK 21 C13 cells during AD virus production.



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

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  $1.82 \times 10^5$  cells  $\text{ml}^{-1}$ , the cells were infected with 9 ml of gE<sup>-</sup> Bartha K-61 strain virus suspension ( $10^{5.9}$  TCID<sub>50</sub>) 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<sub>50</sub>  $\text{ml}^{-1}$  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<sub>50</sub>  $\text{ml}^{-1}$ . Since one-AD vaccine dose requires  $10^{5\pm 0.5}$  TCID<sub>50</sub> (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 (Bajjot *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 oxygentransfer 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<sup>-1</sup>, which is sufficient to grow up to 7 x 10<sup>6</sup> cells ml<sup>-1</sup>. 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<sup>6</sup> cells ml<sup>-1</sup>. 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<sup>7.0</sup> TCID<sub>50</sub> ml<sup>-1</sup> 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<sup>8</sup> cells ml<sup>-1</sup> 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.

### Acknowledgements

This work is funded by the Ministry of Science, Education and Sports of the Republic of Croatia (Grants No. 0058001, 0058010 and TP 05803). We wish to thank Veterina Ltd., Kalinovica, Croatia for their technical support in virus titre assay.

## References

- Baijot B, Duchene M and Stephenne J 1987 Production of Aujeszky vaccine by the microcarrier technology "From the ampoule to the 500 litre fermentor"; *Dev. Biol. Stand.* 66 523–530
- Cruz H J, Freitas C M, Alves P M, Moreira J L and Carrondo M J T 2000 Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells; *Enz. Microb. Technol.* 27 43–52
- Gaurina Srček V, Čajavec S, Sladić D and Kniewald Z 2004 BHK 21 C13 cells for Aujeszky's disease virus production using the multiple harvest process; *Cytotechnology* 45 101–106
- Lojkić M, Markuš-Cizelj Lj, Čajavec S, Zidar V and Gojšić S 1992 Investigation on safety of attenuated Aujeszky's disease vaccine (Ay-Vak Bartha-6) in piglets and sows; *Prax. Vet.* 40 315–322
- Namdev P K and Lio P 2000 Assessing a disposable bioreactor for attachment-dependent cell cultures; *Bio. Pharm.* (Feb) 1–5
- Puentes E, Eiras A, Cancio E, Nores M V, Aquilera A, Seoane R and Regueiro B J 1993 Comparison of the protective efficacy of Aujeszky's disease (pseudorabies) virus glycoproteins obtained from different sources; *Vet. Microbiol.* 35 45–59
- Singh V 1999 Disposable bioreactor for cell culture using waveinduced agitation; *Cytotechnology* 30 149–158
- Weber W, Weber E, Giesse S and Memmert K 2002 Optimisation of protein expression and establishment of the Wave Bioreactor for Baculovirus/insect cell culture; *Cytotechnology* 38 77–85

## Razlozi objavljivanja

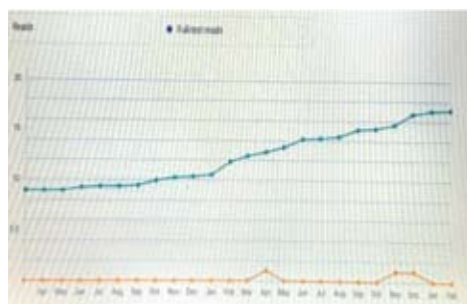
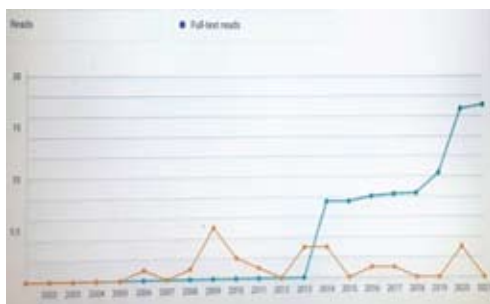
Ostvarena suradnja s PLIVA d.d. započela je tijekom rasprava koje su se vodile na skupu organiziranom od strane Prehrambeno biotehnološkog fakulteta 1992. godine pod naslovom "Prehrambeno-biotehnološki fakultet na raskrižju Sveučilišta i gospodarstva", Zagreb. Stanje gospodarstva bilo je nezavidno, tehnologija zastarjela. Ministarstvo znanosti obrazovanja i športa usvojilo je program potpore tehnološkim projektima, a PLIVA d.d. imala je snažan interes istraživanja za unapređenje i uvođenje novih tehnologija proizvodnje cjepiva. I upravo takvo okruženje u trenutku osnivanja Hrvatske akademije tehničkih znanosti 1993. godine (danas Akademija tehničkih znanosti Hrvatske HATZ) i osnivanje Biotehničkog centra HATZ stvorilo je zajedno sa znanstvenim novacima sufinanciranim od MZOŠ preduvjete za razvoj Tehnologije životinjskih i biljnih stanica na PBF-u. Istraživanje i razvoj se isključivo ostvario u novoosnovanom Laboratoriju za tehnologiju životinjskih i biljnih stanica, Zavoda za biokemijsko inženjerstvo PBF-a. U ovom prilogu prikazano je jedno razoblje istraživanja koje danas pokazuje svu svoju vrijednost, ali i žalosnu istinu da nam je taj dio gospodarstva bitno izmjenjen pa su i daljnja istraživanja u tom pravcu zaustavljena i tako rezultati postali dostupni drugima. Pandemija COVID19 započela je svoje širenje početkom 2019.godine i kasnije zahvatila cijeli svijet. Tehnologija proizvodnje cjepiva danas je primjenom tehnologije stanica mnogo jednostavnija i brža, kao i postupci proizvodnje konačnog proizvoda. Ipak da bi se proizvelo

cjepivo za humanu primjenu potrebno je provesti isti put predkliničkih i kliničkih istraživanja kao i za svaki drugi lijek. Taj postupak je dugotrajan što je i u ovom slučaju stvorilo gužvu i nervozu na svjetskom tržištu u želji da se bude prvi. U druge moguće razloge ovdje ne bih ulazio.

Naša objavljena istraživanja ukazuju na mogućnost znatno brže, jednostavnije i jeftinije proizvodnje cjepiva. Koji proizvođač je do sada primjenio ovu tehnologiju ili će ju primjeniti nikada nećemo znati, ali uočen je veliki interes za objavljeni rad. To ukazuje da nije bitan naziv institucije, znanstveni položaj sveučilišta u svijetu ili istraživački tim, pa čak niti časopis u kojem je rad objavljen, već kvaliteta objavljenih rezultata već dostupnost rezultata u međunarodnim znanstvenim časopisima i potreba svjetskog gospodarstva koje ovim putem dolazi do visokovrijednih informacija bez vlastitih ulaganja. Koliko puta smo u proteklom razdoblju poklonili naše rezultate u utrci za CC radovima i potpunom pomanjkanju želje da se patentima zaštiti naše intelektualno vlasništvo.

U nastavku su prikazani podaci za navedeni rad preuzeti iz RG 12. 2. 2021:

Učestalost pregleda rada po  
godinama 2006 - 2021                      mjesecima 04. 2018. – 02. 2021.



Iz godišnjeg pregleda vidljivo je da je interes za rad započeo 2014. godine, a naročito se intenzivirao 2019 – 2021. Prema mjesecima je također vidljiv interes koji je u skladu s pojavom virusa i to je veljača 2020.