

Cultivation and comparison of BHK-21 anchorage semi-dependent cell line in different production systems

[Yarı yüzey bağımlı BHK-21 hücrelerinin farklı üretim sistemlerindeki veriminin karşılaştırılması]

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ABSTRACT

Aim: The investigation of the different culture systems in order to get high yield of baby hamster kidney (BHK) cell line.

Materials and Methods: Different cultivation systems, vibrofermentor and 5-L stirred tank bioreactors, have been used for the production of BHK cell line. The production systems were evaluated with respect to pH, temperature, viable cell number, aeration rate and stirring speed.

Results: BHK cells were successfully cultivated in vibrofermentor and stirred tank bioreactor (STR) systems. In addition, higher cell concentration was obtained with low aeration rate (0.005 vvm) comparing to those of 0.02 and 0.05 vvm. The cultivation in STR can be operated with low aeration rate due to decrease the expenditure and improve the cell productivity.

Conclusion: In order to improve the cell yield of the anchorage semi-dependent cell line in pilot scale, it should be preferred that the cells are firstly adapted to suspension culture by vibrational stirring and then be cultivated in stirred tank bioreactor which is operated at low aeration rate. These results showed consistency with the previous studies.

Key Words: BHK-21, stirred tank bioreactor, vibrofermentor

Conflict of Interest: Authors have no conflict of interests.

ÖZET

Amaç: Bebek hamster böbrek (BHK) hücre hattında, hücre veriminin artırılması ve pilot ölçekte elde edilmesi için üretim sistemlerinin incelenmesi.

Gereç ve Yöntem: Bu çalışmada, BHK hücre hattının üretimi vibrofermentör ve 5 L karıştırmalı tank biyoreaktör kültür sistemleri kullanılarak gerçekleştirildi. Üretim sistemlerinin; pH, sıcaklık, canlı hücre derişimi, havalandırma ve karıştırma hızı gibi parametreleri değerlendirildi.

Bulgular: BHK hücrelerinin, hem vibrofermentörde hem de karıştırmalı tank biyoreaktörde başarılı bir şekilde üretilebildiği görüldü. Ayrıca, 0.02 ve 0.05 vvm havalandırma hızlarında elde edilenlere göre düşük havalandırma hızında (0.005 vvm) daha yüksek hücre konsantrasyonlarına ulaşıldı. Düşük havalandırma hızlarında çalışan karıştırmalı tank biyoreaktörün hem maliyet hem de hücre verimliliği açısından daha uygun olduğu belirlendi.

Sonuçlar: Yarı-yüzey bağımlı BHK hücrelerinin pilot ölçekli üretiminde yüksek verim elde edebilmek için, öncelikle vibrasyon türü karıştırma ile hücrelerin süspansiyon kültüre adaptasyonu ve ardından düşük havalandırma hızlarında çalıştırılan karıştırmalı tank biyoreaktörlerin kullanılması tercih edilebilir. Elde edilen bu sonuçlar daha önceki çalışmalar ile paralellik göstermektedir.

Anahtar Kelimeler: BHK-21, karıştırmalı tank biyoreaktör, vibrofermentör.

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Industrially well-known expressions systems have become progressively more popular for the production of biopharmaceuticals, using body-forming *Escherichia coli* strains, the yeast *Saccharomyces cerevisiae* and mammalian cells like Chinese hamster ovary cells (CHO) and baby hamster kidney (BHK) cells [1]. Animal tissue and/or cell culture has important implications in biotechnology in the production of vaccines, pharmaceuticals and antibodies (used for diagnosis and treatment) [1-3]. Moreover the culture of some animal cells can produce a correctly processed protein that can be secreted into the culture medium, which greatly facilitates the purification process [4].

With the intension of animal cell culture based pharmaceuticals, the production scale changes from small multiple-unit reactors to 10,000 L single-unit batch reactors. Highly valuable and small demanded products can be produced in small scales however; some “bulk” products for immunization and treatment may possibly require large-scale bioreactors for their cost-efficient manufacturing [2,5-7].

On the industrial scale the adherent cell lines can be cultivated on micro-carriers (*e.g.*, for vaccine production) or adapted to grow in suspension, as in the case of cell lines derived from BHK or CHO [4]. Adaptation ability of many cell types to suspension culture and make use of polymeric additives to decrease shear stress on the cells, have facilitated the application of the suspension cell culture systems. For large scale applications, suspension cell-culture processes are preferred. Moreover, this system provides easier scale-up and process control in homogeneous systems [3].

BHK-21 cells were used in viral studies started in 1960s [8]. Capstick *et al.* [9,10] developed suspension cultures of BHK-21 cells and used them for the propagation of foot and mouth disease virus. Radlett *et al.* [11] greatly improved the growth conditions of BHK-21 cells in suspension culture demonstrating the usefulness of BHK-21 cells for the commercial production of viral vaccines for animals. After that, cell lines have been used for virus detection and production of virus and recombinant proteins in many laboratories throughout the world [12-16]. Cultivation of the BHK cells in submerged systems similar to microorganisms may well be considered an advance for industrial use of animal cells. This BHK cell line has been merely used in veterinary vaccine productions for a long time owing to safety requirements. The foot and mouth-disease vaccine virus was firstly produced in a 1,000 L agitated reactor by using suspended BHK 21 cells and production scaled-up to 10,000 L reactors [5, 17].

Maintaining healthy cell culture and achieving higher viable cell concentration are fundamental factors to acquire high product quality and productivity for the biopharmaceutical manufacturing, consequently many factors should be optimized for the employed production system [3, 5]. In this study, our main focus is to improve BHK An₃₀ cell growth using a 5 L STR. An effort has been made by adapting the BHK An₃₀ anchorage semi-dependent cells to suspended cells in vibrofermentor system and then the cells have been transferred to 5 L STR. Therefore, the possibilities of improving cell growth in an aerated STR have been optimized. The results could be also used for further studies such as monoclonal antibody and recombinant protein production.

Material and Methods

Cell line

Baby hamster kidney (BHK) cells [BHK-21 Strain 35 (IZS, Italy)] were obtained from HUKUK Animal Cell Culture Collection, FMD Institute, Ankara, Turkey. BHK An₃₀ cells were cultivated in growth medium, GMEM (Biochrom, Germany) which was supplemented with 10% (v/v) adult bovine serum (Sigma, Germany), 10% (v/v) tryptose phosphate broth (Applichem, Germany).

Cell growth systems

BHK-21 cells were initially cultivated in T-flasks and then scaled up to 850 cm² polystyrene roller bottles (Greiner Bio-one, Germany) by using 1 rpm stirring speed at 37°C in a humidified atmosphere with 5% CO₂. The initial concentration of the cells was 1×10⁴ cells/cm². In order to adapt the cells in suspension culture, a vibrofermentor (Chemap AG, Switzerland) system was used. The vibrofermentor is a container of various capacities generally spherical or with a hemispherical bottom, equipped with a vibromixer [18]. The experiments were carried out in a water jacketed glassware vessel with a 2 L working volume. Agitation was provided by a vibromixer operating at 180 volts. pH was controlled by aeration rate which was supplied through a diaphragm pump in such a way not to damage the cells. After the 25 days of cultivation in vibrofermentor, the cells were transferred to 5 L STR (Sartorius Biostat B plus, Germany) with a 2 L of working volume. Aeration was supplied with a microsparger (insertion depth 332 mm; hose connector for tubings Di=3.2 mm; pores=20 µm) and different aeration rates *i.e.*, 0.005 vvm, 0.05 vvm, 0.02 vvm, were used at 200 rpm stirring level without controlling the dissolved oxygen throughout the cultivation period. In the STR, in order to prevent bubble forming which may cause cell damage, an antifoaming agent (Antifoam

Sigma, Germany) was used at 0.05% (v/v) level [19]. pH of the medium (7.2 ± 0.2) was controlled by bicarbonate buffer and CO_2 . Culture temperature was maintained at $37^\circ\text{C} \pm 0.1$ by temperature control system. In every 48-72 h, the cells were split and started again with the initial concentrations of 4×10^5 cells/ml in STR.

Morphological studies

The morphology of the cells was regularly detected under inverted light microscope (Olympus, Japan) throughout the work.

Cell Viability

Viable cell concentrations were determined by using the trypan blue dye exclusion method with haemocytometer (Brand, Wertheim, Germany).

Evaluation of oxygen consumption of BHK cells

The dissolved oxygen concentration was measured with a polarographic oxygen electrode (Oxyferm FDA 325, Hamilton, Switzerland). As it is known the oxygen uptake rate (OUR) or the specific oxygen consumption rate (q) is influenced by the cell type, cell density, proliferative state of the culture, and glucose and glutamine concentration. The OUR is a good indicator of cellular activity; under some conditions, it is even a good indicator of the number of viable cells [4,20]. All animal cell cultures are aerobic processes in which the oxygen requirement varies depending on the cell lines used. In the present study, the OUR and the volumetric mass transfer coefficient ($k_L a$) in STR were experimentally determined using dynamic method

by following the change of oxygen concentration when the air was first cut off and then re-passed through the reactor as formerly described by Ruffieux et al. [21].

Data Analysis

The results were evaluated by means of the analysis of variance (one-way ANOVA) at $\pm 95\%$ confidence intervals ($P < 0.05$). This analysis was performed with Minitab® software (Version 16).

Results and Discussion

BHK An₃₀ cells cultivated in roller bottles were used as inoculant for vibrofermentor systems. It has been reported that BHK 21 Strain 35 shows consistent characteristics achieving high cell densities (*i.e.*, 161×10^4 cells/cm²) among short giving cultures with a mixed adherent and suspension morphology [16]. Consequently, in order to adapt monolayer BHK cells to suspension culture, they were cultivated in vibrofermentor. It has been reported that the cells should previously be adapted to suspension culture, which provides some advantages in stirred tank bioreactors which are mostly preferred for pilot and large scale production of pharmaceuticals [22]. Submerged cultures have some benefits such as providing easier manipulation during the incubation period and since it does not require any immobilization agent as in the case of using microcarriers, the manufacturing cost is reasonably lower than the other systems [3,20].

Table 1 depicts the results obtained with 2 L capacity of vibrofermentor containing different working volumes which were changed due to initial cell concentration. Amplitude at 180 volts is suggested as the most

Table 1 Cultivation of BHK An₃₀ cells in vibrofermentor: Cell viability monitoring and working conditions.

| Cultivation time (day) | Passage number | Growth ratio (C_t/C_0) | pH | Working Volume (L) | Amplitude (Volt) |
|------------------------|----------------|----------------------------|-----|--------------------|------------------|
| 0 | M31 S1 | - | - | 1 | 180 |
| 2 | S2 | 3.04 | 7.1 | 2 | 180 |
| 4 | S3 | 7 | 7.0 | 2 | 180 |
| 7 | S4 | 6.8 | 7.0 | 2 | 180 |
| 9 | S5 | 5.75 | 7.2 | 2 | 180 |
| 11 | S6 | 5.5 | 7.2 | 2 | 180 |
| 14 | S7 | 6 | 7.2 | 2 | 180 |
| 16 | S8 | 3.16 | 7.2 | 1 | 180 |
| 18 | S9 | 3.5 | 7.2 | 2 | 180 |
| 21 | S10 | 3.8 | 7.0 | 1 | 180 |
| 23 | S11 | 4 | 7.0 | 1 | 180 |
| 25 | S12 | 4.25 | 6.9 | 1.5 | 180 |

M: monolayer culture passage number; S: Suspension culture passage number; C_t : viable cell concentration before subcultivation (cells/ml); C_0 : Initial viable cell concentration (cells/ml).

convenient agitation for 2 L of culture volume [18]. With this amplitude it poses a good homogenization in the vibrofermentor without any cell damage. Growth ratio progressively increases with each passage from 3 times to around 5 times. pH values changed with a range of 6.9 to 7.2 which was convenient for cell growth. As can be seen from the table, the growth ratio increases during the first 5 days of the cultivation and then decreases gradually since BHK cells are still anchorage semi-dependent in the case of vibromixing. When viable cell concentration reached 2×10^6 cells/ml with a homogeneous spherical morphology, BHK An₃₀ cells were considered as adapted to suspension culture. This result showed well correlation with the previous study [18].

The results obtained from the cultivation of BHK An₃₀ cells in STR with 0.005 vvm showed that, the bubble formation appeared on the top of the liquid surface due to the microsparger usage with low aeration rate in 5 L stirred tank bioreactor. Fig. 1a and Fig. 1b point out that the cell concentration increases up to 1.74×10^6 cells/ml after subcultivation from the initial cell concentration of 4×10^5 cells/ml on the fifth day of the cultivation. Dissolved oxygen (pO_2) concentration showed consistency depending on cell growth and the medium replacements.

Cell clumps appeared during the cultivation of BHK An₃₀ cells in STR with high aeration rate (0.05 vvm) (Fig. 2a; Fig. 2b) showing less increase in cell concentration (Fig 1c). On the other hand, dissolved oxygen concentration

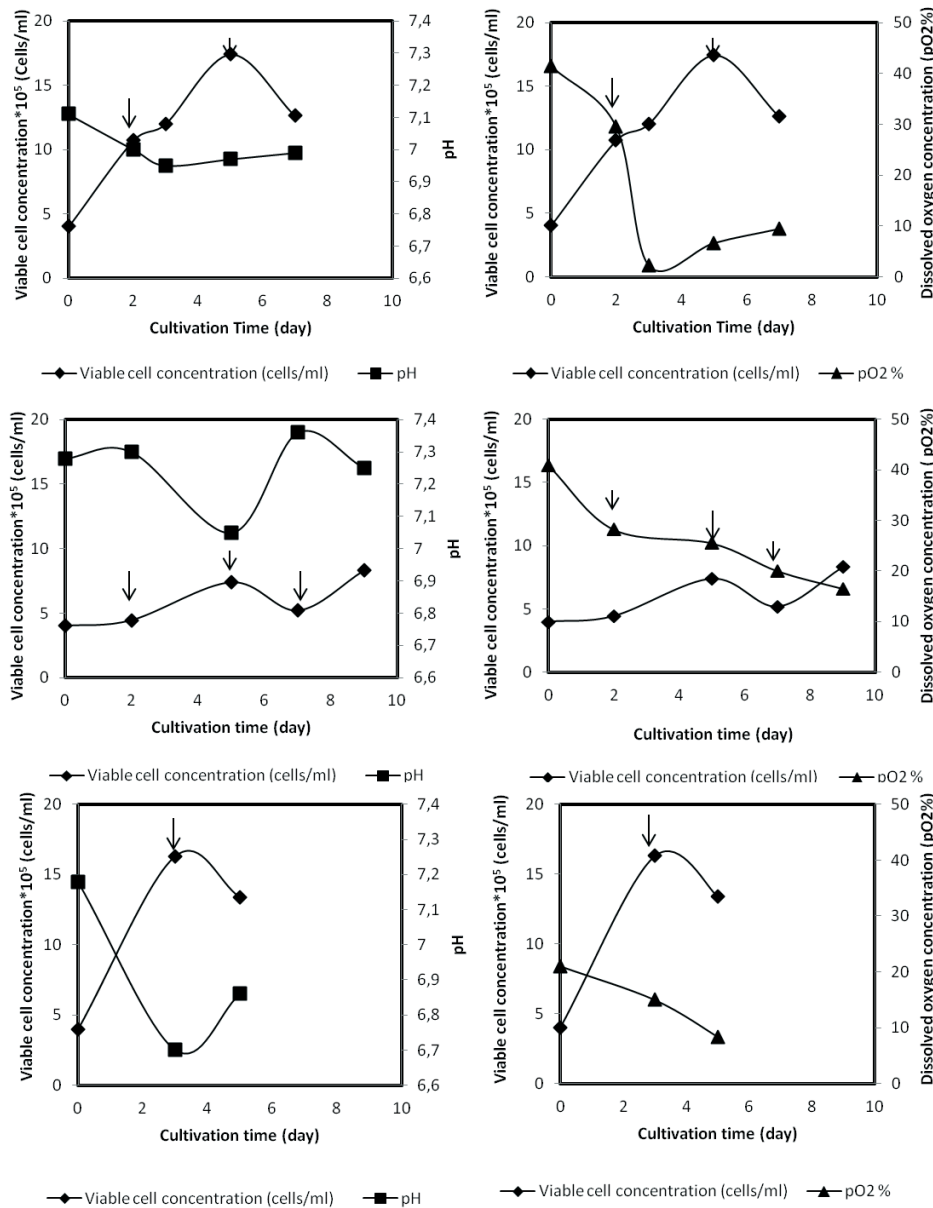


Figure 1 Cultivation of BHK An₃₀ cells in stirred tank bioreactor with various aeration rates (a-b) 0.005 vvm; (c-d) 0.05 vvm; (e-f) 0.02 vvm. Arrows show the days of subcultivation.

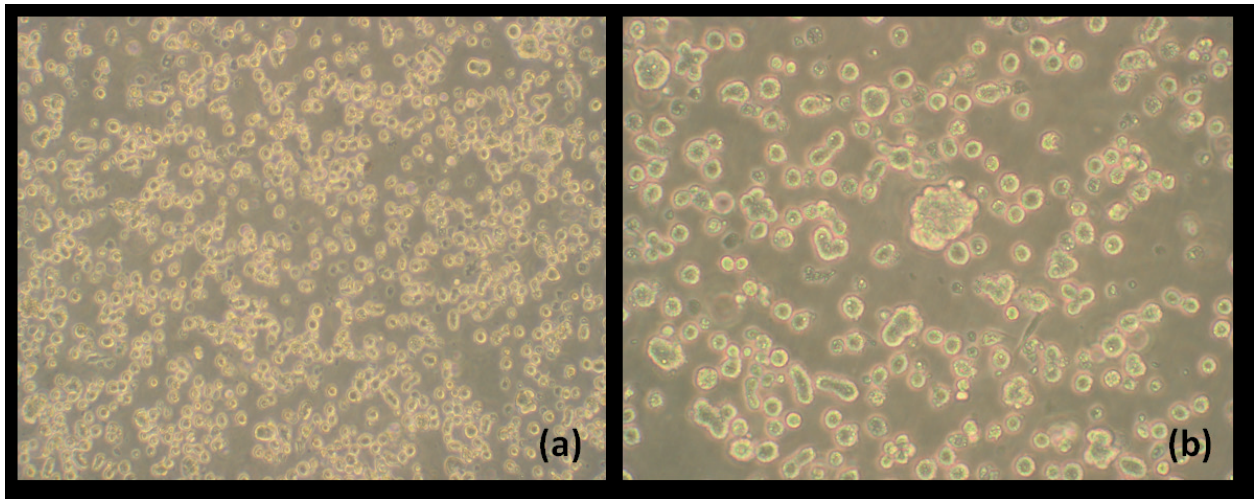


Figure 2 Cell morphology of BHK An₃₀ cells under inverted microscope during the cultivation with high aeration rate: (a) Cell morphology (magnification 10X) and (b) cell aggregates formation in the medium (magnification 20X) (Olympus, Japan).

in the medium decreased gradually and almost remained stable in the medium due to the low growth rate of the cells (Fig. 1d). pH values showed reliability with the cell concentration. In 0.05 vvm aeration, $pO_2\%$ values were higher during the cultivation comparing to the other aeration rates. However, the pH values were higher than the values obtained from the production with 0.005 vvm and 0.02 vvm as a consequence of the lower viable cell concentration. The reasons of low cell concentration might be attributed to the shear stress on the cells and high aeration which caused cell aggregations and therefore prevented the effective mass transfer in the medium [23-26].

Similarly, cell aggregates formed during the cultivation of BHK An₃₀ cells in stirred tank bioreactor with the aeration rate of 0.02 vvm however higher cell concentration was observed (Fig. 1e; Fig. 1f) comparing to that of 0.05 vvm. On the other hand, dissolved oxygen concentration in medium decreased concurrently along with the cell growth. Besides, as anticipated, pH values showed consistency with the cell concentration. Since the rapid cell death occurred after the third day of the cultivation, the process was stopped on the fifth day of the cultivation.

Different ways of agitation have been introduced in the literature for animal cell culture [6,23]. In this study, vibromixing and stirring were used to provide homogenization in the reactor and to improve the mass transfer in the medium. The total viable cell concentrations obtained in vibrofermentor and stirred tank bioreactor operated with different aeration rate, are summarized for comparison in Table 2. Statistical analysis of the results revealed that in terms of viable cell concentration there were not any significant differences between the vibrofermentor and STR with 0.005 and 0.02 vvm aeration rates ($p>0.05$). However,

the use of aeration rate with 0.05 vvm in STR showed significant differences comparing to the other operation systems ($p<0.05$). Studies on animal cell damage due to mechanical agitation and sparging aeration have shown that mechanical damage of freely suspended animal cells is related, in many cases, with gas bubbles, especially the bursting bubbles at the air-medium interface [2,24-26]. The maximum cell concentration decreased concurrently with increasing aeration rate (Table 2). Similar observations were also reported by Al-Rubeai *et al.* [27], who recommended that high hydrodynamic stress which is caused by heavy agitation or bubble bursting, may lead to the aggressive and rapid destruction of the cells just after the beginning of cell death.

The determined OUR, specific oxygen uptake rate (qO_2) and $k_1 a$ values for BHK An₃₀ cells are $4.5 \times 10^{-8} \text{ mMO}_2 \text{ h}^{-1}$, $1.13 \times 10^{-10} \text{ mMO}_2 \text{ cell}^{-1} \text{ h}^{-1}$ and $77.2 \times 10^3 \text{ h}^{-1}$, respectively (Fig. 3). The results obtained are in agreement with the previous studies reported [2, 20].

In conclusion, in order to improve the cell yield of the anchorage semi-dependent cell line in pilot scale one can prefer that the cells must first be adapted to suspension culture by vibration stirring and then be cultivated in stirred tank bioreactor which is operated at low aeration rate due to decreasing the expenditure and recovering the cell productivity. However, since the stirred tank bioreactor provides easier process control, the parameters should also be evaluated by controlling the pO_2 level in the bioreactor and also by using a shear protective agent such as Pluronic F68 to reduce bubble cell damage to improve efficiency of the STR.

Conflict of Interest: Authors have no conflict of interests.

Table 2 Comparison of different cultivation systems with respect to the maximum cell concentration reached in this study.

| Culture system | Working volume (L) | Cell harvesting when the cells reached the maximum cell concentration (day) | Maximum cell concentration (cells/ml) |
|-------------------------------------|--------------------|---|---------------------------------------|
| Vibrofermentor | 1.5 | 25* | 2 · 10 ⁶ |
| Stirred tank bioreactor (0.005 vvm) | 2 | 5 | 1.74 · 10 ⁶ |
| Stirred tank bioreactor (0.02 vvm) | 2 | 3 | 1.63 · 10 ⁶ |
| Stirred tank bioreactor (0.05 vvm) | 2 | 9 | 8.33 · 10 ⁵ |

* The time when the cells were fully adapted to suspension culture.

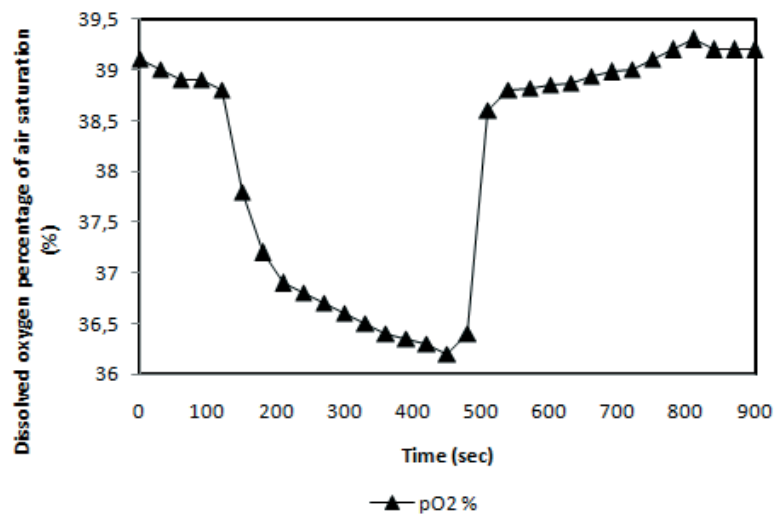


Figure 3 Time course of dissolved oxygen concentration during the measurement of $k_L a$.

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