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OPTIMIZATION OF INITIAL CULTURE STAGE OF VERO AND HEK293 CELL LINES



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Introduction. Laboratory production of viral material in small quantities is performed, as a rule, using adherent cell lines and culture flasks of varying surface area. The need to increase product yield leads to either an increase in the number of flasks or a switch to other accumulation systems, such as roller bottles. One factor influencing the efficiency of cell adhesion and homogeneous monolayer formation is the rotation frequency of the roller bottle. There is a lack of available research data on the impact of rotation frequency on these parameters and determination of its optimal value, particularly based on cellular morphology.

Objective. To optimize the initial stage of roller cultivation for Vero and HEK293 cell lines, taking into account the effect of roller bottle rotation frequency on cell adhesion during seeding and monolayer formation.

Materials and methods. Experiments were conducted using two monolayer cell lines, Vero and HEK293. Seeding concentrations were taken from the cell line passports, amounting to 4×10^4 cells/cm². Each cell line was seeded onto roller bottles and cultured according to the range of rotation frequencies (0.2, 0.3, 0.4, 0.5, and 0.6 rpm) using a Celrol Mid roller (Wiggens) in a RWD D180 CO₂ incubator. Following 1, 2, and 3 days of cultivation, the quality of cell adherence to the growth surface and monolayer formation was assessed by a TC5400 microscope (Meiji Techno).

Results. During cultivation of the Vero cell line, the rotation frequency up to 0.6 rpm did not significantly affect cell adhesion to the surface. The most homogenous cell distribution was observed at rotation frequencies of 0.4–0.5 rpm. The HEK293 cell culture is more sensitive to mechanical disturbances of the nutrient medium; as a result, at rotation frequencies above 0.2 rpm, abnormally rounded cell shapes and impaired adherence to the growth surface were observed. Furthermore, continued cultivation at this rotation frequency did not lead to the formation of a homogenous monolayer due to slow alternation between the respiration and nutrition phases. Consequently, after cell adherence to the surface, the rotation frequency of the roller bottle should be increased.

Conclusions. For the Vero cell line, the optimal rotation frequency was established to be 0.4–0.5 rpm. For the HEK293 cell line, the rotation frequency should be at least 0.2 rpm during the first day followed by its increase to 0.5 rpm after 24 h. The tested cultivation conditions enable an efficient growth of these cell lines for the production of viral biomass.

Keywords: cell cultures; cell cultivation; adherent cultures; Vero cell line; HEK293 cell line; roller bottles; roller cultivation; adhesion; monolayer formation; cell morphology; cell biomass production

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ОПТИМИЗАЦИЯ НАЧАЛЬНОГО ЭТАПА КУЛЬТИВИРОВАНИЯ КЛЕТОЧНЫХ ЛИНИЙ VERO И НЕК293

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Введение. Наработка в лабораториях вирусного материала в малых количествах, как правило, проводится с использованием адгерентных линий и культуральных флаконов различной площади. Необходимость увеличения выхода продукта приводит или к увеличению количества флаконов, или смене системы накопления, например на роллерные бутыли. Одним из факторов, оказывающих влияние на эффективность адгезии клеток и формирование однородного монослоя, является частота вращения роллерной бутыли. При этом отмечено небольшое количество исследований, касающихся оценки влияния частоты вращения и определения ее оптимального показателя, особенно на основе морфологии клеток.

Цель. Оптимизировать начальный этап роллерного культивирования клеточных линий Vero и HEK293 с учетом влияния частоты вращения роллерной бутыли на прикрепление клеток при посеве и формирование монослоя.

Материалы и методы. Для проведения экспериментальной работы были использованы 2 монослойные клеточные линии: Vero и HEK293. Посевные концентрации были взяты из паспортов клеточных линий и составляли 4×10^4 кл/см². Клеточную линию засевали на роллерные бутыли и культивировали согласно диапазону частот вращения 0,2, 0,3, 0,4, 0,5 и 0,6 об/мин с использованием роллерной установки Celrol Mid, Wiggens в CO_2 -инкубаторе D180, RWD. Через 1, 2 и 3 сут культивирования оценивали качество прикрепления клеток к ростовой поверхности и формирование монослоя путем просмотра под микроскопом TC5400, Meiji Techno.

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ОРИГИНАЛЬНАЯ СТАТЬЯ | МОЛЕКУЛЯРНАЯ БИОЛОГИЯ

Результаты. При культивировании клеточной линии Vero частота вращения до 0,6 об/мин не оказывала значительного влияния на адгезию клеток к поверхности. Наиболее равномерное расположение клеток наблюдали при частоте вращения 0,4–0,5 об/мин. Культура клеток НЕК293 более чувствительна к механическим воздействиям питательной среды, и при частоте вращения свыше 0,2 об/мин наблюдали атипично округлые формы клеток и нарушение полноценного их прикрепления к ростовой поверхности. При этом дальнейшее культивирование на данной частоте вращения не приводило к формированию однородного монослоя из-за медленного чередования «фазы дыхания» и «фазы питания». Следовательно, после прикрепления клеток к поверхности необходимо увеличение частоты вращения роллерной бутыли.

Выводы. Для клеточной линии Vero оптимальной частотой вращения является 0,4–0,5 об/мин, для клеточной линии HEK293 в первые сутки необходимо устанавливать 0,2 об/мин, а через сутки увеличить до 0,5 об/мин. Апробированные условия культивирования позволяют выращивать данные клеточные линии для наработки вирусной биомассы.

Ключевые слова: культуры клеток; культивирование клеток; адгерентные культуры; клеточная линия Vero; клеточная линия HEK293; роллерные бутыли; роллерное культивирование; адгезия; формирование монослоя; морфология клеток; наработка клеточной биомассы

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Финансирование: работа выполнена без спонсорской поддержки.

Соответствие принципам этики: исследование не требовало заключения локального биоэтического комитета. В работе использованы клеточные линии НЕК293 и Vero, предоставленные Институтом синтетической биологии и генной инженерии ФГБУ «ЦСП» ФМБА России.

Потенциальный конфликт интересов: авторы заявляют об отсутствии конфликта интересов.

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INTRODUCTION

The majority of modern biotechnological processes rely on the use of adherent cell lines, such as MRC5, Vero, MDCK, and HEK293, as substrates for virus accumulation [1].

The Vero cell line was derived in 1962 from kidney cells of a female African green monkey. This is a continuously cultivable cell line suitable for long-term maintenance without acquiring tumorigenic properties [2]. Due to the low expression of interferon and biosafety, this was the first cell line among those approved by the World Health Organization for use as a substrate for vaccines, continuing to maintain its significance up to the present [3].

The HEK293 cell line represents immortalized human embryonic kidney cells transformed by adenovirus serotype 5 genes. This transformation made HEK293 cells particularly useful as a substrate for accumulating recombinant proteins that require post-translational modifications. The presence of integrated *E1A* and *E1B* genes on chromosome 19 allows for the generation of recombinant adeno-associated particles. Unlike the Vero cell line, HEK293 exhibits genomic instability [4, 5].

Economic and social factors, such as the SARS-CoV-2 pandemic, have stimulated the demand for large volumes of antigen-accumulation substrate biomass and new preventive biopreparations [6, 7].

One of the main challenges in scaling up monolayer cell lines for industrial purposes consists in the need to increase the amount of harvested cell biomass [8]. Four methods for growing large volumes of cell cultures currently exist: static [9], dynamic [10], suspension-based [10, 11], and the use of micro- and macronutrient carriers [10, 12].

In comparison with other cultivation methods, roller (dynamic) cultivation offers a number of advantages:

- economical use of nutrients and reagents with higher titers of virus-containing material per unit volume compared to cultivation in culture flasks and cell factories. The increase in growth surface area by several times is achieved through ribbed vessel walls, while maintaining a compact size of the culture flask and minimal increase in medium volume. The manufacturers recommend filling a roller bottle (1900 cm²) with 300–400 mL of nutrient medium. For comparison, a smooth-wall roller flask of similar volume (area 850 cm²) contains up to 250 mL; therefore, more than doubling the surface area increases the medium volume by less than 1.5 times;
- lower cost of required equipment for biomass production, especially when using roller installations that fit into a standard CO₂ incubator or thermostat, compared to systems employing bioreactors and micronutrients:
- comparable number of operations performed by personnel when working with rollers and when working with culture flasks, yet providing for a larger growth surface area [13].

Although rotation frequency is one of the key factors affecting cell adhesion and monolayer formation [14], studies on this topic are scarce. When developing a methodology for rabies virus cultivation on Vero cell culture using roller bottles, the study [15] indicated an indirect relationship between rotation frequency and the quality of cell adhesion to the growth surface. This work evaluated changes in cell morphology during cultivation stages relying solely on proliferation index measurements. This study provided an assessment of

relationships between rotation frequency and the final cell number and division intensity [15]. For the HEK293 cell line, a specific cultivation protocol was mentioned: starting at 0.25 rpm followed by switching to 1 rpm for transfection with adeno-associated virus. At the same time, the authors offered no justifications for altering the rotation frequency range [16].

It should be noted that information on optimal rotation frequency rates during cultivation, their influence on cell adhesion during initial growth stages, as well as on increasing cell biomass growth and ultimate cell yield, is rather limited.

In this work, our objective was to optimize the initial stage of roller cultivation for Vero and HEK293 cell lines, taking into account the effects of roller bottle rotation frequency on cell adhesion during seeding and monolayer formation.

MATERIALS AND METHODS

In this study, HEK293 and Vero cell lines provided by the Institute of Synthetic Biology and Genetic Engineering of Centre for Strategic Planning of FMBA of Russia were used. Roller bottles with a growth surface area of 2125 cm² (maximum capacity of 2300 mL, diameter of 122 mm, ribbed walls, TC-treated polystyrene produced by Greiner) and culture flasks with an area of 175 cm² (TC-treated polystyrene, ServiceBio, China) were used as vessels for cultivation.

For the HEK293 cell line, Dulbecco's Modified Eagle Medium (ServiceBio, China) was used as the nutrient medium, supplemented with 10 mL of penicillin-streptomycin antibiotic solution (PanEko, Russia) per liter of ready-to-use medium, 1% v/v GlutaMAX solution (Gibco, UK), and 10% fetal bovine serum (Capricorn, UK).

For the Vero cell line, Earle's salt-modified medium (PanEko, Russia) was used, supplemented with 1% gentamicin sulfate (PanEko, Russia), 146 mg glutamine (PanEko, Russia), and 7.5% Embryo Replacement FetalClone III (HyClone, USA).

Initial seeding concentrations for both studied cell lines, determined according to recommended data in the cell culture passports, were identical and comprised 4×10^4 cells/cm² or 2.13×10^5 cells/mL. A cell suspension containing 85 × 10⁶ ± 10% cells was added to each roller bottle. The total volume of complete nutrient medium in the roller bottle was 400 mL (working volume according to manufacturer recommendations). Cultivation was carried out in a CO_a incubator D180 (RWD) using a Centrol Mid roller assembly (Wiggens) at constant rotational speeds of 0.2, 0.3, 0.4, 0.5, and 0.6 rpm. These ranges were selected based on previously conducted studies on cultivating various cell lines in roller bottles [15, 16]. The presence of adherent cells, their morphology, and monolayer formation were assessed using microscopes, models TC5600 and TC5400 (Meiji Techno, Japan) at magnifications of ×10 objective lens and ×10 ocular lens.

The final cell count was determined using a C200FL cell counter and viability analyzer (Life Science, China). Prior to counting, the cells were dissociated with a 0.25% trypsin solution (PanEko, Russia) and resuspended in the appropriate nutrient medium described earlier for each cell culture. A sample of the cell suspension was diluted with 0.4% trypan blue dye (AbiDye, Russia) in a ratio of 1:1. Counting was repeated three times, and the average concentration of viable cells was calculated.

RESULTS AND DISCUSSION

When cultivating the Vero cell line at roller bottle rotation frequencies ranging 0.2–0.5 rpm, on day 1, cell adhesion was observed in all investigated roller bottles, with more than 90% of the seeded cells attached. At a rotation frequency of 0.6 rpm, fewer cells adhered to the plastic growth surface, leading to a lower-quality monolayer characterized by heterogeneity and inability to produce viral material. The quality criteria included the presence of a complete monolayer of cells without pronounced granularity under bright-field microscopy, absence of defects on the monolayer surface, and multi-layer cell growth filling the growth space. This high adhesive capacity of cells is attributed to an elevated integrin expression associated with tissue origin characteristics, since epithelial cells tightly arrange themselves together, forming lavers.

Cells retained their characteristic morphology consistent with the cell line passport image. Although a tendency towards monolayer formation was observed at rotation frequencies of 0.2-0.5 rpm, on days 2 and 3 of cultivation, at a rotation frequency of 0.2 rpm, a notable aggregation of cells appeared on the monolayer surface. At rotation frequencies of 0.3-0.4 rpm, the number of cell aggregates decreased proportionately with an increase in rotation frequency. At 0.5 rpm, we observed the absence of cell aggregates on the surface, which were floating within the medium bulk. This phenomenon suggests that as an increase in rotation frequency (faster alternating cycles of feeding and breathing) creates more favorable conditions for cell growth. However, at rotation rates exceeding 0.5 rpm, the flow of culture medium induced by gravity and roller motion might disturb cell aggregates, causing them to detach from the surface.

For a greater visual clarity, photographs of Vero cells at a roller installation rotation frequency of 0.5 rpm were taken, illustrating the process of monolayer formation during cultivation (Figure 1). The color background differs in the photos due to different settings of the LED light source (built-in filter block) of the microscope used for photography, as well as changes in the color of phenol red in the nutrient medium caused by acid pH.

Figure 1 presents an image of cells 5 h after seeding, depicting their massive adhesion to the plastic growth surface and their typical polygonal morphology.

Figure 2 illustrates the process of monolayer formation during cultivation. We observed gradual filling of the growth space by cells and tightening of intercellular contacts due to limited growth space. The ribbed roller surface caused blurring closer to the edges of the field of view; as a result, capturing the entire developing monolayer in a single frame was not possible.

Following three days of cultivation, a dense monolayer with a confluence greater than 90% was obtained (Fig. 3), with no signs of cell aggregate clusters or degradation of the monolayer observed.

Thus, a roller bottle rotation frequency of 0.4–0.5 rpm ensured normal cell culture growth, since monolayer formation was associated with the least formation of cell aggregates. Cells attached and grew more evenly, and the forming monolayer did not deform. According to the literature data [15], cells indeed adhere better at a rotation frequency of 0.4–0.5 rpm. Therefore, after the onset of monolayer formation, increasing the rotation frequency is unnecessary. When culturing the Vero cell line within a pH range of 7.0–7.4, no significant differences

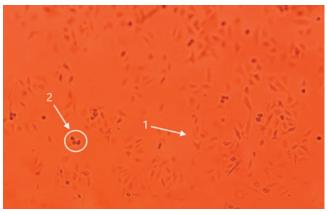


Photo taken by the authors

Fig. 1. Adhesion of Vero cells after 5 h of cultivation at a roller bottle rotation frequency of 0.5 rpm: 1 — cells attached to the surface; 2 — floating cells; magn. ×100

in cell adhesion to the growth surface were observed. In addition, temperature fluctuations within $\pm 2^{\circ}\text{C}$ of the standard temperature (37°C) during cultivation of the Vero cell line had no significant effect on cell adhesion.

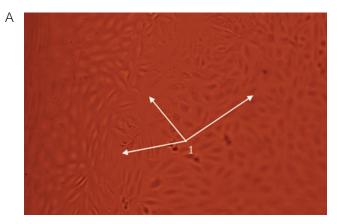
The use of the selected optimal rotation frequency of the roller bottle during cultivation resulted in a cell biomass output reaching $386 \times 10^6 \pm 10\%$ cells per roller bottle.

For the HEK293 cell line, adhesion was observed at rotation frequencies ranging 0.1–0.4 rpm, indicating its lower adhesion capacity compared to the Vero cell line and its greater sensitivity to external mechanical disturbances due to genetic peculiarities and different tissue origins. At a rotation frequency of 0.2 rpm, the cells exhibited typical morphology: a flattened, slightly elongated shape with visible protrusions (Figure 4).

When culturing HEK293 cells for 6 h at a roller bottle rotation frequency of 0.4 rpm, the cells displayed atypical morphology (Figure 5). Despite attaching to the growth surface, their shape remained round, and upon subsequent division, small punctuate cell clusters formed. This phenomenon is presumably linked to strong mechanical impacts exerted by the culture medium, influenced by gravitational forces during roller rotation, which affect the cell membrane and destabilize connections between surface structures and chemical groups of the culture plastic [17, 18].

Meanwhile, when culturing HEK293 cells for 5 h at a roller bottle rotation frequency of 0.5 rpm, less than 50% of the seeded cells adhered, with most retaining a round shape similar to cells grown at rotation frequencies above 0.2 rpm (Figure 6). This is similarly explained by the strong influence of external mechanical forces during roller bottle rotation on cell shape and their ability to adhere to the growth surface. Upon increasing the rotation frequency to 0.6 rpm, no cell adhesion to the surface was observed.

Given that a roller bottle rotation frequency of 0.2 rpm provided the strongest cell adhesion to the growth



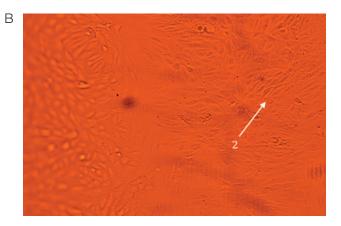


Photo taken by the authors

Fig. 2. Formation of Vero cell line monolayer on day 1 (A) and day 2 (B) of cultivation at a roller bottle rotation frequency of 0.5 rpm: 1 — areas of plastic growth surface; 2 — tight cell-cell contacts; magn. ×100

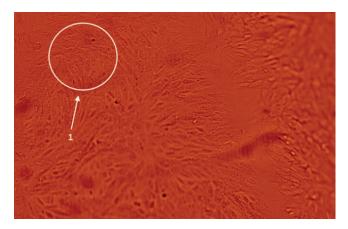


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Fig. 3. Monolayer formed by Vero cell line after three days of cultivation at a roller bottle rotation frequency of 0.5 rpm: 1 — dense cell monolayer; magn. ×100

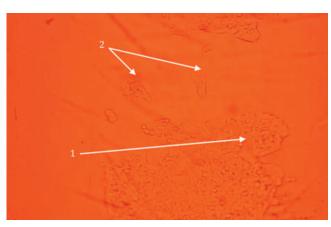


Photo taken by the authors

Fig. 4. Morphology of HEK293 cell line after 8 h of cultivation at a roller bottle rotation frequency of 0.2 rpm: 1 — cells attached to the growth surface; 2 — cell extensions; magn. ×100

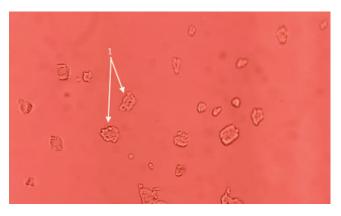


Photo taken by the authors

Fig. 5. Morphology of HEK293 cell line after 6 h of cultivation at a roller bottle rotation frequency of 0.4 rpm: 1 — round clusters of growing cells; magn. ×100

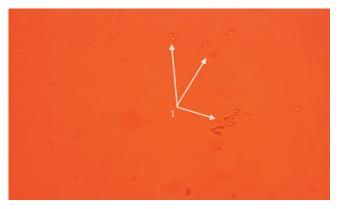


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Fig. 6. Morphology of HEK293 cell line after 5 h of cultivation at a rotation frequency of 0.5 rpm: 1 — attached cells; magn. ×100

surface while preserving cell morphology, monolayer formation was tracked under these conditions.

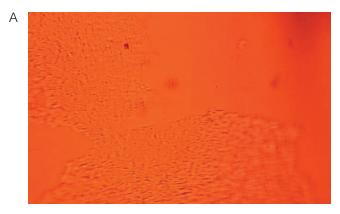
Upon culturing the HEK293 cell line at $37 \pm 2^{\circ}\text{C}$ and a rotation frequency of 0.2 rpm for 1 and 2 days, a tendency toward monolayer formation was observed (Figure 7). Despite active growth processes, a complete monolayer did not form on the remaining surface of the culture plastic; instead, multilayer cell growth occurred.

In the photographs, the color background varies due to changes in the color of phenol red in the nutrient medium when pH becomes more acidic. Since the roller surface was ribbed, causing blurred focus in the vicinity of the field-of-view edges, capturing the entire developing monolayer in a single frame was impossible.

By day 3, at a constant roller rotation frequency of 0.2 rpm, no complete monolayer formation had been

observed; simultaneously, areas not occupied by cell biomass were seen. Individual regions showed foci of multilayer cell growth with sharply demarcated boundaries between the monolayer and the plastic growth surface. Several detached floating cells in the nutrient medium were noted; the corresponding data is presented in Figure 8.

Therefore, after complete cell adherence to the plastic growth surface, the rotation frequency of the roller device was increased to 0.5 rpm to accelerate the transition between the feeding phase and the respiration phase. During slower transitions toward the respiration phase, cells might dry out and die later, leading to defects in the monolayer. In the sites with died cells, the absence of a direct physical contact between the cells leads to reactivated growth and mitotic processes in the cells. The most optimal



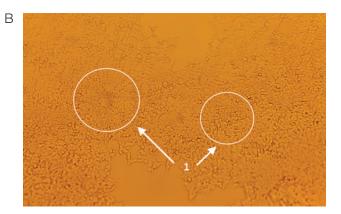


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Fig. 7. Formation of HEK293 cell line monolayer during cultivation on day 1 (A) and day 2 (B) at a rotation frequency of 0.2 rpm: 1 — sections showing a tendency towards multilayer cell culture growth; magn. ×100

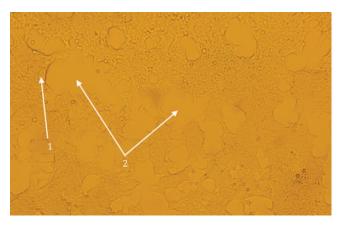


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Fig. 8. Disruption of HEK293 cell line monolayer formation during 3-day cultivation at a roller bottle rotation frequency of 0.2 rpm: 1 — multilayer cell growth; 2 — plastic growth surface sections; magn. ×100

rotation frequency for cell growth was established to be 0.5 rpm.

Additionally, we determined that, on day 2 of cultivation after cell seeding, the optimal increase in rotation frequency should not exceed 0.5 rpm; otherwise, there is an increased risk of deformation of the forming monolayer due to intense mechanical stress arising from the rotation of the roller apparatus and gravitational movement of the nutrient medium. The data obtained suggest that when culturing the HEK293 cell line, the rotation speed of the roller device should be changed to obtain a homogeneous monolayer.

Cultivation under the conditions proposed in this work, i.e., at 37 \pm 2°C during day 1 at a rotation frequency of 0.2 rpm followed by an increase to 0.5 rpm on day 2, yielded a complete, high-quality monolayer (as depicted in Fig. 9). The monolayer consists of cells exhibiting a characteristic flattened shape upon attachment to the growth surface, with

a tendency toward the formation of large cell growth areas.

During the first day of cultivation at a roller bottle rotation frequency of 0.2 rpm, cells adhered to the growth surface, further forming growth areas of the cell culture (Figure 9A). Following the change in rotation frequency to 0.5 rpm, active cell growth was observed across the entire growth surface (Figure 9B) without areas of multilayer cell growth or sharp boundaries between the cell clusters and plastic.

By day 3 of cultivation at a rotation frequency of 0.5 rpm, a dense monolayer had formed, visually homogeneous, without focal points of multilayer cell growth or detached cells from the surface (Figure 10).

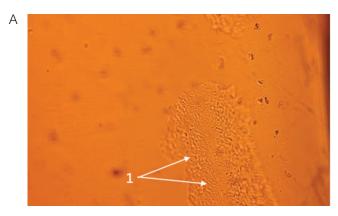
Our analysis of the cultivation conditions for the HEK293 cell line showed that pH values in the range of 7.0–7.4 and temperature variations within $\pm 2^{\circ}\text{C}$ of the standard range did not have a significant impact on cell adhesion to the growth surface. Using the proposed methodology with an increase in roller bottle rotation frequency on day 2 of cultivation, the yield of cell biomass reached 825 \times 10 6 \pm 10% cells per roller by day 3 of cultivation.

Our results agree well with the data obtained by other researchers and illustrate a specific case of applying the cell lines under study. When selecting equipment for cultivation of the HEK293 cell line and lines with similar attachment features to the growth surface, it is advisable to consider the minimum rotation frequency of rollers in the roller assembly.

Future studies into cell adhesion to the growth surface for other cell lines will improve the efficiency of obtaining cell biomass as a substrate for accumulation and production of virus-containing materials.

CONCLUSION

As a result of our experimental research, the initial stage of roller cultivation for two cell lines — HEK293 and



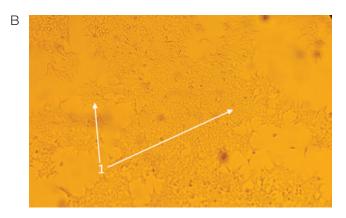


Photo taken by the authors

Fig. 9. Formation of HEK293 cell line monolayer during cultivation for: A — 1 day (rotation frequency 0.2 rpm); B — 2 days (rotation frequency 0.5 rpm); 1 — areas of cell culture growth with a tendency towards homogeneous monolayer formation; magn. ×100

Vero — was optimized. The optimal rotation frequency for the Vero cell line was found to be 0.4–0.5 rpm throughout the entire cultivation period. In order to form a high-quality monolayer of HEK293, the initial rotation frequency should be 0.2 rpm, followed by an increase to 0.5 rpm.

The difference in rotation frequency for these two cell lines is explained by their tissue-specific characteristics. The Vero cell line demonstrates a higher degree of adhesion to the growth surface and resistance to external mechanical disturbances, compared to the HEK293 cell line.

The data obtained have practical significance for scaling up processes of cell and viral biomass production in monolayer cultivation, thus presenting interest to specialists involved in developing experimental viral preparations using adherent cell lines.

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Photo taken by the authors

Fig. 10. Homogeneous monolayer formed by HEK293 cell line after three days of cultivation at 0.2 rpm, followed by a change in rotation frequency to 0.5 rpm on day 2 of cultivation: magn. ×100

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