International Journal of Pharmacy and Natural Medicines

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RESEARCH ARTICLE

Optimization and sub-culturing of Verocell culture

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ABSTRACT

Vero cells are predominant animal cell line widely accepted for viral vaccine manufacturing. Vero cells cannot produce the interferon (antiviral protein) due to inherent genetic defects. This is major advantage of Vero cells used as a substrate for virus propagation in vaccine manufacturing. The regulatory authorities such as World Health Organization (WHO) and Chinese Pharmacopeia are recognized the Vero cells can be used in producing of human biological products. Vero cell lines are stable cell lines, low probability of malignancy, easy to establish the cell bank with different generations because these cells are continuous cell lines. The advances in technology, presently anchorage dependent cells can be produced very large scale, using microcarriers in suspension cultures. This article give an outline of the Vero cells adaptation procedures to serum free media and optimization of adherent cell lines suspension cell cultures using microcarriers technology.

Keywords: Vero cells, Animal cell cultures, Cell biology,Adherent cell lines, Sub culturing, Scale up of cells, bioprocessing, suspension culture, Microcarriers (Cytodex-1)

ARTICLE INFO

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PAPER QR-CODE

ARTICLE HISTORY: Received 05 August 2018, **Accepted** 19 November 2018, **Available Online** 15 December 2018

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Citation: *C M Anuradha et al.,* Optimization and sub-culturing of Verocell culture. *Int. J. Pharm. Natural Med.,* 2018, 6(2): 180-183.

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1. Introduction

Vero cells are continuous and anchorage dependent cell lines, derived from Kidney epithial cells of an African green monkey (Cercopithecus aethiops). This cell lineage developed by Yasumura and Kawakita at 27 March 1962, Chiba University in Chiba, Japan. Vero cell line is one of the leading animal cell line used for scientific research, manufacturing of biopharmaceuticals, and viral vaccines. Most of the biological products (vaccines, monoclonal

antibodies, hormones and recombinant proteins ect.) are produced with the support of different animal cell lines by employingrecombinant technology (SaschaKiesslich et al. 2020; Xiaofeng et al. 2017). Vero cells are adherent cell lines, which will grow on suitable surfaces when cells are inoculated into appropriate growth medium. These adherent cells will survive when attached to an inert surface of culture vessels. Vero cells are round in shape when cells were in suspension; after cells attached to the surface morphology changed to elongated in shape then cells will grow, and maintain its function (Nicole C. Ammerman et al 2008).

The advances in cell culture technologies, different culturing vessels are available for anchorage dependent culturing systems, which include Tissue culture flasks, Roller bottles, Stack plate propagators and multilayer (Cellfactorry, Cell Stacks) and Microcarriers beads (Otto-Wilhelm Merten et al 2015; Caroline Faria Bellani et al 2020). Microcarriers are small beads, made up of with glass or plastic. Multiple types of micro carriers are developed and broadlyit is categorized into positively charged or no-charged microcarriers.

The microcarriers are coated with dextran, collagen, spheres, and polystyrene ect. whichis useful for cell attachment and establishing the primary cell cultures. The microcarriers cell culture technology is extensively used for manufacturing of viral vaccines (Polio, JEV, Rabies ect.) by using Vero cells (Jong Hyun Nam et al 2009; Khaled Trabelsi et al 2006). In this study, Vero cells are initially grown in tissue culture flasks with serum medium. Then cells are adapted to serum free medium by sequential passages. Further, to produce high cell densities, Vero cell suspension culture is optimized and cells are grown on micro carriers in spinner flasks with serum free medium.

2. Materials and Methods

Revival of Verocells

Vero cells were revived on to the MEM Earle's medium suspended with 10% FBS. Vero cells were thawed rapidly by holding the vial at 35 ± 0.5 ° C in a water bath. After thawing, the content is aseptically added drop wise in 50 ml centrifuge tube containing serum free tissue culture medium. After brief centrifugation (1000 rpm/10 minutes), supernatant is discarded and cell concentrate re-suspended in fresh medium. Aseptically, the contents of the vial were then added to MEM medium in T-75cm 2 tissue culture flask. The flask was incubated at 35 ± 0.5 ° Cunder 5% CO. The cells were observed daily under microscope for the formation of monolayer. Once the complete monolayer is formed the cells were passaged further at a split ratio of 1:3 or cell seeding density.

Vero cell sub-culturing

Vero cells monolayer with more than 80% confluence contains tissue culture flasks are passage into fresh flask using growth medium (medium with 10% FBS). Sub culturing of cells are performed to maintain the cells in log phase or increase the cell densities. During the sub culturing, cells are washed with PBS and trypsinzed the cells using enzyme Trypsin with EDTA(0.25%). These cells are quickly transferred to new flasks contain growth medium based on the split ratio. The cells were observed daily till the monolayer reaches 75 - 80 % confluence, and then cells will be expanded further. Serial expansion of the T75 cm and 225cm flasks were performed. Cell count, viability, sterility(by microscopic observation) is performed at each level.

Adaptation of Vero cells onto serum free medium

Vero cells were adapted to grow on serum free medium to avoid the animal proteins. For this cells were sequentially adapted to grow in reduced concentration of FBS and increasing the concentration of serum free medium. Briefly, the cells were passaged onto to medium containing 50% of stock (MEM Earles supplemented with 10% FBS) and 50% SFM (medium without FBS). In the next passage the concentration of stock further reduced to 25% and 75% of SFM. Finally, cells were sub cultured onto the medium containing 100% serum free medium and no FBS. Once the complete monolayer is formed, further cells were passaged for one more generation onto serum free medium (Ara T. Nahapetian et al 1986).

Optimization of sinner flask culture

Cytodex-I micro carrier (Sigma) was used throughout the study to prepare the Vero cell monolayer on beads suspended in the SFM medium in the spinner flask. Spinner flask culture is used as the development of suspension culture process byoptimizing the concentration of Cytodex-I beads and Vero cells for maximum attachment, agitation, medium pH and the incubation time (Kjell Nilsson 1988; Kjell Nilsson 2017).

Different concentration (1gm / 1L to 10gm/1L) of micro carrier was used to seed the Vero cells. Cytodex I beads were hydrated before adding to the medium in spinner flask with 50mL of PBS, pH-7.2 for 1.0 gms of Cytodex beads in sterile beaker for three hours at 37 ± 0.5°C. The buffer was slowly aspirated from the top with pipette; this process was repeated 10times. Finally the microcarriers were equilibrated in the Serum free medium for 20 minutes at 37±0.5°C. The Cytodex beads were then added to the spinner flask containing the medium. Vero cells harvested from T175 cm2 flask were added at a concentration of 4x 10 cells /mg of beads. Final volume of the medium is made to 700 ml and flask is kept on a magnetic stirrer and placed in incubator at 37 ± 0.5 ° C, 30 rpm.

The flask is monitored closely for the agitation and pH and a sample is withdrawn after 24 hours under aseptic conditions in laminar hood to observe the degree of attachment of Vero cells on the beads. After 96 hours of incubation, allow the beads were settled down and then slowly remove the medium from the tope using sterile tube connection with peristaltic pump.

The beads with cell monolayer were trypsinzed with 0.25 % trypsin – EDTA solution. The cells were counted by nucleic counted method. The yield of the cells from different concentration of beads was compared and used for

determination of final concentration of beads to be used for the spinner flask process (Chun Fang Shen et al 2019; Allen Kuan-Liang Chen et al 2011). Based on the cell kinetics, Cytodex-I beads concentration 3.0 gm/1L yields the optimum cell attachment and growth on the beads.

3. Results and Discussion

Although microcarriers is an advanced technique, it is based on the prior knowledge of the cell type, growth characteristics and information about the cells morphology, plating efficiency and other growth properties in traditional monolayer culture is in valuable when optimizing cell propagation on microcarriers. In this system, maximum attachment of the cell inoculum on the microcarriers and results in a rapid, homogeneous growth of cells to higher possible densities (Tarapong Srisongkram 2022; Anju Verma et al 2020). To determine such a technique we selected Cytodex –I micro carrier beads for Vero cells as this beads have higher binding efficiency and do not form aggregate in tissue culture medium, even at large concentration of 10gms/1L.

To understand the effect of micro carrier (Cytodex-I) centration on the final cell density, several optimization experiments were performed with different conditions were used for hydration of the beads as it was observed in the absence of hydration of beads showed very poor attachment. The prolonged hydration of beads in PBS pH 7.2 followed by equilibration in the medium in which they are to be suspended increased the attachment efficiency significantly.

In our study the optimal bead concentration was found to be between 2.5–5.0 mg/mL, Cytodex-I beads at 3mg/mL i.e., 3gm/L concentration optimization is considered of this parameter is important both in terms of sufficient surface area for cell growth (Table-1).

The other parameter which influences the cell attachment efficiency is cell density of inoculum. Vero cells survival and growth rate depends on the inoculation density and conditioning effects. At low plotting efficiency of the cells tends to be very sensitive to culture under low-density conditions. The starting cell number is the inoculation density affects both the proportion of the beads brining cells at the plateau stage and the total cell yield (Figure-1).

The interaction between the cells and the substrate surface is critical where cell adhesion occurs by divalent cation and basic protein which occur between the solid surface and the cell membrane. Under optimal conditions, cells attach and spread onto the carrier's surface and gradually grow out to a confluent monolayer. In our study we found that 20-40 cells/ bead as seeding concentration results in maximum utilization of the micro carrier.

For the development of micro carrier process, we performed our initial studies using 1L capacity spinner flask for cell culture optimization process. This spinner flask is small volume compact system that provides quick and accurate determination of optimal parameters for cell growth. This system in combination with macro carrier based culture allowed used to closely monitor the growth of cells further it allowed us to collect samples under aseptic conditions making it possible to monitor the cell expansion, microscopic counting. Vero cells attached well on the beads surface and formed confluent monolayer in at least 90% of micro carrier the cell growth at different time interval, shows a typical growth of Vero cells on micro carrier system demonstrating that there is no drop in cell density below the starting number during lag phase(first 24 hr in the culture), which obtained occurs when culture conditions are not optimal. In addition, the final cell density is generally more than 10 cells/mL after 96 hrs of culture.

Table-1: Vero cell concentrations produced different concentrations

Experimen t	Cytodex I Conc.	Attachment (%)	Cell density (m/ml)
Run-1	3 gm/1L	85%	5.56
Run-2	3 gm/1L	85%	5.48
Run-3	3 gm/1L	85%	5.82
Run-4	6 gm/1L	LT 60%	2.81
Run-5	6 gm/1L	LT 60%	2.12
Run-6	6 gm/1L	LT 60%	2.21
Run-7	10 gm/1L	LT 60%	2.31
Run-8	10 gm/1L	LT 60%	2.25
Run-9	10 gm/1L	LT 60%	2.36

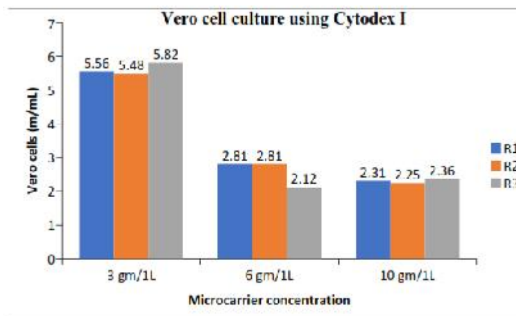

Figure-1

4. Conclusion

Micro carrier's technology able to produce high cell density of animal cell cultures under optimized conditions. The production of higher cell densities (only in hours) is impartment beneficial parameter in biological products manufacturing purpose. Vero cells adaptation to serum free media and suspension culture using Cytodex-1 microcarriers (1L Spinner flask) is demonstrated successfully. Further, studies will be conducted to optimize the maximum utilization of micro carriers surface by studying the factors influence the cell attachment, growth medium support to suspension culture, agitation conditions, and fluid shear stress.

Acknowledgements

The authors would like to thank Professor Suresh Kumar Chitta for technical assistance

Conflict of Interest

Authors have no conflict of interests.

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