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RESEARCH ARTICLE

Factors effects the Growth of Chinese Hamster Ovary (CHO) cell on Microcarriers Culture

Abdel Nasser A. And El-Moghaz*
Department of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University and Biotechnology Center-EIPICO-Egypt
Tel:+20106276329
E-mail:moghazy88@hotmail.com

ABSTRACT

Anchorage-dependent mammalian cells are used extensively in the production of biopharmaceuticals. CHO cells were successfully cultured on microcarriers in the bioreactors. Various factors influencing cell attachment including cell seeding density, microcarrier concentration, cell to microcarrier ratio, agitation speed and agitation profile were investigated. It was found that the optimumagitation rate was 40-60 rpm. A further increasing to 150 rpm resulted a significant reduction of growth with increasing the doubling time and reducing the exponential phase. An initial cell/bead inoculums of 25-30 ensured an even distribution of cells on the available microcarriers with a low proportion of unoccupied beads. An optimal growth rate occurred by an intermittent stirring regimen of 3 min stirring at 40 rpm per 30 min. Cell attachment to the electrostatic surface of the Cytodex 1 microcarrier was highly dependent on pH. This study showed that the rate of stirring influences greatly the growth and final yield of cells. Low agitation rate with initial intermittent stirring 40/30 minutes resulted in high cell densities and high of cell attachment. The growth rate of CHO cell increased with increasing concentration of cell inoculation, and therefore, the specific growth rate increases, too, quickly with increasing inoculation concentration.

***KEYWORDS**: Microcarriers-CHO-cytodex 1-culture agitation- anchorage-dependent cell-Spinner flask culture*

INTRODUCTION

Mammalian cells have been widely used as hosts for producing recombinant therapeutic proteins such as vaccines, hormones, and antibodies due to their ability to express a wide variety of proteins with a glycosylation profile that resembles that of the natural human protein [1-4]. Most mammalian cells and many other cells are anchorage-dependent and need suitable surfaces on which to grow [5]. Culture of adherent cells on the surfaces of bottles, flasks or other containers produces yields limited by available surface area [6]. One approach that has been increasingly employed is the use of microcarrier[7]. Microcarrier culture helps to make it possible to achieve a high yield culture of anchorage-dependent cells. In microcarrier culture cells typically grow as monolayer on the surface of small spheres, which are usually suspended in culture medium by gentle stirring [8]. The microcarriers can be made of various biocompatible materials such as agarose, dextran, cellulose or polyethylene polymers [9]. Cell attachment to these charged microcarriers are mediated by ionic attractions. These microcarriers are suspended in the culture medium by gentle agitation and a homogeneous environment is obtained [10]. The scale-up is done by increasing the fermentor volume. Use of microcarriers in simple suspension culture systems makes it possible to achieve yields of several million cells per milliliter. The culture environment is easily controlled. Since the cells are located on the surface they are subjected to mechanical stress [11]. Microcarrier cultures are used extensively for viral vaccine production, primarily using VERO cells (an African green monkey kidney cell line), although other cells lines such as MRC-5 (a human fetal lung fibroblast cell line) are also used [12]. Microcarriers have also been investigated for production of recombinant therapeutics using either CHO 13 or baby hamster kidney (BHK)[14] cells. In addition, microcarriers have been used to culture hepatic cells [15], pancreatic islets [16], and a variety of fibroblast cells [17]. The aim of this work was to investigate the effects of microcarrier concentration, cell seeding

density and agitation speed on the rate of CHO cell growth onto Cytodex 1 to attempt to optimize conditions for large-scale production.

MATERIALS AND METHODS

Cell-line

The mammalian cell-line CHO (Chinese Hamster Ovary) cells form biotechnology center-EIPICO-Egypt was used in this study.

Culture medium and chemicals

Dulbecco's Modified Eagle's medium (DMEM), glutamine, streptomycin, penicillin, gentamycin and tyrosine were from (Sigma-Aldrich). Cytodex 1 microcarrier was from (GE-Healthcare). Fetal bovine serum was form (Biosera).

Growth in static culture

The CHO cells were maintained on 150cm T-flasks in 50 ml medium at 37°C, 5 % CO_2 Cells required for inoculation spinner flask cultures were harvested at the day of confluence. The medium from each T-flask was removed. The cells were washed in phosphate buffered saline (PBS). Removal of cells from surface of the T-flask was effected by treatment with 5 ml of 1% trypsin pulse 0.2% EDTA in PBS. After treatment for 10 min at 37°C, trypsinization was halted by addition of 10ml medium. The cells were centrifuged and resuspended in fresh medium. The concentration of the detached cells was determined in a haemocytometer. Cell viability density of cells wer analysed by the trypan blue exclusion method. The amount of cell suspension to reach a concentration of $1x10^5$ viable cells/ml in the final volume of 100 ml was calculated and the inoculum added to the equilibrated spinner flasks.

Microcarrier preparation

Microcarriers were prepared according to the manufacturer's protocol. Briefly, microcarriers were swollen and hydrated in PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) overnight. After hydration, the PBS was removed and replaced with fresh PBS (50 ml/g microcarriers) and the microcarriers were sterilized by autoclaving at 121°C for 20 min. Prior to use, the PBS was removed aseptically and the sterilized microcarriers were then dispensed into spinner flasks containing growth medium to give a final concentration of 5 mg/ml.

Spinner flask cultures

Cell suspension at concentration 0f $1x10^5$, was inoculated into stirring flask containing appropriate concentrations of microcarriers previously hydrated, sterilized and equilibrated in pre-warmed serum containing medium. The bead concentration used were 0.5mg/ml. After 3 hours of intermittent stirring (2 min. of stirring at 30-40 rpm followed by 30 min. statically), medium was added to 3/4 of the final volume and intermittent stirring was performed during 3 hours. Then, the rest of the medium was added and the agitation speed adjusted to 40 rpm. Medium was replaced according to the requirements of each individual experiment; as soon as the pH of the medium decreased to approximately 7.1, 50% of its working volume was replaced by fresh medium.

Cell count

Daily samples were taken to determine cell number and morphology. For cell counting 1 ml carrier suspension was removed from the spinner flask and transferred to a test tube. When the carriers had settled the supernatant was removed and the carriers were resuspended in 1 ml lysis buffer (0.1% crystal violet in 0.1 M citric acid). After a minimum incubation period of 1.5 h the released nuclei were counted using a microscope and a haemocytometer. Data about the cell concentration were used to calculate the cell growth rate and cell attachment. The cell attachment was measured six hours after inoculation and was calculated as cell concentration on the microcarriers divided by the viable cell concentration used for inoculation.

RESULTS

A significant number of animal cell lines have an absolute requirement for adhesion to a solid substratum for growth and optimal synthesis of the product of interest. Anchorage-dependent cells grow in monolayers, and both yields in biomass and product are limited by the size of the available adhesion area. With microcarrier technique anchorage-dependent cells attaches to and grows on the surface of small particles that are kept in suspension in the culture medium by gentle agitation.

Effect of stirring rate

The growth properties of microcarrier culture of CHO agitated at 20, 40, 60, 100 and 150 rpm was studied. The results of these experiments showed that the optimal growth rate of CHO cells on microcarrers was between 40-60 rpm. It was observed that the doubling time of CHO cells was increased by increasing agitation rpm reached to 36 hours at 150 rpm compared to 24 hours with 40 rpm. It was also observed that, the exponential phase of culture grown at 150 rpm was reduced from 4 days to under one days followed by a rapid reduction in cell concentration as cells were dislodged from the Cytodex microcarriers beads (Fig. 1).

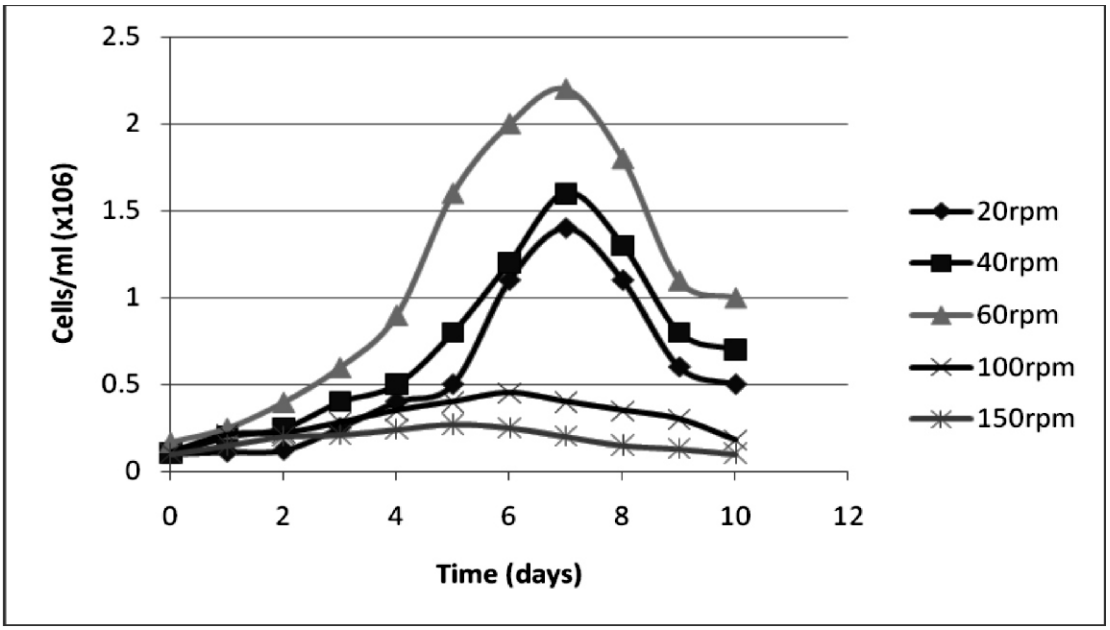

Fig. 1. Effect of agitation on microcarrier culture of attachment CHO to Cytodex 1

Effect of microcarrier concentration

To investigate the effect of microcarrier concentration on the final cell density, experiments were carried out using five different microcarrier concentration, 1, 2, 5, 10 and 20 mg/ml. The optimal bead concentration was 5 mg/ml. Increasing the microcarrier concentration to 20 mg/ml resulted a significant reduction of cell growth (Fig. 2).

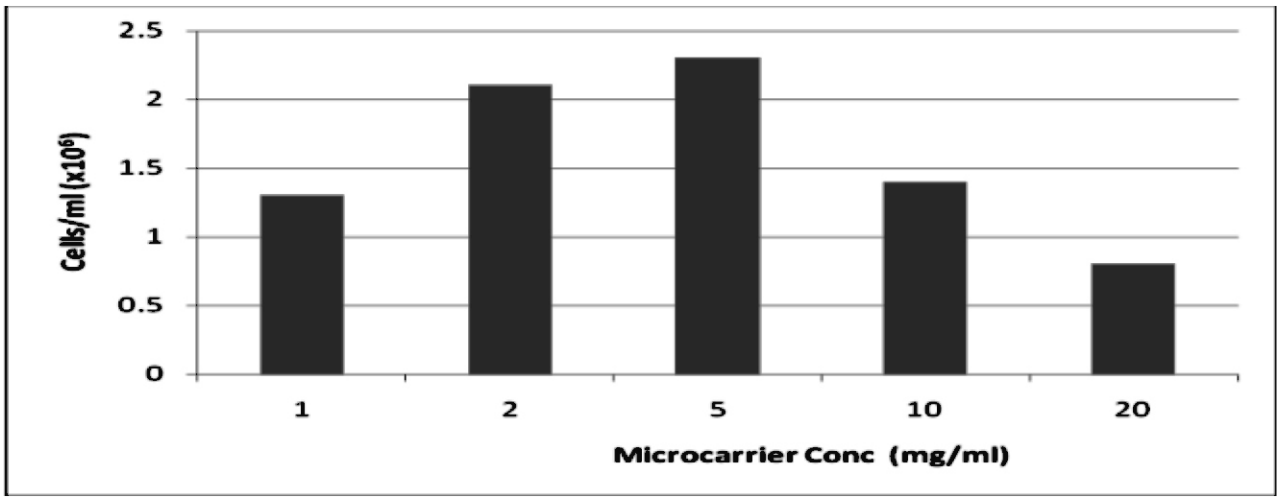

Fig. 2. Effect of microcarrier concentration on final cell density

Effect of agitation speed on attachment efficiency of cells

To investigate the effect of initial stirring procedure on the attachment efficiency of cells to microcarriers, culture

containing 5 mg Cytodex 1 final volume were inoculated with 10^5 cells/ml final volume and were either stirred immediately or cultured in reduced culture volume with intermittent stirring. Under this conditions, the rate and proportion of cells attached to the microcarriers was increased when the culture remains static with gentle intermittent stirring during the early attached stage (Fig. 3).

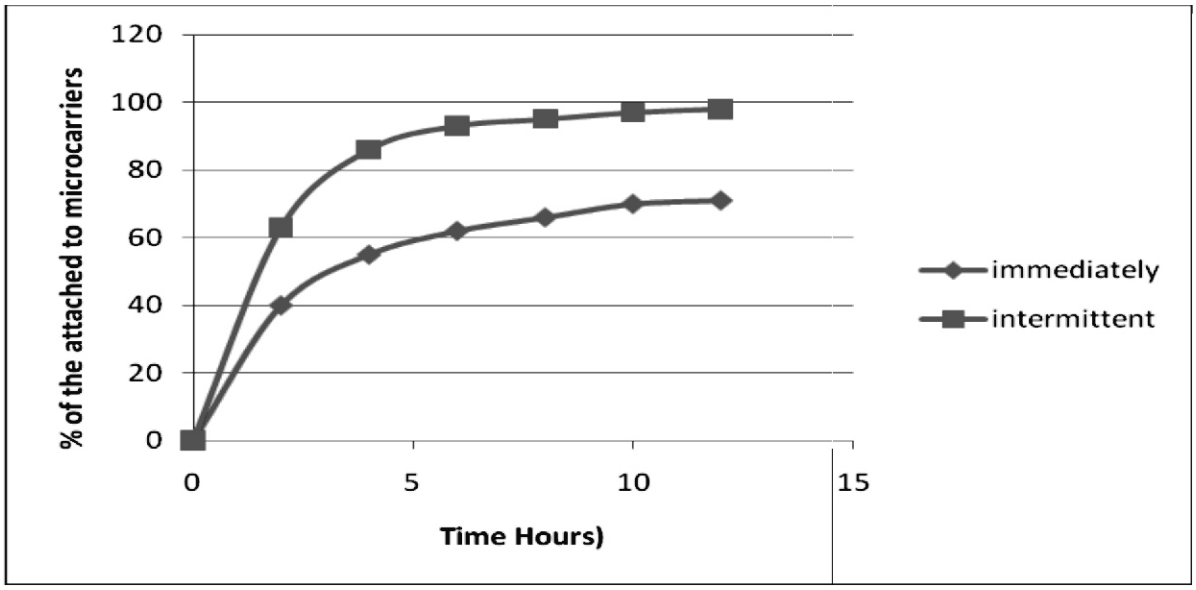

Fig. 3. The effect of initial culture procedure on the attachment efficiency of cells

Effect of the cell density at inoculation

The starting cell number, or inoculation density, affects both the proportion of beads bearing the cells and the total cell yield. It seems that the growth rate was increased with increased concentration of cell inoculation and the optimal ratio of cells/bead was between 25-30 cells/bead (Fig. 4).

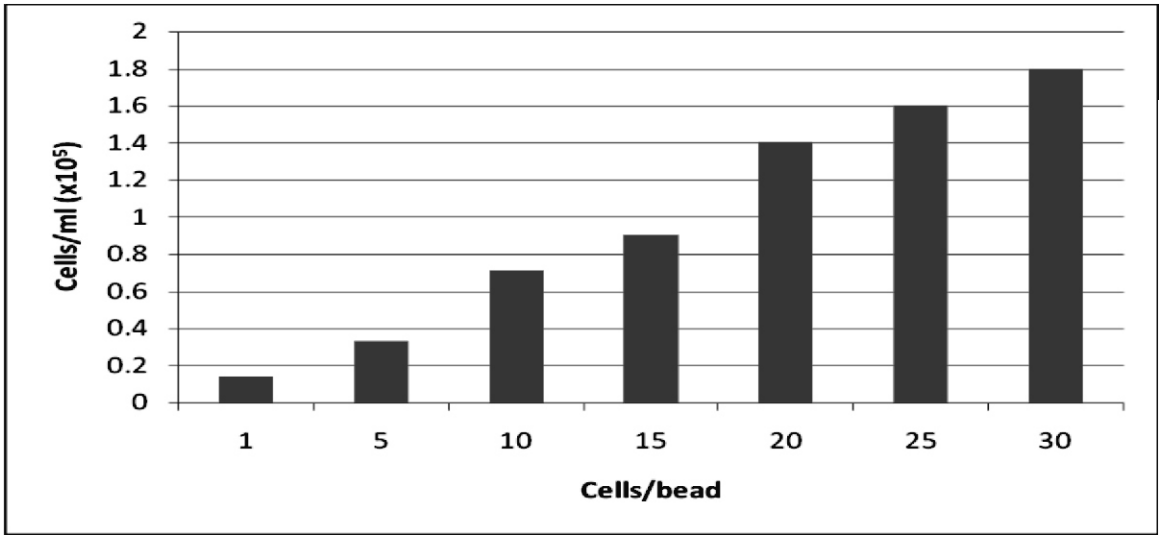

Fig. 4. Effect of starting cell density at inoculation

The effect of pH of the culture medium

A series of experiments to examined the effect of culture pH on growth rate of CHO over Cytodex 1microcarrier. The obtained results showed that the optimum pH was in the region of 7.0 to 7.2 where similar growth patterns were achieved. Higher pH at 7.6 and lower pH at 6.8 showed a reduction of growth rates as well as viable cell

number (Fig. 5).

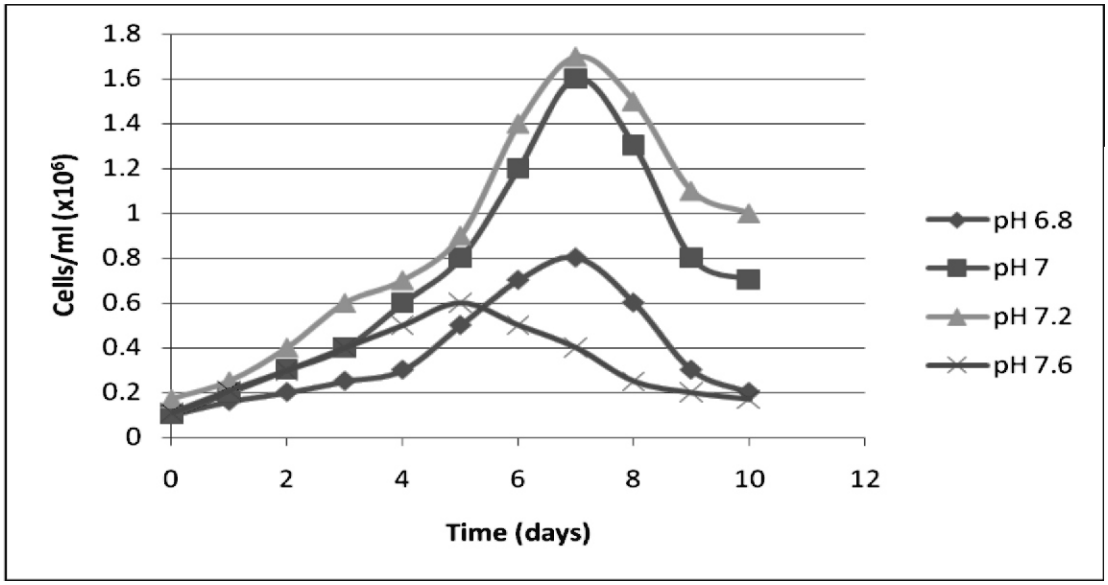

Fig. 5. Effect of pH of culture medium on the growth of CHO cells on microcarriers

DISCUSSION

Chinese hamster ovary cells (CHO) are the most popular commercial platform for the production of therapeutic proteins with much development going into the use of such cell lines for increasing product yields [18]. As much as improvements in specific productivity are important in cell lines, growth characteristics also have a significant impact on the process. A good cell line proliferative capacity and a high integral viable cell number can result in high volumetric recombinant protein production rates. Thus the mammalian biopharmaceutical industry has research interests directed towards the development of cell lines with high proliferation rate that can be grown to high densities and have high production capabilities [19]. Microcarrier culture introduces new possibilities allows practical high-yield culture of anchorage-dependent cell. This technology is replacing conventional monolayer cell culture methods as the extremely high surface area [20]. The effects of microcarrier concentration, agitation rate, and inoculation density on CHO cell growth in Cytodex 1 microcarrier cultures were investigated. Microcarrier cell culture technology was used to produce virus vaccines, interferons, plasminogen activators and urokinases, cytokines, hormones such as animal and human growth hormones and a variety of factors such as platelet derived growth factor (PDGF), epithelial growth factor (EGF), tumor necrosis factor (TNF), interferon and others are produced from such microcarrier facilities.

This study was investigated the effects of agitation rate, concentration of Cytodex 1 and density of inoculums on the growth rate of CHO cell under these conditions.

It has been found that the optimal agitation rate was between 40-60 rpm. Increasing agitation rate to 100 and 150 rpm resulted a significant reduction of CHO cell growth with increasing the doubling time to about 36 hours and reducing the exponential phase to less than 24 hours followed by a rapid reduction in cell concentration as cells were dislodged from the cytodex microcarriers beads due to mechanical damage by high agitation rate.

Sun *et al.* [21] studied the influence of the agitation rate in the range of 20 to 45 rpm on and they reported that at 45 rpm, cell adhesion rate was significantly decreased. They concluded that optimal agitation rate was 30 rpm. Gregoriades, et al. [22] observed that at high levels of agitation, hydrodynamic damage to cells could occur from microcarrier-microcarrier interactions (collision), microcarrier-eddy interactions, and possibly from collisions of microcarriers with the impeller. Also they indicating that cells on microcarriers were more susceptible than suspended cells to damage from hydrodynamic forces. It is obvious from figure 2 that the growth rates depend on the inoculation density and 25 to 30 cells per bead are required for maximum utilization of Cytodex. Increasing microcarrier concentration to 20mg/ml showed decrease the growth rate of CHO cells. When cell culture on a

microcarrier belong to monolayer growth, when the whole surface of the microcarrier is covered with cells, a maximum number of cell densities is reached, which is called the maximum cell density on this microcarrier culture. Mukhopadhyay *et al* [23] studied the kinetics of cell adhesion on Cytodex 1, 2 and 3 microcarriers and they found the highest adhesion rate at a cell-to carrier ratio of 15 cells per bead. Mendonca and Pereira [24] and Julien [25] reported that high concentrations of microcarriers in bioreactor culture reduced the ability of cells to attach due to increased shear stress emanating from collisions between microcarriers. This study reveals that the optimal rate of cell attached to microcarriers occurred by an intermittent stirring regimen of 3 min stirring at 40 rpm per 30 min. This stirring regimen appeared to maximize cell-to-bead attached and minimized cell aggregation. When the cell-microcarrier mixture was contained in reduced volume with intermittent stirring, cells showed greater chance of coming into contact with a microcarrier. This behavior is the result of a lower number of collisions between cells and microcarriers when both were at lower densities in addition to stronger hydrodynamic forces caused by higher stirring of the culture medium after the first 3 hrs. Griffuthis *et al.*, [26] has observed that the modified initial culture procedure was essential for good results when growing human fibroblasts and heart muscle cell respectively.The results obtained by shirangami *et al*., [27] were disagreeing with the previous results because they reported that the result of cell attachment to a microcarrier was best in the case of continuous agitation. Controlling pH is particularly important when using microcarrier culture because cultures can rapidly become acidic at high cell density. pH is very important during using microcarrier culture because cell attached to carriers with an electrostatic surface is highly depends on the right pH. This study showed that the high influences of pH in cell survival, attached and growth of CHO cells and the cells were susceptible to increasing or decreasing the pH of culture medium. Higher at pH 7.6 and lower at pH 6.8 values resulted in decreased growth rates and viable cell numbers. The reduction of cell growth at pH 6.8 may due to accumulation of lactate. Also, an alkaline pH prevents/prolongs attachment and high pH kill the cells.

In conclusion the agitation rate in spinner flask, ratio of inoculated cells and microcarriers concentration showed significant effects on CHO cell growth on Cytodex 1microcarriers. It was shown that a high cell-to-microcarrier, low agitation rate with initial intermittent stirring resulted in high cell densities. It was fund that intermittent stirring/non-stirring regimen of 40/30 minutes resulted in an optimal rate of cell attached. The rate of stirring influences greatly the growth and final yield of cells, an effect related to the integrated shear factor. Slower stirring speeds reduce shearing forces on cells attached to the microcarriers but, when the rate is too slow, growth was reduced. The yield of cells from microcarrier culture was directly related to the surface area for growth and hence the concentration of microcarrier. The optimum pH was 7.2 however, the higher at pH 7.6 and lower at pH 6.8 values resulted in decreased growth rates as well as viable cell numbers.

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