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Full Length Article

Optimization of Physico-chemical Factors for Augmenting Biomass Production of Baby Hamster Kidney Cells (BHK-21) in Roller Bottle

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Abstract

Physical (seeding density, incubation period, incubation temperature and rolling speed) and chemical (amount of cell culture growth medium and fetal calf serum) factors were optimized for enhancing biomass production of Baby Hamster Kidney cells (BHK-21) cells in roller bottle. The roller culture bottles (480 cm^2) were used for the propagation of the cells. Seeding of 10 million cells per bottle showed complete monolayer with maximum cell density (4.7×10^7) within 60 h that was significantly higher than those of other seeding densities of the cells ($P< 0.05$). The BHK-21 cells grew well at 35°C and 37°C within 60 h post incubation and formed confluent monolayer. However, the cell count declined significantly when the roller bottles were incubated at 39°C ($P< 0.05$). The bottles kept at rolling speed of 3 rpm yielded maximum count of the cells while higher or lower rotation speed showed adverse effects on the cell growth. Feeding of the cells with 100 mL of the growth medium (5% fetal calf serum) showed optimum growth of the cells. It is concluded that BHK-21 cells in roller bottle (480 cm^2) grew well and showed more than 4.7×10^7 cell count/bottle under optimized conditions such as 10 million seeding cell density, incubation time 60 h, incubation temperature 37°C, 3 rpm, and 100 mL growth medium.

Keywords: BHK-21 cells; Roller bottles; Micro-carriers; Cell density

Introduction

Animal cells are widely used in the cell biology, cancer research, diagnostics and pharmaceuticals. Cell culture is historically used for the isolation and propagation of viruses (Butler, 2003; Liu *et al*., 2012; Zhang *et al*., 2013). Cell culture is categorized as primary cell culture and cell lines. Primary cells offer a method of choice for the isolation of viruses from the clinical specimen while cell lines are routinely used for achieving high biological titer of animal viruses (Castillo *et al.,* 1991; Li *et al*., 2012). The working with cell lines is less labor intensive and easy to handle as compared to primary culture and hence provide economical means for propagation of viruses. A number of cell lines of mammalian origin i.e., BHK-21, MDCK, MDBK, HeLa, and Vero are employed in the animal virology laboratories. All of the cell lines vary in their spectrum and degree of sensitivity for the virus growth (Freshney, 1998). The cell lines which yield a high titer virus are preferred for the mass propagation of the viruses (Kretzmer, 2002).

Baby Hamster Kidney cells are adhesive and cancerous fibroblast, derived from kidney of a hamster (Macpherson and Stoker, 1961). Adherent BHK-21 cells are commonly cultured on flasks (glass, plastics), roller drums, multilayer dishes, and roller bottles (Ryan, 1979). The selection of culture method depends upon the available resources and the requirement of the procedure. While dealing with the production of veterinary vaccines, we prefer the method that would yield excessive number of cells which is a basic requirement for the augmentation of virus cultivation. In present study, various physico-chemical factors have therefore been optimized to achieve maximum cell density.

Materials and Methods

Growth and Maintenance of BHK-21 Cells

BHK-21 cells were procured from the frozen cell repository of the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. The cryopreserved cells were revived using standard procedure (Rehman *et al.,* 2007). Glasgow minimal essential medium (GMEM) was used for the propagation of cells supplemented with (Biomedical; USA) containing penicillin (1000 IU/mL), streptomycin (200 µg/mL), gentamicin (50 µg/mL), and amphotericin B (5 µg/mL) and5 % fetal calf serum (FCS). The cells were maintained on 175 cm^2 cell culture flasks by growing at 37°C and 5% CO_2. In order to subculture the cells each time, the spent medium was

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discarded from the flask. Cell monolayer was washed with pre-warm PBS and 3 mL of the trypsin-versene solution was added. Flask was incubated at room temperature for 5 min and 7 mL of the growth medium was added. Complete disaggregation of the cells was achieved by repeated pipetting to have a single cell suspension. The cells were routinely sub-cultured in a split ratio of 1:3 to attain a confluent monolayer within 48 h. The counting of disaggregated cells was performed using improved Neubauer hemocytometer chamber (Swayne *et al.,* 1998). The live dead ratio of the cells was also calculated through trypan blue dye exclusion method (Freshney, 1998). The cell stocks with a live dead ratio of more than 90% were used in the subsequent experiments. After counting, the appropriate cell concentration was achieved by diluting the cells in the growth medium.

Physical Factors

Seeding density: A total of nine roller culture bottles (Duran, GmbH, Germany) with a surface area of about 480 cm^2 were used to rule out the possible effect of seeding density on the number of the harvested cells. Three bottles each were inoculated with 5, 10 and 15 million cells per bottle. All of the bottles were supplied with 100 mL of growth medium containing 5% FCS and incubated at 37°C for 48 h in the roller incubator. The cells were harvested by removing the spent medium followed by washing of the cell monolayer with pre-warm PBS. 5 mL of the trypsin-versene solution was added and bottles were placed in the roller incubator for 5 min. The growth medium (5 mL) was added and cells were completely disaggregated by repeated pipetting. The cell counting was performed following Swayne *et al.* (1998).

Incubation period: Effect of incubation period on the cell yield was determined. A total of 15 cell culture bottles seeded with 10 million cells per bottle and supplemented with 100 mL of the growth medium containing 5% FCS were prepared and incubated at 37°C in the roller incubator. After an incubation of 24 h 3 bottles were removed and processed for the cell counting as mentioned in the Section 1.0. Subsequently, 3 bottles were removed after each next 12 h interval and processed for the cell counting following Swayne *et al.* (1998). The experiment was conducted for a maximum of 72 h growth time.

Incubation temperature: Three cell culture bottles containing 10 million cells and 100 mL of growth medium supplemented with 5% serum were prepared and incubated at 33°C for 48 h. Afterwards, 3 bottles each prepared in the similar manner were incubated at 35, 37, and 39°C for 48 h. Each time the bottles were removed after the completion of incubation time and processed for the cell counting following Swayne *et al.* (1998).

Rolling speed: Effect of rolling speed on the attachment and proliferation of BHK-21 cells in the roller culture system was monitored. Three bottles containing 100 mL of the growth medium supplemented with 5% FCS were inoculated with 10 million cells each and placed in the roller incubator whose rolling speed was adjusted to 1 revolution per minute (rpm). The bottles were incubated at 37°C for a period of 48 h. Subsequently, keeping all of the other parameters constant, three bottles each were placed in the incubator adjusted to the rolling speed of either 2, 3, 4 and 5 rpm for the same duration. At the end of incubation period, cell quantitation was performed from each bottle following Swayne *et al.* (1998).

Chemical Factors

Growth medium: The glass roller bottles (Duran, GmbH, Germany) with a surface area of about 480 cm^2 were used for the culture of BHK-21 cells in the present and succeeding experiments. A total of 15 roller flasks with an inoculum density of 10 million cells/bottle were used. Three of the bottles were fed with 25, 50, 100, 125 and 150 mL of the growth media (5% FCS) each. The lids were tightly closed and all of the bottles were incubated at 37°C for 48 h in roller incubator. The cell numbers in each bottle were quantified following Swayne *et al.* (1998).

Fetal calf serum: In order to test the effect of FCS supplementation on the growth of BHK-21 cells, a total of 15 bottles each containing 10 million cells were used in the experiment. Each of the bottle received 100 mL of the growth medium with variable serum concentration. Three bottles each were supplied with the growth medium containing 5, 7, 9 or 11% of the serum, while 3 bottles received the medium without serum. The bottles were incubated in the roller incubator at 37°C for 48 h and processed for cell counting following Swayne *et al.* (1998).

Results

There are various physical and chemical factors affecting growth of BHK-21 cell line on culture substrate. Effect of different volumes of Glasgow minimum essential medium (GMEM) with 5% fetal calf serum (FCS) was monitored on growth of BHK-21 cells in roller culture bottles. Seeding cell density in each bottle was 10 million and the results in the form cell count / bottle of the medium are shown in Table 1. Mean density of the cells grown in each volume of the growth medium was significantly different from each other ($p < 0.05$). There was minimum cell growth in the bottles having 25 mL of medium and was significantly less than the cell density observed in the bottles having 50 ml or more amount of the medium. The cell density in bottles having medium more than 100 ml were not significantly different but each of the cell density was significantly higher than that of bottle having medium ≤ 50ml medium at $p<0.05$.

Effect of different concentrations of FCS in GMEM was monitored on biomass/ growth of BHK-21 cells in roller culture bottles. Cell density in each bottle

Table 1: Effect of growth medium quantity on the scale up of BHK-21 cells in roller culture bottles

*Amount of growth medium (mL)	Cell count (log_{10})			
	Bottle 1	Bottle 2	Bottle 3	**Mean ± SD
25	2.1×10^6 (6.32)	2.5×10^6 (6.39)	2.4×10^6 (6.38)	$6.36^a \pm 0.038$
50	3.8×10^6 (6.57)	3.6×10^6 (6.55)	4.0×10^6 (6.60)	$6.57^b \pm 0.026$
100	4.3×10^7 (7.63)	4.3×10^7 (7.63)	4.5×10^7 (7.65)	$7.63^c \pm 0.011$
125	4.6×10^7 (7.66)	4.9×10^7 (7.69)	4.6×10^7 (7.66)	$7.67^c \pm 0.018$
150	4.5×10^7 (7.65)	5.0×10^7 (7.69)	4.6×10^7 (7.66)	$7.67^c \pm 0.020$

*Glasgow minimal essential medium (GMEM) **Mean and standard deviation of log values

Note: Seeding density was 10 million cells per bottle, 5 % serum was added in the growth medium and the bottles were incubate at 37°C for 48 h. The mean values having similar superscript are not significantly different ($p< 0.05$)

Table 2: Effect of serum concentration on the yield of BHK-21 cells cultured in the roller bottles

Serum concentration (%)	Cell count (log_{10})			
	Bottle 1	Bottle 2	Bottle 3	Mean ± SD
0	2.6×10^5(5.41)	2.5×10^5 (5.39)	2.1×10^5 (5.32)	$5.37^a \pm 0.047$
5	3.7×10^7 (7.56)	3.8×10^7 (7.57)	3.7×10^7 (7.56)	$7.56^b \pm 0.005$
7	4.7×10^7 (7.67)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.67^b \pm 0.005$
9	4.9×10^7 (7.69)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.68^b \pm 0.010$
11	4.9×10^7 (7.69)	4.7×10^7 (7.67)	4.8×10^7 (7.68)	$7.68^b \pm 0.010$

Note: Seeding density was 10 million cells per bottle, 100 mL of M-199 medium was added in each bottle and the bottles were incubated at 37°C for 48 h. The mean values having similar superscript are not significantly different ($p< 0.05$)

Table 3: Effect of rolling speed on the cell proliferation in the roller culture system

Speed in revolution per minute (rpm)	Cell count (log_{10})			
	Bottle 1	Bottle 2	Bottle 3	Mean± SD
1	2.3×10^4(4.36)	2.5×10^4 (4.39)	2.6×10^4 (4.41)	$4.39^a \pm 0.025$
2	6.8×10^4 (4.86)	6.9×10^4 (4.83)	7.0×10^4 (4.84)	$4.83^b \pm 0.015$
3	4.8×10^7 (7.68)	4.7×10^7 (7.67)	4.9×10^7 (7.69)	$7.68^c \pm 0.010$
4	2.2×10^3 (3.34)	2.1×10^3 (3.32)	2.2×10^3 (3.35)	$3.33^d \pm 0.015$
5	3.5×10^2 (2.54)	3.0×10^2 (2.47)	2.8×10^2 (2.44)	$2.49^e \pm 0.051$

Note: Seeding density was 10 million cells per bottle; 100 mL of the growth medium supplemented with 5% serum was added in each bottle and the bottles were incubate at 37°C for 48 h. The mean values having similar superscript are not significantly different ($p< 0.05$)

was 0.01 million cells/mL of the medium and the results in the form of cell count / bottle as shown in Table 2. Mean density of the cell grew in each percentage of FCS was significantly different ($p>0.05$). The bottle having 0% of FCS was significantly less than the cell density observed in bottle having 5 % FCS ($p>0.05$). The cell density in the bottles having more than 5 % FCS were not significantly different but cell density in each of the bottles was significantly higher than that of bottle having 0 % FCS.

Effect of different rolling speeds on growth of BHK-21 cells in roller culture bottles was monitored. The seeding cell density in each bottle was 10 million irrespective of its speed of revolution and the results in the form cell count / bottle as shown in Table 3. Mean density of the cells grown at each revolution speed was significantly different as $p>0.05$. The cell growth in bottle revolving at 05 rpm was significantly less than the cell density observed in bottle revolving at 03 rpm ($p>0.05$). The cell densities in bottles rotated at ≥ 02 rpm were not significantly different but each of the cell densities was significantly lesser than that of bottle rotated at 03 rpm.

Effect of different seeding cell densities of initial inoculum on growth of BHK-21 cells in roller culture bottles was monitored. Inoculum of initial cell density was 5 million, 10 million and 15 million and the results in the form cell count / bottle as shown in Fig. 1. Mean density of the cells grown in the bottle having different seeding cell density was significantly different ($p>0.05$). The minimum cell growth in bottles having 5 million of seeding cell density and was significantly less than the cell density observed in bottle having 10 million and 15 million ($p>0.05$). The cell density in bottles having 10 or 5 million seeding cell density were not significantly different but was significantly higher than that of bottle 05 million seeding cell densities ($p< 0.05$).

Effect of different times of incubation on growth of BHK-21 cells in roller culture bottles was monitored. Seeding cell density in each bottle was 10 million irrespective of time of incubation and the results in the form of cell count / bottle was shown in Fig. 2. Mean cell density of each time period of incubation was different as $p<0.05$. The cell growth in bottle provided 72 h of incubation time was significantly less than the cell density observed in bottle provided with 60 h of incubation ($p>0.05$). The cell density in bottles endow with 48 and 60 h were not significantly different but each of the cell density was significantly higher than that of bottle incubated either for 24 or 72 h ($p>0.05$).

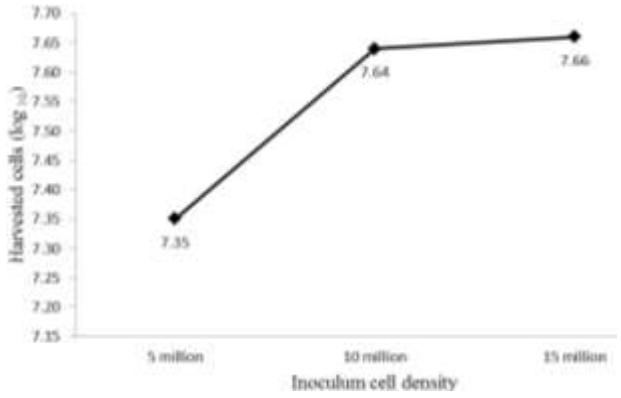

Fig. 1: Growth rate of BHK-21 cells cultured in roller bottles at various seeding density

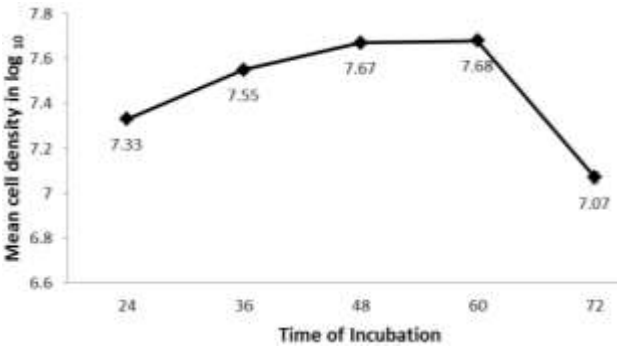

Fig. 2: Effect of Incubation time on the growth of BHK-21 in roller bottles

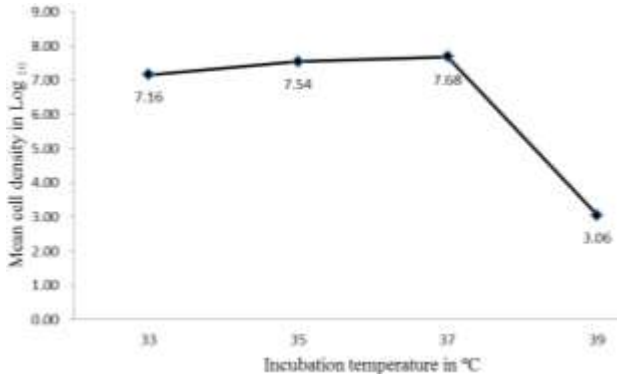

Fig. 3: Effect of temperature on the multiplication of BHK-21 cells cultured in roller bottles

Effect of different incubation temperature on growth of BHK-21 cells in roller culture bottles was monitored. Cell density in each bottle was 10 million cells per bottle irrespective of its incubation temperature and the results in the form cell count / bottle are shown in Fig. 3. The growth of cells in bottle incubated at 39°C was significantly lesser than cell density observed in bottle incubated at 37 and 35°C ($p>0.05$). The cell density in bottles at 35 and 37°C (7.68) was non-significantly different but each of the cell density was significantly higher than that of bottle incubated at 39°C. Remarkable different result was observed in bottles incubated at 39°C.

Discussion

Baby hamster kidney (BHK-21) cell line (adherent cells) grew well in glass/plastics culture vessels such as roux flask, roller bottles (reagent bottle) and micro-carrier culture system. The cells grew, adhered and formed monolayer on the surface of roux flask, inner surface of roller bottle and on surface of micro-carriers. BHK-21 cells routinely grow and adhere on the plastic and glass surfaces. Adherent cells secrete adhering molecules such as fibronectin; plays a major role in the adhesion of many cell types such as fibroblasts, hepatocytes, macrophages, etc., on glass or plastic surface and also form cell to cell adhesion. Cations such as Ca^{2+} and Mg^{2+} are found to attach on glass surface from one side and bind to fibronectin from other side (Freshney, 1998). Cell attachment can therefore be disrupted by using chelating agent such as versin or trypsin solution or both of these (versin trypsin solution). These chemicals can hydrolyse the fibronectin molecules and detach the cells from the substrate and also dissociate from the neighboring cells. Some adherent cells lose their adherent property due to lack of their own surface adhesion molecules (knock out gene expression) or change in the environment. These cells are known as non-adherent cells and can be grown in suspension form (Ferrari *et al.,* 1990). There are several physical and chemical factors that modulate growth and viability of BHK-21 cells. The chemical factors are provided through growth/maintenance media that offer nutrients to the cells needed for their growth and survival. There are number of growth media (M-199, DMEM, F12, GMEM, etc.) that are commercially available for propagation of mammalian cells. A growth medium contains inorganic salts, carbohydrates, amino acids, vitamins, fatty acids and lipids, proteins and peptides, serum, supplements (L. Glutamine, Sodium pyruvate and non-essential Amino acids). The carbohydrates are glucose, galactose, maltose or fructose. Media containing higher concentration of sugars are able to support the growth of a wider range of cell types such as high glucose in GMEM favors the rapid multiplication of BHK-21 cells. All of these are required in the cell matrix for attachment and as enzyme cofactors. Cells require pH conditions in the range 7.2 - 7.4 (Anand *et al*., 2009). It was observed that volume of the growth medium (25 to 100 mL) was directly proportional to growth of the cells but further increase in the volume did not show any significant improvement in the cell density (Table 1). It could be due to limited space available for the attachment and proliferation of the cells (Kurano *et al.,* 1990). Growth medium (volume) augments growth of adherent cells in still culture (Sathya *et al.,* 2008) and non-adherent cells in the spinner flasks (Ryan, 1979). It was

observed that medium without enrichment additive did not support the cell growth. Commonly used additives in the maintenance cell culture medium are fetal calf serum (FCS), goat serum, horse serum, allontoic fluid, colostral whey, oval albumin, etc. (Kruman *et al.,* 1984; Rehman *et al.,* 2007). Addition of 5% FCS showed maximum cell growth. However, the medium supplemented with 7% and 9% of FCS showed non-significant effect on the cell density (Table 2). Serum in the medium is an important source of growth factors such as vitamins, minerals, anti-trypsin agents, adherent molecules, growth hormones, etc. The vitamins are precursors for numerous co-factors. Many vitamins especially B group (riboflavin, thiamine, biotin, etc.) are necessary for cell growth and their proliferation. Trace elements are zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a detoxifier and helps to remove oxygen free radicals (Paranjape *et al.,* 2004). Sometime, the serum is contaminated with infectious agents such as bovine diarrheal virus, mycoplasma, chlamydia, etc., that show detrimental effect on the growth and maintenance of the cell line. It is therefore important to screen batches of serum for their ability to support the growth of cells.

There are several physical factors (seeding density, incubation time and temperature, rolling speed, etc. affecting cell growth and viability of the BHK-21 cells. Seeding cell density significantly enhanced the count of BHK-21 cells. Ten million seeding cell density showed optimum growth of the cells after 48 h of incubation at 37^{o}C (Fig. 1). High yield of hybridoma cells was obtained by increasing the inoculation density (Sadettin and Bernhard, 1990; Sathya *et al.,* 2008). However, low seeding cell density and longer incubation time showed confluent monolayer of the cells. For still culture glass/plastic roux flasks are used. Space is the limiting factor for obtaining high cell density. Roux flask (175 cm^2) yielded 1.5×10^7 cells per bottle/35 mL of growth medium, while roller bottles (480 cm^2) yielded 4.6×10^7 cells per bottle/100 mL of the growth medium (Table 3). Roller bottles slowly rotate (approximately 0.5 rpm to 3 rpm) on motorized racks or drums and are widely used for producing large quantities of cells. Roller bottles employ simple technology but require an investment in the appropriate equipment (Ryan, 1979). Incubation temperature is important for cell growth. Incubation temperature (37°C) showed optimum cell growth while lower incubation temperature showed poor cell growth (Fig. 3). Incubation of cells at low temperature reduces metabolic activities and results low production of interferons by Chinese Hamster Ovary cells (Evelyn *et al.,* 2006: Alireza *et al.,* 2007). High temperature also decreased the cell yield that might be due to the mitigation of enzymatic activities of the mammalian cells. Similarly incubation period also affected the cell growth. Incubation time of 60 h showed maximum cell density, while increasing incubation period up 72 h proportionally reduced the cell growth (Fig. 2). The decline in cell yield after achieving confluence could be due to the accumulation of metabolic wastes or shortage of the vital ingredients like glutamine in the spent medium (Radlett *et al.,* 1971). Many anchorage-dependent cells can be adapted to grow on micro-carriers to achieve high cell density. While considering the cell propagation as monolayer culture, the roller bottles are thought to be a better choice (Kunitake *et al.*, 1997; Kretzmer, 2002). The major advantage of roller culture flasks over the flat surface bottles is that it provides increased surface area for the attachment of cells (Sadettin and Bernhard, 1990). However, rolling speed is a critical factor for the cell growth. The maximum cells were harvested at a rolling speed of 3 rpm while the cell yield was relatively less at low or high rolling speeds (Table 3). Medium insufficiency at low speed and attachment failure at high speed could be possible reasons for low cell yield. BHK-21 cells grow on suspending micro-carrier in mechanically stirred vessels (spinner flasks), bioreactors or fermentors (Kretzmer, 2002).

It is concluded that optimized conditions such as 100 mL growth medium supplemented with 5% FCS, 3 rpm rolling speed, 60 h of incubation time and 37^{o}C incubation temperature supported the 4.5 × 10 7 BHK-21 cells in roller bottles (480 cm^2) that was three times more than that of the cells (1.5×107) in roux flask (175 cm^2).

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