Optimization of physical parameters for attachment and growth of Vero cells on Cytodex 1 and Cultispher G microcarriers.

By

BILLY YU-CHUNG NG

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the degree of

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OPTIMIZATION OF PHYSICAL PARAMETERS FOR ATTACHMENT AND GROWTH OF VERO CELLS ON CYTODEX 1 AND CULTISPHER G MICROCARRIERS

BY

BILLY YU-CHUNG NG

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Declaration

I declare that this thesis is a report of the research carried out during the period of September, 1991 to March, 1995, in Department of Microbiology, at The University of Manitoba under the supervision of Dr. Michael Butler. The work completed has not been submitted for any other degree, and reported by other persons in any literature.

Abstract

The attachment of Vero cells on Cytodex 1 (Cyt 1) and Cultispher G (CuG) microcarriers was determined with 6 parameters. They were stirring mode, agitation, pH, serum concentration, inoculum size, and bead concentration. It was found that Vero cells attached differently to both bead types. A faster attachment rate of 28.2 $x10^{-2}$ min^{-1} was obtained in Cyt 1 compared to 1.02 $x10^{-2}$ min^{-1} in CuG cultures. Agitation, pH, and serum concentration were the most influential factors for cell attachment to Cyt 1, whereas stirring mode was to CuG. This suggests that Vero cells employed different attachment mechanisms to both bead types. On the other hand, at the attachment phase, cell aggregation was absent in Cyt 1 cultures but significant in CuG cultures, especially under continuous stirring. Intermittent stirring improved not only cell attachment, but also cell distribution and bead occupancy in CuG cultures. A staining protocol of Neutral red was used to reveal cells on semi-transparent CuG beads.

Under the same parameters, growth of Vero cells on Cyt 1 and CuG beads was determined after optimal attachment was achieved. Optimal conditions for growth were different from those for cell attachment. For Cyt 1 and CuG batch cultures, high agitation speed and low serum concentration were detrimental to growth, while an increase in inoculum density and bead concentration did not lead to better microcarrier surface area usage. 1 g/L Cyt 1 and 1.72 g/L CuG cultures yielded 1.0 $x10^6$ and 1.7 $x10^6$ cells/ml respectively as their maximum cell densities, indicating CuG provided more surface areas and therefore higher cell yield. For fed-batch Cyt 1 cultures, higher cell densities were achieved but other limiting factors were possibly present. Oxygen transfer was improved by increasing agitation speed to 75 rpm, higher cell yields were obtained at a range of bead concentrations (1-5 g/L).

Cyt 1 beads were also shown to transfer cells on the basis of bead-to-bead bridging and to be reused for cell growth in serial subculturing.

Acknowledgments

To my Supervisor, **Dr. M. Butler**, who gave me this project in which I have learned more than I expected. There are many people that I am very grateful to. Among them, they are my colleagues who I worked with and along on day-to-day basis. They accepted me and sometimes my mistakes. Without these individuals, this research might have not been the same.

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1.0. Introduction

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Abbreviations

CHO cells	Chinese Hamster Ovary cells
CS	Calf serum
Cyt 1	Cytodex 1 microcarrier
CuG	Cultispher G macroporous microcarrier
DME	Dulbecco's modified Eagle's medium
DME/F-12	DMEM/Ham's nutrient mixture F-12
HEPES	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
Iscove's modified DME	Iscove's modified Dulbecco's modified Eagle's medium
μm	micrometer (10^{-6} m)
meq/g	milliequivalent of mole of charge per gram
nm	nanometer (10^{-9} m)
PBS	Phosphate Buffer Saline
PBS-EDTA	PBS plus Ethylenediamine tetraacetic acid
RPMI	Rosewell Park Memorial Institute

Chapter 1. Introduction

1.0. Introduction

1.1. History of tissue culture development

Growth of animal cells *in vitro* was first reported by Harrison, (1907). Dissected nerve tissue of frog embryos was allowed to grow in clotted lymph fluid on the underside of a microscope cover slip for several weeks, this was the " Hanging drop technique ". Later, Burrows adopted this technique with the use of plasma clots to grow cells of warm blooded animals. In 1912, Carrel used chick embryo extracts to supply growth factors and plasma fibrin matrix to serve as attachment substratum for cells. Also, he introduced the "Carrel flask" to prolong the growth of a wide range of mammalian cells under aseptic conditions. The discoveries of antibiotics (penicillin and streptomycin) in 1940s further reduced the chances of contamination, particular in cultures with undefined compositions of embryo extracts and fluid. At that time, cultures were basically tissue explant cultures composed of different cell types. Growth was also limited to the periphery of the tissue explants where new cells proliferated outwardly.

The use of an enzyme (trypsin) in subculturing cells was developed by Moscona and Moscona, (1952), it subsequently allowed true "cell culture" to be distinguished from "tissue culture". Freshney, (1983) defined that cell culture was composed of single cell type and isolated by either mechanical, chemical or enzymatic dissociation means to yield a dispersed cell culture (single cell culture).

In 1950s, Earle and Eagle developed a medium for human cervical carcinoma cells, known as HeLa, and chemically transformed mouse L cells on the basis of analyses of nutrient requirements of these cells. This chemically defined medium was a mixture of amino acids, sugars, minerals and vitamins dissolved in water with addition of chemically undefined serum. In 1961, Hayflick and Moorhead established a human diploid cell line, WI-38, that had a maximum population doubling of approximately 50 and showed senescence prior to death. This finding suggests that normal cell lines had a limited

potential for cell division and are therefore mortal. In contrast, more and more continuous cell lines had been cultured with the provision that they were immortal and easier to handle than normal cells.

Another significant contribution was made by Kohler and Milstein, (1975) in the establishment of a hybridoma cell line which was the product of cell fusion between myeloma and lymphocyte cells. Cells derived from this process had the characteristics of both cell lines: the abilities of infinite growth of cancer cells and of secretion of monoclonal antibodies of lymphocytes with single specificity. With the use of recombinant DNA technology in animal cells, more cell lines were manipulated and emerged with desirable traits and functions. In mammalian cell based processes, the primary concerns were to yield cells easy to produce in mass with abilities to synthesize, process, modify, and secrete biologicals or recombinant gene products that were invaluable in diagnostic and therapeutic areas, but could not be expressed in a prokaryotic cell system.

1.2. Cell Attachment

1.2.1. Introduction to Cell Adhesion

For anchorage dependent cells, subculturing or inoculation involves detachment and reattachment of cells. Cell attachment is important in determining the survival and subsequent growth of the cells. The objective of any cell culture is to ensure a fast cell attachment and spreading, followed by immediate exponential growth, thus reaching high cell density in the shortest time possible.

1.2.2. Physical Theory of Cell-Substratum Adhesion

In 1940's, the phenomenon of cell-substratum adhesion was discussed and characterized in terms of physical charge interactions (Curtis, 1980, and Barngrover, 1986). It accounts for two distinct forces on negatively charged surface: electrostatic and

van der Waals forces in which both are active over a range of about 100 nm. (Lenard et al., 1987). On positively charged surface, the electrostatic force attracts cells. The first microcarriers were associated with positive charge groups, DEAE (a tertiary amine), to facilitate cell-bead attachment of overall negatively charged cells (Fig.1.2.1.).

However, the physical theory fails to address the existence of other important factors. First, it assumes the charge distribution was uniform over the cell surface, which is found to be incorrect (Pharmacia, 1991). Second, it ignores the absorption of serum protein onto the substratum, which is about a 2-5 nm thick layer (Grinnell, 1978). In fact, most of the studies are carried in serum containing media or with cells known to synthesize the similar proteins required for cell adhesion. On the other hand, the strength of adhesion can be changed by modifying the pH and ionic strength of medium since both affected the extent of negative charge on the surfaces.

1.2.3. Specific Chemical Binding

The chemical theory targets the deficiency of the physical theory. It emphasizes that the adhesion process is mediated by specific adhesion proteins absorbed in the extracellular matrix and involvement of other cell components (i.e. cell surface receptors and cytoskeleton movement).

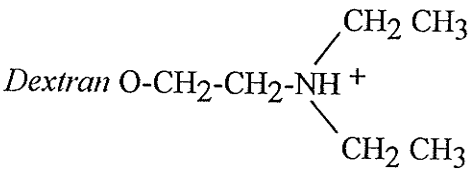
(a) Extracellular Matrix Macromolecules

(i) Fibronectin

In the late 1970's, an adhesion simulating factor was isolated and purified from sera of different origins (porcine, calf, avian, and human) and of different ages (fetal, newborn, calf, adult) which was demonstrated to facilitate cell adhesion despite the presence of varied numbers of non-specific proteins (Grinnell, 1978). This factor was later termed fibronectin (*in Latin , Fibra=Fiber; nectere=Link*). Fibronectin is an elongated

(Olden et al., 1980), multifunctional glycoprotein found in extracellular matrix and in body fluid (Ruoslahti et al., 1982, and Akiyama et al., 1990). This glycoprotein can be synthesized by fibroblasts, endothelial cells and other cells (Kleinman et al., 1981). In fact, fibronectin is the major protein (40-50% of total membrane proteins) found in the membrane as fibrillar network (Olden et al., 1980, and Ruoslahti et al., 1982). It promotes cell adhesion as well as spreading (Hughes et al., 1979). The binding activity resides in a recognition sequence identified as RGD (Arg-Gly-Asp) and recently reported as RGDS ((Arg-Gly-Asp-Ser) by others (Juliano, 1987, and Ruoslahti and Pierschbacher, 1987). In addition, other studies suggest that a distinct synergistic site is also required for mediating efficient adhesion and cytoskeletal organization (Ruoslahti and Pierschbacher, 1987). Fibronectin also possesses domains with affinity to bind with other molecules (i.e. gangliosides, collagen, glycosaminoglycans, proteoglycans, etc.) (Akiyama et al., 1990). It regulates the distribution of receptors and localization of fibronectin in adhesion contact sites (Chen et al., 1986).

Fig.1.2.1. Chemical composition of N,N-diethylaminoethyl (DEAE)- linked to Dextran of Cytodex 1 (Pharmacia, 1981).



Recently, it was reported that the role of fibronectin is also involved in the cytokinesis of the cells during cell division (Orly and Sato, 1979., Bottenstein and Sato, 1979). Cells depleted of fibronectin may become binucleated.

(ii) Vitronectin

Vitronectin, also called serum spreading factor, is an adhesive plasma glycoprotein (Underwood and Bennett, 1989). In fact, vitronectin mediated spreading occurs independently of fibronectin mediated spreading (Akiyama et al., 1990). Like fibronectin, vitronectin has binding sites for collagen, glycosaminoglycans, and cells. The cell binding domain also possesses the RGD sequence (Akiyama et al., 1990). Recently, vitronectin was reported to be more effective in cell adhesion and spreading than fibronectin (Steele et al., 1992). Also, vitronectin was reported to be 5-50 fold excess of fibronectin in culture bovine serum (Underwood and Bennett, 1989).

(iii) Glycosaminoglycans and Proteoglycans

Glycosaminoglycans are polysaccharides made of repeated units of disaccharides and mostly negatively charged because of sulphate groups (Ruoslahti and Pierschbacher, 1987). Proteoglycans have a protein core joined by glycosaminoglycans (Barngrover, 1986). They are believed to stabilize or facilitate the attachment process.

(b) Integrins

Integrin is heterodimeric glycoprotein receptors located in the plasma membrane (Akiyama et al., 1990). Each one is divided into three domains: extracellular, membrane-spanning, and cytoplasmic (Pharmacia, 1991). The integrins have their amino termini in the extracellular region binding to the RGD ligands of adhesive proteins, this process requires divalent ions such as Ca^{++} and Mg^{++} which can be sequestered by EDTA during PBS-EDTA washing. The cytoplasmic ends are composed of carboxyl termini which have high affinity for talin, a cytoplasmic protein with affinity to cytoskeletal actin filaments (Ruoslahti and Pierschbacher, 1987). Therefore, the receptors may serve as a connection between extracellular matrix and cytoskeleton.

1.2.4. Cell attachment to microcarriers

During an industrial process, cell cultures may be required to be harvested and reinoculated a few times during scale up (Fig.1.2.2.). Therefore, attachment is as crucial as growth to ensure the efficient use of microcarriers and homogeneity of cell confluence on the beads at any time of the cell culture. In fact, cell attachment depends on complex interactions of physical, chemical, biochemical, and biological factors that are attributed to the entities of cell line, substratum, and culture environment (Fowler and Whish, 1987). The combinations of these cell, substratum, and culture environment factors are infinite and therefore each microcarrier system can be specific. Nevertheless, the general mechanisms of cell attachment in vitro have been described (Grinnell, 1978). Cell attachment onto a substratum surface can be divided into 3 stages: (1) cell-substratum contact, when cells and substratum are brought into the vincinity of each other, (2) cell-substratum attachment, a process where cells are made passively attached onto the surface of a substrate by means of physical (charge), and physiochemical (pH, osmolality) and biochemical (ligand-receptor) interactions, and (3) cell spreading, at which attached cells actively involve in stabilizing the attachment via biochemical factors (attachment factors) and biological (cytoskeletal) movements of the cells.

Figure 1.2.2. The sequence of a microcarrier cell culture.

(1) Cell attachment → (2) Growth → (4) Harvest/ Virus Infection/ etc.
↖ (3) Subculturing ↙

Nevertheless, the diversity of cell types, medium compositions, and some physical parameters (i.e. vessel type and stirring apparatus) as well as the nature and type of microcarriers do not yield a universal attachment condition for all microcarrier cell

cultures, therefore a specific optimization of attachment conditions is required for different cell lines and microcarrier systems.

1.3. Growth characteristics of animal cells *in vitro*

Cells isolated from animal tissue grow *in vitro* as primary cultures. Subsequent passage from primary culture is a secondary culture. The passage number (P) denotes the number of subcultures taken from the cells of a primary source.

For anchorage dependent cells, growth is generally grouped into 3 phases: lag, exponential and decline phases (Butler, 1993). Cells harvested from a culture after chemical and enzymatic treatment (i.e. EDTA washing and trypsinization) reattach and spread on a surface before growth can proceed. The combinations of medium, serum components, nature of the substratum, physiological state of the cells, cell inoculation density and other physical and physiochemical environments would govern cell attachment and subsequent growth. A lag phase is characterised by a drop or no change in cell density on Day 1. It occurs as cells adapt to new conditions or recover from trypsinization. Cell growth may require the production and secretion of cell-specific attachment and growth promoting factors. Cells grow at their highest rates in the exponential phase. The doubling time is the time required to multiply an existing population of cells by a factor of 2, which is calculated from the growth period in the exponential phase. As cells continue to grow exponentially, the nutrients are depleted while inhibitory metabolites accumulate in medium making the culture environment less favorable to the cell growth. Eventually, the growth rates of cells decline and reach a level that equals the rate of cell death. At this time, cells may reach saturation on the surface (i.e. confluence). This stationary state is called a "plateau" where any cell growth replaces dead cells sloughed off from the surface. Normal diploid cell lines are usually restricted to a monolayer of cells on a substratum. In contrast, some other adherent cell lines, particularly of transformed origin, can form

regional foci of cells and even multilayers provided that there are sufficient amounts of nutrients and low levels of inhibitory metabolites. Eventually, cell densities decline as a result of no cell growth and lack of nutrients.

Suspension cells follow the same basic phases of growth.

1.4. Culture Modes

Cultures reach different cell densities depending on the culture mode, or simply a regime of nutrient supply and waste removal (Butler, 1993).

For anchorage dependent cells, a closed system is a batch mode without any incoming and outgoing exchanges for medium and oxygen, etc. At inoculation, the nutrient concentration is high and inhibitory metabolite concentration is low. As cells grow, the chemical composition of the medium changes as nutrients are replaced with inhibitory wastes. There is a critical concentration for nutrients and waste on cell growth before cells enter a stationary phase. Cells eventually die.

In a fed batch mode, cells are periodically resupplied with nutrients and inhibitory metabolites simultaneously removed. Therefore, cells are exposed to a "feast-famine phenomenon" which has an affect on the growth phase. In other words, cells are exposed to fluctuating patterns of growth promoting and inhibitory conditions that can affect the maximum cell densities. Nevertheless, maximum cell densities are higher in a fed-batch mode than in a batch mode.

For a perfusion system, nutrients are delivered continuously while wastes are taken out from the culture. As a result, the conditions in the perfused culture are more consistently maintained. Cell yield can reach higher levels dependent upon nutrient supply and availability of surface area for cell growth. Theoretically, cell densities could be achieved in cultures in an increasing order of batch, fed batch, and perfusion mode.

1.5. Culture reactor

The doubling times of animal cells are extremely slow (18-24 h) in comparison to those of microorganisms (i.e. bacteria, yeast and fungi). To avoid the risk of contamination and takeover of undesirable microorganisms, a reactor system for animal cells was handled under aseptic conditions.

1.5.1. Multiple Unit System

Traditionally, anchorage dependent cells have been cultured in glass roller bottles mounted on a rotator in which medium is circulated inside at slow speed (Pharmacia, 1981). Recently, tissue culture flasks (T-flasks) were introduced, which are mostly made of plastic and optimally charged for cell attachment of both anchorage dependent and independent cell lines. Greater cell yields are simply achieved by increasing the numbers of flasks. Since each unit is designed as an independent reactor, the process is labor intensive and prone to contamination. As a result, T-flasks and roller bottles are used extensively for stock cell passages and seeding stocks for cultures in the beginning of a scale-up process.

1.5.2. Single Unit System

The shortcoming of the multiple unit system is reduced in the single unit system. Scale-up can be performed by increasing the microcarrier concentration or volume of the system. Fewer subculturing steps are required to obtain greater cell yields.

(a) Bioreactors

At present, there have been a number of bioreactors developed for the cultivation of animal cells, they are classified into 3 major groups utilizing different mechanisms (Appendix A). They are stirred tank, column and membrane reactors.

The early bioreactors are similar to the stirred tank for microorganisms with some modifications. The fragility and sensitivity of animal cells do not allow high agitation speeds that are normally used in microbial fermentors. Therefore, an environment with minimal agitation to achieve homogenous mixing (of microcarriers or suspension cell) is required. Direct sparging (bubbling) may cause cell damage (Handa-Corrigan et al, 1989) and foaming in serum containing medium. A suspension culture is likely to maintain a homogenous microenvironment as opposed to static culture which may have gradients. Mass production of animal cells and their biologicals have been conducted mainly in fermentors under controlled conditions. Parameters such as pH, dissolved oxygen, temperature, suspension or agitation, and perfusion rate are controlled by on-line control devices which are essential to achieve maximum cell densities.

Systems using an entirely different mechanism of suspending anchorage dependent cells are also available, such as fluidized bed bioreactors. This is designed to have medium fluid pumping through a bioreactor in laminar flow pattern at a high rate to counteract the force of gravity on the biomass support particles.

(b) Spinner bottles

Spinners resemble stirred tank bioreactors in their mode of agitation. A spinner bottle is made of a glass vessel with two side arms and a stirring device comprising a shaft, a magnetic stirring bar and a paddle blade. The stirring bar, the paddle blade, and part of the shaft is attached to a cap assembly submerged into the fluid phase. Agitation is activated by a magnetic stirrer platform beneath the spinners. Agitation speed was set at particular rate measured in terms of revolution per minute (rpm).

The temperature (37^{o}C) and pH are maintained in an incubator. pH is buffered through a combination of dissolved CO_2 from the gas phase and HCO_3^- in the liquid

phase, the oxygen supply is through surface aeration from overhead space. High nutrient levels can be maintained by regular medium replacement (50-100% volume).

1.6. Cells

The cell is the basic unit of life. It is also the key component in animal cell technology where it serves as the biomachinary to convert nutrients and growth factors to cell biomass, biologicals (secondary metabolites), or tertiary metabolites (i.e. the expression of exogenous genes after viral infection and insertion).

1.6.1. Classifications of Cells

Cells can be classified into many categories according to different characteristics. Two major classes, normal and transformed cells, are used. The definition of these classes is based on a number of criteria as set by Ham and McKeehan, (1979).

- Karyotype: it refers to the number and morphology of chromosomes. Normal somatic cells are diploid. Any morphological or constitutional change (i.e. chromosome fragmentation) is regarded as heteroploidity or aneuploidity.
- Multiplication potential or population doubling: Finite life span is associated with normal cells with a limited number of cell divisions prior to senescence. The culture then enters a decline phase and eventually dies. Cells from an embryonic state will have a greater multiplication potential than the same cells at an adult state, implying a finite growth capacity is genetically programmed in each cell. On the other hand, infinite growth and multiplication is seen in transformed cells as they can be subcultured indefinitely.

- Anchorage dependency: Cells from normal tissues have to attach to a surface prior to subsequent growth is allowed. Transformed cells lose some of the phenotypic characteristics of normal cells and often can be grown in suspension without the requirement of surface substratum. However, some transformed cells re-acquire a need for attachment once certain attachment factors are added.
- Malignancy: The injection of normal cells into the immunosuppressed animals will not result in the formation of tumors. In contrast, some transformed cells can induce malignant tumor formation.
- Density-dependent inhibition of multiplication: Normal cells yield a monolayer of cells on a surface at confluence. In contrast, transformed cells are capable of forming multilayer of cells or foci of cells on top of a monolayer of cells.

Nevertheless, some cells have some characteristics of both normal and transformed cells as defined above. The crucial characteristics of transformed cells is the ability for infinite growth. Cells that are capable of infinite growth are therefore "established" and classified as a "continuous cell line".

1.7. Microcarriers

1.7.1. History of Microcarrier development

Since Enders et al., (1949) reported the growth of poliovirus in non-neuronal cells in culture, mass production of viral vaccines was initiated. The cell substrates considered suitable and safe for such vaccine production were normal, diploid, and anchorage dependent cells. Their growth required a surface substratum. Therefore the larger the surface area available, the more cells could be produced and subject to viral infection. However, laboratory scale tissue culture flasks (T-flasks) and roller bottles were not

amenable to scale up. A large number of flasks were required in order to achieve a high production of cells. Each flask or bottle acted as an independent bioreactor for cell growth (i.e. a multiple system). Also, such a process required excessive aseptic manipulations (i.e. numerous operations of inoculation or harvest and reinoculation) and stringent culture condition control thus it was labour intensive and prone to contamination.

The development of single unit systems including microcarrier technology was precipitated by demands for better cell culture system for mass production. van Wezel, (1967) developed the concept of cell growth on spherical beads in suspension in a stirred tank bioreactor. DEAE-Sephadex A-50 beads originally designed for ion exchange chromatography are made of dextran matrix bound with DEAE (N,N-diethylaminoethyl group), yielding a net positive charge for cell attachment. This allows a favorable surface area to volume ratio for cell growth. However, the bead concentration capable of supporting growth without any adverse effect of cell detachment was limited to 1 g/L (gram dry weight of bead per liter of medium). Later, it was identified that an apparent toxic effect was attributed to an unfavorable charge density of the beads. By decreasing the charge density from 6.5 as originally found in the DEAE-dextran bead to 1.8-2.0 meq/g, cell growth at higher concentrations of beads was allowed (Levine et al., 1977). Subsequently, these reduced charge DEAE dextran beads were commercially developed (i.e. Cytodex beads).

Following the principle of maximizing surface area for cell growth, the development of macroporous microcarriers was initiated. The introduction of a porous structure inside an existing microcarrier further increases available surface area per unit volume of microcarrier.

1.7.2. General Characteristics of Microcarrier Design

(a) Solid Microcarriers

The success of microcarriers was based on the fact that they comprise features and properties that can be tailored to suit the attachment and growth of cells in different culture systems and culture practices.

- Density: Unlike prokaryotes, fragile animal cells do not have a cell wall to protect themselves from high shear forces under agitation. Desirable agitation should be optimal without causing injuries to the cells and sedimentation of beads. The optimal bead density is 1.03 g/ml (Butler, 1987) to allow bead suspension at low agitation. Beads of higher density tend to settle whereas lighter beads float; both of these characteristics are undesirable.
- Diameter: The surface areas of a spherical bead is a function of its diameter. The ratio of surface area to bead volume is critical in designing the optimal microcarrier. A higher ratio is achieved with a smaller diameter, but the number of cells produced on each bead will be lower as less surface area is available. On the other hand, a smaller ratio results in substantial bead volume with respect to the medium volume, therefore minimizing the capacity for scale-up. In order to maximize the number of cells produced and minimize the volume the beads occupy, a bead diameter of 100-400 μm is found to be suitable.
- Charge: The optimum range is determined to be 1.8-2.0 meq/g. Also, it is found that the polarity of the charge was not a significant determinant of cell attachment (Barngrover, 1986).
- Size Distribution: Distribution of bead size is reported to affect the cell distribution on the beads since small beads tended to attract more cells (Butler, 1987) and resulted in an uneven cell population on the beads.

- Transparency: Since cells grow on the bead surfaces, the transparency of bead assists in microscopic observation. This is important to monitor cell-bead attachment, growth and cell morphology on the beads.
- Rigidity: Since cells have been reported to exert mechanical forces on the beads, they should be strong enough to maintain their configuration during cell attachment and growth. However, cells on rigid beads may be injured in the event of collisions inside the stirred tank bioreactor.

(b) Macroporous Microcarriers

The characteristics of macroporous microcarriers are somewhat different from those of solid microcarriers. These microcarrier may protect cells from undesirable shear forces imposed on the surface in stirred tank bioreactors. Like solid microcarriers, characteristics of density, size distribution and diameter are of primary concern; additionally other parameters need to be optimized in order to support a cell culture at higher cell yields.

Pore size and void fraction are important for successful inoculation and subsequent growth. Pore size should be as big as cells (10-20 μm) to allow them to migrate inwards, interconnecting channels surrounding pores allow medium to pass through. Void fraction denotes the percentage of void space to the total bead volume.

Nature of the beads is important to allow cell attachment. Also, cell harvesting is easier if the matrix of the macroporous microcarriers can be digested by enzymes, thus releasing cells or cell aggregates. As a result, collagen and gelatin (denatured form of collagen) are the mostly commonly used substrates as they are easily digested by trypsin and collagenase. These substances are found naturally in interstitial areas and basement membrane and may be involved in the growth regulation of cells. Furthermore, they are

closely associated with attachment proteins (i.e. fibronectin, vitronectin, and proteoglycans) and carbohydrates (i.e. glycosaminoglycans).

1.7.3. Different types of Microcarriers

(a) Solid Microcarriers

A variety of microcarriers with different materials have been made. Surface chemistry has been modified to suit attachment and growth of specific cell lines, particularly of cells which could not attach to the standard Cytodex 1 beads. In some cases, the bead surface is coated with or whole bead is made of natural materials such as collagen and gelatin. Appendix B lists a number of solid beads and their characteristics.

(b) Macroporous Microcarriers

Several of these beads are in the market. The first macroporous microcarrier was developed by the Verax Corporation as porous matrix particles. These Verax microspheres are made of native collagen with added weight for a fluidized bed bioreactor. Another cross-linked gelatin macroporous microcarrier marketed as Cultispher® G is produced by Percell Biolytica. Other artificial substrates such as polystytrene and cellulose are also used. These substances can be crosslinked with chemicals during manufacture to enhance the mechanical strength especially when the beads are highly porous. Appendix C lists the macroporous microcarriers using different compositions and structure characteristics.

1.7.4. Advantages of Microcarriers

(a) Solid Microcarriers

Microcarriers have several advantages in cell culture technology. These include:

- high available surface area to medium volume ratio.

- Light weight design for easy suspension in medium.
- Surface charged or coated with specific biomaterials and other chemicals (i.e. collagen, gelatin, insulin, and ligands of cell adhesion proteins) for attachment and growth purposes.
- transparency for ease of monitoring in cell-bead attachment, growth, and cell morphology.
- Homogeneity / even mixing to yield better mass transfer of nutrients.
- reduction or elimination of microenvironments (i.e. gradients of nutrients and waste products in vicinity of cells).
- ease of culture sampling and growth monitoring.
- amenable to scale up by increasing unit volume or unit bead concentration.

(b) Macroporous Microcarriers

In addition to those advantages of solid microcarriers, other distinct features of macroporous microcarriers are:

- relative higher surface area to volume ratios than solid beads, therefore high cell yield potentials.
- protection of cells residing into the porous space from hydrodynamic shear stresses caused by bead-bead, bead-reactor components, and bead-microeddy interactions.
- reduced sensitivity to higher agitation speed.
- reduced sensitivity to sparging.
- retention of tissue-like architecture.

1.8. Cell Substrate and Microcarriers for Project Investigation

A transformed cell line (Yasumara and Kawakita, 1963), African Green Monkey kidney cells or commonly known as Vero cells, is selected as the cell substrate in this

project. They have been extensively studied (Levine et al., 1979, Pharmacia, 1981, and Nahapetian et al., 1986, Reuveny and Thoma, 1986, Nilsson., 1989, and Reuveny, 1990) and licensed for vaccine production such as Polio (Montagnon et al., 1984, and van Wezel et al., 1984). Vero cells when attached to a substratum are fibroblastic-like and capable of infinite growth and multilayer formation. They are hypodiploid as 66% of the cells have a modal chromosome numbers below that of diploidity (ATCC, Rockville, MD). Although reverse transcriptase has not been detected in the cells, there are a number of conflicting reports on the tumorigenicity of the cells (Montagnon et al., 1984., van Wezel et al., 1984., Pertricciani, 1987).

The optimization of attachment and growth of a variety of cells of different origin on Cytodex beads is given by Pharmacia, (1981), which shows that 80 cell lines can grow on these beads. On the other hand, studies on the recently developed macroporous microcarrier, Cultispher G are limited. Therefore in this project, solid microcarriers (Cytodex 1) and macroporous microcarriers (Cultispher G) are chosen to study the optimal conditions of Vero cells in cell-bead attachment and growth. The general characteristics of both bead types are given below in Appendix D.

1.9. Aims

The aims of the project are as follows:

1. To study the optimal conditions of different parameters for cell-bead attachment of Vero cells onto Cyt 1 and CuG beads. To study the effect of cell-cell aggregation on cell-bead attachment under CuG and bead-free environments. To assess and compare results in terms of attachment kinetic rates or aggregation rates, bead occupancy, and cell distribution.

2. To study the growth patterns and yields of Vero cells in Cyt 1 and CuG cultures in batch mode under different parameters.

   To study the growth patterns and yields of Vero cells in Cyt 1 and CuG cultures in fed-batch mode at high bead concentrations, and at higher agitation rates (75-100 rpm) on these cultures.

   To study the critical agitation speed (above 100 rpm) in Cyt 1 and CuG cultures at low and high bead concentrations.

3. To determine the feasibility of bead-to-bead transfer of Vero cells in Cyt 1 culture.

4. To examine the possibility of serial subculturing of Vero cells on "spent beads" of Cyt 1, on which cells had previously grown.

Chapter 2. Materials and Methods

2.1. General Materials and Methods

2.1.1. Cell Line

African Green Monkey Kidney cells (Vero), strain CCL81, were obtained from the American Type Culture Collection (ATCC). The cells were received at passage number 121 and frozen in a cryovial containing Medium 199 (M199) supplemented with (+) 10% Fetal bovine serum (FBS) and 10% Dimethyl sulphoxide (DMSO). Immediately upon receipt, cells in the cryovial were thawed rapidly in a 37°C water bath and subsequently diluted with 10 ml of M199 + 5% FBS or Dulbecco's modified Eagle's medium (DME) + 5% calf serum (CS) in a centrifuge tube (15 ml). They were then centrifuged at 1000 rpm for 5 minutes (min). After the supernatant was discarded, the cell pellet was resuspended with 10 ml DME + 5% CS and inoculated into a 25 cm^2 tissue culture flask (T25).

New cells were passaged in tissue culture flasks at least 3 times before they were used for experiments. The cell line was tested periodically for mycoplasmal infection by Rh Pharmaceuticals (Biotechnology Division) Inc., Winnipeg. All tests proved negative.

2.1.2. Basal Medium

The basal medium used for cell growth was Dulbecco's modified Eagle's medium (DME) containing 25 mM (4.5 g/L) glucose and 4 mM (0.58 g/L) glutamine. This produced higher cell yields than other media tested such as RPMI 1640, DME/F-12 in 1:1 (v/v), M199 and Iscove's modified-DME media in T25 cell growth experiment. All media were supplemented with 5% calf serum (CS). Calf serum was preferred to fetal bovine serum for economic reason. Also, no significant difference in growth performance of cells between two sera was observed in batch T25 cultures.

2.1.3. Cell Subculturing

Stock cells were cultivated in T-flasks, T75 or T150, with 35 or 50 ml of DME + 5% serum (v/v), respectively. When cells reached approximate 75 % confluence (maximum achievable cell density) after 3 days, the spent medium was removed and cells were washed twice with Dulbecco's phosphate buffer saline (PBS) in 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA). Afterwards, a 1 or 2 ml of 0.25% Trypsin (w/v)/ 1 mM EDTA was added to T75 or T150, respectively. The T-flasks were then incubated at room temperature for 3 to 5 minutes until cells showed signs of detachment. Cell harvest was assisted by tapping the T-flasks to release all the cells from the surface. Then, cells were resuspended in 9 ml of culture medium by aspirating a few times inside the T-flasks before transferring to a centrifuge tube. The cell suspension was centrifuged at 1500 rpm for 5 min. After the removal of the supernatant, cell pellet was resuspended with complete medium (5-7 ml). A split ratio of 1/7 to 1/10 was used for each subculture, that is, taking approximately 1 ml of cell suspension (total 7 to 10 ml) and adding it into a new T-flask with 35 or 50 ml of complete medium. This resulted in an inoculation density of 0.1 $x10^6$cells/ml. The inoculated T-flasks were swirled to distribute the cells in the medium, before putting into an incubator, in which the atmospheric composition was 90% air with 10% CO_2. The same procedure was repeated when cells reached the third day or high confluence on the surface of the T-flasks.

A similar protocol was used to inoculate spinner cultures.

2.1.4. Cell Counting Methods

(a) Haemocytometer (Improved Neubauer)

The haemocytometer had two identical chambers for manual cell counting. Cell / nuclei counts were based on the number of cells / nuclei present in 4 corner squares. The cell density (cells/ml) was calculated as the total number of cells / nuclei divided by 4 and multiplied by 10^4 and any dilution factor on the sample.

(b) Trypan Blue Exclusion

Trypan blue dye is used to assess the viability of a population of cells. It is based on a functional test of the permeability of cell membrane (Cook and Mitchell., 1989): Non-viable cells which are most likely to have damaged cell membrane and therefore greater permeability to extracellular substances become blue. Viable cells with an intact membrane exclude the dye. Therefore, cells appear in blue may be compromised in membrane integrity and therefore considered non-viable. The viability (%) is expressed as a ratio of the number of viable cells to the total number of cells times 100 (Sigma, 1994).

For stock cells, 0.25-0.5 ml cell suspension was transferred to an eppendorf tube (1.5 ml) and mixed with 0.5-0.75 ml of 0.4% (w/v) Trypan blue in PBS (pH 7.2) to make up the total volume to 1 ml. Resuspension of the mixture by gentle aspiration or shaking was done before cells were applied onto the haemocytometer.

The exact procedure of Trypan blue exclusion method on Cyt 1 and CuG beads is given in section (Appendix E)

(c) Crystal Violet Stain (Nuclei count)

Sanford et al, (1951) assessed cell density by counting the nuclei released from the lysed cells after the addition of a hypotonic crystal violet stain mixture, which was made up of 0.1% (w/v) crystal violet in distilled water with 0.1 M citric acid and 1% (v/v) Triton X-100. Crystal violet stain was only conducted on bead sample. A 1 ml bead sample was added to an eppendorf tube which had 0.9 ml medium supernatant removed. The stain mixture (0.9 ml) was added to the sample to make up the total volume to 1 ml. The sample was incubated at 37^{o}C for an hour with occasional shakings. At the end, the sample was aspirated with a pipette to release nuclei. The released nuclei were applied onto the haemocytometer and counted as were cells. The expression of cell density from nuclei counts was identical to that in section 2.1.4. (a) as the number of nuclei were

regarded to be equivalent to the number of cells. However, viability assessment can not be done with this stain method.

(d) Comparison of two cell counting methods

Cell enumeration by Crystal violet and Trypan blue exclusion methods was inconsistent (Appendix F).

2.1.5. Microcarrier Preparation

Approximately 500 ml of fresh PBS at pH 7.3 was added to 10 g (dry weight) of each bead type (Cyt 1 and CuG) in siliconized glass bottles (500 ml). The beads were allowed to rehydrate overnight with occasional shakings. The PBS was removed the following day and the beads were washed once with fresh PBS. Finally, the beads were soaked in PBS to a total volume of 300 ml, making the bead concentration at 10 g/300 ml. The bottles were then sterilized by steam autoclaving at 121°C for 20 min.

Originally, it was reported by the manufacturers that CuG bead number was 2.5 $x10^6$ /g (Hyclone, 1992b) and Cytodex 1 was 4.3 $x10^6$ /g (Pharmacia, 1991). Thus an equivalent bead number in cultures was established in spinners by using 1 g/L Cyt 1 and 1.72 g/L CuG. However, subsequent information released (Hyclone, 1994) indicates the CuG bead number at 0.9 $x10^6$ /g. Therefore, the cell/ bead inoculum ratio was changed from 10 cells/bead for both bead types to 10 and 28 cells/bead for 1 g/L Cyt 1 and 1.72 g/L CuG bead respectively.

2.1.6. Spinner Cultures

(a) Spinner Modifications

Both 100 ml and 250 ml spinner bottles (Bellco) were used. 100 ml spinners were modified prior to use. Firstly, the length of the paddle blades (L X W dimension: 5 X 2

cm) was reduced by 2 to 3 mm on both ends to avoid any direct contact with the interior wall of the spinners during stirring. Secondly, the space between the paddle and the shaft was filled with silicone sealant to prevent the inclusion of beads during stirring. No modifications were made to the 250 ml spinners, since they had a greater internal diameter which allowed greater clearance of the paddle.

(b) Experimental Setup

The spinners were siliconized using Sigmacote® (Sigma) and left to evaporate overnight. The siliconized spinners were then rinsed with distilled water before the cap assembly, including the stirring component, and screw caps for the side arms were put on. The shaft was set at a small distance (2-5 mm) above the convex structure on the bottom of the spinners. Spinners were autoclaved at 121°C for 20 min with 10 ml of distilled water or PBS in the bottom to produce an inner steam pressure.

The liquid was decanted from the spinners before use. Bead suspension and medium at 37°C were added to the spinners to make up the total volume to 100 ml. After PBS-EDTA washing, cells were released from T-flasks by trypsinization. The cell density and the viability of the cell suspension were determined before cells were inoculated into the spinners. The spinners were then put onto a Bellco's remote stirrer module (7766-10009) which was magnetically driven inside the incubator. The screw caps on the side arms were loosened to allow gas diffusion from the incubator's atmosphere.

(c) pH maintenance and Refeeding

Since the buffer system by HCO_3^{-1}/CO_2 was weak, cultures reaching high cell densities became acidic through the production of lactic acid. A small quantity (0.1-0.2 ml) of 2N NaOH was added to cultures periodically to maintain the pH in the range of 7.1-7.3.

For some of the cultures, a medium refeeding regime was performed by letting the beads settle on the bottom and then replacing 50% of the top spent medium with fresh complete medium.

(d) Effect of spinner size on growth

Maximum cell density was influenced by the size of spinner used (Appendix G).

2.1.7. Medium Preparation

Fresh double distilled reverse osmosis water was used to dissolve basal DME powder (Cat.no. 430-2100). Glucose (4.5 g/L), glutamine (0.58 g/L), and phenol red (15 mg/L) were added. The mixture was allowed to stir in a designated bucket until all the chemicals were completely dissolved. Then, sodium bicarbonate at 3.7 g/L was added. pH of the medium was adjusted to 7.2-7.3 by the addition of 2M hydrochloric acid (HCl). The medium was sterile filtered and dispensed into sterile 500 ml glass bottles using a peristaltic pump (Watson-Marlow) and a silicone tubing through a 0.2 μm cutoff sterile filter (Millipore). The freshly made medium batch was kept at 4°C and away from light source in a walk-in refrigerator.

2.2. Materials and Methods for Cell Attachment Experiments

2.2.1. Culture Conditions

The effects of stirring mode, agitation, pH, serum concentration, inoculum ratio and bead concentration on attachment of Vero cells to Cyt 1 and CuG beads were determined by measuring the free cell concentrations with times in the attachment phase. Standard conditions were established for Cyt 1 and CuG cultures to test for cell attachment. Both Cyt 1 and CuG cultures were set at 5 g/L bead concentration. DME medium + 5% calf serum (v/v) in spinners was equilibrated to pH 7.3 and allowed to stir at 40 rpm during the experiments except as noted.

Beads were directly dispensed into spinners without any medium and serum washings. Cell clumps are defined as those with 3 or more cells aggregating together. The cell inocula used had cell clumps ranging from 5 to 20% of the total populations whose size was between 3 to 5. Cyt 1 cultures were stirred continuously at 40 rpm while CuG cultures were allowed to stir intermittently at 40 rpm. Due to a difference between Cyt 1 and CuG in the number of beads per gram basis (see Table 1.8.1.), each CuG culture would be inoculated with approximately 4.7 times more cells/bead than that of Cyt 1 cultures under the same inoculum level.

For experiments conducted on stirring mode, some CuG cultures were established at 1.72 g/L and stirred continuously or intermittently. They were primarily tested for the effect of continuous stirring at different speed (30, 40, 75, and 100 rpm) and of intermittent stirring at 40 rpm. On the other hand, cultures at 5 g/L CuG were mainly tested for the effects of different parameters on cell-bead attachment under intermittent stirring.

For pH experiments on both bead types, 25 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) was used to replace 3.7 g/L sodium bicarbonate in DME to maintain pH range, pre-incubation was not required. Additionally, DME with

HEPES had an extra 1.4 -1.5 g/L NaCl added to adjust the osmolality to 310-320 mOsm/kg and 10 M NaOH to adjust pH to set range. During the experiment, all screw caps were tightened in the spinners to prevent CO_2 diffusion.

For serum concentration experiments, pre-incubated beads (Cyt 1 or CuG) were made by stirring fresh beads in spinner with DME + 10% serum (v/v) at 40 rpm for an hour. Subsequently, the beads were collected into a centrifuge tube, which had been previously siliconized to prevent bead sticking. The beads were allowed to settle, the supernatant was withdrawn and replaced with 15 ml of DME medium. Gentle shaking was made to mix the beads and medium, the medium supernatant was later removed after the beads had been centrifuged at 1500 rpm for 1 min. Prior to cell inoculation, the whole contents were transferred to a new spinner containing DME medium without serum.

For microcarrier-free cultures, the procedure was the same. All cultures were supplied with DME + 5% serum (v/v) equilibrated to pH 7.3 and stirred continuously at 40 rpm.

2.2.2. Culture Sampling and Counting

After spinners were inoculated with cells and put on a magnetic driven stirrer module, time intervals were noted. For every time interval, the spinners were allowed to stop stirring and the beads settled by gravity. For CuG cultures immediately after each stirring period, CuG beads were allowed to settle. Within 1 to 1.5 minutes, 20 µl of the supernatant sample was withdrawn from each culture in microcarrier-free supernatant zone and transferred into an eppendorf tube (1.5 ml). Also, microcarrier samples (0.1-0.2 ml) were taken for microscopic observations of cell attachment onto the beads or cell aggregate formation in the background. Immediately, the spinner cultures were put back into the incubator and time intervals were noted again.

The counting process was aimed at the assessment of the disappearance of free cell concentrations in the background. Cells were microscopically counted with the use of the haemocytometer (Improved Neubauer). Both cells and cell aggregates within the 9 squares under a grid layout were scored. At least 100 cells were scored in each count except at the very late attachment phase at which the cell concentrations were very low. The variation for majority of counts was 10 to 15% in the early time points.

The method of measuring cell aggregation could not be done accurately using conventional cell haemocytometer counting, as cell aggregates were not presented evenly inside the counting chambers. Instead, cell aggregation was accessed indirectly by measuring the free cell concentrations at different times. It is based on the idea that the decrease in free cell concentrations in a bead-free culture was due to cell aggregation. Therefore, the rate of cell aggregation was measured and expressed as the rate at which single cells were converted to cell aggregates. In other words, cell aggregation rates were equal to cell disappearance rates that were also used to represent cell attachment rates in Cyt 1 and CuG cultures. Upon sampling, spinners were taken out and swirled gently before 0.5 ml of medium supernatant containing cells was pipetted out. Since cell aggregates were defined as 3 or more cells associating together, their presence in the haemocytometer was ignored during free single cell counting.

The Cell Aggregate Index was used to determine the state of cell aggregation in cultures under the condition tested. The Index was obtained by counting cell aggregates onto a haemocytometer and expressed as the average number of cell aggregates per haemocytometer count (30 µl) ± standard error of the average. A representative sample (0.2 ml) was pipetted out from a culture at 2 h and put into an 1.5 ml eppendorf tube, a 30 µl was taken from the sample after gentle shaking and applied onto the center of the chambers of the haemocytometer. Cell aggregates would be allowed to distribute onto the chamber freely and later counted within the gridded squares. It is assumed that the counts

of cell aggregates would reflect the state of cell aggregation in the spinner culture. The way cell aggregates spreading throughout the chamber was highly influenced by the application, therefore care should be given in comparing the results.

2.2.3. Analysis of Attachment rate, k, (min^{-1})

(a) Measurements of Cell Attachment

The disappearance of free cell concentrations at different times was used to reflect the cell attachment pattern on the beads, therefore the higher the rate at which free cell concentrations were disappeared in the background; the greater the rate at which cells attached onto the beads.

(b) Curve Fitting of data points

The cell concentrations for Cyt 1 cultures at t = 0 were based on the values determined by haemocytometer counting of the inocula. The cell concentrations for CuG cultures were obtained by counting cells collected from each culture immediately after inoculation.

Since data points on the semi-logarithmic scale expressed as free cell concentrations (ordinate) against times (abscissa) appear to yield either (1) linear or (2) biphasic relationships. Two mathematical equations obtained from the SigmaPlot Scientific Graphing Software were selected to model such relationships. The curve fitting equation of single exponential decay, $y = a \cdot e^{-b \cdot x}$ (where a and b are constants and x and y are variables) was used to curve fit data, which appeared as linear lines on semi-logarithmic scale. On the other hand, the equation of double exponential decay, $y = a \cdot e^{-b \cdot x} + c \cdot e^{-d \cdot x}$, (where b and d are constants) was used to curve fit the results that appeared as a biphasic curve on semi-logarithmic scales. Patterns of the data points on graphs were assessed

prior to the selection of appropriate curve fitting equation. For the results, all plots are shown in a linear scale with single or / and double exponential decay curve fitting.

All plots of cell aggregation in bead-free cultures are shown in a linear scale and curve-fitted by the single exponential decay equation.

(c) Calculations of Attachment rate

The attachment rate, k, is calculated from the equation,

$$k = - \ln [(Co-Ca) / Co] / t,$$

where k, is attachment kinetic rate (min^{-1}),

Co, is the initial free cell concentration on the curve at t = 0, (cells/ml),

Ca, is the attached cell concentration on the curve at t = t, (cells/ml), and

t, is the elapsed time (min).

Since Ca is not measured during the attachment phase, the equation can be simplified as,

$$k = [- \ln (Cf / Co)] / t,$$

where Cf, is the free cell concentration on the curve at t = t, (cells/ml).

From plots with - ln (Cf/Co) against t (min), the slopes represented attachment rates, k. Plots with data curve-fitted by single exponential decay appear to be linear, therefore the attachment rates are equal to the slopes at all times, suggesting that the attachment rates are unchanged during the period of measurements. On the other hand, plots with data curve-fitted by double exponential decay are composed of two phases, the first attachment rate, k, is determined from calculating the linear regression of the linear segment (first phase) of plots through the origin. In most cases, the linear segment is found to be 10-15 min long, suggesting that initial attachment rate is maintained for about 10-15 min before it gradually decreases. The attachment rate of the second phase is

usually an order of magnitude lower compared to that of the first phases and therefore not reported.

The b value of both curve-fitting equation is corresponded to the attachment rate. In fact, b of single exponential decay is equal to k, it is because the equation, $y = a \cdot e^{-b \cdot x}$, is identical to, $k = [- \ln (Cf / Co)] / t$, when a, b, x, and y are substituted by Co, k, t, and Cf. However, b of double exponential decay, $y = a \cdot e^{-b \cdot x} + c \cdot e^{-d \cdot x}$, is slightly different from the k. Such variation may be related to the effect of the presence of the second exponential in the equation.

2.2.4. Neutral Red Staining

(a) Preparation of Neutral Red solutions

0.5% (w/v) neutral red stock was made by dissolving Neutral red in warm PBS (pH 7.0). The solution was warmed in a 37°C water bath before being filtered. Fresh 0.1% Neutral red (working stock) was prepared by diluting pre-warmed 0.5% stock with PBS prior to filtration. pH of the solutions was adjusted to 5.0-6.5. Both 0.1 and 0.5% solutions were wrapped with aluminum foil and stored at room temperature.

(b) Neutral Red Staining of microcarrier samples

0.2-0.3 ml of sample was taken from each spinner culture and transferred to an eppendorf tube. Immediately, an equal volume of Neutral red was added. After 15-20 minute incubation at room temperature, the top supernatant was removed to approximate 0.1 ml or near the bead volume and replaced with 1.4 ml PBS to wash the beads. After 5 minutes, the top supernatant was discarded and replaced with another 1.4 ml PBS. For another 15 minutes, the supernatant was removed. During incubation with the stain and PBS washings, occasional swirling of the sample was necessary to resuspend the mixture.

(c) Microscopic observation of stained microcarrier samples

The sample was transferred onto the haemocytometer chamber using a wide-mouth and siliconized plastic tip, which could be made by cutting the top 7-10 mm end of an ordinary pipette tip. Under microscopic observation, the viable cells appeared red on the transparent Cyt 1 beads while they were somewhat obscured from the semi-translucence of CuG beads. The distribution pattern of the cells on the beads and bead occupancy are determined visually through the intensity of the red stain on the surface of the beads.

Therefore, Neutral Red staining was used to assess qualitatively the cell/ bead distribution and semi-quantitatively the bead occupancy on CuG. Bead occupancy was determined by scoring beads with one or more "red spots", or stained cells located on the matrix surfaces at different focal lengths. The opaqueness of CuG beads limited the accuracy of the measurements, if cells were located on the back of the beads where they were obscured.

Pictures shown were photographed under either 40 or 100 time magnifications using a Nikon camera (model F601M) mounted on a phase contrast microscope (Labophot-2, Nikon) with Kodak films (35 mm DX100/21^{o}). The number of stained cell aggregates shown in the pictures did not represent the actual numbers in relation to those of the surrounding beads in the cultures, as some cell aggregates were removed during the PBS washings. Sometimes, at 100X magnification the morphology of the cells on beads can be described.

2.3. Materials and Methods for Growth Experiments

2.3.1. Culture conditions for Cyt 1 and CuG cultures on the basis of 100 ml spinner and Trypan blue (TB) counts

Those batch culture experiments conducted in section 3.4. were based on 100 ml spinners and trypan blue cell counts. Effects of different parameters on growth of Vero cells on Cyt 1 and CuG beads are similar to those studied for cell attachment, they are stirring mode, agitation, pH, serum concentration, inoculum (cell/ bead) ratio, and bead concentration.

For most experiments, medium used was DME + 5% serum (v/v) at pH 7.3. The bead concentrations and inoculum for Cyt 1 and CuG were 1 and 1.72 g/L, respectively, and 5 $x10^4$ cells/ml except as noted. The total working volume was made up to approximately 100 ml. Continuous stirring at 40 rpm was set for all Cyt 1 cultures, while intermittent stirring was established for all CuG cultures at 40 rpm at 3 min/ 30 min for the first 6 hr before switching to continuous stirring. High speed cultures were switched to higher agitation (60 or 80 rpm) on Day 1.

For the experiment of stirring mode on Cyt 1, cultures were set up in 250 ml spinners and cell densities were based on nuclei counts.

2.3.2. Culture conditions for Cyt 1 and CuG cultures on the basis of 250 ml spinners

Conditions for fed-batch experiments conducted differed in spinner size and cell counting method. 250 ml spinner and CV counts were used. DME + 5% serum (v/v) as the basal medium was also used for medium replacement at which 50% of the bulk medium of the spinners was replaced with fresh complete medium. Other culture conditions were same as those reported in 2.3.1. Specific procedures are listed as follows.

(a) Effects of bead concentration and agitation (Fed-batch cultures)

Unlike other cultures at low bead concentration in which bead volume occupied a small fraction (3% at 1 g/L Cyt 1 and 5% at 1.72 g/L CuG) of the total working volume in spinners, fed-batch cultures at high bead concentrations (5 g/L Cyt 1 and 8.6 g/L CuG) required higher strength medium to compensate the high bead volume (15% and 25%, respectively). DME medium (two-fold concentrated) was distributed to 250 ml spinners. $NaHCO_3$ (7.5 g/L), distilled water and serum (v/v) were then added to reconstitute the medium. Beads were supplied in proportion according to the design (Tables 2.3.1a. and 2.3.1b.). Finally, cells were trypsinized from T150 and inoculated at 10 cells/ bead for Cyt 1 and 28 cells/ bead for CuG cultures, which were set at 40 rpm continuous stirring. All cultures was replaced with 50% complete medium (125 ml) since Day 2. Cell densities were based on CV nuclei counts.

Table 2.3.1a. Experimental setup of Cyt 1 cultures at different conditions.

	Bead Concentration			
Compositions (ml)	1 g/L (NF)	1 g/L (F)	3 g/L (F)	5 g/L (F)
Bead	5	5	15	25
2X Medium	119	119	119	119
Serum	11.9	11.9	11.9	11.9
$NaHCO_3$	12.3	12.3	12.3	12.3
Double distilled H_2O	100.5	100.5	88	75.4

N: Non fed cultures; F: Fed cultures

Table 2.3.1b. Experimental set up of CuG cultures at different conditions.

	Bead Concentration			
Composition (ml)	1.72 g/L (NF)	1.72 g/L (F)	5.16 g/L (F)	8.6 g/L(F)
Bead	8.6	8.6	25.8	43
2X medium	119	119	119	119
Serum	11.9	11.9	11.9	11.9
$NaHCO_3$	12.3	12.3	12.3	12.3
Double distilled H_2O	96.9	96.9	77.2	57.4

NF: Non fed cultures; F: Fed cultures

With Cyt 1 cultures at different bead concentration that were set at 70 and 100 rpm, all cultures were initially stirred for 6 hr at 40 rpm before they were switched to higher speed.

(b) Critical agitation speed (Fed-batch cultures)

The agitation regimes for both Cyt 1 and CuG cultures were gradual speed increase and rapid speed increase. All cultures were allowed to be stirred continuously at 40 rpm for 24 h, the high speed cultures were switched to 100 rpm on Day 1. From Day 2, the gradual speed increase regime increased the speed by an increment of 25 rpm/day to 300 rpm on Day 9, while the rapid speed increase regime increased the speed by an increment of 100 rpm/day to 400 rpm on Day 4. Medium replacement was conducted from Day 2 with 50% change of medium volume. Cell densities were based on TB cell counts

(c) Bead-bead transfer of Vero cells on Cyt 1

The experimental setup was based on DME + 5% serum (v/v) in 250 spinners at different bead concentrations, from 1 to 3 g/L. One 1 g/L culture (1 g/L +) was subject to

two additions of fresh beads on Days 5 and 9. Beads were added into the culture at 1 g/L equivalence. Medium replacement started on Day 2 with 50% volume change. The same conditions were used for Cyt 1 cultures at higher agitation speeds. Cell densities were based on TB cell counts.

(d) Serial subculturing on Cyt 1

Two Cyt 1 cultures at 1 g/L were inoculated with 10 cells/ bead and one was subject to trypsinization on Day 4 when cells were near the end of the exponential growth. The protocol of trypsinization was given in Appendix H. Cells along with fresh DME medium were later introduced into the culture to reinoculate the spent beads. Cell densities were based on TB cell counts.

Chapter 3. Results

3.1. Cell Attachment to Cytodex 1 Microcarriers

3.1.1. Introduction

Cell attachment can also be analyzed in three aspects: attachment kinetic rate, the initial rate at which cells attach to the microcarriers, bead occupancy, the percentage of beads that receives one or more cells at the end of the attachment phase; and cell distribution on microcarriers, the distribution of inoculating cells on microcarriers at the end of the attachment phase.

3.1.2. Results

Standard conditions of Cyt 1 cultures were set at 5 g/L beads in 100 ml spinners with DME medium + 5% calf serum (v/v). Cultures were allowed to equilibriate pH to 7.3 and temperature to 37°C through overnight incubation. They were generally inoculated with 10 cells/ bead and stirred at 40 rpm except as noted.

The cell-bead attachment rate, k, was measured with respect to five parameters: pH, agitation, serum concentration, inoculum size and bead concentration. Table 3.1.1. summarizes the k values of cultures under these parameters. Cell-bead attachment was assessed by specific rates (min^{-1}) as well as by measuring the times (T_{50} or T_{90}) taken for 50% or 90% of cells to attach. In this study, T_{50} of the cultures under different parameters are presented in Table 3.1.2..

Bead occupancy during the course of attachment was measured through microscopic examination. All beads (95-100%) were occupied by cells under conditions tested except as noted. Cell distribution patterns were only observed under the microscope and found to be favorable under the conditions that were optimal for cell-bead attachment.

(a) Stirring Mode

Cell attachment to Cyt 1 was compared in an intermittent stirring mode to a

Table 3.1.1. Summary of Attachment kinetic rates, k, ($x10^{-2}$ min^{-1}) of Vero cells on Cyt 1 beads under different parameters [a].

pH	6.8	7.1	7.3	7.8	
k	8.4 ± 0.10	11.2 ± 0.10	10.6 ± 0.10	4.2 ± 0.10	
Speed (rpm)	40	60	80		
k	14.0 ± 0.38	10.7 ± 0.21	4.0 ± 0.23		
Serum Conc. (%)	0	0 [b]	2.5	5	10
k	28.2 ± 0.10	13.1 ± 0.15	11.2 ± 0.36	8.6 ± 0.19	3.9 ± 0.10
Inoculum ratio [c] (cells/bead)	10	20	40	80	
k	1.68 ± 0.10	1.61 ± 0.10	1.68 ± 0.10	1.34 ± 0.10	

Bead Conc. (g/L)	1	3	5
fixed cell/bead ratio [d]	2.0 ± 0.10	6.0 ± 0.15	9.05 ± 0.33
fixed inoculum [e]	3.9 ± 0.15	7.0 ± 0.17	10.2 ± 1.6

[a] Use of either curve fitting equation was based on the appearance of the plots on semi-logarithmic scale, in which plots could be identified as lines or biphasic curves. The double exponential decay equation was used to curve fit most of the plots in Figs.3.1.2a., 3.1.3a., 3.1.4a. (except 10% serum), 3.1.6a., and 3.1.7a. while the single exponential decay equation was used to curve fit the plots in Fig.3.1.5a., the 1 g/L culture at Fig.3.1.6a, and the 10% serum culture in Fig.3.1.4. The cell-bead attachment rate, k, was calculated on the analysis of the linear regression on the linear segment (first phase) of the curves through the origin and expressed as the value ± standard error (SE) in the unit of $x10^{-2}$ min^{-1} (n = 4)

[b] 0% serum represents the beads were pre-incubated with DME medium supplemented with 10% calf serum and followed by a medium washing before being inoculated into a culture with DME medium only.

[c] Cultures were conducted at 1 g/L bead concentration.

[d] The cultures were inoculated with cells according to a fixed ratio of approximately 10 cells/ bead at each bead concentration.

[e] All cultures were inoculated at $25x10^4$ cells/ml regardless of the bead concentration.

Table 3.1.2. Summary of times (min), T_{50}, taken to reach 50% cell attachment of Vero cells on Cyt 1 cultures under different parameters [a].

pH	6.8	7.1	7.3	7.8	
T_{50}	9.6	8	8.4	16.8	
Speed (rpm)	40	60	80		
T_{50}	5	7	24.5		
Serum Conc. (%)	0	0 [b]	2.5	5	10
T_{50}	3	4.8	5.2	7.9	18
Inoculum ratio [c] (cells/bead)	10	20	40	80	
T_{50}	42.8	42.8	43	52	

Bead Conc. (g/L)	1	3	5
fixed cell/bead ratio [d]	28	11.8	8.7
fixed inoculum [e]	24	10.4	4.6

[a] The 50% cell attachment efficiency was determined by the time at which 50% of the inoculum was measured on the curve fit plot.

[b,c,d,e] refer to those in Table 3.1.1.

continuous stirring mode at 40 rpm. Under intermittent stirring at 40 rpm for 3 min every 30 min non-stirring time (3 min/ 30 min), bead occupancy after 2 h was between 80-90% because of poor cell distribution over the beads. In addition, bead aggregates were formed as beads were bridged together by cells. Under continuous stirring at 40 rpm, cultures were shown to have 100% bead occupancy, improved cell distribution, and absence of bead aggregates within first hour. Therefore, continuous stirring was the optimal stirring mode. As a result, all subsequent experiments were conducted with a continuous stirring mode.

(b) Agitation

The effect of the agitation speed on cell attachment was determined in an experiment in which the initial stirring speed was varied from 40 to 80 rpm (Fig.3.1.2a). In a preliminary experiment, it showed that 40 rpm was the minimal speed to achieve complete bead suspension in the spinners without bead settlement. The results indicate that increasing the agitation speed from 40 to 80 rpm caused a decrease in the rate of cell attachment from 14 to 4.0 $x10^{-2}$ min^{-1} (Fig.3.1.2b.). The corresponding T_{50} values were 5 and 24.5 min, respectively. Also, it was shown that average attached cell/ bead ratios were lower in cultures at 80 rpm than at 40 rpm control. For all cultures, attached cells on the beads eventually flattened after 6 h. The bead occupancy was at 95-100% in all cultures regardless of agitation speed tested. This experiment shows that 40 rpm is the optimal stirring speed for maximizing cell attachment rate. All subsequent cultures were therefore stirred at 40 rpm.

(c) pH

The optimal pH for cell attachment to Cyt 1 beads was determined in an experiment in which pH of the culture medium was varied from pH 6.8 to 7.8

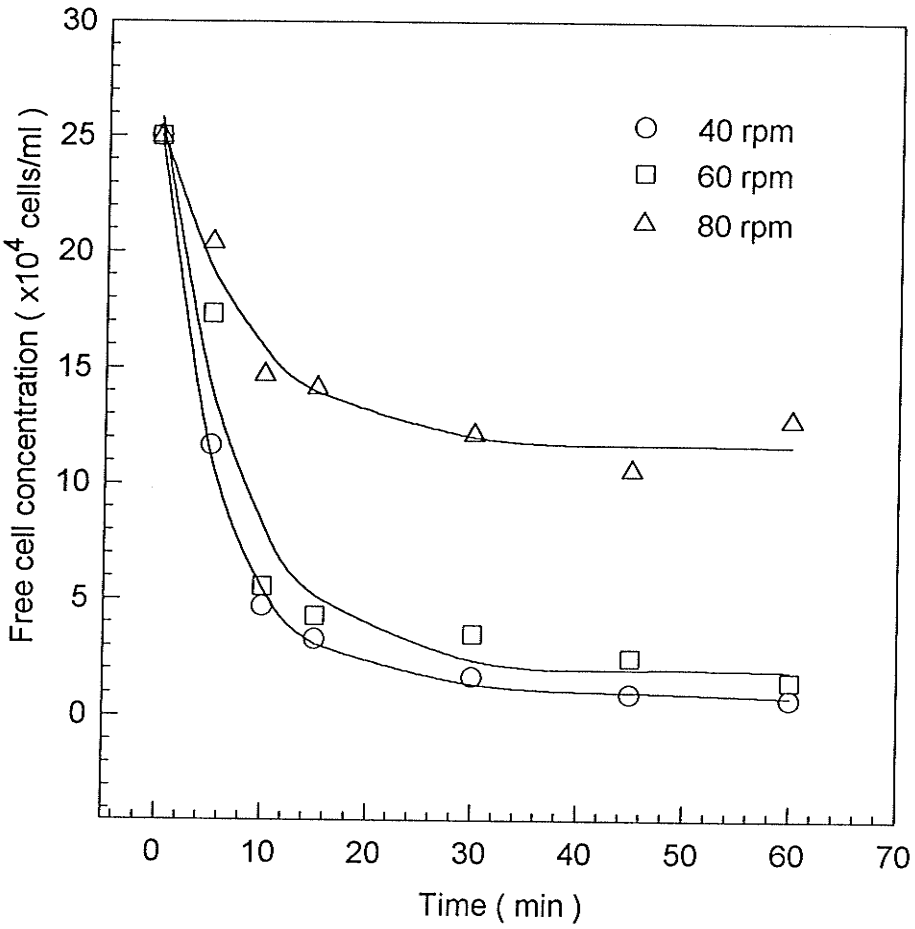


Fig.3.1.2a. Effect of agitation speed on cell attachment onto 5 g/L Cyt 1. All cultures pH adjusted to 7.3 were supplemented with 5% calf serum (v/v). (n = 1)

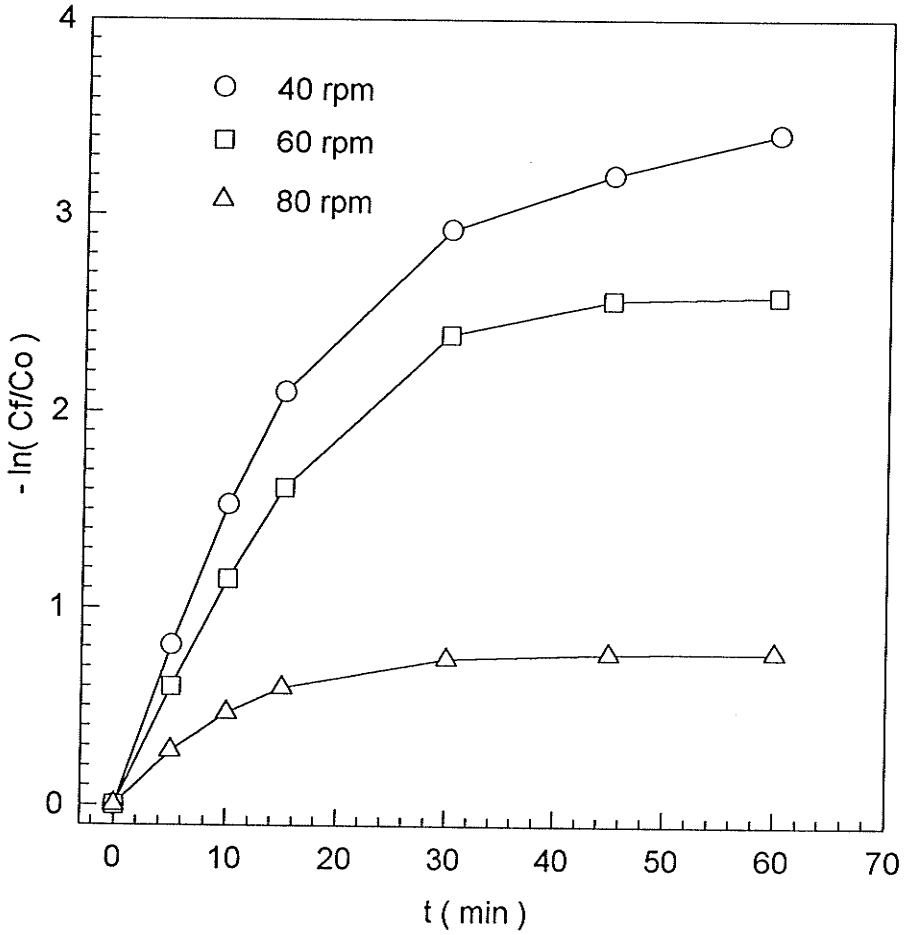


Fig.3.1.2b. Effect of agitation speed on cell attachment onto 5 g/L Cyt 1. Data were taken from Fig.3.1.2a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the linear segment of the plot through the origin. (n = 1)

(Fig.3.1.3a.). pH of each culture was controlled by the use of 25 mM HEPES and adjusted with the use of 10 N NaOH. In Fig.3.1.3b., rapid cell attachment rates, k, are observed at pH 7.1 and 7.3 at 11.2 and 10.6 $x10^{-2}$ min^{-1} respectively, while at pH 6.8 it is relatively slower at 8.4 $x10^{-2}$ min^{-1}. The culture at pH 7.8 not only yielded the slowest rate at 4.2 $x10^{-2}$ min^{-1} but also produced a substantial number of cell aggregates during the attachment phase. As a result, it was observed that the cell distribution on the beads was affected by a wide range of attached cell/ bead ratios. It was not known that whether cell aggregates were the results of either high pH or suboptimal charge density on the bead surfaces. Either explanation could result in greater cell-cell interactions and lower cell-bead interaction. Also, 6 h after inoculation attached cells were observed to remain rounded only on beads at pH 7.8.

The effect of pH on cell aggregation in microcarrier-free cultures is presented in section 3.3.. Cell aggregation rates are compared to the attachment rates of Cyt 1 cultures. It is found that the rates of cell aggregation are 10-fold or more lower than the attachment rates, in excess of ten times.

Alternatively, cell attachment rate could also be measured by assessing times for 50% cell attachment (T_{50}). T_{50} for cultures at pH 6.8, 7.1. and 7.3 were 8-9.6 minutes while it was doubled at pH 7.8. Therefore, this experiment shows the optimal pH to be 7.1 to 7.3. High pH is detrimental to both attachment rates and cell distribution, therefore preincubation or adjustment of pH to desired level is necessary in spinner cultures prior to cell inoculation. pH for subsequent cultures was allowed to equilibriate to 7.3 after overnight incubation.

(d) Supplemented Serum Concentration

The effect of serum supplementation on cell attachment to Cyt 1 beads was determined in an experiment in which the serum concentrations ranged from 0 to 10%

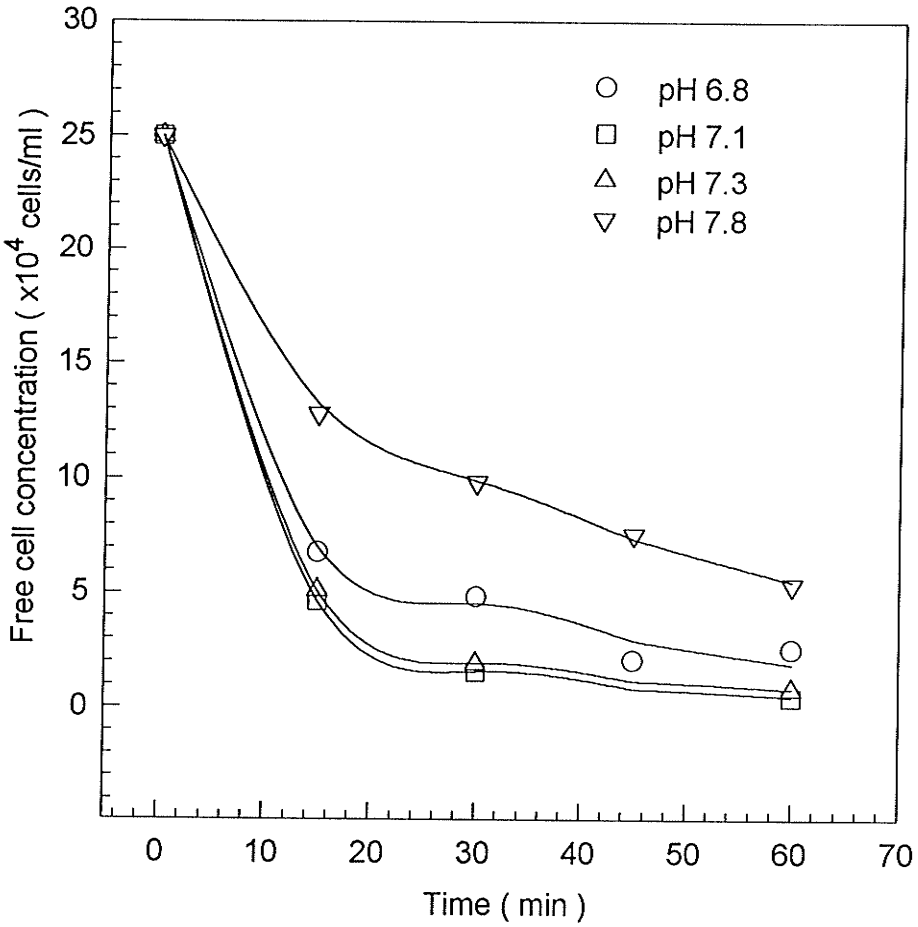


Fig.3.1.3a. Effect of pH on cell attachment onto 5 g/L Cyt 1. DME medium buffered by 25 mM HEPES containing no sodium bicarbonate was pH adjusted by the addition of NaOH. All cultures were supplemented with 5% calf serum and stirred at 40 rpm. (n = 1)

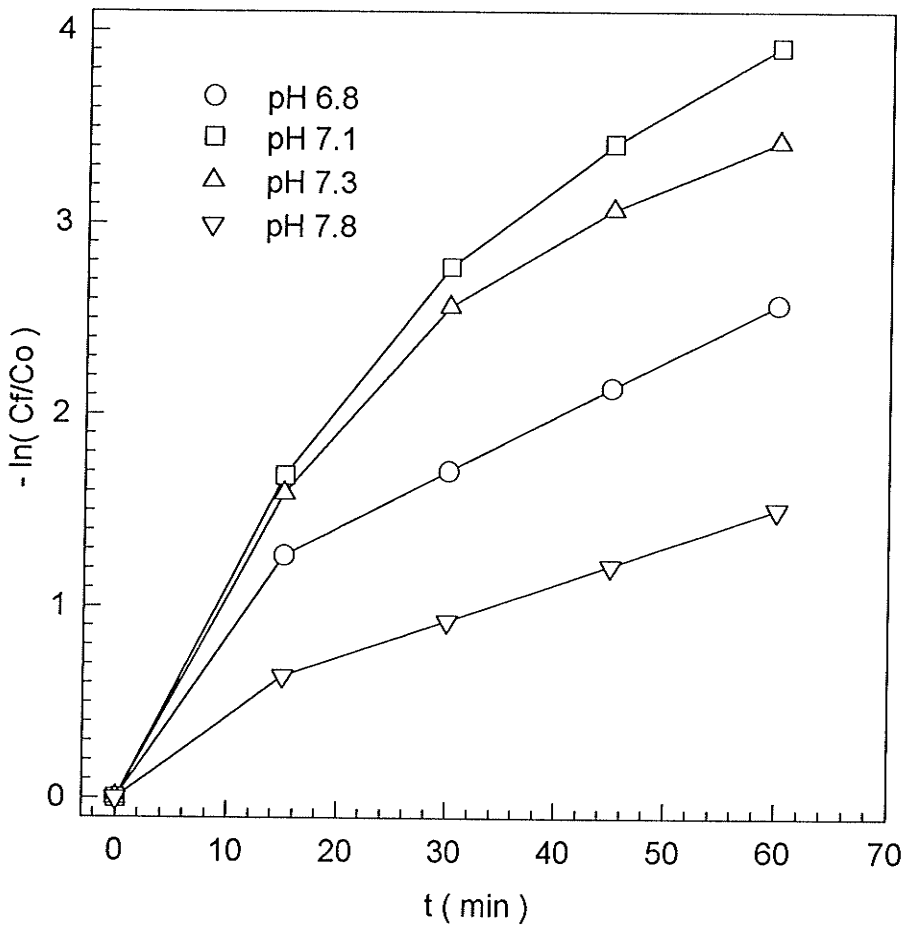


Fig.3.1.3b. Effect of pH on cell attachment onto 5 g/L Cyt 1. Data were taken from Fig.3.1.3a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the linear segment of the plot through the origin. (n = 1)

(v/v) of the medium (Fig.3.1.4a.). It is shown that the initial rates of cell attachment are inversely proportional to the concentrations of serum supplemented to the medium (Fig.3.1.4b.). The k value of the culture at 0% serum was 28.2 $x10^{-2}$ min^{-1} while at 10% serum it was seven time less at 3.9 $x10^{-2}$ min^{-1}. For cultures at 2.5 and 5% the initial attachment rates were at 11.2 and 8.6 $x10^{-2}$ min^{-1} respectively, significantly less than that of the culture at 0% but greater than that at 10%. A culture with beads preincubated with medium + 10% serum prior to experiment at 0% showed a rate at 13.1 $x10^{-2}$ min^{-1} which was similar to that at 2.5%. Under all tested concentrations, optimal cell distribution was maintained. The T_{50} showed a similar trend with shorter time intervals required for cell attachment in cultures with no or low serum concentrations. Therefore, serum concentration at 0% concentration is demonstrated to yield the fastest attachment rate. Despite the negative effect on initial attachment rate, serum contains attachment and spreading proteins important for the spreading of attached cells on the beads. Cells inoculated into cultures with 0% serum showed no or little degree of cell spreading after 6 h.

However, it was noted that there was no advantage of having 0% serum at the early stage, since 90% cell attachment was achieved for all cultures at different serum concentrations in the first hour. On the other hand, serum contains important mitogenic substances that are essential to cell growth. Therefore, in order to simplify the culture conditions for cell attachment and subsequent growth, serum was supplemented in all subsequent cultures at 5% at the time of cell inoculation (see section 3.4.).

(e) Inoculum Size

The effect of the inoculum (cell/ bead) ratio on cell attachment and distribution on Cyt 1 beads was investigated with 1 g/L cultures inoculated at 10 to 80 cells/ bead

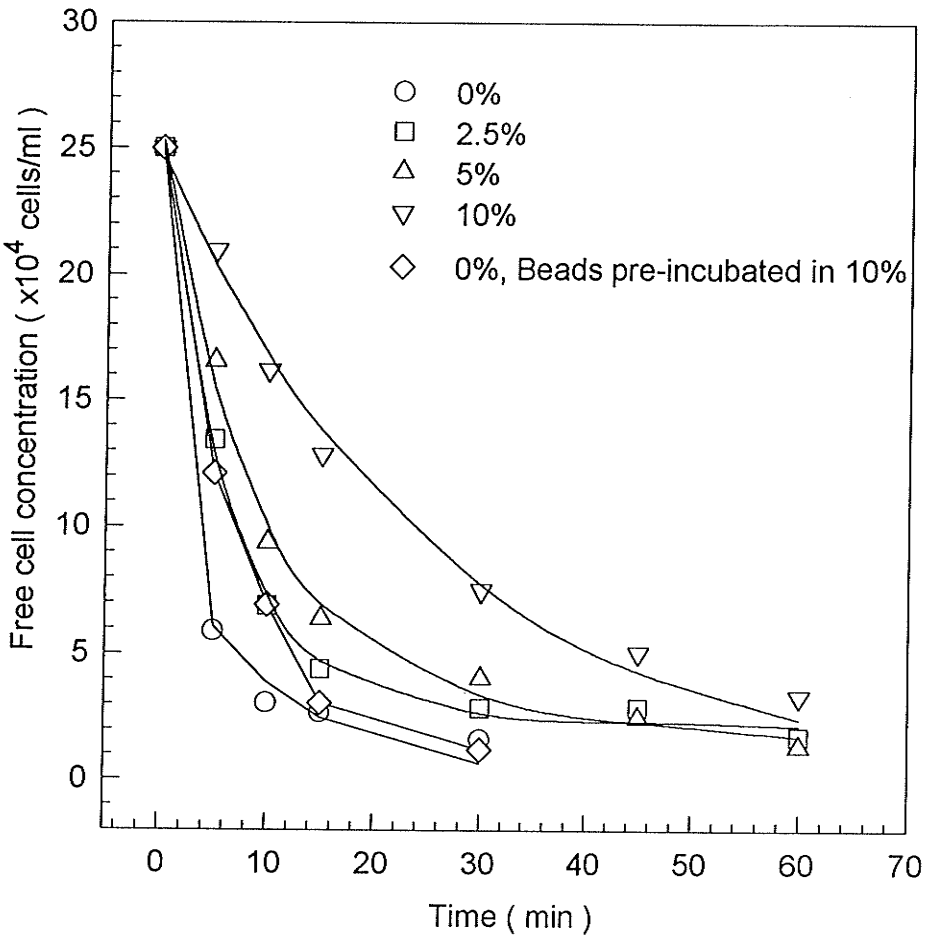


Fig.3.1.4a. Effect of supplemented serum concentration on cell attachment onto 5 g/L Cyt 1. Cultures were pH adjusted to 7.3 and stirred at 40 rpm. Beads in one culture (◊) were pre-incubated for one hour in spinner which had DME supplemented with 10% serum, and after a simple wash with same volume of DME then inoculated into a new spinner with DME only. (n = 1)

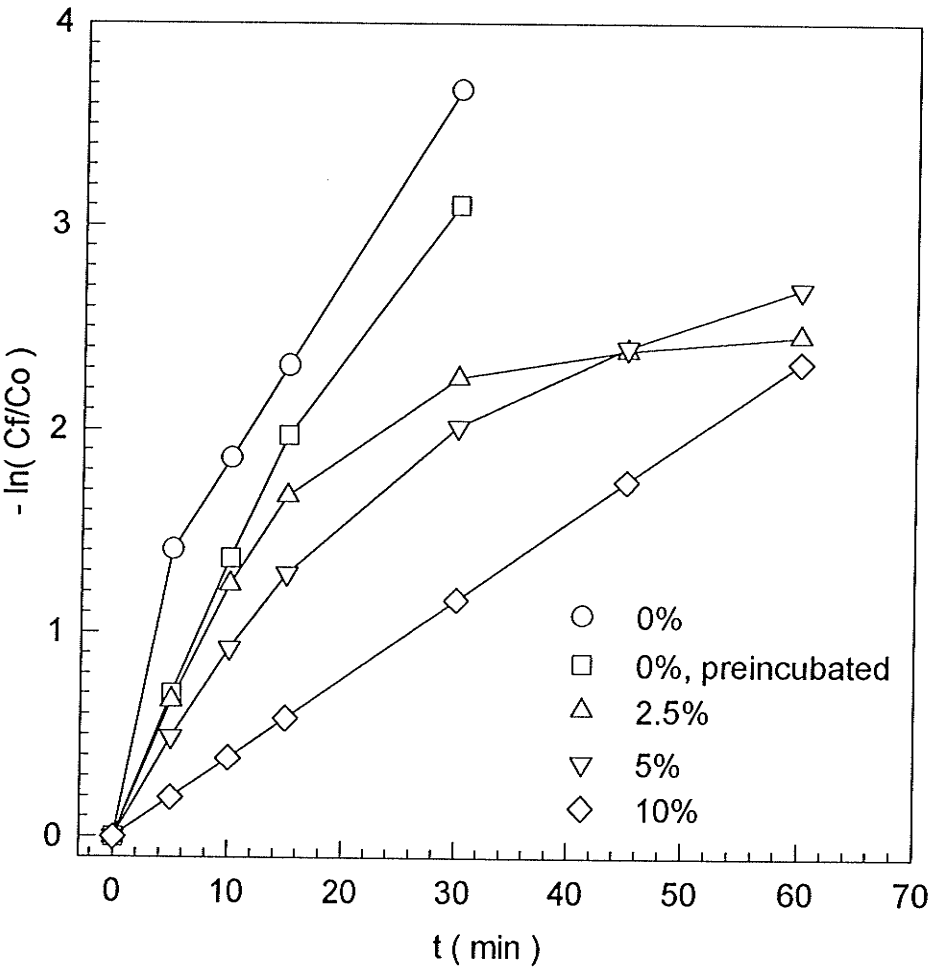


Fig.3.1.4b. Effect of supplemented serum concentration on cell attachment onto 5 g/L Cyt 1. Data were taken from Fig.3.1.4a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the linear segment of the plot through the origin. (n = 1)

(Fig.3.1.5a). In our previous studies, it was determined that both 5 and 10 cells/ bead ratios yielded favorable cell distribution pattern and 100% bead occupancy. 10 cells/ bead was chosen as the standard because there was a short or no lag phase in spinner and Celligen™ bioreactor system (New Brunswick). No significant difference is found in attachment rates of 1 g/L cultures at 1.6-1.7 $x10^{-2}$ min^{-1} having 10, 20, and 40 cells/ bead inoculum ratios (Fig.3.1.5b.). Culture with 80 cells/ bead ratio had a relatively slower rate at 1.34 $x10^{-2}$ min^{-1}. Bead occupancy was 100% and cell distribution was optimal at all inoculum ratios. On the other hand, the T_{50} was about 43 minutes for cultures at 10-40 cells/ bead, while it was 52 minutes for culture at 80 cells/ bead (Fig.3.1.5c.). Therefore, it shows that the ratio 10 cells/ bead is the minimum level of the range tested to yield optimal cell attachment rate and distribution. As a result, 10 cells/ bead was selected as the inoculum ratio for subsequent cultures.

(f) Bead Concentration

The effect of bead cocentration on cell attachment was determined on cultures with 1, 3 and 5 g/L Cyt 1 microcarriers. Bead concentration was tested under two conditions: (a) the cell/ bead ratio was constant at 10 cells/ bead. Each culture therefore had a different initial inoculum (Fig.3.1.6a.), and (b) the inoculum level was fixed at 25 $x10^4$ cells/ml. Each culture had a different cell/ bead ratios (Fig.3.1.7a).

Under condition (a), the results indicate that the rate of cell attachment for 5 g/L culture is the highest at 9.05 $x10^{-2}$ min^{-1}, the rates for 3 and 1 g/L cultures are at 6.0 and 2.0 $x10^{-2}$ min^{-1}, respectively (Fig.3.1.6b). The corresponding T_{50} under condition (a) for 1, 3, and 5 g/L cultures were 28, 11.8, and 8.7 minutes, respectively (Fig.3.1.6c.). 95-100% bead occupancy and optimal cell distribution was achieved. In fact, the attachment rate was increased proportionally to the bead concentration. For instance, the attachment rates

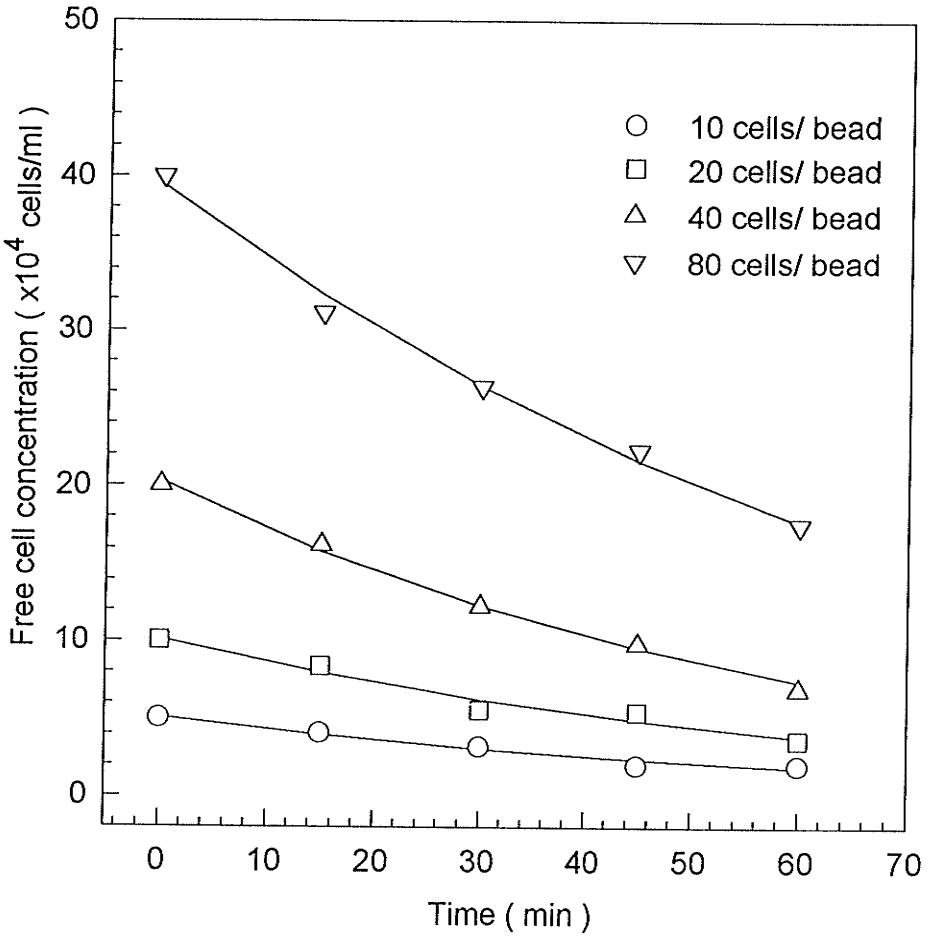


Fig.3.1.5a. Effect of inoculum ratio on cell attachment onto 1 g/L Cyt 1. All cultures were pH adjusted to 7.3 and supplemented with 5% calf serum and stirred at 40 rpm. (n = 1)

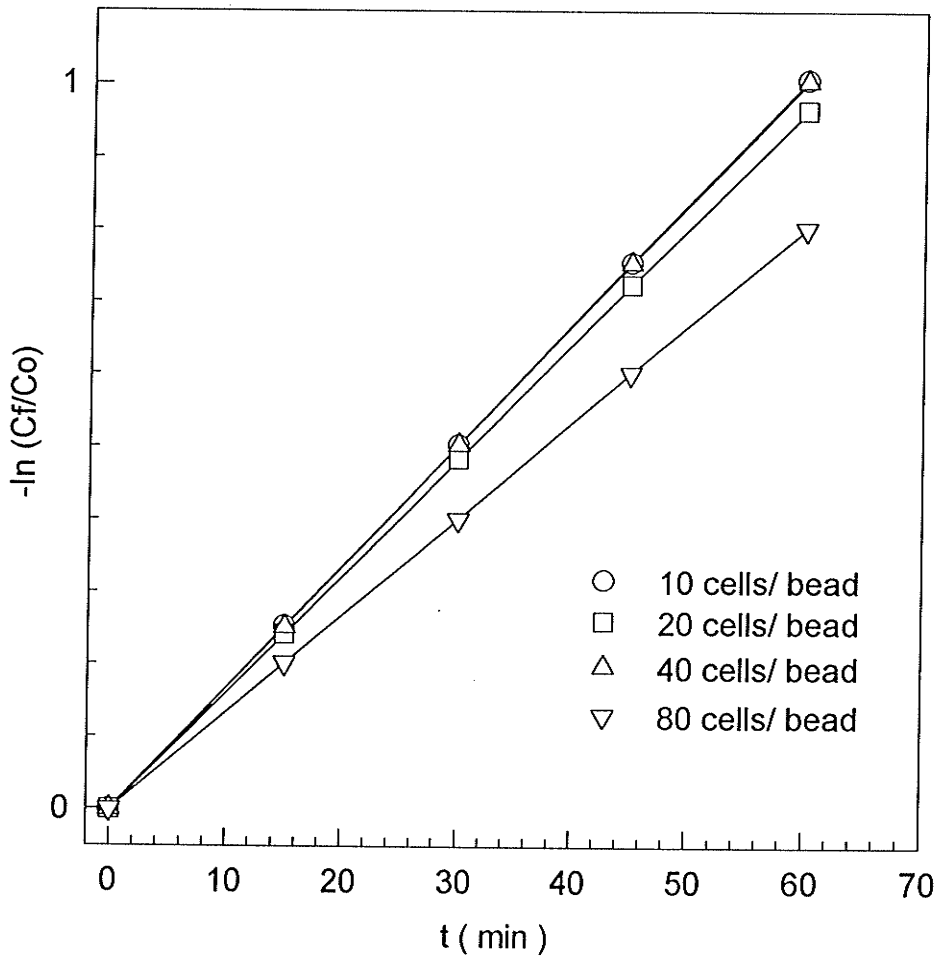


Fig.3.1.5b. Effect of inoculation ratio on cell attachment onto 1 g/L Cyt 1. Data were taken from Fig.3.1.5a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression on slope of the plot through the origin. (n = 1)

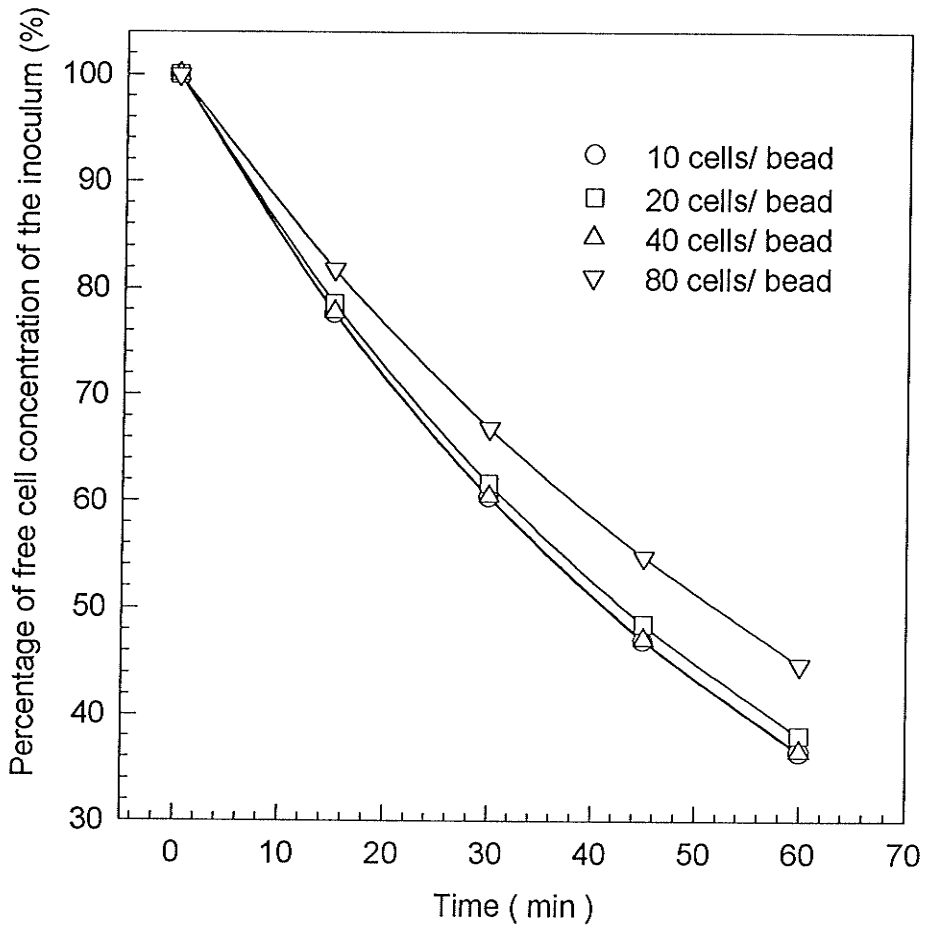


Fig.3.1.5c. Effect of inoculum ratio on cell attachment onto 1 g/L Cyt 1. Free cell concentrations are expressed as percentages of inoculum and plotted against time. Conditions were same as those in Fig.3.1.5a. (n = 1)

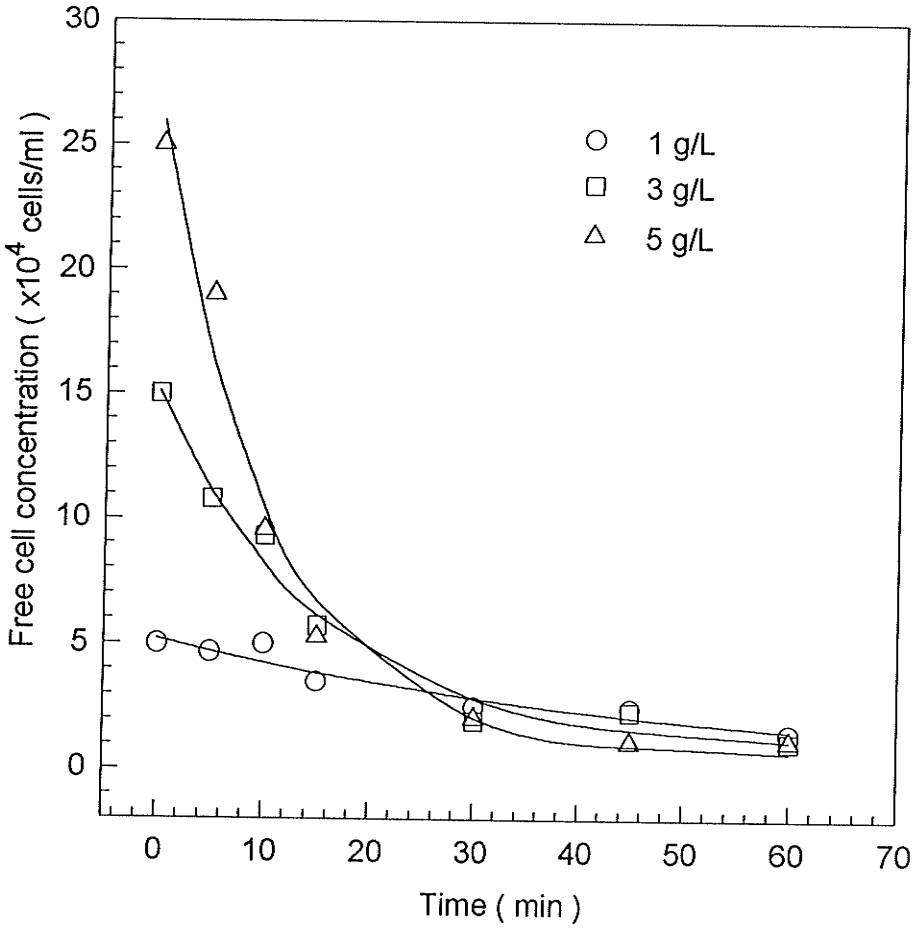


Fig.3.1.6a. Effect of bead concentration on cell attachment onto Cyt 1. All cultures were inoculated with 10 cells/ bead, therefore having different inoculum levels. Medium at pH 7.3 was supplemented with 5% calf serum (v/v) and stirring was set at 40 rpm. (n = 1)

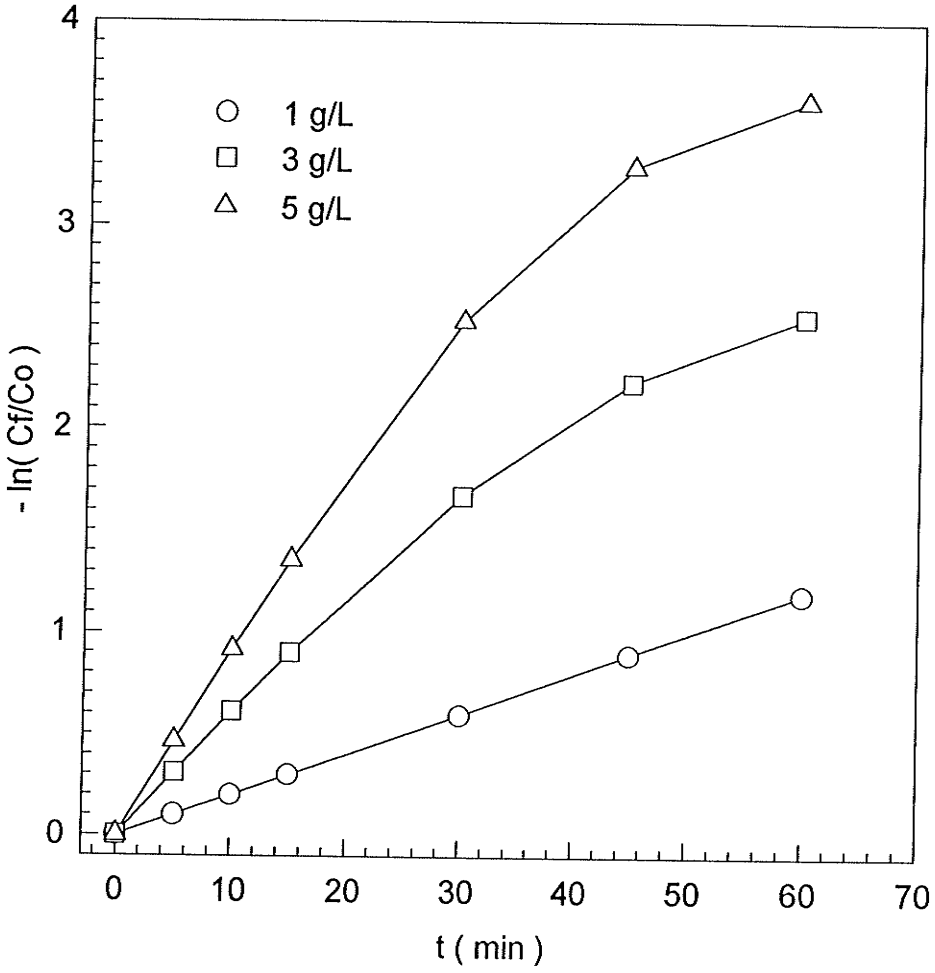


Fig.3.1.6b. Effect of bead concentration on cell attachment onto Cyt 1. Data were taken from plots in Fig.3.1.6a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the linear segment of the plot through the origin. (n = 1)

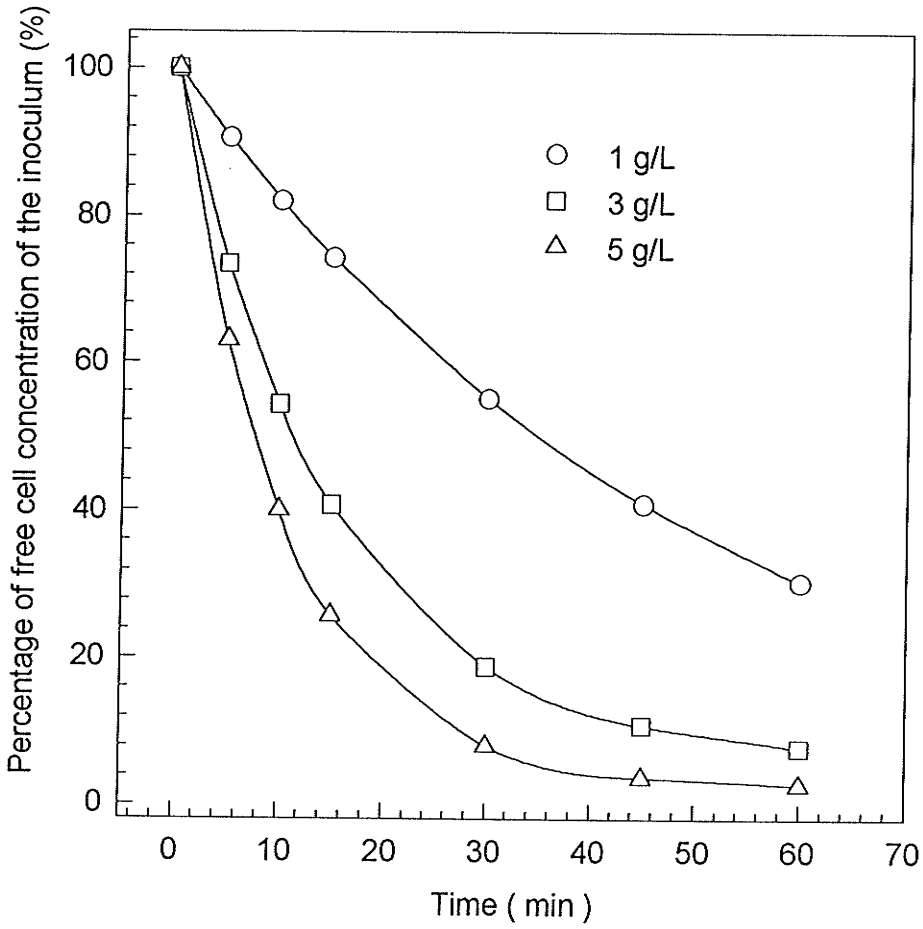


Fig.3.1.6c. Effect of bead concentration on cell attachment onto Cyt 1 beads. Free cell concentrations are expressed as percentages of the inoculum and plotted against time. Conditions were same as those in Fig.3.1.6a. (n = 1)

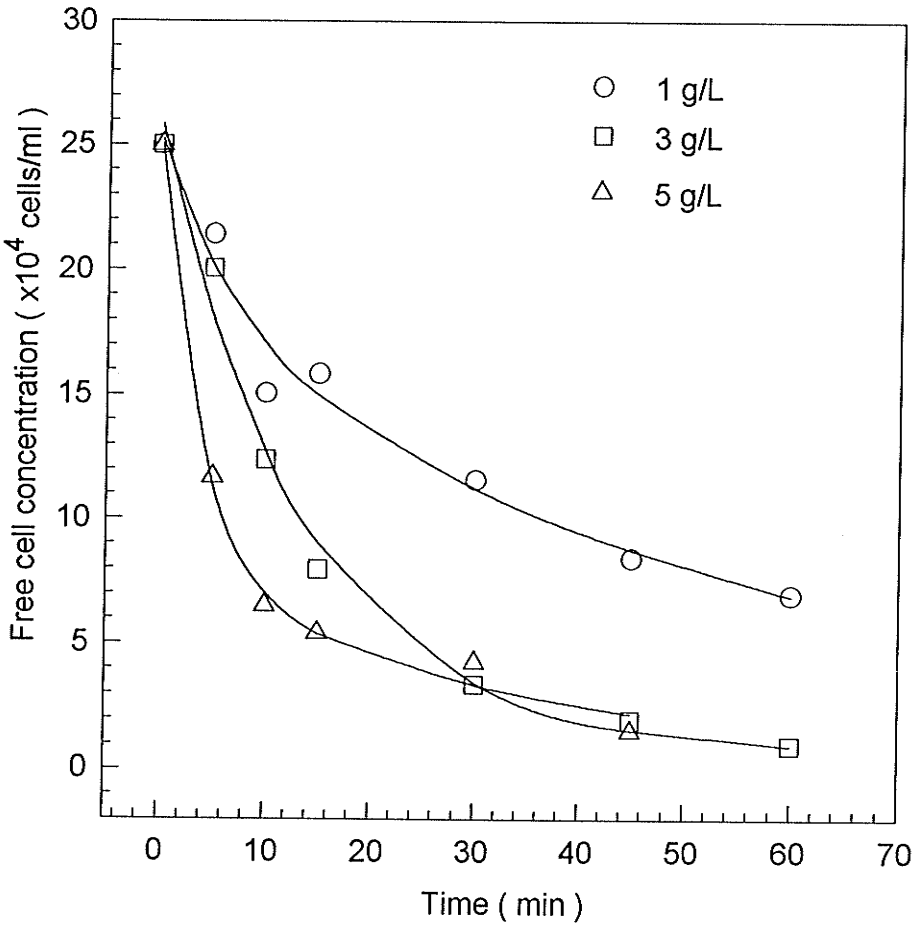


Fig.3.1.7a. Effect of bead concentration on cell attachment onto Cyt 1. All cultures were inoculated with the same inoculum level at different bead concentrations, therefore yielding different inoculum cell/ bead ratios. Medium at pH 7.3 was supplemented with 5% calf serum (v/v) and stirring set at 40 rpm. (n = 1)

of Cyt 1 culture at 1 g/L were 3 and 5 times lower than those at 3 and 5 g/L, respectively. Even though the cell/ bead ratio was kept the same in cultures at different bead concentrations, there was greater chances of cells coming into contact with beads at higher bead concentrations when there were more cells and beads being mixed in spinners. 5 g/L culture therefore has the highest collision frequency and attachment rate followed by 3 and 1 g/L cultures.

Under condition (b), the results show that the rate of cell attachment for 5 g/L culture was at 10.2 $x10^{-2}$ min^{-1}, while the rates for 3 and 1 g/L cultures are at 7.0 and 3.9 $x10^{-2}$ min^{-1}, respectively (Fig.3.1.7b.). Indeed the same order of attachment rates was found as those under condition (a). Cell-bead attachment rates here were limited by the available bead surfaces. The corresponding T_{50} for 1, 3, and 5 g/L cultures were 24, 10.4 and 4.6 minutes, respectively. 95-100% bead occupancy and optimal cell distribution were achieved.

Both conditions (a) and (b) suggest that the highest cell attachment rate is obtained at 5 g/L culture.

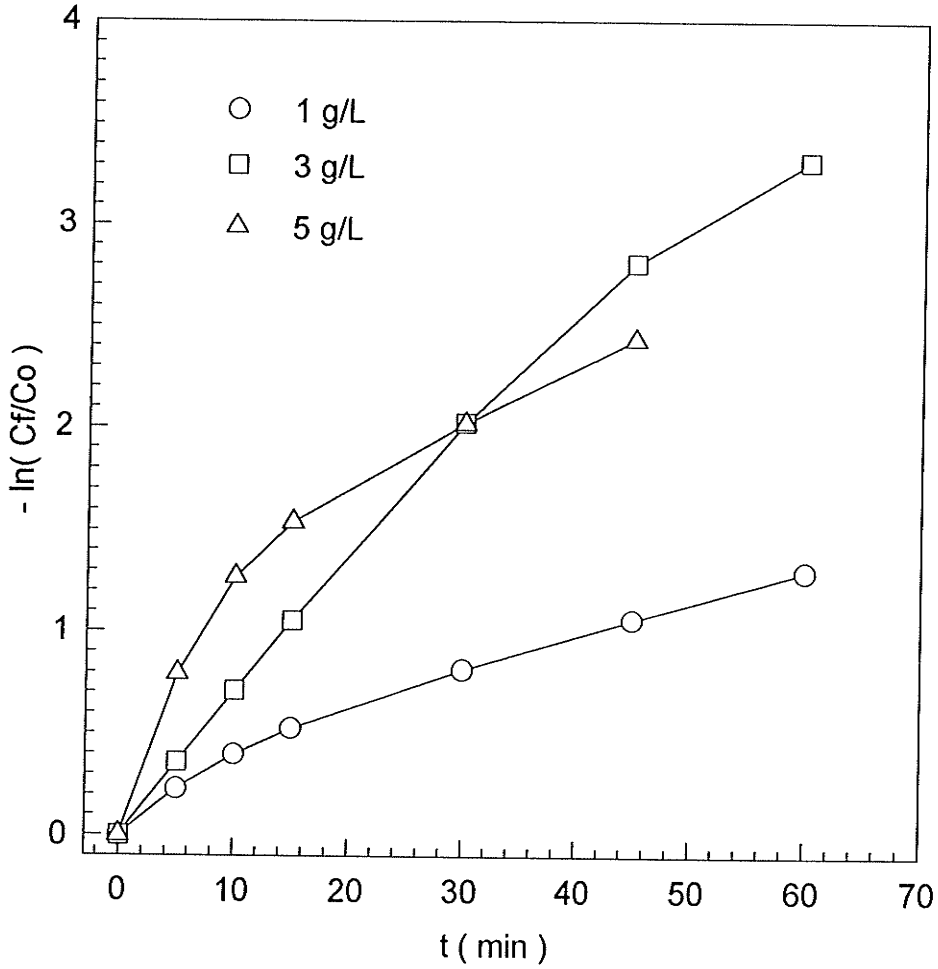


Fig.3.1.7b. Effect of bead concentration on cell attachment onto Cyt 1. Data were taken from Fig.3.1.7a. and plotted as - ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the linear segment of the plot through the origin. (n = 1)

3.1.3. Discussion

Some of the following assumptions about cell-bead attachment were made during the attachment phase in this study.

- The inoculum consisted of single cells with no substantial cell aggregation.
- Cell growth was insignificant during the attachment period.
- Cells and microcarriers were well suspended in the spinners upon stirring.
- Every cell had the chance to attach to microcarriers through cell-bead contacts.
- Detachment and reattachment of cells would not occur after cell-bead attachment was successful.
- Cell aggregation did not occur.

Since cell aggregation during the course of cell-bead attachment could affect the cell/ bead distribution on microcarriers, a population of non-aggregated cells was critical at the beginning of the attachment phase. In this study, the inoculum consisted mostly of single cells, some as doublets, and few as triplets as observed under a microscope (see Fig.3.3.1a. in section 3.3.). Cell aggregates which were defined as three or more cells were found to be less than 5-20% of the total cell population. Therefore, the cell inocula in the experiments were composed of non-aggregated cells. Furthermore, for all conditions tested in Cyt 1 cultures, it was found that cells remained mostly single (<20%) during the course of the experiments as determined by microscopic observations.

When single cells disappeared from supernatants, they were presumed to be attached onto the microcarrier surface. Himes and Hu, (1987) reported that disappearance of suspension CHO cells corresponded to cell attachment on DEAE-charged microcarriers. Therefore, cell attachment to beads was measured by the rates of disappearance of free cells from supernatants in the cultures and were calculated as cell-bead attachment rates.

(a) Stirring Mode

Clark and Hirtenstein, (1981) reported that using intermittent stirring improved the attachment of cells onto Cyt 1 beads. However in our studies, intermittent stirring yielded poor cell distribution and bead occupancy. This effect was likely to have been caused by differential settling velocities of cells and beads.

(b) Agitation

Higher agitation speed would result in greater hydrodynamic shear stress (Cherry and Papoutsakis, 1988, and Croughan and Wang, 1989). The results indicate that the net effect of increasing speed resulted in a decrease in attachment rates. This effect was minimal at 60 rpm but significant at 80 rpm. It was because cell-bead contacts would be reduced at higher speeds. Furthermore, the detrimental effect of the high agitation speed would be more significant to the already attached cells which remained rounded. Therefore, initially those rounded cells having least attachment bonds with the bead surfaces may be damaged or removed from the surfaces under increasing stress.

(c) pH

pH influenced cell-bead attachment in Cyt 1 cultures. Both the surfaces of the beads and cells were charged by opposite polarity and therefore attracted to each other. Charge on Cyt 1 beads originated by derivatizating the dextran matrices with DEAE (Diethylaminoethyl), which when combined with free hydrogen ions (H^+) became protonated and therefore positively charged. Such a protonation process is shown to be affected by pH (Himes and Hu, 1987). The charge density on Cyt 1 beads was reported to be 1.5 meq/g (Pharmacia, 1981), changes of pH in physiological range may affect the charge interactions between cells and beads. At higher pH the charge density was low (Himes and Hu., 1987) and a lower attachment efficiency was obtained (Forestell et al.,

1992). Therefore, charge density of Cyt 1, which was affected by pH, determines the cell-bead attachment.

Our results confirmed previous studies by Pharmacia, (1981) and Nilsson, (1989), showing pH 7.1 to 7.3 was optimal for cell attachment.

(d) Supplemented Serum Concentration

Some cells require the presence of attachment factor(s) to facilitate attachment and subsequent spreading on a substratum. Serum as a universal source of attachment proteins was reported to affect significantly the kinetic rate of cell attachment onto charged-beads (Himes and Hu, 1987). The results reported in our experiments indicated serum hampered cell attachment despite its content of attachment proteins. Mukhopadhyay et al., (1993) reported that binding of serum proteins significantly lowered the charge density on bead surfaces, therefore attributing to slower attachment rate. The competition between serum proteins (attachment specific and non-specific) and cells for binding sites on bead surfaces could explain the observed effect of serum in lowering cell attachment rates.

(e) Inoculum Size

Inoculum (cell/ bead) ratio was determined as an important factor to obtain an optimal cell distribution (including bead occupancy) as well as the cloning efficiency (ability of cells to survive and multiply into colonies) on microcarriers (Hu et al., 1985a). Assuming the cell distribution is governed by Poisson distribution in the attachment phase, a higher inoculating cell/ bead ratio in a culture will lead to a lower frequency of empty beads. Cell/ bead inoculum size commonly ranged from 5 to 100. It was found by Butler and Thilly, (1982) found that 7 cell/ bead would be the minimum level without incurring significant number of empty beads for MDCK cells, therefore higher bead occupancy. Our optimal inoculum ratio was 10 cells/bead.

(f) Bead Concentration

Increasing bead concentration provides more surface area and thus increases the chance of cell-bead contacts. Similarly, increasing cell/ bead inoculum ratios increases the chance of cell-bead contacts. With increase in frequency of cell-bead contacts, cell-bead attachment rates were correspondingly increased.

The effects of agitation speed and microcarrier concentration were closely related to growth (Croughan et al., 1988). Forestell et al., (1992) had found agitation speed higher than 40 rpm and bead concentration higher 5 g/L could reduce the average attached cell per bead at the end of attachment phase.

3.1.4. Conclusions

Levine et al., (1979) reported that optimizing the charge densities on beads would improve cell attachment and growth. Not surprisingly, charge interactions are the underlying mechanism of cell-bead attachment. The most influential parameters in our study affecting the overall cell attachment efficiency are pH and speed, these respective physiochemical and physical factors may directly or indirectly influence the charge interactions and contact frequency between cells and beads. Serum represented the respective biochemical factor to actively stabilize and complete a full cell attachment. Although there was a significant difference in cell-bead attachment rates under most conditions tested, 90% of cells were attached with bead occupancy at 95-100% after 60 min (T_{90}) (Figs.3.1.2a., 3.1.3a., and 3.1.4a.). This suggested that cell-bead attachment could occur over a wide range of culture conditions.

The variations found in some of the cell attachment rates (Table 3.1.1.) under identical conditions indicate that the process is most likely subject to changes especially from the state of the cells at which the experiments are conducted. Cells had undergone trypsinization which could affect the surface membrane differently each time, therefore

leading to a different membrane characteristics. Therefore results were compared from cultures that received the same inoculum.

It is concluded that the optimal conditions for Vero cell attachment to Cyt 1 cultures are when cultures are stirred continuously at 40 rpm, in DME without serum supplementation at pH 7.3, with an inoculum ratio of 10 cells/ bead and 5 g/L bead concentration. However, these conditions for maximizing cell-bead attachment are not necessarily the optimal conditions for growth (see section 3.4.).

3.2. Cell Attachment to Cultispher G Microcarriers

3.2.1. Introduction

There have been few reports on the slow attachment rate of cells on gelatin based microcarriers (Wissemann and Jacobson, 1985, Tao et al., 1987, and Nikolai and Hu, 1992) . In the work presented here, attempts were made to determine the optimal attachment conditions of Vero cells on CuG under different parameters.

3.2.2. Results

All results were curve fitted by the single exponential decay equation, suggesting that the specific attachment rate was constant throughout the time period of measurements. A simple neutral red staining procedure was developed to reveal cell aggregates as well as cells on the optically opaque CuG beads, it was also used to aid in measuring bead occupancy and assessing cell distribution on beads. It was reported that neutral red was taken up by viable cells via non-ionic absorption through the cell membrane and revealed on the nucleus (Modha et al., 1993) or appeared in the form of lysosomes in cytoplasm (Allison and Young, 1969). Neutral red revealed that attached cells remained round or less flattened on CuG beads than those on Cyt 1 beads regardless of the serum concentrations. Unlike the chemically toxic MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) recommended by Hyclone, (1994) for staining cells, neutral red was simpler, easier, safer, and faster to use.

The cell-bead attachment rate, k, was investigated with respect to six parameters: Stirring mode, agitation, pH, serum concentration, inoculum ratio and bead concentration. Bead occupancy and cell distribution were also assessed microscopically. T_{50} and T_{90} were used to study the cell-bead attachment. The results are summarized as attachment rates and T_{50} in Tables 3.2.1. and 3.2.2., respectively.

Standard conditions of CuG cultures were set at 5 g/L beads in 100 ml spinners

Table 3.2.1. Summary of Attachment rates, k values ($x10^{-2}$ min^{-1}) of Vero cells on CuG beads under different parameters [a].

Stirring Mode	Intermittent	Continuous
k	0.864 ± 0.10	0.546 ± 0.10

Intermittent Stirring Regime [b]	3 min/ 15 min	3 min/ 33 min	3 min/ 66 min
k	0.679 ± 0.10	0.828 ± 0.10	0.917 ± 0.10

Speed (rpm)	40	60	80
k	0.854 ± 0.10	0.700 ± 0.10	0.610 ± 0.10

pH	6.8	7.1	7.3	7.8	8.0
k	0.646 ± 0.10	0.802 ± 0.10	0.866 ± 0.10	0.857 ± 0.10	0.874 ± 0.10

Serum Conc. (%)	0	0 [c]	2.5	5	10
k	0.877 ± 0.10	1.14 ± 0.10	0.817 ± 0.10	0.827 ± 0.10	0.767 ± 0.10

Inoculum ratio (cells/bead)	14	28	56	112
k	0.744 ± 0.10	0.755 ± 0.10	0.844 ± 0.10	0.952 ± 0.10

Bead Conc. (g/L)	1	3	5
fixed cell/bead ratio [d]	0.552 ± 0.10	0.668 ± 0.10	1.02 ± 0.10
fixed inoculum [e]	0.829 ± 0.10	0.865 ± 0.10	0.927 ± 0.10

[a] The single exponential decay equation was used to plot all the graphs shown. The cell-bead attachment rate, k, was calculated on the analysis of the linear regression on the linear segement (first phase) of the curves through the origin and expressed as the value ± standard error (SE) in the unit of $x10^{-2}$ min^{-1} (n = 4).

[b] Intermittent Stirring Regime: the figure before the slash refers to the time in which the cultures was allowed to stir, whereas the figure after the slash refers to the time the culture remained stationary. 3 minute was chosen as the stirring time for all cultures.

[c] 0 % serum represents the beads in that culture preincubated with DME medium supplemented with 10% calf serum and followed by a medium washing before being inoculated into a culture with medium only.

[d] The cultures were inoculated with cells according to a fixed ratio of approximately 56 cells/ bead at each bead concentration.

[e] All cultures were fixed inoculated at 32 $x10^4$ cells/ml regardless of the bead concentrations.

Table 3.2.2. Summary of times (min), T_{50}, taken to reach 50% Cell attachment of Vero cells on CuG beads under different parameters [a].

Stirring Mode	Intermittent	Continuous		
T_{50}	82	na		
Intermittent Stirring Regime [b]	3 min/ 15 min	3 min/ 33 min	3 min/ 66 min	
T_{50}	83	88	78	
Speed (rpm)	40	60	80	
T_{50}	83	101	112	
pH	6.8	7.1	7.3	7.8	8.0
T_{50}	111	87	82	84	80
Serum Conc. (%)	0	0 [c]	2.5	5	10
T_{50}	80	58	84	84	89
Inoculum ratio (cells/bead)	14	28	56	112	
T_{50}	104	88	83	72	
Bead Conc. (g/L)	1	3	5		
fixed cell/bead ratio [d]	132	106	67		
fixed inoculum [e]	83	82	78		

a,b,c,d, and e refer to footnotes in Table 3.2.2.

na not assessed

with DME medium + 5% serum (v/v), spinners were allowed to equilibrate pH to 7.3 and temperature to 37°C through overnight incubation. Cultures were inoculated with 56 cells/ bead and stirred intermittently at 40 rpm for first 6 h except as noted.

(a) Stirring Mode

(i) Continuous Stirring

The effect of continuous stirring at 40 rpm was tested in cultures at 1.72 and 5 g/L CuG. The attachment rate in CuG cultures is at 0.55 $x10^{-2}$ min^{-1} which was significantly lower compared with 9.2-16.2 $x10^{-2}$ min^{-1} in Cyt 1 cultures (Table 3.1.1.). Results suggest that charge interactions were suboptimal for CuG beads and Vero cells.

Under continuous stirring, the shear stress generated would further weaken the existing charge interactions between cells and CuG beads and therefore result in poor attachment rates. High shear stress is generated by a high agitation speed. This is consistent with the fact that cultures at higher agitation speed (75 and 100 rpm) showed poor cell attachment and bead occupancy (10-35%). In addition, these cultures had persistent cell clumps beyond 48 h.

(ii) Cell aggregation

Under continuous stirring, cell aggregates were formed in the cultures during the attachment phase (Fig.3.2.1a.). The size of the cell aggregates progressively increased in cultures while the number of free cells decreased with time. Cell aggregation continued beyond 12-16 h after inoculation. Furthermore, some cell aggregates became compact in appearance at 24 h after cell inoculation (Fig.3.2.1b.). At 48 h, however, almost all cell aggregates disappeared. Bead occupancy on day 1 under 40 rpm continuous stirring was variable, ranging from 50 to 75% in all cultures. In addition, there was an uneven cell distribution on the beads. Some beads received 1 to 5 cells/ bead; some were populated

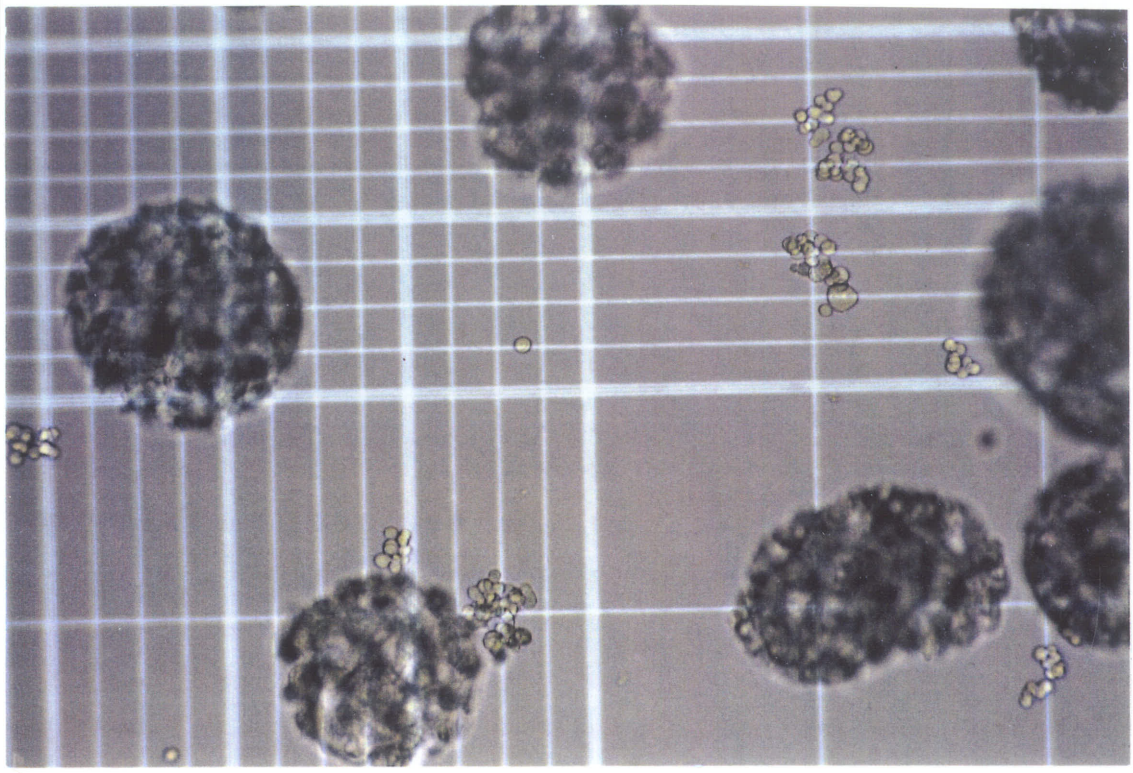


Fig.3.2.1a. Cell aggregates in the background of 1.72 g/L CuG culture stirred continuously at 5 h. Culture was inoculated with 5 $x10^4$ cells/ml and stirred at 40 rpm. Sample was photographed under 100X magnification. Cells were represented by cell aggregates.

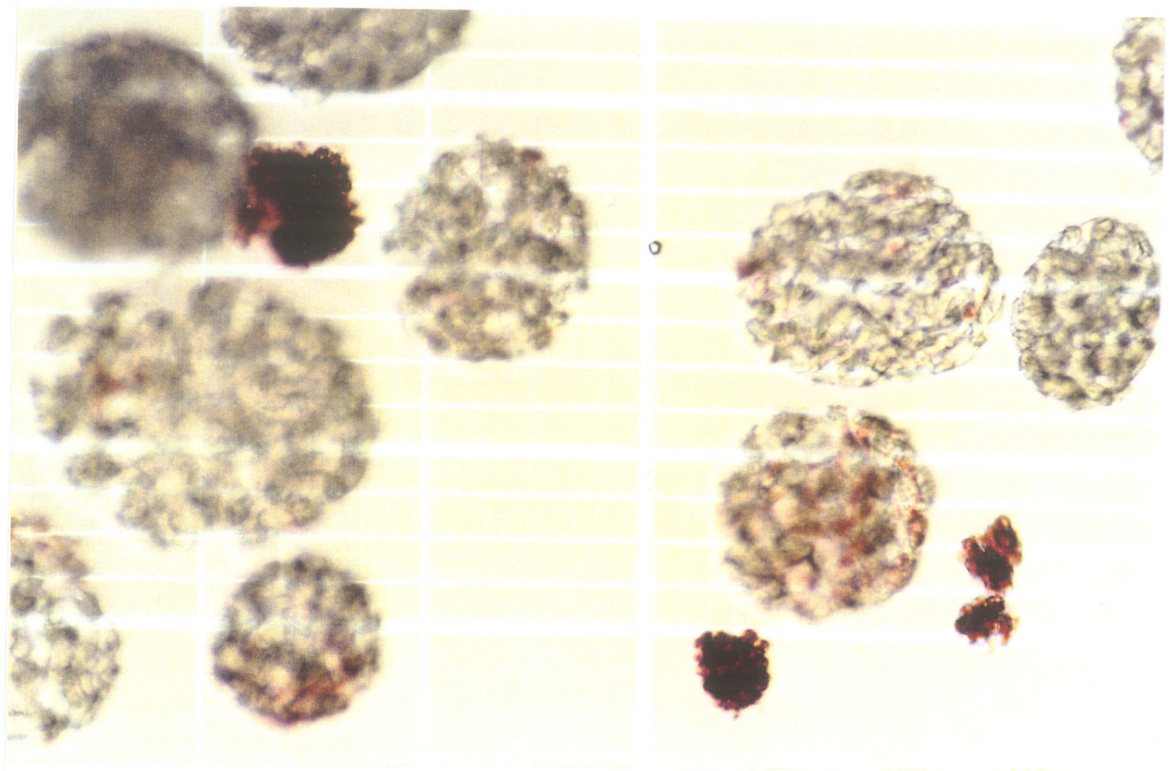


Fig.3.2.1b. Cell aggregates in the background of 1.72 g/L CuG culture stirred continuously at 24 h. Culture was inoculated with 5 $x10^4$ cells/ml and stirred at 40 rpm. Sample was neutral red stained and photographed under 100X magnification. Viable cells are represented by red spots on the beads or cell aggregates.

with significant number of cells, estimated at 50 to 100 cells in the form of cell aggregates attaching and localizing only small regions of the beads (Fig.3.2.2.). Therefore, continuous stirring at 40 rpm was considered suboptimal for cell attachment on CuG. On the other hand, the appearance of cell aggregates was reduced to 24 h after inoculation with cultures at 30 rpm (Fig.3.2.3.), suggesting that attachment of cell aggregates was favored under lower hydrodynamic stress condition. Nevertheless, the disadvantage of 30 rpm is that some beads settled on the bottom of the spinners and adhered strongly to the bottom surface.

Continuous stirring at speeds higher than 40 rpm was attempted to improve cell attachment and reduce cell aggregation. Although smaller cell aggregates were observed, higher speeds (75 and 100 rpm) decreased cell attachment onto CuG beads with only 10-35% beads populated with cells at 24 h (Fig.3.2.4.). As far as cell-bead attachment is concerned, 30 rpm is the optimal continuous stirring speed in spite of poor cell bead occupancy (75-90%) and significant cell aggregate formation within 24 h.

In contrast, cell aggregates were found to be produced in smaller number and size under intermittent stirring with majority of the cells being non-aggregated in CuG cultures, suggesting the stirring mode is an important factor in determining the degree of cell aggregation. Cell aggregates found under intermittent stirring were mostly between 3 to 5 cells although some were larger (5-10 cells).

(iii) Intermittent Stirring

Intermittent stirring was tested in 1.72 and 5 g/L CuG cultures. Cultures were stirred intermittently at 40 rpm for 3 min every 30 min stationary period. In Fig.3.2.5., intermittent and continuous stirring were compared in cultures with 5 g/L CuG beads. The cell attachment rate was much higher at 0.86 $x10^{-2}$ min^{-1} under intermittent stirring as

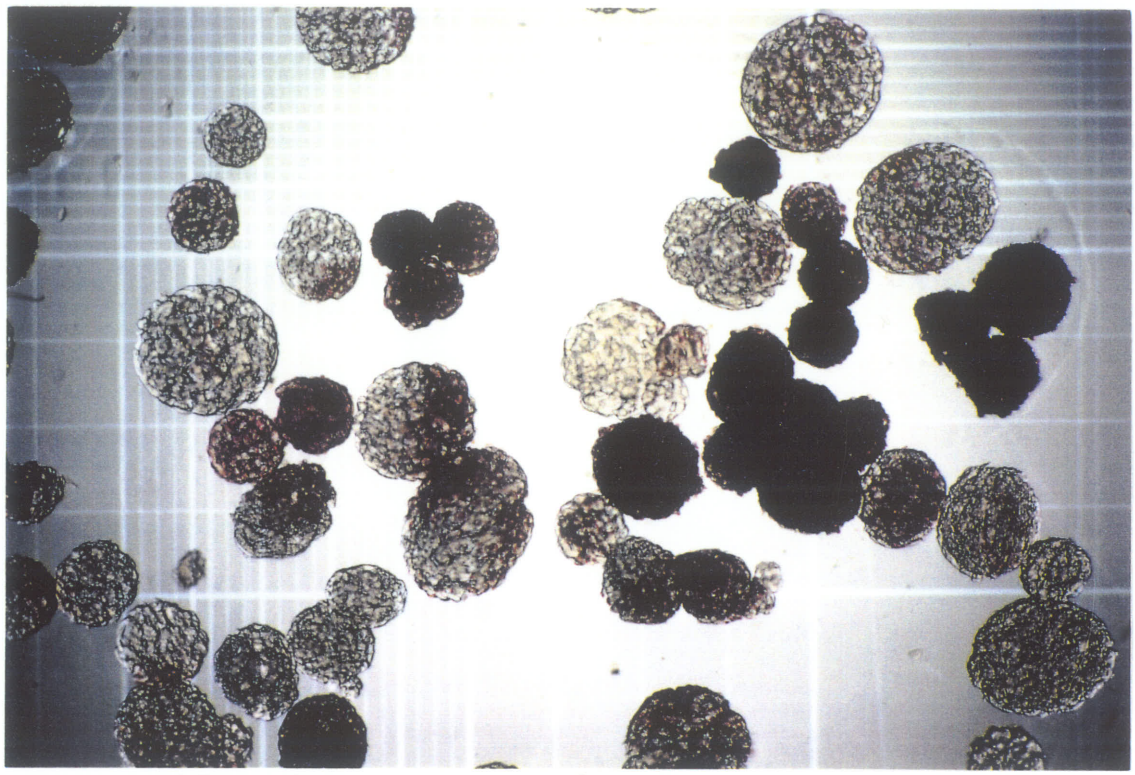


Fig.3.2.2. The attachment of cell clumps on 1.72 g/L CuG beads at 24 h. Culture was inoculated with 5 x10^4 cells/ml and stirred at 30 rpm. Sample was neutral red stained and photographed under 40X magnification.

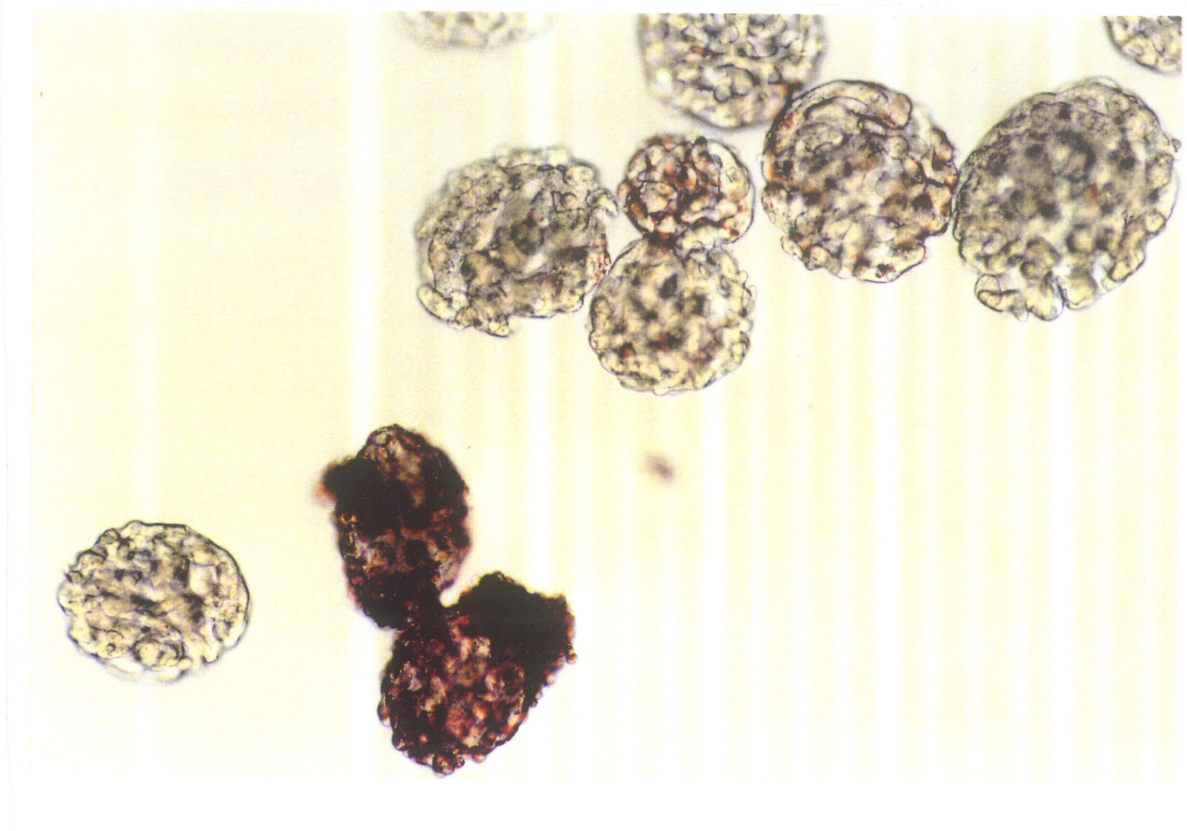


Fig.3.2.3. The appearance of 1.72 g/L CuG continuously stirred at 30 rpm at 24 h. Culture was set up similar to Fig.3.2.2. Absence of cell clumps in the background is also revealed.

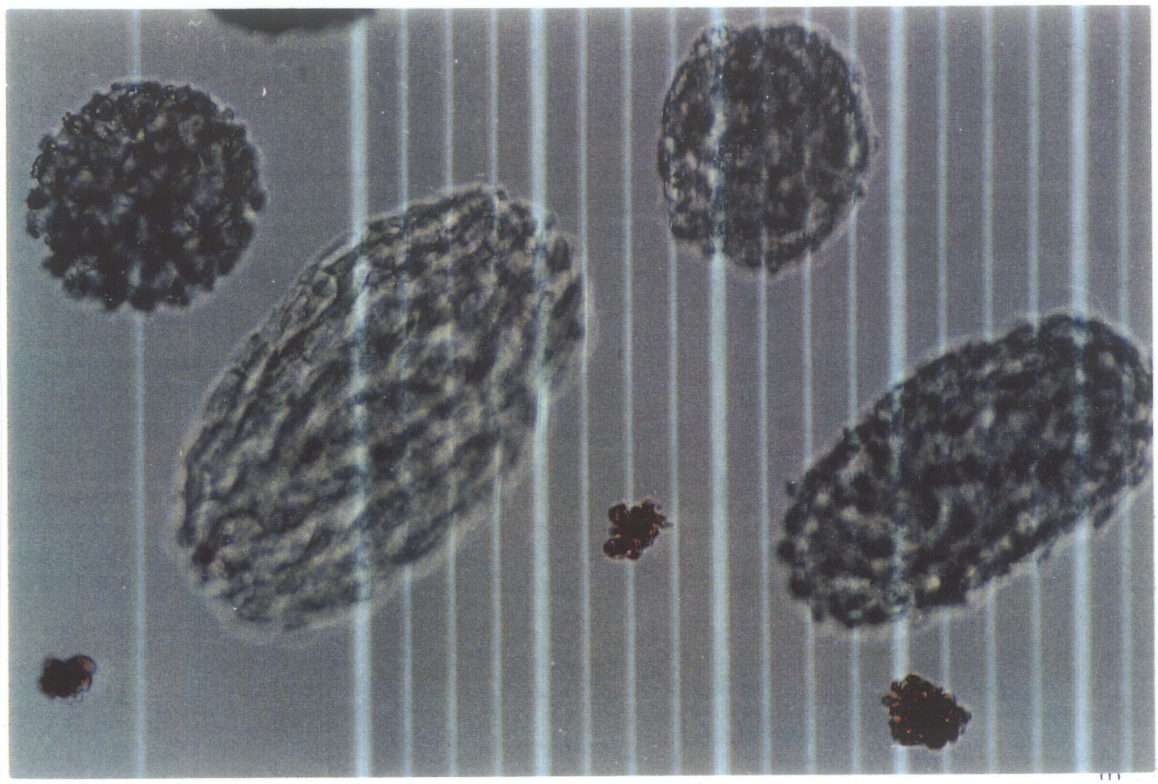


Fig.3.2.4. The appearance of cell aggregates in the background and poor bead occupancy of 1.72 g/L CuG culture at 75 rpm at 24 h. Sample was neutral red stained and photographed under 100X magnification. Culture was set up similar to Fig.3.2.1a.

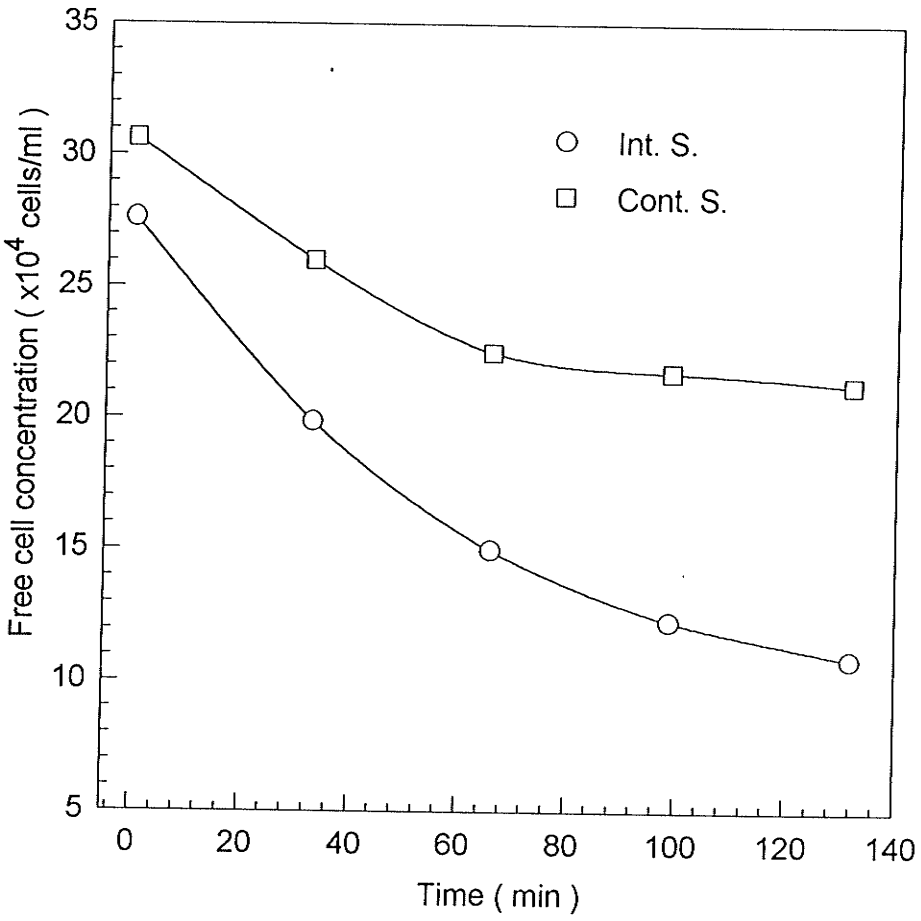


Fig.3.2.5. Effect of stirring mode on cell attachment onto 5 g/L CuG. Cultures were set at 40 rpm under intermittent (Int. S.) or continuous stirring (Cont. S.). Medium was supplemented with 5% serum (v/v) and pH adjusted at 7.3. (n = 1)

opposed to 0.55 x10^{-2} min^{-1} under continuous stirring. Cell/ bead distribution was significantly improved by intermittent stirring compared to all cultures tested under continuous stirring modes. In other words, cells and small aggregates formed were attached and distributed evenly among the beads (Figs.3.2.6a, b, c, and d.). The time for 50% cell attachment (T_{50}) under intermittent stirring was 82 min. Also, the time (T_{90}) for 90% cell attachment was achieved in 1.72 and 5 g/L CuG cultures after 6 h intermittent stirring (data not shown). Therefore, intermittent stirring rather than continuous stirring is considered optimal for cell-bead attachment on CuG beads. As a result, all subsequent CuG cultures was stirred intermittently for the first 6 h.

(iv) Intermittent Stirring Regime

The effect of intermittent stirring regimes on cell attachment and distribution was determined in an experiment in which the intermittent stirring time was set at 3 min while the stationary times were varied from 15 to 33 to 66 min for three cultures (Fig.3.2.7.). Stirring regime was expressed as alternative stirring and non-stirring periods specified by the designated times; for instance, 3 min/ 15 min indicates that the culture was being stirred for 3 min every 15 min non-stirring period. From the stirring regimes tested, it was found that 3 min/ 15 min induced more cell aggregates to form (Table.3.2.3.), in addition to a slower attachment rate at 0.68 x10^{-2} min^{-1}. Whereas cultures at 3 min/ 33 min and 3 min/ 66 min yielded attachment rates of 0.83 and 0.92 x10^{-2} min^{-1} respectively with reduced cell aggregation (number and size). T_{50} values were similar among cultures tested at three regimes, ranging from 78 to 88 min. However, poor cell distribution was observed in culture at 3 min/ 66 min culture. Therefore 3 min/ 33 min is considered the optimal intermittent stirring regime for CuG cultures. As a result, subsequent CuG cultures were set at 3 min/ 30 min under intermittent stirring for the first 6 h.

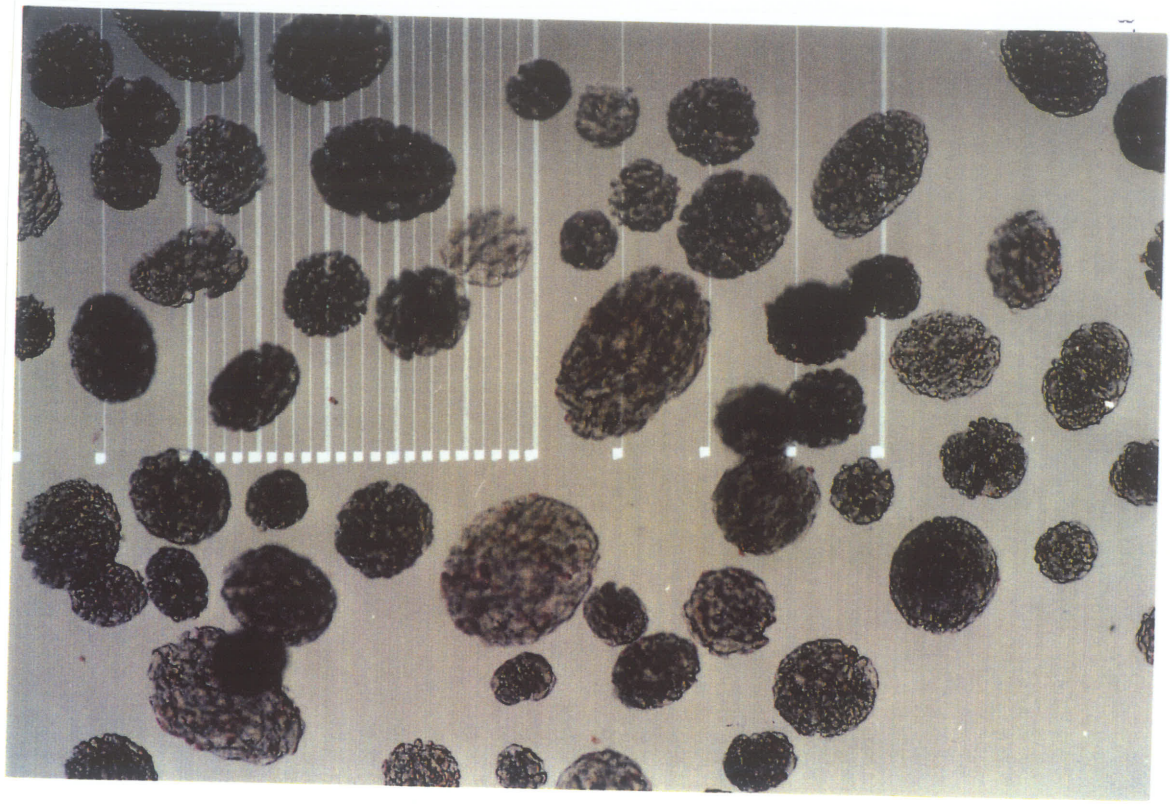


Fig.3.2.6a. The appearance of even cell/ bead distribution on the CuG beads at 7 h under intermittent stirring at 40 rpm. Culture inoculated with 5 x10^4 cells/ml and 1.72 g/L CuG beads was stirred intermittently at 3 min/ 30 min for 7 h before switching to continuous stirring. Sample was taken and neutral red stained, and photographed under 40X magnification.

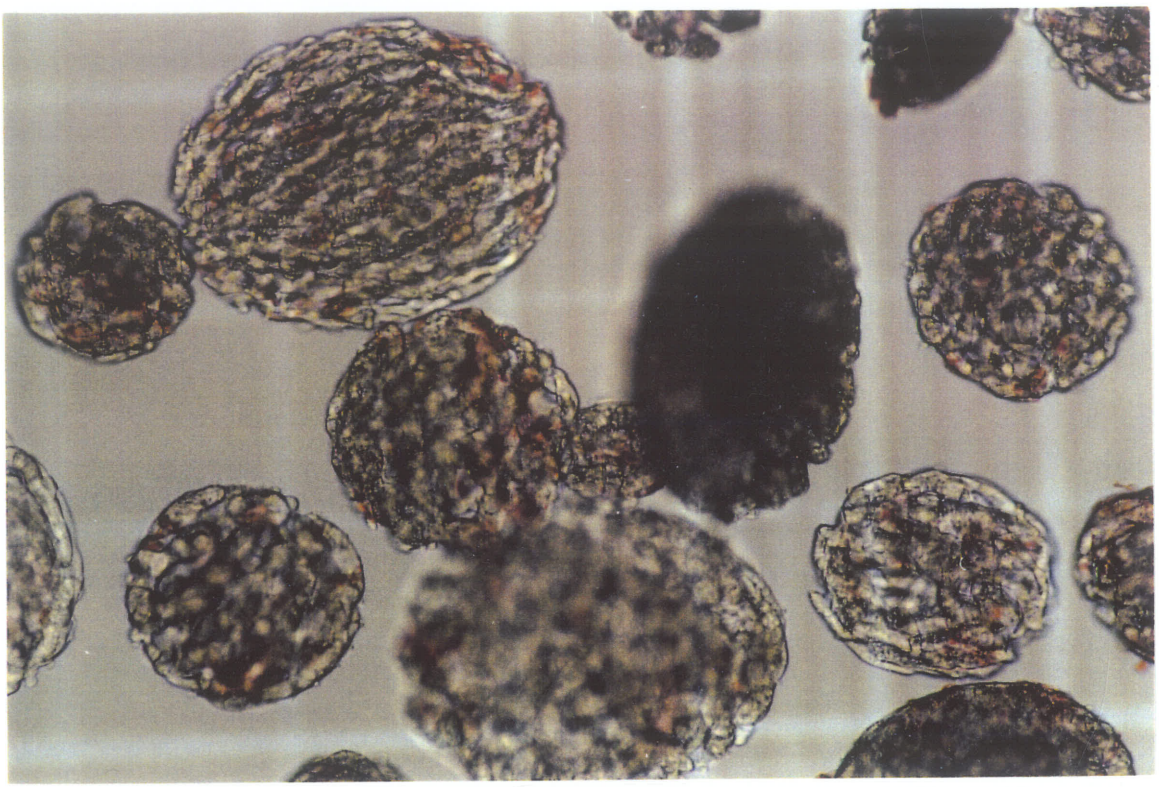


Fig.3.2.6b. The appearance of even cell distribution on the CuG beads at 7 h under intermittent stirring at 40 rpm. Culture inoculated with 5 x10^4 cells/ml and 1.72 g/L CuG beads was stirred intermittently at 3 min/ 30 min for 7 h before switching to continuous stirring. Sample was taken and neutral red stained, and photographed under 100X magnification.

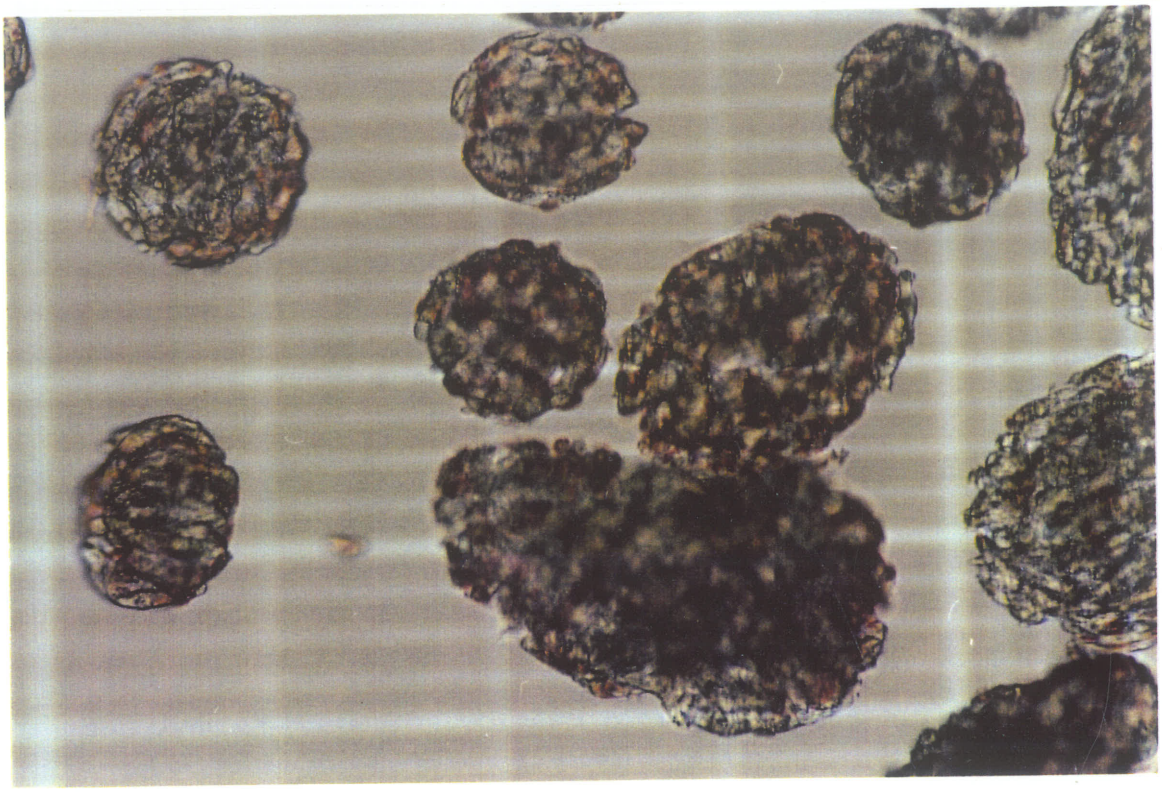


Fig.3.2.6c. The appearance of even cell distribution on the CuG beads on Day 1 under intermittent stirring at 40 rpm for first 7 h. Culture inoculated with 5 x10^4 cells/ml and 1.72 g/L CuG beads was stirred intermittently at 3 min/ 30 min for 7 h before switching to continuous stirring. Sample was taken and neutral red stained, and photographed under 100X magnification.

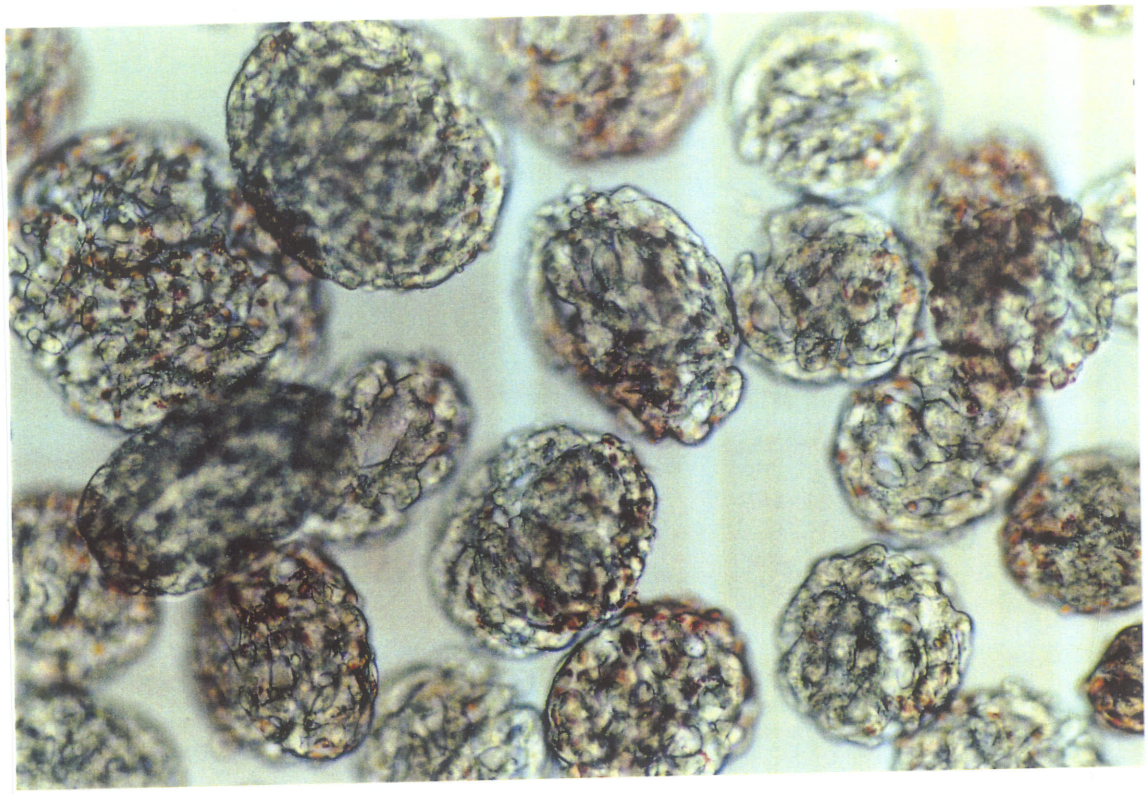


Fig.3.2.6d. The appearance of even cell distribution on the CuG beads on Day 1 under intermittent stirring at 40 rpm. Culture inoculated with 25 x10^4 cells/ml and 5 g/L CuG beads was stirred intermittently at 3 min/ 30 min for 7 h before switching to continuous stirring. Sample was taken and neutral red stained, and photographed under 100X magnification.

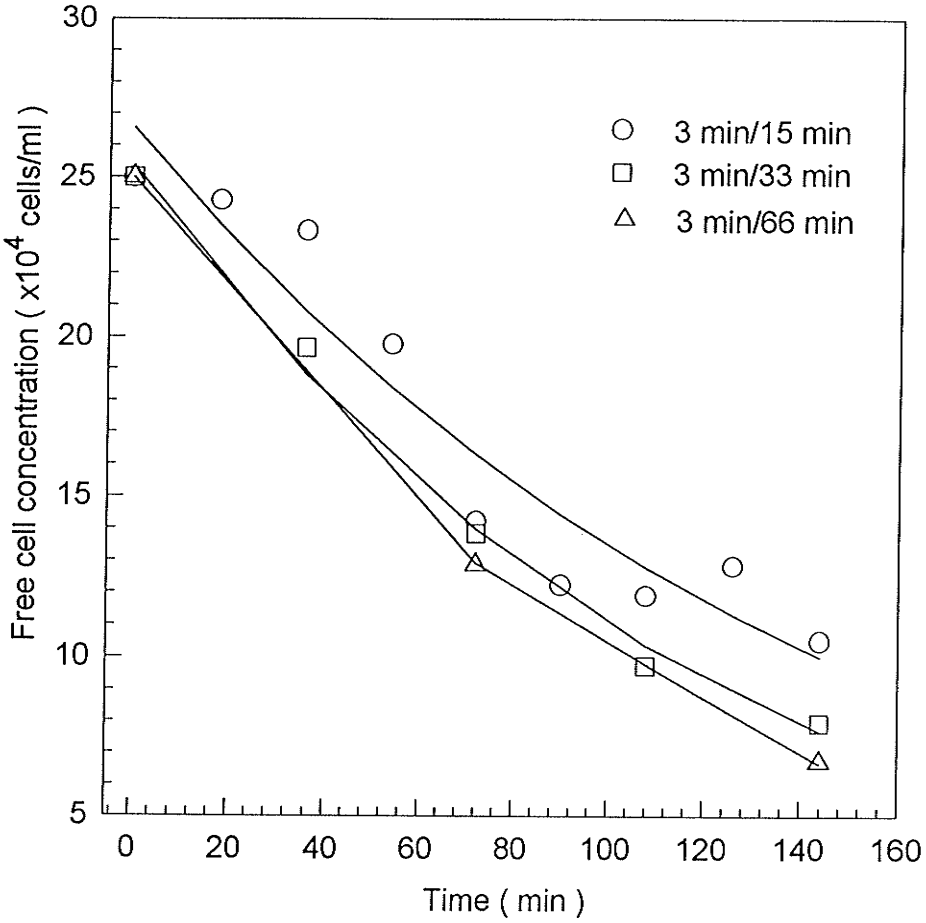


Fig.3.2.7. Effect of intermittent stirring regime on cell attachment onto 5 g/L CuG. 3 min/ 15 min, 3 min/ 33 min, and 3 min/ 66 min regimes had stirring time for 3 min for all cultures and variable non-stirring periods of 15, 33, and 66 min. Culture conditions were same as those in Fig.3.2.5. (n = 1)

Table 3.2.3. Cell Aggregate Index of 5 g/L CuG cultures under different intermittent stirring regimes [a].

3 min/ 15 min	3 min/ 33 min	3 min/ 66 min
86 ± 3	33.5 ± 2.5	40.5 ± 4.5

a Cell Aggregate Index is expressed as the average number of cell aggregates counted on haemocytometer (30 µl). Procedure refer to Chapter 2.

(b) Agitation

The effect of agitation speed in conjunction with intermittent stirring on cell attachment to CuG was determined in three cultures with stirring speed ranging from 40 to 80 rpm. As in Cyt 1 cultures, 40 rpm was the minimum speed to ensure adequate suspension of beads. Therefore 40, 60 and 80 rpm were tested on CuG cultures. It was shown in Fig.3.2.8. that higher stirring speeds under intermittent stirring yielded slower attachment rates. 0.85, 0.70, and 0.61 $x10^{-2}$ min^{-1} were the initial attachment rates of 40, 60 and 80 rpm cultures, respectively. The times of 50% attachment (T_{50}) were measured as 83, 101, and 112 min for cultures at 40, 60 and 80 rpm respectively. Bead occupancy at 6 h was 95-100% and cell distribution was optimal under all conditions tested. Therefore, 40 rpm was the optimal agitation speed under intermittent stirring for cell attachment to the CuG beads. Subsequent CuG cultures were intermittently stirred at 40 rpm for the first 6 h.

(c) pH

The effect of pH on cell attachment to CuG was investigated in cultures with pH ranging from 6.8 to 7.8. pH was maintained in cultures for at least 2 h with the use of 25 mM HEPES in DME medium without sodium bicarbonate. Fig.3.2.9. indicates that at pH 7.1-7.8 there is no significant difference in the attachment rates ranged between 0.80-0.87 $x10^{-2}$ min^{-1}. Another independent culture at pH 8.0 also showed no difference in the attachment rate to those above. However, the pH 6.8 culture yielded a slightly lower rate at 0.65 $x10^{-2}$ min^{-1}. There was no significant difference in the degree of cell aggregate formation in all cultures as observed microscopically. The times for 50% cell attachment (T_{50}) were 82 to 87 min for cultures at pH from 7.1 to 7.8 while it was 111 min for the culture at pH 6.8. Bead occupancy was between 95-100% in all cultures. The results indicate that pH does not significantly influence cell-bead attachment over a range of 7.1

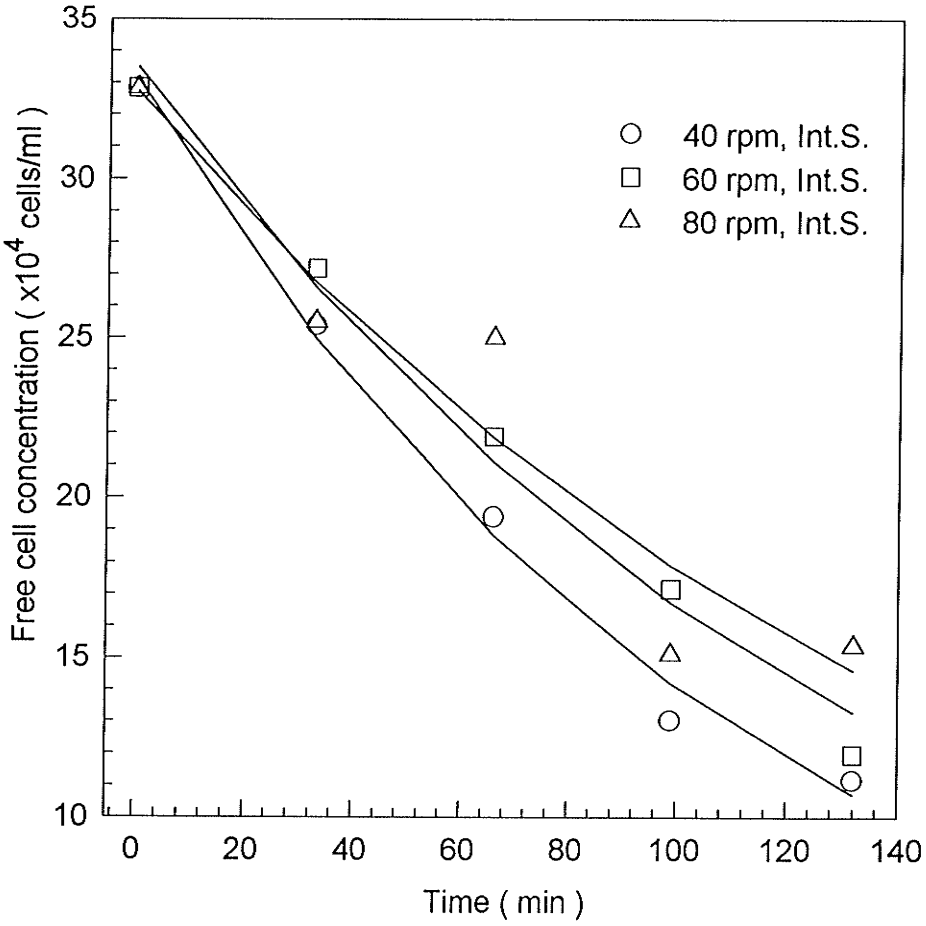


Fig.3.2.8. Effect of agitation speed under intermittent stirring on cell attachment onto 5 g/L CuG. Cultures were set up in conditions as in Fig.3.2.5. (n = 1)

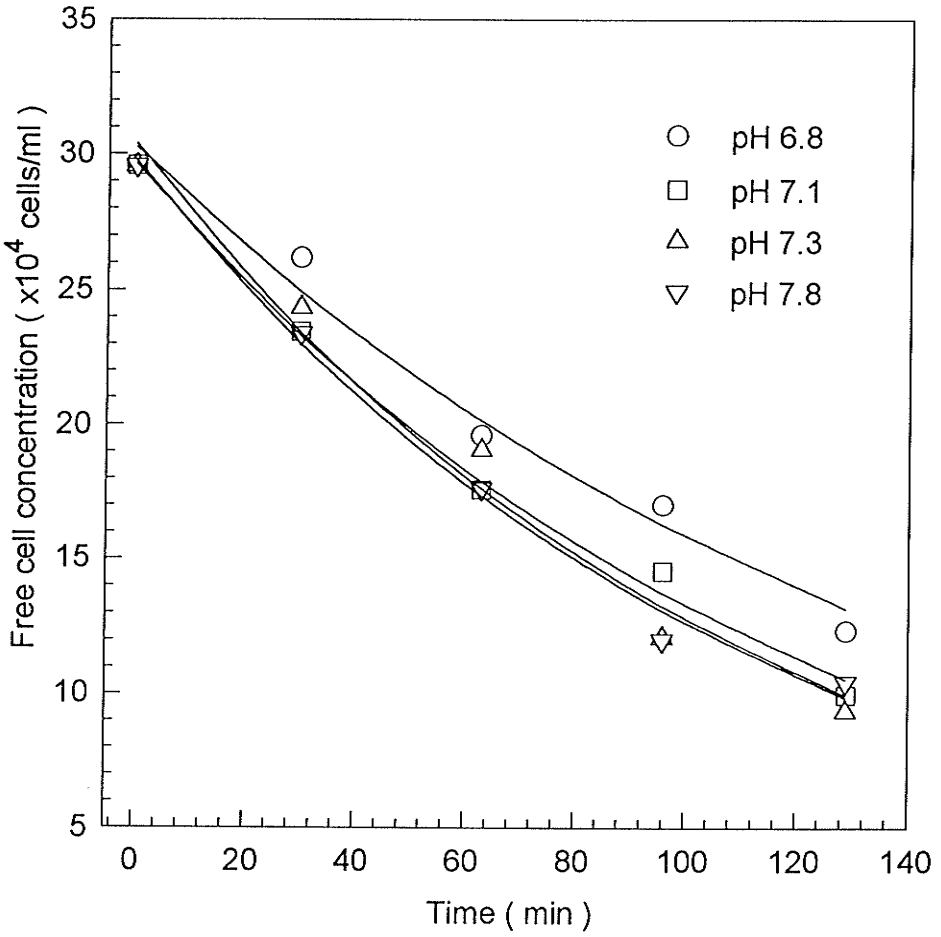


Fig.3.2.9. Effect of pH on cell attachment onto 5 g/L CuG. 25 mM HEPES was used to replace 3.7 g/L sodium bicarbonate for pH controls in cultures. Medium was supplemented with 5% serum (v/v) and adjusted at different pH range with the use of 10 M NaOH. (n = 1)

to 7.8. Therefore, pH 7.3 was selected as the optimal pH. As a result, all subsequent CuG cultures were allowed to equilibrate to pH 7.3 by incubation overnight with a 10% CO_2 overlay.

(d) Supplemented Serum Concentration

The effect of serum supplementation in the medium on cell attachment onto CuG was determined in experiments in which the cultures were supplemented with serum from 0 to 10%, and one additional culture in which beads were pre-incubated in DME at 10% serum for 1 h before they were subject to medium at 0% serum (Fig.3.2.10a). Under intermittent stirring, there was no significant difference in the attachment rates from 0.77 and 0.88 $x10^{-2}$ min^{-1} in cultures at 0-10% serum concentrations (Fig.3.2.10b.). However, preincubation of beads with serum resulted in a higher attachment rate at 1.14 $x10^{-2}$ min^{-1} than any untreated cultures. The times for 50% cell attachment (T_{50}) were between 80 to 89 min in cultures with 0 to 10% serum supplemention while it was 58 min in the culture with preincubated beads. Even though the attachment rate increased with the pre-incubation, T_{90} values were 6 h for all cultures with or without preincubation. A high bead occupancy (95-100%) and optimal cell distribution at 6 h were also observed in all cultures. Therefore, results indicate that attachment rates are not affected by supplemented serum concentration in cultures from 0 to 10%. 5% serum supplementation was used for subsequent cultures because of its importance to growth (see section 3.4).

(e) Inoculum Size

The effects of inoculum (cell/ bead) ratio on cell attachment and distribution onto CuG beads were tested on four CuG cultures from 14 to 112 cells/ bead (Fig.3.2.11a.). Attachment rates varied from 0.74 $x10^{-2}$ min^{-1} in culture at 14 cells/ bead to 0.95 $x10^{-2}$ min^{-1} in culture at 112 cells/ bead (Fig.3.2.11b). The culture at 112 cells/ bead however

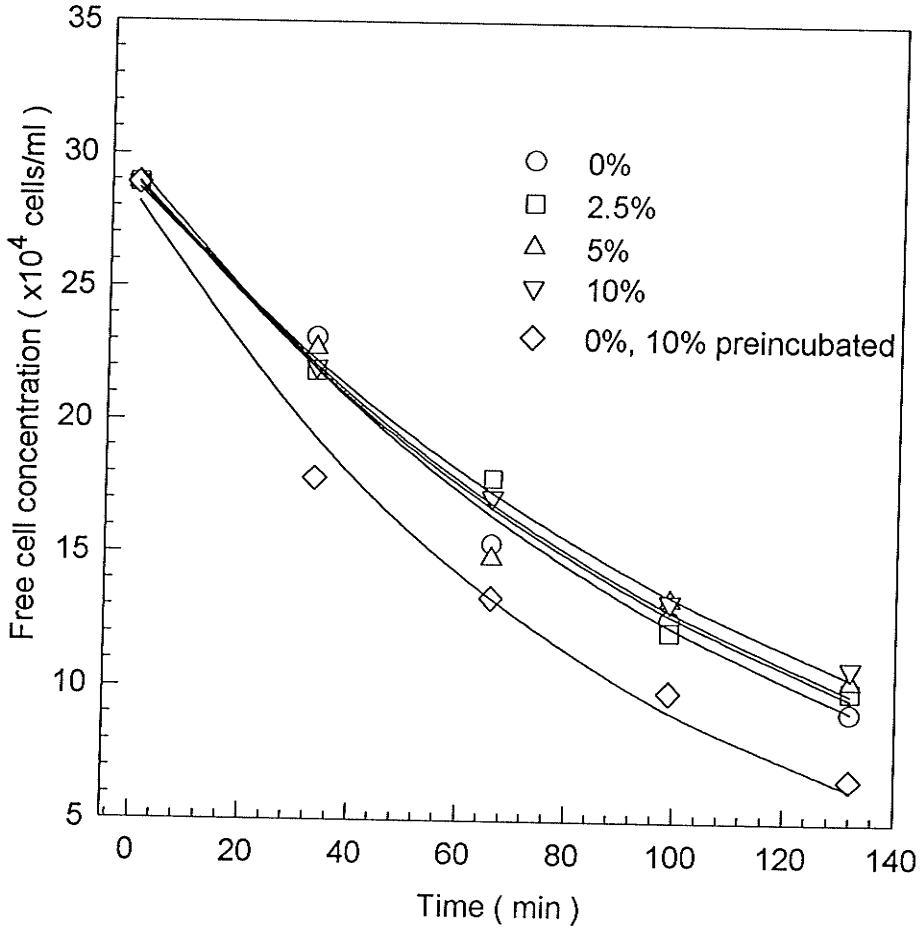


Fig.3.2.10a. Effect of supplemented serum concentration on cell attachment onto 5 g/L CuG. Culture conditions were same as in Fig.3.2.5. (n = 2).

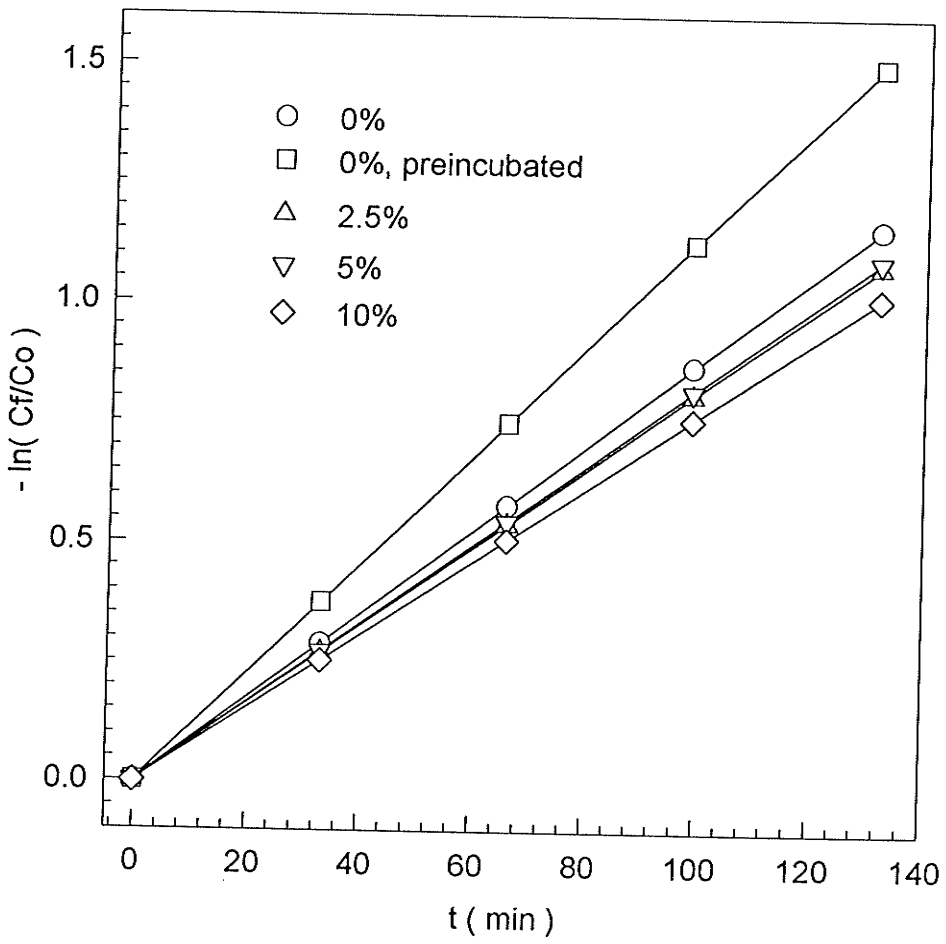


Fig.3.2.10b. Effect of supplemented serum concentration on cell attachment onto 5 g/L CuG. Data were taken from plots in Fig.3.2.10a. and plotted as -ln (Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the slopes of the plots. (n = 2)

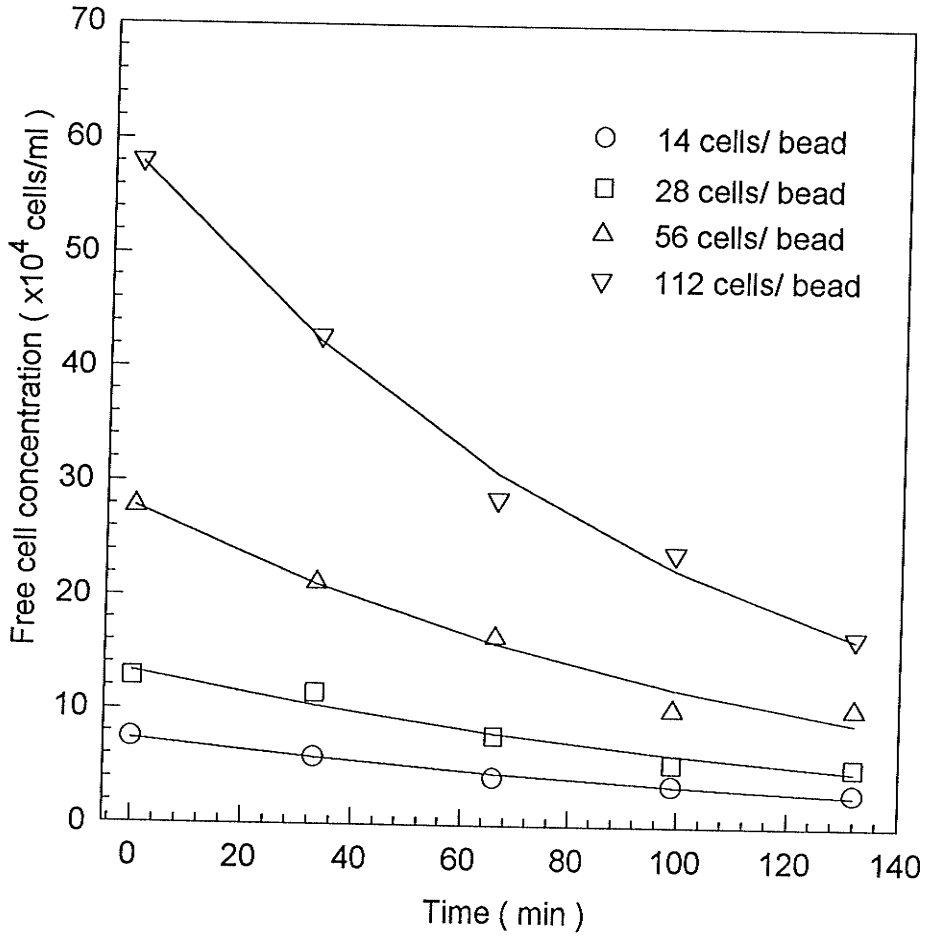


Fig.3.2.11a. Effect of inoculum ratio on cell attachment onto 5 g/L CuG. Culture conditions were identical to those in Fig.3.2.5. (n = 1)

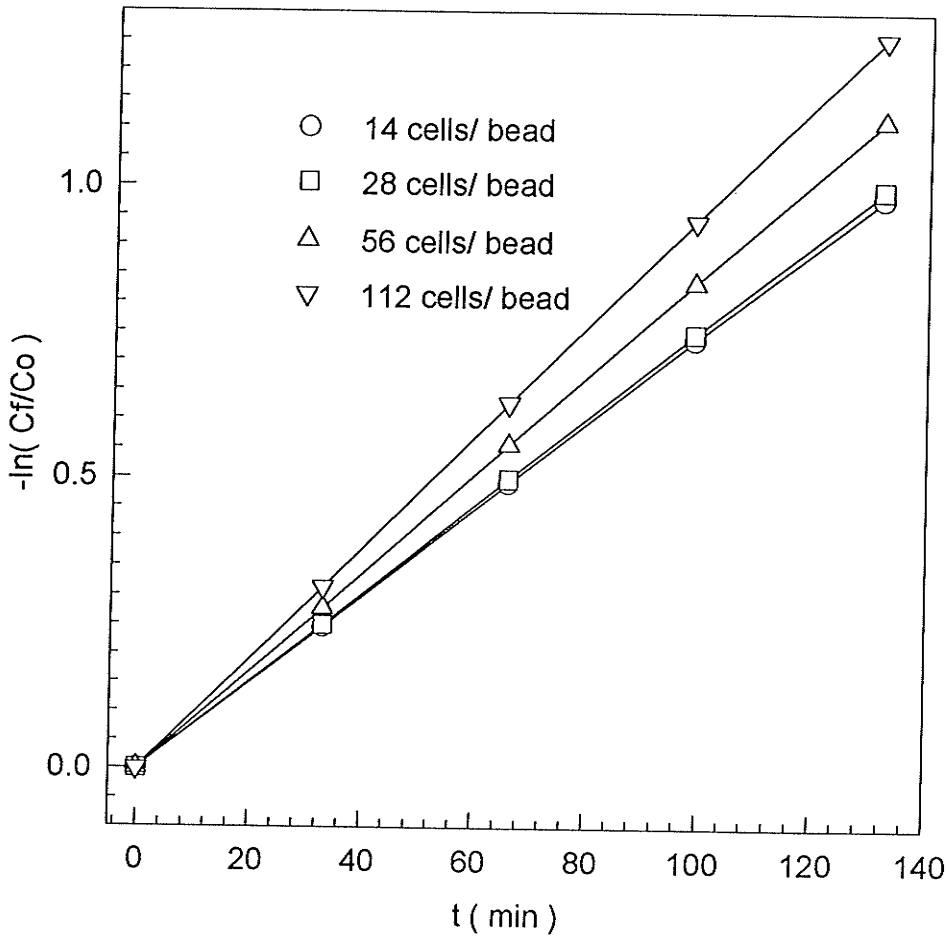


Fig.3.2.11b. Effect of inoculum ratio on cell attachment onto 5 g/L CuG. Data were taken from Fig.3.2.11a. and plotted as -ln(Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the slopes of the plots. (n = 1)

resulted in extensive formation of cell aggregates, whereas cultures at 14 to 56 cells/ bead cultures resulted in smaller extent of cell aggregation. 50% cell attachment (T_{50}) ranged from 72 to 104 min. Bead occupancy was 80-90% in the cultures at 14 cell/ bead and 95-100% in cultures at 28 cells/ bead and above. In Fig.3.2.11c. the free cell concentrations expressed as percentages of the inoculum were shown to reach approximately 30-40% of the inoculum for cultures at 14-112 cells/ bead at 130 min. Cell distribution was less optimal in the culture at 14 cells/ bead, and optimal at 28 cells/ bead and above. Therefore, results suggest that both cultures at 14 and 112 cells/ bead yield less optimal cell attachment than cultures at 28-56 cells/ bead. The subsequent cultures were set at 56 cells/ bead.

(f) Bead Concentration

The effect of bead concentration on cell attachment was determined in cultures ranging from 1 to 5 g/L CuG. The bead concentration was tested under two conditions: (a) with a constant 56 cell/ bead ratio at each bead concentration, and (b) with fixed inoculum at 32 $x10^4$ cells/ml at each bead concentration.(Figs.3.2.12a., and 3.2.13.).

Under condition (a), the attachment rate was the highest in culture at 5 g/L CuG at 1.02 $x10^{-2}$ min^{-1}, followed by 3 g/L at 0.67 $x10^{-2}$ $min^{-1}$and 1 g/L at 0.55 $x10^{-2}$ min^{-1} (Fig.3.2.12b.). Higher bead concentrations will lead to higher cell collision frequencies even though the inoculum ratio is constant at each bead concentration. In fact, the attachment rate was improved only by two-fold when the bead concentration was increased by five-fold from 1 to 5 g/L. The full cell concentrations of cultures at 1, 3 and 5 g/L decreased to 50, 45, and 25% of the inoculum respectively at 130 min (Fig.3.2.12c.). T_{50} were 132, 106 and 67 min for cultures at 1, 3, and 5 g/L. Bead occupancy was between 95-100% and cell aggregation was low in all cultures. The results suggest that 5 g/L is the optimal bead condition. After 6 h all cultures were shown to achieve 90% cell

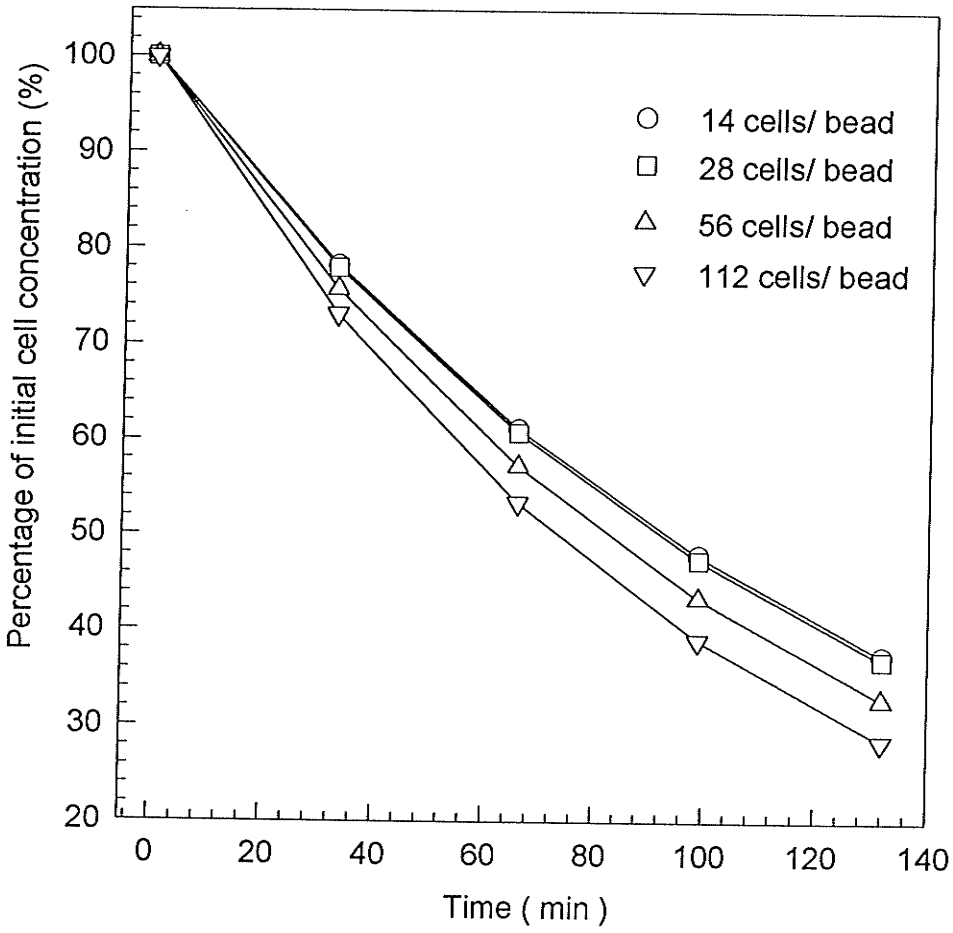


Fig.3.2.11c. Effect of inoculum ratio on cell attachment onto 5 g/L CuG. Free cell concentrations are expressed as percentages of inoculum and plotted against time. Conditions were same as those in Fig.3.2.11a. (n = 1)

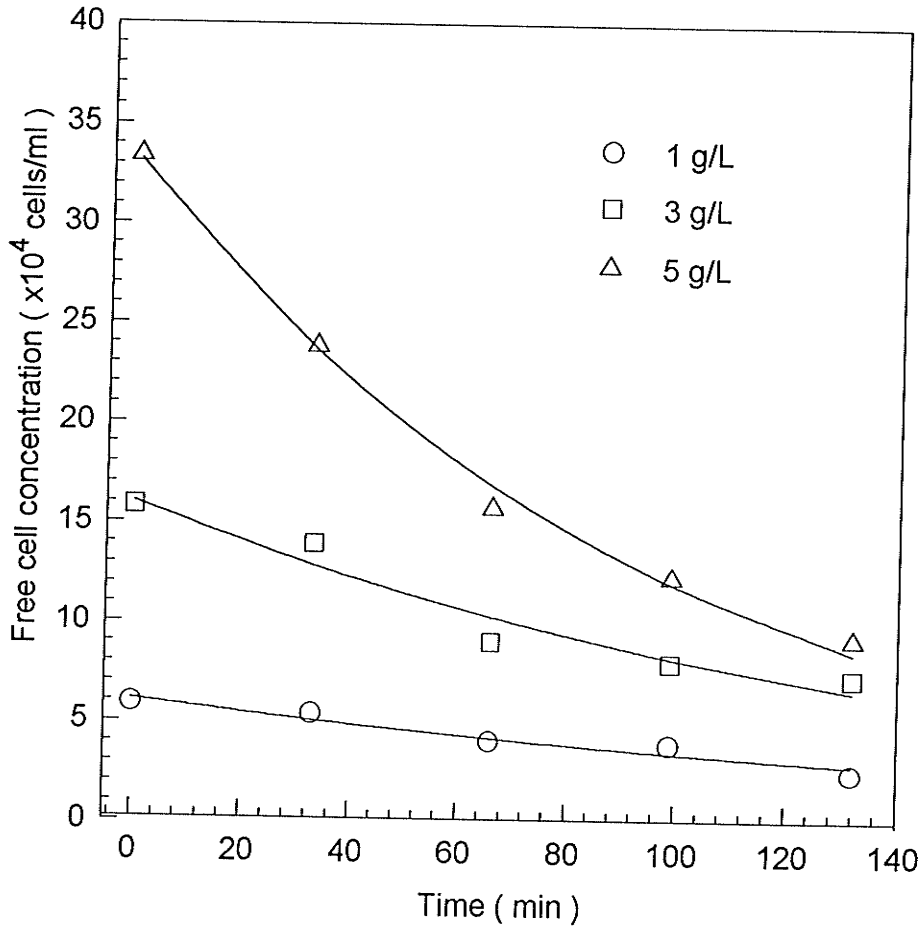


Fig.3.2.12a. Effect of bead concentration on cell attachment onto CuG. Cultures were inoculated with fixed cell/ bead ratio, therefore starting at different inocula. Conditions were same as in Fig.3.2.5. (n = 1)

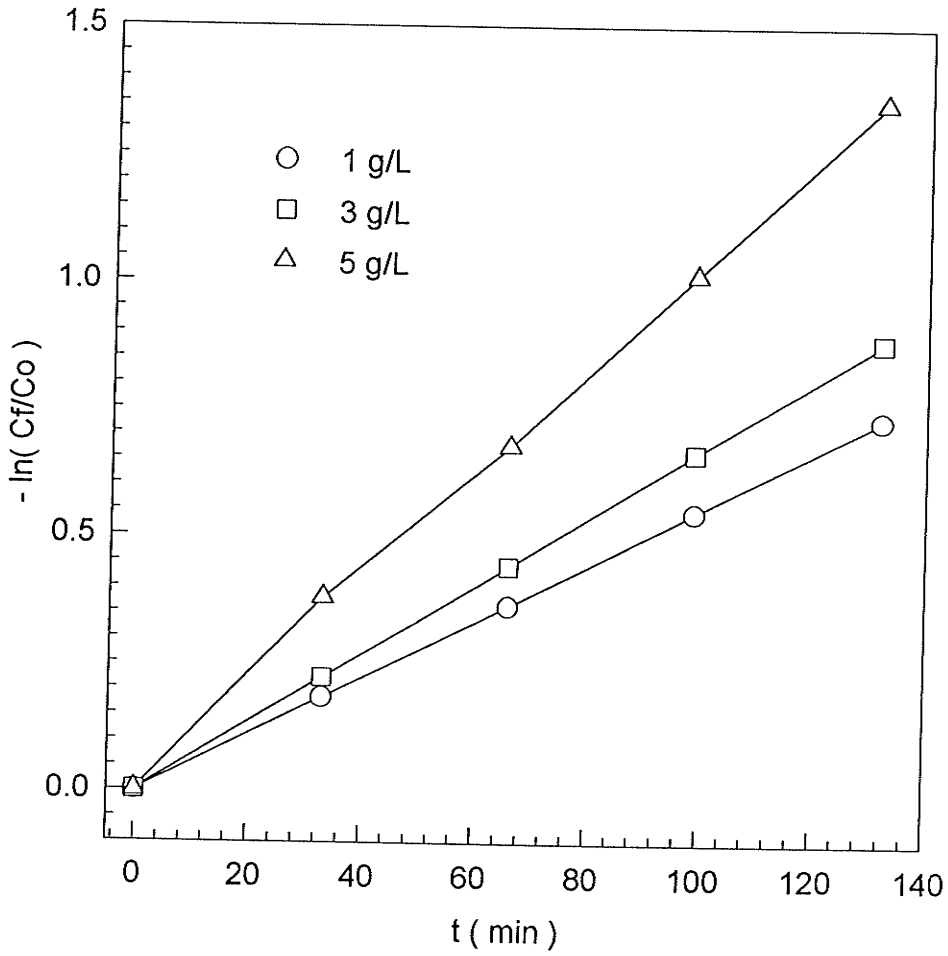


Fig.3.2.12b. Effect of bead concentration on cell attachment onto CuG. Data were taken from Fig.3.2.12a. and plotted as -ln(Cf/Co) against t (min). Cell attachment rate, k, is determined from calculating the linear regression of the slope of the plot. (n = 1)

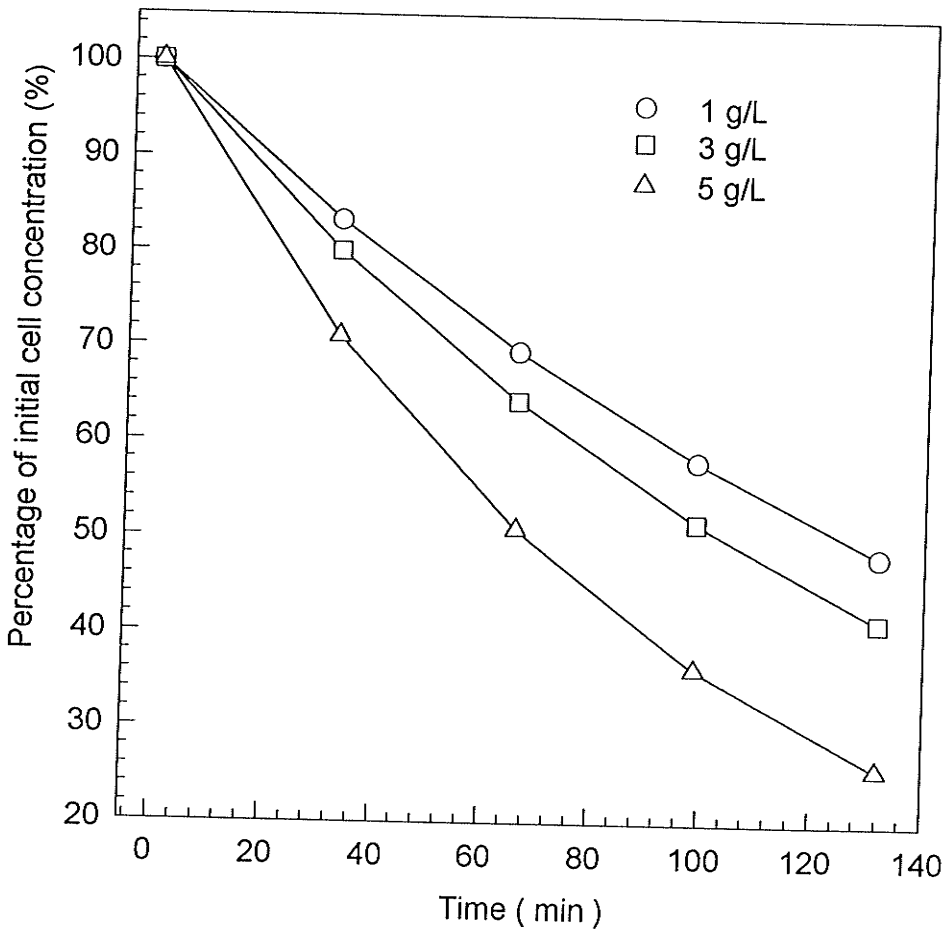


Fig.3.2.12c. Effect of bead concentration on cell attachment onto CuG. Cultures were inoculated with fixed cell/ bead ratio, therefore starting at different inocula. Free cell concentrations are expressed as percentages of the inoculum and plotted against time. Conditions were same as in Fig.3.2.5. (n = 1)

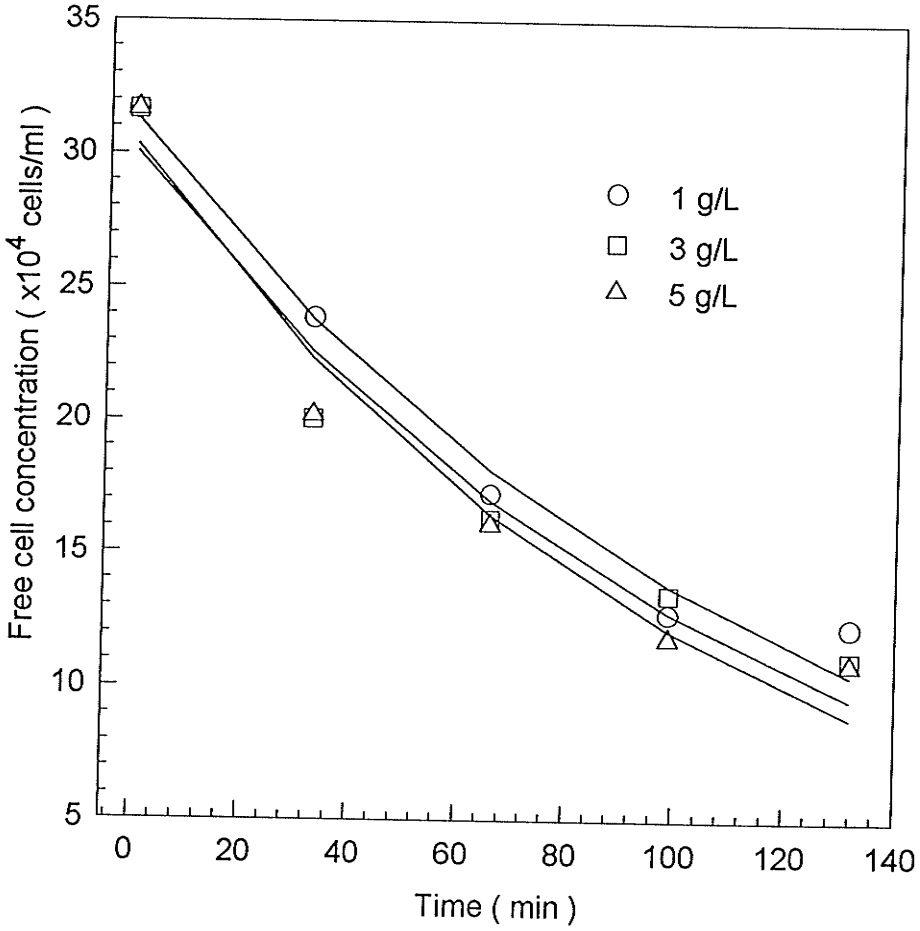


Fig.3.2.13. Effect of bead concentration on cell attachment onto CuG. Cultures were inoculated with fixed inoculum level regardless of bead level, therefore yielding different cell/ bead ratios. Conditions were same as in Fig.3.2.5. (n = 1)

attachment

Under condition (b), attachment rates for all cultures at different bead concentrations were between 0.83 to 0.93 $x10^{-2}$ min^{-1} (Fig.3.2.13). No increase in cell attachment rate occurred by increasing the bead concentration over a fixed inoculum level. However, the decrease in bead concentrations led to an increase in cell/ bead ratios for 1 and 3 g/L cultures. The high cell/ bead ratio, led to significant cell aggregate formation at 1 g/L and 3 g/L but minimal aggregate formation at 5 g/L. Bead occupancy was 95-100% in all cultures even though there were cell aggregates in the supernatants of 1 and 3 g/L cultures. The T_{50} values were between 78 to 83 min for cultures at 1 to 5 g/L. Therefore, the optimal bead concentration for cell attachment is at 5 g/L.

3.2.3. Discussion

(a) Stirring Mode

(i) Continuous Stirring

In addition to lower cell-bead attachment rates, poor bead occupancy and cell distribution occurred in all CuG cultures under continuous stirring. It is believed that this was due to an immediate step of formation of cell aggregates which were subsequently attached to beads (24-48 h). Because the cell aggregate/ bead ratios were lower than the cell/ bead ratios of the inoculum, this influenced the final bead occupancy and cell/ bead distribution.

Nikolai and Hu., (1992) and Lim et al., (1992) investigated attachment of Vero cells on two similar macroporous microcarriers, CuG and in-house synthesized gelatin beads, the cell attachment rates to both bead types were much lower compared to those of charged beads and also found to be significantly different from each other. Under continuous stirring, T_{90} were approximately 3 and 9 h for the CuG and the homemade gelatin bead cultures. On the other hand, Tao et al., (1987) reported that FS-4 cells attached to charged Cyt 1 beads at twice the rate compared to gelatin-coated Cyt 3 beads.

Differences in attachment rates may be related to the nature and density of charge on the beads. The gelatin beads used by Lim et al., (1992), which were synthesized by a modified process used for CuG beads, were charged at 0.5-0.6 meq/g under physiological pH compared to about 1.5 meq/g on DEAE-charged Cyt 1 (Pharmacia., 1981). The charge density of CuG beads may be too low and therefore suboptimal for cell-bead attachment between cells and beads. On the other hand, the different chemical nature of charge groups may influence the attachment between cells and substratum surface, as it was demonstrated by Reuveny et al., (1983) that the primary amino derivatized beads had resulted in faster cell attachment and spreading compared to tertiary amino derivatized beads. Charges on CuG are likely attributed to the amino acids of gelatin which are

chemically different from the Diethyaminoethyl (DEAE) groups derivatized on dextran matrices of Cyt 1 beads.

(ii) Cell Aggregation

In our studies, continuous stirring resulted in substantial numbers of Vero cell aggregates in CuG cultures after inoculation. The factors affecting cell aggregation are not understood. It may be related to the presence of serum factors and divalent ions (calcium and magnesium) present in the medium. Therefore, cell disappearance rates represented the combined rates of cell-bead attachment and cell-cell aggregation. In other words, the actual cell-bead attachment rates would be lower than rates calculated on the basis of declining single cell concentrations. The number of cells that contributed to cell aggregates and their size could not be accurately quantified. Fig.3.2.14. shows the possible mechanisms of disappearance of free cells. As a result, it was difficult to identify exactly how many free cells followed path (2a) or (2b) and (2c) or (2d) under continuous stirring during the course of cell attachment. Average attached cell/ bead ratios were lower than the inoculum ratios on the beads at 6 h, this suggests that cells formed cell aggregates rather than attached to the beads.

Fig.3.2.14. Conditions of cell attachment onto (1) Cyt 1, and CuG under intermittent stirring and (2) CuG under continuous stirring.

(1) Disappearance of free cells in culture → Cell-Bead attachment

(2) Disappearance of free cells in culture →
- **a)** Cell-Bead attachment (cell-bead contacts)
- **b)** Cell Aggregation (cell-cell contacts) → **d)** Cell aggregates
- **c)** Cell aggregate-Bead attachment (speed dependent)

(iii) Intermittent Stirring

Results show that intermittent stirring achieved the optimal cell attachment on CuG beads in terms of cell attachment rate, bead occupancy, and cell distribution. Intermittent stirring is believed to minimize the effect of shear stress produced by stirring and to maximize the contacting times between cells and beads during the non-stirring phase. Besides, intermittent stirring allows significantly less and smaller cell aggregates to form. The attachment rates under intermittent stirring were significantly higher than those under continuous stirring but still much lower than those found in Cyt 1 cultures (Tables. 3.1.1., and 3.2.2), usually in excess of 10-fold. This may reflect the lack of charge interactions for cell-bead attachment.

The more even cell distribution and full bead occupancy shown in photographs (Fig.3.2.6a,b,c, and d) were largely attributed to a direct result of prolonged cell-bead contacts and low extent of cell aggregation under intermittent stirring.

Until recently, only few reports suggest the use of intermittent stirring or a stationary method to facilitate cell attachment on macroporous microcarrier cultures during the initial phase. Kennard and Piret., (1994) reported the use intermittent stirring at 2 min/ 30 min for Cu-GH beads in 500 ml spinners before switching to continuous stirring at 3 h. Also, Ong et al., (1994) demonstrated attachment of hybridoma cells onto charged macroporous microcarriers, Cellsnow EX ™ in serum-free medium under an intermittent regime of 2 min/ 30 min. On the other hand, Kratje et al., (1994) showed a 4 hr stationary phase (without stirring) to achieve 65% of cell attachment to macroporous microcarriers in a fluidized bed bioreactor. Literature from Hyclone., (1994) also suggested the use of intermittent stirring may increase attachment rates. These reports did not state the reason intermittent stirring was preferred to continuous stirring in their systems. To our knowledge, this study is the first to identify the effect of intermittent stirring and other

parameters on cell attachment onto CuG beads in terms of the cell attachment rate, bead occupancy, cell/ bead distribution pattern.

(iv) Intermittent Stirring Regime

Cell-bead attachment was shown to be optimal at 3 min/ 33 min for optimal cell attachment rate, bead occupancy and even cell distribution. When beads had settled on the bottom of the spinners, only the top bead layers were exposed to descending cells. A redistribution during stirring period was necessary to ensure that beads underneath could be shuffled to the top layer, otherwise cells would attach differentially to certain beads and yield poor cell distribution as in the culture at 3 min/ 66 min. 3 min/15 min regime. These stirring regimes approached the continuous stirring state and cell aggregates were found in significant numbers.

(b) Agitation

Increasing the agitation speed to 60 and 80 rpm lowered the attachment rates, but to a lesser extent than those on Cyt 1 cultures, suggesting that speed was not such an important factor on cell attachment under intermittent stirring.

(c) pH

Previous results showed that the attachment rate in Cyt 1 cultures was adversely affected at pH 7.8. In contrast, attachment rates in CuG cultures were not altered at pH from 7.1 to 7.8. This suggests that cell attachment onto CuG beads could be via different a mechanism than involved in Cyt 1. It may be related to the nature of gelatin matrix of CuG beads, which is different from the charged dextran of Cyt 1 beads.

(d) Supplemented Serum Concentration

Under intermittent stirring, cell attachment was not affected in CuG cultures with serum concentrations ranging from 0 to 10%. Sayer et al., (1987) used gelatin-coated Cyt 3 beads for MDCK cell attachment in serum-free medium, suggesting that gelatin could substitute for serum-based attachment factors.

However, serum was essential for cell attachment to CuG under continuous stirring. Cell aggregates formed were unable to attach to CuG beads in medium without serum. Beads were unoccupied even after 24 h.

Interestingly, CuG beads preincubated with serum showed higher attachment rates than those untreated. This may suggest that serum proteins particular attachment factors when combined with the natural gelatin matrices would improve attachment. Furthermore, cell aggregation may be reduced in the absence of serum. Kleinman et al., (1981) reported that gelatin with unfolding structure would increase its affinity to attachment factors such as fibronectin. Therefore, it may enhance the adhesiveness of cells to bead surfaces.

(e) Inoculum Size

Cell aggregation depends upon cell-cell contacts. Thus, increasing the cell/ bead ratio would likely induce cell aggregation than improve cell-bead attachment. Therefore, cell inoculation at high cell/ bead ratio was detrimental to overall cell-bead attachment rates.

(f) Bead Concentration

Under condition (a), the results suggest that increasing collision frequency did not effectively increase attachment rate because only a fixed surface area of settled beads at the bottom that came into contact with cells. Also, the lack of electrostatic attraction between cells and beads would be attributed to the slow attachment rates despite greater numbers of available cells and beads.

Under condition (b), cell-cell contacts were greatly increased and substantial cell aggregates were produced at 1 and 3 g/L cultures. In considering the effect of cell aggregation, the actual attachment rates for both 1 and 3 g/L culture could be lower than that of 5 g/L culture.

3.2.4. Conclusions

(a) Possible mechanisms of Cell Attachment onto CuG beads under Intermittent Stirring

Cell attachment to CuG beads is shown to be a much slower process than to Cyt 1. The following is a series of events that may occur alone or in combinations during cell-bead attachment under intermittent stirring. In Table 3.2.4., the comparisons of cell attachment rates onto CuG under both continuous and intermittent stirring are shown.

(i) Stirring Mode rather than Charge Interactions

Unlike cell attachment onto Cyt 1, charge interactions are not believed to be involved significantly in CuG cultures under intermittent stirring. Conditions such as pH and serum that affected substantially cell attachment in Cyt 1 cultures had no effect in CuG cultures. This suggests that the mechanism of cell attachment onto CuG beads is different from Cyt 1.

The stirring mode used affected cell attachment substantially. It is proposed that intermittent stirring allows a balance between non-stirring periods in which cell-bead contacts are made and stirring periods in which an even distribution of both beads and unattached cells are allowed. In addition, the cell-bead contacts at stationary phase are benefited by the absence of shear stress. Every bead also has an equal chance of settling on the top layer of bead sediments during the course of intermittent stirring and non-stirring periods. Under intermittent stirring, cell aggregation is significantly reduced. Any cell aggregates formed are quickly attached to beads at stationary periods, so there was only a

Table 3.2.4. Comparisons of Cell attachment onto CuG beads under continuous and intermittent stirring.

	Continuous Stirring	Intermittent Stirring
Attachment Phase [a]	- 24 h	- 6 h
Cell Aggregation	- progressive and substantial	- very small extent
Cell Distribution at 24 hr	- wide range (cell/ bead ratios) - some clump-attached beads - regional occupancy across beads	- uniform cell/ bead among beads - no clump-attached beads - even distribution occupancy across beads
Cell aggregates [b] at 24 hr	speed dependent - no or very small cell clumps at 30 rpm - small cell clumps at 40 rpm - substantial numbers at 75 & 100 rpm	

a Attachment Phase refers to the time at which 90% cell attachment was achieved.

b The measurement was conducted by counting cell clumps of a 0.1 ml sample on Petri disc under microscopic observation.
1) no or very small cell clumps: 0-5 cell clumps
2) small cell clumps: 5-10 cell clumps
3) substantial numbers: 20->50 cell clumps

small number of free cell aggregates suspending in the medium. Full bead occupancy and optimal cell/ bead distribution occur under intermittent stirring.

(ii) Cell Entrapment and Immobilization

Ong et al., (1994) suggested that on charged porous carriers cell immobilization was facilitated by mechanical entrapment besides electrostatic interactions. Entrapment would occur under intermittent stirring. Cells can be trapped into the pores and then immobilized at the stationary periods. Entrapment is likely to be a random process and probably significant because the size of the beads (20-30 times of cells) and number (4500 beads/ ml at 5 g/L) of CuG beads are involved. The optimal intermittent regime results in an even distribution of cells across the bead matrices (Figs.3.2.6b., and 3.2.6d.). It may be possible that cells attach to CuG beads independent of any charge interactions under intermittent stirring in which the extended cell-bead contacts may compensate for the lack of charge interactions. Therefore, charge interactions may be predominant for Cyt 1 cultures while entrapment immobilization may be the primary factor for CuG cultures in cell-bead attachment.

(iii) Substratum Surface Modifications

Medium components such as attachment factors and divalent ions (calcium and magnesium), which have been known to stabilize attachment and spreading of cells on beads, may be required to establish optimal substratum condition for cell-bead attachment. Although the cell attachment to CuG was improved in culture with serum preincubation, the rate was incomparable to those of Cyt 1 beads. On the other hand, cells may be actively involved in optimizing the culture conditions for cell attachment. Mediating factors produced by cells in conjunction with serum attachment factors may be essential for the attachment process.

(b) Optimal Conditions for Cell attachment

The critical parameter for Vero cell attachment on CuG is shown to be the stirring mode employed at the attachment phase. However, even with optimal conditions established for each parameter, it is noticed that the cell attachment rates of CuG cultures are not comparable with those of Cyt 1 cultures. In fact, the attachment rates are still ten-fold or more lower compared to those of Cyt 1 beads.

It is concluded that the optimal conditions for Vero cell attachment onto CuG beads are when intermittent stirring at 3 min/ 30 min at 40 rpm, in DME without serum supplementation at pH 7.3, with an inoculum ratio of 56 cells/ bead and 5 g/L bead concentration. These optimal conditions are for cell attachment and not on growth, which is covered in section 3.4.

3.3. Cell Aggregation in Microcarrier-free spinner cultures

3.3.1. Introduction

The observation of the formation of cell aggregates of anchorage dependent cells is not novel, they have been successfully cultured (Tolbert et al, 1980., Litwin, 1992., and Moreira et al, 1994). However, the mechanism of their formation and their effects on cell growth compared to those attached cells on microcarriers are not clear.

Formation of cell aggregates was considered detrimental to microcarrier cultures, as cells were preferably attached to cells than to beads. Cell aggregation reduces the ratio of available single cells to beads. This not only affects attachment rates, but also bead occupancy and cell distribution at the attachment phase. Such negative effects could be extended to subsequent growth of cells in the cultures and therefore the efficiency of the microcarrier cultures. In previous experiments, cell aggregates were commonly found in CuG cultures under continuous stirring and to a small extent under intermittent stirring. In contrast, among all conditions tested for Cyt 1 cultures, only the culture at pH 7.8 yielded cell clumps. In this chapter, cells were inoculated into spinners without beads to encourage cell aggregate formation.

3.3.2. Results

Photographs in Fig.3.3.1a-d. show that at different times cell aggregates are slowly being formed while free single cells are simultaneously diminished in the background of a microcarrier-free spinner culture. All plots were curved fitted by the single exponential decay equation. Cell aggregation rate, k, was determined by measuring the rate at which free single cells disappeared in the supernatant to form cell aggregates. Data were taken from individual plot (Cf against t) and plotted as - ln (Cf/Co) against t (min). The cell aggregation rate was based upon the analysis of linear regression of the linear segment of the plot.

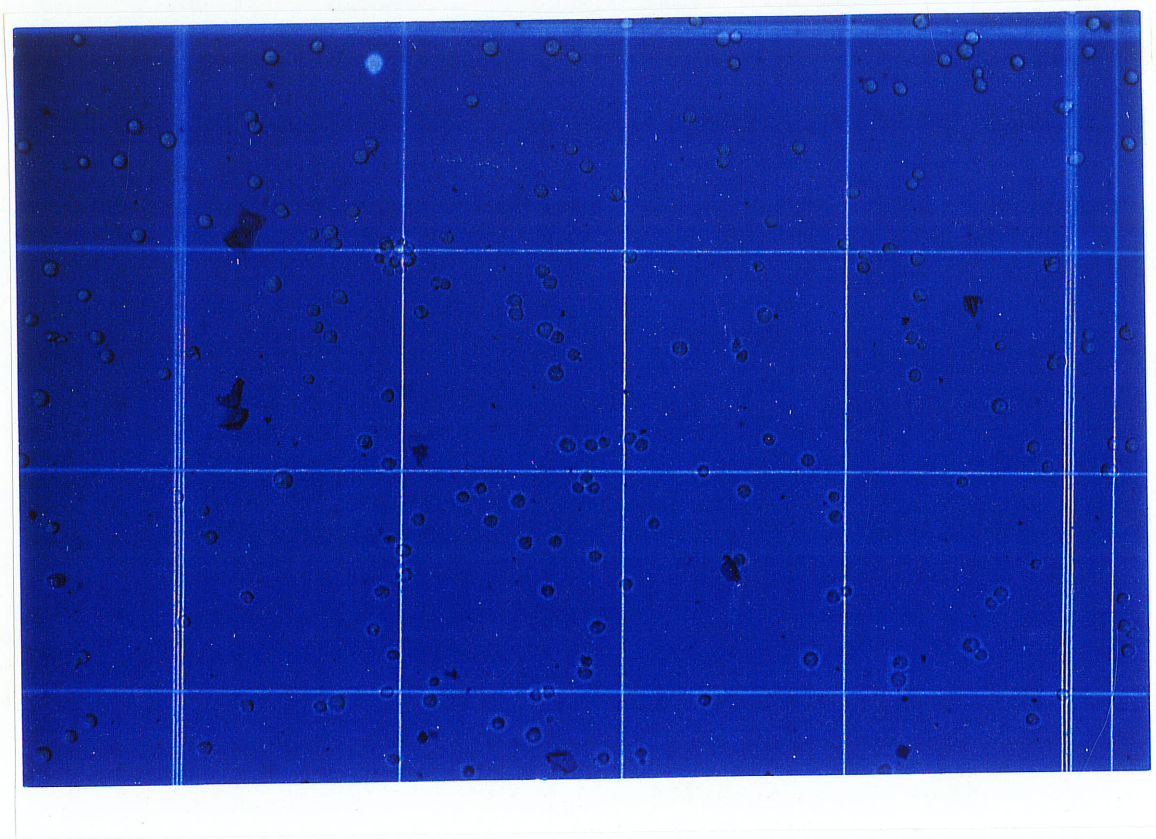


Fig.3.3.1a. The state of Vero cell inoculum used prior to cell inoculation to cultures with or without beads. Cells were mixed with 0.4% Trypan blue and photographed on haemocytometer at 100X magnification.

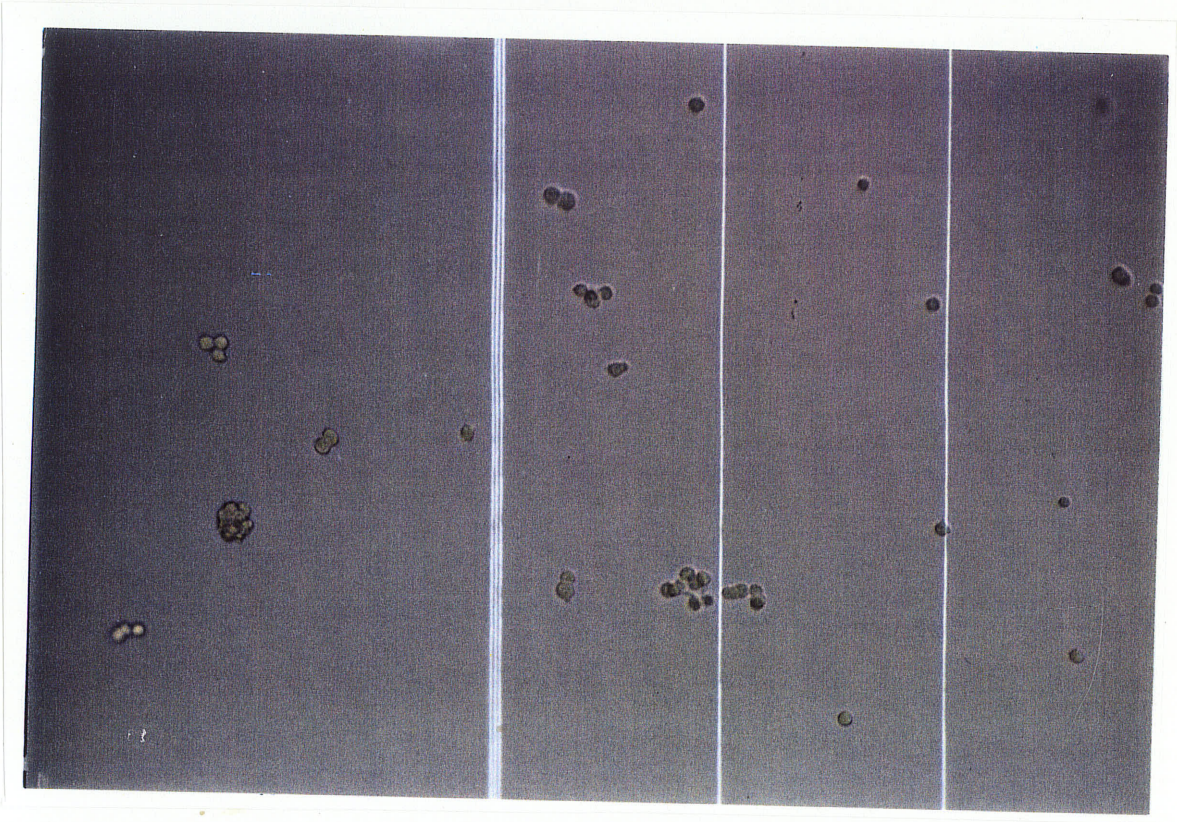


Fig.3.3.1b. The state of Vero cells in a microcarrier-free spinner 2 h after inoculation. Culture was supplied with DME + 5% serum at pH 7.3.

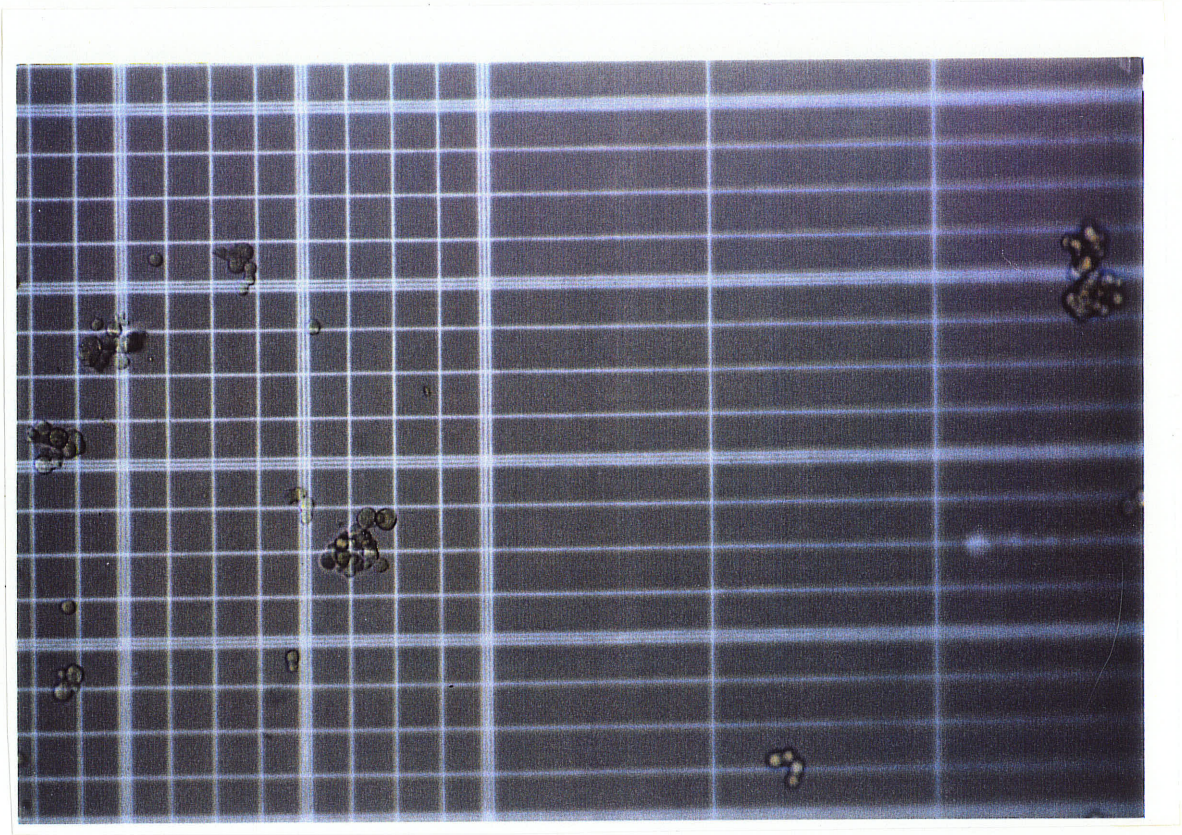


Fig.3.3.1c. The state of Vero cells in a microcarrier-free spinner 5 h after inoculation. Culture was supplied with DME + 5% serum at pH 7.3.

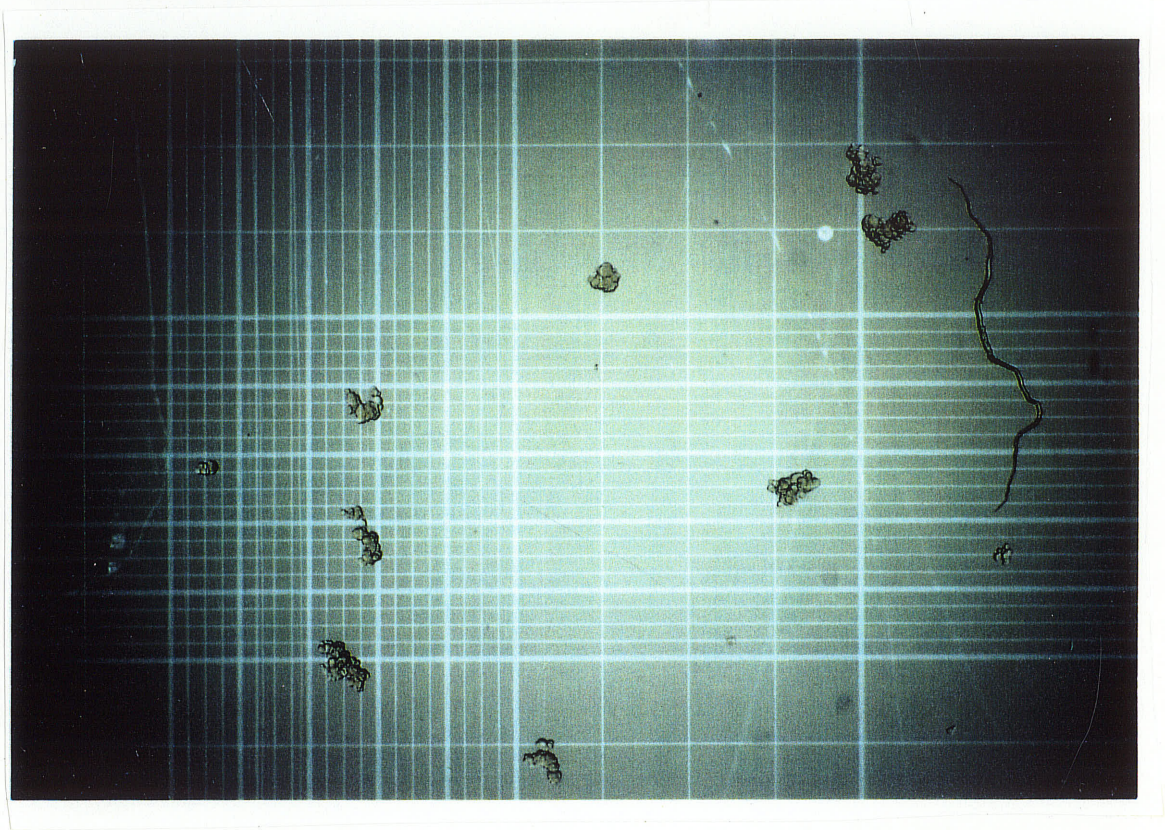


Fig.3.3.1d. The state of Vero cells in a microcarrier-free spinner 12 h after inoculation. Culture was supplied with DME + 5% serum at pH 7.3.

(a) pH

The effect of pH on cell aggregation in microcarrier-free condition under continuous stirring was determined in 100 ml spinners. Culture conditions were maintained with the use of 25 mM HEPES which was identical to those cultures with beads in sections 3.1. and 3.2.. pH ranged from 6.8 to 7.8. Cell aggregation rates, which are calculated the same way as the cell-bead attachment rates, are based upon the disappearance rate of single cells. Results showing trends of cell aggregation are given in Fig.3.3.2.. In Table 3.3.1., the averages of two independent experiments indicated that pH affected the cell aggregation to a small degree. pH 6.8 yielded the slowest aggregation rate at 0.73 $x10^{-2}$ min^{-1} which was significantly different from cultures at 7.1-7.8 at between 0.84-0.93 $x10^{-2}$ min^{-1}. However, no significant differences were found between aggregation rates determined in cultures at 7.1-7.8.

(b) Supplemented Serum Concentration

The effect of supplemented serum in medium on cell aggregation was tested in cultures with serum added at 0 to 10% to the medium. It was found that in the absence of serum, the cell aggregation rate ($0.45x10^{-2}$ min^{-1}) is significantly lower than those with serum at 0.8 $x10^{-2}$ min^{-1} (Fig.3.3.3.). Therefore, this suggests that serum has a role in cell aggregation.

(c) Comparison of rates of cell attachment onto Cyt 1, CuG, and rates of cell aggregation onto bead-free cultures.

Fig.3.3.4. shows the results of the cell-bead attachment rates and cell aggregation rates in Cyt 1 and CuG or microcarrier-free cultures, which were supplied with DME + 5% serum (v/v) at pH 7.3. The results are based upon the measurements of disappearance of single cell concentrations in the cultures. This shows that the cell attachment rate to Cyt

Table 3.3.1. Summary of Cell aggregation rate, k_{ag}, ($x10^{-2}$ min^{-1}) of Vero cells under continuous stirring in different tested conditions [a].

pH	6.8	7.1	7.3	7.8
k_{ag}	0.73 ± 0.1	0.93 ± 0.4	0.84 ± 0.6	0.92 ± 1.0

Serum Conc. (%)	0	2.5	5	10
k_{ag}	0.45 ± 0.1	0.83 ± 0.1	0.78 ± 0.1	0.79 ± 0.1

[a] Cell aggregation rate was determined by measuring the rate at which free single cells disappeared in the supernatant to form cell aggregates. Data were taken from individual plot (Cf against t) and plotted as - ln (Cf/Co) against t (min). The cell aggregation rates were based upon the analysis of linear regression of the linear segment of the plots. (n = 5)

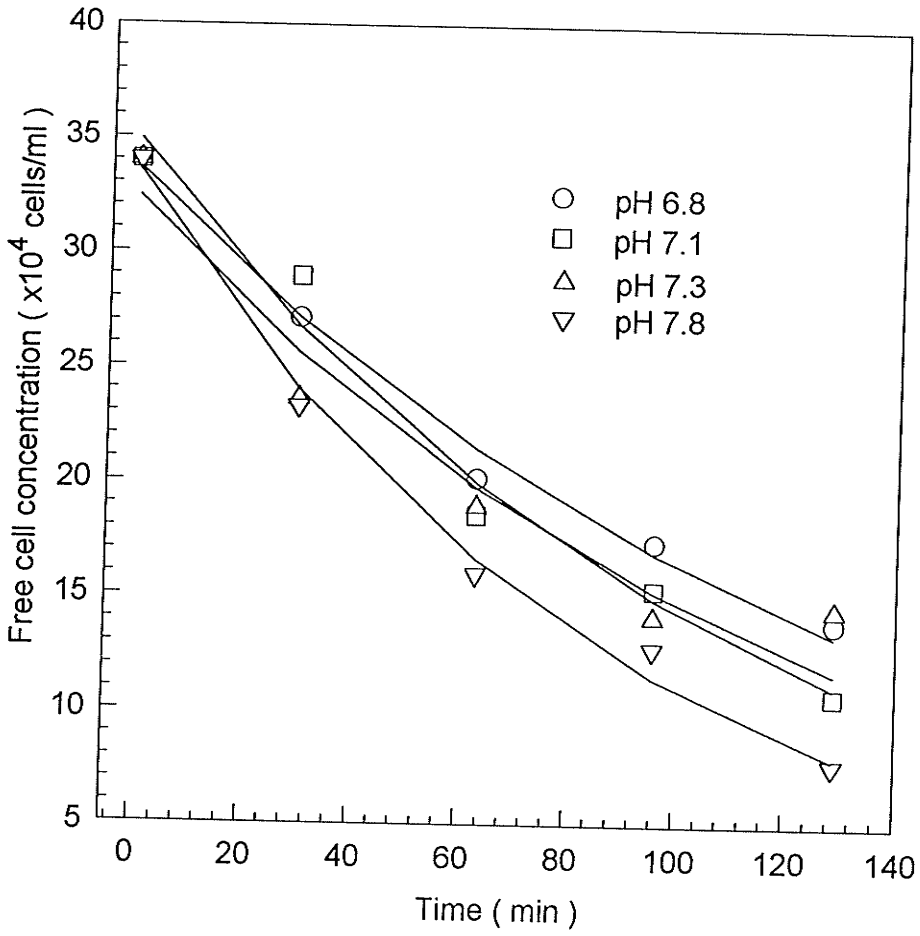


Fig.3.3.2. Effect of pH on cell aggregation of Vero cells in microcarrier-free spinner cultures. pH was maintained by using 25 mM HEPES. Cultures were supplied with DME + 5% serum and continuously stirred at 40 rpm. (n = 1)

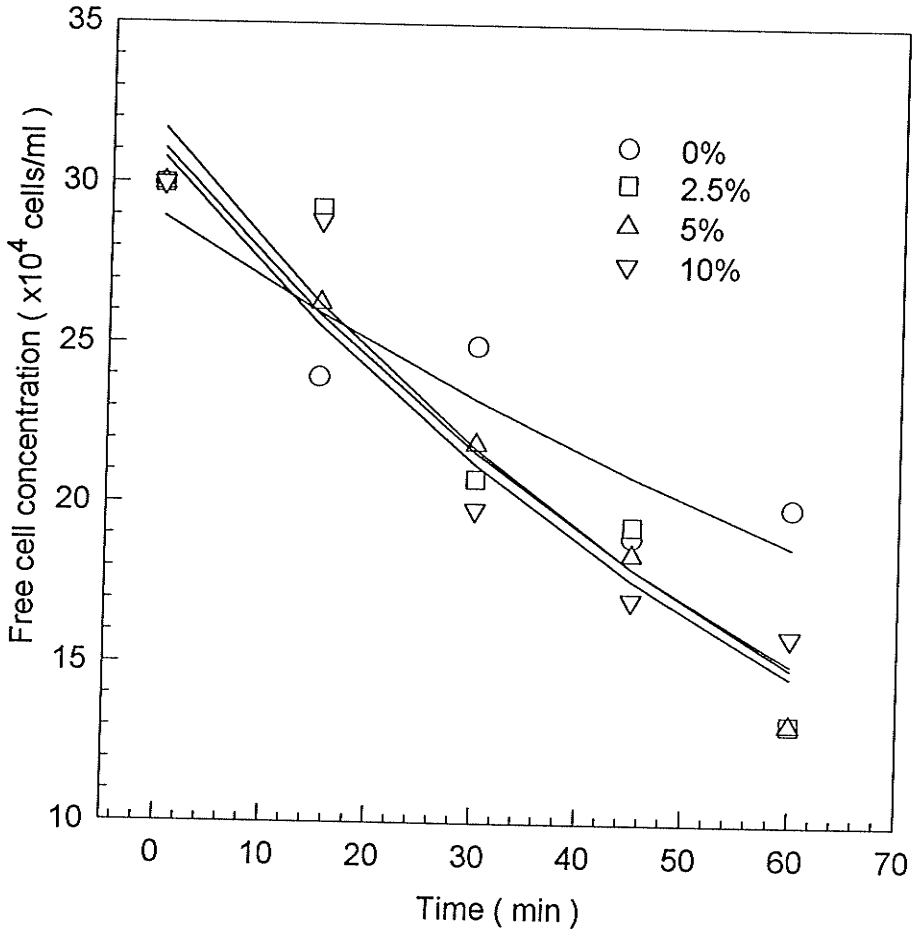


Fig.3.3.3. Effect of supplemented serum concentration on cell aggregation of Vero cells in microcarrier-free spinner cultures. Culture conditions were identical to those in Fig.3.2.2. (n = 1)

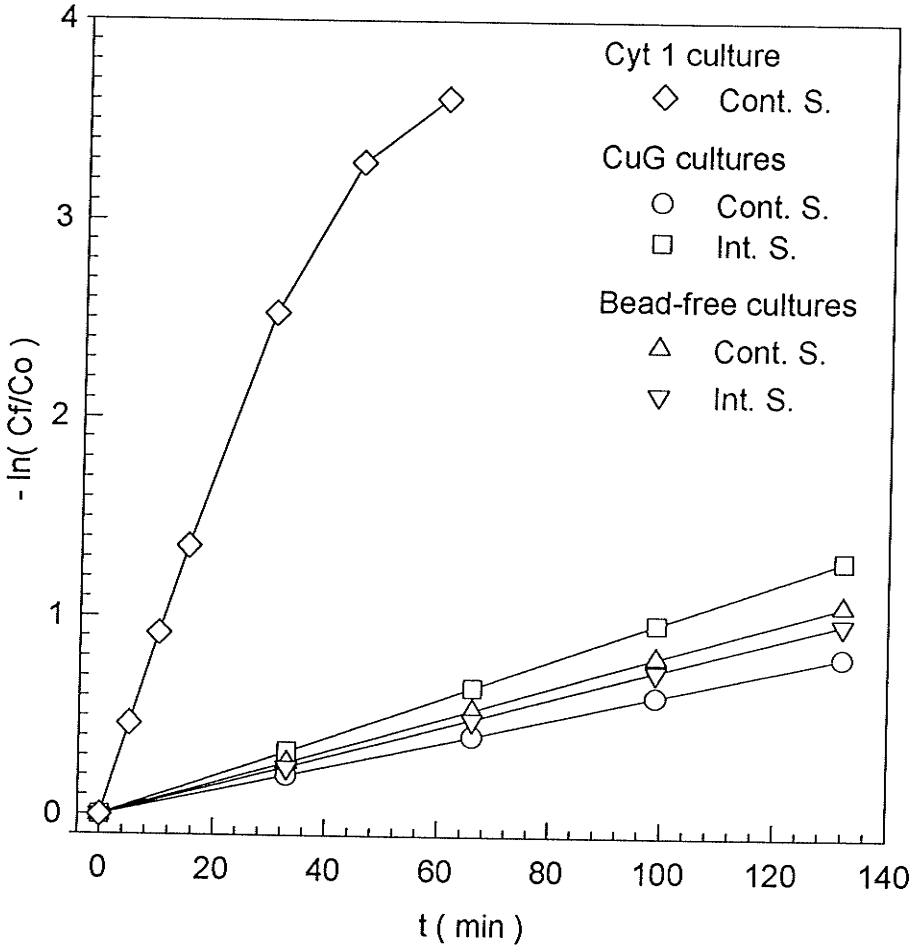


Fig.3.3.4. Effect of stirring mode on Cell attachment rates of Cyt 1, CuG cultures and cell aggregation rates in bead-free cultures. Cultures were supplied with DME + 5% serum (v/v) at pH 7.3 and stirred at 40 rpm continuously or intermittently. Measurements of cell disappearance were based on single cell concentrations in each count. (n = 1)

1 was much higher than to CuG, in the order of 10-fold. Table 3.3.2. lists the respective rates for cultures under different conditions. Intermittent stirring for CuG culture not only produced fewer and smaller cell aggregates but also faster attachment rate in comparison to that under continuous stirring. The calculated cell attachment rate of CuG beads under continuous stirring would have been lower if the measurements had accounted for cell aggregates in addition to single cells.

The extent of cell aggregation was assessed by measuring the cell concentrations that contributed to cell aggregates. Fig.3.3.5. shows that both continuously stirred cultures with and without CuG beads resulted in a significant increase in cell aggregation from 20 to 50% of total cell counts. On the other hand, under intermittent stirring CuG cultures showed a decline in the degree of cell aggregation to 10% while bead-free culture resulted an increase to 30%. This supports microscopic observations that cell aggregation occurred to smaller degree in CuG cultures under intermittent stirring. It is possible that those small aggregates being formed under intermittent stirring may have a high turnover rate for cell-bead attachment than for cell aggregation so that only a relatively small number was present in each count.

Fig.3.3.2. Summary of cell-bead attachment rates of Cyt 1 and CuG cultures and cell aggregation rates of bead-free cultures.

Culture Type	Stirring Mode	Cell attachment, k, or Cell aggregation rates, k_{ag}, ($x10^{-2}$ min^{-1})
Cyt 1	Cont. S.	9.05
CuG	Cont. S.	0.60
	Int.S.	0.98
Bead-free	Cont.S.	0.81
	Int.S.	0.74

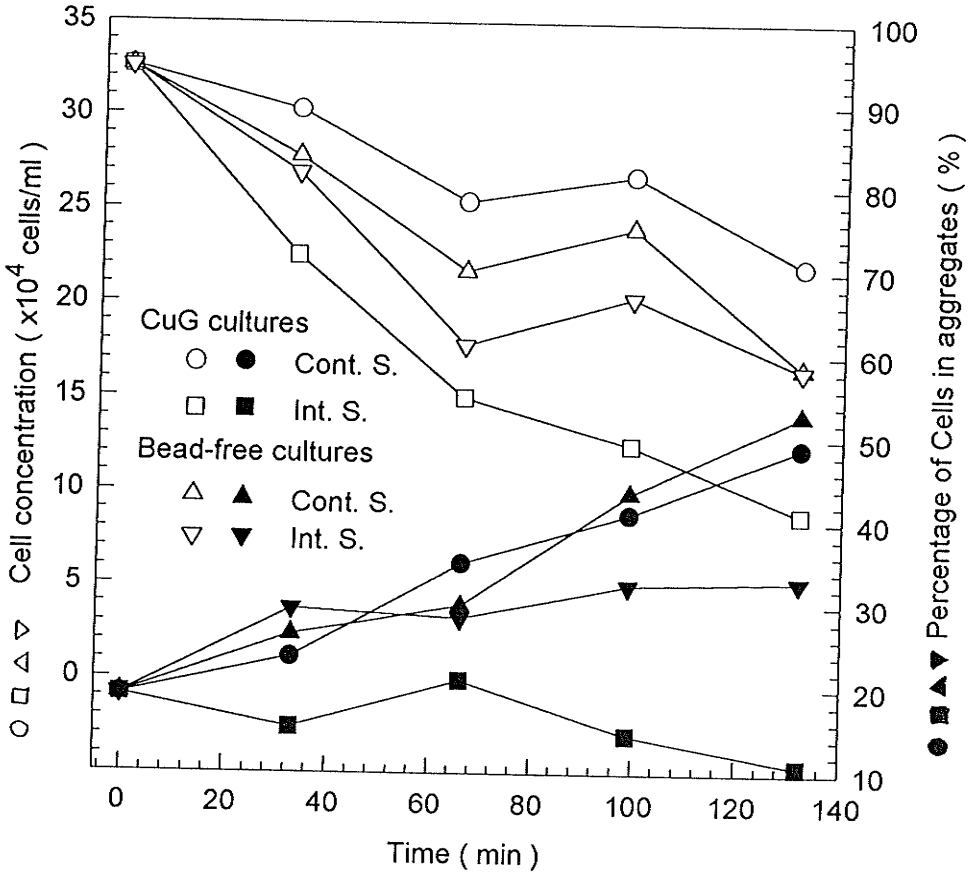


Fig.3.3.5. Effect of stirring mode on Cell attachment rates of Cyt 1, CuG cultures and cell aggregation rates and bead-free cultures. Cultures were supplied with DME + 5% serum (v/v) at pH 7.3 and stirred at 40 rpm continuously or intermittently. Measurements of cell disappearance were based on total cell counts, single cells and cell aggregates. Cell aggregation was expressed as the percentage of cells which appeared as cell aggregates in cell counts. Cell aggregates were defined as 3 or more cells associating together. (n = 1)

3.3.3. Discussion

(a) Influence of method used

The same method of measurment of free cell concentration adopted from microcarrier cultures is more prone to errors when cell aggregation rate concentrations were determined. The curve fitting showed variability of data points. Cell aggregation is a slow process. In the haemocytometer, cell clumps were sometimes difficult to distinguish from a loose association of free cells. Steps during sampling and counting may contribute to poor reproducibility.

Another disadvantage of the free cell concentration measurement is the absence of any indication on the degree of cell aggregation. The aggregation rates did not distinguish small (3-5) or large (5-10) cell aggregates

(b) Influence of cells used

Cell aggregation is dependent on the surface properties of the cell membrane. Trypsinization is known to modify the cell membrane properties including its adhesive properties (Cassiman and Bernfield, 1981), and it may influence the ability of cells to aggregate. Furthermore, cells used in all the experiments showed a varying degree of cell aggregation in the inoculum, ranging from 5 to 20% of total population.

(c) Mechanism of cell aggregation

It is known that the overall charge on cells is negative, but regional segments of the cell membrane could be positively charged. Cell aggregation between two or more negatively charged cells would be likely to occur when the charge repulsion is overcome by other factors. Attachment proteins are possibly involved in this process, as was demonstrated by higher aggregation rate in the presence of serum. Divalent ions such as

calcium and magnesium, which are important in stabilizing the ligand-receptor reactions for cell-bead attachment, may also participate in cell-cell aggregation.

The rate of cell aggregation in the presence of microcarriers may not be equal to the rate of cell aggregation without microcarriers. Only cell-cell contacts are possible in microcarrier free cultures, while in microcarrier cultures cells can interact with beads or other cells. With Cyt 1 beads, cells preferentially attach to beads rather than undergo cell aggregation because of the strong electrostatic charge interactions which promotes cell-bead attachment upon cell-bead contact. As a result, cells are capable of fast attachment rates on Cyt 1 beads within few minutes of inoculation (Table 3.1.2.). Cell aggregation becomes minimal and insignificant. However the degree of cell aggregation would be increased if the cell-bead interactions were reduced. For instances, increasing pH to 7.8 may lead to significantly reduced cell-bead interactions and therefore poor cell-bead attachment. At the same time, a significant level of cell aggregation was possibly the result of an increase in cell-cell interactions. The effect of such high pH on surface membrane properties of cells may be substantial, as the results show that it increased the ability of cells to aggregate significantly (Fig.3.3.2.). High inoculum ratio (0.8×10^6 cells/ml or 186 cells/ bead) was found to produce a number of cell aggregates in a 1 g/L Cyt 1 culture. Here it is believed that the surface area of the beads became saturated with only a fraction of the total cell population, thus cell-bead interactions for the remaining suspended cells were limited. In addition, cell-cell contacts were greatly increased in cultures with higher inoculum ratios.

For CuG beads under continuous stirring, cell-bead contacts may not be immediately converted to a secure cell-bead attachment as in Cyt 1 cultures, since the binding forces between cells and CuG beads are lower than those in Cyt 1 cultures. The charge on the CuG beads is much lower than that of Cyt 1 beads due to the chemical nature of the surfaces. Thus, in CuG cultures cell aggregation is in competition with cell-

bead attachment. In our studies, the balance seems to be affected strongly by stirring mode. Continuous stirring favored the interactions between cells, whereas intermittent stirring favored cell-bead interactions.

3.3.4. Conclusions to cell attachment and cell aggregation

Under optimal conditions, the cell attachment rate to Cyt 1 was 28.2×10^{-2} min^{-1} (Table 3.1.1.), while the equivalent rate of attachment to CuG was 1.14×10^{-2} min^{-1} under intermittent stirring and 0.55×10^{-2} min^{-1} under continuous stirring (Table 3.2.1.). Cell aggregation rates ranged from $0.45\text{-}0.83 \times 10^{-2}$ min^{-1} (Table 3.3.1.). Therefore, the rate of cell aggregation is insignificant to the rate of cell attachment to Cyt 1 but comparable to the rate of cell attachment to CuG.

3.4. Growth of Vero cells on Cyt 1 and CuG beads in spinners

3.4.1. Introduction

The growth phase, which immediately follows the cell attachment phase, is when cells start to multiply on the surfaces of the beads. For cell culture processes, it is desirable (1) to reduce lag phase (no growth) of cells after attachment and induce immediate exponential growth, (2) to maintain optimal growth for extended period before stationary phase is reached, (3) to maximize the cell densities and multiplication ratios (number of doubling), and (4) to produce and maintain healthy viable cells at all stages from growth to stationary phases. Therefore, the overall efficiency of a microcarrier system is dependent on optimal conditions for all culture phases including attachment to growth phases. However, conditions important for cell attachment are not necessarily the same as those for growth. In this chapter, the effect of those conditions, which had been tested on cell-bead attachment (sections 3.1 and 3.2), were also studied on growth of Vero cells in Cyt 1 and CuG cultures. Optimal cell attachment and distribution were established in all cultures at the attachment phase before the investigation of the effects of different conditions on growth began.

3.4.2. Results

(a) Stirring Mode

The effect of initial stirring mode on growth of Vero cells on Cyt 1 and CuG beads was determined in experiments in which cultures under continuous and intermittent stirring were compared. For Cyt 1 cultures, 1 g/L beads in 250 ml spinners were suspended in 250 ml DME + 5% serum (v/v) at about pH 7.3. Cultures were stirred either continuously at 40 rpm or intermittently for 3 min / 30 min at 40 rpm for 4 h before switching to 40 rpm continuous stirring. Cell densities were based on CV nuclei counts. The results showed that the growth pattern of both cultures is identical and their final cell densities reached

about 1 x10^6 cells/ml (Fig.3.4.1.). Under intermittent stirring, although most cells were attached to Cyt 1 beads within 2 h, cells were poorly distributed and bead occupancy was 90%. At late growth phase, full confluence was reached on Cyt 1 beads of both cultures with a monolayer of cells. In fact, the variation in cell confluence among Cyt 1 beads under two conditions became less distinguishable at the stationary phase. Beads initially populated with more cells would have less surface areas for growth, whereas beads with less cells would have a longer period of exponential growth before surface limitation occurred. Therefore, the final cell density was only dependent upon the surface areas. Continuous stirring is desirable for cell-bead attachment in Cyt 1 cultures. An initial intermittent stirring in Cyt 1 culture at cell attachment phase is shown to have no impact on growth compared to continuous stirring. As a result, subsequent cultures were set under continuous stirring.

For CuG beads, under both initial stirring modes and two bead concentrations, 1.72 and 5 g/L, were tested in 100 ml spinners. Intermittent stirring was established during the initial 6 hr attachment phase in which 90% cell attachment was achieved. The stirring regime was set at 3 min/ 30 min at 40 rpm before cultures were switched to continuous stirring. Cell densities were based on TB cell counts. Cultures at both bead concentrations under continuous stirring yielded poorer growth compared to those under intermittent stirring (Fig.3.4.2.). Continuous stirring, was shown to yield large cell aggregates prior to cell attachment, lead to long attachment phase (24 h) and poor bead occupancy (50-75%), and poor cell distribution. As a result, the microcarrier culture was not efficient in the usage of surface area. A differential degree of confluence among CuG beads under continuous stirring on Day 5 was indicated by neutral red stain (Fig.3.4.3.). Both 1.72 and 5 g/L cultures had a lag phase and a poor growth span. After the lag phase, growth resumed to a rate similar to that under initial intermittent stirring, but was reduced before reaching the final cell densities, 0.82 and 1.2 x 10^6 cells/ml for 1.72 and 5 g/L cultures

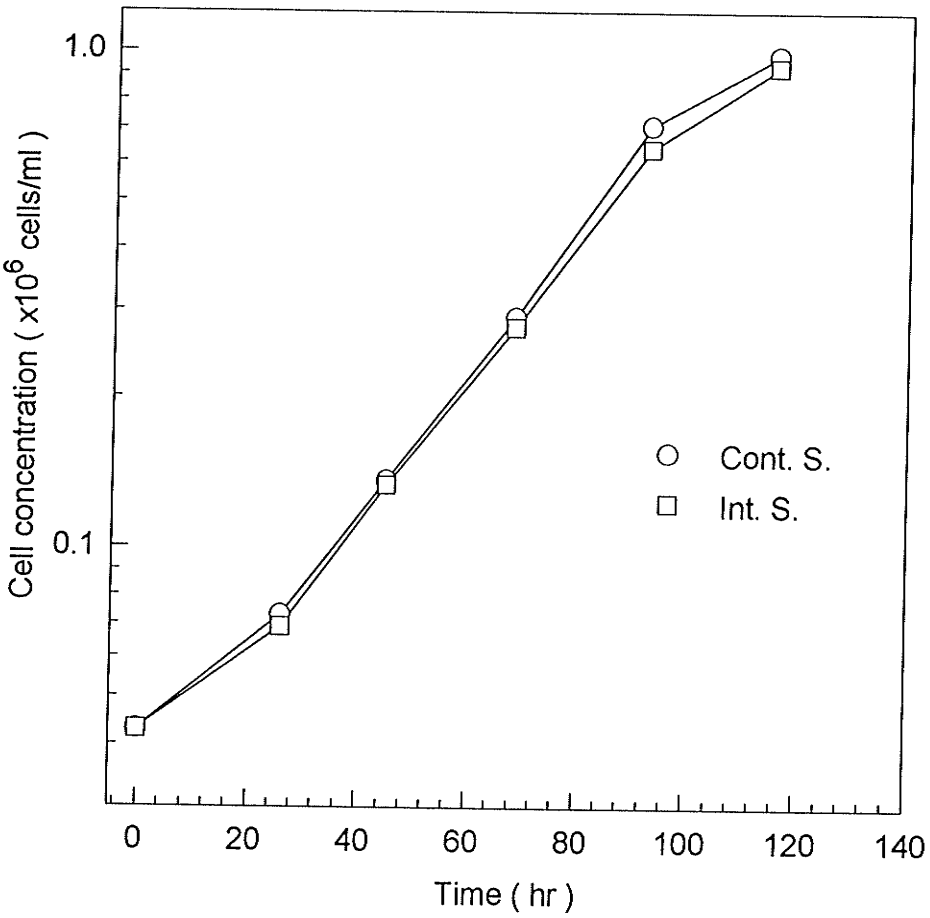


Fig.3.4.1. Effect of initial stirring mode at attachment phase on growth of Vero cells on 1 g/L Cyt 1. Cultures were supplied with 250 ml DME + 5% serum (v/v) in 250 ml spinners and stirred either continuously or intermittently at 40 rpm. The intermittent stirring regime was set at 3 min/ 30 min for 4 h before it was switched to 40 rpm continuous stirring. Nuclei stain counts were used to represent the cell concentrations in the cultures. (n = 1)

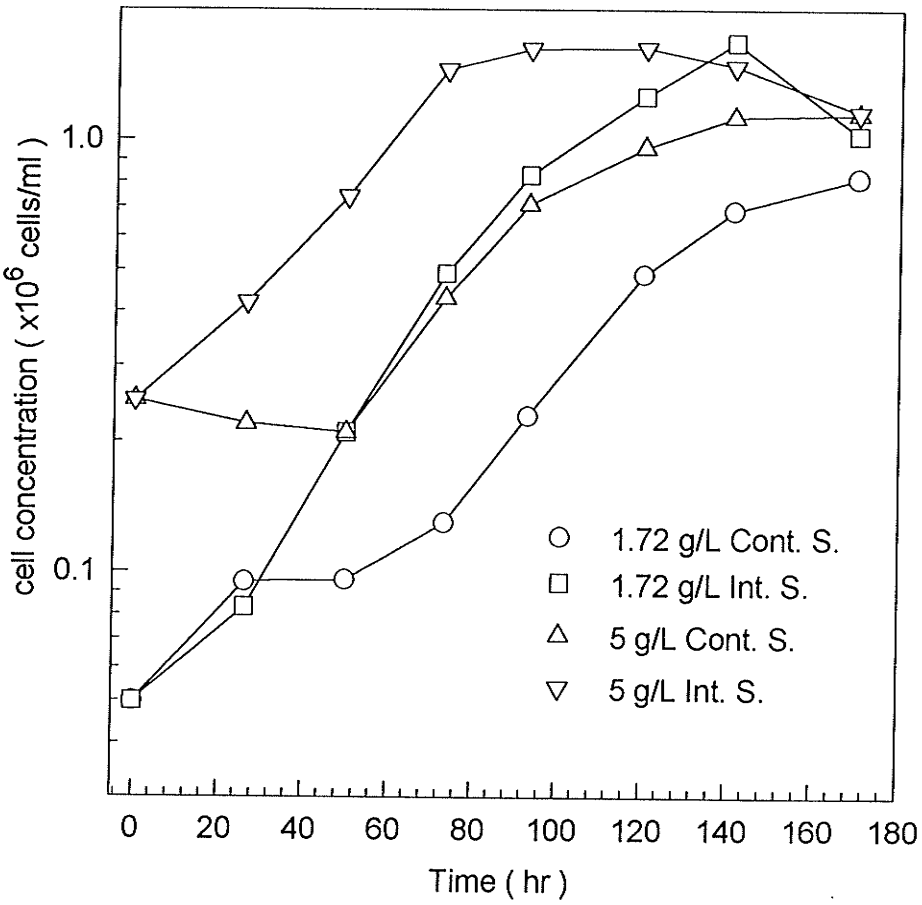


Fig.3.4.2. Effect of initial stirring mode on growth of Vero cells on CuG beads. Cultures were set at 1.72 and 5 g/L and initially stirred either continuously or intermittently. Those cultures initially under intermittent stirring were set at 3 min/ 30 min for first 6 h before switching to continuous stirring. All cultures were supplied with DME + 5% serum (v/v) and pH adjusted to 7.3. (n = 1)

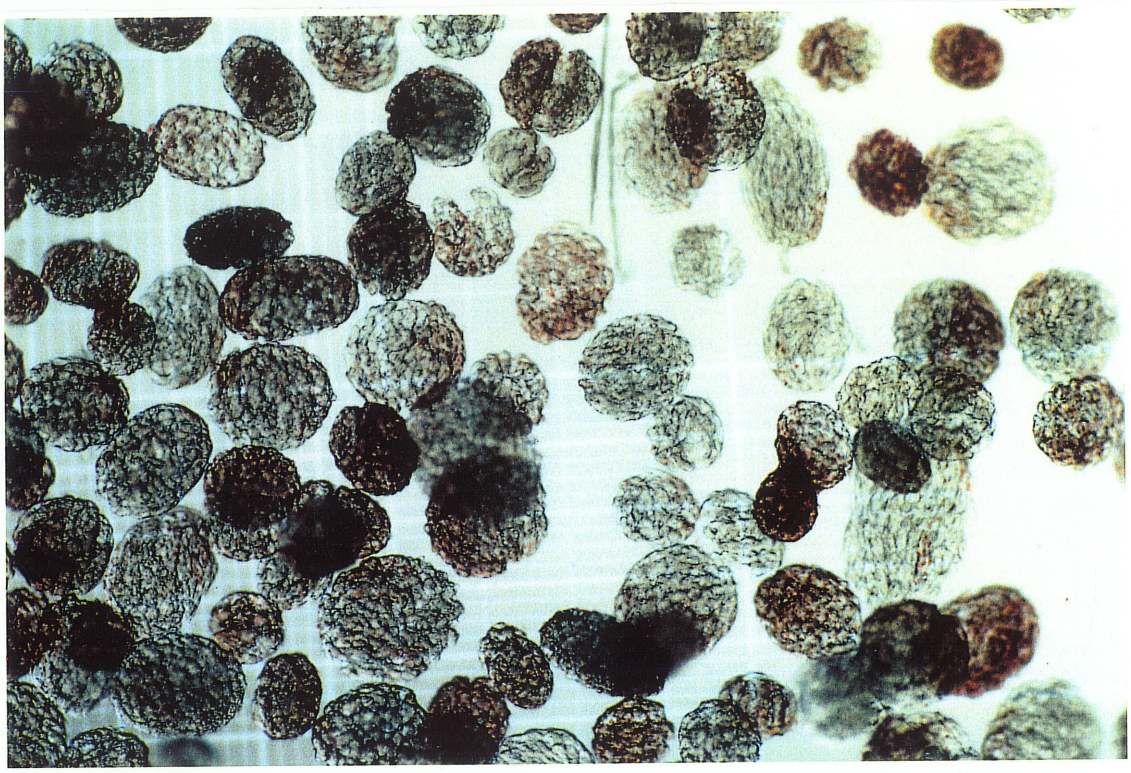


Fig.3.4.3. Effect of continuous stirring on Vero cell growth on 5 g/L CuG on Day 5. Culture was supplied with DME + 5% serum (v/v) and stirred continuously at 40 rpm after inoculation. Bead samples were photographed under 40X magnification.

respectively under continuous stirring.

On the other hand, cultures under intermittent stirring yielded optimal cell attachment and distribution. No lag phase was produced and uniform confluence was maintained among beads as revealed by neutral red stain (Fig.3.4.4.a-d). The final cell densities reached by 1.72 and 5 g/L were 1.7 and 1.6 x10^6 cells/ml respectively under intermittent stirring.

Therefore, continuous stirring through attachment to growth phases is proved to be detrimental to cell growth on CuG beads. As a result, subsequent cultures were stirred intermittently at 3 min/ 30 min at 40 rpm for the first 6 hr before switching to continuous stirring at 40 rpm.

(b) Agitation

(i) Batch Cultures

The effect of agitation speed on cell growth in Cyt 1 and CuG cultures was investigated in experiments in which 100 ml spinners with beads at 1 g/L (Cyt 1) and 1.72 g/L (CuG) were tested. Since 40 rpm is the optimal agitation speed for cell-bead attachment in Cyt 1 and CuG cultures, this agitation speed was used on all cultures. After 24 h, some cultures of both bead types were switched to 60 or 80 rpm from 40 rpm. Cell densities were based on TB cell counts. For Cyt 1 beads, increasing the stirring speed to 80 rpm showed to slow down cell growth and yield lower maximum cell density (Fig.3.4.5.) Cultures at 40 and 60 rpm resulted in a similar growth pattern and reached maximum cell densities of 1 x10^6 cells/ml as opposed to 0.74 x10^6 cells/ml at 80 rpm. Therefore, optimal cell growth could be maintained at 40 rpm or 60 rpm. As a result, 40 rpm was selected as the continuous stirring speed for subsequent cultures.

For CuG beads, intermittent stirring was allowed at 40 rpm for the first 6 h for all cultures before switching to continuous stirring. Both cultures at 40 and 60 rpm yielded

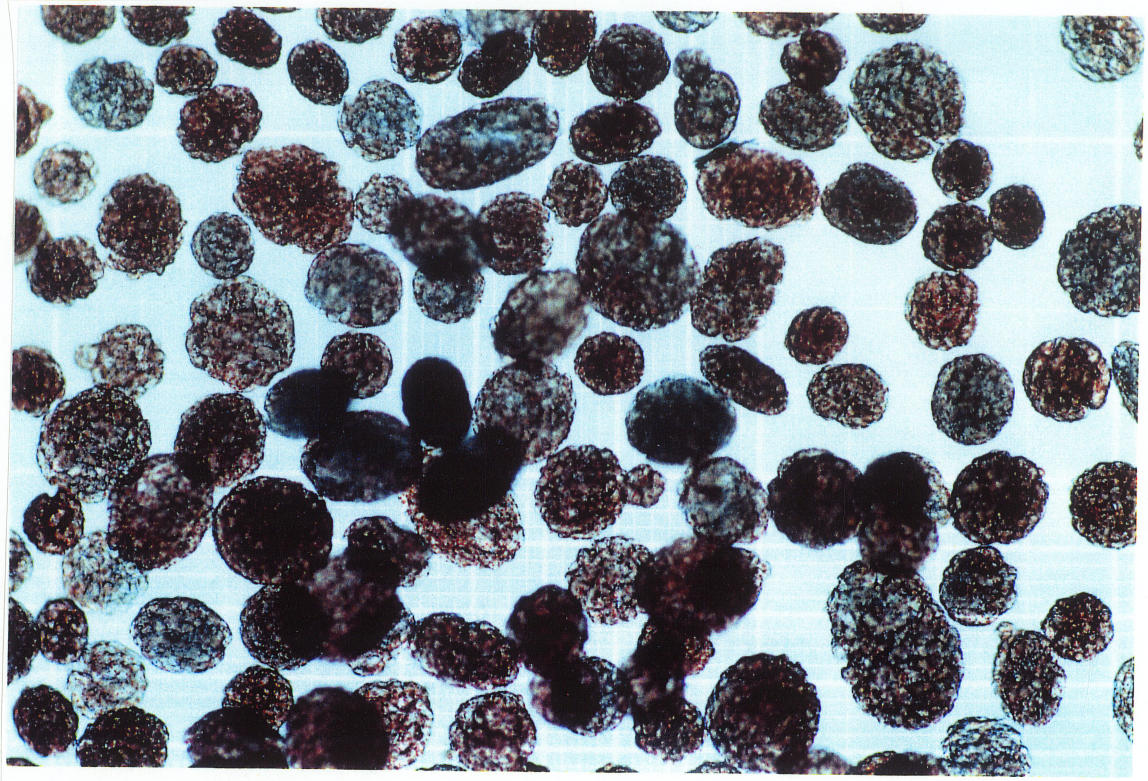


Fig.3.4.4a. Effect of initial intermittent stirring on Vero cell growth on 1.72 g/L CuG on Day 4. Culture was initially stirred under intermittent stirring at 3 min/ 30 min for 6 hr at 40 rpm before switching to 40 rpm continuous stirring. Culture was also supplemented with 5% serum in the medium. Bead samples were photographed under 40X magnification.

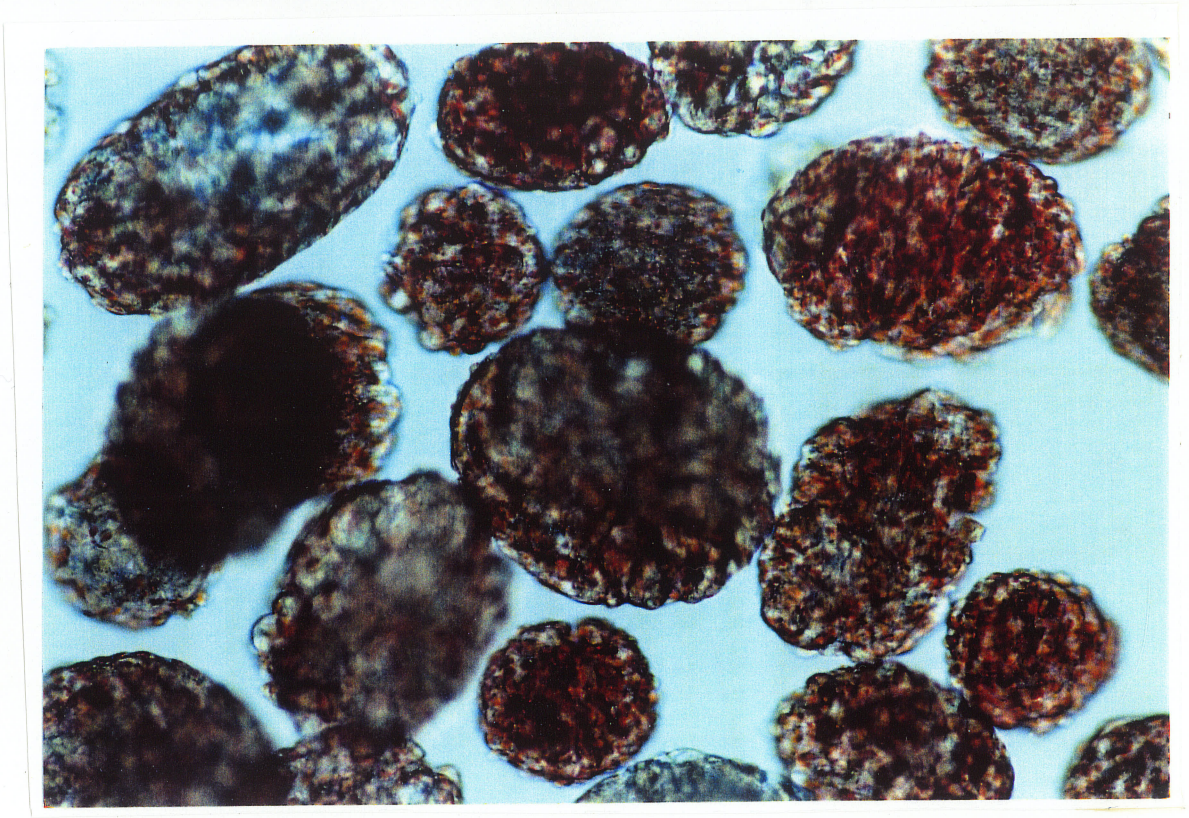


Fig.3.4.4b. Effect of initial intermittent stirring on Vero cell growth on 1.72 g/L CuG on Day 4. Culture condition was identical to Fig.3.4.4a. Bead samples were photographed under 100X magnification.

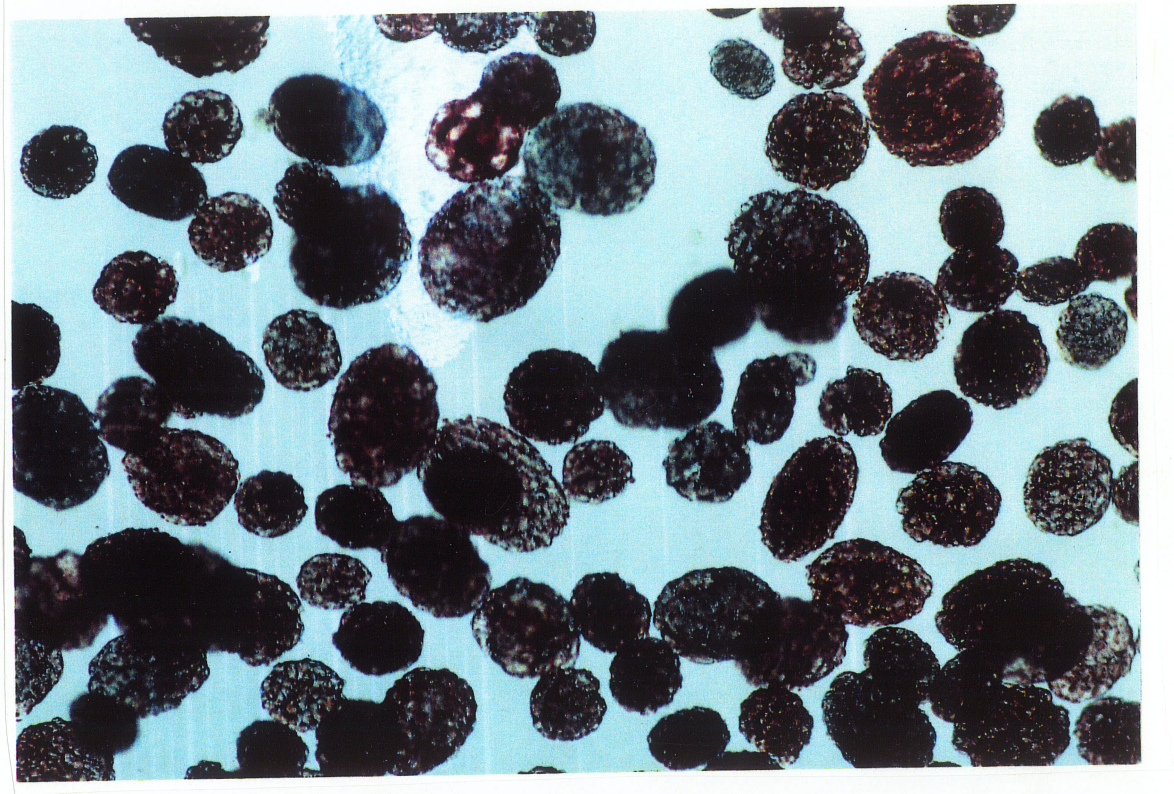


Fig.3.4.4c. Effect of initial intermittent stirring on Vero cell growth on 1.72 g/L CuG on Day 6. Culture condition was identical to Fig.3.4.4a. Bead samples were photographed under 40X magnification.

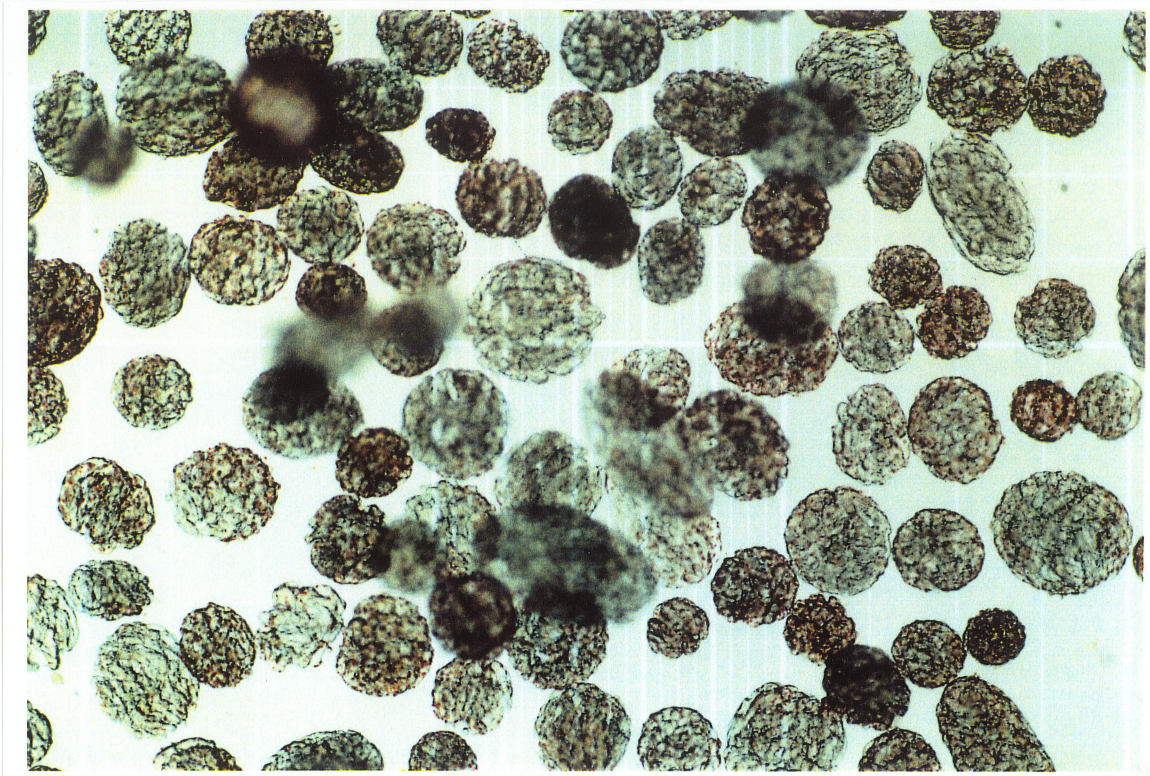


Fig.3.4.4d. Effect of initial intermittent stirring on Vero cell growth on 5 g/L CuG on Day 3. Culture condition was similar to Fig.3.4.4a. Bead samples were photographed under 40X magnification.

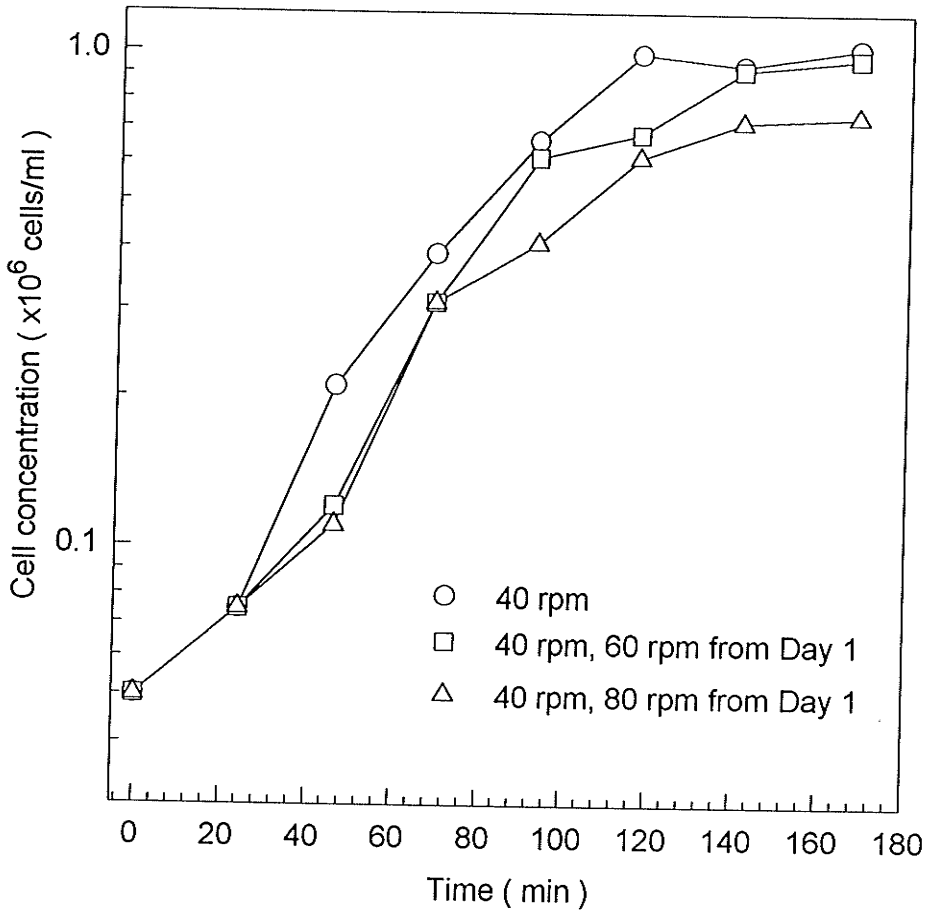


Fig.3.4.5. Effect of agitation speed on growth of Vero cells on 1 g/L Cyt 1. Cultures were supplied with DME + 5% serum (v/v) and stirred at 40 rpm for first 24 h. Afterwards, high speed cultures were switched to 60 or 80 rpm. (n = 1)

approximately 1.7 and 1.6 x10^6 cells/ml as opposed to 1.42 x10^6 cells/ml at 80 rpm. (Fig.3.4.6.).

Therefore, agitation at speed higher than 60 rpm is detrimental to cell growth and maximum cell densities in both bead types. Such detrimental effect on cells is likely coupled with the increasing depletion of nutrients and accumulation of metabolite by-products which are contributing to adverse conditions for the ability of cells to counteract the effect of shear stress.

(ii) Critical Agitation Speed

It was of interest to identify the maximum agitation speed that did not affect cell growth. Agitation was tested in experiments in which the speed was increased gradually (at 25 rpm/day) or rapidly (at 100 rpm/day). All Cyt 1 and CuG cultures in 250 ml spinners were allowed to be stirred continuously at 40 rpm for 24 h. Afterwards, the high speed cultures were switched to 100 rpm. From Day 2, they were then replaced with 50% medium. Cell densities were based upon TB cell counts.

In the first experiment in which the agitation speed was increased at an increment of 25 rpm/day from 100 rpm on Day 2 up to 300 rpm on Day 9 in both Cyt 1 and CuG cultures at low bead concentration. For Cyt 1 cultures (Fig.3.4.7.), growth pattern between the control and high speed culture was similar and maximum cell densities reached were at 1.5 and 1.2 x10^6 cells/ml respectively. Cell concentrations were significantly different on day 9 when the high speed culture was exposed to 275 rpm for 24 hr. For CuG cultures (Fig.3.4.8.), growth pattern between the control and high speed cultures was similar and maximum speed from 100 to 300 rpm did not affect Vero cell growth on CuG beads. Therefore, the critical agitation speed for Cyt 1 culture is 275 rpm but for CuG culture is >300 rpm.

On the other hand, in another experiment in which the speed of Cyt 1 and CuG

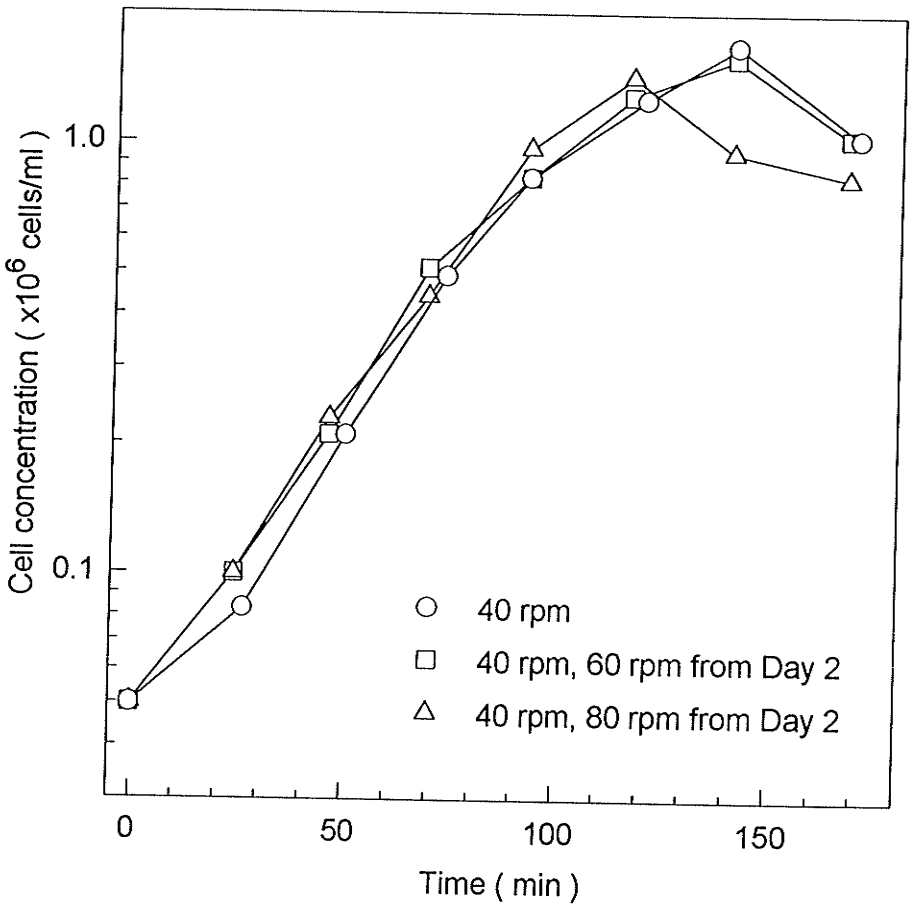


Fig.3.4.6. Effect of agitation speed on growth of Vero cells on 1.72g/L CuG. Intermittent stirring was established for all cultures at 3 min/ 30 min at 40 rpm for first 6 h before they were switched to 40 rpm continuous stirring. High speed cultures were switched to 60 or 80 rpm on Day 1 (24 h). Also, cultures were supplied with DME + 5% serum (v/v). (n = 1)

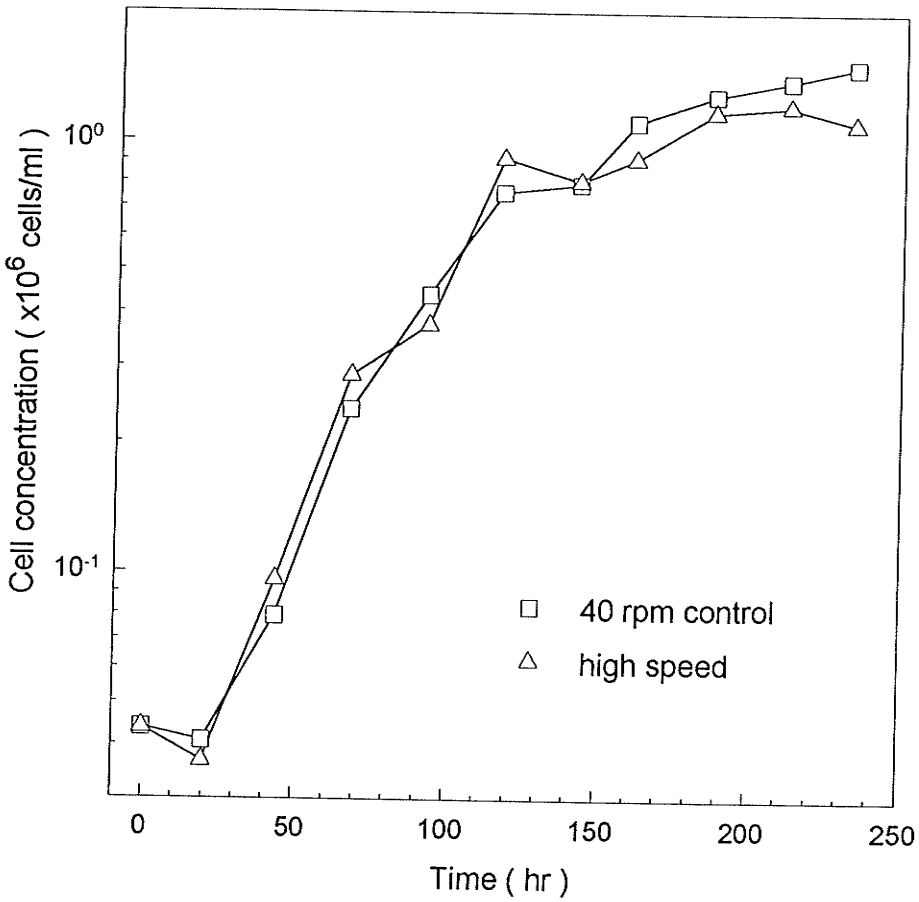


Fig.3.4.7. Effect of high speed agitation on growth of Vero cells on Cyt 1 beads. All 1 g/L cultures were supplied with DME + 5% serum (v/v) and stirred initially at 40 rpm, on Day 1 the high speed culture was switched to100 rpm and increased at an increment of 25 rpm/day from Day 2 to 300 rpm on Day 9. (n = 1)

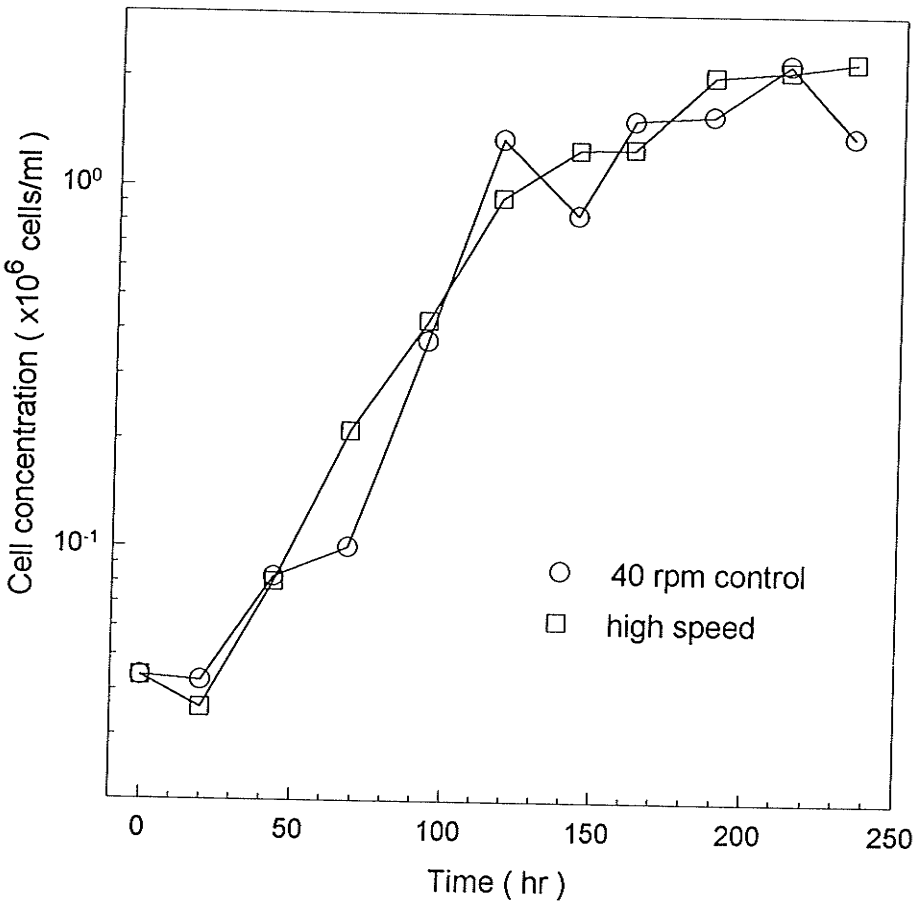


Fig.3.4.8. Effect of high speed agitation on growth of Vero cells on CuG beads. All 1.72 g/L cultures were supplied with DME + 5% serum (v/v) and stirred initially at 40 rpm, on Day 1 the high speed culture was switched to100 rpm and increased at an increment of 25 rpm/day from Day 2 to 300 rpm on Day 9. (n = 1)

cultures was increased rapidly at 100 rpm/day from 100 rpm on Day 2 up to 400 rpm on Day 4. For Cyt 1 beads cultures (Fig.3.4.9.), results show that in cultures at 1 and 5 g/L the critical speed was 300 rpm. Subsequently, cell yields were further decreased in both cultures after 24 h at 300 rpm and <300 rpm, respectively. At 400 rpm, cell densities dropped very significantly and empty beads were predominant in both Cyt 1 cultures. For CuG beads (Fig.3.4.10.), results indicate that in cultures at 1.72 and 8.6 g/L the critical speed was at 300 rpm and < 300 rpm, respectively. Subsequently, cell yields at both bead concentrations leveled and declined much more gently at 400 rpm than those Cyt 1 cultures. It suggests that CuG beads has greater tolerance for shear stress than Cyt 1 beads at extremely high speed.

(c) pH

The pH buffering was based on the coupling of sodium bicarbonate in DME medium and CO_2 in the gaseous phase. pH at equilibrium was showed to be 7.3 after overnight incubation overlay with 10% CO_2. Since the pH was not maintained at 6.8-7.8 by sodium bicarbonate, it was attempted to use a stronger buffer reagent, N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], HEPES in cultures. Cultures were supplied with DME medium in which HEPES was added and pH adjusted to the desired value with 10N NaOH. In addition, the side arms of the spinners were tightened and wrapped with parafilm to prevent any CO_2 diffusion. It was found that pH was maintained only for a period of 2 h before cultures at both extreme ranges converged to pKa (7.5) of the buffer. In fact, color changes indicated that cultures at 6.8 became increasingly alkaline while at 7.8 increasingly acidic. Therefore, pH would not be maintained at values other than 7.5 for extended time periods in spinners.

With the use of Celligen™ bioreactor (New Brunswick, NJ) , optimal pHs for growth in both Cyt 1 and CuG cultures were at 7.1 and 6.8 (unpublished result). These pH

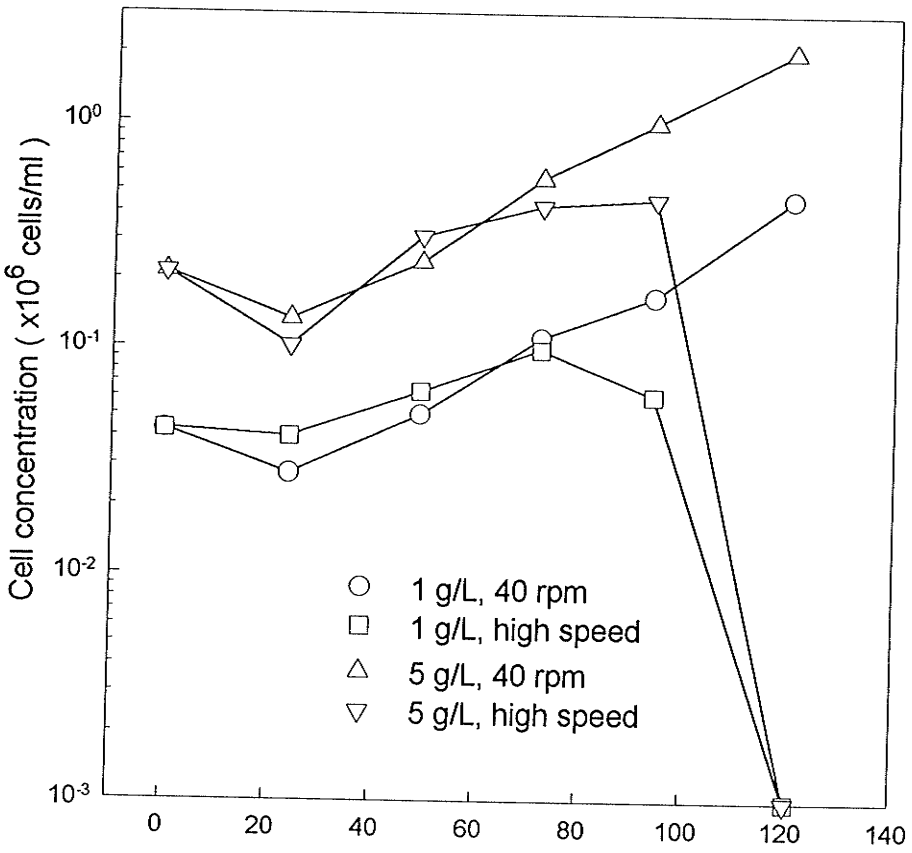


Fig.3.4.9. Effect of high speed agitation on growth of Vero cells on Cyt 1 beads. All cultures were supplied with DME + 5% serum (v/v) and stirred initially at 40 rpm, on Day 1 the high speed culture was switched to 100 rpm and increased at an increment of 100 rpm/day from Day 2 to 400 rpm on Day 4. (n = 1)

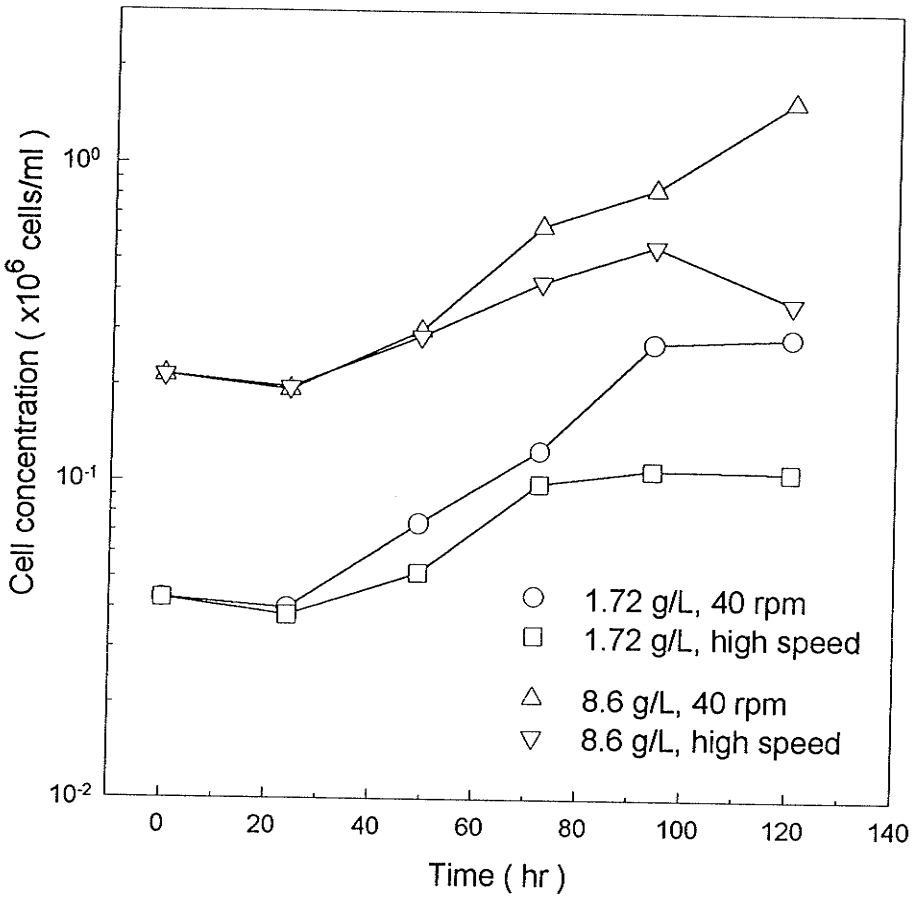


Fig.3.4.10. Effect of high speed agitation on growth of Vero cells on CuG beads. All cultures were supplied with DME + 5% serum (v/v) and stirred initially at 40 rpm, on Day 1 the high speed culture was switched to100 rpm and increased at an increment of 100 rpm/day from Day 2 to 400 rpm on Day 4. (n = 1)

ranges, however, could not be steadily maintained in spinners. In sections 3.1. and 3.2., the optimal pH was determined to be 7.3 for Vero cell attachment onto Cyt 1 and CuG beads. Efforts were made by adjusting pH of spinner cultures to 7.1-7.3 with 2N NaOH once growth was initiated by cells.

(d) Supplemented Serum Concentration

The effect of supplemented serum upon cell growth in Cyt 1 and CuG cultures was determined in experiments where the concentration was varied from 0 to 10% at the time of inoculation. Cell densities were based on TB cell counts. For Cyt 1 cultures, results show that growth is dependent on serum supplementation. Cultures at 5 and 10% yielded a similar growth pattern and reached maximum cell densities of 1 $x10^6$ cells/ml, compared to 0.8 $x10^6$ cells/ml for culture at 2.5% (Fig.3.4.11.). Cells of the culture at 0% remained rounded on the bead surfaces and yielded negative growth. Therefore, optimal supplemented serum concentration for growth is found to be 5 % on Cyt 1. Subsequently, cultures were supplemented with serum at 5% of the medium.

For CuG cultures, intermittent stirring was established initially for all cultures for the first 6 h. Results in Fig.3.4.12. indicate that the growth pattern is dependent upon serum supplementation. In the absence of serum, cells attached and grew at a slower rate to 0.56 $x10^6$ cells/ml. Those cultures containing serum at 1 and 2.5%, reached maximum densities at 0.85 and 1.2 $x10^6$ cells/ml, respectively. On the other hand, cultures at 5 and 10% provided the maximum growth, with a maximum density of 1.7-1.8 $x10^6$ cells/ml. Therefore, optimal growth is obtained in cultures with medium supplemented at 5% serum. As a result, subsequent cultures were supplemented with serum at 5%.

(e) Inoculum Size

The effect of inoculum (cell/ bead) ratio on growth of Vero cells in Cyt 1 and CuG

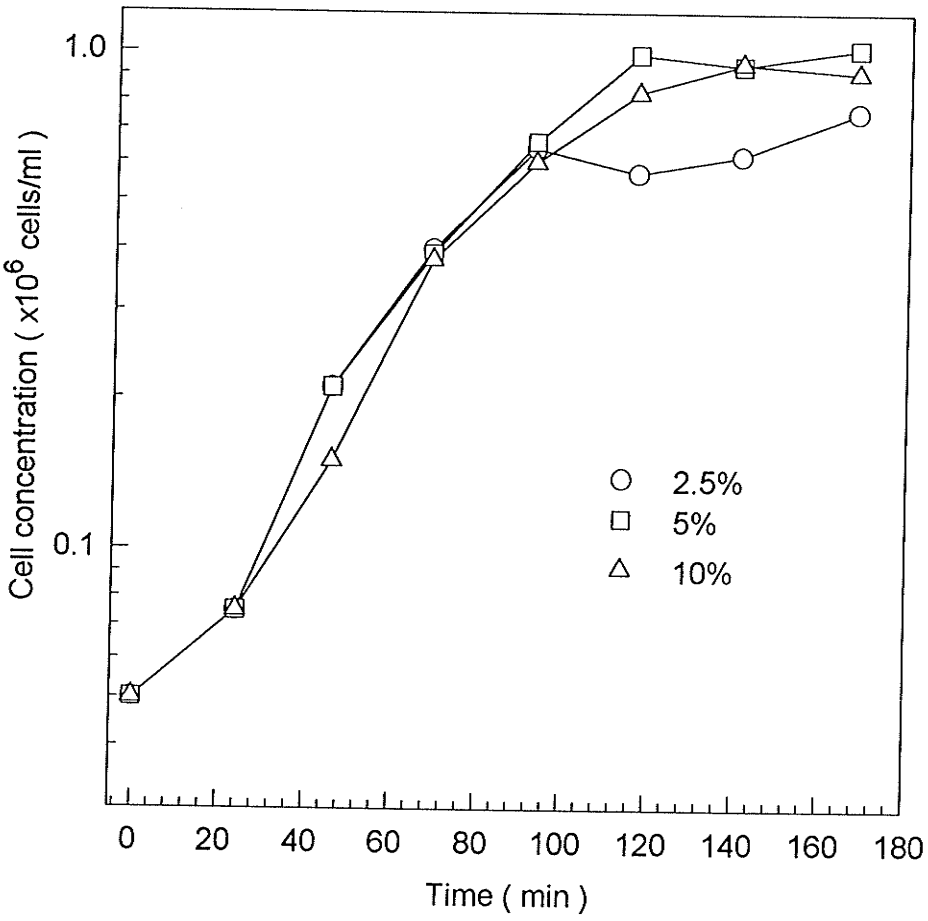


Fig.3.4.11. Effect of supplemented serum concentration on growth of Vero cells on 1 g/L Cyt 1. Cultures were supplemented with serum at different concentrations (v/v) and stirred continuously at 40 rpm. (n = 1)

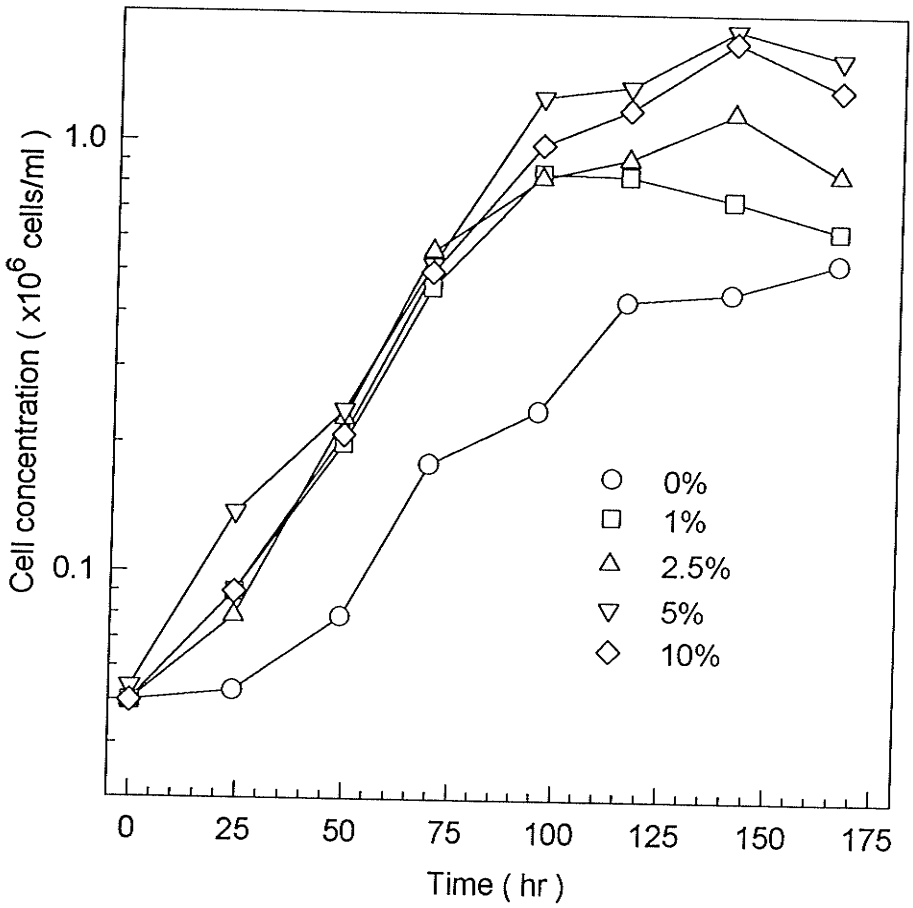


Fig.3.4.12. Effect of supplemented serum concentration on growth of Vero cells on CuG. Intermittent stirring was established for all cultures at 3 min/ 30 min for the first 6 h before switching to 40 rpm continuous stirring. (n = 1)

cultures was also determined. Cell densities were based on TB cell counts. For Cyt 1, cultures were inoculated with cells from 10 to 80 cell/ bead. Results in Fig.3.4.13. show that the increase of inoculum ratio from 10 to 40 in 1 g/ L cultures did not lead to an increase in maximum cell densities, they all reached 1-1.1 $x10^6$ cells/ml on Day 6. On the other hand, culture at 80 cell/ bead yielded 1.5 $x10^6$ cells/ml over the same period. However, the multiplication ratio (number of doubling obtained from inoculum to final cell yield) is the least among all cultures. From Table 3.4.1., an inoculum ratio at 10 cell/ bead results the highest multiplication ratio and comparable maximum cell density over others. Therefore, 10 cell/ bead is the optimal inoculum ratio. Subsequently, Cyt 1 cultures were set at 10 cells/bead.

Inoculum ratio was also tested on 1.72 g/L CuG cultures, ranging from 8 to 645 cell/ bead. Cell attachment was successful for all cultures under 6 hr intermittent stirring. Bead occupancy was maintained between 95-100% for all cultures except that at 8 cell/ bead which had approximately 90%. Results show similar growth patterns in CuG cultures was observed as in Cyt 1 cultures. There seemed to be a limit for cell growth regardless of the inoculum densities. Results in Fig.3.4.14. show that even in a wide range of cell/ bead inoculum ratios, the maximum cell densities of the cultures were confined to a relatively narrow range of maximum cell densities, from 1.6 $x10^6$ cells/ml at 30 cells/ bead to 1.9 $x10^6$ cells/ml at 645 cells/ bead. While a maximum cell density of 1 and 1.32 $x10^6$ cells/ml was found in cultures at 8 and 16 cells/ bead, respectively, it is believed such limitation of growth is affected by something other than physical factors. Since surface area is significantly greater in CuG beads compared to Cyt 1 beads, it is likely that nutrients including oxygen and pH are critical for expanding growth beyond a monolayer. Culture at higher inoculum density yielded a smaller multiplication ratio, whereas, those at lower inoculum density yielded higher multiplication ratio. Table 3.4.2. indicates that the optimal balance of values of maximum cell density and multiplication ratio is from culture at 30

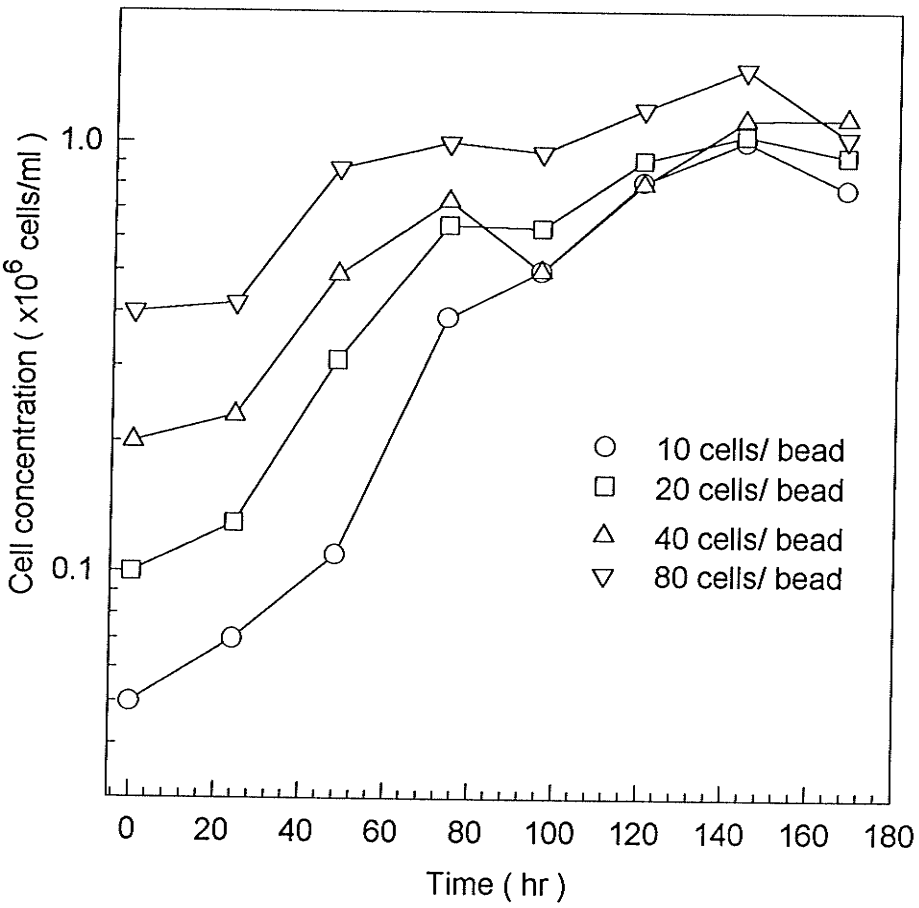


Fig.3.4.13. Effect of inoculum ratio on growth of Vero cells on 1 g/L Cyt 1. Cultures were supplemented with DME + 5% serum (v/v) and pH adjusted to 7.3. They were all stirred continuously at 40 rpm. (n = 1)

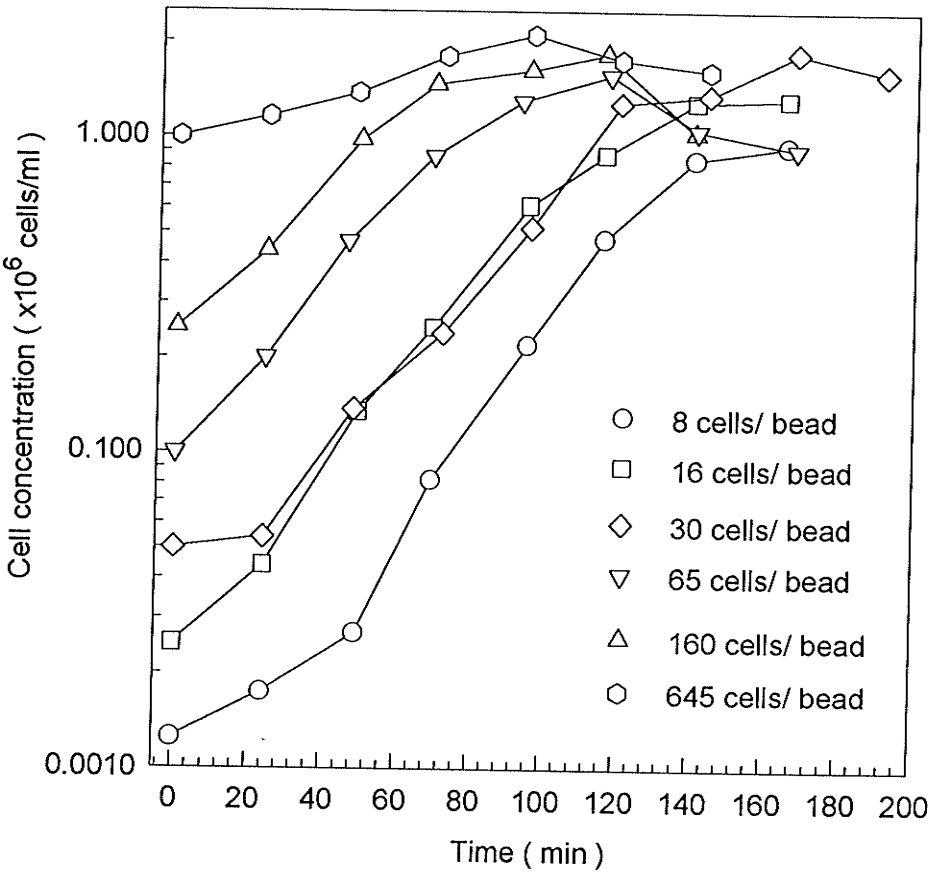


Fig.3.4.14. Effect of inoculum (cell/ bead) ratio on growth of Vero cells on 1.72 g/L CuG. Cultures were supplied with DME + 5% serum (v/v) and stirred intermittently at 40 rpm for first 6 h before switched to 40 rpm continuous stirring. (n = 1)

Table 3.4.1. Summary of growth of Vero cells on 1 g/L Cyt 1 at different cell inoculum ratios.

Inoculum (cell/ bead)	Inoculum ($x10^6$ cells/ml)	Maximum Cell density ($x10^6$ cells/ml)	Maximum cell/ bead	Multiplication Ratio [a] (number of doublings)
10	0.05	1.01	235	4.3
20	0.10	1.04	242	3.4
40	0.20	1.13	263	2.5
80	0.40	1.48	344	1.9

a Mulitplication ratio refers to the number of doubling obtained from inoculum to final cell yield, which is calculated as n in the equation of $Xf = Xi \cdot 2^n$, where Xf and Xi are final and initial cell densities, respectively.

Table 3.4.2. Summary of growth of Vero cells on 1.72 g/L CuG at different cell/ bead ratios.

Inoculum (cell/ bead)	Inoculum ($x10^6$ cells/ml)	Maximum cell density ($x10^6$ cells/ml)	Maximum cell/ bead	Multiplication ratio (number of doublings)
8	0.0125	0.94	607	6.2
16	0.025	1.32	853	5.7
30	0.05	1.85	1195	5.2
65	0.10	1.57	1014	4.0
160	0.25	1.83	1182	2.9
645	1.00	1.90	1227	0.9

cell/ bead. As a result, subsequent cultures were set at 28-30 cell/ bead or at 0.05 $x10^6$ cells/ml.

(f) Bead Concentration

(i) Batch cultures

The effect of bead concentration on growth of Vero cells in Cyt 1 and CuG cultures was determined. Cell densities were based on TB cell counts. For Cyt 1 cultures, the bead concentration was varied from 1 to 5 g/L. Results indicate that the increase in bead concentration (surfaces) did not lead to proportional increase in cell densities (Fig.3.4.15.). In contrast, 5 g/L culture reached a lower cell density at 0.7 $x10^6$ cells/ml compared to 1 $x10^6$ cells/ml in 3 and 1 g/L cultures. From Tables 3.4.3a and 3.4.3b., the 1 g/L culture achieved the optimal cell density and multiplication ratio despite the least available surface areas. In fact, it yielded approximately 235 cell/ bead at its maximum cell density.

For CuG cultures, bead concentration was tested in experiments, ranging from 0.43 to 5 g/L under continuous and intermittent stirring. As reported in sub-section 3.4.2. (a), continuous stirring was shown to be detrimental to cell growth in cultures at different bead concentrations (Fig.3.4.16a) in comparison to those under intermittent stirring (Figs.3.4.17a.). The initial 6 h intermittent stirring allowed proper cell attachment and distribution in cultures with concentrations from 0.43 to 5 g/L as revealed by neutral red stain on the beads. Results show that the desirable conditions for attachment were also important to subsequent growth. Bead concentrations under initial intermittent stirring improved cell densities compared to those under continuous stirring. The maximum cell/ bead ratio was highest in culture at 0.43 g/L under both continuous and intermittent stirring (Figs.3.4.16b., 3.4.17b.), reaching approximately 2000 and 2500 respectively. Although at 1.72 g/L the maximum cell/ bead ratio was 2.3 times lower compared with

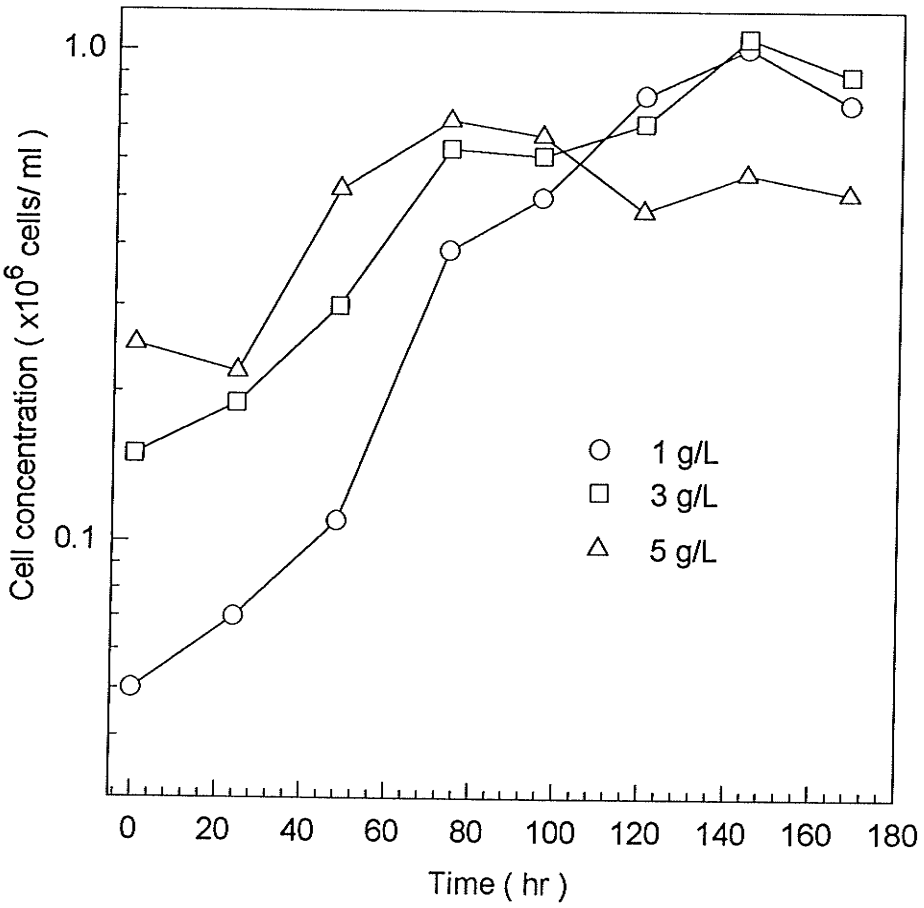
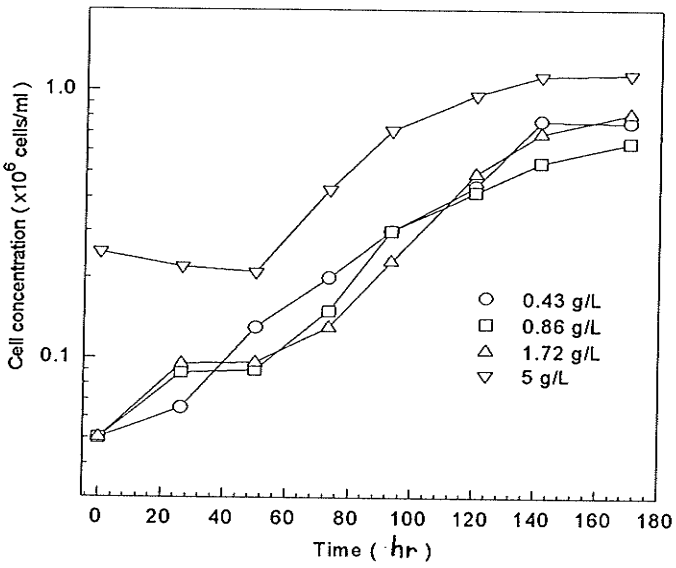


Fig.3.4.15. Effect of bead concentration on growth of Vero cells on Cyt 1. Cultures were supplied with DME + 5% serum (v/v) and stirred continuously at 40 rpm. (n = 1)

(a)



(b)

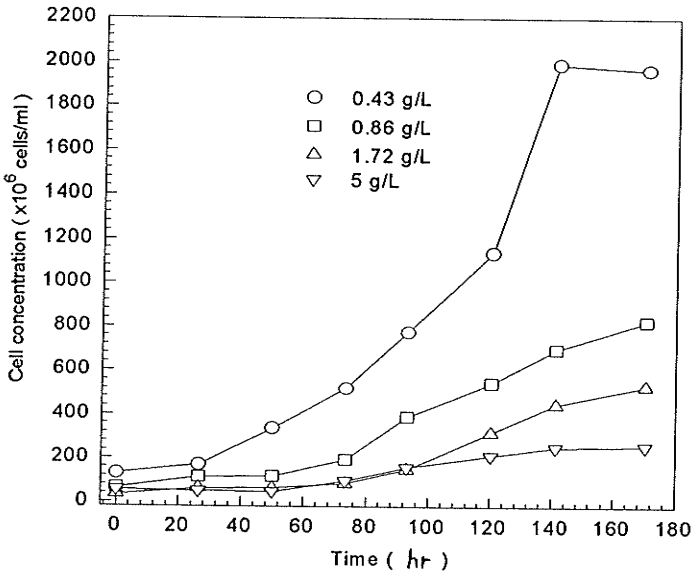
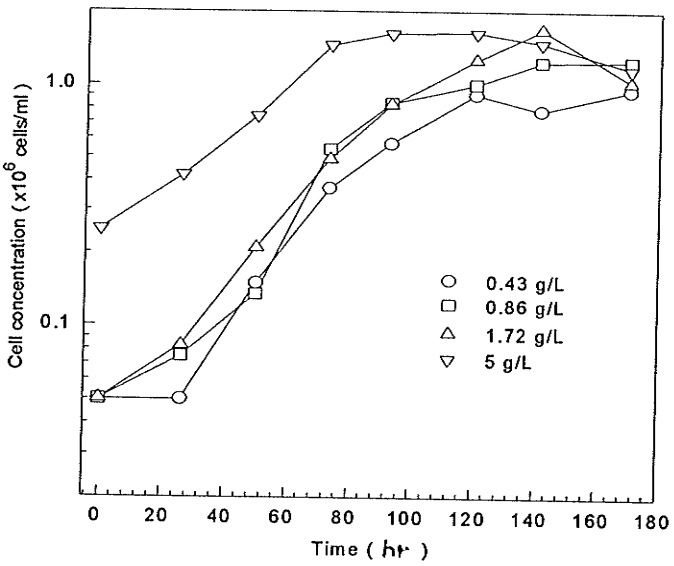


Fig.3.4.16. (a) Effect of bead concentration on growth of Vero cells on CuG under continuous stirring at attachment phase. Cultures were supplied with DME + 5% serum (v/v) and stirred continuously at 40 rpm. (b) Effect of bead concentration on growth of Vero cells on CuG is expressed as cell concentration (cell/ bead) against time (h). (n = 1)

(a)



(b)

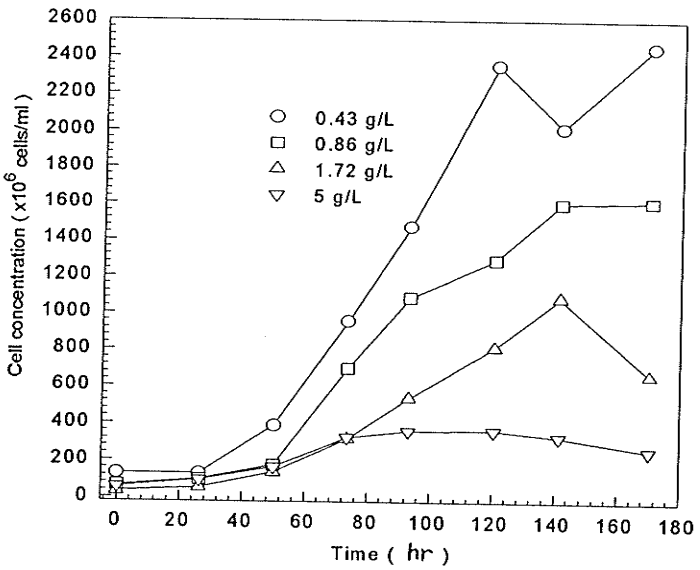


Fig.3.4.17. (a) Effect of bead concentration on growth of Vero cells on CuG under initial intermittent stirring at attachment phase. Cultures were supplied with DME + 5% serum (v/v) and allowed to stir intermittently at 3 min/ 30 min at 40 rpm for first 6 h before switching to continuous stirring at 40 rpm. (b) Effect of bead concentration on growth of Vero cells on CuG is expressed as cell concentration (cell/ bead) against time (h). (n = 1)

Table 3.4.3a. Summary of growth of Vero cells on Cyt 1 at different bead concentrations.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Maximum Cell Density ($x10^6$ cells/ml)	Multiplication Ratio (number of doublings)
1	0.05	1.01	4.3
3	0.15	1.06	2.8
5	0.25	0.72	1.5

Table 3.4.3b. Summary of growth of Vero cells on Cyt 1 at different bead concentrations.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Inoculum (cell/ bead)	Maximum cell/ bead
1	0.05	10	235
3	0.15	10	82
5	0.25	10	34

compared with 0.43 g/L, the culture yielded a much higher cell density at 1.7 x10^6 cells/ml that was comparable to 1.6 x10^6 cells/ml at 5 g/L. Also, it again suggests that surface areas in CuG beads is not limiting but nutrients and pH and others (i.e. oxygen) are possible limiting factors. The optimal results of maximum cell density and multiplication ratio derived from culture at 1.72 g/L under intermittent stirring, are 1.7 x10^6 cells/ml and 5.1 respectively (Tables 3.4.4a and 3.4.4b.), compared to the same culture under continuous stirring at 0.82 x10^6 cells/ml and 4.0 respectively (Tables 3.4.5a. and 3.4.5b.)

(ii) Fed-batch cultures

The effect of bead concentration on growth was tested in fed-batch cultures where bead concentrations ranged from 1 to 5 g/L in Cyt 1 and from 1.72 to 8.6 g/L in CuG cultures (250 ml spinners). The feeding regime involved 50% medium replacement daily from Day 2. All cultures were supplied with DME + 5% serum (v/v) and stirred continuously at 40 rpm. Cell counts were based on CV counts. Results in Fig.3.4.18. showed that medium feeding led to a higher cell yield in cultures at 1 g/L Cyt 1. The growth pattern for both cultures was similar up to 6 days but at that point the fed cultures entered a second phase of slow growth. The maximum cell densities of fed and non-fed Cyt 1 cultures were 1.5 and 3.6 x10^6 cells/ml respectively. For cultures at 3 and 5 g/L Cyt 1, the growth pattern was similar and with cell yields of 5.3 and 5.8 cells/ml. The optimal multiplication ratio, in Table 3.4.6a., was from Cyt 1 cultures at 1 g/L in fed-batch mode, which was also the most efficient culture in surface area usage.

Fig.3.4.19. show that the growth patterns of fed batch CuG cultures were similar to the Cyt 1 cultures. A biphasic growth was present in both bead systems. Growth pattern remained the same for both 1.72 g/L CuG cultures for first 5 days, before the non-fed culture started to gradually slow down and level while the fed culture extended the

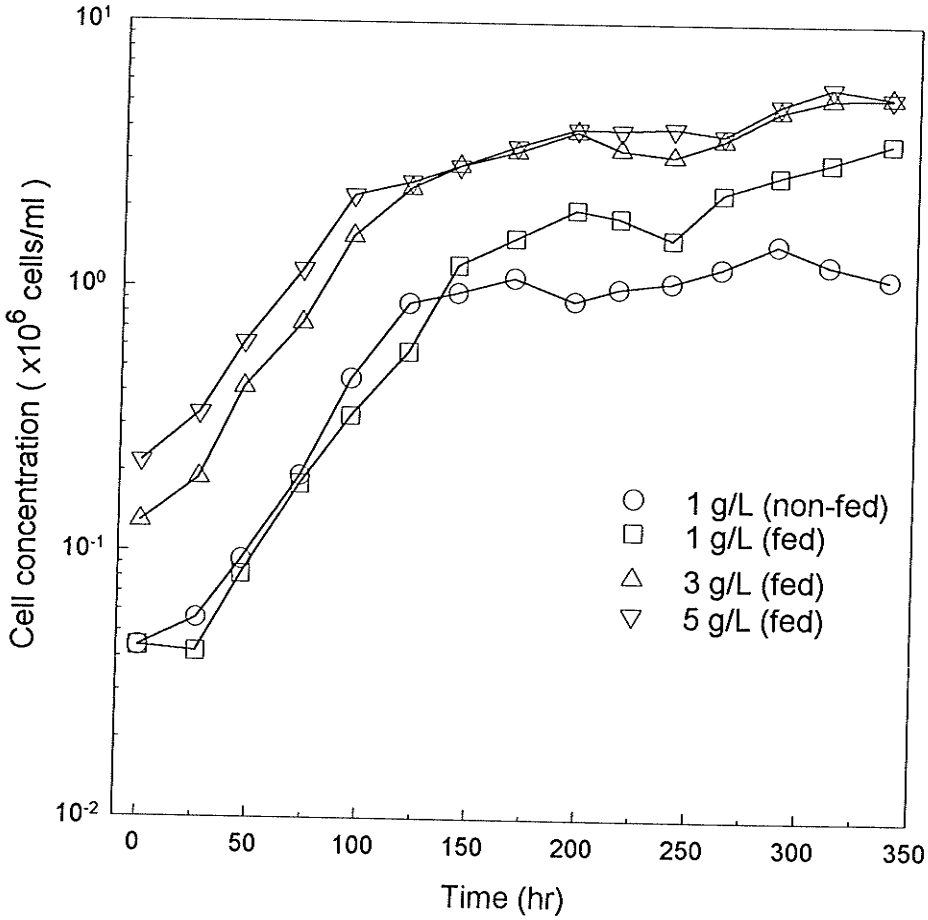


Fig.3.4.18. Effect of bead concentration on growth of Vero cells on Cyt 1 beads. All cultures were stirred continuously at 40 rpm in 250 ml spinners and refed daily with 50% complete medium since Day 2. Total cell concentrations were based on nuclei counts. (n = 1)

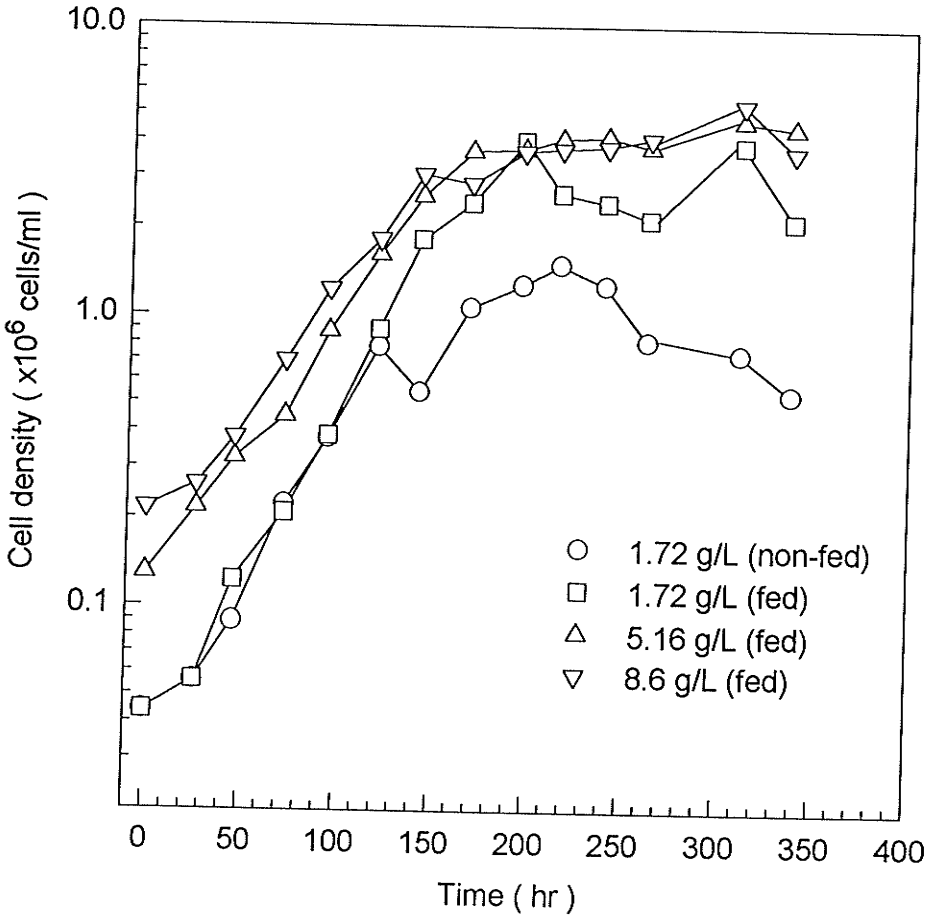


Fig.3.4.19. Effect of bead concentration on growth of Vero cells on CuG beads. All cutlures were stirred continuously at 40 rpm in 250 ml spinners and refed daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

Table 3.4.4a. Summary of growth of Vero cells on Cyt 1 at different bead concentrations under intermittent stirring.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Maximum Cell Density ($x10^6$ cells/ml)	Multiplication Ratio (number of doublings)
0.43	0.05	0.95	4.2
0.86	0.05	1.25	4.6
1.72	0.05	1.68	5.1
5	0.25	1.63	2.7

Table 3.4.4b. Summary of growth of Vero cells on CuG at different bead concentrations under intermittent stirring.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Inoculum (cells/ bead)	Maximum cell/ bead ratio
0.43	0.05	129	2455
0.86	0.05	65	1615
1.72	0.05	32	1085
5	0.25	56	363

Table 3.4.5a. Summary of growth of Vero cells on CuG at different bead concentrations under continuous stirring.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Maximum Cell Density ($x10^6$ cells/ml)	Multiplication Ratio (number of doublings)
0.43	0.05	0.77	3.9
0.86	0.05	0.64	3.7
1.72	0.05	0.82	4.0
5	0.25	1.15	2.2

Table 3.4.5b. Summary of growth of Vero cells on CuG at different bead concentrations under continuous stirring.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Inoculum (cells/ bead)	Maximum cell/ bead ratio [a]
0.43	0.05	129	1989
0.86	0.05	65	827
1.72	0.05	32	529
5	0.25	56	256

[a] Due to both poor cell distribution across the beads at the attachment phase and differential cell confluence at subequent growth phase, the maximum cell/ bead ratios shown were merely average estimates from calculating the division of cell concentration ($x10^6$ cells/ml) by the number of beads (beads/ml) in the systems.

Table 3.4.6a. Summary of growth of Vero cells on Cyt 1 beads at different bead concentrations in fed-batch cultures.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Maximum Cell Density ($x10^6$ cells/ml)	Multiplication Ratio (number of doublings)
1 (NF)	0.044	1.47	5.1
1 (F)	0.044	3.57	6.4
3 (F)	0.13	5.31	5.4
5 (F)	0.22	5.8	4.8

NF: Non-fed (batch) culture
F: Fed-batch culture

Table 3.4.6b. Summary of growth of Vero cells on CuG beads at different bead concentrations in fed-batch cultures.

Bead Conc. (g/L)	Inoculum ($x10^6$ cells/ml)	Maximum Cell Density ($x10^6$ cells/ml)	Multiplication Ratio (number of doublings)
1.72 (NF)	0.044	1.51	5.1
1.72 (F)	0.044	3.90	6.5
5.16 (F)	0.13	4.73	5.2
8.6 (F)	0.22	5.43	4.7

NF: Non-fed (batch) culture
F: Fed-batch culture

exponential growth for 2 more days. The non-fed culture reached maximum cell density at 1.5 x10^6 cells while the fed culture reached about 4.0 x10^6 cells/ml. On the other hand, the higher bead concentration cultures, 5.16 and 8.6 g/L had identical growth pattern and reached 4.7 and 5.4 x10^6 cells/ml, respectively. From Table 3.4.6b., the optimal multiplication ratio was from CuG culture at 1.72 g/L in fed-batch mode, which was also the most efficient culture in surface area usage.

(iii) Effect of Bead Concentration and Agitation

The effects of both bead concentration and stirring speed on Vero cell growth were studied on Cyt 1 cultures. 250 ml spinner cultures at three different bead concentrations (1, 3, and 5 g/L) were supplied with DME + 5% serum (v/v) and subject to agitation from 40 to 100 rpm. Cell densities were based on CV counts. Results show that growth patterns were similar to those in (ii), cultures at 3 and 5 g/L reached their higher cell densities than that at 1 g/L (Figs.3.4.20). The increase in agitation from 40 to 75 to 100 rpm (Figs. 3.4.21, 3.4.22) did not lead to detrimental effect shown in batch culture from 40 to 80 rpm (sub-section 3.4.2. (b)), which was possibly related to cells under better medium condition and to wider gap between the vessel and the paddle in 250 ml spinners. The maximum cell densities reached under different bead concentration and agitation are shown in Fig.3.4.23. Cell yields are indeed improved at bead concentration at higher agitation (75 and 100 rpm), with the best found at 75 rpm. Therefore, it suggests that growth is not affected by agitation provided that other factors such as medium replacement is given.

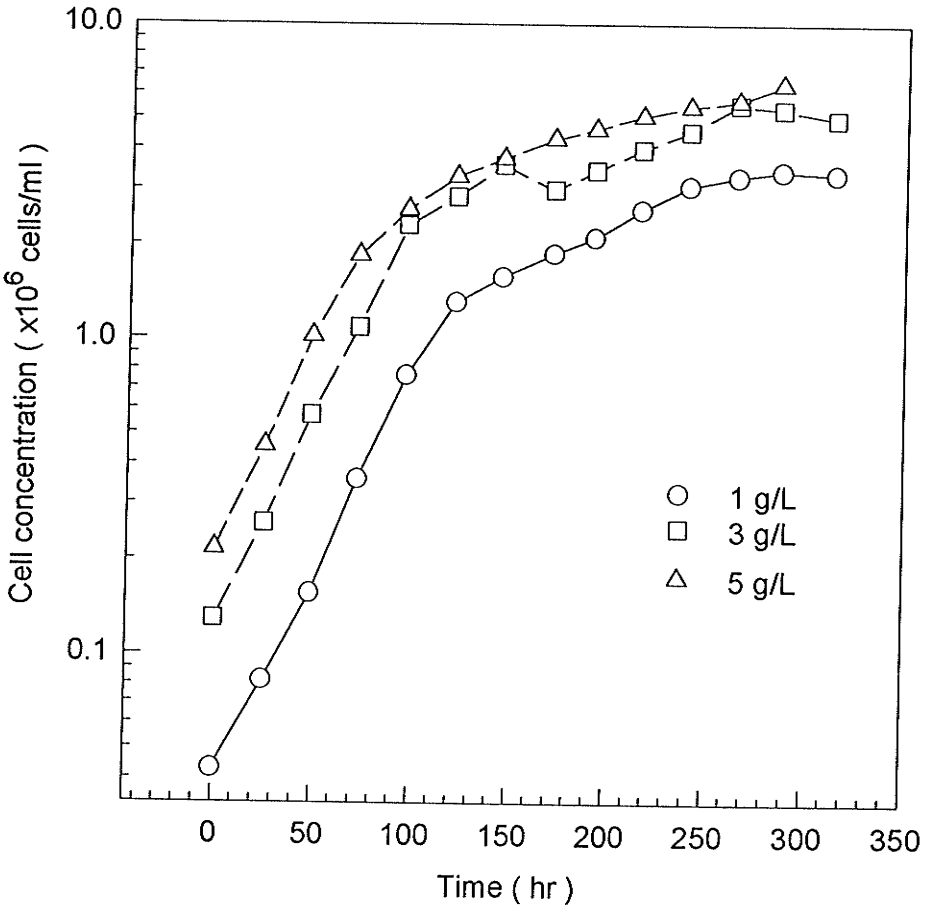


Fig.3.4.20. Effect of bead concentration on growth of Vero cells on Cyt 1 beads at 40 rpm in 250 ml spinners. All medium-fed cultures were replaced daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

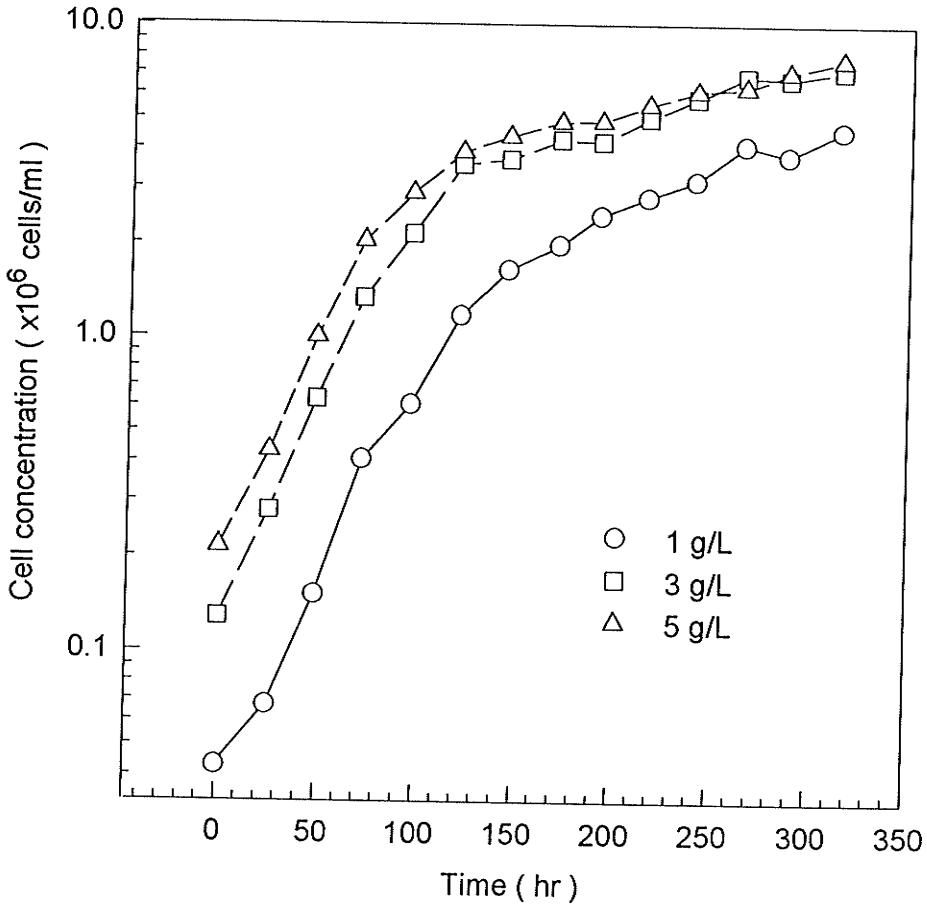


Fig.3.4.21. Effect of bead concentration on growth of Vero cells on Cyt 1 beads at 75 rpm in 250 ml spinners. All medium-fed cultures were replaced daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

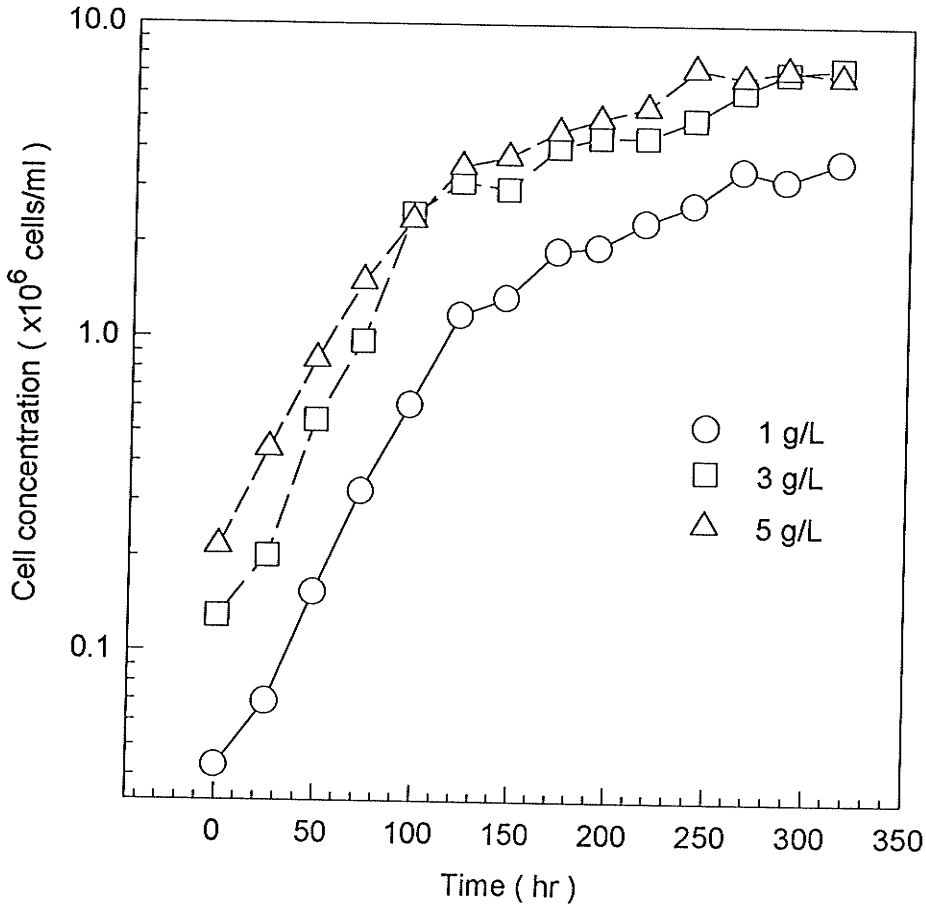


Fig.3.4.22. Effect of bead concentration on growth of Vero cells on Cyt 1 beads at 100 rpm in 250 ml spinners. All medium-fed cultures were replaced daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

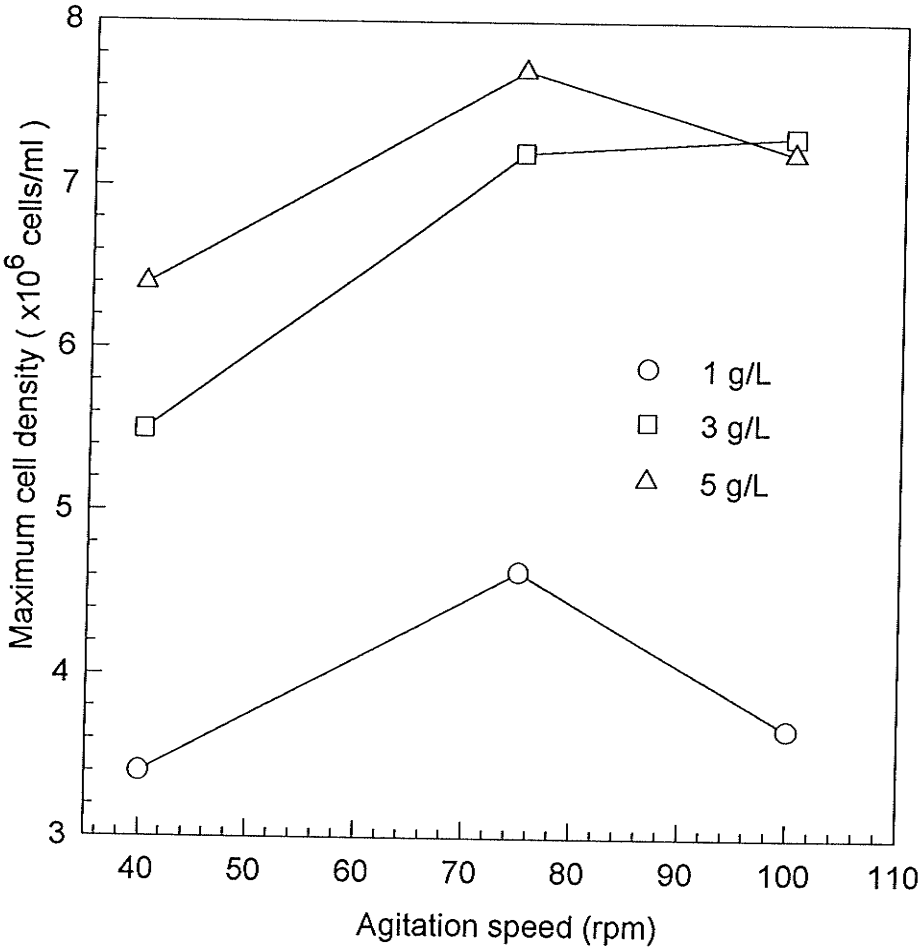


Fig.3.4.23. Summary of effect of agitation speed on maximum cell densities on Cyt 1 cultures at different bead concentrations. Data were taken from those cultures in Figs.3.4.20., 3.4.21., and 3.4.22.. (n = 1)

3.4.3. Discussion

(a) Stirring Mode

Our results were in contrast to Clark and Hirtenstein, (1981) who reported that growth in Cyt 1 culture under initial intermittent stirring yielded better growth and final cell densities over those under continuous stirring. In contrast to Cyt 1, the effect of initial stirring mode was significant in determining the growth patterns and maximum cell densities in CuG cultures. As indicated in section 3.2., intermittent stirring significantly improved cell-bead attachment and subsequently allowed an efficient growth phase.

(b) Agitation Speed

Agitation can affect cultures in several ways:

- Agitation ensures bead suspension and mixing in a homogenous state without incurring settlement of beads and differential gradients of culture microenvironment.
- Degree of shear stress is related to the agitation speed, higher agitation produces stronger hydrodynamic stress on the cells.
- The effect of bead-bead collisions may be characterized by the turbulent collision severity, defined as the product of collision kinetic energy and frequency, both of which are affected by agitation (Cherry and Papoutsakis, 1988).
- The mass transfer coefficient of oxygen, Kla, is proportional to changes in agitation (Fleischaker and Sinskey, 1981), since oxygen is one of the critical nutrient requirements in cell culture at high cell densities, changes in agitation will affect oxygen transfer and growth.

(i) Batch Cultures

Results suggest that at the highest speed, the detrimental effect outweighed any beneficial effect. Those attached and elongated cells were capable of withstanding greater

shear stress at 60 rpm but not at 80 rpm. Similarly, Croughan and Wang, (1989) reported a slight increase in removal of FS-4 cells from Cyt 1 beads at 60 rpm compared to a culture at 35 rpm. However at 150 rpm cell detachment from beads was greatly increased.

Each macroporous bead is designed to separate cells from direct physical effect of shear stress at high speed agitation. Those cells that were initially immobilized onto the surfaces were still subject to the shear stress. This was shown by an increase in cell debris in a culture at 80 rpm.

(ii) Critical Agitation Speed

Results clearly suggest that CuG beads provided a better physical environment from the stress and collisions at higher agitation speed than Cyt 1 beads, and cultures at high bead concentration was more sensitive to stress than cultures at low bead concentration.

The physical effect of high microcarrier concentration on cultures at high agitation was detrimental. Likewise, Croughan et al., (1988) reported that the detrimental effect of bead concentration occurred at high agitation but not a mild agitation. They also stated that the specific death rates was dependent on two factors: a constant bead-eddy and a changing bead-bead interactions, in which the latter was directly influenced by the bead concentration present in the culture.

(c) Supplemented Serum Concentration

Serum, is an undefined complex mixture of attachment factors, nutrients, immunoglobulins, and mitogenic factors (Griffiths, 1986). Despite its high cost, serum is one of the essential factors for cell growth. Also, there have been concerns about the effect of batch variations of serum on cell growth (Clark et al., 1980). It is logical and

economical to reduce the amount of serum in a culture to a minimum level without compromising the cell growth potential.

It was reported by Orly and Sato, (1979) that fibronectin in serum not only promoted cell adhesion but was also involved in cell division and proliferation of rat follicular cells. Otherwise, binucleated cells were significantly increased in the absence of either serum or fibronectin. In our studies, Vero cells attached to Cyt 1 beads at all serum concentrations tested (0-10%). However, at 0% serum cell densities declined, suggesting that DME alone was not providing sufficient nutrients for cell spreading and proliferation.

Sayer et al., (1987) demonstrated that gelatin-coated Cyt 3 beads with or without fibronectin coating treatment allowed optimal MDCK cell attachment and growth over charged beads (Cyt 1 and Ventreglas), suggesting that (1) gelatin may serve a similar role as fibronectin in cell adhesion and proliferation or / and (2) cells may behave biologically differently to the gelatin on Cyt 3 than onto other charged beads. Gelatin (denatured collagen) has molecular structures (ligands) that are recognized by cell receptors. The reactions of ligand-receptor may trigger a cascade of chemical signals inside the cells that are functionally similar to those produced by serum factors. In other words, growth may be allowed in serum-free medium if the substratum matrices produce a similar effect to those of essential serum components. It was shown that Vero cells managed to attach and multiply on CuG beads at a slower rate in serum-free condition but not on Cyt 1 beads.

(d) Inoculum Size

It suggests that surface area would be a limit to cultures started at high inoculum density. Given limited surface area on the beads, cells only reached confluence with a monolayer of cells on the beads.

As cells bear the inherent costs of production from the beginning up to this stage, it became strategic and economical to select an inoculum density that would yield the

maximum multiplication ratio in shortest times possible. 10 cells/ bead for Cyt 1 and 28 cells/ bead for CuG seemed to balance the requirements of these two factors.

(e) Bead Concentration

(i) Batch Cultures

The increase in Cyt 1 bead concentration did not lead to increase in maximum densities but instead a substantial decline in the culture. It is believed that the lower cell yield is related to the bead concentration in the culture, in which bead collision frequency was significantly increased at high bead concentration. On the other hand, the increase in CuG bead concentrations resulted increased cell yields until reaching maximum cell level at 1.7 $x10^6$ cells/ml in 1.72 and 5 g/L cultures. Also, our result was consistent with Nikolai and Hu, (1992) that the average Vero cell per CuG bead was 2600 in an 0.5 g/L culture.

Although the increase in bead concentration (therefore surface area) was more detrimental, results seem to indicated that nutrients are likely to be limiting in these cultures as well.

(ii) Fed Batch

The cell yields between non-fed and fed Cyt 1 and CuG cultures indicate that nutrients are limiting factors for cell growth.

In contrast to the detrimental effect of high bead concentration (5 g/L) in Cyt 1 culture in batch mode (i), cell yield was significantly improved in culture with 50% medium replacement. Therefore, it suggests that cells under a more healthy state could withstand the physical effect of high bead collision frequency. For CuG beads, cell yields were similar and comparable to those in Cyt 1 at high bead concentration. It implies that

factors other than surface area are limiting to cellular growth to a higher level at high bead concentration.

Even though these Cyt 1 and CuG cultures were replaced with 50% medium from day 2, there are other possible factors that would cause such inhibition, pH and oxygen are not controlled in spinner system that can be easily upset at high cell densities. The second phase of growth (in which growth rate was slower) would be related to oxygen deficiency or oxygen demand exceeding the supply capacity via surface aeration.

(iii) Effect of Bead Concentration and Agitation

Factors other than surface areas are involved in the limitation of growth for cultures in batch mode. The possibilities may be the lack of nutrients including oxygen or accumulation of metabolite by-products. These undesirable effects are believed to be alleviated by increasing agitation speed in addition to medium replacement.

3.4.4. Conclusions

Typically, 1 g/L Cyt 1 reached 1 x10^6 cells/ml while 1.72 g/L CuG at 1.7-1.8 x10^6 cells/ml. At confluence, cells appeared as a monolayer on Cyt 1 beads. Although cells were difficult to identify even in stained CuG beads, it was suggested that cells were capable of growing as multilayers or aggregates inside the voids (Adema et al., 1990). Maximum cells/ bead levels achieved were around 344 and 2500 for 1 g/L Cyt 1 and 0.43 g/L CuG cultures, respectively, indicating that surface area provided by CuG was several-fold of that in Cyt 1.

The optimal conditions for cell growth are different from those for cell attachment for Cyt 1 and CuG beads. It is concluded that optimal growth would be obtained in Cyt 1 and CuG cultures after cell-bead attachment was made successfully under optimal conditions. A continuous stirring throughout the attachment and growth phases for Cyt 1

cultures while an initial 6 h intermittent stirring for CuG cultures before switching to continuous stirring were desirable. Serum concentration, inoculum density, and bead concentration were set at 5%, 10 and 30, 1 and 1.72 g/L for Cyt 1 and CuG cultures, respectively.

3.5. Scale-up cultures of Cyt 1 beads

3.5.1. Introduction

Effect of bead-bead transfer of Vero cells in Cyt 1 cultures was studied as an alternative way to increase cell density in cultures through periodic feeding of fresh beads. On the other hand, serial subculturing is also an important practice during scale-up of cell culture to achieve high cell densities in a fermentation process at the final production stage. Cells harvested at each subculture were used as inoculum to a culture of bigger volume or higher bead concentration. The subculturing process becomes easier if the "spent beads", which have been used previously for cell growth, can be reused.

3.5.2. Results

(a) Bead-to-bead transfer of Vero cells

The effect of adding fresh Cyt 1 beads to a Cyt 1 culture was studied. A 1 g/L Cyt 1 culture in 250 ml spinner was supplied with DME + 5% serum (v/v). At Days 5 and 9, fresh beads (1 g/L) were added into the culture (1 g/L +). Therefore, the final bead concentration became 3 g/L. Results of the 1 g/L + culture are compared with those controls at 1 and 3 g/L. Cell densities were based on CV nuclei counts.

Results in Fig.3.5.1. show cell yield in 1 g/L + was significantly higher than 1 and 3 g/L throughout the experiment. Maximum cell densities were 3.4, 5.5, and 6.6 $x10^6$ cells/ml for 1, 3 and 1 g/L + cultures. In the 1 g/L + culture, it was observed that prior to the second addition of beads (Day 9) the original beads present from Day 0 were confluent but these added on day 5 were only partially (50-90%) covered with cells. Therefore, there was inconsistency in the overall confluence among beads, which may lead to the appearance of empty beads. It is believed that the bead-to-bead transfer of Vero cells is based on the bridging of fresh beads with cell-laden beads.

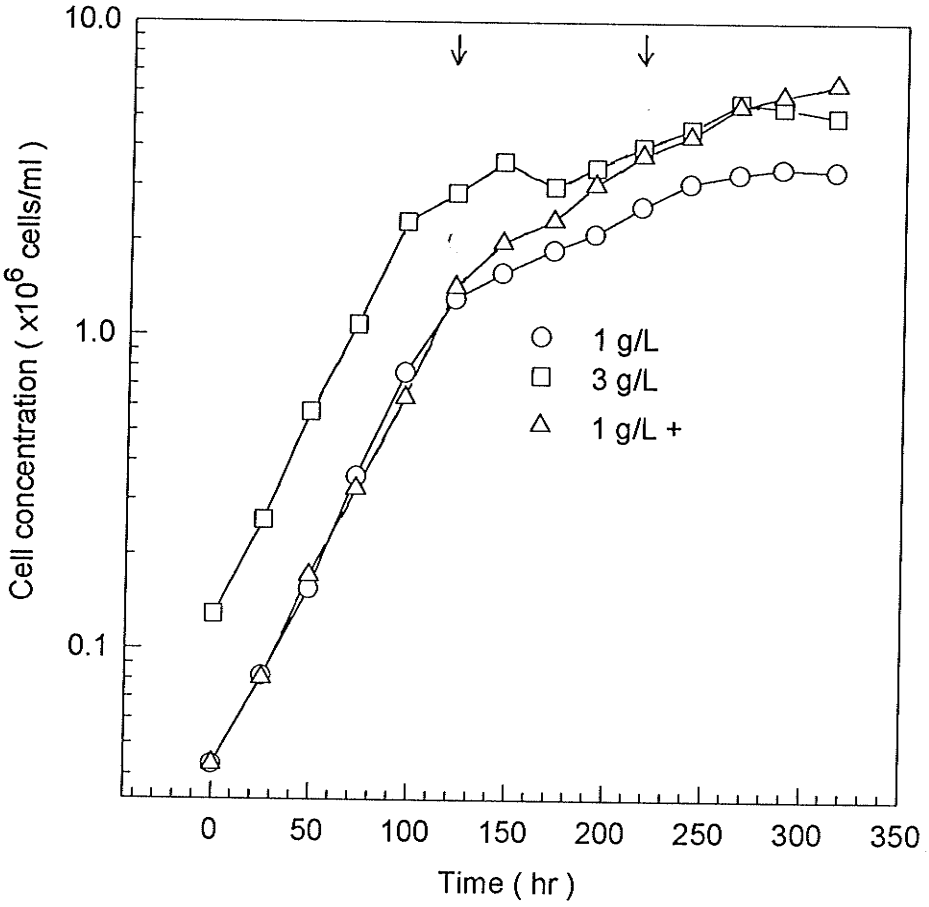


Fig.3.5.1. Effect of bead addition on growth of Vero cells on Cyt 1 beads at 40 rpm in 250 ml spinners. 1 g/L equivalent of beads was added into a culture (1 g/L +) on Days 5 and 9 (indicated by arrows). All cultures were replaced daily with 50% complete medium from Day 2. Cell densities were based on CV nuclei counts. (n = 1)

In a second experiment in which the agitation was increased to 75 and 100 rpm, the optimal agitation speed (75 and 100 rpm) tested on all 1 g/L + Cyt 1 cultures also resulted in improved cell yields over cultures at 1 g/L but still lower than those at 3 g/L (Figs.3.5.2 and 3.5.3.). Both inconsistent confluence and low bead occupancy (70%) occurred in 1 g/L + cultures at the late growth phase where substantial number of beads were clear from cells than that at 40 rpm.

Results in Fig.3.5.4. could show that cell yields were increased in 1 g/L + cultures, but they were still relatively lower than those at 3 g/L at optimal agitation range (75 and 100 rpm), therefore the efficiency of microcarrier usage was poor.

(g) Serial subculturing of Vero cells on Cyt 1 beads

The feasibility of Vero cells growing on "spent Cyt 1 beads" was studied with the use of a trypsinization protocol, which is listed in Chapter 2. Results in Fig.3.5.5. show that cells were capable of growing on the spent beads for the second time. Growth rate was similar to that of the first time at the exponential growth phase. The final cell densities are also comparable between the cultures. Therefore, it suggests that cells could reuse the surface without showing a detrimental effect on cell yield.

3.5.3. Discussion

(a) Bead-to-bead transfer of Vero cells

The sequential addition of fresh beads into an existing low bead concentration culture is an alternative to a culture at high bead concentration, if transfer of cells from bead to bead could occur. Under the conditions used in the experiments, bead-to-bead transfer was basically dependent on bead bridging, because of inconsistent cell confluence it was also limiting to the number of bead additions.

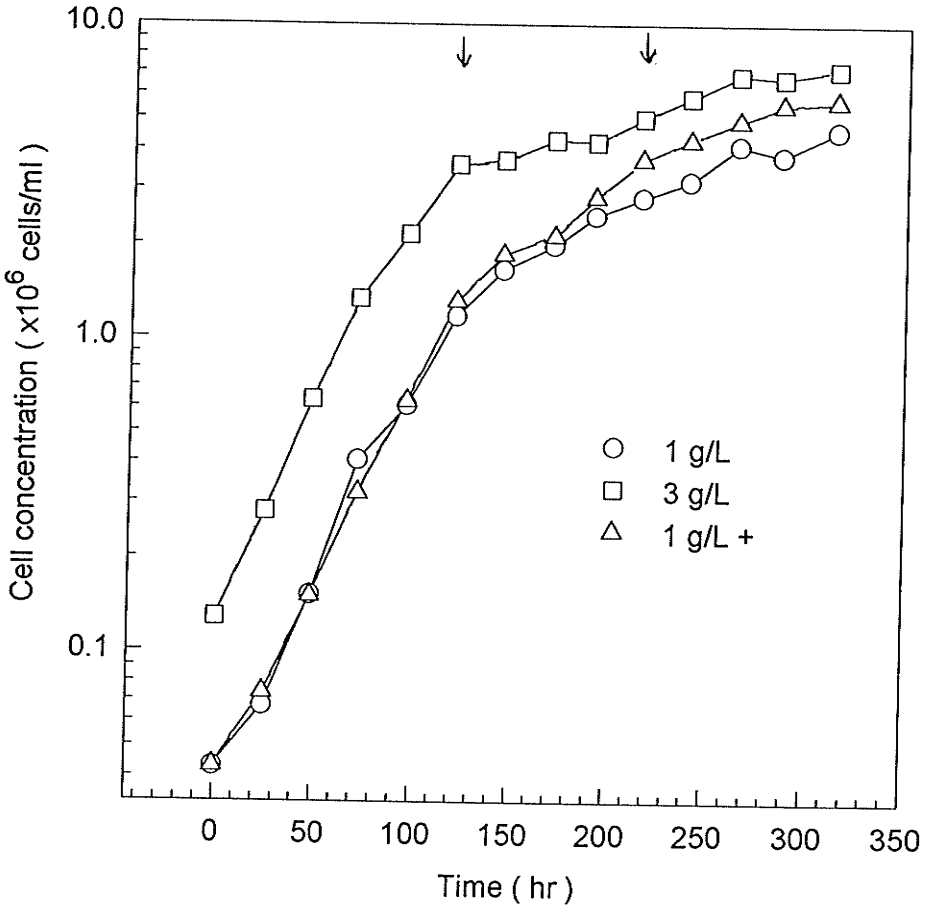


Fig.3.5.2. Effect of bead addition on growth of Vero cells on Cyt 1 beads at 75 rpm in 250 ml spinners. 1 g/L equivalent of beads was added into a cullture (1 g/L +) on Days 5 and 9 respectively (indicated by arrows). All cultures were replaced daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

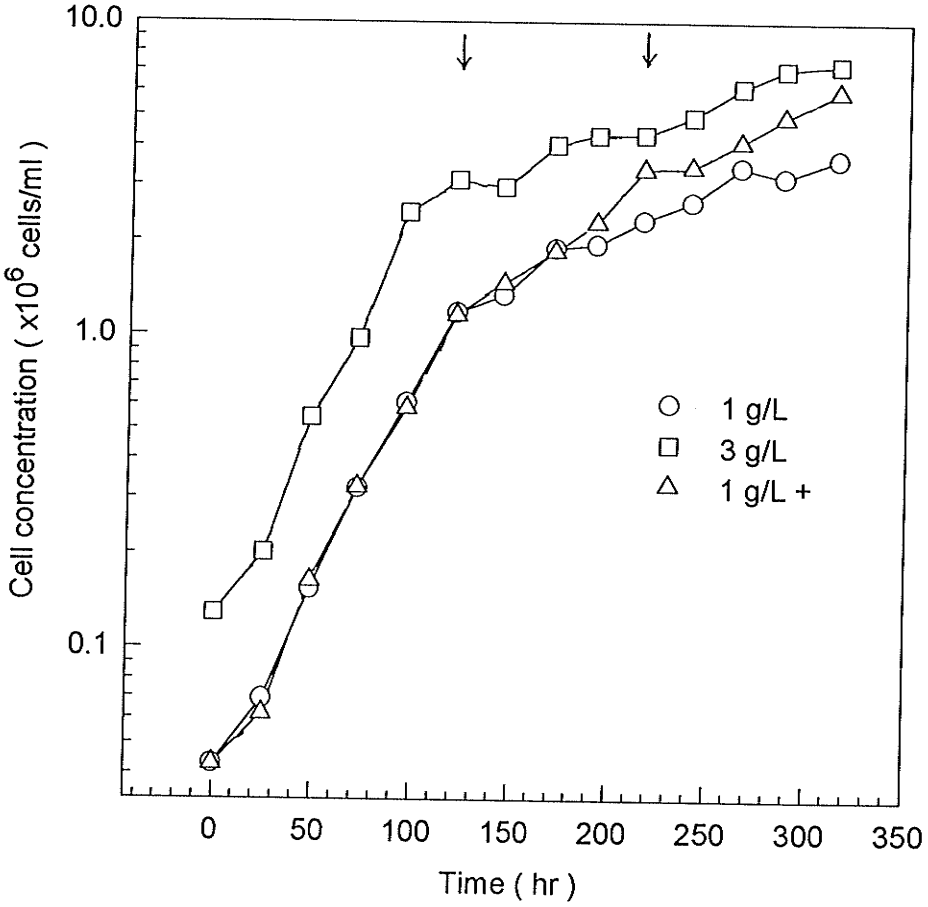


Fig.3.5.3. Effect of bead addition on growth of Vero cells on Cyt 1 at 100 rpm in 250 ml spinners. 1 g/L equivalent of beads was added into a cullture (1 g/L +) on Days 5 and 9 respectively (indicated by arrows). All cultures were replaced daily with 50% complete medium since Day 2. Cell densities were based on CV nuclei counts. (n = 1)

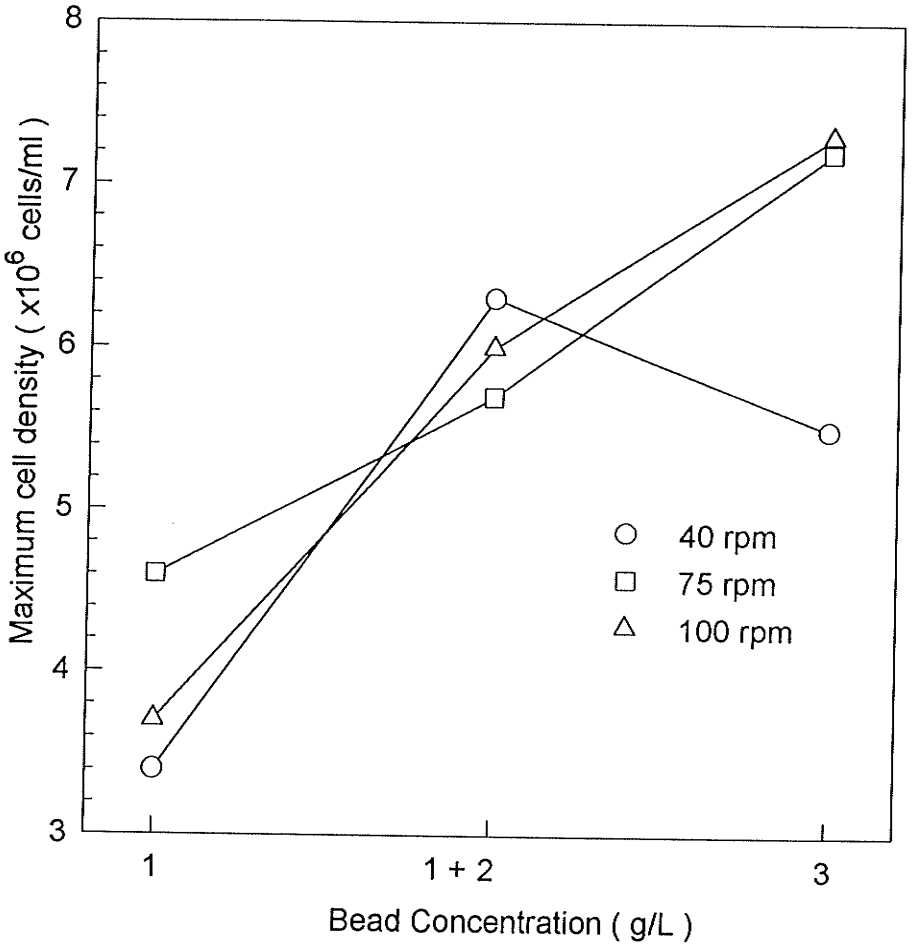


Fig.3.5.4. Summary of effect of bead addition on maximum cell densities of Cyt 1 cultures at different agitation speed. Data were taken from Figs.3.5.1., 3.5.2., and 3.5.3. The 1 g/L + cultures indicates here as 1 + 2 for their final bead concentration. (n = 1)

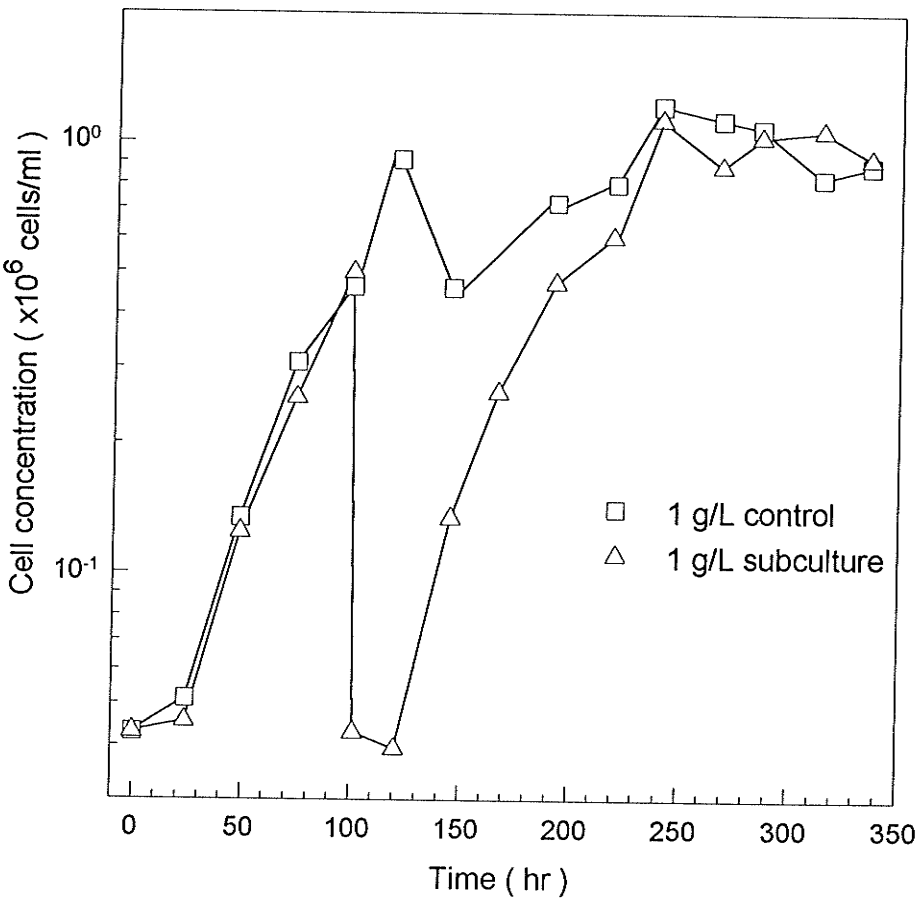


Fig.3.5.5. Serial subculturing of Vero cells on Cyt 1 beads. Cells in exponential growth on the beads were transfered to a centrifuge tube and were trypsinized with trypsin in a 1:1 ratio to the bead volume for 15 min at 37°C on shaker at 120 rpm. The enzymatic reaction was stopped by the addition of complete medium. Cells were collected in the supernatatns separated from the settling beads through serial dilution with complete medium. The beads were returned to the spinner with appropriate numbers of cells. (n = 1)

Crespi and Thilly, (1981) examined bead-to-bead transfer of two epithelial cell lines, LLC-MK_2 and CHO-K1, on charged beads. They demonstrated that the growth rates of two cell lines were kept constant upon series dilutions of spent beads (1/5 and 1/20 respectively) with fresh beads. The mechanism of bead-to-bead transfer was not clear but it may be likely related to processes of detachment and reattachment, since low calcium media were used for both cell lines.

(g) Serial subculturing of Vero cells on Cyt 1 beads

Vero cells can utilize the spent beads on which cell had grown previously. It is in agreement with Hu et al., (1985b) that they serially subcultured FS-4 cells and Vero cells in DEAE-charged bead systems.

Although the procedure used was preliminary, trypsinization could be refined inside the spinner in order to minimize steps involved. In our experiment, the turnover of spent bead is 100%, in a real scale-up operation they will represent only a fraction of the total beads after dilution with fresh beads.

4.0. General Conclusions

4.1. Cell Attachment

The 6 parameters (stirring mode, agitation, pH, serum concentration, inoculum size, and bead concentration) were tested for their physical effects on attachment of Vero cells onto Cyt 1 and CuG beads in spinners. The parameters that significantly affected attachment on Cyt 1 beads were agitation speed, pH, and serum concentration. In contrast, these parameters affected cell-bead attachment very differently on CuG beads. Only stirring mode was shown to improve the overall attachment efficiency (cell attachment rate, bead occupancy, and cell distribution). The cell attachment rates of Cyt 1 cultures were much higher than CuG cultures, in excess of 10-fold. These suggest that cell attachment on both bead types was facilitated by different mechanisms.

The attachment mechanisms involved in both bead types were different. It has been known that charge interactions were the main factor in cell attachment on Cyt 1 beads, while they were insignificant on CuG beads. Instead, it is believed that cells attached to the porous CuG beads through immobilization under intermittent stirring, assisted possibly by ligand-receptor interactions, and entrapment.

Therefore, the optimal conditions for cell attachment onto Cyt 1 are when the beads are stirred continuously at 40 rpm, in DME without serum supplementation at pH 7.3 , with an inoculum of 10 cells/ bead and 5 g/L bead concentration. Although the presence of serum slowed down attachment rates, it was required for full cell spreading at the end of the attachment phase.

On the other hand, the optimal conditions for cell attachment onto CuG are when the beads are stirred intermittently at 3 min/ 30 min at 40 rpm for 6 hr, in DME without serum supplementation at pH 7.3 , with an inoculum of 56 cells/ bead and 5 g/L bead concentration.

4.2. Cell Aggregation

Cell aggregation was very rare in Cyt 1 cultures, but very common in CuG cultures especially under continuous stirring. Cell aggregation was undesirable as cells prefered to attach to one another than to beads, this would affected not only the attachment rate but also bead occupancy and cell distribution on the beads. The only parameter that reduced cell aggregation in CuG cultures was determined to be stirring mode. Under intermittent stirring, besides a low degree of cell aggregation, better cell attachment, distribution and bead occupancy were obtained.

The reasons for cell aggregation are somewhat related to serum concentration and pH of the medium. High serum concentration and high pH were shown to enhance the aggregation process, reflected by higher aggregation rates.

4.3. Cell Growth

(i) Batch Cultures

After optimal cell attachment, the same parameters tested for their effects on cell attachment were also determined for their effects on growth except stirring mode and pH. The latter was determined by a Celligen™ bioreactor in which pH was maintained. Growth can be assessed by both maximum cell densities and multiplication ratio (number of doubling from inoculum to maximum cell yield). The results indicated that optimal conditions for growth were different from those for cell attachment. Unlike cell attachment, growth depended on serum supplementation. High agitation speed and low serum concentration were equally detrimental. Increase in both inoculum density and bead concentration did not improve the efficiency of microcarrier usage. In typical 1 g/L Cyt 1 and 1.72 g/L CuG cultures, 1 and 1.7 $x10^6$ cells/ml were achieved.

The optimal growth conditions are 1 g/L Cyt 1 and 1.72 g/L CuG in DME + 5% serum (v/v) at pH 7.3, stirred at 40 rpm and inoculated with 10 and 30 cells/ bead, respectively.

(ii) Fed-batch Cultures

With medium replacement, cell densities were also improved at all bead concentration levels. This suggests that nutrients were essential for cells to reach higher densities. Nevertheless, other limiting factors were still present. By increasing agitation speed, oxygen transfer would be enhanced. The results indicated Cyt 1 cultures at 75 rpm yielded relatively higher cell densities than their 40 rpm counterparts.

On the other hand, at much higher agitation speed, cells attached to CuG beads showed greater tolerance to shear stress than Cyt 1 beads. The results clearly indicated that CuG beads hae potentials of yielding higher cell densities and greater tolerance to shear stress.

4.4. Scale-up cultures

An increase in cell yield in Cyt 1 cultures occurred through (1) a limited number of bead addition and (2) capability of cells to multiply on "spent beads".

4.5. Final Comments and Future Prospects

It is shown that intermittent stirring delivers better cell attachment and distribution on the CuG bead. However, there is a disadvantage of using intermittent stirring especially in large scale bioreactors. The problems of heat and oxygen diffuision will arise during intermittent non-stirring periods and result differential gradients. As a result, some cells may be deprived of oxygen and subject to higher temperature than normally encountered under continuous stirring. Therefore, the remedy is to optimize the charge on the surface

of these porous beads for continuous stirring in bioreactors at the attachment phase, so that they may achieve comparable attachment rates as in Cyt 1. Other parameters such as the pore size and agitation are also critical of successful cell attachment. In fact, there are two commerical macroporous microcarriers marketed as Cellsnow (Kirin, Japan) and Cytocell (Asahi, Japan) that are charged by polyethyleneimine and N,N,-diethylaminoethyl group, respectively. At present, there have been no reports on their performance on cell attachment compared to other non-charged macroporous microcarriers.

The physiology and metabolism of cells on CuG beads and probably other macroporous beads should also be investigated with their metabolic relation to their underlying substratum substance. It is very interesting that CuG could support growth of Vero cells in a considerable level in DME based medium, suggesting that the cell-gelatin interactions in CuG beads was physiologically beneficial to cell growth than the cell-dextran interactions in Cyt 1 beads (Figs.3.4.11. and 3.4.12.).

The development of serum-free medium should be taken more pragmatically. The cell culture conditions are critical to downstream purifications and concentration processes of virus or other biologicals. The serum proteins from the cultures complicate these processes, with serum-free medium fewer contaminating proteins (only cellular source) are obtained at the end of the frementation stage. However, it is known to us that some of the supplements that are used to replace serum are extremely expensive, one example is fibronectin. The cost of making a serum-free medium for same growth efficiency could be significantly higher than that from a serum-based medium. In overall, cell attachment is the interactions between cells, medium (plus serum), and surface substratum. Therefore, working with cells and medium is not sufficient to improve the attachment and growth efficiencies. In fact, Cinatl et al., (1994) developed a protein-free medium which further reduced protein supplements required for Vero cell growth. The main focus is on the optimization of both the surface substratum and the medium components for the cells. In

addition, they also reported that the cell yields and virus titers produced were comparable in this protein-free medium to those conventional serum-containing media. Most importantly, the cost of this medium would be significantly reduced.

The use of spinners is only regarded as an intermediate research level for large scale cultures in bioreactor, in which all control parameters are well maintained. Therefore spinners are used in large number to establish some background information on cell attachment and growth. High cell densities, however, can not be successfully maintained in spinners. In the well controlled bioreactors, optimization for microcarrier cultrues are finalized. In order to reach high cell densities, a systematic study of medium design at different perfusion rates is very crucial. At high cell densities, the metabolic consumption rates of some of the essential nutrients have to be analyzed for a specific medium replacement regime. That is, instead of replacing spent medium with fresh complete medium, supplements in concentrated form are added and little medium is taken out from the system.

The ultimate goal of microcarrier cultures is to yield products produced at a desirable rate. Vero cells, are known for licensed Polio virus production, should be tested for their productivities on CuG. Nikolai and Hu., (1992) successfully infected Vero cell-laden CuG beads with Vesicular stomatitis virus on the exterior and interior pores, it is likely that other viruses could infect cells in interior of the beads.

I could forsee CuG beads or the like will be used as extensively as Cyt 1 for virus / biologicals production in the near future, provided that they are further improved for cell attachment.

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Appendix A

A 5.1. Summary of stirred tank, column, and membrane biorectors (Prokop and Rosenberg, 1989).

Reactor type	Type of support/substrate	Suspension (S) or Anchored (A) cell lines
(1) Stirred Tank Bioreactor a. flat turbine b. paddle - stirrer bar/paddle - angled blades - marine propeller - helical mixer c. vibromixer d. cavity (cage) type e. hanging stirrer	Possible for all: Microcarriers, microcapsules, porous support particles/beads	A and S for all except A for d only
(2) Column Reactor a. column b. bubble column c. column with draft tube d. fluidized bed e. packed bed	 rotating vertical stack plate rotating horizontal disc porous support or beads porous support or beads porous support or beads glass spheres or ceramic or glass tubes	 A for all except A and S for (b) and (c)
(3) Membrane Reactor a. hollow fiber b. flat membrane c. microencapsulation d. gel matrix	 capillary (extracapillary or lumen) no support capsule gel bead	 A and S for all except S for (b)

Appendix B

A 5.2a. Commercially available solid microcarriers of different compositions and surface modifications (Butler., 1987).

Type	Trade Mark	Composition	Company
Dextran	Superbeads	DEAE-dextran	Flow Labs, USA
	Cytodex 1	DEAE-dextran	Pharmacia, Sweden
	Cytodex 2	Quaternary amine coated dextran	Pharmacia
	Microdex	DEAE-dextran	Dextran Products, Canada
Plastic	Biosilon	Polystyrene-charged	Nunc, Denmark
	Biocarriers	Polyacrylamide/DMAP	Biorad, USA
	Cytospheres	Polystyrene-charged	Lux, USA
	Acrobeads	Polyacrolein-various coatings	Galil, Israel
Gelatin	Cytodex 3	Gelatin-coated dextran	Pharmacia
	Geli-beads	Gelatin	K C Biologicals, USA
	Ventregel	Gelatin	Ventrex, USA
Glass	Bioglas	Glass-coated plastic/latex	Solohill Eng., USA
Cellulose	DE-52/53	DEAE-cellulose	Whatman, UK

A 5.2b. Characteristics of microcarriers listed in A 5.2a.

Name	Density (g/cm^3)	Diameter (μm)	Surface area (cm^2/g)	Number of beads /g
Superbead	1.05	~150	250	$3.5x10^5$
Cytodex 1	1.03	131-220	4400	$6.8x10^6$
Cytodex 2	1.04	114-198	3300	$5.8x10^6$
Microdex	1.03	~150	250	$3.5x10^5$
Biosilon	1.05	160-300	225	$3.5x10^5$
Biocarriers	na	120-180	4700	na
Cytospheres	1.04	160-230	250	$2.1x10^5$
Acrobeads	1.04	100-200	500	$7.1x10^5$
Cytodex 3	1.04	133-215	2700	$4.8x10^6$
Gelibeads	na	60-230	3300-5300	$4x10^6$
Ventregel	1.03	150-250	4300	$3.6x10^6$
Bioglas	1.03-1.04	150-210	350	$5x10^5$
DE-52/53	1.03	40-50 x 80-400	1000	$2.7x10^6$

na: not available

Appendix C

A 5.3. Characteristics of different Macroporous Microcarriers.

Trade Name	Material (μm)	Diameter (μm)	Pore diameter volume (%)	Void volume(%)
Cultispher G (Percell Biolytica, Sweden)	Gelatin	170-270	10-20	50 [a]
Informatrix (Biomat Corp., USA)	Collagen-glycosaminoglycan	500	40	99.5
Microsphere (Verax Corp., USA)	Collagen	500-600	20-40	75
Siran (Schott Glaswerke, Germany)	Glass	300-5000	10-400	60
Cellsnow (Kirin Brewery, Ltd., Japan)	Cellulose-matrix [b]	500-3000	na	>97%
Cytocell [c] (Pharmacia Bioprocess Technology AB., Sweden)	Cellulose	180-210	30	na

[a] The void volume given was estimated by Cahn., (1990), Adema et al., (1990), and Griffiths., (1990).

[b] Types EX : Polyethyleneimine charged
CX : Type 1 collagen coated
PX : RGDS peptide (Arg-Gly-Asp-Ser) linked

na: not available

[c] Cytocell is manufactured and marketed as Asahi carrier by Asahi Chemical Ltd. in Japan and distributed exclusively by Pharmacia Bioprocess Technology AB outside Japan.

Appendix D

A 5.4. Characteristics of Cytodex 1 and Cultispher G.

	Cytodex 1	Cultispher G
Matrix	cross-linked dextran	cross-linked gelatin [a]
Charge group	DEAE	—
Density (g/ml) [b]	1.03	1.04
Size (μm) d_{50} [b]	190	220
d_{5-95} [b]	140-278	170-270
Number of microcarrier per gram dry weight [c]	4.3 x10^6	0.9 x10^6
Swelling factor (ml /g dry weight) [b]	20	13-15
Pore size (μm)	—	10-20
Void fraction (%)	—	50
Transparency (degree)	full	semi

[a] The gelatin of CuG beads was crosslinked by glutardialdehyde to render more structural strength and thermal resistance.

[b] The measurements of density and size were taken in 0.9% NaCl for Cytodex and in PBS for Cultispher G beads. d_{50} represents medium diameters while d_{5-95} refer to diameters in 5 and 95 percentiles.

[c] The listed CuG bead number (bead /g) was based on the facsimile recently received from Hyclone (1994), in which the bead number was in contrast to early manufacturer's specification (2-3 x 10^6/g), and Hyclone's Application Notes 108 and 109 (4.0 x 10^6/g), but similar to 8 x10^5 beads/g (Shiragami et al., 1991., Nikolai and Hu., 1992).

Appendix E

A 5.5. Procedure of sampling and cell enumeration of Vero cells on Cyt 1 and CuG beads by Trypan blue exclusion methods.

1. Spinners were swirled to suspend beads and ensure good mixing. A pipette was used to draw an 1 ml sample from the spinner to an eppendorf tube (1.5 ml). The position of pipette sampling in the spinner was kept at about the same height with the magnetic bar and in between the shaft and the vessel's wall.

2. Beads were allowed to settle by gravity and tapping with a pipette against the lower portion of the eppendorf tube.

3. The medium supernatant was discarded.

4. Immediately, an 1.4 ml PBS-EDTA was added to the sample to wash the beads. The tube was allowed to invert gently to resuspend the contents during washing.

5. #2 and #3 were repeated for PBS-EDTA supernatant disposal.

6. For both Cyt 1 and CuG bead sample, 1 ml of 0.25% trypsin/ 1mM EDTA at 37°C was added.

7. The sample was shaken to suspend the beads in trypsin before going into incubator at 37°C.

8. After 1 min, the sample was gently shaken. Subsequently, the sample was at least shaken twice during incubation to resuspend Cyt 1 beads or cell clumps from CuG sample.

9. For 5 to 10 minute incubation, the sample was taken out and shaken for few times before an 0.5 ml of complete medium was added. The incubation time was dependent on the cell density in the sample.

10. The sample was again shaken and centrifuged at 1500 rpm for 5 minutes.

11. After centrifugation, the supernatant was withdrawn down to 0.5 ml and replaced with 0.5 ml Trypan blue (0.4% w/v in PBS) to make up 1 ml.

12. The tube was rapped against the index finger 10 times and shaken 15 times.

13. A cover slip was first breathed onto and then rubbed back and forth against the haemocytometer in order to secure the placement.

14. Finally, the sample was pipetted 5 times with a 200 µl pipette before putting onto a haemocytometer.

Appendix F

A 5.6. Comparison of two cell counting methods

In Fig.5.6.1., crystal violet (CV) nuclei counts were shown to be consistently higher than trypan blue (TB) cell counts. Such difference in cell density was statistically substantial at all the time points. The errors (reflected by error bars) incurred were much higher at the beginning because of low cell / nuclei densities, subsequently they were found to be within 10% of the averages of two or more counts. Since TB cell counts are based on real cell counts, therefore they are considered more direct and reliable than CV nuclei counts. As a result, TB cell counts are used to represent cell densities on both Cyt 1 and CuG beads on subsequent spinner cultures. Those experiments in which results are based on CV nuclei counts will be specified and compared accordingly as if they were cell counts.

Counting of nuclei stained by crystal violet was adopted as means of cell enumeration by the inventor of charged-microcarriers (van Wezel et al., 1967). Since then this method has been extensively used by others who work on microcarriers and regarded as reliable methods (van Wezel, 1981), until it was discovered in our laboratories that nuclei counts and actual cell counts are not identical. The difference between cell and nuclei counts should not be too surprising as there are cells known to be binucleated at one or certain stages in their cell cycle. In studies conducted in our laboratories, commonly used cell lines, such as L-929, Vero, and CHO, were found to result a significant difference between two cell counting methods (unpublished results by Berry and Butler), therefore it suggests that binucleation is more extensive in cell lines than it was originally recognized. The mechanism of this phenomenon is not the objective of this study, and should refer to the Ph.D. thesis of J. Berry. Nevertheless, it is suspected that lack of nutrients or mitogenic factors are linked to binucleation of cells. Orly and Sato, (1979) reported that rat follicular cells became binucleated when fibronectin or serum was

absent. From our results, the difference between two counting methods was consistent with times. Therefore, it may also be related to factors other than nutrients factors. Cytokinesis is possibly taking relatively longer time after nuclear division has been complete in the cells. Such lapse between two cellular functions would lead to the difference in nuclei and cell counts.

Such difference in counting methods has a very significant effect on the fundamental measurements of any kind involving cells, from specific consumption rates to specific productivites of biologicals.

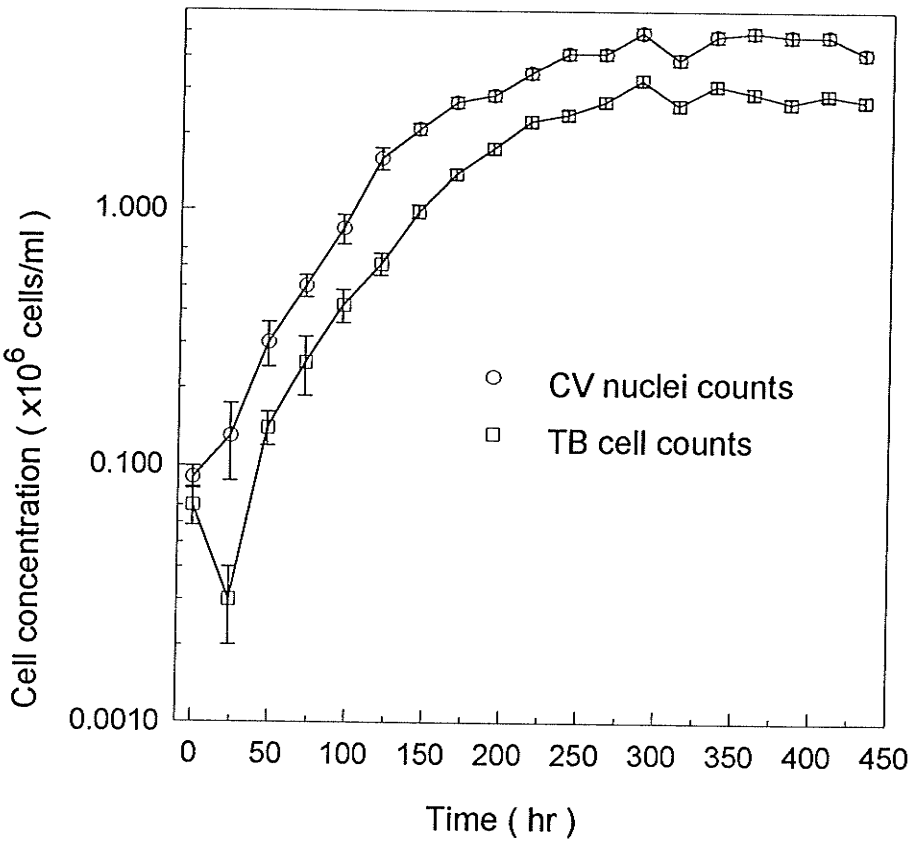

Fig.5.6.1. Measurements of cell densities in an 1 g/L Cyt 1 culture by Crystal violet (CV) and Trypan blue (TB) cell methods. The culture was supplied with DME + 5% serum (v/v) and stirred continuously at 40 rpm. Medium replacement (50%) was conducted from Day 2. (n = 1)

Appendix G

A 5.7. Effect of spinner size on growth

Since spinners are not equipped with any air control, oxygen supply to the medium is via gaseous diffusion from the overhead space inside the spinner. As a result, the surface area at the air-liquid interface is critical for aeration. Traditionally, the relationship of the available surfaces (S) to the medium volume (V) is expressed as S/V ratio, which reflects its effect on cell growth (Hyclone., 1992a). Similarly, it was reported that maximum cell yields of Vero cells were proportional to a lower V_m/V_I or a higher S/V ratio, where V_m and V_I were the volumes of medium and of vessel, respectively (Hirtenstein et al., 1982). Alternatively, the oxygen transfer rate (Kla) and the cellular respiration rate are compared in systems at certain cell density. Katinger and Scheiver., (1985) showed that in a culture with volume above 1 liter the oxygen utilization rate of the cells at 1 $x10^6$ cells/ml exceeded the oxygen transfer rate by gaseous diffusion from overhead space.

The effect of spinner size on growth of Vero cells was determined in experiments in which cultures at 1 g/L Cyt 1 and 1.72 g/L CuG were set up in spinners at different sizes, ranging from 100 to 500 ml. Cultures were supplied with DME + 5% serum (v/v) and stirred at 40 rpm continuously. 50% medium replacement was conducted on all cultures from Day 4. For Cyt 1 beads, results in Fig.5.7.1. show that growth yields were significantly lower in cultures in 250 ml and 500 ml spinners than that in 100 ml spinner. In fact, the maximum cell density reached was 2.8 $x10^6$ cells/ml in 100 ml compared with 2.0 $x10^6$ cells/ml in both 250 and 500 ml spinners. Their maximum cell densities in medium-fed Cyt 1 cultures reached over 1 $x10^6$ cells/ml, therefore the overall oxygen demand at 2.9 $x10^6$ cells/ml would be much higher than that at 1 $x10^6$ cells/ml. The S/V ratios of spinner at different sizes are summarized at Table 5.7.1. It shows that the S/V ratio is 0.25 cm^{-1} in 100 ml and 250 ml spinners compared to 0.16 cm^{-1} in 500 ml spinner. Although the S/V ratio is the same for 100 and 250 ml spinners, oxygen may be more deficient / limiting

in cultures at 250 and 500 ml so that higher cell yields were inhibited. Similarly, Hu et al., (1986) reported that oxygen was important to support high cell yield, with growth of Vero cells resumed in the presence of oxygen-enriched air.

On the other hand, the effect of spinner size on growth was also studied on CuG in which only 100 and 250 ml spinners sizes were tested. Results in Fig.5.7.2. show that there is no difference in the maximum cell density achieved in two cultures. Both cultures reached approximately at 3.4 and 3.7 $x10^6$ cells/ml respectively. It suggests that the spinner size (100-250 ml) had no effect on growth of Vero cells on CuG beads. Besides, greater surface area, Vero cells may metabolize differently on CuG beads, owing to its natural gelatin matrices.

The use of 100 ml spinners yielded easy preparation and handling. As a result, subsequent cultures for cell attachment and growth were studied with 100 ml spinners.

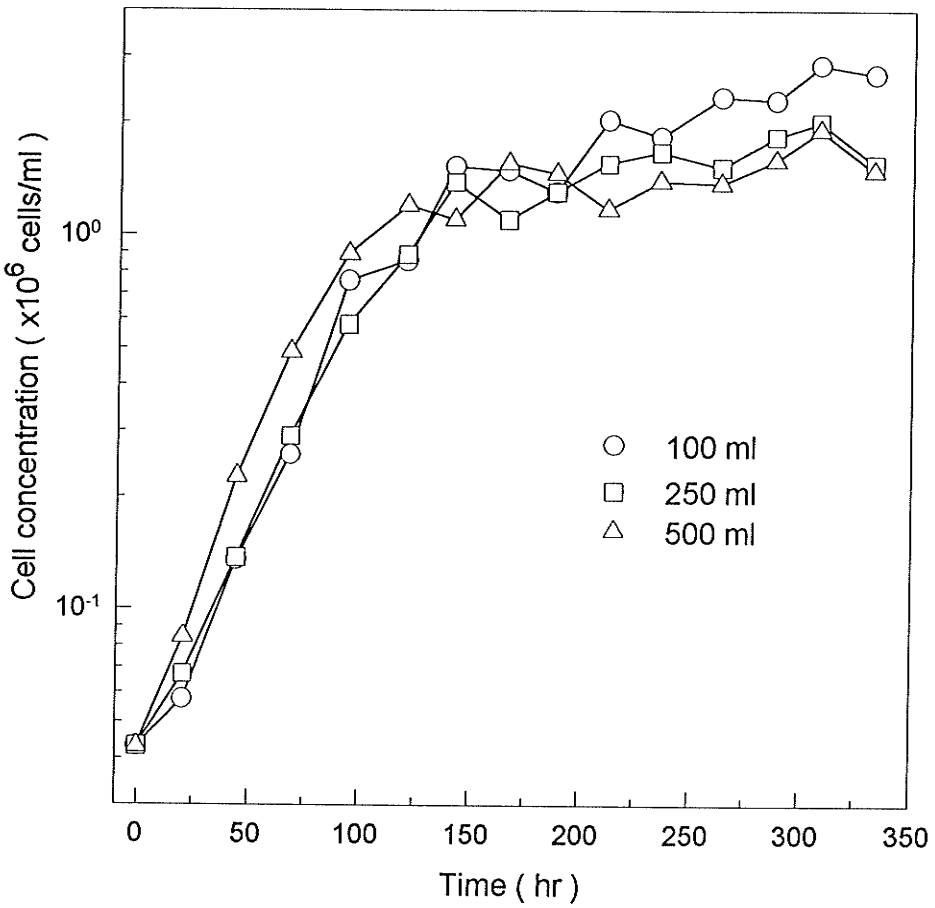

Fig.5.7.1. Effect of spinner size on Vero cell growth in 1 g/L Cyt 1 cultures. Spinners at different size (100-500 ml) were supplied with 100, 250 and 500 ml of DME + 5% serum (v/v). Agitation was at 40 rpm and 50% medium replacement was taken from Day 2. Cell enumeration was based on TB cell counts. (n = 1)

Table 5.7.1. Summary of surface-to-volume ratio, S/V, (cm^{-1}) of spinners [a].

	Spinner		
	100 ml	250 ml	500 ml
Internal diameter (cm)	5.6	7.9	10
Paddle diameter (cm)	4.5	5.2	7
S/V (cm^{-1})	0.25	0.25	0.16

a Surface-to-volume is based on the measurements of the surface areas of the overhead space (cm^2) over the volume of the medium (cm^3) in the spinners.

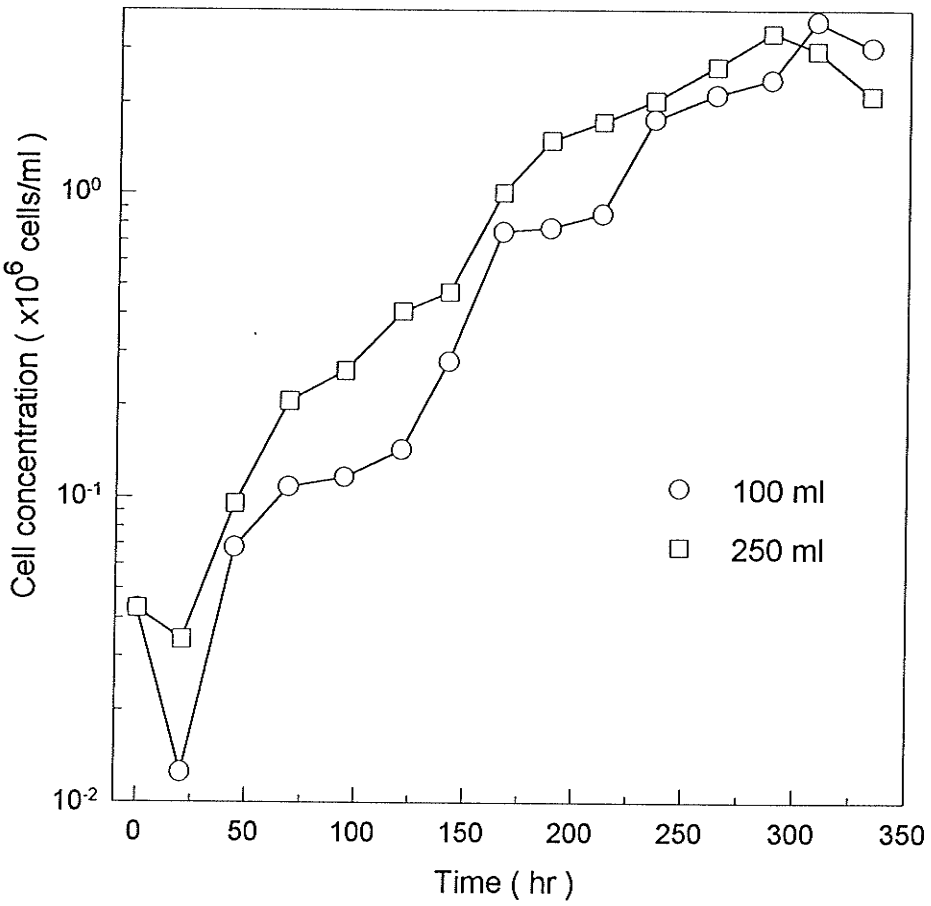

Fig.5.7.2. Effect of spinner size on Vero cell growth in 1.72 g/L CuG cultures. Spinners at different size (100 and 250 ml) were supplied with 100 and 250 ml of DME + 5% serum (v/v). Agitation was at 40 rpm and 50% medium replacement was taken from Day 2. Cell enumeration was based on TB cell counts. (n = 1)

Appendix H

A 5.8. Trypsinization procedure on 1 g/L Cyt 1 spinner culture in Serial subculturing.

1. Let beads settle on the bottom of the spinners.
2. Decant as much medium as possible.
3. Wash beads with 25 ml PBS-EDTA (0.02% w/v), which is 5 times the volume of bead.
4. Decant as much PBS-EDTA supernatant as possible
5. Transfer the beads to a centrifuge tube (25 ml).
6. Add 5 ml of warm 0.25% trypsin (w/v)/ 1 mM EDTA solution to the tube.
7. Incubate the mixture for 15 min at 37^{O}C on a shaker (New Brunswick) at 120 rpm.
8. Add complete medium to neutralize trypsin and dilute the contents.
9. Let the beads settle before collecting the supernatants.
10. Keep diluting the contents until there is no sign of significant numbers of cells present in the tube.
11. The supernatants are pooled together and centrifuged at 1500 rpm for 5 min.
12. Cells are enumerated and inoculated at 10 cells/ bead into the spinner with fresh DME + 5% medium.