Chapter 6

Scale-Up of Suspension and Anchorage-Dependent Animal Cells

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1. Introduction

In this chapter, scale-up is described in a laboratory context (10–20 L), but the principles and techniques employed have been successfully adapted so that cells are now grown industrially in unit volumes of up to 8000 L for vaccine, interferon, and monoclonal antibody production. The need to scale-up cell cultures has been expanded from the historical requirement for vaccine manufacture to include not only interferon and antibodies, but many important medical products such as tissue plasminogen activator and a range of hormones and blood factors. The low productivity of animal cells, resulting from their slow growth rate and low expression of product, plus the complexity of the growth conditions and media, led to attempts to use recombinant bacteria to express mammalian cell and virus proteins. However, this has proven unsuitable for many products, mainly because of incomplete expression and contamination with bacterial toxins, and more importance is now being put on expression of recombinant proteins from mammalian cells. This has allowed the use of faster growing and less fastidious cell lines, such as CHO, and amplification of product expression by multiple copies of the gene.

2. Principles of Scale-Up

Animals cells are grown in two completely separate systems. For those cells that grow individually in suspension, the range of fermentation equipment developed for bacteria can be readily modified. This is a great advantage, since these culture vessels are economic in terms of space, the environment is homogeneous and can be critically controlled, and scale-up is relatively straightforward. Many cell types, however, will only grow when attached to a substrate or, in some cases, will only produce significant levels of a product when grown in this mode. Scale-up of substrate attached cells is far more difficult to achieve and has given rise to a wide range of alternative culture systems.

Two approaches to scale-up can be taken. The first is volumetric—a simple increase in volume while retaining the same cell density or process intensity. The second method is to increase the cell density/unit vol 10–100-fold by means of medium perfusion techniques. Cell densities of over 10^8/mL can be achieved in a variety of systems, but they are difficult to volumetrically scale-up because of difficulties in supplying media in great enough, and constant enough, volumes. Compromise is possible with large-scale (100–500 L) cultures operating at just 10–20 times above the conventional cell densities (1–3 x 10^6/mL) by means of special perfusion devices, such as the spin filter.

The environmental factors that can most readily be controlled are pH (and redox) and oxygen. The limiting factor in scale-up, particularly in cell density, is usually oxygen. Surface aeration used in small cultures soon becomes inadequate, since the volume (and therefore depth) of medium increases. Bubbling of air/oxygen mixtures into cultures, with turbulent stirring/agitation, is the most efficient means of effecting mass transfer. Unfortunately, cells are fragile, compared to bacteria and only slow stirring and bubbling rates can be used, which are often inadequate for maintaining a sufficient oxygen supply. To overcome this problem, most cultures rely on several oxygenation methods, and many ingeneous methods have been developed for this purpose (*1*).

Two further points should be taken into account during scale-up. The first of these is the increased risk of contamination and the proportionally higher costs of culture failure. The second is a question of logistics in the preparation of medium and particularly cell inocula. It is a small matter to harvest 10^8 cells and inoculate them in a good physiological state into a new culture. An inoculum of 10^{10} cells takes a long time to prepare, cells can be left for long periods in damaging conditions, and media can lose its tem-

perature and set pH while these handling procedures are carried out. The objective is to keep both the process and the culture system as simple as possible, having everything well prepared and ready, and not to be over-ambitious with regard to scale. This will ensure that cultures are initiated with cells in good physiological condition and reduce the risks of microbial contamination.

3. Methods

3.1. Suspension Culture

3.1.1. Culture Vessels (Fig. 1)

The simplest means of growing cells in agitated suspension is the spinner culture vessel. The culture pot has a magnetic bar, usually placed a few millimeters from the bottom of the vessel, and is placed on a magnetic stirrer. As long as the bar is able to rotate freely and the stirrer is of sufficient quality to maintain constant stirring speeds, and not overheat, this methodology works extremely well for growing most cells up to densities of 1–2 x 10^6/mL. These glass spinner vessels are available from a wide range of suppliers (e.g., Bellco, Wheaton) in sizes from 0.2–20 L (2). A modification of this principal for shear-sensitive cells is the spinner vessel using surface agitation as exemplified by the BR-06 Bioreactor (Techne). Spinner vessels are only satisfactory up to a certain size—between 2 and 10 L depending upon the cell line and its required use. Above 10 L, glass vessels become inconvenient to handle and the progression to *in situ* stainless steel vessels should be considered. The other reason for change is that, with scale-up, the need to control the culture environment and carry out specialized manipulations (e.g., perfusion, media changes, and so on) increases. For this level of sophistication, a fermentation system needs to be used. The main differences are that (a) stirring is by a direct-drive mechanism with a motor, and (b) the vessel has a complex top that allows the inclusion of a range of sensors, probes, feed supply lines, and sampling devices for contamination-free monitoring and control. Fermenter kits (laboratory scale) are available in the range from 1to 40 L and cost in the region of $4500–37,500 (£3,000–25,000) (with control of stirring speed, temperature, pH, and oxygen).

Culture vessels for animal cells should have the following features:

1. Curved or domed bottom to increase mixing efficiency at the low stirring speeds that are used (100–350 rpm).

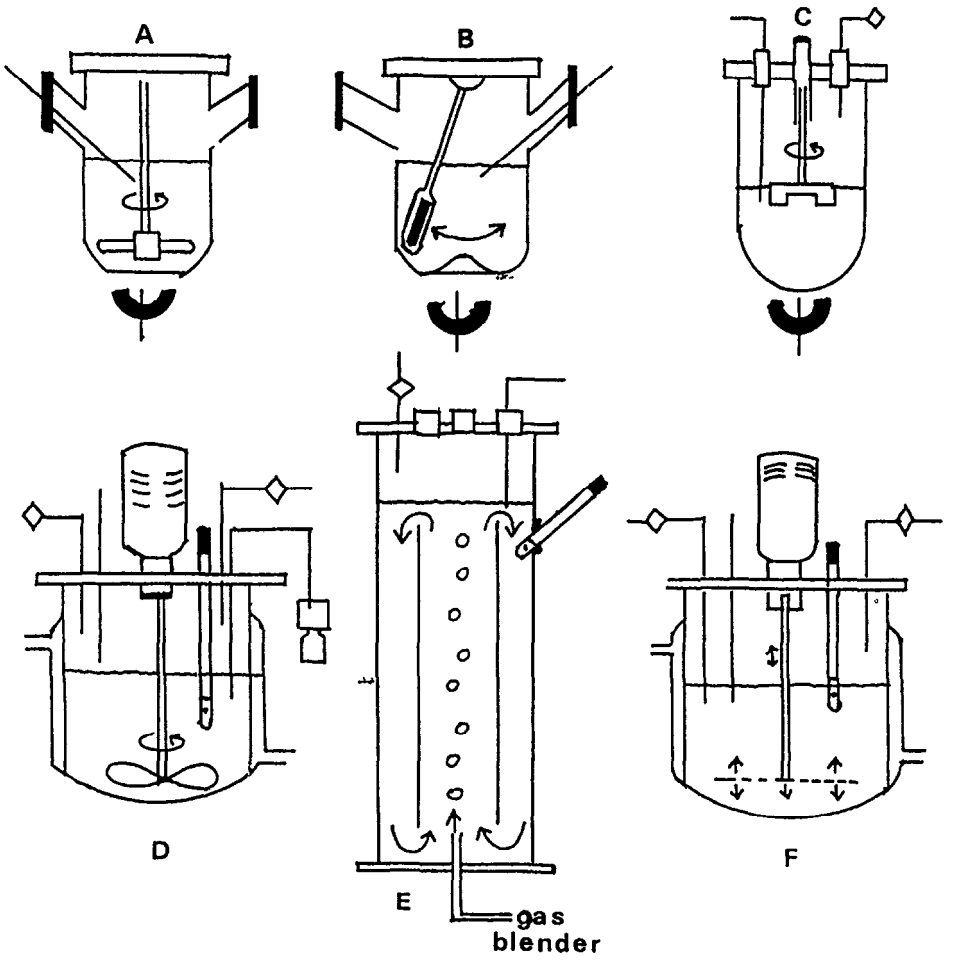

Fig. 1. Range of culture apparatus for suspension cells (A) magnetic bar spinner culture; (B) Techne MCS stirrer; (C) surface stirrer (Techne BR-05/06); (D) small scale fermenter with marine impeller; (E) airlift fermenter; (F) vibro-fermenter (Chemap).

2. Water jacket temperature control to avoid the use of immersion heaters that give localized high temperatures. Electrically operated silicone pads are also suitable at volumes up to 5 L—the only disadvantage is the reduction in visibility into the vessel.
3. Absence of baffles and other sharp protrusions that cause turbulence. The interior is finished to a high grade of smoothness to minimize mechanical damage and for cleanliness.
4. An aspect ratio (height to diameter) of 2:1 maximum, and preferably no more than 1.5:1.

5. Suitable impeller to achieve nondamaging bulk flow patterns (e.g., modified marine or pitched blade impellers) with top drive, so that there are no combinations of moving parts that would grind up the cells.

Some animal cells, including many hybridoma lines, are very sensitive to the mechanical effects of stirring. For such cells, there are two alternative means of mixing besides stirring.

1. Vibromixer—this is a nonrotary device using a plate that vibrates in the vertical plane a distance of 0.1–3 mm. Conical perforations in the plate affect the mixing (Vibro-fermenter, Chemap).
2. Airlift—medium is circulated in a low velocity bulkflow pattern by being lifted up a central draft tube by rising air bubbles, and recirculated downwards in the outer ring formed by the draft tube. This system forms a near ideal mixing pattern and allows near-linear scale-up to at least 1000 L. Unfortunately, the apparatus, with a 12:1 aspect ratio, is very high and a 30-L fermenter needs 3-m ceiling height. Airlift fermenters are available commercially either as complete systems (e.g., LH Fermentation) or as disposable 570 mL U (Cellift—Fisher Scientific).

3.1.2. Culture Procedure for Suspension Cells

1. Inoculum should be prepared from a growing suspension of cells (i.e., in mid to late logarithmic phase). Stationary phase cells are either slow to start in a fresh culture or do not grow.
2. Prewarm to 37°C, and equilibrate the pH of the culture medium with the CO_2/air gas mix, before inoculating the cells.
3. Inoculate cells at over 1×10^5/mL. Recommended level is $2–3 \times 10^5$/mL for many cell (hybridoma) lines.
4. Stir the culture within the range 100–300 rpm. This speed depends upon the individual type of culture reactor. Stir at a speed sufficient to keep the cells in homogeneous suspension. Do not use speeds that allow cells to settle out at the bottom of the culture.
5. Monitor cell growth at least daily by taking a small sample, either through a special sampling device or removing the vessel to a laminar flow cabinet and using a pipet, and carry out a viable cell count (Trypan blue stain and a hemocytometer).
6. pH—if the culture is closed (i.e., all ports stoppered with no filters), then the pH will fall. You should remove the culture to a cabinet and gas the head space with air. If the culture is very acid, sterile sodium

bicarbonate (5.5%) can be added or, when the cells have settled out, remove 50% of the medium and replace with fresh (prewarmed) medium. Return culture to stirrer. It is preferable to have inlet and outlet filters, so that there is continuous head-space gassing, initially (i. e., first 24 h) with 5% CO_2 in air, followed by air only. Suitable filters are nonwettable with a 0.22 μm rating.

7. After 3–4 d, the saturation density of 1–2 x 10^6/cells/mL should be attained. Most suspension cells will then die at a rapid rate unless harvested or maintained with medium changes.

3.1.3. Special Procedures

1. Airlift—follow the above protocol except, instead of stirring, a gas flow rate of approximately 5–20 cc air/L/min is used for mixing.
2. Increase cell density by perfusion. To perfuse a culture (i.e., the continuous or semicontinuous addition of fresh medium and withdrawal of an equal volume of spent medium) means that methods of separating the cells from the medium are needed. There are basically two techniques that can be used, spin filter and hollow fibers.

3.1.3.1. Spin Filter. The problem with most filtration techniques is that the filter rapidly becomes blocked with cells. A spin filter, so called because it is attached to the stirrer shaft, reduces the problem of blockage, because as it spins it produces a boundary effect on its surface that reduces cell contact. Also, they normally have a large surface area and, thus, have a low flow rate at any one point. A porosity of about 6–10 μm is needed, and stainless steel mesh can be used. This is thin enough to be cut to form a cone in which the join can be double-folded and machine-pressed (Fig. 2). This allows a perfusion rate in the order of 1–2 vol/d. This device will allow cell densities of over 10^7 cells/mL to be achieved—higher if the culture system has full pH, and oxygen monitoring and control (3).

3.1.3.2. Hollow-Fiber Cartridges. Hollow-fiber units are available at both ultrafiltration and filtration grade. For the purposes of withdrawing medium and returning the cells to the culture in a loop outside the vessel filtration grade (0.22 μm) is sufficient. The scheme is shown diagrammatically in Fig. 3. Many fiber cartridges are not steam sterilizable, but polysulfone and teflon fibers are. The quantity of medium withdrawn must be balanced by adding fresh medium from a reservoir. Using flow rates of up to 1 vol/h, cell densities in the region of 2–5 x 10^7/mL can be achieved, but oxygen is a limiting factor and an additional filtration cartridge should be put in the external loop as an oxygenator.

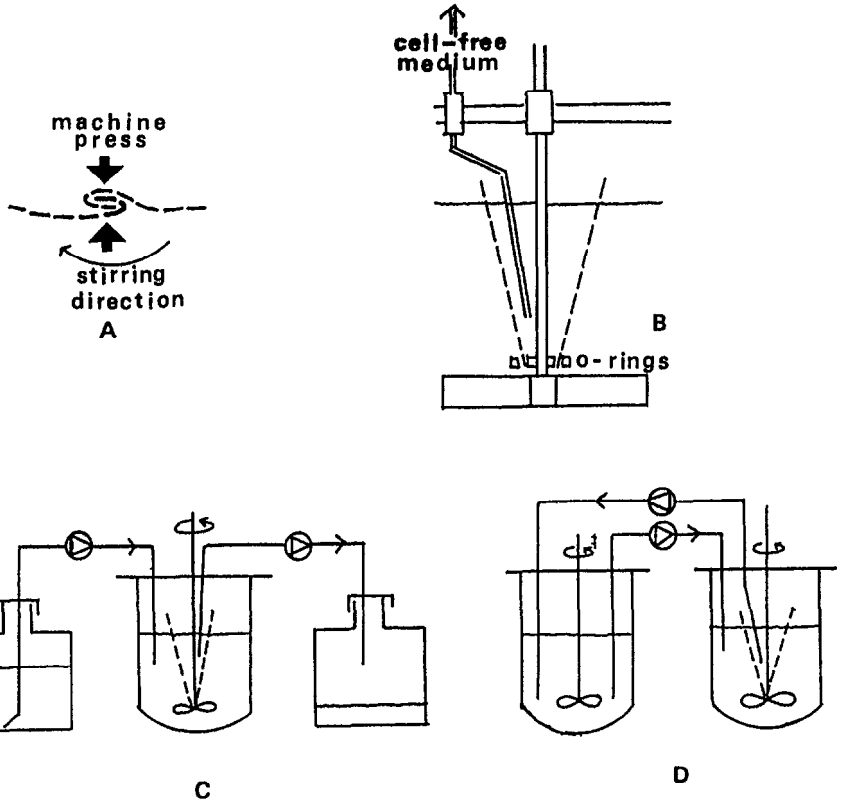

Fig. 2. Spinfilters: (A) construction using folded interleaving edges; (B) diagrammatic representation of a spin filter; (C) open perfusion systems; (D) closed (recirculating perfusion system).

3.1.4. Notes–Suspension Culture

1. Perfusion—media usually becomes limiting because of acid buildup and low oxygen, and this leaves a surplus of the other nutrients, such as glucose and amino acids. In the open perfusion systems described above, the medium probably only yields 3–5 x 10^5 cells/mL. If, however, the medium is circulated in a closed loop after passing through a reservoir in which violent agitation/aeration and addition of NaOH occurs (possible because it is a cell-free environment), then yields of 1–2 x 10^6/mL are achieved in media such as Eagles' MEM (*4*).
2. Media—serum is a very high cost addition to culture media, but alternatives such as specialized serum-free media that include a range of growth factors usually work out as expensive, or more so. Although cultures may have to be initiated in a complex media, as cell density

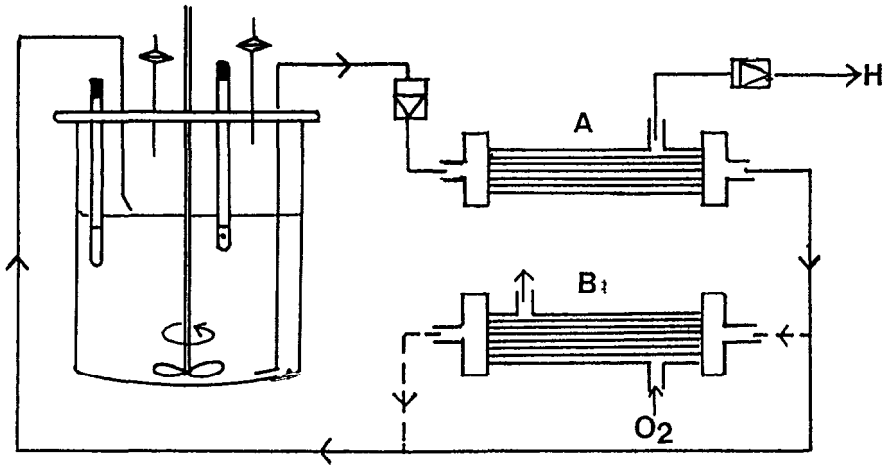

Fig. 3. Hollow-fiber cartridges in perfusion loop for (A) removing spent medium (H) and returning cells to the culture and (B) oxygenating the medium.

increases, so cells become less dependent upon serum and growth factors. Thus, at densities above 5×10^6–10^7/mL, serum concentration can be drastically reduced (to 2%) or even excluded. If serum-free medium is used, cells are more susceptible to damage by stirring and air bubbles, but this can be offset to a certain extent by adding pluronic F68 (polyglycol) at 0.1%. (*See* review (4) for other means of cutting the costs of media.)

3. Contamination/sterility—the larger the scale, the more expensive a culture failure becomes. Carry out stringent quality-control procedures by testing growth media several days before it is to be used for bacterial contamination. Do not take shortcuts on the support equipment, but use specialized tubing connectors and sampling devices supplied by fermenter equipment companies. Also, do not overuse the air filter (6–10 sterilization cycles maximum), and do not allow them to get wet (either during autoclaving or with media or condensation).
4. Suspension culture—many cells either attach to the surfaces of the vessel or form unwanted clumps. Media for suspension culture should have a reduced calcium and magnesium-ion concentration (special formulations are commercially available) because of the role of these ions in cell attachment. Attachment to the vessel can be discouraged with a pretreatment of a proprietary silicone solution (e.g., Repelcote, Hopkins & Williams).

3.2. Anchorage-Dependent Culture

3.2.1. Materials

Anchorage-dependent culture systems are far more difficult to scale-up than suspension cultures because of the additional requirement of providing the extra surface area in an economical (in terms of space) way and still maintain homogeneity throughout the system. For this reason, suspension culture, in which a 1-L stirred vessel is conceptually similar to a 1000-L vessel, is always the preferred culture method. The first step in scale-up (Fig. 4) usually involves the change from stationary flasks (available in sizes up to 200 cm^2) to roller bottles (sizes up to 1750 cm^2). The larger size of roller bottle will yield in the range of 2–5 x 10^8 cells, and therefore, for most purposes, a multiplicity of rollers has to be used. The next step in scale-up is to use roller bottles that have an increased surface area resulting from the inclusion of glass tubing (Belco-Corbeil, Chemap Gyrogen) or plastic spiral films (Sterlin). By this means, the surface area within a roller bottle can be increased to 8500 cm^2 (spiral film) and 15,000 cm^2 (glass tubing). An alternative to investing in specialized, and costly, roller culture equipment is to use plastic multi-tray units (Nunc). Each tray has a surface area of 600 cm^2 and units of 6, 10, and 40 (24,000 cm^2) plates can be obtained. Two systems that allow a huge unit scale-up of substrate attached cells are immobilized beds (e.g., constructed of glass spheres) and microcarrier culture (cells growing on 200 μm spheres that are stirred in suspension culture apparatus). Microcarriers can provide 5000 to 50,000 cm^2/L and are currently being used in commercial production systems at the 1000-L scale (a total of 15 x 10^6 cm^2 surface area, which has the potential of supporting 15 x 10^{11} cells).

3.2.1.1. Roller Culture. Reutilizable glass or disposable plastic roller cultures are used. The most commonly used sizes are 750–850 cm^2 and 1500–1700 cm^2. Complete modular systems holding up to 48 large bottles can be purchased either free-standing, for use in hot rooms, or within an incubator cabinet.

3.2.1.1.1. PROCEDURE. The following procedure is based on a 1500 cm^2 (24 x 12 cm) roller bottle.

1. Add 200–300 mL of medium.
2. Add 1.5 x 10^7 cells (observe previously listed advice on preparation of inocula and medium).
3. Revolve the culture at 15 rph.

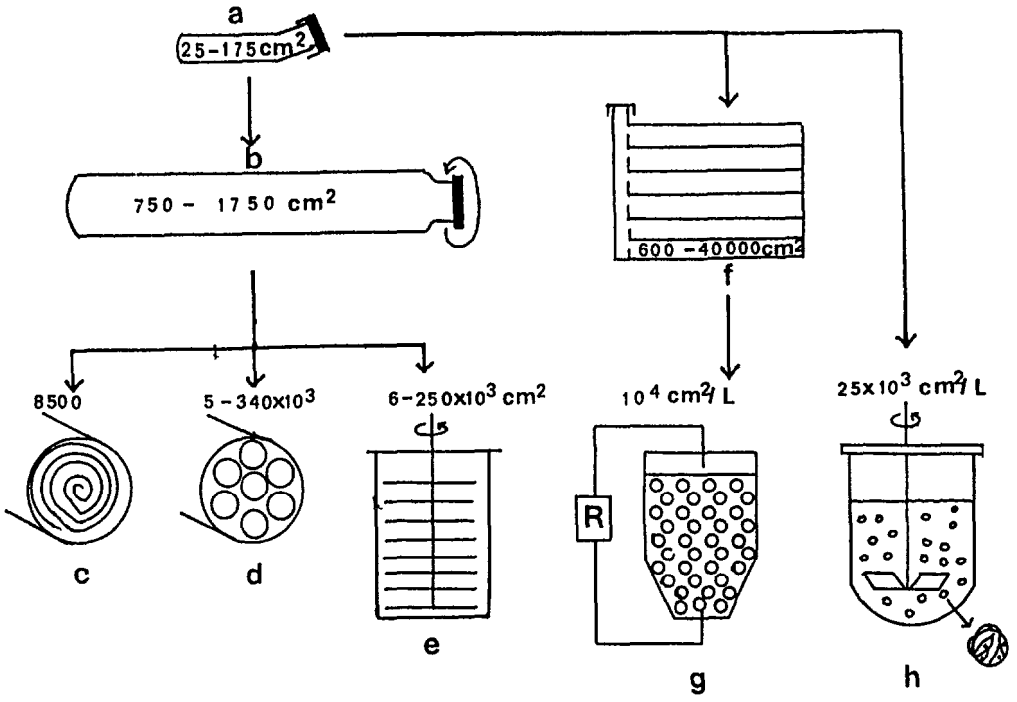

Fig. 4. Scale-up of anchorage-dependent cultures: (a) flask; (b) roller bottle; (c) plastic spiral (Sterlin); (d) glass tubing (Belco-Corbeil); (e) stack plates; (f) multi-tray units (NUNC); (g) immobilized bed (glass spheres); (h) microcarrier culture. The figures define the surface area for each culture vessel.

4. Cell growth can be observed under an inverted microscope with a long-distance objective.
5. After 3–5 d, the cell sheet will be confluent yielding from 1.5 x 10^5 (human diploid) to 5 x 10^5 (heteroploid cells, e.g., HeLa) cells/cm^2.
6. Pour off the medium, wash the cell sheet with prewarmed phosphate buffered saline, and add 50 mL trypsin (0.25%). Place culture back on roller, and allow to revolve for 10–20 min. The cells will detach and can be harvested, diluted in fresh medium and serum, and passaged on.

This outline protocol can be considerably modified. An advantage of this method is that the medium volume:surface area ratio can be altered easily. Thus, after a growth phase, and when a product is to be harvested, the medium volume can be reduced to 100 mL in order to obtain higher product concentration.

3.2.1.2. Glass Bead Immobilized Beds (3,5). Apparatus—this type of culture system is easily fabricated in the laboratory (Fig. 5). A suitable

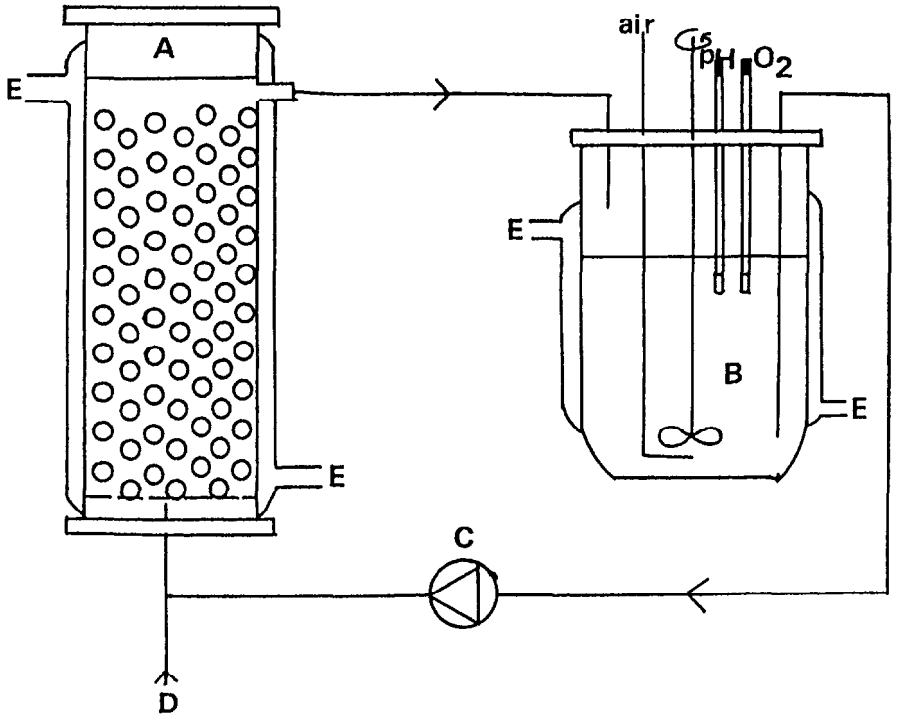

Fig. 5. Glass-sphere immobilized-bed culture: (A) immobilized bed; (B) reservoir with stirrer, pH and oxygen probes, air sparging; (C) pump; (D) inlet for inoculum/trypsin; (E) water at 37°C.

cylindrical or funnel-shaped glass container is packed with borosilicate glass spheres (minimum diameter 3 mm, optimum diameter 4 or 5 mm). Medium is perfused by means of a peristaltic pump from a reservoir (which ideally is a spinner flask or small fermenter), which can be monitored and controlled for pH, oxygen, and so on. The productive capabilities of the system are give in Table 1.

3.2.1.2.1. PROCEDURE

1. Prepare growth medium, and add to reservoir (2 mL/cm^2 culture surface area).
2. Equilibriate the system for temperature and pH, and circulate the media through the packed bed (allow sufficient time for the solid glass spheres to reach 37°C).
3. Inoculate cells (1–2 x 10^4/cm^2) into a volume of medium equal to the void volume of the bed (250 mL/kg 5 mm spheres).
4. Allow the cells to attach (3–8 h depending upon cell type), but the culture can safely be left overnight (16 h) at this stage.

Table 1
Physical Characteristics of Glass Sphere Beds

Bead diameter	3 mm	5 mm
Surface area (cm^2)		
Total	7400	4600
Available (70%)	5200	3200
Void medium vol (cc)	295	250
Total vol (cc)	675	625
Cell count (x $10^5/cm^2$)	0.78	2.50
(x 10^8/kg)	4.0	8.0

5. Start medium perfusion, initially at a rate of 1 linear cm of column/10 min, but as the culture progresses, this rate is increased to a maximum of 5 cm/min.
6. Cell growth can only be monitored by indirect measurement, and the glucose utilization rate is the most convenient. An alternative is oxygen utilization rate. Growth yields should be determined for the particular cell line and nutrient, so that an approximation of cell numbers can be made (e.g., glucose utilization is usually within the range of 2–5 x 10^8 cells produced/g glucose).
7. When the culture is estimated to be confluent (after 5–7 d), drain the medium, give the bed a phosphate-buffered saline wash, and add trypsin/versene to harvest the cells. The efficiency of cell detachment can be increased by intermittently draining and pumping back the trypsin solution. The bed acts as a depth filter, and to recover a high percentage of the cells, the bed should be washed through several times with medium after the trypsin has been drained off. (NB., 5 mm beads allow a better drainage and cell recovery than 3 mm.)
8. Wash out the culture bed immediately with a detergent, so that cell debris does not become fixed onto the glass beads.

This culture method is basically very simple, and the apparatus is cheap and reutilizable. It has a large potential scale-up and has been proven at the 100 L vol scale (30 L bed of 3 mm beads) (5). It is perhaps most suitable for harvesting a secreted cell product over a long period of time, rather than acting as a source of cells resulting from the difficulties of removing cells from the bed.

3.2.1.3. Microcarrier Culture. The advantage of this methodology is that the cells, when growing on small carriers, can be treated as a suspen-

sion culture with all the advantages of large unit scale-up, homogeneity, and easily controlled environmental conditions. The range of microcarriers commercially available is extensive (Table 2) (*2,6*), and at least one type is suitable for all cell types, however demanding. The decision of which one to use is influenced by whether a dried powder or already-prepared sterile solution is preferred, the cost/cm^2, whether a special derivitized surface is needed for a particular cell, or whether one wishes to harvest cells by dissolving the carrier (gelatin, collagen) and thus producing a higher quality cell suspension. Experience has shown that it is worthwhile to evaluate several types in small-scale cultures for each particular cell line, since significant differences in cell yield and longevity of culture (before cell detachment) are seen.

3.2.1.3.1. CULTURE APPARATUS. Modifications of suspension culture vessels are used. Spinner flasks with a magnetic bar are unsuitable, but versions are available with large paddle-type impellers (Bellco) or specially modified stirring actions (e.g., Techne MCS). Stirring rates are much slower (20–70 rpm) than for suspension cells, and thus more efficient mixing at low speeds is required. Scale-up in laboratory fermenters can also use the large-bladed paddles, but there are several modifications of the marine impeller available that are very efficient (e.g., SGI ascenseur).

3.2.1.3.2. PROCEDURE. Microcarrier culture is not a difficult technique, but it does require more critical attention to experimental detail than most methods and the use of the correct culture vessels. The following procedure is based on using Cytodex 3 (Pharmacia) or Dormacell 2.3 (Pfeifer & Langen) at 3 g/L. Prepare the microcarriers according to manufacturer's recommendations.

1. It is essential that the medium with microcarriers be prewarmed and stabilized before inoculating the cells. Cell attachment to moving spheres requires conditions to be just right. It is even more important with this method than with previously described ones to initiate the culture with growing (logarithmic cells) and not stationary-phase cells, and cells that have been rapidly prepared and are in good physiological condition (i.e., have not been standing in trypsin, and so on, for extended periods).
2. Inoculate at $2 \times 10^4/cm^2$ into 30–50% of the final volume. Stir at the minimum speed to maintain homogeneity (20–30 rpm) for 4–8 h.
3. When the cells have attached (expect 70–90% plating efficiency), the volume can be increased to the full working volume. (Stirring speed may also have to be increased to give complete mixing.)

Table 2
Commercially Available Microcarriers

Name	Manufacturer	Type	cm^2/g^a
Acrobead	Galil	Derivitized	5000
Bioglas	Solo Hill Eng.	Glass/latex	350
Bioplas	Solo Hill Eng.	Polystyrene	350
Biosilon	Nunc	Polystyrene	255
Cytodex 1,2	Pharmacia	Dextran	6000
Cytodex 3	Pharmacia	Collagen	4600
Cytosphere	Lux	Polystyrene	250
Dormacell	Pfeifer & Langen	Dextran	7000
Gelibead	Hazelton Lab.	Gelatin	3800
Mica	Muller-Lierheim	Polyacrylamide	[b]
Micarcel G	Reactifs 1BF	Polyacrylamide	5000
Microdex	Dextran Prod.	Dextran	250
Superbeads	Flow Labs.	Dextran	6000
Ventregel	Ventrex Lab.	Gelatin	4000
Ventreglas	Ventrex Lab.	Glass/polystyrene	300

[a]A guideline only as different types vary 2–5-fold in cell yield/cm^2.
[b]Porous matrix beads.

4. A great advantage of the microcarrier system is that samples can be readily removed and microscopically examined. Unstained preparations will show whether or not cells have attached, spread out, and then begun to grow. Cell counts can be made by the standard nuclei-counting procedure, which releases the stained nuclei from the attached cells.
5. As the culture progresses, the stirring rate can be increased to prevent cell-to-cell attachment, bridging microcarriers and causing clumps to form. A maximum of 75 rpm should be arrived at.
6. In nonenvironmental controlled cultures, the media will turn acid after 3–4 d and a partial (50–70%) media change should be carried out. Stop stirring, allow beads to settle (10 min), siphon off spent medium, add fresh (prewarmed) medium, and start stirring, gradually increasing the rate.
7. Cells can be harvested when confluent by allowing the carriers to settle out, giving a serum-free wash, allowing the carriers to settle out again, decanting off as much of the free fluid as possible, adding trypsin, and restarting stirring, but at slightly faster speed (75–125 rpm). After 20 min, allow the beads to settle out for 2 min. Cells can either

be removed by decanting, or the mixture can be filtered through a coarse sintered glass filter that allows passage of the cells, not the microcarriers. If gelatin or collagen carriers are being used, then cells can be released by treatment with trypsin/EDTA (which solubilizes gelatin) or collagenase.

3.2.2. Scaling-Up

This can be achieved by increasing the culture volume, and increasing the microcarrier concentration from the suggested 3 to 5–15 g/L. If higher concentrations are used, then it is imperative to have a perfused system with full environmental control. The easiest means of perfusing is the spin filter (as described for suspension cells), but a much larger pore size can be used (60–100 μm). This allows much faster perfusion rates to be attained (1–2 vol/h). Perfusion from a reservoir that is adequately gassed is an efficient means of oxygenating the culture. Spin filter systems are commercially available (LH Fermentation, New Brunswick).

4. Conclusion

Microcarrier is undoubtably the most efficient scale-up method for anchorage-dependent cells currently available. It allows volumetric (to 1000 L) and density scale-up with a spin filter (to 5×10^7 cells/mL). The equipment must be specially designed for the procedure, but it can also be used for suspension cells, or as reservoir vessels for other high process intensity systems (e.g., hollow fiber). A disadvantage is the high substrate cost $1800–2250/kg (£1200–£1500/kg) and the fastidious nature of the technique.

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