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High cell density and productivity culture of Chinese hamster ovary cells in a fluidized bed bioreactor

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Abstract

A recombinant Chinese hamster ovary clone was cultivated in a 2L Cytopilot Mini fluidized bed bioreactor using Cytoline 1 microcarriers and a 10L B. Braun stirred tank bioreactor with Cytodex 1 microcarriers. Cytoline 1 is a macroporous polyethylene microcarrier and Cytodex 1 is a solid DEAE-dextran microcarrier. Cytoline 1 microcarriers in the fluidized bed bioreactor were gently mixed by an uplifting flow. Circulation and sparging in Cytopilot Mini were separated from the fluidized microcarrier bed. Cytopilot Mini bioreactor with Cytoline 1 microcarriers offered 2.3 times more surface area than the stirred tank bioreactor. The 2L fluidized bed bioreactor accommodated approximately half the cells in the 10L stirred tank bioreactor. Moreover, Cytopilot Mini had approximately three times more product output rate and 5.5 times higher specific productivity than the stirred tank bioreactor.

Introduction

Until recently, a batch process is still the most widely used culture system for mammalian cells. Historically, mammalian cell culture launched on vaccine production and vaccine products are administered in small doses. The adoption of well-established bacterial stirred tank bioreactor as a culture system satisfied the demands for vaccine products. The current interest of mammalian cell culture has shifted to the production of complex recombinant proteins that require eukaryotic post-translational processing. With seven products from mammalian cell cultures on the market, many more under various stage of development, and the fact that the dose of protein products is usually larger than vaccines, ever greater emphasis has been placed on how the proteins can be efficiently manufactured from mammalian cell cultures. It is generally recognized that a high volumetric productivity can be attained by employing perfusion cultures. In many, but not all, cases, not only did cell density increase tremendously in perfusion cultures by various means of cell retention, but an increase in cell-specific productivity was also noted.

Many of the mammalian cells are anchorage-dependent or -preferred. In favor of a perfusion culture system, a variety of new bioreactor configurations, modifications and microcarriers have been introduced in the last ten years. Spinfilters are the simplest modification to stirred tank bioreactors and have been successfully applied to the cultures of many cell lines and products. In addition, cell immobilization, packed bed, gravity settler, acoustic settler, membrane, hollow fiber, and fluidized bed culture systems have also been used in perfusion cultures. Inaccessibility to real-time cell density and non-homogeneity limited further application of several of the above bioreactor configurations. Many solid and porous microcarriers are commercially available for the large-scale cultures of anchorage-dependent cells. Cytodex 1, a solid microcarrier, has a proven history of supporting the cultures of a large number of mammalian cells for more than 30 years. Porous microcarriers can offer much more

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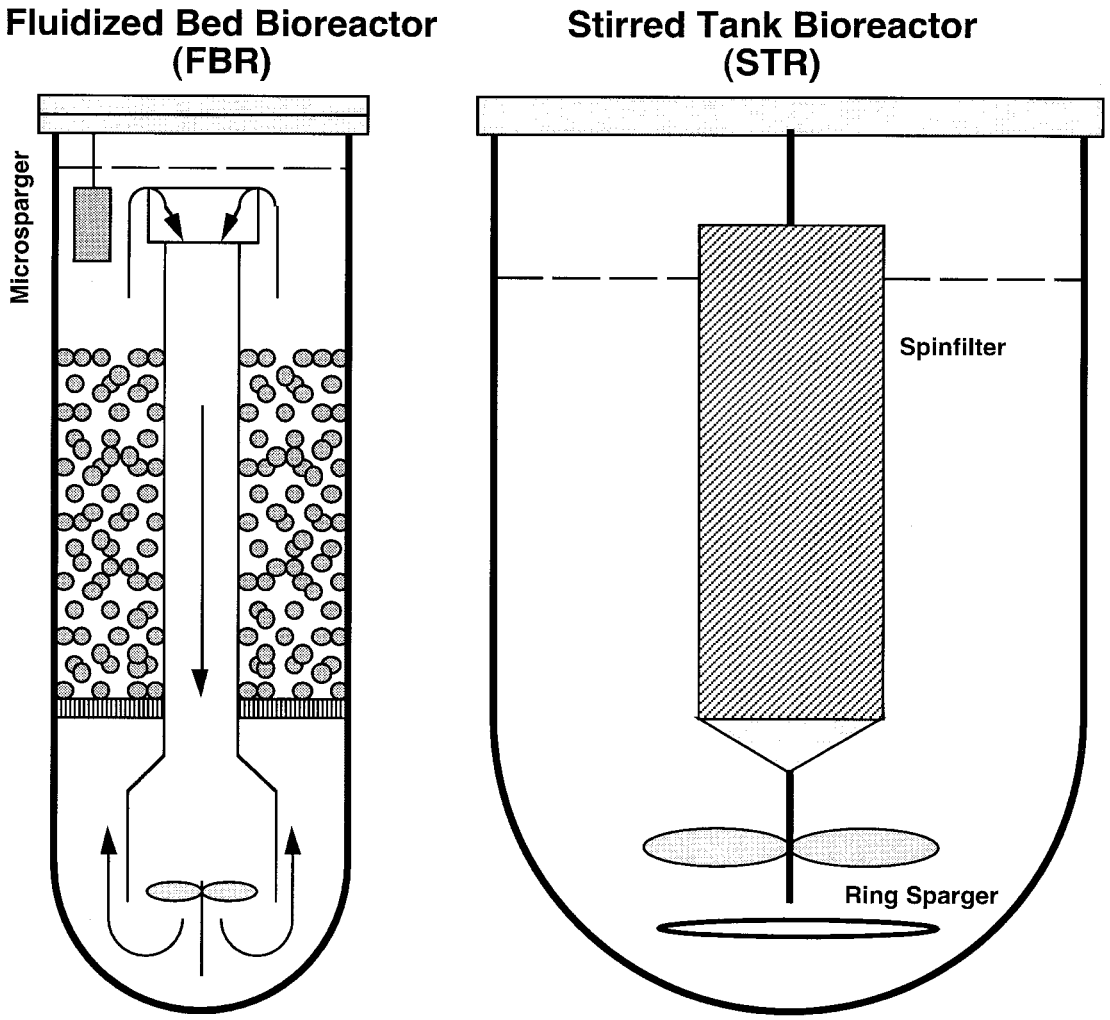

Figure 1. Diagram of the FBR and the STR.

surface area than solid microcarriers and usually will give rise to a high cell density. Cultispher (Percell Biolytica/Hyclone), Cytopore and Cytoline (Amersham Pharmacia Biotech) are examples of porous microcarriers.

A stirred tank bioreactor with a spinfilter has limited duration of operation due to the spinfilter fouling. Conventional impeller agitation and gas aeration cause concerns on cell and microcarrier damages. Modifications and improvements on the agitation and aeration methods have not yet radically solved the problems, especially at high cell density. Fluidized bed bioreactors use weighted-microcarriers. The microcarriers are suspended by their relative density and upward flow. Upon proper bioreactor design, agitation and aeration can readily be separated from the microcarriers. Fluidized bed bioreactors were shown to have high cell density and productivity (Griffiths et al., 1992).

This study reports the comparison of CHO cell perfusion cultures in a spinfilter stirred tank bioreactor and a fluidized bed bioreactor, Cytopilot Mini. The CHO culture in the fluidized bed bioreactor demonstrated a much higher cell density and productivity.

Table 1A. Cell densities and productivities in the FBR and the STR

Bioreactor	hours	cells/ml	Total Cells	total m^2	cells/m^2
FBR	903–1311	3.28×10^7	0.98×10^{10}	40.5	2.43×10^8
STR	557–1140	2.63×10^6	2.11×10^{10}	17.6	1.20×10^9

Table 1B. Productivities in the FBR and the STR

Bioreactor	hours	mg/L/day	mg/day	total cells	mg/billion cells/day
FBR	903–1311	22.82	34.2	0.98×10^{10}	3.49
STR	557–1140	1.65	13.2	2.11×10^{10}	0.63

Materials and methods

Cell line, media and cell cultures

A CHO clone (i.e. CHO.OAP.46), expressing a novel human gene product, was used in this study. This novel human gene was recently isolated from a chromosome 1 cosmid library (Smits et al., 1997). Because of unknown function of the gene product, three different names [i.e. osteoblast derived antiviral protein (OAP), extra cellular matrix protein 1 (ECM1) and osteoblast derived growth factor (ODGF)] have been used to describe the protein. The sequences of this novel gene have recently been published (Smits et al., 1997) and the protein expressed from CHO cells was glycosylated. For the purpose of consistency, the name OAP was used throughout this study.

Routine T-flask passage was carried out every 3-5 days at 1:2 – 1:4 split ratio in MEM alpha$^-$ medium supplemented with 5% dialyzed fetal bovine serum (DiFBS) and 100 μM methotrexate (MTX). Seed cultures in T-flasks and spinners were prepared in HGS-CHO-3 medium, a D-MEM/F12 based medium, containing 1% regular fetal bovine serum and supplemented with glucose and glutamine.

Microcarriers and preparations

Cytodex 1 (Amersham Pharmacia Biotech, Uppsala, Sweden) is a positively charged solid microcarrier, made of DEAE-dextran with a density of 1.03 g/cm^3, size around 190 μm, and 4400 cm^2/g area. Cytodex 1 microcarriers were hydrated overnight in 1X PBS. Following triple washings with 1X PBS and autoclaving, the Cytodex 1 microcarriers were conditioned in HGS-CHO-3 medium containing 5% regular fetal bovine serum overnight. Cytoline 1 (Amersham Pharmacia Biotech, Uppsala, Sweden) is a polyethylene macroporous microcarrier and has 1.32 g/cm^3 density and more than 0.3 m^2/g surface area. Cytoline 1 microcarriers were washed and soaked in 0.1 N NaOH overnight and autoclaved in 1X PBS. Upon use, Cytoline 1 was washed once with HGS-CHO-3 medium.

Bioreactor setup

A B. Braun MD10 bioreactor (B. Braun Biotech Inc., Allentown, PA) was equipped with a 100 μm internal spinfilter and had a working volume of 8 L (Figure 1). A digital control unit (DCU) was used to control temperature at 37 °C, pH between 7.0 and 7.2, agitation at 40 rpm and DO at 30%. Cytodex 1 microcarrier load was 5 g/L. The bioreactor was inoculated with one liter microcarrier spinner culture. Initial cell density was 0.38×10^6 cells/ml. Aeration was done with sparging through a ring sparger.

Cytopilot Mini (Amersham Pharmacia Biotech, Uppsala, Sweden) was loaded with 300 ml of Cytoline 1 microcarriers with a medium filling volume of 1.5 L. A B. Braun BIOSTAT A micro-DCU-system (B. Braun Biotech Inc., Allentown, PA) was used to control temperature at 37 °C, pH in 7.0–7.2 and DO at 40%. A separate motor controlled the circulation impeller at 250 – 320 rpm. Cytopilot was inoculated directly from 36 T-225 T-flasks, which gave an initial cell density of 0.82×10^6 cells/ml. After initiating the culture, the reactor was set in packed bed mode during attachment with reduced stirrer speed (100 rpm). The carriers were fluidized every 30 min for 10 seconds at 300 rpm. After 5 hours (supernatant became clear), the microcarrier bed was fluidized. Figure 1 shows a

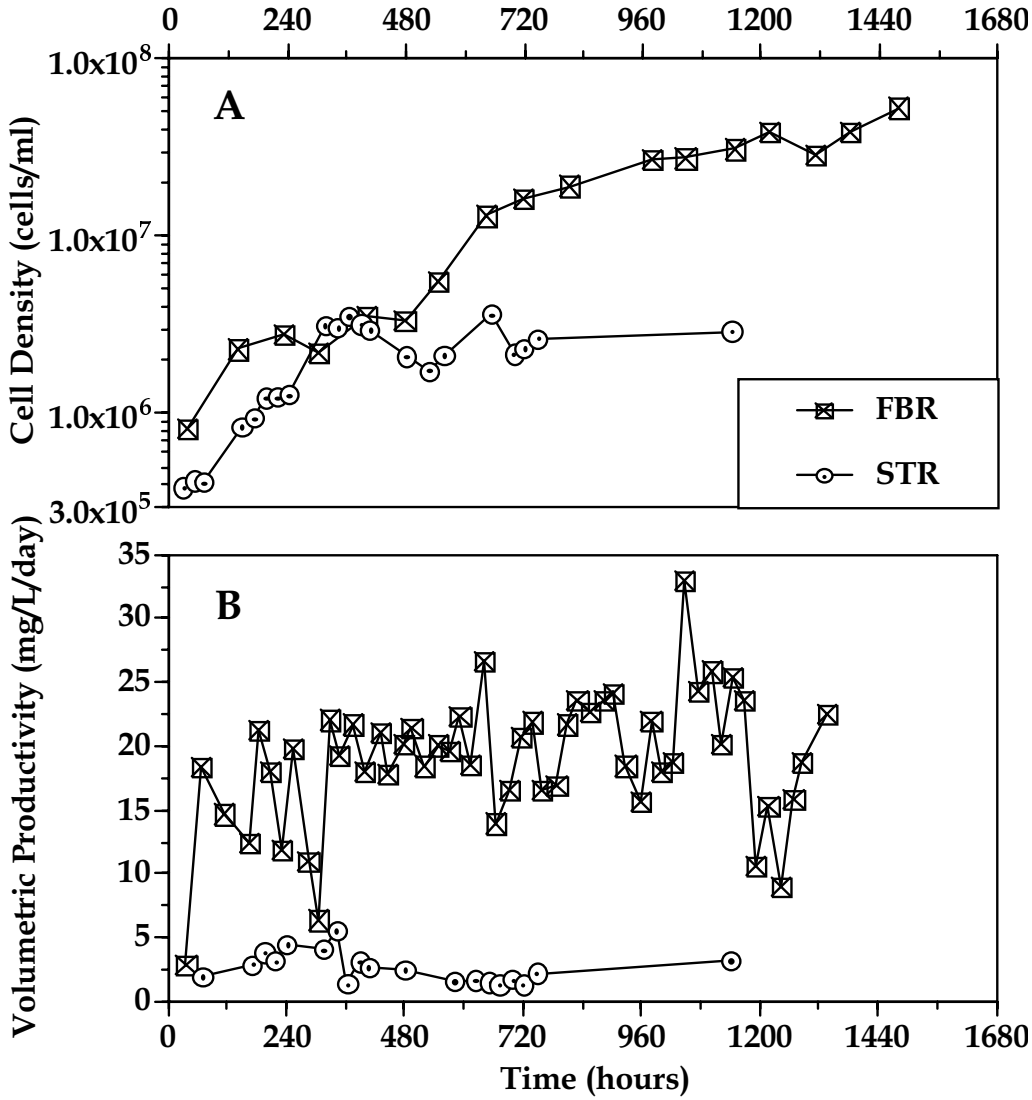

Figure 2. Cell growth and volumetric productivity in the FBR and the STR.

diagram of the fluidized bed bioreactor. Cytoline 1 microcarriers were lifted by an upward flow through a distributor plate and retained in an expanded zone by their density. Microcarrier-free medium was circulated through a draft tube in the center, driven by a marine impeller at the bottom. Aeration was provided by a microsparger below the retention sieve. This configuration guarantees a sufficient nutrient supply throughout the carrier bed.

Product and cell density analysis

OAP concentration was analyzed by reverse phase HPLC (HP1100, Hewlett Packard, Wilmington, DE). Floating cells and viability were estimated by running 1 ml of culture sample through a 75 μm cell strainer and counting the cells on a haemocytometer by trypan blue stain. Cells on Cytodex 1 microcarriers were enumerated by spinning down 1 ml well-mixed microcarrier containing culture sample and counting cells with a haemocytometer after crystal violet stain. Cell density in Cytoline 1 culture was estimated by dispensing known number of Cytoline 1 microcarriers in 2 ml of crystal violet and 2 ml of Triton-X-100 (0.1% Triton-X-100 and 100 mM citric acid). Crystal violet treated samples were vortexed vigorously and counted with a haemocytometer. Using Coulter Multisizer II, the Triton-X treated sample was used for confirming

cell counting by the crystal violet method. Results from both cell counting methods were consistent. Conversion of number of cells per Cytoline 1 microcarrier to cells per ml bed volume was based on 440 Cytoline 1 microcarriers per ml of bed volume.

Results

Cell densities in perfusion cultures

The same CHO clone was prepared for the cultures in a B. Braun MD10 stirred tank bioreactor (STR) and a fluidized bed bioreactor (FBR), Cytopilot Mini. A pronounced difference was noticed for the cell densities in the FBR and STR. The STR was a homogenous system. The cell density in the STR was expressed in number of cells per ml of working bioreactor volume. With regard to the Cytopilot Mini, fluidized bed volume was dependent on the flow rate and the microcarrier load could vary independently with the bioreactor working volume. The cell density in Cytopilot Mini was thus showed as cells per ml of Cytoline 1 microcarrier volume. Figure 2A showed cell density in the FBR and the STR. Cell density in the FBR continued to increase for more than 1440 hours and reached 5.3 $\times$ 10^7 cells/ml, while cell density in the STR reached and maintained a plateau cell density of about 2.5 $\times$ 10^6 cells/ml after 315 hours. Table 1A was generated in an effort to compare cell densities from the two bioreactors despite of the different basis cell densities were expressed. Between 903 and 1311 hours of the FBR run, an average cell density in the FBR was 3.28 $\times$ 10^7 cells/ml. For the STR, the average cell density was around 2.63 $\times$ 10^6 cells/ml. The total number of cells in the 2L FBR was approximately half of that in the 10L STR (Table 1A) and had potential to increase further. In terms of total number of cells as producing units, the compact FBR was equivalent to approximately half of the 10L STR. The potential of the cells in the FBR to grow further could be partially explained by the total surface area presented in the two bioreactors. With the microcarriers and their loads described in Materials and Methods, it was calculated the FBR provided 40.5 m^2 compared to 17.6 m^2 in the STR. The small FBR actually offered 2.3 times as much surface area as the STR. Consequently, the number of cells in each square meter of microcarrier surface area for the FBR was approximately five times lower than that for the STR. If surface area was the sole source of limiting factors, a further increase in cell density from the FBR could be expected. Non-attached cells were under 2% throughout the FBR run.

Productivity and product formation

Given the evidence that the 2L Cytopilot could accommodate approximately half the cells in the 10L stirred tank bioreactor and provide separated circulation and sparging, how was cell performance and bioreactor productivity? Fig. 2B shows the volumetric productivities from the two bioreactors. Volumetric productivities are good measurements of how fast a product can be made and how efficient a bioreactor is utilized to produce such a product. In the STR, volumetric productivity reached 5.6 mg/L/day during the growth period. After the maximal cell density being reached, productivity dropped and maintained at around 1.65 mg/L/day. For the FBR, volumetric productivity remained consistently around 22.8 mg/L/day. The volume used for the FBR productivity calculation was just a working volume (i.e. 1.5 L in this study). A more realistic comparison would be made on daily product output. Using data approaching the end of each run for which the time in hours was listed in Table 1B , the 2L FBR could give 34.2 mg/day and the 10L stirred tank bioreactor only generated 13.2 mg/day. Using average total cell numbers for the same period of time, overall specific productivity was 0.63 mg/billion cells/day for the STR and 3.49 mg/billion cells/day for the FBR. Not only did the FBR contain approximately half the cells in the stirred tank bioreactor, but the average cell specific productivity in the FBR was also 5.5 times higher than that in the STR.

Discussion

Given the superiority of the FBR over the STR in cell numbers and productivity, how is the FBR really different from the STR? Close contact of cells inside the porous microcarriers might promote product formation. Fluidized bed bioreactor configuration made possible a high microcarrier load, thus a large surface area in a compact bioreactor. Isolated sparging and agitation caused less stress to cells. One question remains, if the surface area in the STR increases by increasing microcarrier load or using porous microcarriers, would it enhance the performance of the STR.

The highest Cytodex 1 load reported in the literature was 20 g/L, which was a four times load increase.

However, a 20 g/L Cytodex 1 load was technically difficult to implement without adverse effects, such as agitation and aeration in the dense microcarrier culture. In a bubble-free system, VERO cell density increased with the increase of microcarrier load and reached 9×10^6 cell/ml at 15 g/L Cytodex 3 (Reiter et al., 1990); but it was still much lower than that from our FBR run.

Porous microcarriers can provide an environment of increased surface area and close cell contact. A study on stirred tank bioreactor CHO cell cultures on 4 g/L Cultispher-G, a porous microcarrier, and 3 g/L Cytodex 3 showed the porous microcarrier culture had a 4.5 fold cell density increase to 13.5×10^6 cells/ml (Mignot et al., 1990). However, there was no significant increase in volumetric productivity, even though bubble-free aeration was employed in the cultures to eliminate cell damage from sparging. Further optimization on the porous microcarrier cultures only brought up a 2 fold increase in volumetric productivity. Given the increase in cell density, specific productivity would be lower in the porous microcarrier culture, as was contrary to our comparison between the FBR and the STR. Another culture of CHO cells on a low Cultispher-G load of 0.5 g/L yielded 2.16×10^6 cells/ml (Nikolai and Hu, 1992). In addition, a comprehensive study of embryonic kidney cells (293) in multi-tray, fixed-bed, spinner with Biosilon microcarriers, spinner with Cytodex 3 microcarriers, bioreactor with Cultispher-G porous microcarriers, and Servacel microcarriers showed little difference on system productivity (mg/L/day) (Lazar et al., 1993). A similar result was seen in our CHO cell cultures in the STR with Cytopore 2 (Amersham Pharmacia Biotech) macroporous microcarriers (data not shown). This evidence indicates the performance of a STR might not be able to increase to the level of a FBR by just a boost in total microcarrier surface area.

On the other hand, fluidized bed bioreactors were shown to have high cell density and productivity (Vournakis and Runstadler, 1991). Cell density of BHK cells in a fluidized bed bioreactor system with porous borosilicate glass was indirectly estimated at 6.6×10^7 cells/ml (Kratje et al., 1994). A Verax system one fluidized bed bioreactor with Verax porous microcarriers showed significantly higher cell number and productivity than fixed bed, CSTR, T-flask, stirred and airlift bioreactors (Griffiths et al., 1992). Although the Verax system one fluidized bed bioreactor, verax porous collagen microcarriers and hybridoma cells were used instead in the study, cell density and specific productivity reached 1.5×10^7 cells/ml and 32 mg/billion cells/day respectively.

Equally important is the availability of Cytoline 1 for the FBR. In the prototype of the FBR, Cytopilot (Reiter et al., 1990; Reiter et al., 1991), maximal cell density of CHO cells increased from $3.5 \times 10^7$to 1.8×10^8 cells/ml by changing microcarriers from Cultispher-GLD to prototype Cytoline.

We thus speculate that the superiority of the FBR had to do with FBR configuration. We further speculate that it was the gentle mixing and isolated oxygenation that brought up the advantages of increased surface area and close contact in porous microcarriers to a high cell number and productivity.

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