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Cultivation of HEK 293 cell line and production of a member of the superfamily of G-protein coupled receptors for drug discovery applications using a highly efficient novel bioreactor

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Abstract

A process of producing a receptor in HEK-293 cells used for the drug discovery program at Pfizer Inc. has been successfully developed with a novel BelloCell bioreactor to replace the conventional 2-D cell culturing devices including Cell Factories and roller bottles. A single BelloCell-500 has produced $> 1.4 \times 10^9$ HEK-293 cells, which are equivalent to those produced by 12 roller bottles, with substantially easier operation, single inoculation, less inoculum, less medium consumption and better space utilization. The receptor expression levels are better than those obtained by the traditional process. 3.7 pmoles of radioligandY mg^{-1} protein were attained in the bioreactor compared to 2.3 pmoles of radioligandY mg^{-1} protein in roller bottles. This may be attributed to the three dimensional attachment during cell growth. A 92% cell recovery from the bioreactor has been attained using Acutase or Trypsin treatment followed by four washes. It has been proven to be a viable and efficient device to produce adherent cells and express target components of interest for drug discovery applications.

Introduction

Whole cell or cell extracts used for cellular binding and functional assays are essential for drug discovery programs in pharmaceutical industry. As required, cell culture technology plays an important role to achieve high cell density and high yield of active target cell components.

Currently 2-D bioreactors such as roller bottles, Cell Factory™ and Cell Cube®, etc, have been most commonly and reliably used for adherent cell cultures. However, the operation is usually tedious, time consuming, with inefficient utilization of space and gives low productivity due to relatively small available surface area. Microcarrier technology has been applied successfully in the cultivation of adherent cells and the production of many important biological materials. It has advantages of easy sampling, homogeneous distribution, relatively large surface area and performing perfusion culture with spin filter. However, the microcarrier culture system can usually achieve only limited cell densities of 1.8×10^6 cells ml^{-1}, because of oxygen transfer limitation and other problems such as filter clogging and bead to bead collision effect under high shear agitation (Mendonca and Pereira, 1998).

Above all, the agitation and bead collision often result in serious adverse effect on the activity of cellular target component. Therefore, 2-D device is still the most commonly used device in industry for this type of application. We typically use T-flasks for the earliest seed stage, followed by the propagation stage using Cell Factories™ (or Cell Cubes®) and then proceed to the final cultivation stage using roller bottles which are operated by a Cellmate automatic system in our laboratory. The process not only is lengthy, expensive and labor intensive but also occupies a lot of lab space, even though the labor has been drastically reduced in the roller bottle stage.

Chang and Ho (2003) and Wang et al. (2002) have recently developed a novel disposable bioreactor named BelloCell® with many unique advantages over conventional devices. Hu et al. (2003) has successfully applied this device to cultivate insect cells and produce baculovirus. In this study we have evaluated this device along with our on-going standard process using Cell Factories/roller bottles to cultivate HEK-293 and express a specific G-protein coupled receptor (GPCR), designated as Receptor X. GPCRs participate in a wide range of cell signal pathways and are believed to play a role in a variety of disease states. As a result, they have been attractive targets for new drug discovery.

Materials and methods

Cell/receptor and media

HEK-293. Cells were obtained from ATCC and stored frozen at −150 °C in freeze medium (10% DMSO and 90% heat-inactivated FBS).

Receptor. Receptor X belongs to the Superfamily of G-protein coupled receptors.

Media. The growth medium used consists of 500 ml MEM without glutamine, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 × non-essential amino acids, 25 mM HEPES, 300 μg ml^{-1} G418, 50 μg ml^{-1} zeocin.

Analytical methods

Glucose, glutamine, lactic acid, ammonia and H+ (pH) were measured by Bioprofile 100 Analyzer (Nova Biochemical).

Receptor activity. Receptor binding studies were performed according to standard techniques (Seeger et al., 1995). Briefly, frozen HEK-293/Receptor X cell paste was homogenized in 50 mM Tris buffer pH 7.4 containing 2 mM $MgCl_2$ using a Brinkman Polytron Model PT3000 (setting 15,000 rpm, 15 s). The homogenate was centrifuged for 10 min at 40,000 × *g*. The supernatant was discarded and the pellet was re-suspended in 50 mM Tris buffer pH 7.4 containing 2 mM $MgCl_2$ at a concentration of 3.75 mg ml^{-1}. Incubation was initiated by the addition of tissue homogenate (200 μl) to wells of a 96 well V-bottom polypropylene plate containing varying concentrations of radioligand Y (0.1 – 10 nM) and buffer or unlabeled competitor to determine non-specific binding for a final volume of 250 μl. After 60 min of incubation at room temperature, assay samples were filtered onto Brandel GF/B filters that had been presoaked in 0.5% polyethylenimine and dried, using a Skatron cell harvester and washed with ice-cold 50 mM Tris buffer (pH 7.4). Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin as the standard. Radioactivity was quantified by liquid scintillation counting (Betaplate, Wallac Instruments). Kd and Bmax values were determined using Lundon software.

Cell size, count and viability. For cell size, count and viability of free cells, the standard dye exclusion method was applied using Cedex (Innovatis, Germany). For attached cells, a Crystal violet dye (CVD) nucleus staining method was used. Two disks each were sampled from three different locations of the bottle and placed in one 1.5 ml Eppendorf vial and added 1.0 ml CVD reagent. Vortexing and incubation for over 1 h ensured that the cell membranes ruptured and the nuclei were released from the disks. Then a hemocytometer was used to do the nucleus count, from which the cell count was attained.

Cell releasing agent. Accutase (Innovative Cell Technologies, Inc) in DPBS-0.5 mM EDTA solution or 0.05% trypsin in 0.53 mM EDTA was used.

Cell Factories™/Cellmate and operation

Cell Factories™ (Nunclon® 10 chamber Cell Factory). Each cell factory™ is molded from high quality polystyrene with tissue culture treated

surface. The total growth area is 6320 cm^2. Inoculated the cell factory with 2.5×10^5 viable cells ml^{-1} (total volume 1000 ml) and incubated at 37 °C with 7% CO_2 and 70% humidity for 72 h.

Cellmate® (The Automation Partnership, England)/roller bottle. Roller bottles used were BIOCOAT® (Becton Dickinson) which is PDL coated and has a surface area of 850 cm^2.

Each roller bottle was inoculated with 2.5×10^5 viable cells/ml (total volume 200 ml), purged for 4 s with CO_2, sealed tightly and incubated at 37 °C rotating at 2.5 rpm. After 48 h a complete medium change was performed, purged once again with CO_2 and returned to the incubator for additional 24 h. Cells were harvested at 72 h, by removing growth medium, adding 40 ml Dulbecco's Phosphate Buffered Saline, and scraping cells. The scraped cells were centrifuged at 4 °C and 4000 rpm for 10 min. The saline was decanted and cells were frozen.

BelloCell-500 and operation

A BelloCell-500, a disposable bioreactor bottle, purchased from Cesco Bioengineering Co, consists of a compressible Bello chamber holding 6.5 g of BioNOC matrices and a lower compressible Bello chamber. BioNOC matrices are composed of 100% polyethylene terephthalate non-woven fabric with a treated surface area of about 2000 $cm^2\ g^{-1}$ (see Cesco product literature or www.cescobio.com.tw). As shown in Figure 1, the bottle was mounted onto a control console named BelloStage, which was placed in a CO_2 incubator. The temperature was controlled at 37 °C and CO_2 was controlled at 5% initially and then adjusted later up or down as needed for pH control between 6.8 and 7.4. The bottle was filled with 500 ml medium and inoculated with 2.5×10^5 cell ml^{-1}. After inoculation, the BelloStage controlled the up/down speed at 2.0 mm s^{-1}. The top holding time of 20 s assured the cell attachment to the matrices for the first 2–5 h, then the up/down speed was reduced to 1.5 mm s^{-1}, the down holding time was changed to 60 s and the top holding time was suspended to allow sufficient time for oxygen transfer from the air to the cells. During the entire run the substrate and metabolite concentrations including glucose, glutamine, ammonia and lactate were monitored once every day. The medium was replaced or glucose concentrate was added as needed to maintain a glucose level of 0.2–1.5gl^{-1} and/or lactic acid or ammonia at $<1.5gl^{-1}$. The cell density was periodically measured by a CVD nucleus staining method with disk samples taken from the bottle. As the cell density reached the desired level ($>3 \times 10^6$ cells ml^{-1}), the run was terminated and bottle removed for processing to release the cells from the matrices.

Cell releasing agent and cell detachment method

The bottle was demounted from BelloStage and the medium was discarded. Then the bottle was pressed and locked in the compressed position with a bottom cap. The matrices were washed with 300 ml PBS–EDTA (5 mM) solution twice before 300 ml of an Accutase solution (in DPBS–0.5 mM EDTA) or 300 ml of a 0.05% trypsin solution (in 0.53 mM EDTA) was added. It was then placed in

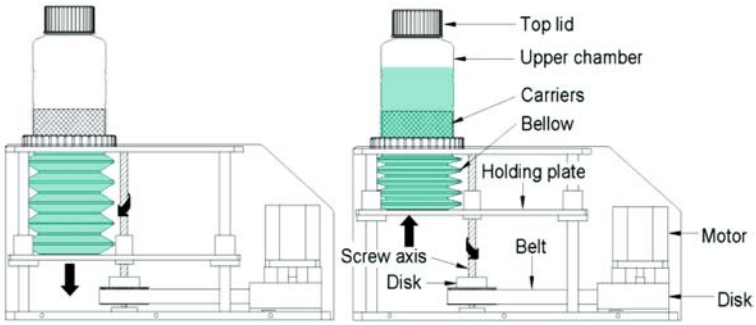

Figure 1. Schematic diagram of BelloCell® and BelloStage®.

the incubator for 10–20 min after a gentle mixing, followed by 15 min of incubation after the releasing agent was decanted. Washing solution and releasing agent were saved to recover possibly detached cells and to reuse the releasing agent. The empty bottle was then tapped against a soft object vigorously around the matrices for 2–3 min. The bottom cup was removed and 450 ml of washing buffer solution was added immediately and the bottle was placed in the BelloStage. A setting was used of 2 mm s^{-1} up and down speed for 3 min without holding time. The solution was poured out and saved for cell harvesting. The washing step was repeated three to four times until $<5\%$ cells remained in the wash.

Results and discussion

Cell growth in Cell Factories^{TM} and roller bottles

At 72 h the cells reached $>80\%$ of confluence on the surface of Cell Factories^{TM}. The cells were then trypsinized with 0.05% trypsin and a cell density of 1.1×10^6 cells ml^{-1} or a total cell count of 1.1×10^9 was obtained. The cells were then inoculated to each 850 cm^2 (200 ml medium) roller bottles at a cell density of 2.5×10^5 ml^{-1}. At 80 h, the roller bottles were harvested and a cell density of 5.75×10^5 cells ml^{-1} or 1.15×10^8 cells in total were obtained per bottle. The cell viability was $>95\%$ and receptor Bmax was 2.3 pmol mg^{-1} proteins.

Cell growth in BelloCell®

In parallel to the experiment mentioned above using the standard Cell Factories^{TM}/roller bottles, the seed obtained from the same lot of T-flasks was used to inoculate a BelloCell at a cell density of 2.5×10^5 cells ml^{-1} each (500 ml medium). After 12 days of cultivation and five medium changes, the run was terminated and cells were harvested via detachment. The total count of the recovered cells was 1.29×10^9 (= wet cell weight of 3.3 g) while the total cell count was 1.4×10^9. This indicates that a 92% detachment efficiency was achieved using an Accutase solution. The cell viability was 90% and receptor Bmax was 3.7 pmol mg^{-1} protein, which was higher than 2.3 pmol/mg protein obtained by 2-D growth in a roller bottle. This is in agreement with the finding by Luo and Yang (2004) that hybridoma cells grown in 3-D matrices had higher monoclonal antibody (Mab) productivity than in the 2-D culture system.

Figure 2 shows the profiles of glucose concentration, glucose uptake rates (GUR) and total cell count during the course of the culture. Figures 3 and 4 show the profiles of pH, glutamine and metabolites including lactate and ammonia. The data show that lactate and ammonia were kept below 1.2gl^{-1} and glutamine above 0.5 mmol l^{-1} by changing for fresh medium five times during the run. The first change did not occur until the fourth day because of a slow initial growth phase. To

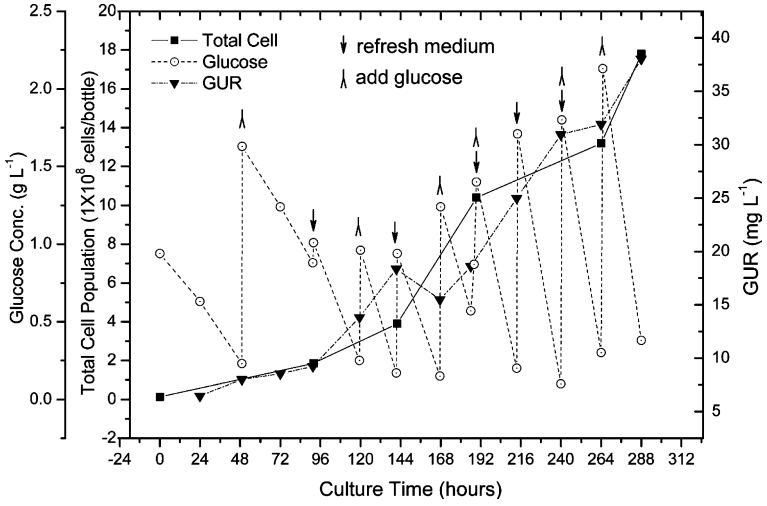

Figure 2. Cell growth, glucose concentration, and GUR of a 293 culture in the Bellocell® culture system.

avoid the glucose from depletion, concentrated glucose solution was added as needed. In this study, the glucose concentration was maintained between 0.1 and 1.5gl^{-1}. No optimum concentration was yet determined for maximizing the receptor X expression. Table 1 summarizes the results of the cultivation of HEK-293 expressing receptor X in the BelloCell reactor and standard roller bottles. Results showed that one BelloCell® was equivalent to 12 roller bottles in term of cell production and to 20 roller bottles, in terms of receptor expression. The cell density based on the surface area was less in the BelloCell® than in roller bottles (1.08 vs. 1.35×10^5 cells cm^{-2}). This indicated that the surface of matrices was not yet fully covered and it may support further cell growth.

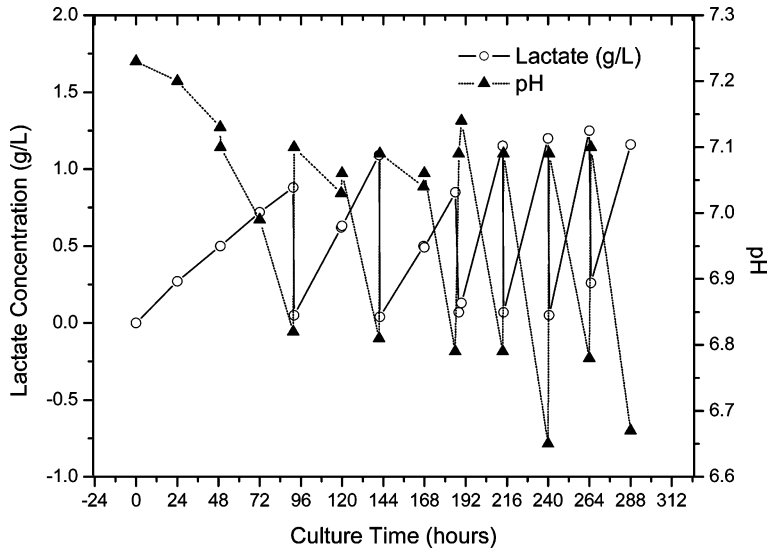

Figure 3. Lactate concentration and pH.

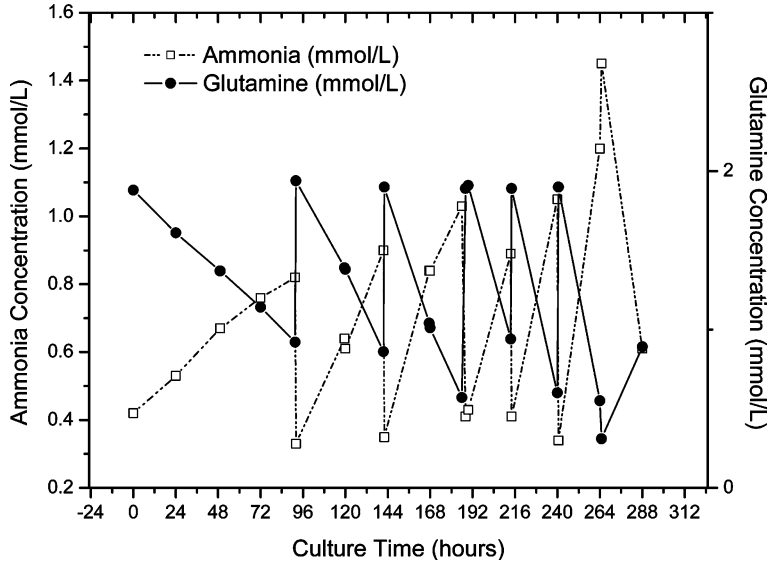

Figure 4. Glutamine and ammonia concentrations.

Table 1. Comparison of HEK293 Cell growth and Receptor X production in Cell Factory®/20 roller bottles and BelloCell-500 Bioreactor.

	Cell Factory/20 Roller bottles	BelloCell/BioNOC matrices
Description	One 10 layer/cell factory/1000 ml medium/6320 cm^2 surface area 20 roller bottles/850 cm^2 surface area each/200 ml medium each	1000 ml bottle/500 ml medium/100 cm^3 (6.5 g) carriers
Total surface area (cm^2)	17,000	13,000
Mode of operation	Batch	Semi-batch
Medium	MEM/10% FBS	MEM/10% FBS
Inoc density $\times$ 10^5 (cells ml^{-1})	2.5	2.5
Inoc density $\times$ 10^4 (cells cm^{-2})	1.47	0.96
Total Inoc $\times$ 10^8 (cell#)	2.5	1.25
Maximum cell density $\times$ 10^6 (cells ml^{-1} of medium)[a]	0.58	2.8
Cell density $\times$ 10^5 (cells cm^{-2} surface area)	1.35	1.08
Total cell number $\times$ 10^9	2.3	1.4
Total cells increased, fold	9.2	11.2
Cell number per bottle $\times$ 10^9	0.115	1.4
Total medium used, l	4	3
Run time, day	6.5	12
Specific Receptor X activity (p moles radioligand Y mg^{-1} protein)	2.3	3.7
Total Receptor X activity (p moles radioligand Y)	1113	1090
Receptor X activity per bottle	55.65	1090

[a]Maximum cell density achieved in the experiments conducted.

The GUR curve at harvest shown in Figure 2 is still increasing since the cells have not yet reached the maximum growth space. The growth rate was apparently faster on the 2D than 3D surface (9.2-fold increase in 6.5 days vs. 11.2-fold in 12 days). Most interestingly, the specific activity of the receptor X was significantly greater in 3D than in 2D cultures. Recently Luo and Yang (2004) has shown a similar result of producing Mab in a 3D culture with hybridoma cells. Figure 2 suggests that this may be attributed to the slow initial growth rate in 3D space with much less cell density based on the surface area (0.96 vs. 1.47×10^4 cells cm^{-2}). It was also caused by slower rate toward the end of the run as the cells substantially filled the space of the matrices. Each BelloStage® can hold 4 BelloCells and only occupies a small space of the incubator. The total medium required to produce the same amount of receptor was 31% less in the BelloCell® than in the Cell Factories™ /roller bottles. However, there was no effort to maximize the medium utilization efficiency in this study. Above all, the labor saving, such as smaller inoculum size and only one BelloCell to operate, was unparalleled compared to roller bottles or other methods for producing the same amount of product. In a 2D production process it is required to optimize several operating conditions such as inoculum size, roller bottle rotation speed and medium volume to obtain the desired cell mass. In our experience, the range of each of the operating variables seems relatively wider in comparing the BelloCell® system to the other systems. Therefore it does not require a lengthy process development to derive optimal conditions before proceeding to the production stage.

Cell detachment

Two cell releasing agents were examined in this study. The result indicates that both Accutase and

trypsin can equally detach the cells at cell recovery efficiencies of about 92% with only one treatment and four washes. No further recovery was attempted. Based on previous experience, trypsin may damage some other receptors but not the specific receptor in this case.

Conclusion

BelloCell® is a novel disposable bioreactor requiring no air sparging, no agitation, no pumping and provides a low shear environment and an easy means for stable cell immobilization. It has been successfully utilized to grow HEK-293 and produce active receptor X for our drug discovery program. A high cell yield of 1.4×10^9 cells and 3.7 pmol mg^{-1} protein of Receptor X per bottle were attained using 3 l medium. This was equivalent to those produced in 12 roller bottles with our standard process using Cell Factories/roller bottles in term of cell mass or 20 in terms of receptor X. Moreover, the medium utilization efficiency for BelloCell can be higher than that for the standard process. Above all the space and labor savings were unmatchable. The detachment efficiency is not perfect and remains for further improvement. We are convinced that the BelloCell® system can be of benefit to replace or supplement our standard process for the adherent cell cultures used in our drug discovery application.

The system has also been shown equally effective and efficient for many other adherent and suspension cell cultures (Chang and Ho, 2003). The application remains to be further explored and developed.

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