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January 2014

Applications

Technical application notes on cell culture and fermentation

Table of Contents

Cell culture

Fermentation

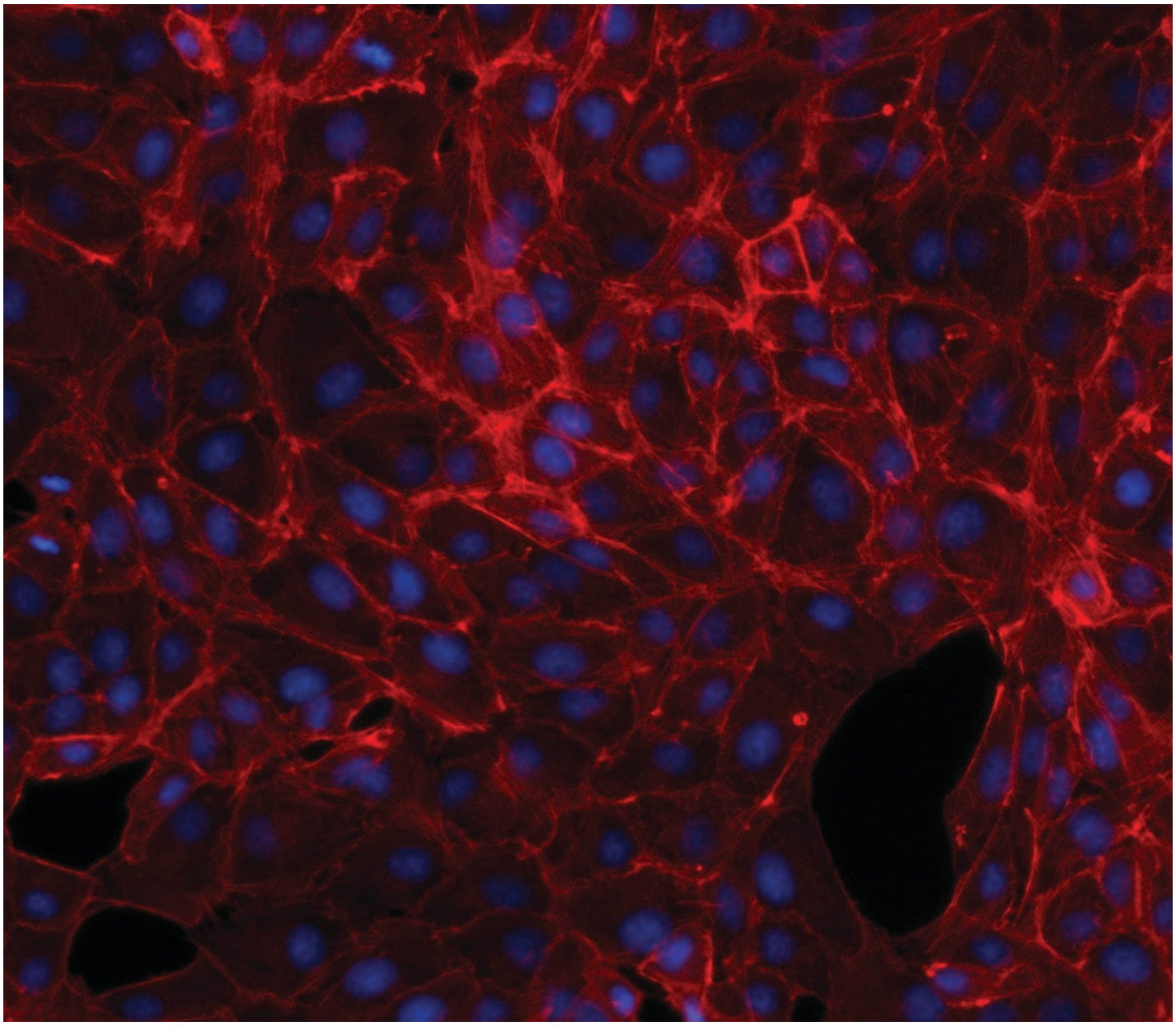
Title	Cell line	Product	Doc.	Page
Continuous Separation of *E. coli* Fermentation Broth Using a CEPA® LE Laboratory Centrifuge System	*E. coli*	CEPA LE Centrifuge	319	65
Amino Acid Fermentation: Evaluation of Scale-Down Capabilities Using DASbox Mini Bioreactors	*E. coli*	DASbox® Bioreactor	290	67
Scalability of Parallel *E. coli* Fermentations in BioBLU® f Single-Use Bioreactors	*E. coli*	BioBLU® 0.3f Single-Use Vessel	293	70
High Cell Density *E. coli* Fermentation using DASGIP® Parallel Bioreactor Systems	*E. coli*	DASGIP® Parallel Bioreactor System	294	74
Fed-Batch Bioethanol Production Process using the New Brunswick™ BioFlo®/CelliGen® 115	Yeast	BioFlo®/CelliGen® 115 Fermentor/Bioreactor	318	78
Anaerobic Yeast Fermentation for the Production of Ethanol in a New Brunswick™ BioFlo® 310 Fermentor	Yeast	BioFlo® 310 Fermentor	311	82
Using Redox Measurements to Control Anaerobic Yeast Fermentation in a New Brunswick™ BioFlo® 310 Fermentor	Yeast	BioFlo® 310 Fermentor	317	85

External application notes and publications

Title	Cell line	Product	Doc.	Page
Hybridoma and CHO Cell Culture Using the New Brunswick™ S41i, An Environmentally-Friendly Low Emission Incubator Shaker, *Bioprocessing J.*, Fall 2012	CHO, Hybridoma	New Brunswick™ S41i CO_2 Incubator Shaker	J113	89
A Comparative Bioreactor Vessel Study: Conventional Reusable Glass & Single-Use Disposables for the Production of Alkaline Phasphotase, *Bioprocessing J.*, Spring 2013	CHO	CelliGen® BLU Single-Use Bioreactor System	J121	93
Expansion of MRC-5 Cells on Microcarriers via Serial Passage in Stirred Vessels	MRC5	CelliGen® 310 Bioreactor	006B	102
Expansion of Vero Cells on SoloHill Hillex II Microcarriers via Serial Passage in Stirred Vessels	Vero	CelliGen® 310 Bioreactor	003B	108
Microcarrier-Based Expansion of Adipose-Derived Mesenchymal Stem Cells in Shake Flasks, *Bioprocessing J.*, Winter 2013	Stem Cell	S41i CO_2 Incubator Shaker	J124	114

Other

Title	Product	Doc.	Page
Isobutanol from Renewable Feedstock - Process Optimization by Integration of Mass Spectrometry to two 8-fold DASGIP® Parallel Bioreactor Systems	DASGIP® Parallel Bioreactor System	295	122
Vero Cell-based Vaccine Production: Rabies and Influenza Cell lines, Media and Bioreactor Options	CelliGen® 310 Bioreactor		126



Cell Culture

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APPLICATION NOTE No. 331 I October 2013

Hypoxic Cell Culture in the New Brunswick™ Galaxy® 170R Incubator: Normal Growth, Morphological Changes

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Abstract

New Brunswick Galaxy 170 series incubators have a wide array of options that allow for not only CO_2 and temperature control, but also for O_2 concentration control which can be used to create a hypoxic internal environment. Cancer cells are known to be resistant to the toxicity of O_2 deprivation. To demonstrate the low O_2 capability of this system, we grew prostate cancer (LNCaP) cells in 2 % O_2 and observed their growth and morphology over time. In contrast to the effects of hypoxia on normal cells, LNCaP cells were able to grow normally and displayed morphological changes.

Introduction

Oxygen is a critical regulator of cellular homeostasis and as such, oxygen deprivation is lethal to normal cells. In contrast to normal tissues, solid tumors often have regions of significantly reduced oxygenation due to an inconsistent and disorganized blood supply at the center of the tumor. Tumor O_2 deprivation, or hypoxia, can result in chemotherapeutic drug resistance and gene expression changes in cancer cells. Decades of cancer research have established that tumor cell growth, survival, motility, the recruitment of blood vessels (angiogenesis), energy metabolism, and cellular differentiation are affected by hypoxia[3]. Patients with tumors displaying high levels of hypoxia often have poor prognoses and negative clinical outcomes[4]. This effect has been documented for many deep tissue tumors such as breast, prostate, ovarian and uterine cancer[2].

A number of human tumor cell lines have been used to model the effects of hypoxia in an effort to target these drug-resistant cells with new therapeutics. One such cell line, LNCaP, was derived from a bone metastasis from a terminal prostate cancer patient. These cells have been widely used in prostate cancer drug discovery and are uniquely sensitive to androgen (e.g. testosterone and dihydrotestosterone) levels. Danza and colleagues have shown that LNCaP cells previously stimulated with androgens before growth under hypoxic conditions (2 % O_2) grow faster than cells maintained in normoxia (20.9 % O_2)[1]. We chose to use a simplified version of this model to establish a protocol for setting up low-O_2 cell culture in a standard tissue culture laboratory. Since standard laboratories do not have access to controlled substances, we did not stimulate the cells with androgen before growth in hypoxic conditions.

Figure 1: The New Brunswick Galaxy 170R CO_2 incubator

We show here that low-O_2 cell culture conditions are easy to establish using the New Brunswick Galaxy 170R incubator with 1 - 19 % O_2 control. LNCaP cells grew well in both normoxia and hypoxia in this system and morphological changes were noted in low O_2 conditions. With available O_2 control from 0.1 - 19 %, the Galaxy 170R incubator provides an excellent environment with which to culture cells in a range of gas conditions.

Table 1: Materials, media and cells

Material	Supplier	Order no.
RPMI-1640	ATCC®	30-2001™
Penicillin-Streptomycin 10,000 U/mL	Life Technologies®	15140-122
Fetal bovine serum (FBS)	Life Technologies®	10437-028
1x Dulbecco's Phosphate buffered saline	Life Technologies®	14190-144
Trypsin-EDTA	HyClone®	SV30031.01
16 % Paraformaldehyde (w/v) methanol-free	Pierce™	28906
rhodamine-conjugated phalloidin	Molecular Probes®	R415
NucBlue® Fixed Cell ReadyProbes®	Molecular Probes®	R37606
T75 T-flasks*	Eppendorf	0030 711.106
6-well dishes*	Eppendorf	0030 720.105

* Currently available in China, India and Italy only

Materials and Methods

Consumable Materials

Table 1 details the consumable reagents and materials that were used in this study.

Cell Culture

Cell culture was carried out using two Galaxy 170 incubators (Eppendorf). First, the Galaxy 170R with High temperature disinfection, 4 split inner doors and 1 - 19 % O_2 control was used for culture in hypoxic conditions (Figure 1). Second, the Galaxy 170S with High temperature disinfection and 4 split inner doors was used for normoxic conditions. Setpoints on both units for temperature and CO_2 concentration were 37 °C and 5 %, respectively. For hypoxic conditions, the Galaxy 170R was set at 2 % O_2 and allowed to stabilize at setpoint for 72 h before cells were introduced into the incubator. To monitor O_2 concentration, the Galaxy 170R was connected to a computer using a RS-232/RS-422 converter (Eppendorf). Using BioCommand® SFI (Eppendorf), O_2, temperature and CO_2 process values were tracked for the course of the experiment. The O_2 concentration inside the Galaxy 170R never exceeded 2.1 %.

LNCaP clone FGC cells were acquired from the American Type Culture Collection® (ATCC®, USA #CRL-1740™) and were grown in RPMI medium supplemented with 1 % Penicillin-Streptomycin and 10 % FBS. Cells were grown in T75 flasks until 80 % confluency. At target density, the cells were disassociated from the surface by trypsinization and neutralization with FBS. After pelleting by centrifugation at 120 x g for 3 min, cells were resuspended in complete growth medium and counted using a Vi-CELL® automated cell counter (Vi-CELL XR; Beckman Coulter, Inc., USA #731050). Cells were seeded in quadruplicate 6-well dishes at a density of 300,000 cells/well and two plates were placed in the same position in both the normoxia and hypoxia incubators.

According to the protocol established by Danza and colleagues, 3 wells from both hypoxic and normoxic conditions were counted every 3 days for 9 days, generating cell concentrations per well for days 3, 6 and 9[1]. At each timepoint, the wells were also photographed using phase contrast microscopy on an Olympus® IX51 inverted microscope equipped with an Infinity2 CCD camera (Lumenera®, Canada) . The data were analyzed and statistical tests including a 2-way analysis of variance (ANOVA) were performed in Microsoft® Excel®.

Fluorescent Marker Staining

After 6 days in normoxia or hypoxia, two wells were fixed in 4 % paraformaldehyde and stained using the phallotoxin, phalloidin (which selectively binds f-actin) and the double-stranded DNA intercalator, 4',6-diamidino-2-phenylindole (DAPI; NucBlue® Fixed Cell ReadyProbes® reagent). Staining was performed exactly as the manufacturer recommended. The cells were photographed using an EVOS® LED florescence microscopy system (Life Technologies, USA #AMF4300).

Results and Discussion

LNCaP Cells Grow Normally Under Hypoxic Conditions

As the graph in Figure 2 details, LNCaP cells grown at 2 % O_2 showed growth dynamics indistinguishable from those grown in normal atmospheric O_2 (20.9 %). It is possible that we did not see the previously published growth bias in hypoxia because we did not stimulate the cells with androgens prior to exposure to O_2 deprivation. Importantly, no differences were seen in the attachment of the cells in hypoxia, as evidenced by the phase contrast micrographs in Figure 3.

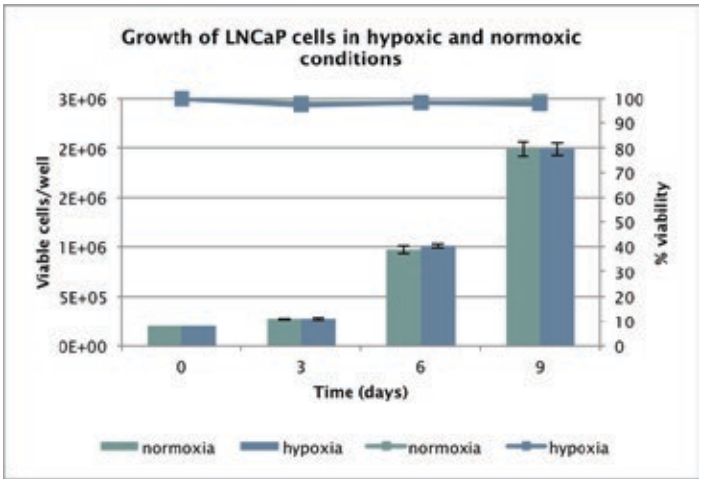

Figure 2: Growth and viability of LNCaP cells grown in hypoxia and normoxia. This graph shows the viable cell density and % viable cells in each well. Each data point represents the mean of 3 wells. Error bars indicate standard error of the mean. ANOVA analysis revealed that no significant difference was observed between the two growth conditions.

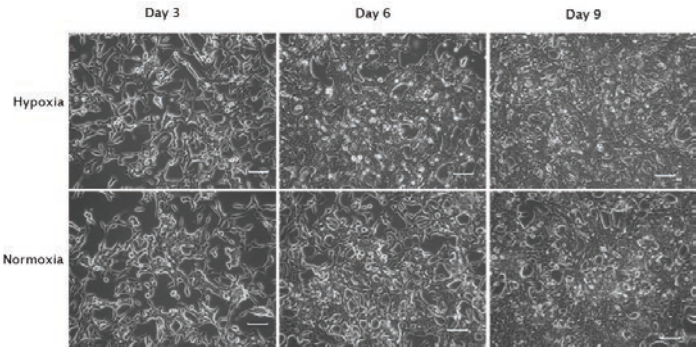

Figure 3: LNCaP cells grown in hypoxic conditions display normal attachment and homogenous growth. In the top row, LNCaP cells grown in 2 % O_2 show similar density as compared to those grown in atmospheric O_2 (bottom row). Photos were taken at 100 x magnification and the scale bar in each panel represents 100 µm.

Interestingly, we noted a significant morphology difference between the cells grown in hypoxic and normoxic conditions. Grown in atmospheric O_2 conditions, LNCaP cells are known to form large clusters or colonies of cells wherein they display no contact inhibition.

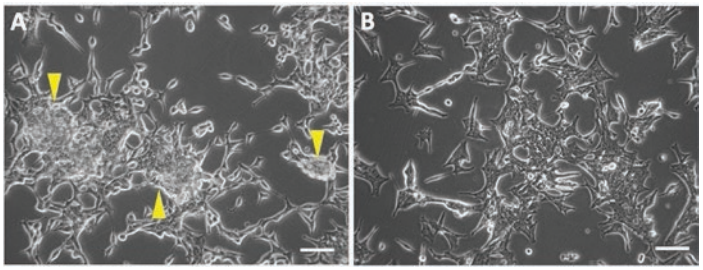

Figure 4: Growth pattern of LNCaP cells in normoxic (A) vs. hypoxic (B) conditions. Grown in atmospheric O_2 conditions, LNCaP cells make large 3-dimensional clusters (yellow arrowheads) whereas in hypoxic conditions, cells appear more spread out and do not form large colonies. Photos were collected at 100 x magnification and the scale bar in each panel represents 100 µm.

These colonies are themselves loosely attached to the substrate and can become quite large (see yellow arrowheads in Figure 4A). In contrast, LNCaP cells grown in 2 % O_2 do not form such 3-dimensional structures and tend to cover the substrate and grow in more of a packed monolayer (Figure 4B). The cells also appear more spread out and seem to display tighter adherence as evidenced by the length of time required for the cells to detach during enzymatic disassociation. To document these morphological changes, we stained cells grown in both O_2 concentrations with rhodamine-conjugated phalloidin and DAPI to visualize the actin cytoskeleton (red, Figure 5) and the cell nucleus (blue, Figure 5). As documented in Figure 5, large clusters of cells are observed in normoxic conditions while a flatter monolayer is seen in hypoxic conditions. Furthermore, the cytoplasm-to-nucleus ratio in the cells grown in 2 % O_2 appears to be larger than in those grown in 20.9 % O_2, although this observation was not quantified. We conclude that morphological changes have occurred in LNCaP cells grown with O_2 deprivation that result in diminished colony forming behavior and a flatter appearance.

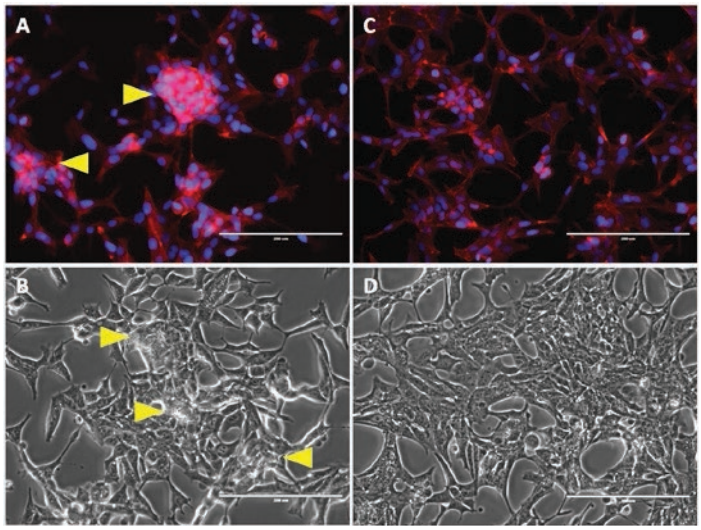

Figure 5: Growth characteristics of cells in hypoxia and normoxia. Panels A (fluorescence) and B (phase contrast) show examples of cells grown in 20.9 % O_2 for 3 days. The yellow arrowheads denote areas where 3-dimensional colonies are observed, as evidenced by the overlapping cell nuclei (blue) and high concentration of actin (red). Panels C and D show monolayers grown in 2 % O_2 where 3-dimensional growth is not seen. In addition, cells grown in hypoxia appear flatter and with larger cytoplasmic volume. The images in this figure were collected at 100 x using an EVOS LED imaging system; scale bars represent 200 µm.

Conclusion

We have shown that LNCaP cells grown in hypoxic conditions display normal growth as compared to those grown in atmospheric O_2 concentrations. Growth in 2 % O_2 also resulted in morphological changes and changes in growth characteristics such as colony formation. The ease of setup and the tight O_2 concentration control displayed by the Galaxy 170R incubator provided the ideal conditions for this experiment.

This study is a demonstration of the low O_2 feature that is available on the Galaxy 170 series incubators. Experimental conditions including O_2 concentration have not been optimized.

References

[1] Danza, G. D. (2011). Notch signaling modulates hypoxia-induced neuroendocrine differentiation of human prostate cancer cells. *Mol Cancer Res*, 230-238.

[2] Hockel, M. V. (2001). Tumor Hypoxia: Definitions and Current Clinical, Biologic, and Molecular Aspects. *J Natl Cancer Inst*, 93(4), 266-276.

[3] Kim, Y. L. (2009). Hypoxic Tumor Microenvironment and Cancer Cell Differentiation. *Curr Mol Med*, 9(4), 425-434.

[4] Wilson, W. H. (2011). Targeting hypoxia in cancer therapy. *Nature Reviews*, 11, 393-410.

Ordering Information

Description	International order no.	N. America order no.
Galaxy® 170R With high temp disinfection 1 - 19 % O_2 control Split inner doors, 4	170R230120_ B04	170R120120_ B04
Galaxy® 170S With high temp disinfection	CO170S-230-1000	CO170S-120-1000
8-port RS-232 to USB converter	P0460-7750	P0460-7750
BioCommand® SFI	M1291-1001	M1291-1001
Easypet® 3	4430 000.026	4430 000.026
Centrifuge 5810 R With 4 x 500 mL rotor, 120 V	022628179	022627082

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APPLICATION NOTE No. 320 I November 2013

Pitched-Blade vs. Spin Filter vs. Packed-bed Basket: CHO Cell Culture Comparison

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Abstract

In the following application note, the pitched-blade impeller, the spin filter impeller and the packed-bed basket impeller are discussed, highlighting the uses and advantages for each type. Then examples of actual CHO cell cultures are given for each impeller type; showing the perfusion capability when using the spin filter or packed-bed basket impeller and the resulting higher cell densities over the pitched-blade impeller.

Introduction

In the world of bioprocess, there are many tools and methods that can be used to culture mammalian cells, each with their own strengths, weaknesses and purposes. One of the most critical decisions that is made before a bioprocess system purchase is which impeller type is ideal for a particular cell culture. In this application note, three impeller types were compared using CHO cell culture: The pitched-blade impeller, the spin filter with marine-blade impeller and the packed-bed basket impeller. All experiments were performed using a New Brunswick™ CelliGen® 310 benchtop bioreactor.

The pitched-blade impeller has three flat blades set at approximately a 45 ° angle which produces both axial and radial flow. Right handed or left handed blades are options that can be considered depending on which direction you would like your axial flow. Pitched-blade impellers are low shear impellers, designed to gently mix both suspension cells and cells attached to a microcarrier. Typically, these impellers are used for mammalian, insect or other shear-sensitive cell lines, but have also been used in highly viscous fermentation cultures with bacteria and fungi, as well as some biofuel processes. When using a pitched-blade impeller, a culture is typically grown in a batch-style run (no media is added or removed) or fed-batch-style run (a culture is started at a lower working volume and more media is added later during the run). A perfusion-style run (fresh media is continuously added and old media is removed) is possible, however, unless a filtering device is attached with this system to prevent the cells from being removed, cells will be depleted with the harvested ("waste") media.

Figure 1: Pitched-blade impeller (left) and spin filter with marine blade (right)

A spin filter is a cylinder-shaped cage that spins with the impeller shaft and is covered with a screen designed to prevent cells from being collected with the waste media. Typically, underneath the spin filter, a marine blade is attached to the impeller shaft. When attached to the vessel, media is added so it covers the spin filter almost to its top, with a specially designed harvest tube that can reach the media inside the spin filter. When used, this device can keep cells in the vessel while old media is perfused out from inside of the spin filter. The spin filter is offered with two screen sizes, 10 µm openings for suspension cultures and 75 µm openings for microcarrier cultures. The marine-blade impeller attached underneath the spin filter provides gentle mixing but, due to its unidirectional flow, has a lower KLa than the pitched-blade. The spin filter is perfect for cultures that secrete proteins or compounds of interest since the desired product can be collected with the media while the cells are left to continue to produce. This also helps with downstream processing as cells will not have to be removed

with centrifugation or filtration. It should be noted that at very high density cultures the spin filter may eventually get clogged with cell debris and require cleaning, which can limit run time.

Figure 2: Packed-bed basket with Fibra-Cel disks

The packed-bed basket impeller, combined with Fibra-Cel® disks, is a system perfect for manufacturing high-yield secreted products from both attachment and suspension cultures with perfusion. Fibra-Cel is a solid supported fiber-mesh matrix microcarrier used predominantly for secreted products with perfusion. Fibra-Cel allows for long-term, high-density cultures without the risk of clogging. Fibra-Cel can be used for both anchorage-dependent cultures and suspension cultures due to its electrostatically-treated material and woven nature that traps the cells in a single step within 15 - 60 minutes (no need to stop agitation). The basket consists of two horizontally positioned, perforated metal screens that isolate a section in the interior of the vessel that is filled with Fibra-Cel. The impeller consists of a hollow tube (draft tube) with three smaller discharge tubes radiating from the top. When media is filled over the three tubes at the top of the impeller and it is spun, the centrifugal force exerted on the media forces out the liquid, causing a gentle suction at the bottom of the impeller, which brings media from the bottom of the vessel to the top. The media then gently flows through the Fibra-Cel packed-bed from the top to the bottom. Gases are sparged into the vessel through the central draft tube; this method oxygenates the media but prevents bubbles from interacting with the cells growing inside the Fibra-Cel packed-bed, thus, preventing bubble shear.

Eppendorf also offers other impellers for various bioprocess needs. Some impellers offered but not explored in this application note include the Rushton-type impellers; which are ideal for fermentation cultures with bacteria, yeast and fungi that require higher dissolved oxygen level (oxygen transfer rate) but are not sensitive to mechanical shearing damage; and the Cell-Lift impeller; which is an ultra-low-shear impeller that provides uniform circulation for microcarrier cultures and a bubble free environment for the cells.

Materials and methods

Table 1: Materials, media and cells

Material	Supplier	Catalog no.
CelliGen® 310 Control Station	Eppendorf	See ordering information, page 6
4 TMFC (0 - 1 SLPM)	Eppendorf	
2.5 L water jacketed vessel (with motor)	Eppendorf	
2.5 L pH/DO Sensor Kit (with cables)	Eppendorf	
2.5 L Pitched-Blade Impeller Kit	Eppendorf	
2.5 L Spin Filter Impeller Kit (10 µm)	Eppendorf	
2.5 L Basket Impeller Kit	Eppendorf	
YSI 2700 Select™ analyzer	YSI® Life Science	2700D
Vi-CELL® XR	Beckman Coulter®	731050
Media and cells		
Fibra-Cel® Disks	Eppendorf	M1292-9988
Freestyle® CHO-S	Life Technologies®	R800-70
CD CHO media	Gibco®	10743
L-glutamine	JRH Biosciences®	90114
Penicillin/streptomycin 100x	Gibco®	15140-122
D-(+)-Glucose	Sigma-Aldrich®	G5146
Sodium Bicarbonate	Thermo Fisher Scientific® Chemical	S631-3

Bioreactor conditions

Figure 3: CelliGen 310 with packed-bed basket impeller

During all three of the following CHO bioprocess examples, a CelliGen 310 Bioreactor with four 0-1 Standard Liters Per Minute (SLPM) Thermal Mass Flow Controllers (TMFC) were used. A TMFC is a device that monitors specific gas flow and is used by the cabinet to automatically control the gases flowing into the vessel. The vessel was a 2.5 L glass, water-jacketed vessel with a magnetic drive motor. The water jacket provides uniform temperature distribution with gentle heating and cooling for the culture while the magnetic drive motor provides a sterile vessel environment. All three culture types utilized 3 gas mixing (Air, O_2 and CO_2) for DO and pH control with a base addition (Pump 2, 0.3 M sodium bicarbonate solution). Table 2 shows all of the settings for each loop used during all three runs. Both the DO and pH were controlled using the cascade parameters seen in Tables 3 and 4.

Table 2: Loop settings

Loop	Setpoint
Agitation	See each example
Temperature	37 °C
pH-1	7.20 (Deadband 0.05)
pH-2	Off
DO-1	50
DO-2	Off
Air	Auto
O_2	Auto
Gs3Flo	Off
CO_2	Auto

Table 3: DO-1 cascade

	Start setpoint	@ DO start output %	End setpoint	@ DO end output %
Air	0.0	0.0	0.5	60
O_2	0.0	10	1	100

Table 4: pH-1 cascade

	Start setpoint	@ pH start output %	End setpoint	@ pH end output %
Pump 2	0.0	0.0	100	100
CO_2	0.0	0.0	0.3	-50

Cells were grown in CD CHO media supplemented with 8 mM of L-glutamine and 1 % penicillin/streptomycin and kept at a total working volume of ~1.6 L. Each vessel was inoculated at identical densities of 0.3 x 10^6 cells/mL. Glucose was added to the perfusion media as needed. Cell counts were performed on the pitched-blade and spin filter reactors using a Vi-CELL®. A YSI® 2700 Biochemical Analyzer was used to determine glucose and lactate concentrations for all three reactors.

Results

Pitched-blade culture

The pitched-blade reactor was run at an agitation speed of 80 rpm. It was cultured as a batch-style reactor so no media was added or removed throughout the process run. As you can see from Figure 4, viable cell concentration continued to rise until all of the glucose was consumed from the media at which point the cell viability began to drop. Lactate levels increased until the drop in glucose concentrations caused a shift in cellular metabolism which caused the cells to consume lactate.

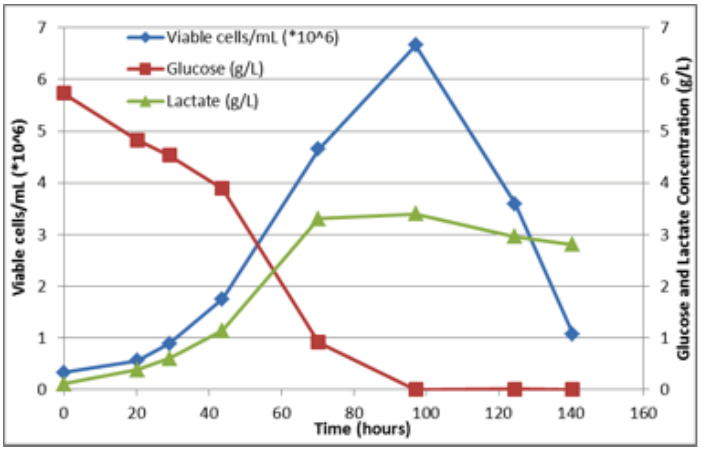


Figure 4: The pitched-blade viable cell concentration and glucose and lactate concentrations. Viable cell concentration begins to decrease when all the glucose is consumed in the vessel due to it being a batch-style run.

Spin filter culture

The spin filter reactor was run at an agitation speed of 100 rpm with a 10 µm filter screen. With the spin filter, the culture was run using continuous perfusion. One of the CelliGen 310 cabinet pumps was calibrated and run at varying rates of input as needed to maintain a glucose level above 1 g/L and to keep waste metabolites low. Another pump was cascaded to a level sensor so media was automatically removed from the vessel anytime it reached a volume over 1.6 L. Since the media being removed was from inside the spin filter, the cells were retained outside of the 10 µm spin filter cage. Figure 5 shows that the cells achieved a high density and viability with perfusion using the spin filter. Although the spin filter can achieve 3X, the run ended due to the high cell concentration eventually clogging the spin filter.

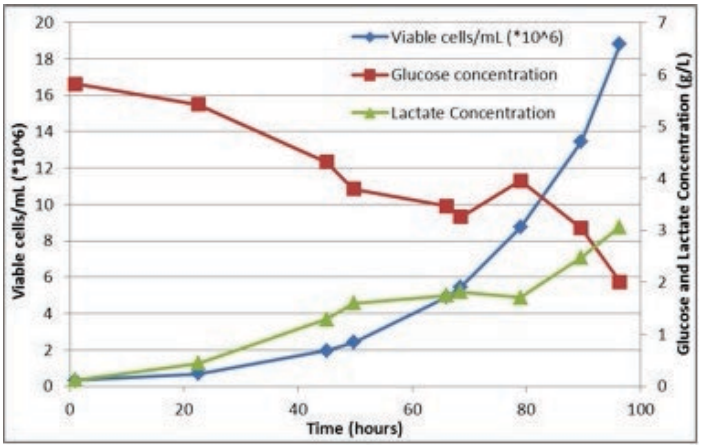
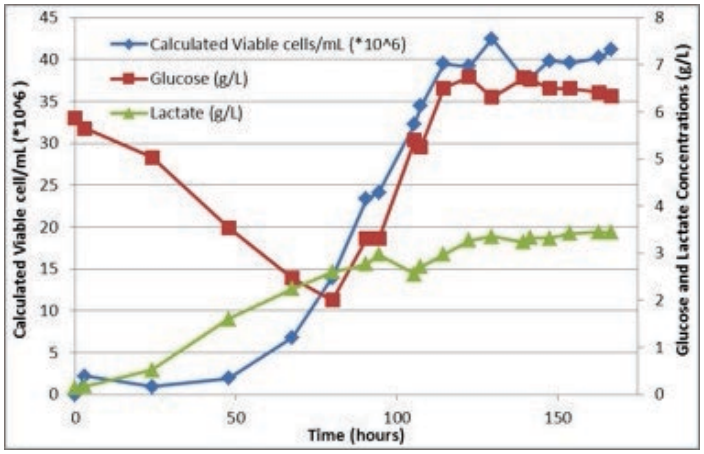


Figure 5: The spin filter viable cell concentration and glucose and lactate concentrations. Perfusion prevented glucose from being totally consumed from the vessel and lactate levels from getting too high.

Packed-bed basket culture

The packed-bed basket impeller was run at an agitation speed of 100 rpm and the basket was filled with 70 g of Fibra-Cel disks. This culture, like the spin filter, was run using continuous perfusion using the same methods as described above, except that media was removed from a normal harvest tube, not from inside of the basket. Since all of the cells were trapped in the Fibra-Cel disks and could not be counted using standard methods, the cell number was determined using the amount of glucose consumption. Due to glucose levels being too high during the run, the cells transitioned from a log phase to stationary phase resulting in a plateau in cell growth, as seen in Figure 6. Higher cell numbers were expected.

Figure 6: Packed-Bed Basket results showing calculated viable cells as well as glucose and lactate concentrations. Perfusion prevented



glucose from being totally consumed from the vessel and lactate levels from getting too high.

Discussion

Each impeller and cell culture method results in a different growth pattern and it is necessary to determine what is best for the desired process. When comparing the viable cell growth curves for each of the impellers (Figure 7), it can be seen that each results in a different cell concentration and rate of growth. More importantly, as discussed earlier, some of the impellers/methods allow for perfusion (Packed-bed Basket and Spin filter) resulting in higher and possibly continually sustainable cultures.

The pitched-blade impeller provided a simple way to grow a low-density culture, but it is not possible to grow the culture to a higher density without extra cell separation

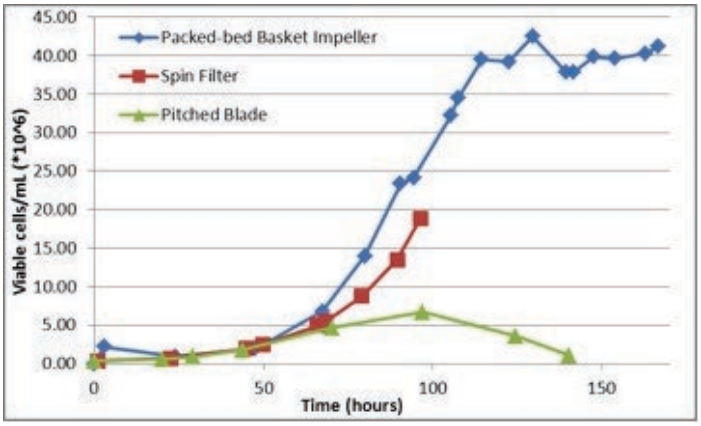


Figure 7: A comparison of viable CHO cell concentration for all three impeller experiments. The packed-bed basket impeller provided long term, high-density cell growth. The spin filter also provided high density cell growth compared to the pitched-blade impeller. Since the pitched-blade impeller was run as a batch-style reactor, a lower viable cell density was reached which eventually drops due to all the glucose being consumed in the vessel.

equipment to allow for perfusion. The spin filter resulted in almost 4X the number of cells as the pitched-blade impeller due to its ability to run in perfusion mode. The perfusion process usually does not last as long as the Fibra-Cel basket due to the tendency of clogging at very high cell densities. However, the cost of the spin filter is much less than that of the Fibra-Cel basket. It is reusable and it does not rely on consumable Fibra-Cel disks. The packed-bed basket impeller resulted in 8X the number of cells as the pitched-blade impeller and over 2X the spin filter. The packed-bed impeller culture also grew faster than the spin filter culture which was most likely due to the lack of direct physical agitation and bubble shear on the cells while they are trapped in the Fibra-Cel disks. Table 5 shows a general list of the advantages for each impeller type. Every cell line is different and what will work best for each culture and purpose can vary. Table 6 is a general guide for choosing impellers based on some common cell lines. The CHO cell cultures in this paper were not optimized and are just a general example of what can be expected for each impeller type.

Table 5: Advantages for each impeller type

Impeller	Advantages
Pitched-Blade Impeller	> Axial and Radial flow > Simple design > Suspension or Microcarrier attached cultures
Spin Filter Impeller	> Easy to use with perfusion > Capable of higher cell densities
Basket Impeller	> Higher cell densities without the risk of clogging > Gentler environment for cells

Table 6: A general guide to choosing impellers by cell line

Cell line	Rushton, Rushton-Like	Pitched-Blade	Marine Blade	Spin Filter	Cell Lift	Basket
Human						
HEK 293		■	■	■	■	■
HeLa		■	■	■		■
HL60		■	■	■		■
Lncap		■	■	■		■
THP-1		■	■	■		■
UMSCC		■	■	■	■	■
HFF		■	■	■	■	■
KB		■	■	■	■	■
MRC-5		■	■	■	■	■
Hybridoma						
DA4.4		■	■	■		■
123A		■	■	■		■
127A		■	■	■		■
GAMMA		■	■	■		■
67-9-B		■	■	■		■
SP20		■	■	■		■
Primate						
Vero		■	■	■	■	■
COS-7		■	■	■	■	■
Rat Tumor						
GH3		■	■	■		■
9L		■	■	■		■
PC12		■	■	■		■
Mouse						
3T3		■	■	■		■
MC3T3		■	■	■		■
NS0		■	■	■	■	■
Hamster						
CHO		■	■	■	■	■
BHK		■	■	■	■	■
Zebrafish						
ZF4		■	■	■	■	
AB9		■	■	■	■	
Insect						
SF9		■		■		■
Hi-5		■		■		■
Sf21		■		■		
Bacteria						
Streptomyces	■	■				
Bacillus	■					
Escherichia coli	■					
Yeast						
Saccharomyces cereviseae	■					
Baker's yeast	■					
Pichia pastoris	■					
Candida albicans	■	■				
Algae						
Red/Green		■	■			

References

1. Mirro, R, and K. Voll. 2009. *Which Impeller Is Right for Your Cell Line?*. BioProcess Int. 7:52-57.

Ordering Information

Product	Description	International order no.	N. America order no.
Voltage Option	Cabinet voltage	M1287-1020 (200V)	M1287-1010 (120V)
CelliGen® 310 Control Station	Cell culture control cabinet	M1287-2110	M1287-2110
4 TMFC (0 - 1 SLPM)	Gas flow control	M1287-2020	M1287-2020
2.5 L water jacketed vessel (with motor)	Cell culture vessel	M1287-0310	M1287-0310
2.5 L pH/DO Sensor Kit (with cables)	pH and Dissolved oxygen sensors	M1287-0400	M1287-0400
2.5 L Pitched-Blade Impeller Kit	Pitched-blade impeller	M1287-5068	M1287-5068
2.5 L Spin Filter Impeller Kit (10 µm)	Spin Filter Impeller	M1287-1125	M1287-1125
2.5 L Basket Impeller Kit	Basket impeller	M1287-1140	M1287-1140
Fibra-Cel® Disks	Microcarrier	M1292-9988	M1292-9988

For information on products used in this application note or other sizes and options available please contact your local sales representative.

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www.eppendorf.com

APPLICATION NOTE No. 312 I April 2012

Growing CHO Cells in a New Brunswick™ CelliGen® BLU Benchtop, Stirred-Tank Bioreactor Using Single-Use Vessels

Guozheng Wang, Wenying Zhang, Rich Mirro and Vikram Gossain, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

The study presents a typical protocol for the setup and operation of the Eppendorf New Brunswick CelliGen BLU single-use, stirred-tank bioreactor, a versatile new benchtop system for the culture of a wide range of mammalian cells. This bioreactor has been designed to provide research and production facilities with a single-use vessel which combines the benefits of both traditional stirred-tank design and single-use technology, capable of seamless process scale-up. The system can be operated in batch, fed-batch or continuous modes. A procedure for culturing Chinese Hamster Ovarian (CHO) cells in a 5.0 L vessel, using CD CHO serum-free medium in a batch culture is described.

Introduction

Historically, stirred-tank fermentors and bioreactors have been the trusted design for culturing all types of submerged cultures including suspension and anchorage-dependent mammalian cells, insect, yeast, plant and microbial cultures. The tried and tested tank design offers scalability and proven reproducibility which is pivotal for cost-saving process development and productivity. In the last decade, there has been an increasing acceptance and use of single-use technologies, due to their convenient operation and low start-up cost. Single-use systems eliminate the need for cleaning and sterilization, reduce validation requirements, provide rapid turn-around between runs, and significantly reduce the risk of cross contamination and microbial contamination because the culture vessel is only used once and then discarded. Although single-use, stirred-tank systems in the 75 – 2000 L scale have been on the market for some time, as have small-scale single-use bags that are gently rocked rather than stirred, until now there has been no single-use stirred-tank system for small-scale work. The new Eppendorf New Brunswick CelliGen BLU fills that void, offering a proven stirred-tank design as well as the benefits of single-use technology in a benchtop system.

Materials and Methods

Single-Use Vessels
BioBLU® single-use vessels are offered in 5.0, 14.0 and 50.0 L total volume capacities. The vessels are delivered preassembled with pitched-blade impeller, porous microsparge, and all the necessary tubing, filters, and connectors; and come sterilized, ready for use right out of the package. All components in product contact are made of materials that meet USP Class VI standards and have been tested for leachables and extractables, making these vessels appropriate for cGMP environments. In this protocol, we describe use of a CelliGen BLU with 5.0 L vessel.

Rapid set up, easy operation, and elimination of autoclaving and cleaning between runs. These are a few of the many advantages of the new BioBLU 5.0, 14.0 and 50.0 L stirred-tank bioreactors for growth of mammalian cultures.

Controller

CelliGen BLU's compact control station is designed to provide advanced process management and monitoring capability, ranging from three fixed-speed pumps for additions and harvesting, to a powerful controller with 15 in. industrial color touchscreen monitor. Multiple options, including gas flow control, a weight scale, validation packages and more, enable customization to your needs.

The control station used in this protocol was configured with one 2 – 100 cubic centimeters per minute (ccm) Thermal Mass Flow Controller (TMFC) for direct sparging of gases and an integrated gas overlay with 0.1 – 3.0 Standard Liters Per Minute (SLPM) flow rate also regulated by a TMFC. Both the gas flow and gas overlay are capable of 4-gas mixing for automatic pH and Dissolved Oxygen (DO) control. Pumps, temperature control, agitation, as well as all of the other process loops, were controlled and monitored through the powerful Reactor Process Controller (RPC) firmware installed in the controller. DO was monitored using a noninvasive reusable polarographic DO probe; and pH was monitored using a non-invasive optical pH probe and fluorescence sensor.

Inoculum Preparation

One 2.5 mL vial of CHO cells was thawed and used to inoculate a 125 mL shake flask which contained 25 mL of serum-free CD CHO medium (Life Technologies® 10743-029) which was pre-warmed to 37 °C.

On day 4, when the viable cell density reached 1.5 x 10^6 cells/mL, the cells were transferred into a 500 mL shake flask which contained 100 mL of freshly made, pre-warmed medium and allowed to incubate for 3 additional days at the same conditions as earlier. The cells were then transferred to two 1 L shake flasks, each containing 250 mL of the freshly made medium. The inoculum was grown in the shake flasks until cell density reached 2.0 – 3.0 x 10^5 cells/mL, with greater than 90 % cell viability, sufficient for the bioreactor inoculation.

Bioreactor Set-Up and Inoculation

One day before the cells reached inoculation density, the growth medium was warmed to 37 °C and the DO probe was polarized. For this study, 3.0 L of sterile CD CHO serum-free medium was prepared by pre-warming at 37 °C for 24 hours in a CO_2 incubator. During this time, the DO probe was connected to the controller for at least 6 hours to enable polarization, as per the manufacturer's recommendation.

Once the medium was warmed and the inoculum grown to sufficient starting density, the CelliGen BLU bioreactor vessel was removed from its sterile packaging and the heat blanket supplied with the unit was wrapped around the outside of the vessel. Next, the vessel containing the cell culture medium was connected to one of the bioreactor vessel's inlet lines using a tube welder. (A tube welder is offered as an optional accessory to the CelliGen BLU. A pre-sterilized medium filter with an attached quick connect or Luer connection can also be used if a tube welder is not available). Since this was a batch process, all of the medium was pumped into the bioreactor vessel. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made.

pH and DO were calibrated through the touchscreen controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values shown on the next page. Once the parameters were at their setpoints, the inoculum flasks were connected to the addition line in a sterile manner using a tube welder and contents were pumped into the bioreactor vessel.

Operational Parameters

Cultivation of animal cells in an environment optimal for manufacture of desired end products require monitoring and control of a substantial number of physical and chemical parameters. Physical parameters include temperature, fluid flow (gas flow and liquid flow) rates and agitation rates. Chemical parameters include the dissolved oxygen (DO) concentration and pH.

Control Setpoints

Temperature	37 °C
pH	7.0
DO	40 %
Agitation	80 rpm

pH Control Parameters

pH control was set to Auto mode, which automatically adds base solution or CO_2 gas to the system based on culture demands.

Dead-band	0.10
PID values	Factory set default values
Base	Sodium bicarbonate, 7.5 % solution
Base Solution Transfer tubing	Narrow bore silicone tubing with Luer-connection (1/18 in. ID & 1/4 in. OD)
Vessel inlet	1/8 in. inlet tubing in the vessel headplate

Dissolved Oxygen (DO) Control
DO control was set to Auto mode, which automatically regulates gas mixing based on culture demand. PID values: factory set default values.

Gas Control
The gas control was set to 4-gas mode, which automatically maintains DO and pH. The gas flow rate was based on the vessel size.

Up until day 3, gases were introduced into the vessel headspace only through the overlay port at a rate of 0.30 L/min using 4-gas mixing to maintain pH and DO. On day 3, and for the remainder of the run, 5 – 10 ccm of gas were directly sparged into the system using a porous sparger and automatic 4-gas mixing. The overlay gas flow in the vessel headspace was kept at the previous settings.

A built-in sampling device enabled sterile sampling. Daily offline measurements of glucose and lactate concentration were read using a YSI® 2700, and cell density and cell viability was measured using an Automated Cell Counting System (New Brunswick NucleoCounter®).

All data was logged via BioCommand® Batch Control PC-compatible Supervisory Control and Data Acquisition (SCADA) software (New Brunswick).

Results and Discussion

As shown in Figure 1, the CHO cells in this study grew steadily, reaching a maximum viable cell density of 5.55 x 10^6 cells/mL on day 5.

Cell viability, shown in Figure 2, ranged between 97.1 and 97.9 % through Day 5, until the nutrient source, glucose, was depleted from the medium, as shown in Figure 3.

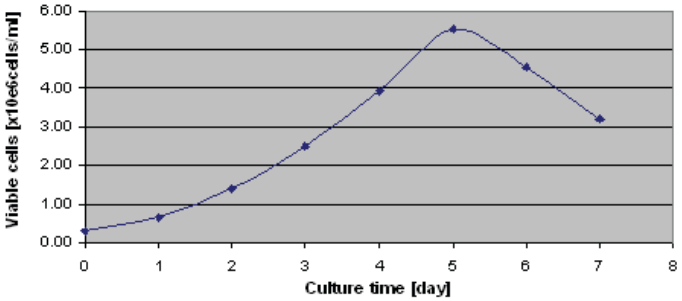

Figure 1. Cell growth over the 7-day run.

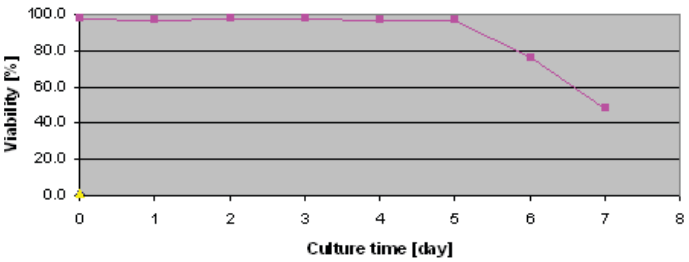

Figure 2. Cell viability remained high through day 5.

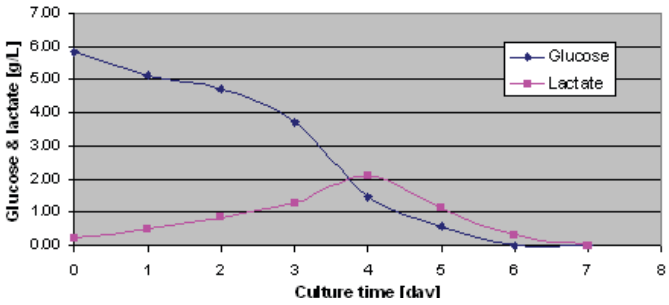

Figure 3. Glucose consumption and lactate production.

As expected, lactate production steadily increased as the available glucose in the medium was consumed. As glucose in the medium became exhausted, consumption of lactate as a secondary carbon source also declined[1].

This data presented here, and in Table 1, demonstrates that the CelliGen BLU bioreactor is an easy-to-use, efficient system for the culture of CHO cells. No effort was made to optimize either the medium or the cell culture process control parameters. This study was only intended to document a general guide to bioreactor setup and operation, and present typical results you could expect to achieve with your mammalian cell line. For protocols on other cell lines, or for additional information on the CelliGen BLU, see eppendorf.com.

Day	Total [10^6 cells/mL]	Viable [10^6 cells/mL]	Viability [%]	Glucose [g/L]	Lactate [g/L]
0	0.31	0.30	97.9	5.83	0.23
1	0.69	0.68	97.1	5.14	0.52
2	1.42	1.39	97.6	4.711	0.87
3	2.57	2.51	97.6	3.74	1.27
4	4.02	3.92	97.5	1.47	2.10
5	5.70	5.55	97.3	0.59	1.12
6	5.98	4.52	76.6	0.00	0.32
7	6.71	3.21	47.8	0.00	0.01

Table 1.

References

[1] **A single nutrient feed supports both chemically defined NS0 and CHO fed-batch processes: Improved productivity and lactate metabolism.** Ma N, Ellet J, Okediadi C, Hermes P, McCormick E, Casnocha S. *Biotechnol Prog.* 2009; 25 (5): 1353-63.

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APPLICATION NOTE No. 257 I November 2012

Single-Use Scalability: CHO Cell Culture using 5 to 50 L New Brunswick™ CelliGen® BLU Benchtop, Stirred-Tank Bioreactors

Nick Kohlstrom, Joseph Capone, and Ma Sha, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

This study illustrates a protocol for the scale up of CHO cells using New Brunswick™ CelliGen BLU stirred-tank bioreactors equipped with 5-Liter (L) and 50-Liter (L) single-use vessels. CelliGen BLU is a versatile benchtop system for the culture of a variety of cell lines. This bioreactor has been designed to provide research and production facilities with single-use vessels that combine the benefits of both traditional stirred-tank design and single-use technology, capable of seamless process scale-up. Eppendorf has recently launched the CelliGen BLU 50 L system to address larger volume batch demands.

Introduction

Historically, stirred-tank bioreactors have been the standard for culturing all types of submerged cultures including suspension and anchorage-dependent mammalian, insect, yeast, plant and microbial cultures. This well-tested vessel design offers scalability and reproducibility, which enhance productivity and provide cost savings in process development. In the last decade, there has been an increasing acceptance of single-use technologies, due to their convenient operation and low start-up cost. Single-use systems eliminate the need for cleaning and sterilization, reduce validation requirements, provide rapid turn-around between runs, and significantly reduce the risk of cross contamination and microbial contamination. Until recently, the CelliGen BLU single-use bioreactor system has been limited to 5 L and 14 L sizes. The new 50 L CelliGen BLU vessel is a direct response to customer feedback, accommodating much larger process volumes while maintaining the benefits of single-use technology in the same proven, rigid-walled, stirred-tank design, all in a benchtop platform. All three vessel sizes have the capability to be operated in batch, fed-batch or perfusion style. This protocol describes a cell culture process using Freestyle™ Chinese Hamster Ovarian (CHO-S) cells (Invitrogen® Corp.) starting from the smaller 5 L vessel and finishing up in the larger 50 L vessel.

Materials and Methods

Single-Use Vessels

CelliGen BLU single-use vessels are now offered in 5.0 L, 14.0 L, and 50.0 L volumes. The vessels are delivered pre-assembled with a pitched-blade impeller. The vessels have either a porous micro-sparge or a macro-sparge element configuration (selected at time of purchase), and also include all the necessary tubing, filters, and connectors. The vessels come sterilized and ready for use right out of the package. All components in contact with cell culture are made from materials that meet USP Class VI standards and have been tested for leachables and extractables, making these vessels appropriate for cGMP environments. In this protocol, we describe the use of 5 L and 50 L CelliGen BLU vessels with pitched blade impellers and the macrosparge element configurations. The 5 L culture was conducted in a batch style while the 50 L culture was completed as a fed-batch.

The CelliGen BLU 50 L stirred-tank bioreactor offers many advantages for mammalian cell culture: it sets up rapidly, it is easy to operate, and it eliminates cleaning and autoclaving between runs

Controller

The CelliGen BLU compact control station is designed to provide advanced process management and monitoring capability with a powerful Reactor Process Controller (RPC) with 15" LCD color touchscreen monitor. The controller includes three integrated pumps, and other options enable customization to meet a customer's needs, including high- or low-flow thermal mass flow controllers (TMFC) for gas flow control, scales, and validation packages.

The control station used in this protocol for the 5 L vessel was equipped with three low-flow TMFCs (draw at 0.002-1.0 standard liters per minute [SLPM]) for direct sparging to control gases including air, oxygen, nitrogen, and CO_2. The controller was also equipped with an integrated gas overlay function controlled by a single TMFC with a regulated flow of 0.05 - 5.0 SLPM. The control station used for the 50 L vessel utilized the same design with the high gas flow option with TMFCs ranging between 0.04 - 7.5 SLPM. Both the sparge and overlay were capable of 3-gas or 4-gas mixing for automatic pH and Dissolved Oxygen (DO) control. For this protocol, both the 5 L and the 50 L set-ups were operated in 3-gas mode in conjunction with 0.1 SLPM of air as an overlay. The pumps, temperature control, agitation, and all other process loops were controlled and monitored through the RPC firmware installed in the controller. DO and pH were monitored non-invasively; DO was monitored using a traditional, stainless-steel polarographic probe, while pH was monitored using an optical probe with fluorescence sensor.

Inoculum Preparation

For the 5 L bioreactor inoculation, Freestyle Chinese Hamster Ovarian (CHO-S) cells were used to inoculate a 125 mL shake flask which contained 30 mL of serum-free CD CHO medium (Invitrogen) supplemented with 8mM L-glutamine (JRH Biosciences) and 1 % Penicillin-Streptomycin (Invitrogen).

This initial shaker culture was expanded to a one liter shake flask containing 240 mL; the inoculum was then grown until day 4, when the viable cell density reached 3.16 x 10^6 cells/mL with a viability of 99.3 %, a density sufficient for transfer into the 5 L bioreactor.

Bioreactor Set-Up and Inoculation

Inoculation of 5 L Bioreactor

The DO probe was connected to the controller for at least 6 hours for polarization.

On inoculation day, the CelliGen BLU bioreactor vessel was removed from its sterile packaging and the heat blanket was wrapped around the outside of the vessel. Next, the vessel containing the cell culture medium was connected to one of the bioreactor vessel's inlet lines using a quick connect. A Luer connection or tube welder can also be used with the CelliGen BLU. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made.

Approximately 2 L of sterile CD CHO serum-free medium was pumped into the vessel and warmed to 37° C. After the growth medium was stabilized at 37° C, the traditional polarographic probe (DO probe) was calibrated to an electronic zero , and then spanned after the agitation was set at 50 rpm and the airflow was set to 100 % at 1 SLPM for ~20 minutes (may vary depending on how long it takes the raw value to stabilize). The optical pH calibration was performed using the pH probe raw data (located on the vessel and packaging; preconfigured for optical pH using fluorescence sensor technology), and an offline sample was taken to re-zero the medium within the bioreactor (after the fluorescence spot was hydrated ~20 mins). DO and pH were calibrated through the touchscreen controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values represented within the following pages below. Once the parameters were at their setpoints, the inoculum flasks were connected to the addition line in a sterile manner using a quick connect and the cells were pumped into the bioreactor vessel for a total volume of ~2 L with an inoculation density of 0.3 x 10^6 cells/mL.

Inoculation of 50 L Bioreactor

Medium warming and DO polarization were conducted in a similar fashion to the preparation prior to the inoculation of the 5 L vessel.

Once culture growth within the 5 L bioreactor had achieved sufficient density (4.84 x 10^6 cell/mL; viability 99.4 %), the 50 L CelliGen BLU vessel was removed from its sterile packaging and the heat blanket was wrapped around the outside of the vessel. Next, the bag containing the cell culture medium was connected to one of the 50 L bioreactor vessel's inlet lines using a quick connect. Since this portion of the experiment was a fed batch process with starting volume less than 20 L, only the initial 17.8 L of the medium was pumped into the bioreactor vessel. All additional connections to the controller including sparge, overlay, RTD, pH, and agitation were also made. The base pump was also calibrated and primed for use with 20 % Sodium Bicarbonate for pH control.

The polarographic probe on the 50 L vessel was calibrated to an electronic zero once the growth medium was stabilized at 37° C, and then spanned when the agitation was set at 50 rpm and the airflow was set to 100 % at 7.5 SLPM

(~20 minutes to stabilize the raw value). The optical pH calibration was performed using the pH probe raw data, and an offline sample was taken to re-zero the medium within the bioreactor. DO and pH were calibrated through the touchscreen controller, and all process setpoints were entered on the touchscreen using the Control Setpoint values shown on the next page. Once the parameters were at their setpoints, the 5 L harvest line was connected to the inoculation/addition line on the 50 L in a sterile manner using a quick connect, and then the calculated 1.2 L of high density CHO cells were pumped in for a total volume of 19 L with final starting cell density of 0.3 x 10^6 cells/mL. The 50 L vessel was then fed with an additional 21 liters of pre-warmed Gibco CD CHO serum-free medium on day 5 to support high cell growth and viability.

Operational Parameters

Cultivation of animal cells for manufacturing of desired end products requires monitoring and controlling of a substantial number of physical and chemical parameters. Physical parameters include temperature, gas flow rates, fluid flow rate, and agitation speed. Chemical parameters include the dissolved oxygen (DO) concentration and pH.

5 L and 50 L Control Setpoints

Temperature	37° C
pH	7.1
DO	50 %
Agitation	50 rpm (clockwise)

5 L and 50 L pH Control Parameters

Both vessels' pH control was set to Auto mode, which automatically adds base solution or CO_2 gas to the system based on culture demands. Base addition was utilized for pH control on the 50 L culture due to the higher density expected at end of the 50 L run but was not needed for the 5 L run.

pH Dead-band	0.05
PID values	factory set default values
Base (50 L only)	Sodium bicarbonate, 20 % solution

5 L and 50 L Dissolved Oxygen (DO) Control

DO control was set to 3-gas Auto mode, which automatically regulates gas mixing based on culture demand. Factory-set default PID values were used.

5 L and 50 L Gas Control

The gas control was set to 3-gas Auto mode for both bioreactors, automatically maintaining DO and pH. For the 5 L bioreactor, the low flow limit set at 0.002 SLPM with a high flow limit set at 1.00 SLPM, and for the 50 L Bioreactor, the low flow limit was set at 0.04 SLPM with a high flow limit set at 7.5 SLPM. In addition, overlay air flow was supplied to both bioreactors at 0.10 SLPM.

Gases were introduced via the macrosparge element for aeration supplementation to maintain DO and pH and into the vessel headspace through the overlay port during the entire run for both the 5 L and the 50 L bioreactors.

A built-in sampling device enabled sterile sampling. Daily off-line measurements of glucose and lactate concentration were read using an YSI® 2700; cell density and cell viability were measured using an Automated Cell Counting System (Vi-CELL®).

Results and Discussion

All vessel data was logged via BioCommand® Batch Control Supervisory Control and Data Acquisition (SCADA) software (Eppendorf).The bioreactors' total cell density and viability are shown in Figure 1. CHO cells exhibited steady and consistent growth on both the 5 L and 50 L bioreactors. Cell growth reached a viable cell density of 4.82 x 10^6 cells/mL on day 4 in the 5 L, and 8.58 x 10^6 cells/mL on day 8 in the 50 L vessel. Cell viability was maintained around 99 % for the entire culture duration. As expected, lactate production steadily increased as the available glucose in the medium was consumed (Figure 2).

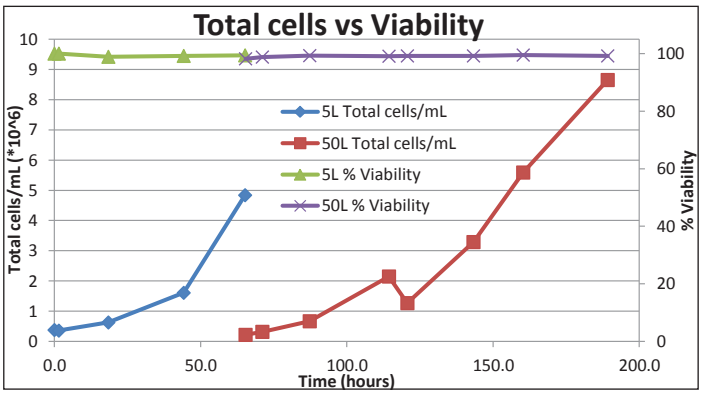

Figure 1. CHO Cell growth and viability plots for the scale-up from 5 L to 50 L CelliGen BLU bioreactors in a combined cell culture process of 8 days. The dip of cell count seen on the 50 L graph represents the single feeding event and cell density dilution resulted from 21 L media addition introduced on day 5.

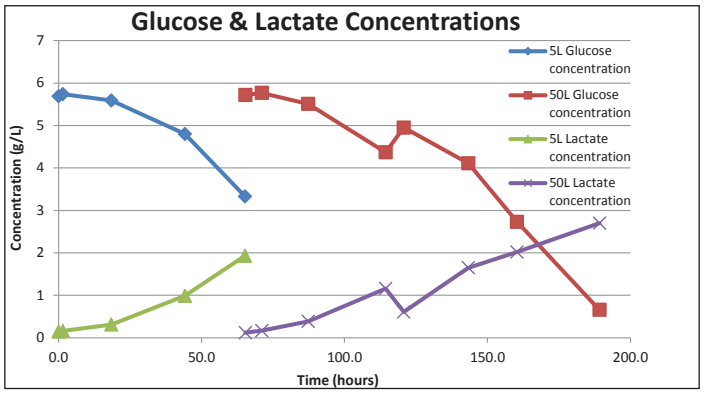

Figure 2. Glucose consumption and lactate production in the 5 L and the 50 L vessels.

The data presented here demonstrates that the CelliGen BLU bioreactor is an easy-to-use, efficient system for the scale-up of CHO cell culture up to the 50 L vessel size.

No efforts were made to optimize either the medium or the cell culture process control parameters.

This study was only intended to document a general procedure for CelliGen BLU bioreactor setup and operation, and present typical results one could expect to achieve with mammalian cell line.

Although it is possible to perform such scale-up using a single CelliGen BLU controller, two separate CelliGen BLU systems were used for this application note. For protocols on other cell lines, or for additional information on the CelliGen BLU, see eppendorf.com.

References

1. **Productivity Studies Utilizing Recombinant CHO Cells in Stirred-Tank Bioreactors: A Comparative Study Between Pitched-Blade and Packed-Bed Bioreactor Systems.** Taylor Hatton, Shaun Barnett, Abby D. Benninghoff, PhD, and Kamal Rashid, PhD. *Bioprocessing J.*, Volume 11, Issue 2 (Summer 2012)

2. **A single nutrient feed supports both chemically defined NS0 and CHO fed-batch processes: Improved productivity and lactate metabolism.** Ma N, Ellet J, Okediadi C, Hermes P, McCormick E, Casnocha S. *Biotechnol Prog.* 2009 Jul 27.

Ordering Information

Description	International order no.	North America order no.
CelliGen® BLU Control Station 100 - 120V, 50/60Hz, high flow sparge, overlay w/TMFC, and scale	M1374-120-HSA (120V) M1374-230-HSA (230V)	M1374-120-HSA
CelliGen® BLU Control Station 100 - 120V, 50/60Hz, low flow sparge, overlay w/TMFC, and scale	M1374-120-LSA (120V) M1374-230-LSA (230V)	M1374-120-LSA
CelliGen® BLU 5L vessel Single-use 5.0L vessel with macrosparge (pack of 1)	M1363-0121	M1363-0121
CelliGen® BLU 14L vessel Single-use 14.0L vessel with macrosparge (pack of 1)	M1363-0122	M1363-0122
CelliGen® BLU 50L vessel Single-use 50.0L vessel with macrosparge (pack of 1)	M1363-0129	M1363-0129
CelliGen® BLU vessel kit Includes heat blanket, RTD temperature sensor, pH and DO probes, with cables and needle-free syringes	M1363-0105 (5L kit) M1363-0114 (14L kit) M1374-0151 (50L kit 120V) M1374-0150 (50L kit 230V)	M1363-0105 (5L kit) M1363-0114 (14L kit) M1374-0151 (50L kit)
Eppendorf Research® plus, adjustable pipette Single channel pipette – 1 -10 mL	3120 000.089	3120000089
Easypet® Pipette dispenser – suitable for pipettes from 0.1 to 100 mL	4421 000.013	022230204

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APPLICATION NOTE No. 254 I July 2012

CHO Cell Culture with Single-Use New Brunswick™ CelliGen® BLU Packed-Bed Fibra-Cel® Basket

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Abstract

The objective of this study was to compare New Brunswick CelliGen BLU single-use packed-bed bioreactor and the traditional glass vessel counterpart used in New Brunswick CelliGen 310. Alkaline phosphatase (ALKP)-secreting Chinese Hamster ovary (CHO) cells were used to measure ALKP production in each bioreactor. Overall, the results from these comparisons suggest that there is no significant difference between the reusable and single-use FibraCel basket systems for bench-scale production of recombinant proteins. Productivity of cells and collection of secreted proteins will not be hindered by the implementation of single-use bioreactor systems.

Introduction

The packed-bed basket technology, developed by New Brunswick Scientific (acquired by Eppendorf Inc. in 2007), provides a shear free environment for production of animal cells. At present, little information is available on the utility of the New Brunswick CelliGen® BLU single-use bioreactor system for the production of secreted proteins, especially in perfusion mode of operation. Thus, this study was conducted to measure the growth and productivity of alkaline phosphatase (ALKP)-secreting rCHO. Two packed-bed bioreactor types were used: 5 L New Brunswick CelliGen® BLU single-use vessel and 2.5 L autoclavable glass vessel both operated by New Brunswick CelliGen® 310 console in perfusion mode. The perfusion process provides a homeostatic environment for optimal cell growth similar to that experienced by cells in vivo, where waste products are constantly removed and fresh nutrients are replenished. Cells cultured in packed-bed bioreactors are not exposed to hydrodynamic forces, thus, allowing for maximum cell growth and protein expression[1]. The objective of this study was to compare the two types of bioreactors to determine if any differences are observed between the productivity of the two bioreactors.

Materials and Methods

Culture procedures
In order to evaluate the impact of these bioreactor systems on protein production, we utilized a recombinant alkaline phosphatase-secreting CHO cell line (rCHO), a proprietary cell line provided by CDI Bioscience, Inc. (Madison, WI). The rCHO cells were engineered with the IPTG-regulated RP Shift vector so that the rCHO cells stop replicating and shift to protein production when induced with IPTG. Serum free CD-CHO medium (Gibco, Life Technologies, Grand Island, NY) was used throughout these experiments. The media contains 6.3g/L glucose and was supplemented with 8 mM L-glutamine and 100µg/ml of an antibiotic/antimycotic solution (Invitrogen, Life Technologies). Frozen rCHO cells were thawed and transferred to T-75 flasks with CD-CHO medium and allowed to expand. Once a sufficient number of cells were achieved, sterile disposable spinner flasks were utilized to further expand the cells. Subculture of the cells continued until a sufficient number of viable cells was achieved for use as a seed culture at the density of 5 x 105 cells/ml. Two New Brunswick CelliGen® 310 advanced bench-top stirred-tank bioreactors were utilized to grow the rCHO cells. One of the New Brunswick CelliGen® consoles was connected to an adaptor kit (available from Eppendorf) for use of the New Brunswick CelliGen® BLU single-use vessel.

Table 1: Comparison of perfusion volumes

Perfusion	Glass	BLU
Day 1	0.5 L	1 L
Day 2	1 L	2 L
Days 3 - 15*	2 L	4 L

* Perfusion occurred every other day.

Table 2: Bioreactor parameters (setpoints)

Parameter	Glass	BLU
Temperature	37° C (± 0.1° C)	37° C (± 0.1° C)
Agitation	120 rpm (± 5 rpm)	120 rpm (± 5 rpm)
DO	35 % (± 1 %)	35 % (± 1 %)
pH	7.1 (± 0.01)	7.1 (± 0.01)
Gas flow	0.5 slpm	1.5 slpm

Packed-bed basket impeller operated in perfusion mode
Two experimental trials were performed using the packed-bed vessels in perfusion mode: 2.5 L total volume autoclavable vessel (1.7 L working volume) and a 5 L total volume single-use vessel (3.5 working volume, pre-loaded with 150g of Fibra-Cel® disks). The perfusion process was initiated once the cells reached the exponential growth phase as shown in table 1. Both experimental trials had the following parameters shown in table 2.

Biomarkers of cell growth and productivity
Cell productivity was assessed by measuring activity of the secreted ALKP protein using an enzyme assay (AnaSpec, Freemont, CA) according to the manufacturer's protocol. For simplicity unit measurements were used in this study. A unit (U) of ALKP activity was defined as the amount of enzyme that *hydrolyzes 1µmol* of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of *1* ml in *1* minute at *37° C*. The YSI 2700 Select Biochemistry Analyzer (YSI, Inc., Yellow Springs, OH) was utilized to monitor the glucose and lactate levels in the culture media every 24 hr for the duration of each trial.

Results and Discussion

Glucose utilization and lactate production
Glucose is the main energy source for cell proliferation and ALKP production. Thus, glucose levels were expected to directly correlate with ALKP production in each experiment. Because lactate is a secondary energy source, lactate levels were expected to decline following this initial increase and the utilization of glucose in the media. Lactate metabolism is beneficial to the system by reducing a major metabolic by-product from the system[2,3]. Glucose levels measured at the time of induction (day 3) were nearly 0 g/L in both experiments (Fig. 1). Media lactate concentrations increased in response to decreasing glucose availability. The use of lactate as a secondary energy source can also be observed as lactate levels decrease at each 2 L perfusion.

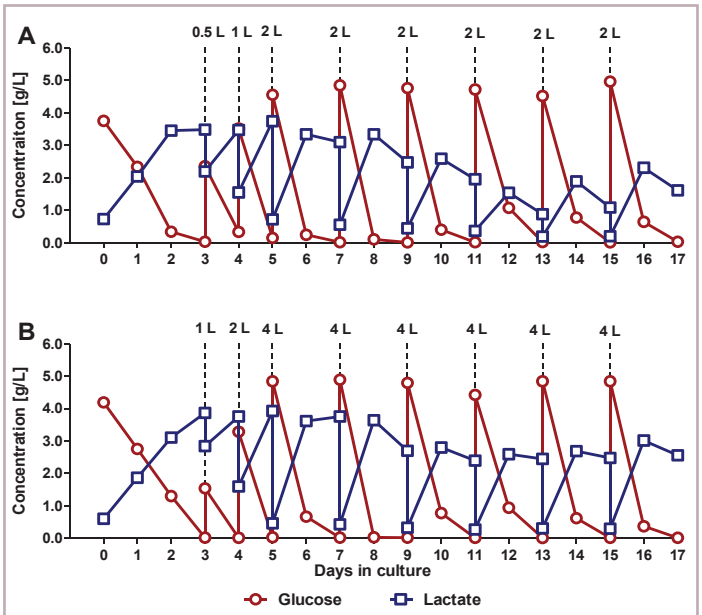

Figure 1. Glucose consumption and lactate production by rCHO cells cultured in two packed-bed bioreactor system. Values shown are the amounts of glucose and lactate measured in the culture media at each media exchange. The time and volume of the media exchange is indicated at each dashed line. Induction of ALKP activity by IPTG began on culture day 5 and continued every two days throughout the remainder of the experiment. Results of two experimental trials are shown (A, reusable; B, single-use).

Comparison of bioreactor systems for ALKP production

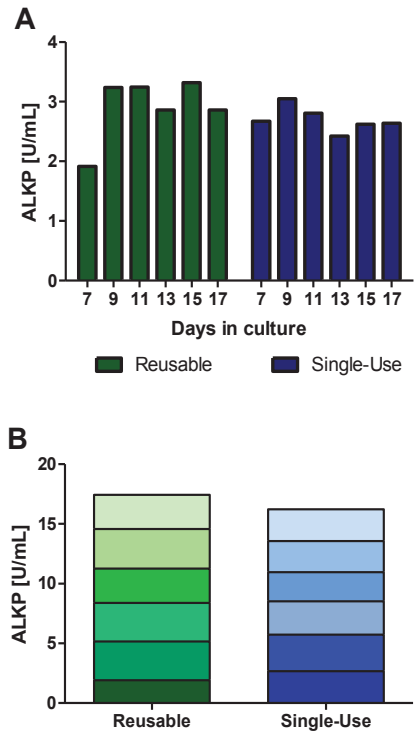

Figure 2. ALKP production by rCHO cells cultured in two packed-bed bioreactor system. (A) ALKP concentration in culture media measured each day of each experimental trial. IPTG induction of ALKP began on culture day five and continued every two days for the remainder of the experiment. (B) Stacked bar charts show the cumulative production of ALKP throughout the experiment, with each bar representing a perfusion.

The average total ALKP production per experiment trial is shown in Figure 2; overall, there is not a significant difference in ALKP production between the two bioreactor systems. The total amount of ALKP measured after five media exchanges in the reusable vessel was 17.44 U/mL and 16.22 U/mL in the single-use vessel.

In summary, these results demonstrated comparable yields in ALKP production (within the usual biological fluctuations) between the two packed-bed bioreactor systems when operated in perfusion. Given the greater productivity of cells cultured in the packed-bed bioreactor and the multitude of advantages of this system operated in perfusion mode, researchers desiring to scale up mammalian cell culture for protein production should strongly consider utilization of the New Brunswick CelliGen® BLU packed-bed, single-use bioreactor system.

Note: This application note study was partially funded by Eppendorf Inc.

References

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APPLICATION NOTE No. 255 I July 2012

Hybridoma and CHO Cell Culture using the New Brunswick™ S41i, an Environmentally-Friendly, "Low Emission" Incubator Shaker

Nick Kohlstrom, George Wang, Linette Philip and Ma Sha, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

In this study, the New Brunswick S41i CO_2 incubator shaker's mammalian cell culture capability was first verified by culturing CHO cells. This was followed by a comparative performance evaluation against two leading incubator shakers on the market. The New Brunswick S41i provided equivalent performance on the growth rate and viability of mouse hybridoma cells. Comparison of CO_2 gas consumption was also conducted. Due to the superior "green" engineering and advanced control of critical parameters, the New Brunswick S41i demonstrated up to 10 times lower gas consumption compared to the competitive units while delivering uncompromised performance.

Introduction

Cars aren't the only source of CO_2 emissions; laboratory equipment, such as CO_2 incubators, could be releasing over 20,000 liters of CO_2 gas per year. Eppendorf® established the epGreen initiative to reduce the environmental impact of our products. Most of the CO_2 gas consumed by incubators is released to the environment. Eppendorf's new incubator shaker, the New Brunswick S41i, releases extremely low amounts of CO_2 under normal cell culture conditions without sacrificing performance. This study evaluates the New Brunswick S41i's performance culturing hybridoma and Chinese hamster ovary (CHO) cells. The study also investigates the New Brunswick S41i's CO_2 gas consumption compared to competitive units. The data reveals that the New Brunswick S41i consumes 5 to 10 times less CO_2 than competitors, resulting in a 5 to 10 times smaller carbon footprint. Superior engineering minimizes gas leakage with a tightly sealed inner glass door protected by sturdy outer door, tightly sealed motor drive boots as well as a sealed incubation chamber. The performance evaluation, based on the comparison of cell culture growth rates, cell densities, and percent viabilities, demonstrates the New Brunswick S41i's industry leading performance. This new CO_2 incubator includes a robust New Brunswick triple eccentric drive shaker for accurate and stable parameters required to grow non-adherent cells. The shaker drive is optimized for high performance within a humid and carbon dioxide rich environment.

Materials and Methods

Instruments

> New Brunswick S41i equipped with high-temperature disinfection
> CO_2 incubator shaker from competitor 1
> CO_2 incubator shaker from competitor 2
> Vi-CELL® analyzer (Beckman Coulter, Germany)
> YSI® 2700 analyzer (YSI Life Science, USA)
> New Brunswick Galaxy® gas analyzer
> Omega® FMA-1608A thermal mass flow-meter (Omega Engineering, USA)
> Eppendorf consumables
- Research® plus, single channel pipette
- epT.I.P.S®
- Easypet®

Media and cells

> DG44 CHO cell (Invitrogen)
> EX-CELL® CD CHO serum-free medium for CHO cells (Sigma)
> Hybridoma cell DA4-4; ATCC:HB57
> DMEM (ATCC)
> Fetal Bovine Serum 5% (Gibco)
> Penicillin-Streptomycin 100x (Gibco)

CHO culture protocol

CHO cells were grown in EX-CELL CD CHO serum-free medium supplemented with 1% penicillin-streptomycin antibiotic. Six 250mL Erlenmeyer flasks were each inoculated with 60mL of stock culture at a concentration of 3 x 10^5 cells/mL. All flasks were prepared from the same stock culture. Erlenmeyer flask was placed in six different locations on the shaker platform and the results were averaged. The flasks were incubated at 37°C in a mixture of 5% CO_2, 95% air and agitated at 130 RPM (4.69 rcf).

CHO cells were grown for a period of 14 days. A sample was taken on days 3, 5,7,10, 12 and 14 and was analyzed for glucose concentration, cell concentration and viability using YSI 2700 and Beckman Coulter Vi-CELL.

Hybridoma culture protocol

DA4-4 hybridoma cells were grown in DMEM medium supplemented with 5% FBS and 1% Penicillin-Streptomycin. Six 250mL Erlenmeyer flasks were each inoculated with 45mL of stock culture at a concentration of 2 x 10^5 cells/mL. All shake flasks were prepared from the same stock culture and were equally distributed in six different locations in the New Brunswick S41i, competitor 1 and competitor 2. The flasks were incubated at 37°C in a mixture of 5% CO_2 and 95% air. The units were agitated at 95 RPM (2.52 rcf).

Hybridoma cells were subcultured on day 2 and 4 to a concentration of approximately 2 x 10^5 cells/mL. A sample was taken every day from each of the flasks and analyzed for glucose concentration, cell concentration, and viability using YSI 2700 and Beckman Coulter Vi-CELL, respectively.

Gas consumption

The New Brunswick S41i and competitor incubator shakers 1 and 2 were programmed at 37°C, 95 RPM and 5% CO_2 and were allowed to equilibrate for at least 12 hours. Inline CO_2 gas pressures were set at the lowest values recommended by each manufacturer. An offline gas analyzer was used to verify the CO_2 levels within each incubator. A thermal mass flow-meter was used to record volumetric gas consumption over a time period of 48 hours on each unit. Tests were repeated three times and the average values are reported below.

Results

1.) Growth assessment of CHO and hybridoma

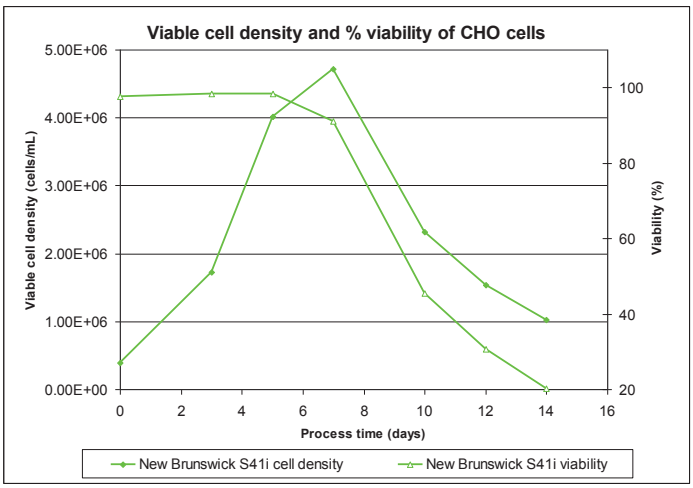

Figure 1: Average viable cell concentration and viability of CHO culture in the New Brunswick S41i.

Viable cell density of CHO cells reached a maximum of 4.72 x 10^6 cells/mL by day 7. The cell viability was maintained at approximately 98% up to day 5 and dropped steadily thereafter (Figure 1).

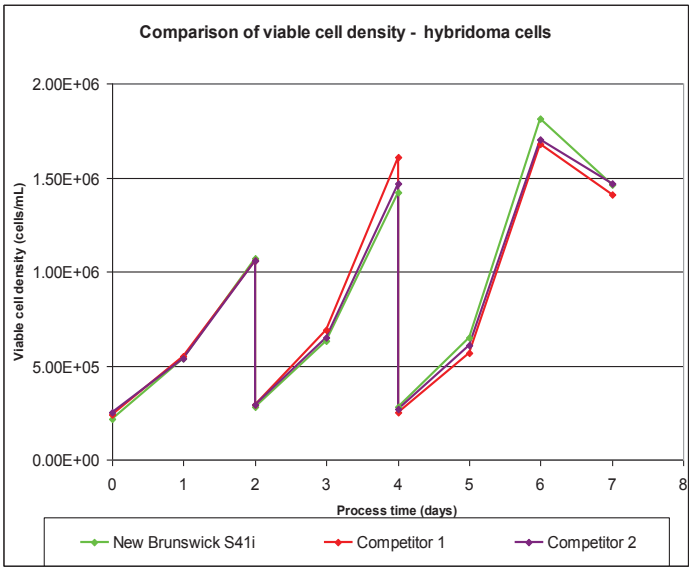

Figure 2: Comparison of average viable cell densities of hybridoma cultures grown in New Brunswick S41i and competitors 1 and 2.

In comparison to CHO cells, hybridoma culture was able to maintain a high average viability of approximately 95% through day 6, due to the subculturing of cells during log phase of growth on days 2 and 4. The maximum viable cell density of 1.81 x 10^6 cells/mL was achieved on day 6 (Figures 2 and 3).

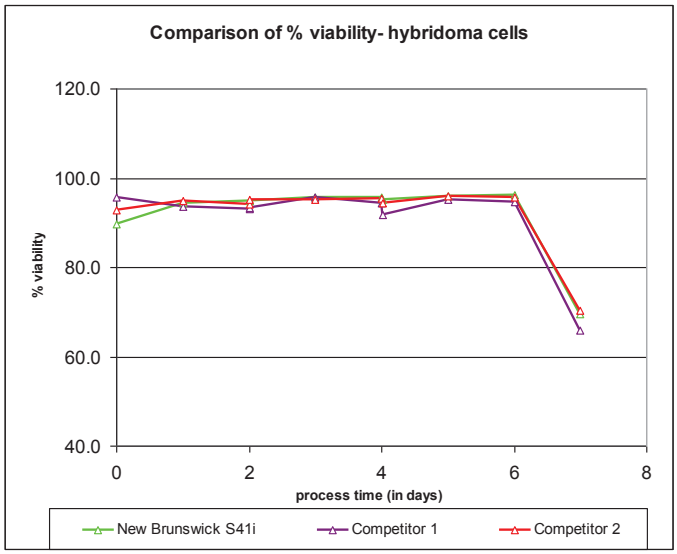

Figure 3: Comparison of average percentage viabilities of hybridoma cultures grown in New Brunswick S41i and competitors 1 and 2.

2.) Measurements of gas consumption

The measurement of CO_2 consumption at 5% CO_2 setpoint revealed that the competitive units evaluated consumes much higher CO_2 gas over the same period as compared to the New Brunswick S41i (Figure 4).

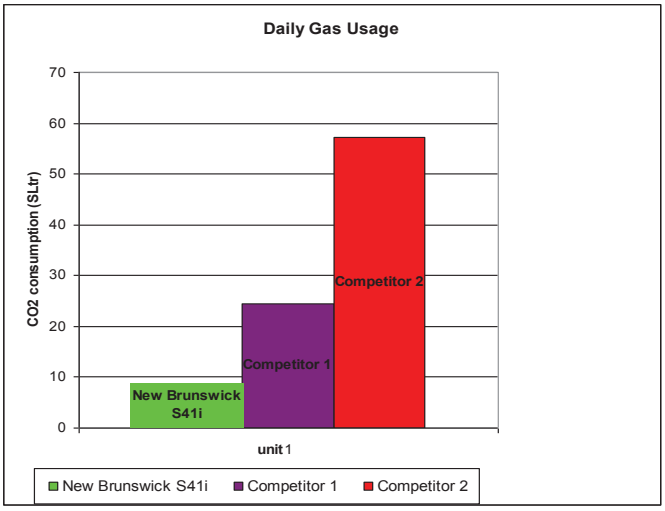

Figure 4: Average CO_2 gas consumption of tested units in standard liters (Sltr) over a 24 hour period

Discussion & Conclusion

The rising need to create more eco-friendly products and customer demand for higher efficiency were taken into consideration during the development of the New Brunswick S41i. This study validates the performance of the new CO_2 incubator with a New Brunswick shaker built inside by growing two cell lines which are very commonplace in research and production. Process and media were not optimized for either cell lines in this study.

In conclusion, the results show that the New Brunswick S41i is competent at growing mammalian cells while reducing environmental impact down to a minimum. The New Brunswick S41i combines a robust triple eccentric drive shaker within a CO_2 incubator, to provide accurate and stable parameters required for the growth of non-adherent cells.

Ordering Information

Description	International order no.	North America order no.
New Brunswick S41i CO_2 incubator shaker with high-temperature disinfection	S41I-230-0100	S41I-120-0100
Galaxy gas analyzer Electronic CO_2 gas analyzer	P0628-6150	P0628-6150
eppendorf Research® plus, adjustable Single channel pipette – 1-10 mL	3120 000.089	3120000089
Easypet® Pipette dispenser – suitable for pipettes from 0.1 to 100 mL	4421 000.013	022230204

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APPLICATION NOTE No. 258 I July 2013

Hybridoma culture using New Brunswick™ CelliGen® 310 with Packed-bed Fibra-Cel® Basket Impeller

Ray Rose, Stacey Willard, Ma Sha, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

The Fibra-Cel packed-bed basket technology has been established as an excellent method for the growth of suspension and anchorage-dependent cell lines. The three dimensional structure of the Fibra-Cel disk provides an excellent solid-support matrix for the entrapment or attachment of animal cells, allowing constant perfusion of nutrients in a low-shear environment. In this application note, we show that Hybridoma cells can be successfully cultivated in high densities in the 2.5 L packed-bed Fibra-Cel basket controlled by a CelliGen 310 bioreactor.

Introduction

Packed-bed bioreactor cell culture is generally accepted as one of the best methods to simulate the conditions of animal cell growth in vivo since cells are maintained in a low-shear environment with constant refreshment of nutrients and removal of waste. The growth of attachment-dependent cells on Fibra-Cel has been shown to increase both cell and product yields. In particular, Hybridoma cells are inherently sensitive to waste buildup and the implementation of packed-bed Fibra-Cel growth conditions in addition to perfusion production methods has greatly increased yields. To demonstrate that the CelliGen 310 2.5 L basket impeller bioreactor is capable of robust, reproducible high density Hybridoma culture under perfusion conditions, two independent trials were conducted using the suspension-adapted DA4.4 hybridoma cell line.

Figure 1. Left: The packed-bed basket impeller including Fibra-Cel disks. Right: The CelliGen 310 bioreactor with 2.5 L vessel.

Materials and Methods

Inoculum preparation

DA4.4 Hybridoma cells (ATCC® #HB-57; Manassas, VA) were grown in 1 L shake flasks at 37 °C with 5 % CO_2 and agitation set at 95 rpm. Culture medium was prepared using Gibco® Hybridoma-SFM complete DPM powder supplemented with 5 % Hyclone® Fetal Bovine Serum and 1 % Gibco liquid Pen/Strep before sterile filtration using a 0.2 µm Millipore® Millipak® gamma gold filter into sterile Hyclone bags (5 L and 10 L, as necessary). Medium was stored at 2 – 8 °C until use. The 1.75 L vessel working volume was inoculated with a target total of 4.1 x 10^8 cells. Actual viable cell numbers were 3.5 x 10^8 cells (2.2 x 10^5 cells/mL) for the first run and 4.8 x 10^8 cells (3 x 10^5 cells/mL) for the second run. The table below shows the origin of the materials used in this study.

Material	Supplier	Catalog #	Lot #
Hybridoma-SFM complete DPM powder	Gibco	12300-067	949234
Pen/Strep 100X liquid	Gibco	15140	1092590
Hyclone Fetal bovine serum	Hyclone	SH30070.03	AWC99936
D (+) - Glucose Hybri-Max powder	Sigma®	G5146-10k	071M01453V
45 % Glucose solution	Sigma	G8769	54K2371
Fibra-Cel	Eppendorf	M1292-9988	Trial 1: 78690 Trial 2: 1100081

Bioreactor conditions

For both runs, hybridoma cells were cultured in the same vessel, using the same CelliGen 310 cabinet for 9 consecutive days, using the basket impeller system packed with 75 g of Fibra-Cel disks.

CelliGen 310 Setpoints	
Agitation	80 rpm
Temperature	37 °C
pH	7.15 Dead band 0.04
DO	50 %
Gas supplied	4-gas mix control (N_2 off; CO_2 fo pH control)
Gas flow conditions	0.4 SLPM
Vessel	2.5 L glass water jacketed
Fibra-Cel	75 g

Perfusion was initiated for each bioreactor on day 3 and continued through day 9. Initially, the main objective was to increase the perfusion rate to maintain a glucose concentration above and near 1 g/L. For the second bioreactor experiment, the perfusion rate was adjusted to match the first bioreactor rate in order to make the two runs as identical as possible. The tables illustrate the experimental parameters and perfusion volumes for both trials.

Day	Perfusion volume (L)
3	0.73
4	1.81
5	4.25
6	5.5
7	4.25
8	4.75
9	5

Biochemistry analysis

Daily off-line measurements of glucose concentration were performed using a YSI® 2700 analyzer (YSI, Inc., Yellow Springs, OH). The glucose consumption was calculated for each time point and plotted as an average of the two independent trials. Error bars indicate standard error of the mean.

Results and Discussion

As presented in the graph below (Figure 2), the rate of glucose consumption across both trials is indicative of reproducible growth of hybridoma cells in this environment. We conclude that the use of Fibra-Cel in the basket impeller system on the CelliGen 310 is an excellent method for high density hybridoma culture. In a batch run with the CelliGen pitch blade bioreactor, hybridoma cells usually peak at approximately 5 g/day of glucose consumption. The packed-bed basket impeller system presents significantly higher productivity with glucose consumption peaking at, on average, 25 g/day. In addition, if growth conditions are maintained by continued fresh media perfusion and glucose concentration is never allowed to fall below 1 g/L, hybridoma can be continuously cultured in the basket many days after the 9 day window provided in this study; this further increases productivity and decreases overall antibody production costs. No optimization of growth conditions were attempted for either bioreactor run.

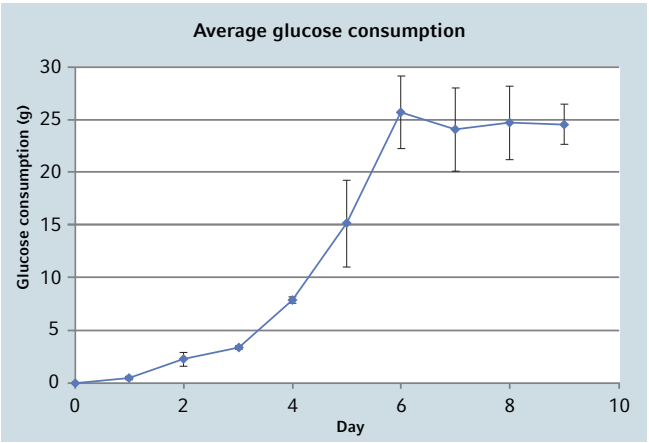

Figure 2. The glucose consumption was calculated daily for each bioreactor and the mean is presented. Error bars indicate standard error of the mean. Comparable consumption was observed across the two bioreactors.

Ordering information

Description	North America Order no.	International Order no,
New Brunswick™ CelliGen® 310 Bioreactor, 2.5 L System	M1287-1260 (100-120 V)	M1287-1264 (200-240 V)
Fibra-Cel® Disks, 250 grams	M1292-9988	M1292-9988
2.5 L Basket Impeller Kit	M1287-1140	M1287-1140

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APPLICATION NOTE No. 314 I February 2010

Optimization of HEK293 Cell Culture in a New Brunswick™ CelliGen® 115 Bioreactor for the Production of Recombinant GPCR

K. Christopher Min and Yan Jin, Neurological Institute, College of Physicians & Surgeons, Columbia University, New York, NY, USA
and Vikram Gossain, Eppendorf Inc., Enfield, CT, USA

Abstract

Heterologous expression of membrane proteins remains a bottleneck for structural characterization by x-ray crystallography. Such proteins represent approximately 30 % of the proteome and are not sufficiently represented in the Protein Data Bank (PDB)[1]. G-protein-coupled receptors (GPCRs) are an area of particular interest as it is estimated that one third of current FDA-approved drugs act through this class of receptors.

Introduction

We have been studying rhodopsin with an interest in determining the conformational change that leads to signal transduction in this class of receptors. Although there has been some success in expressing select members of the large GPCR family in bacterial systems, the best characterized expression systems have generally been in mammalian tissue culture. In our case, we isolated stable cell lines in which the desired receptor is expressed upon exposure to tetracycline. The cell line was derived from HEK293 cells, which can be grown in suspension. Attempts to scale up production of recombinantly-expressed protein by the use of spinner flasks were unsuccessful.

Based on our initial experiments using tissue culture plates, we had expected approximately 1 mg of recombinant protein for 1 L of cells grown in suspension, but found that expression levels in spinner flasks were closer to 0.1 mg per L. Use of a stirred-tank bioreactor allowed for optimization of cell growth, as described below, and resulted in higher cell densities with concomitant higher levels of expression of our recombinant protein.

The BioFlo/CelliGen 115 bioreactor equipped with a thermal mass flow controller and four-gas mix module.

Materials and Methods

Cell Line

The cell line, HEK293 GnT (N-acetylglucosaminyl-transferase I), was a generous gift from Phillip Reeves and H. Gobind Khorana[2]. It is a derivative of the standard HEK293 cell line that was selected by mutagenesis and ricin treatment to be deficient in N-acetylglucosaminyl-transferase I activity. GPCRs expressed in this cell line have a more uniform pattern of glycosylation which should result in a higher likelihood of crystallization. We also utilized the vector that Reeves, Callewaert et al. have described[2] which places receptor expression under the control of tetracycline exposure to the cells.

Bioreactor

We used the New Brunswick™ BioFlo®/CelliGen® 115 bioreactor equipped with a thermal mass flow controller and four-gas mix module. A pitched-blade impeller was driven by a magnetic motor, and the cultures were grown in a 7.5 L water-jacketed vessel.

Culture Media

DMEM/F12 supplied as a powder from Atlanta Biologicals (Lawrenceville, Georgia, USA) was used as the base media. This was supplemented with sodium bicarbonate (3.7 g/L), Primatone® RL-UF (0.3 g/L), 10 % heat-treated FBS, penicillin G (100 units/mL), streptomycin (100 µg/mL), glutamine (292 µg/mL), dextran sulfate (300 µg/mL), and pluronic F-68 (0.1 % w/v). The media was sterilized by filtration through a 0.2 µm membrane and pumped into the vessel.

Control Software

All equipment was monitored using New Brunswick BioCommand® software with data logging set at one-minute intervals.

Method

On the day before inoculation, the 7.5 L bioreactor vessel was filled with 4 L of phosphate buffered saline. The various ports were connected to appropriate tubing for removal of the saline, introduction of media, introduction of cells, and the pumping of the four-gas mix through the sparger. The pH electrode was calibrated and then disconnected and the protective cover was installed. The oxygen probe was examined and also covered by a protective cover. The jacket of the vessel was filled with water, and the assembly was set in autoclave for a 30-minute sterilization cycle. Afterward the vessel was returned to the tissue culture room and allowed to cool overnight. The following day the calibration of the pH electrode and oxygen sensor was checked after allowing the oxygen sensor to charge by the control unit.

A stable cell line which contains the expression cassette for the GPCR, under the control of a cytomegalovirus promoter/tetracycline-responsive promoter was selected using the neo gene. These cells were maintained in tissue culture plates with DMEM/F12 medium supplemented with 10 % fetal bovine serum, G418, and blasticin. For inoculation of a 7.5 L bioreactor vessel, thirty 15 cm plates were grown to approximately 80 % confluence. On the day of inoculation, 4 L of media were prepared and transferred to the vessel with a peristaltic pump after removal of PBS from the vessel.

All setpoints were programmed from BioFlo/CelliGen 115 control station as follows: temperature at 37 °C and a pH of 7.2. Oxygenation was maintained at 50 % using the four-gas mixture of air, nitrogen, oxygen, and carbon dioxide, and the thermal mass flow controller was set to deliver 0.5 L per minute. The pH was maintained by a combination of carbon dioxide and a solution of 7.5 % sodium bicarbonate that was controlled by pump 2. Agitation with the pitched blade impeller was set to 30 rpm.

The cells were recovered from the tissue culture plates by brief trypsinization and resuspension in the culture medium. The cells were pumped into the vessel with an auxiliary peristaltic pump. A small sample was removed and the starting cell density was determined with a hemocytometer.

Over the next five to seven days, the cell density was checked on a daily basis. Once the density reached 0.8 – 1.0 x 10^6, the culture was supplemented with 40 mL of 20 % (w/v) glucose and 120 mL of 10 % (w/v) Primatone RL-UF. The following day, expression was induced by the addition of tetracycline (2 µg/mL) and sodium butyrate (5 mM) to the culture (Figure 1). One day later, the cells were recovered from the bioreactor and pelleted by centrifugation. A 1 mL aliquot was reserved for analysis by Western Blot to determine the level of expression (Figure 2).

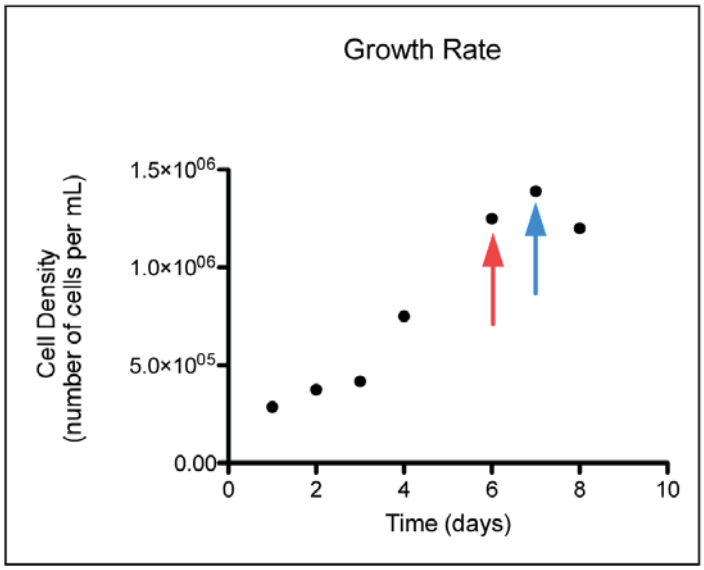

Figure 1. Growth curve for HEK293 cells in a CelliGen 115 bioreactor 7.5 L vessel. The red arrow indicates the addition of glucose and Primatone. The blue arrow indicates the addition of tetracycline and sodium butyrate for the induction of protein expression.

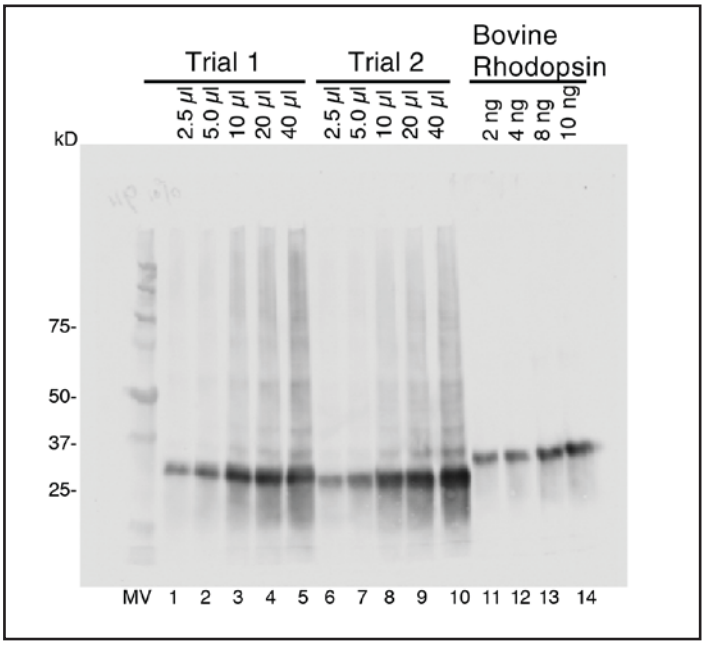

Figure 2. Western Blot with mAb for detection of GPCR protein expressed in HEK293 cells.

Results and Discussion

The expressed GPCR was solubilized by lysing the cell pellet from the small aliquot with a buffer containing 1 % (w/v) dodecyl-maltoside. The expressed GPCR was detected using a Western Blot with a monoclonal antibody, and the signal detected was compared to rhodopsin purified from cow retinae. We detected approximately 1 mg of recombinant GPCR per L of cell culture. The migration of the recombinant protein was probably due to differences in glycosylation. This was a dramatically improved result when compared to cell growth in suspension with spinner flasks where a cell density above 0.5 x 10^6 was hard to achieve. In experiments using the same cell line performed in spinner flasks, the expression level of recombinant GPCR ranged from 0.1 – 0.2 mg/L of culture (Table 1).

Yields	7 L Spinner Flask	BioFlo/CelliGen 115 Bioreactor 4.5 L Run	BioFlo/CelliGen 115 Bioreactor Two 4.5 L Runs
Maximum Cell Density	0.5 x 10^6/mL	1.4 x 10^6/mL	1.4 x 10^6/mL
Protein Culture Volume	0.1–0.2 mg/L	1 mg/L	10 mg/L

Table 1. Recombinant protein (rGPCR) expression comparisons.

A large-scale prep (two 4.5 L runs) was subsequently performed, and 10 mg of purified rGPCR were obtained in a detergent solubilized form. A G-protein activation assay in which uptake of a radio-labeled non-hydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirmed the bioactivity of the recombinant protein. The reaction was started by the addition of GTPγS, and aliquots of the reaction were applied to nitrocellulose filters at various times. In the absence of a receptor, very little spontaneous uptake of the radio-labeled nucleotide was detected. The form of the receptor expressed in the experiments contained mutations in which residues were altered to cause constitutive activation. The receptor expressed in the bioreactor caused an increase in the rate of nucleotide uptake by transducin, as expected (Figure 3).

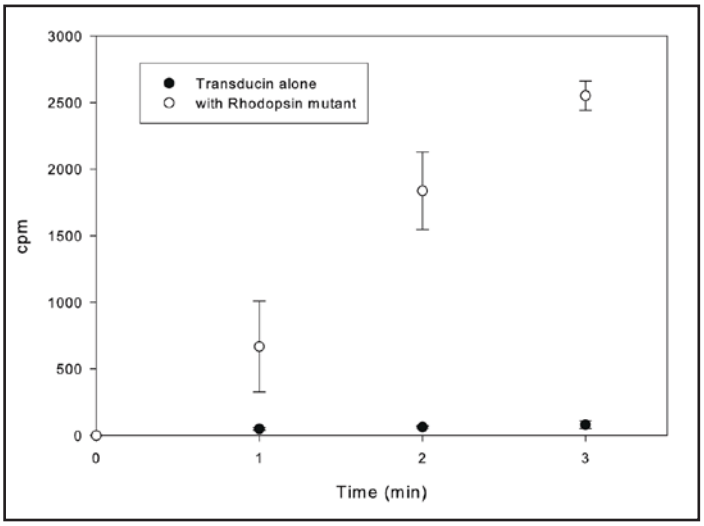

Figure 3. The bioactivity of the expressed rGPCR protein in the bioreactor was measured using G-protein activation assay using [^{35}S]-GTPS binding assay. Uptake of a radio-labeled nonhydrolyzable analog of GTP by transducin was measured as a function of time in the presence of the recombinant receptor, confirming the bioactivity of the recombinant protein.

Conclusion

This study demonstrates that by being able to control the cell culture process parameters using a cell culture bioreactor, both the HEK293 cell density and expression levels of the rGPCR dramatically increased in comparison to using a spinner flask or tissue culture plates. The bioactivity of the rGPCR was good, however a change in the level of glycosylation of the recombinant protein was indicated by the positions of the rGPCR bands relative to the standard protein band in the Western Blots.

References

[1] **Worldwide Protein Data Bank information portal to biological structures:** http://www.rcsb.org/pdb/static.do?p=search/index.html.

[2] **Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line.** Reeves PJ, Callewaert N, et al. *Proc Natl Acad Sci USA* 2002; 99 (21): 13419 -24.

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APPLICATION NOTE No. 316 I July 2008

Sf-9 Insect Cell Culture Using a New Brunswick™ CelliGen® 310 Bioreactor: Using Headspace Air Overlay for Reduced dCO_2

Vikram Gossain, Guozheng Wang and Wenying Zhang, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

This study describes a simple procedure for improving insect cell yields in a benchtop cell culture bioreactor. Here, yields of *Spodoptera frugiperda* (Sf-9) cells were increased by nearly 29 % through monitoring dissolved carbon dioxide (dCO_2) levels in the culture and adding air to the vessel headspace to reduce dCO_2. The method can also be used to maximize yields in a wide range of mammalian cell types.

Introduction

Producing high yields of protein from insect cells usually requires maintaining high levels of dissolved oxygen (DO) in the culture. However, as cell concentrations grow, they generate an ever-increasing level of dCO_2, which can inhibit cell growth. We compared growth of Sf-9 insect cell yields in two runs, first without control of dCO_2 levels and then by continuously flowing air into the vessel headspace above the liquid media level to reduce dCO_2 concentration. This insect cell culture protocol has not been fully optimized to obtain the highest yields possible, but it is meant to serve as a guide for basic procedures and materials.

The benchtop CelliGen 310 bioreactor is a versatile research tool for optimizing cell growth and production of mammalian, insect, and plant cell lines. Multiple connections, easily accessible from the side and rear, provide ability to integrate data from all your ancillary devices, such as dCO_2 sensors, a gas overlay controller, and SCADA software. A large touchscreen interface makes it easy to set up, calibrate, and monitor each run, as well as export data and trend graphs to a PC.

Materials and Methods

Bioreactor

We used a 2.5 L total volume New Brunswick CelliGen 310 benchtop autoclavable bioreactor with a pitched-blade impeller. The CelliGen 310 is a cGMP-compliant system designed for high-density growth of mammalian, plant, and insect cell lines. It comes standard with a large 15-inch industrial color touchscreen interface with an advanced controller to simplify setup, calibration, and control. The bioreactor includes three built-in pumps, pH/DO and level/foam probes, and one thermal mass flow controller (TMFC) for regulating gas flow. The CelliGen 310 used in this study had a TMFC range of 0.1 – 5 L/min (other flow ranges or choice of multiple TMFCs are also available).

We added three optional accessories. A Mettler-Toledo® dCO_2 sensor and transmitter were connected to the CelliGen 310 to measure dCO_2 concentration throughout the process. A New Brunswick accessory gas overlay controller with a flow range of 0.1 – 5 L/min (capable of regulating four gases) was used to regulate addition of air to the vessel headspace. And an optional New Brunswick BioCommand® supervisory software package was also used to automatically log data. Additonally, a gas overlay vessel kit that includes neccessary tubes, filters, and fittings is highly recommended.

Overview

1. Autoclave the vessel with phosphate buffer solution (PBS) for 60 minutes.
2. Remove PBS from the vessel.
3. Add 1.5 L of insect cell media to the vessel.
4. Inoculate 500 mL of insect cell suspension with a starting cell count of 4.0×10^5 cells/mL.

Medium

We used Sf-900 II serum-free media.

Inoculum

A proprietary Sf-9 cell line was supplied by a leading biotechnology company. The inoculum was cultivated in an Eppendorf New Brunswick open-air Innova® 2000 shaker placed inside an incubator for temperature regulation.

Control Setpoints

The following setpoints were keyed into the touchscreen controller prior to inoculation:

> Temperature 28 °C
> DO 40 %
> Agitation 70 rpm, gradually increased to 100 rpm over the course of the run

DO Control

The DO probe provided as part of the CelliGen 310 kit was calibrated at 0 % (obtained by briefly disconnecting the cable), then calibrated at 100 % (obtained using 100 rpm agitation and 5 L/m airflow rate). The control was set to 4-gas mode to automatically maintain the DO setpoint by sparging three gases (air, O_2 and N_2).

pH Control

Although pH control capability is built into the CelliGen 310 bioreactor, it is not generally needed for controlling insect cell growth and was therefore not regulated in this study.

Fed-Batch Control

Pumps were calibrated using standard supplied tubing to keep track of liquid quantities entering and exiting the vessel. Samples were taken several times a day (as described below) to measure glucose and cell density.

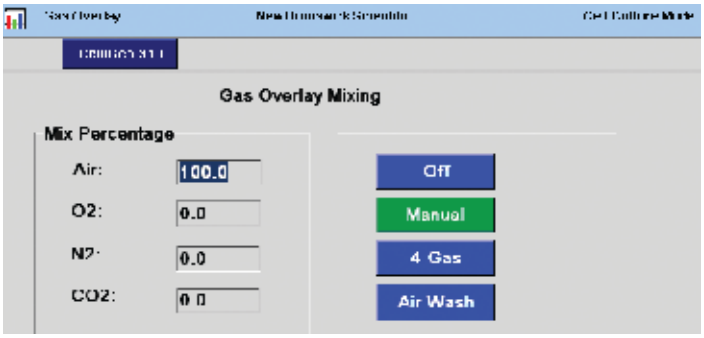

Figure 1: The CelliGen 310's gas overlay option enables addition of up to four gases into the vessel headspace, as shown in this screen capture.

4. Select "Calibrate" and either "15", "30," or "60" seconds as the time interval. Record the quantity of water pumped into a graduated cylinder for the defined time period, and enter that into the "Flow Rate" field, then hit "OK." Your flow rate will be automatically calculated.

Control Program

For these studies, we used one of three built-in pumps. When cells reached a density of 6×10^6 cells/mL, 20 mL/L of Gibco® Yeastolate (Life Technologies® Catalog No. 18200-048) was automatically added by Pump 1. In both runs, O_2 was sparged into the liquid media and controlled by the CelliGen 310's built-in 4-gas controller. In run 1, we added no gas overlay into the vessel headspace. In the second run, all conditions remained the same, but 300 mL/min of air was continuously added to the vessel headspace to reduce dCO_2 concentration (control screen shown in Figure 1).

Results and Discussion

As shown in Figure 2, maximum viable cell density in run 1 reached 9.18 × 10^6 cells/mL on day 9. In run 2, maximum cell density increased by 28.5 %, reaching 11.8 × 10^6 cells/mL on day 6. This study shows that by reducing dCO_2 concentration, maximum cell density not only was significantly improved, but was also achieved at a faster growth rate.

It should be noted that the CelliGen 310 bioreactor can simultaneously control more than 120 process loops (32 loops per vessel, four vessels simultaneously), making it an extremely powerful research tool. It can be operated in batch, fed-batch, or perfusion modes and comes with a choice of four interchangeable vessels (2.5 – 14.0 L) as well as a wide range of specialized impellers to maximize yields. For secreted products, a packed-bed basket option is available to maximize cell productivity regardless of cell type. The system includes multiple analog inputs/outputs for easily integrating data from up to 10 ancillary devices, such as additional TMFCs, sensors, scales, or on-line gas and glucose analyzers for optimized process control. However, we did not take advantage of the full potential of the 310, intending only to provide an easy technique for increasing yields in insect cell culture. This technique is also very useful in maximizing yields in a wide range of mammalian cultures. For system specifications or to request additional information see www.eppendorf.com.

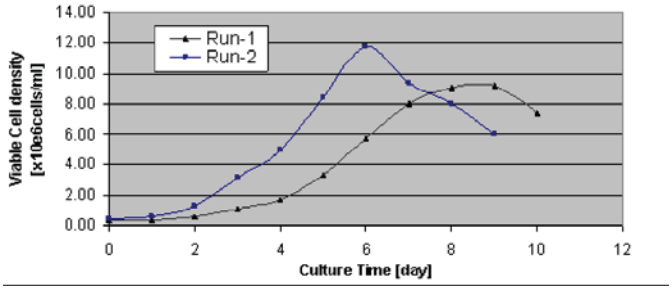

Figure 2: Without air supplementation, run 1 reached a maximum of 9.18 × 10^6 cells/mL on day 9. When an air overlay was added in run 2 to reduce dCO_2 levels, viable cell density increased by nearly 29 %, reaching 11.8 × 10^6 cells/mL on day 6. This shows that when dCO_2 levels were reduced, cell density not only increased, but was achieved at a faster growth rate.

Ordering information

Description	Order No.
New Brunswick™ CelliGen® 310 120V / 2.5 L System	M1287-1260
New Brunswick™ CelliGen® 310 2.5 L Packed Bed Impeller Kit	M1287-1140
New Brunswick™ CelliGen® 310 Gas Overlay (Rotameter)	M1287-3550
New Brunswick™ CelliGen® 310 Gas Overlay Vessel Kit	M1287-3505
New Brunswick™ Fibra-Cel® disks (50g)	M1292-9984
New Brunswick™ BioCommand® Batch Control	M1326-0010
Mettler-Toledo® CO_2 Sensor	P0720-6480
Mettler-Toledo® M400 transmitter	P0620-6581

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APPLICATION NOTE No. 256 I July 2012

Insect Cell Culture Using the New Brunswick™ BioFlo®/CelliGen® 115 Benchtop Fermentor/Bioreactor with Spin Filter Assembly

James Jarvis, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

This application report presents a simple protocol for achieving high-density culture of *Spodoptera frugiperda* (Sf9) cells using a New Brunswick bench-top, autoclavable stirred-tank reactor with a spin-filter assembly. Factors such as substrate concentration and metabolite buildup can be limiting for culture growth and viability at high densities. Using the spin filter in a 3.0 L vessel (2.0 L working volume) attached to the BioFlo®/CelliGen® 115 cabinet, a cell density of 18.24 x 10^6 cells with viability over 90 % was achieved, outperforming the batch or fed-batch process.

Introduction

Stirred-tank bioreactors are widely used for research and industrial applications for cultivating a wide variety of cells types, including insect cells, Hybridoma, CHO, BHK21, HEK293, and others; these cultures manufacture viral vaccines and human therapeutic proteins such as monoclonal antibodies, blood clotting factors, etc.

The BioFlo®/CelliGen® 115 features an easy-to-use control station with color touch-screen monitor and built-in capability to operate in either fermentation or cell-culture modes. Switching between the operating modes automatically adjusts the control settings. Three fixed-speed pumps, temperature and agitation control, and one rotameter with choice of gas flow ranges are available in the BioFlo®/CelliGen® 115 systems. Pre-packaged kits for Basic or Advanced Fermentation and Advanced Cell-Culture simplify ordering. All kits include options for direct-drive or magnetic-drive agitation as well as water-jacketed or heat-blanketed vessels in 1.3 - 14.0 L (total volume) capacities. pH/DO and foam/level controllers can be included depending on the selected kit, or can be added individually as options. Options for additional rotameters or thermal mass flow controllers (TMFC) are also available.

Spodoptera frugiperda, also known as the *Fall Armyworm* or Sf9, are insect cells commonly-used for the production of proteins of interest in pharmaceutical research due to their unique ability to replicate mammalian post-translational modifications such as glycosylation. Insect cells produce a variety of proteins utilizing the Baculovirus Expression System (BEVS). Cell lines such as Sf9, Hi-5, Sf21, etc., are proven to express high levels of end products.

Insect cell culture can be achieved by using batch, fed batch or perfusion methods. For this study, the perfusion method was used in conjunction with New Brunswick spin filter assembly.

The spin filter allows for the removal of exhausted media without removing the cells in suspension, making room for fresh media addition, thus achieving and maintaining the highest culture densities possible.

Figure 1: BioFlo®/CelliGen® 115 systems feature a compact control station capable of either fermentation or cell culture operating modes to accommodate growth of a wide variety of cell types. A built in color touch-screen interface facilitates set-point control and monitoring. The BioFlo®/CelliGen® 115 system shown above is equipped with a 3.0 L water-jacketed vessel with pitched-blade impeller and four rotameters.

Figure 2: New Brunswick Impellers: Pitched Blade, left; Spin Filter with marine blade, right.

Materials and Methods

Bioreactor

For this application, a standard 3.0 L BioFlo®/CelliGen® 115 advanced cell culture kit with a magnetic drive and water-jacketed vessel were used (order no. M1369-1162). A Suspension-Cell Spin Filter with 10µ screen (order no. M1273-3202) was used to grow the insect cells in a continuous, high flow rate perfusion mode. In addition, BioCommand® Batch Control software (order no. M1326-0010) was used to monitor the system and control the feeding schedule.

Medium

This application used an animal-component-free-chemically-defined ESF-921 medium from Expression Systems (Woodland, CA).

Inoculum

The cell stock used was Sf9 cells derived from ATCC CRL-1711 adapted to a serum free environment, obtained from Expression Systems (Woodland, CA). Inoculum was cultivated in a New Brunswick shaker (Innova 40, order no. M1299-0092).

Controller Setpoints

Calibrate pH probe prior to autoclaving. Enter controller set points prior to inoculation and allow the media equilibrate to prior to proceeding. The DO may remain high after calibration and before inoculation due to the absence of cells consuming it. An initial DO value of > 95 % is acceptable; it will decrease as the cells start to metabolize it. Normal set points for *Spodoptera* cells are controlled by the Primary Control Unit (PCU) and are as follows:

Parameter	Setpoint
Temperature	28° C
Dissolved Oxygen	50
pH	6.3
Agitation	100
Gas Control	4-gas mode
Inoculum	4.1×10^5

DO Calibration

The DO probe is calibrated using a standard two-point calibration method: 0 % (often referred to as the zero point) and 100 % (often referred to as span). The zero can be achieved by either disconnecting the DO cable (the electronic zero; used in this process) or by sparging N_2 into the media to achieve a level stable near zero. The 100 % calibration point is achieved by bringing the vessel filled with medium to all of the operational set points, i.e. agitation, temp, etc. DO should be calibrated post-autoclave and pre-inoculation after a six hour polarization period. After calibration, the DO may remain around 100 % until after inoculation.

pH Calibration

The pH probe was calibrated prior to the autoclave cycle outside the vessel using a two-point calibration method with standard pH buffers. The pH7.0 buffer is used to zero the probe and the pH4.0 or 10.0 can be used as the span (Refer to the BioFlo® 115 Instruction Manual).

pH Control

The pH for insect cells normally does not drift much from set point, but at higher culture densities the pH may drop. The pH parameters are maintained by the addition of CO_2 to lower the pH or an 8 % sodium bicarbonate solution to raise the pH. The dead band was set to 0.1 for this run.

Gas Control

The BioFlo®/CelliGen® 115 was set to the 4-gas mode to maintain the DO and pH set points automatically. The cascade in 4-gas mode was set to gas flow and the O_2 control was set to 4-gas mode.

Continuous Feed (Perfusion)

All pumps were calibrated using the standard, supplied tubing to track liquid quantities entering and exiting the vessel. Samples were taken several times a day to measure the density of the culture as well as nutrient consumption.

Controller Setup	
Pump 1	Base addition dependent on pH accomplished through the tri-port adapter in the vessel head plate.
Pump 2	Harvest of spent media accomplished with a level sensor configured to a predetermined level with a dip tube to the interior of the spin-filter. This allows for the extraction of media without the loss of cell density.
Pump 3	Perfusion in of fresh media at a predetermined rate.

Control Program

For this study, a BioFlo®/CelliGen® 115 for the control of the pH, DO, Level sensor, and pumps was used; BioCommand® Plus software was also used to monitor the culture parameters.

Results and Discussion

Insect cells generally have a high demand for oxygen during protein production. Maximum growth rate and high cell densities are achieved by keeping the DO at a constant set point.

Factors such as substrate concentration and metabolite buildup can be limiting factors; these were made more controllable through the abilities of the BioFlo®/CelliGen® 115 Bioreactor with the Advanced cell culture kit (order no. M1369-1162) coupled with a spin filter kit (order no. M1273-3202).

The BioFlo®/CelliGen® 115 system allowed for the growth of insect cells to a final density of 18.24 x 10^6 cells/mL. The inclusion of the TMFCs (Thermal Mass Flow Controller) provided the ability to mix the four gasses according to culture needs and further enhanced the final culture density.

Conclusion

Considering the above results, we can view the viable cell density of 18.24 x 10^6 as proof of the fundamental capabilities of the BioFlo®/CelliGen® 115 system.

The temperature of the system remained steady and was controlled by using un-chilled tap water as the coolant.

DO and pH control remained stable and consistent throughout the experiment.

Overall, The BioFlo®/CelliGen® 115 system performed extremely well. The BioFlo®/CelliGen® 115 advanced cell culture system with spin filter assembly is recommended for insect cell culture to achieve high cell densities.

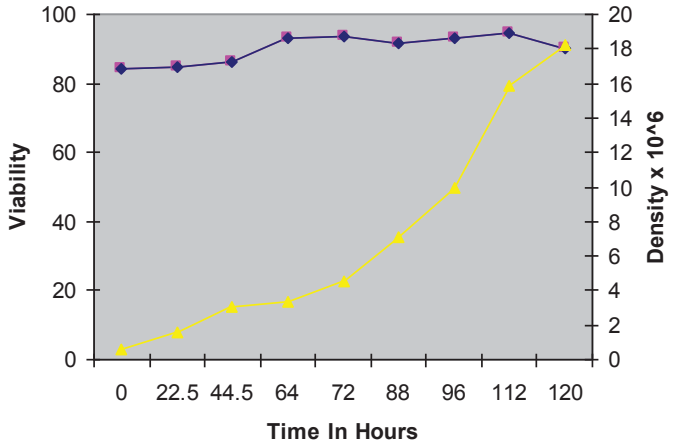

Figure 3: Insect cell Sf9 perfusion culture in a 3 L BioFlo®/CelliGen® 115 bioreactor with Spin Filter Impeller. The cells were inoculated from 1000mL shaker flask culture. The inoculum cell density was 4.9 x 10^5 cells/ml. After two days of the batch process, medium perfusion was started at the rate of 0.5 - 2 L working volumes per day.

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APPLICATION NOTE No. 259 I September 2013

A Novel Method for the Expansion of Mesenchymal Stem Cells using a New Brunswick™ S41i CO_2 Incubator Shaker

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Abstract

The expansion of stem cells, including mesenchymal stem cells (MSC), has been successfully demonstrated using microcarrier-based small bioreactors such as spinner flasks. In this study, we explored a simple alternative for microcarrier-based MSC expansion using conventional shake flasks. This method relies on a new type of CO_2 incubator with built-in shaking capability, i.e. New Brunswick S41i CO_2 Incubator Shaker. The expansion of adipose-derived mesenchymal stem cells (AdMSCs) was compared between shake flasks and spinner flasks using microcarriers. The AdMSCs were seeded at a density of $3x10^3$ cells/cm^2 in both setups, each containing 0.5 g plastic microcarriers and 40 mL of stem cell growth medium.

The cell culture experiments were conducted for 12 days and samples were collected daily for analysis of cell growth, biochemistry and metabolites. Cell density studies revealed that AdMSCs cultured under shake flask conditions achieved excellent growth under 12 day batch-culture conditions.

Lastly, the AdMSCs expanded using the shake flask method remained high quality stem cells, which was evident by CD44 and CD90 stem cell marker assays and their ability to differentiate into either adipocytes or osteocytes.

Introduction

Stem cells are undifferentiated cells which have the capability of self-renewal and the potential to divide for a long period of time. They have the ability to differentiate into various specialized cells when appropriate growth factors and conditions are provided. Stem cells can be broadly classified as: embryonic, adult, and induced pluripotent stem cells (iPS). Adult stem cells can be further characterized by their tissue of origin, such as: hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicle stem cells. Most of the studies performed on adult stem cells utilize either hematopoietic or adipose-derived mesenchymal stem cells[1]. Like other adult stem cells, adipose-derived mesenchymal stem cells (AdMSCs) express all of the common stem cell markers and can be differentiated into various types of specialized cells under appropriate growth conditions. AdMSCs have advantages over other mesenchymal stem cells (MSCs), since they can be isolated in large quantities from fat tissue and are resistant to apoptosis[2].

Although MSCs have enormous advantages for regenerative medicine, drug screening and drug discovery, their applications are limited by the quantities required for industrial or clinical applications[3]. In this study, we developed a simple shake flask culture technique to expand MSCs on microcarrier beads which can be used to scale-up into large-scale bioreactors. The microcarrier shake flask culture, which requires both agitation and CO_2 gas control, was conducted in the Eppendorf New Brunswick S41i CO_2 incubator shaker.

The New Brunswick S41i CO_2 Incubator Shaker, designed for both non-adherent and adherent cell culture applications, combines the precise temperature and CO_2 control of an incubator with the reliable New Brunswick laboratory shaker drive mechanism. Key features include sealed inner/outer doors, high-temperature disinfection, and reduced CO_2 consumption compared to competitor models[4].

Materials and Methods

Initial cell culture in T-Flasks
AdMSCs were obtained from ATCC® (PCS-500-011) at passage 2 and cells were seeded at a density of 5,000 cells/cm^2 into a T-75 cm^2 flask (Eppendorf) using 15 mL of mesenchymal stem cell basal medium (ATCC) supplemented with 2 % fetal bovine serum, 5ng/mL rh FGF basic, 5ng/mL rh acidic, 5 ng/mL rh EGF and 2.4 mM L-Alanyl-L- Glutamine (ATCC).

Cultivation of cells on microcarriers
Prior to start of the experiment, 0.5 g of 125-212 micron polystyrene microcarriers (SoloHill®) (180 cm^2 for a 50 mL culture) was transferred into a siliconized (Sigmacoat®; Sigma) 250 mL spinner flask (Corning®) and shake flasks (Schott®, Duran®) along with 25-30 mL of PBS. The flasks were then autoclaved at 121 °C for at least 30 minutes. Microcarriers were allowed to settle to the bottom of the shake/spinner flasks and the autoclaved PBS buffers were carefully aspirated with the electronic pipetting aid easypet® (Eppendorf) equipped with a 25 or 50 mL pipette. The AdMSCs were initially seeded at a density of 3x10^3 cells/cm^2 into both flasks, each containing 40 mL of basal mesenchymal stem cell medium. For the initial attachment of cells, the agitation speed of the New Brunswick S41i CO_2 incubator shaker and rotation speed of the spinner (housed inside of an Eppendorf Galaxy® 170 R CO_2 incubator) were both kept at 50 rpm and incubated for 2 hrs at 37 °C with 5 % CO_2. After incubation, the cell culture volume was adjusted to 50 mL total with 10 mL of medium containing serum to reach a final FBS concentration of 4 % and targeted final concentration of growth supplements (10 ng/mL final concentration of rh FGF basic, rh FGF acidic & rh EGF and 4.8 mM final concentration of L-Alanyl-L-Glutamine). Following the addition of FBS and growth supplements, the rotation speed of the spinner and the agitation speed of New Brunswick S41i CO_2 incubator shaker were both raised to 70 rpm. After 18 to 24 hrs of incubation, 1 mL of homogeneous samples containing both media and microcarriers were collected for microscopic observations, cell counting as well as biochemistry analysis.

Cell counting
Cells on microcarrier beads were counted by hemocytometer. To accomplish this, microcarrier beads were suspended in citric acid solution containing crystal violet (0.1 % crystal violet in 0.1 M citric acid solution) equal to the volume of supernatant removed from the tube. The contents of the tube were incubated for 1 hr or overnight at 37 °C and vortexed for a few seconds to release the stained nuclei. The nuclei were counted with hemocytometer.

Biochemistry and metabolites analysis
The supernatants collected during cell counting were used for biochemistry and metabolite measurements using an automated YSI® 2950 Bio-analyzer.

Stem cell surface marker assay
To assess the quality of AdMSCs after expansion and to confirm that the stem cell markers were retained during the microcarrier-based culture, CD44 and CD90-specific fluorescent immunoassays were performed using the following procedure. 5 mL samples were collected from both the spinner and shake flasks near the end of microcarrier culture. After the microcarriers settled to the bottom, the supernatants were removed and the microcarrier beads containing cells were gently washed 3 times with PBS at room temperature. Cells on the microcarrier beads were then fixed with 4 % paraformaldehyde for 30 minutes followed again by PBS washing 3 times. Cell-containing microcarrier beads were blocked with 5 % FBS at room temperature for 1 hr and immunostained with FITC-conjugated antihuman CD44 (BioLegend®) and APC-conjugated antihuman CD90 (BioLegend®) antibody solutions, also for 1 hr at room temperature. The beads were washed 5 times with room temperature PBS for 5 min and visualized using an EVOS® FL fluorescence microscope.

Stem cell differentiation assays
AdMSCs were harvested from both shake and spinner flasks into 50 mL tubes. Once the microcarrier beads settled to the bottom of the tube, the supernatants were removed and cells were washed with DPBS. Afterwards, the microcarrier beads were treated with 5 mL of prewarmed trypsin-EDTA solution at 37 °C for 10 min. During incubation, the tubes were occasionally vortexed for 2 sec and then neutralized by adding an equal volume of trypsin neutralizing solution. Microcarrier beads were a llowed to settle to the bottom of the tube and the supernatants were collected as soon as possible. Microcarrier beads were washed 2-3 times with DPBS and as much supernatant as possible was collected into a 50 mL tube. Following washing, AdMSCs were collected to bottom of the tube by centrifugation at 120 xg for 5 min and resuspended in 5 mL of mesenchymal stem cell medium. Cells were seed at a density of 18,000 cells/cm^2 into a 24-well plate. Adipocytes and osteocyte differentiations were performed on those cells using differentiation assay kits from ATCC. Adipocyte and osteocyte differentiated cells were identified by cell-type specific staining with either Oil red O or Alizarin red S kits (ScienCell®) according to manufacturer instructions and visualized using an OLYMPUS® CK40 microscope.

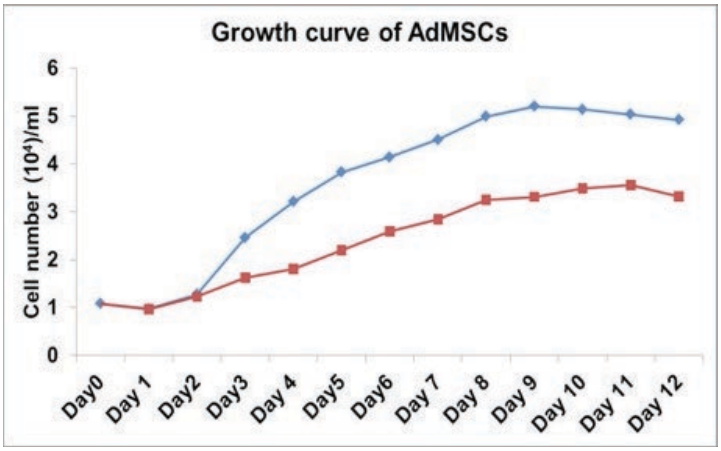
Results and Discussion

To compare between shake flask and spinner flask cultures, AdMSCs were seeded at a density of 3x10^3 cells/cm^2 in both systems. Cell culture studiess were conducted for 12 days and samples were collected for cell growth, biochemistry and metabolite analysis daily. Cell growth studies revealed that AdMSCs cultured under shake flask conditions achieved excellent growth during the 12 day batch culture (Figure 1A). Biochemistry and metabolite analysis revealed that glucose concentrations decreased from 1.09 g/L to 0.548 g/L (for shake flask culture) and 0.798 g/L (for spinner culture), whereas lactate concentrations increased from 0.042 g/L to 0.396 g/L (for shake flask culture) and 0.259g/L (for spinner culture) after 12 days of culture (Figure 1B &C).

The higher glucose consumption and lactate production rate seen in the shake flask culture supports the finding that the stem cells grew at a faster rate under the shake flask conditions. Furthermore, during early growth phase (day 4); the amount of ammonium accumulated in spinner flask culture (2.4 mM) was 1.8-fold higher than shake flask culture (1.3 mM) (Figure 1D). It has been shown that even low level of ammonium (1.9 mM) inhibits MSC growth[5]. The spinner culture has shown ammonium level exceeding 2 mM early and throughout the culture process, which indicates the slower growth by the spinner method could be a result of ammonium toxicity-induced growth inhibition. The fact that spinner culture had elevated ammonium levels early in the culture not seen in the shake flask also indicates possible stem cell damage due to shear force by the spinner rod. The spinner rod was observed to display a “stop & go” motion at low speeds; precise speed control is not possible, especially at low rotation speeds. However, our observations were based on the specific spinner device available at our research facility, the results may not represent typical or average performance from spinner devices available in the market place.

To determine whether or not AdMSCs retained their stem cell properties during their growth under shake flask conditions, immunostaining of stem cell surface markers and differentiation assays were performed. Microcarrier beads that contained AdMSCs were immunostained with stem cell surface marker antibodies such as: FITC-conjugated antihuman CD44 and APC-conjugated antihuman CD90 and revealed that AdMSCs retained stem cell surface markers during growth under shake flask culture condition (Figure 2A&B). For the adipocyte and osteocyte differentiation assays, AdMSCs were collected from the microcarrier beads and seeded into 24 well plates that contained either adipocyte or osteocyte differentiation medium. After 17 days of culture, the plates were stained with Oil Red O or Alizarin Red S staining solutions, respectively. Microscopic observation indicated that most of the AdMSCs from shake flask culture differentiated into either adipocytes or osteocytes successfully (Figure 3A&B).

A.



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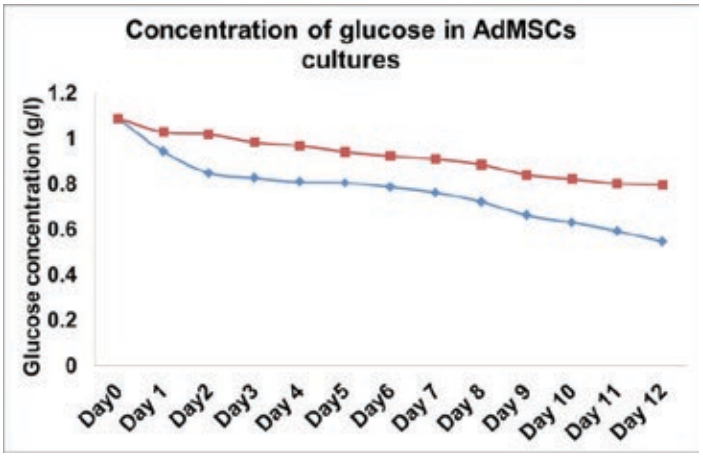

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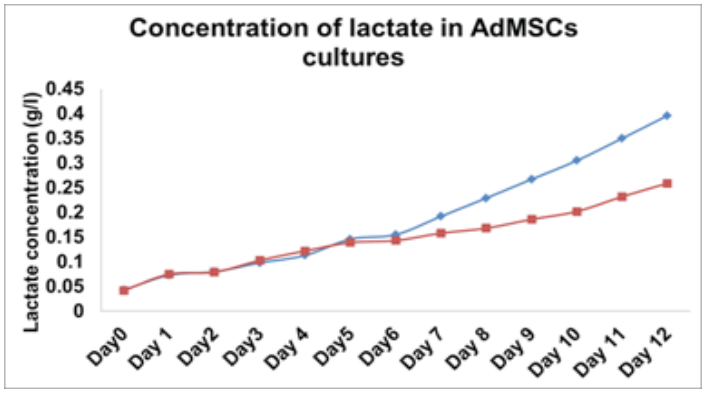

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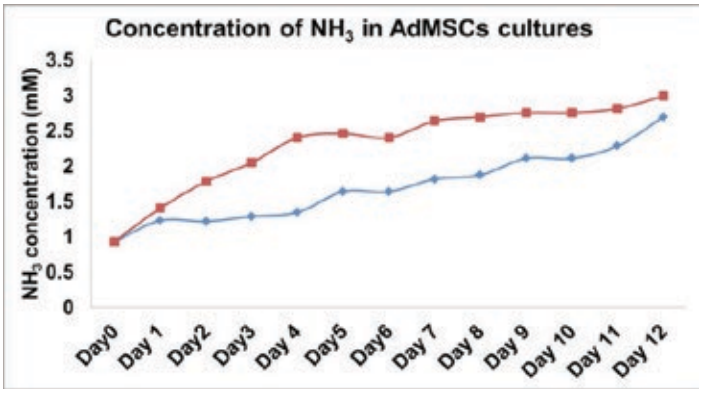
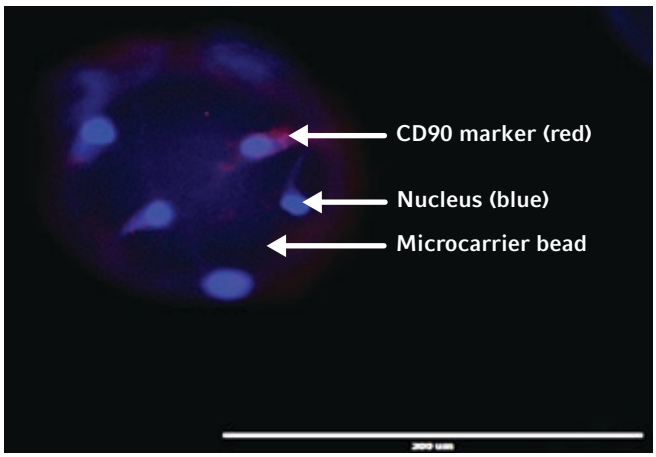


Figure 1. Analysis of AdMSCs growth and metabolism in shake flask and spinner flasks culture conditions: A) growth; B) glucose utilization; C) lactate production and D) ammonium production.(◆) shake flask and (■) spinner flask.

A.



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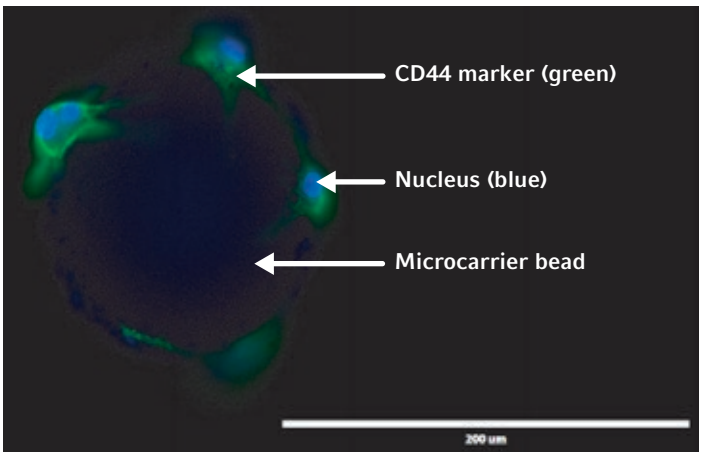
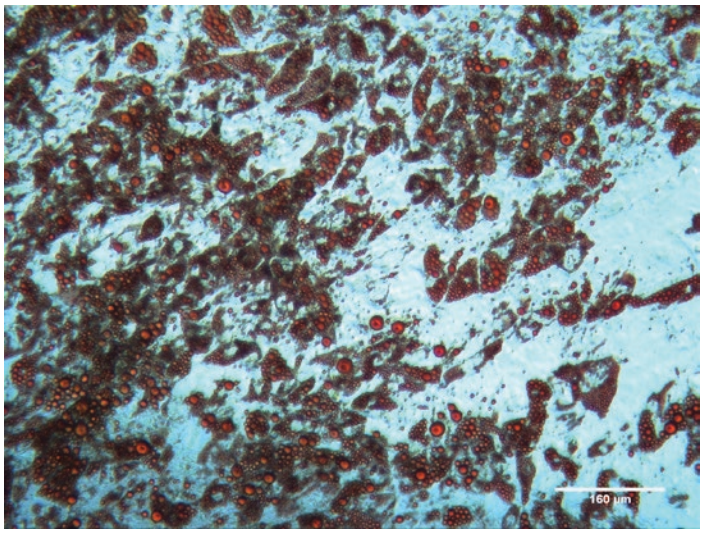


Figure 2. Stem cell marker identification assay for AdMSCs expanded on microcarriers in shake flask. A) AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging. B) AdMSCs on microcarrier beads are positive for CD 44 stem cell marker, as indicated in green by Fluorescence Imaging. Blue color indicates stem cell nuclear staining by DAPI.

A.



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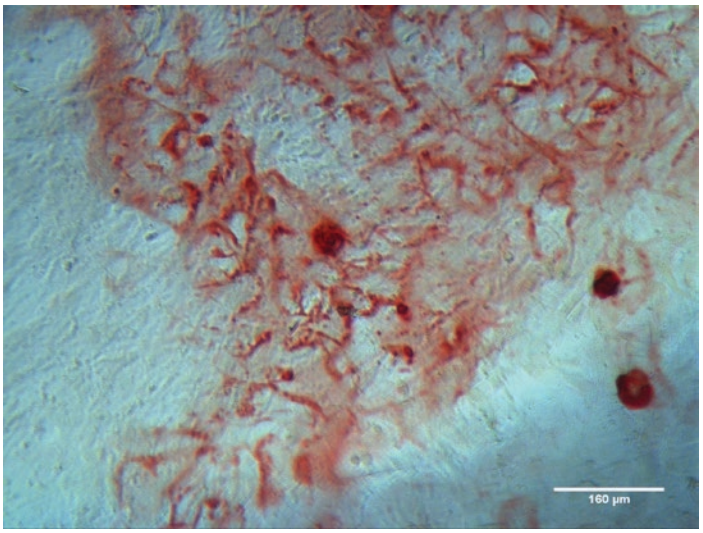

Figure 3. Differentiation assays for AdMSCs expanded on microcarriers in shake flask. A) Adipogenic differentiation formed lipid droplets as indicated by Oil red O positive staining. B) Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by Alizarin Red S positive staining.

Conclusions

Stem cell expansion using shake flask conditions appears to be a viable and simple alternative to the spinner flask system. This novel method relies on a new type of CO_2 incubator with built-in shaking capability, such as the New Brunswick S41i CO_2 Incubator Shaker. The New Brunswick S41i reduces shearing, eliminates potential cell damage by the spinner rod, decrease the risk of contamination associated with inserting a magnetic stirrer base into the CO_2 incubator and reduces experimental complexity. This method also greatly increases the cell culture capacity whereby a large number of shake flasks can be placed in the New Brunswick S41i simultaneously. In the case of spinner flask culture, a typical incubator without active cooling can only handle the heat emitted from a very limited number of magnetic stirrer bases before causing temperature setpoint overshoot, a significant limitation to the scale-up potential of the spinner method. This reinforces the superiority of New Brunswick S41i CO_2 Incubator Shaker as an alternative to incubator/spinner based stem cell culture. This method eliminates a scale-up bottleneck while providing the highest quality stem cell culture for inoculation of large scale industrial bioreactors for the production of clinical material. The shake flask has lower cost and less parts to disassemble, clean, assemble and autoclave.

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Ordering information

Description	International order no.	North America Order no.
New Brunswick™ S41i CO_2 Incubator Shaker, 120 V, 60 Hz		S41I-120-0100
New Brunswick™ S41i CO_2 Incubator Shaker, 230 V, 50 Hz	S41I-230-0100	
Options		
Stacking kit	P0628-6502	P0628-6502
Additional perforated shelf	P0628-6181	P0628-6181
Interchangeable platforms, 612 x 356 mm (24 x 14 in)	M1334-9920	M1334-9920
Universal platform	M1334-9921	M1334-9921
250 mL Dedicated platform	M1334-9922	M1334-9922
500 mL Dedicated platform	M1334-9923	M1334-9923
1 L Dedicated platform	M1334-9924	M1334-9924
2 L Dedicated platform	M1334-9925	M1334-9925
2.8 L Dedicated platform	M1334-9926	M1334-9926
4 L Dedicated platform	M1334-9927	M1334-9927
Galaxy 170 R CO_2 Incubator (High-Temp Disinfection,1-19 % O2 Control)	CO170R-230-1200	CO170R-120-1200
T-75 Tissue Culture Flask 5 flasks per bag, 100 flasks per case (Available in China, India and Italy)	0030 711.106	
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APPLICATION NOTE No. 292 I October 2013

Scalable Expansion of Human Pluripotent Stem Cells in Eppendorf BioBLU® 0.3 Single-Use Bioreactors

Ruth Olmer[1], Christina Kropp[1], Claudia Huether-Franken[2]* and Robert Zweigerdt[1]

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Abstract

The routine application of human pluripotent stem cells and their derivatives in regenerative medicine and innovative drug discovery will require the constant supply of high cell numbers in consistent, high quality. Well monitored and controlled stirred-tank bioreactors represent suitable systems to establish up-scalable bioprocesses enabling the required cell production. The following application note describes the successful cultivation of human pluripotent stem cells in suspension culture using Eppendorf BioBLU 0.3 Single-use Vessels in a DASbox® Parallel Mini Bioreactor System.

Introduction

Human pluripotent stem cells (hPSCs), comprising human embryonic stem cells (hESC) und induced pluripotent stem cells (hiPSC), and their derivatives are considered promising cell sources for novel regenerative therapies [1]. Cell therapies aim at the replacement of cell or tissue loss induced by degenerative disorders such as cardiovascular and neurodegenerative diseases, diabetes and many others, which cannot be healed by currently established, conventional treatments. Moreover, specific human cell types derived from hPSCs by differentiation can be utilized for the development of yet unavailable *in vitro* disease models, novel drug discovery strategies and more predictive drug safety assays.

Most of the envisioned clinical and industrial applications will require billions of lineage-specific cells which cannot be produced by conventional surface-adherent 2-dimensional (2D) cultures. Stirred-tank bioreactors, which are widely used in the biopharmaceutical industry for the generation of recombinant proteins expressed in mammalian (tumor) cell lines, provide numerous advantages for process development, as they allow for online monitoring and control of key process parameters such as pH, oxygen tension and biomass formation. Advanced bioreactor systems which have been developed in a wide range of culture vessels also facilitate the straightforward scale-up to larger process dimensions. However, cultivation and differentiation of hPSCs in stirred bioreactors apparently require the adaptation of cell cultivation from the established 2D surface-adherent culture to 3-dimensional (3D) suspension culture. It was recently demonstrated that hPSCs can be successfully grown as free

Figure 1: DASbox Mini Bioreactor System with BioBLU 0.3 Single-use Vessels

floating, "cell only aggregates" in small-scale suspension [2-4]. Based on this knowhow the transfer to a DASGIP® Parallel Bioreactor System with four individually controlled glass vessels having a working volume of 100 - 250 mL each, was established [5]. Optimization of stirring-controlled aggregate formation from single cell inoculated hiPSCs led to an approximately four-fold cell expansion resulting in 2 x 10^8 cells per vessel (100 mL) using a fed-batch process. However, with regard to the envisioned clinical application of hPSCs, the possibility to utilize single-use culture vessels, which will support the development of GMP-conform processes, is of great interest. Subsequently, aim of this work was to establish a suspension culture of hiPSCs in a DASbox Parallel Mini Bioreactor System equipped with fully instrumented BioBLU 0.3 Single-use Vessels.

Materials and Methods

Experiments were performed utilizing the cord blood derived hiPSC line hCBiPSC2 [6]. Suspension cultures were initiated by detachment and dissociation of hiPSC monolayer cultures with accutase (Life Technologies). Single cells were suspended in mTeSRTM1 (STEMCELL Technologies, Vancouver, Canada) supplemented with the ROCK inhibitor Y-27623 (10 µM). Each BioBLU 0.3 single-use vessel was equipped with probes for pO_2 and pH. The pH probes were calibrated by two-point calibration. pO_2 probe calibration was conducted under process conditions: headspace gassing with 3 sL/h air plus 5% CO_2, stirring at 70 rpm utilizing an pitched-blade impeller [5, 7], 37°C in 100 mL mTeSRTM1; after stable pO_2 values were reached a slope calibration was performed. For culture inoculation 25 mL of a single-cell suspension were added to achieve a density of 5 x 10^5 cells /mL in the final 125 mL culture volume. After 48 h the entire medium was replaced daily (batch feeding) excluding cell loss. For cell counting and other analysis a sampling volume of 3.5 mL was harvested daily without medium replacement to prevent culture dilution. This strategy resulted in subsequent culture volume reduction from 125 to approximately 100 mL during the 7 day process duration. Beside pO_2 and pH, glucose and lactate concentrations, viable cell counts and the expression of pluripotency markers were monitored. Daily viable cell counts were performed via a trypan blue exclusion assay after cell-aggregate dissociation by collagenase B (Roche) treatment. Pluripotency assessment was performed by flow cytometry analysis specific to SSEA4 and TRA1-60.

Results and Discussion

24 h after inoculation of respective single cell suspensions to BioBLU 0.3 Single-use Vessels small cell aggregates with an average diameter of 58.1 ± 23.1 µm emerged in the stirred cultures. These aggregates, which showed a highly homogeneous size distribution throughout the process, increased in size over the cultivation period resulting in an average diameter of 139.25 ± 25.37 µm (figure 2) on day 7. A robust ~4-fold increase in viable cell count was achieved in this fed-batch process resulting in an average cell concentration of 2.1 x 10^6 cells/mL on day 7 and thus a total cell yield of ~2.1 x 10^8 cells per vessel (figure 3). Monitoring the metabolic activity revealed ~47% of glucose consumption and accumulation of 7.4 mM lactate at 48 hours. The

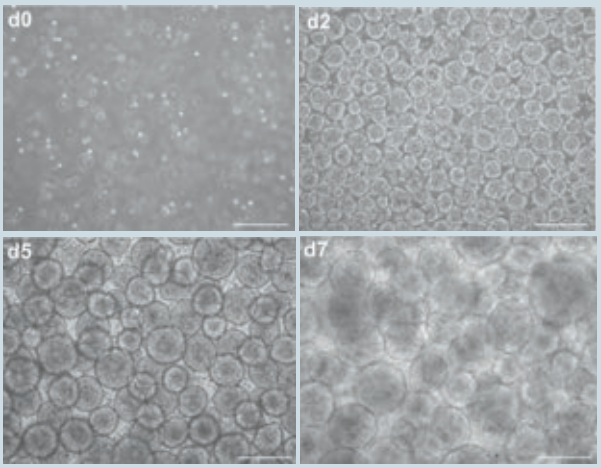

Figure 2: Aggregate formation in stirred suspension culture. After process inoculation by single cell suspension small cell aggregates arose. Increase in aggregate size was observed during the process. Scale bars: 200 µm.

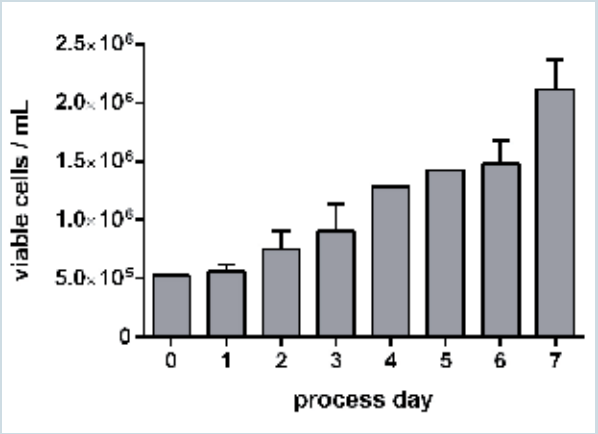

Figure 3: Growth kinetics of hCBiPSC2 in stirred suspension culture. Cells were seeded at 5 x 10^5 cells /mL (d0) and cell counts were determined daily. An up to 4-fold increase in cell count was achieved in individual runs over 7 days resulting in up to 2.3 x 10^6 cells /mL.

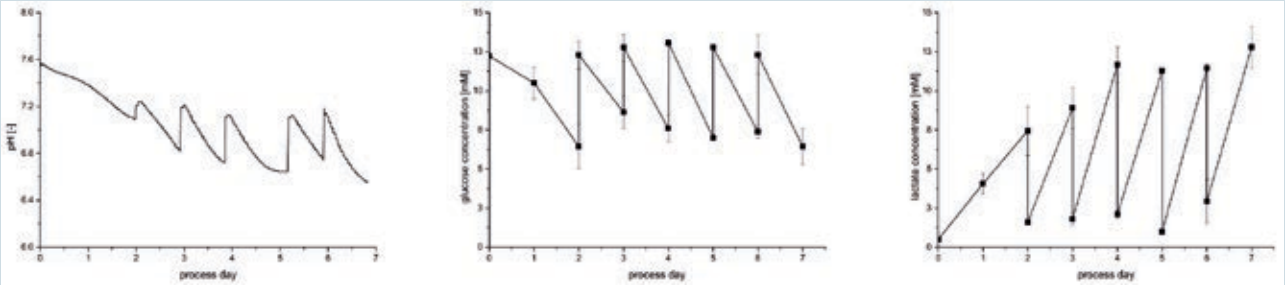

Figure 4: Metabolic activity of hCBiPSC2 in stirred suspension culture. The culture medium was replaced daily starting at 48 h thereby resulting respective glucose and lactate concentration patterns. The pH dropped from 7.4 in fresh medium to lowest values of ~6.8 at the end of a 24 h feeding interval on process days 5-7.

metabolic activity was also followed by online measurements of pH and pO_2. Increasing cell numbers over time resulted in a maximum pH drop to 6.8 (figure 4; as compared to pH 7.4 in fresh medium) and dissolved oxygen levels decreased to 57% (data not shown). The expression of pluripotency-associated surface markers TRA 1-60 and SSEA4 were determined at the process endpoint to evaluate the quality of the expanded hPSCs. Flow cytometry revealed that the majority of the yielded cell population retained expression of these markers i.e. 84% positivity for TRA 1-60 and 90% for SSEA4 (figure 5) was observed suggesting maintenance of pluripotency in this cultivation process.

Conclusion

This set of experiments demonstrates the successful expansion of human pluripotent stem cells applying the DASbox system in combination with BioBLU 0.3 Single-use Vessels. In a 7 day-lasting expansion process in stirred suspension culture cell yields of up to 2.3 x 10^8 cells /100 mL were obtained, which is in good agreement with our previous data in the DASGIP Parallel Bioreactor System, stirred glass vessel system (DS0200TPSS; 100-250 mL working volume) [5]. Notably cells generated by the described process retained expression of established, pluripotency associated cell surface markers. The work confirms the general applicability of the culture system for hPSC expansion in stirred suspension and reveals the DASbox system in combination with BioBLU 0.3 Single-use Vessels to be an excellent platform for further process optimization and future adaptation to lineage-specific hPSC differentiation processes.

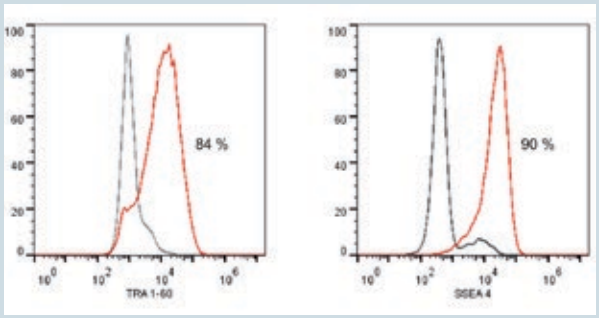

Figure 5: Expression of pluripotency-associated surface markers is retained after expansion in stirred suspension cultures. Flow cytometry revealed that the majority of cells that were harvested at the process endpoint (after day 7) expressed pluripotency-associated surface markers TRA 1-60 and SSEA 4 (gray line represents isotype controls).

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APPLICATION NOTE No. 313 I July 2011

An Update on the Advantages of Fibra-Cel® Disks for Cell Culture

Julia Cino, Rich Mirro and Suzy Kedzierski, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

With a renewed acceptance of perfusion processes in cell culture, both in the laboratory and for production applications[1], New Brunswick packed-bed bioreactors using Fibra-Cel disks are seeing an upsurge in interest. This application note examines Fibra-Cel technology and its many advantages, from increased secreted protein yields to labor savings in applications including rabies vaccine production and production of the first licensed gene therapy drug.

What Is Fibra-Cel?

Eppendorf Fibra-Cel is a solid-support growth matrix (Figure 1) for anchorage-dependent and suspension cell cultures. It is used predominantly in perfusion processes for the production of secreted products—such as recombinant proteins and viruses—and it is currently being evaluated for stem cell research[2].

Since the 1980s, scientists around the globe have been using Fibra-Cel to grow a wide range of cell types (see inset, right), including hybridomas and insect cultures. Originally used in New Brunswick™ autoclavable CelliGen® cell culture bioreactors, Fibra-Cel technology has now been successfully scaled up for commercial production in sterilizable-in-place systems as large as 150 liters. BioBLU® packed-bed, single-use vessels containing Fibra-Cel are also available for those who prefer the advantages of a disposable system.

Manufactured according to cGMP guidelines, Fibra-Cel is composed of two layers of nonwoven material—polyester and polypropylene—which are sonicated together, cut into disks, and electrostatically treated to attract cells and facilitate their attachment to the disks. Normally it takes about six hours for cells to attach to microcarriers (with a normal inoculum of 1×10^6 cells/mL), whereas cells can attach within 15 – 60 minutes on Fibra-Cel disks.

Cells Successfully Used on Fibra-Cel Disks

Hybridoma: DADA4.4, 123A, 127A, GAMMA, 67-9-B

Anchorage-Dependent: 3T3, COS, human osteosarcoma, RC-5, BHK, Vero, CHO, rCHO-tPA, rCHO (hep B surface antigen), HEK 293, rHEK 293, rC127 (hep B surface antigen), normal human fibroblasts, stroma, hepatocytes

Insect Cells: Tn-368, SF9, rSF9, Hi-5between runs

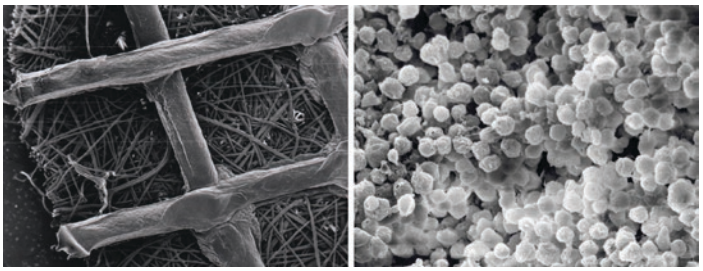

Figure 1. Scanning electron micrograph of Fibra-Cel disks (left); mouse–mouse hybridoma DA4.4 immobilized on Fibra-Cel disks during production at 1×10^8 cells/cm^3 of packed-bed volume (right)

Moreover, the growth process for microcarrier cultures can require extended delays for periodic stoppage of stirring to allow time for cells to become attached. By comparison, the Fibra-Cel bed is inoculated (3×10^5 cells/mL of bed volume) in a single step.

Fibra-Cel in a New Brunswick bioreactor is also advantageous over microcarriers because it enables sustained long-term periods of high-density growth in perfusion mode, without danger of clogging because there are no filters. Perfusion is a mode of cell culture in which a fresh nutrient medium is continuously added to the culture while simultaneously removing the spent medium that contains the product of interest. In a New Brunswick bioreactor, cells growing on or in the Fibra-Cel disk bed are retained within the vessel, inside the packed bed, where they continue the production of the desired product.

The packed bed comprises two horizontally positioned screens that extend to the bioreactor vessel walls. Enclosed between the screens, a bed of Fibra-Cel disks serves as solid support for the growing cells (Figure 2). Cells growing in the disk bed become immobilized on or between the disks, where they remain throughout the culture run, protected from external shear forces. The process is favored for manufacturing because product yields can be increased by as much as tenfold over comparable processes[3]. Once the bioreactor is set up and inoculated, the culture can be maintained to produce proteins for long periods of time thus saving labor, time, and money.

Like New Brunswick's proprietary Cell Lift impeller, rotation of the discharge ports in the proprietary packed-bed impeller creates a low differential pressure at the base of the impeller tube, which circulates the medium throughout the system. The medium receives gases through a sparger located at the bottom of the inner tube, protecting the cells from being exposed to the gas liquid interface. This results in low turbulence and low shear stress on the culture. Exceptionally high cell densities are achievable due to the high surface-to-volume ratio provided by the disk bed, coupled with the ability to use perfusion. In comparison with other cell support systems, it was found that higher titers and cell densities were achieved in trials using Fibra-Cel disks[4].

Figure 2. Basket Impeller with Fibra-Cel disks

Other Benefits

Because higher yields are possible, smaller bioreactors can be used to substantially reduce the initial capital expenditure as well as reduce the utilities required for operation (such as electricity, water, and steam if required). In addition, because the cells remain entrapped, the packed bed eliminates the need for cell filtration to separate cells from the end product, thus simplifying harvesting. Last, product recovery and downstream processing can be more easily controlled because users can determine the volume of harvest material that is to be processed at any given time.

Commercial Production

Bioreactors using Fibra-Cel have been used in the production of a variety of commercial products. One example is Gendicine®, the world's first licensed gene therapeutic drug, which was developed in China by Shenzhen SiBiono GeneTech Co., Ltd (Shenzhen, China). Gendicine is an anticancer treatment used to treat head and neck squamous cell carcinoma (HNSCC). Specifically, Gendicine is a replication-incompetent, recombinant human adenovirus engineered to contain the human wild-type p53 tumor-suppressor gene and is produced by the SBN-Cel cell line, which was subcloned from the human embryonic kidney (HEK) cell line 293. After scaling up the process from roller bottles, New Brunswick's bioreactor with Fibra-Cel technology was selected over a competitor's system because it produced 15× greater yields of viral particles[5].

Other examples of commercial production include end products such as EPO, which can now be commercially produced on the bench using Fibra-Cel technology, eliminating the need for labor-intensive and space-consuming roller bottles. A substantial portion of the world's human rabies vaccine is also produced using New Brunswick's Fibra-Cel technology. Additionally, many of our customers are currently using Fibra-Cel in their proprietary processes to produce interferons, diagnostic test kits, monoclonal antibodies, and hormones.

In summary, Fibra-Cel provides benefits in research laboratories as well as in commercial production. Our customers have found that because yields are high, bioreactors containing Fibra-Cel packed beds can outperform much larger-sized bioreactors, thereby achieving commercial-scale production in a bioreactor with a far smaller footprint. Production space requirements are reduced, as are costs associated with labor, start-up, and operations.

For protocols on other cell lines, or for additional information on the Fibra-Cel, see eppendorf.com.

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APPLICATION NOTE No. 315 I January 2009

Which Impeller Is Right for Your Cell Line? A Guide to Impeller Selection for Stirred-Tank Bioreactors

Rich Mirro and Kevin Voll, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

When growing microbes or animal cells in a stirred-tank reactor, it is critical to choose the impeller type that is best suited to your process. Select the wrong impeller, and you could make chop suey of your filamentous fungi. Pick the right impeller, and you could greatly increase yields of your fussy mammalian cultures such as Chinese hamster ovary (CHO) and Vero kidney epithelial cells. With a wide range of impeller designs to choose from, how do you tell which is right for your application? Here we describe six commonly used fermentor and bioreactor impellers, explain how they work, and identify which may perform best for culturing certain animal, insect, plant, yeast, and bacterial cell lines.

How Blade Orientation Affects Mixing

All impellers are designed to homogenously mix cells, gases, and nutrients throughout the culture vessel. The mixing action evenly distributes oxygen and nutrients to cells for healthy growth, keeps them from settling to the bottom of the vessel, and helps to maintain a uniform culture temperature. Depending on the impeller type you select, mixing will be imparted as a radial flow, axial flow, or a combination of the two. As Figure 1 shows, radial flow occurs when fluid is pushed away from the impeller's axis toward the vessel wall. Axial flow occurs when fluid is pushed up or down along the axis or shaft of the impeller. The orientation of an impeller (left- or right-handed) and its agitating direction determine whether the direction of axial flow is up or down. A right-handed impeller option will push fluid in an upward direction toward the top of the vessel if agitation is clockwise (as viewed from the top). A left-handed option paired with a clockwise agitation will push fluid down toward the bottom of the vessel. Therefore, when positioning blades on an impeller shaft, it's important to know which direction your impeller will be turning (clockwise or counterclockwise). To increase mixing action in some applications, one impeller blade may be oriented for up flow while the other is positioned for down flow. Although there is no right or wrong way to position an impeller blade(s), reversing flow direction could improve yields in some instances.

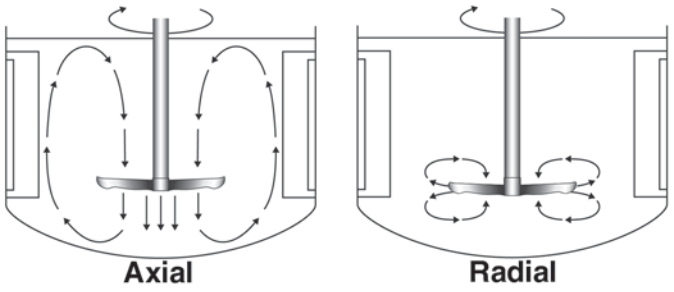

Figure 1: Axial and radial flow models

Rushton Impellers for Fermentation

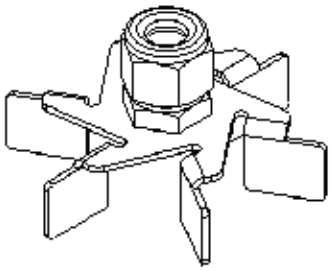

Figure 2: A six-bladed Rushton-type impeller

J. H. Rushton originally called the impellers he designed flat-bladed turbines. So Rushton impeller is today the most common generic term applied to flat-bladed or disk-turbine impellers (e.g., Figure 2). Their blades are flat and set vertically along an agitation shaft, which produces a unidirectional radial flow. Rushton and Rushton-type impellers are commonly used in fermentations of cell lines that are not considered shear-sensitive, including yeasts, bacteria, and some fungi.

Pitched-Blade Impellers for Shear-Sensitive Cells

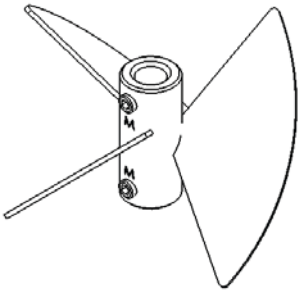

Figure 3: Pitched-blade impeller, right-handed orientation

The blades on pitched-blade impellers (Figure 3) are flat and set at ~45° angles, which produces a simultaneous axial and radial flow. This combination provides better overall mixing and creates a higher oxygen mass transfer rate (KLa) than that of unidirectional marine blade impellers. Pitched-blade impellers are low-shear impellers designed to gently mix the contents of a culture without causing cell damage. They are most often used with mammalian, insect, or other shear-sensitive cell lines growing in suspension or with the aid of microcarriers. These impellers are often used in batch or fed-batch cultures, but they can also be used for continuous and perfusion processes. Because of their proficient mixing design, pitched-blade impellers are also widely used in fermentation processes that involve highly viscous cultures, such as filamentous bacteria and fungi, as well as in some anaerobic biofuels processes.

Gentle Marine-blade Impellers

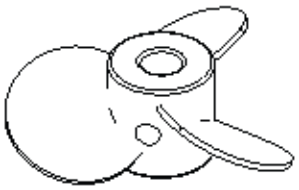

Figure 4: Marine-blade impeller, left-handed orientation

The leading face of the blades on a marine-blade impeller (Figure 4) can be flat or concave, whereas their back sides are convex. This produces an axial flow. Like pitched-blade impellers, marine-blade impellers are used for applications that require gentle mixing without causing cell damage. Due to the unidirectional flow, however, the KLa values of marine-blade impellers tend to be slightly lower than those of impellers that produce both axial and radial mixing.

Spin Filters

Spin filters are retention devices commonly used to keep cells inside a vessel during continuous or perfusion culture. In New Brunswick™ bioreactors, for example, spin filters with low-shear marine blade impellers are designed for suspension and microcarrier applications. A spin-filter kit consists of a screened cage surrounding an impeller shaft with very small filter pore openings that keep cells isolated outside the cage (Figure 5). Inside that rotating cage, a dip tube is provided for continuous withdrawal of culture broth. A media feed tube outside the cage provides a steady supply of fresh nutrients. Although pore openings vary from one manufacturer to the next, New Brunswick spin filters come with 10-µm openings for suspension cultures and 75-µm openings for microcarrier cultures. Because of its gentle mixing nature, a spin filter is typically used with microcarrier-dependent cell lines or those that are highly sensitive to shear. These mechanisms are ideal for use in production of secreted proteins because they keep harvested media cell-free, which simplifies purification in downstream processing. Over time, however, the screen material covering a spin-filter cage will become clogged with cell debris and require replacement. Culture run times are limited by this factor.

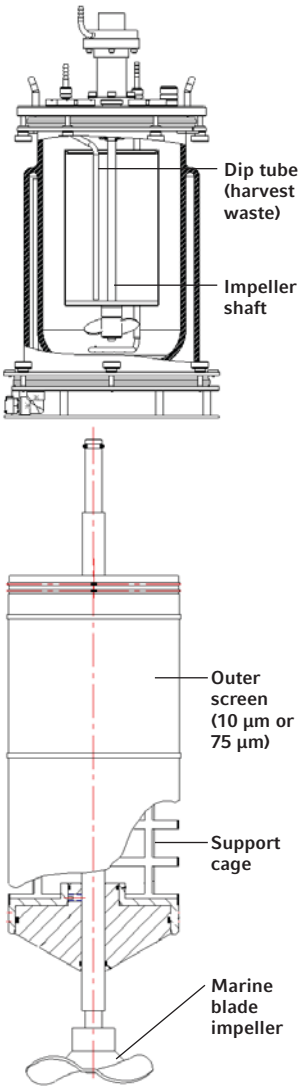

Figure 5: Spin filter assembly

Special Impellers for Microcarrier Cultures

The New Brunswick™ CelliGen® cell-lift impeller (Figure 6) provides uniform circulation for microcarrier cultures. This is an ultra-low-shear impeller in which flow is caused by three discharge ports located on the impeller shaft. Rotation of those ports creates a low-differential pressure at the base of the impeller tube, lifting microcarriers up through the tube and expelling them out through its ports. This continuous recirculation loop keeps cells uniformly dispersed throughout a vessel. Gases are introduced through a ring sparger, which generates bubbles that pass along the impeller

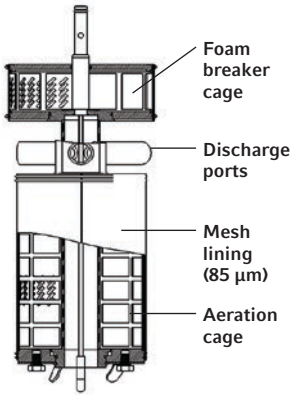

Figure 6: Cell-lift impellers for microcarrier culture

between the exterior of the inner tube and an outer membrane, known as the aeration cage. A mesh lining on the outer membrane of this cage has penetrations that are small enough (85 µm) to ensure that cells growing on the microcarriers cannot pass through. Gas exchange occurs at the membrane–media interface, ensuring that cells remain in a bubble-free environment and are not subjected to shear due to bubble breakage. The bubbles are then expelled through two ports (located at the top of the impeller) into a second screened-in cage. A foam breaker directs air, supplied by a gas overlay, into the cage to break up foam. Cell-lift impellers are typically used in batch and fed-batch processes involving shear-sensitive animal cells. They can also be used for continuous perfusion processes when a decanting column(s) and media feed-in and broth pump-out setup are added.

A General Guide

Impeller designs are almost as varied as the types of cell lines they are designed to help grow. Table 1 lists several cell lines commonly used in fermentation and cell culture processes and matches each with the impeller(s) best suited for its growth.

Packed-Bed Basket Impellers

Another New Brunswick design is the packed-bed basket impeller used in the manufacture of secreted products from either anchorage-dependent or suspension cultures. A basket includes two horizontally positioned, perforated metal screens that extend to the walls of a bioreactor vessel (Figure 7). Enclosed between those screens, a bed of Fibra-Cel® disks serves as a solid support matrix for cell growth.

Cell Line	Rushton and Rushton-Like Impellers	Pitched-Blade Impeller	Marine-Blade Impeller	Spin Filter Impeller	Cell Lift Impeller	Basket Impeller
Human						
HEK 293		■	■	■	■	■
HeLa		■	■	■		■
HL60		■	■	■		■
Lncap		■	■	■		■
THP-1		■	■	■		■
UMSCC		■	■	■	■	■
HFF		■	■	■	■	■
KB		■	■	■	■	■
MRC-5		■	■	■	■	■
Hybridoma						
DA4.4		■	■	■		■
123A		■	■	■		■
127A		■	■	■		■
GAMMA		■	■	■		■
67-9-B		■	■	■		■
SP20		■	■	■		■
Primate						
Vero		■	■	■	■	■
COS-7		■	■	■	■	■
Rat Tumor						
GH3		■	■	■		■
9L		■	■	■		■
PC12		■	■	■		■
Mouse						
3T3		■	■	■		■
MC3T3		■	■	■		■
NS0		■	■	■	■	■
Hamster						
CHO		■	■	■	■	■
BHK		■	■	■	■	■
Zebrafish						
ZF4		■	■	■	■	
AB9		■	■	■	■	
Insect						
SF9		■		■		■
Hi-5		■		■		■
Sf21		■		■		
Yeast						
Saccharomyces cereviseae	■					
Baker's yeast	■					
Pichia pastoris	■					
Candida albicans	■	■				
Bacteria						
Streptomyces	■	■				
Bacillus	■					
Echerichia coli	■					
Algae						
Red/Green		■	■			

Table 1: A general guide to choosing impellers by cell line

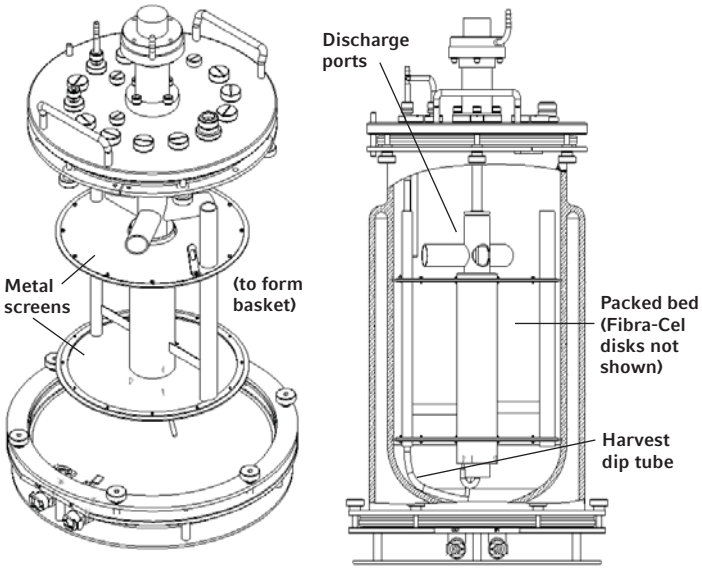

Figure 7: Packed-bed basket impellers for secreted products

Cells growing in the disk bed become immobilized on or between the disks, where they remain protected from external shear forces throughout each culture run. Media circulates by way of a hollow impeller tube with discharge ports positioned above the basket. As with the cell-lift impeller, rotation of these discharge ports creates a low differential pressure at the base of the impeller tube, which circulates media throughout the system. The medium receives gases through a sparger located at the bottom of the inner tube, which protects cells from being exposed to the gas-iquid interface. This results in low turbulence and low shear stress for the culture. Exceptionally high cell densities are achievable with packed-bed baskets because of a high surface-to-volume ratio for cell growth provided by the disk bed coupled with an ability to use perfusion or medium-replacement techniques. Culture periods in excess of three months have been reported[1][2]. By ensuring that cells remain entrapped in the bed, this system also simplifies protein harvesting from the resulting cell-free media.

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[2] **Modified CelliGen Packed Bed Bioreactors for Hybridoma Cell Cultures.** Wang G, et al. *Cytotechnol.* 9, 1992: 41–49.

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APPLICATION NOTE No. 291 I June 2013

Cultivation of Human CAP® Cells: Evaluation of Scale-Down Capabilities using Single-Use Bioreactors

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Abstract

Increasing process complexity coupled with rising cost pressures and rapidly evolving regulatory requirements makes today's process development efforts a special challenge. The pressure of achieving faster time-to-market for new and innovative biotechnological products has led to the need to optimize every element of the total development workflow.

The following application note illustrates how the DASbox® Mini Bioreactor System combined with the BioBLU® 0.3c single-use vessels supports bioprocess development in human cell culture. Scale-down capabilities were investigated by comparison of 500 mL cultures in a DASGIP® Parallel Bioreactor System with 170 mL cultures in the DASbox using the BioBLU 0.3c single-use vessel.

Introduction

Initial bioprocess development involves cell line optimization, clone selection, and screening for media, feed components and strategies, and other process conditions. Shake flasks, the most common vessels used in early cell and microbial work, have served the biotechnology industry well over the decades but their limitations for optimizing cell culture or fermentation conditions are well known. Equipment used during screening should mimic the physical and mechanical characteristics of production-scale bioreactors to the highest degree possible in order to assure consistency throughout development phases. Ideally, these best practices will support the aims of QbD: that quality measures initiated during development are carried forward and manifested in product quality. DASGIP Parallel Bioreactor Systems have the potential to address process consistency and harmonization of unit operations between development and production. Today's state-of-the-art benchtop systems use sensors and information technology to control, monitor, and record critical process parameters such as temperature, pH, dissolved oxygen, and agitation. As in production-scale bioreactors, gassing and feeding proceed according to defined settings.

Figure 1: BioBLU® 0.3c Single-Use Bioreactor for cell culture

CEVEC® Pharmaceuticals GmbH (Cologne, Germany), a global solution provider focussing on the development of top notch human expression systems with highest ethical standards, has established a master cell bank (MCB) of CAP® cells growing in suspension, tested and certified according to ICH guidelines and European Pharmacopeia. The platform expression technologies CAP and CAP-T are based on specific, amniocyte-derived human cell lines. CAP and CAP-T were designed for stable and transient protein production and achieve highest protein yields with authentic human glycosylation patterns. Simple and reliable protocols allow for the fast generation of customized producer cell lines for pharmaceutically relevant proteins based on the parental permanent CAP cells under controlled and optimized conditions. For the required human cell line screening as well as for media optimization, the small working volumes of 100 – 250 mL make the extendable 4-fold DASbox and the BioBLU 0.3c single-use vessel a perfect fit. Bioprocesses are controlled as precise and effectively as they are in larger scale bioreactors while cell material, media and supplements as well as lab space are saved.

Several experiments were carried out aiming at verifying the scale-down capabilities from the DASGIP Parallel Bioreactor System, which CEVEC generally uses in process development, to the Mini Bioreactor System DASbox.
To overcome the risk of cross-contamination and to reduce time for cleaning, sterilization and assembly they evaluated the novel developed BioBLU 0.3c single-use vessel. Which comes with a magnetic coupled stirrer and pitched blade impeller and holds several short and long dip-tubes as well as two standard PG13.5 ports facilitating full industry standard instrumentation. A specifically designed port including a gas permeable membrane allows for DO measurement using a reusable probe which can be plugged easily in directly on the bench. Recuperation of liquid from exhaust gas is carried out via a novel liquid-free operated condenser.

Materials and Methods

To evaluate the scale-down capability of the new DASbox Mini Bioreactor System and the usability of the BioBLU 0.3c single-use vessel experimental series with two different systems were carried out and compared. A 4-fold Parallel Bioreactor System for cell culture was used in 500 mL scale experiments (PBS). The corresponding small-scale approaches were carried out in a (parallel) DASbox system using single-use vessels with 170 mL (DASbox SU).

The recombinant human CAP cells producing a pharmaceutically relevant protein were batch cultivated for 7 d (170 h) in CEVEC's serum-free, chemically defined CAP medium supplemented with 40 mM glucose and 6 mM glutamine at 37 °C. Initial viable cell density was 3*105 cells/mL. The DO set-point of 40 % was maintained by a constant stirrer speed and the oxygen concentration in the inlet gas. Stirrer speed was adjusted to 160 rpm (PBS) and 150 rpm (DASbox SU). The pH value was regulated to 7.1 by addition of 1 M Na2CO3 (feeding, speed rate regulated) and CO_2 (submerged gassing). Inlet gas (air, O_2, CO_2 and N_2) was mixed continuously mass flow-controlled. The bioreactors were equipped with pitched blade impellers and liquid-free operated exhaust gas condensers. The pre-cultures were cultivated in 125 mL Erlenmeyer flasks (Corning) with 25 mL working volume using a shaker incubator (37 °C, 5 % CO_2) agitating at 185 rpm (Multitron 2, Infors AG). The cells were expanded up to a viable cell density of 3*106 cells/mL in the same medium used for bioreactor runs.
The critical process parameters were monitored, controlled and visualized online while additionally offline parameters were added manually for collective analysis and storage in a joint database. Daily samples were taken in place. Viable cell numbers, the concentrations of glucose as well as the target protein were determined via semi-automated trypan blue cell counting (Cedex XS, Roche Innovatis), an automated

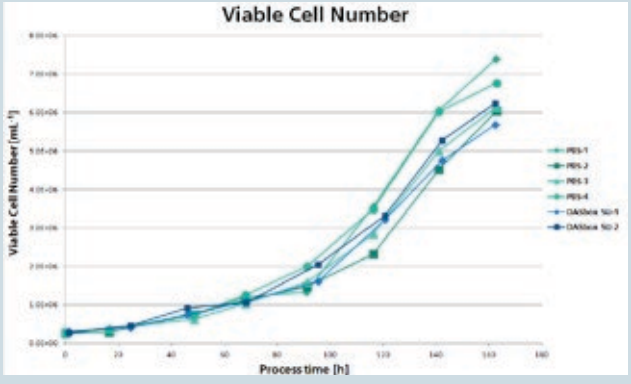

Figure 2: Viable Cell numbers of all experiments with DASGIP Parallel Bioreactor Systems (PBS) and BioBLU 0.3c vessels with average growth rate of 0.02 h^{-1}.

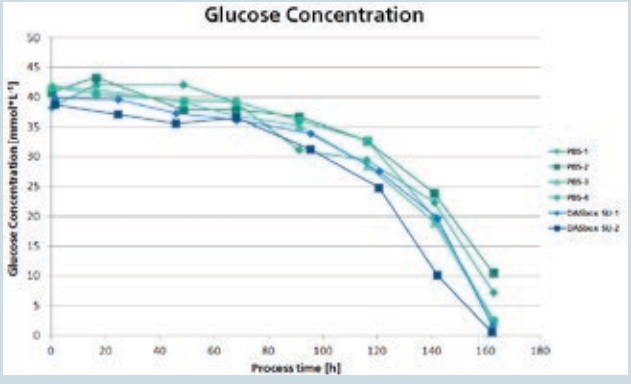

Figure 3: Comparism of metabolic activity by glucose consumption.

glucose biosensor (YSI 7100 MBS, YSI Life Sciences) and ELISA, respectively.

Results and Discussion

The highly comparable results shown in figures 2 and 3 prove the reliability of the process control in both independent experimental series.

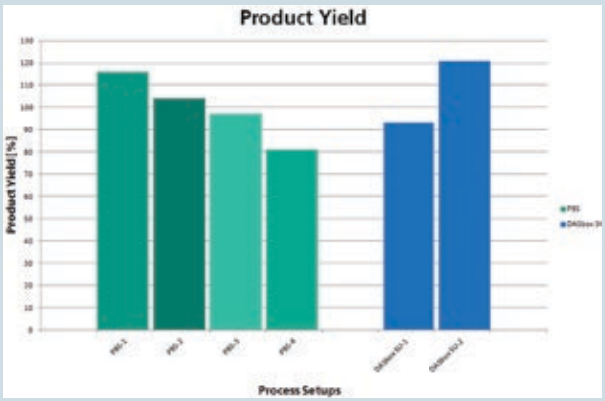

Figure 3: Product yield normalized to the average protein concentration gained using the DASGIP Parallel Bioreactor System (PBS)

The viable cell density increases exponentially within all cultivation studies in a reproducible manner with an average growth rate of 0.02 h-1. The corresponding anti-cyclic glucose consumption thereby illustrates the similar metabolism of the different cultures. Cell viabilities ranged in between 90 – 95 % for each sample. As shown in figure 4 the final product yield reached 80 – 121 % in respect to the average protein concentration gained with the Parallel Bioreactor System (PBS) commonly used at CEVEC. No differences in cell growth, metabolic activity and protein expression could be observed using the BioBLU 0.3 c single-use vessels. The results show the successful scale-down from a 500 mL (PBS) to 170 mL (DASbox SU) bioreactor working volume.

Conclusion

Summarized, the presented results give direct evidence to the scale-down capability of the DASbox Mini Bioreactor System used with single-use vessels. This proves the DASbox to be a superior tool for process development with human cell cultures. The small working volumes save material and consumable costs while utilizing single-use vessels drastically reduce turnover-times and thereby labour costs and development times.

Ordering information	Order no.
DASbox® Mini Bioreactor System for Microbial Applications	
with 4 vessels, max. 5 sL/h gassing, for cell culture applications	76DX04CC
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BioBLU® 0.3c	
4 x mini single-use vessel for cell culture applications	78903501

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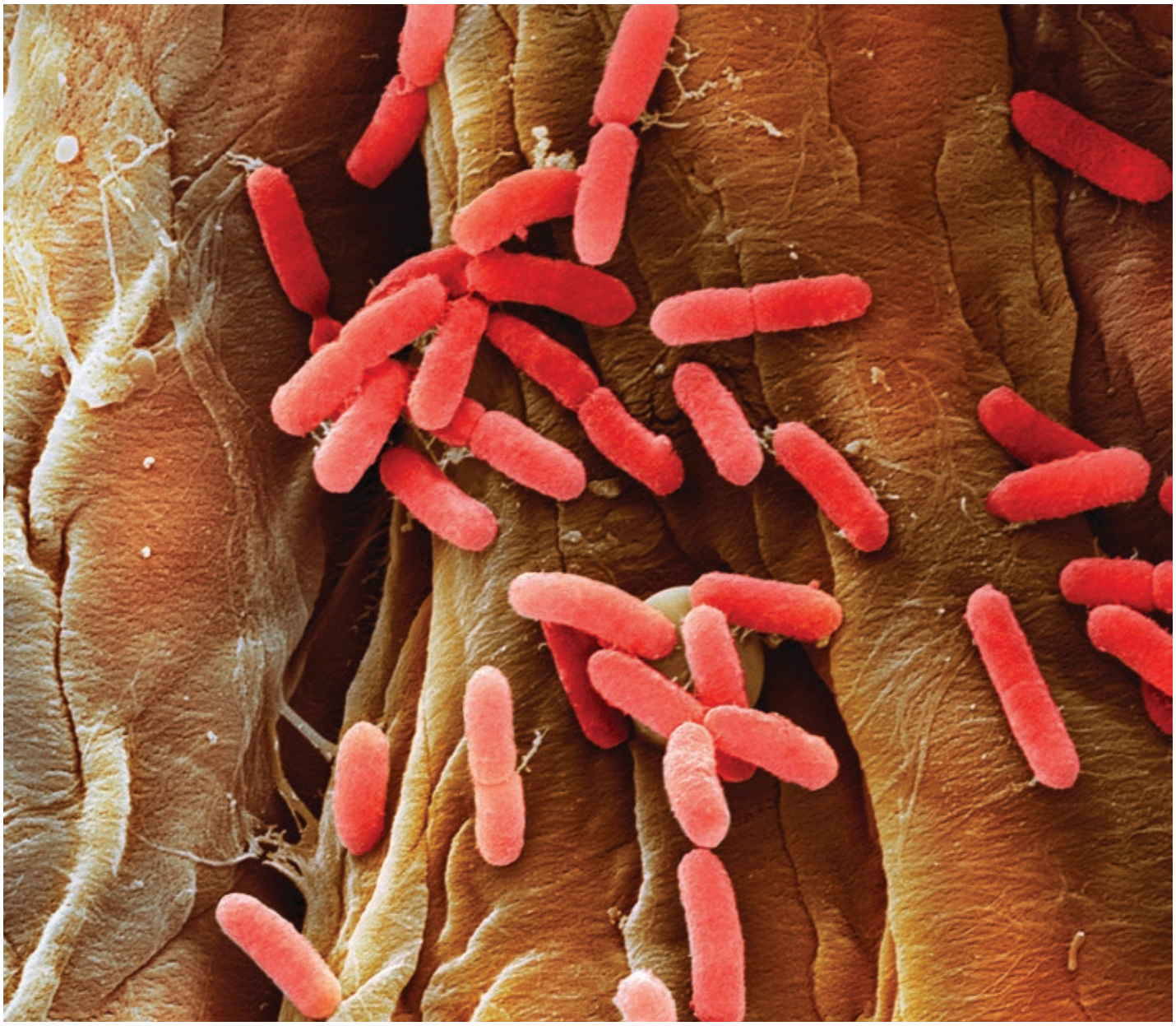

Fermentation

APPLICATION NOTE No. 319 I August 2013

Continuous Separation of *E. coli* Fermentation Broth Using a CEPA® LE Laboratory Centrifuge System

Y. Chen, J. Gerber, G. Hart and J. Capone, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

In this bioprocess laboratory application, 4.2 liters of *E. coli* fermentation broth containing 6 % solids by volume were separated by CEPA LE in 22 minutes. The cellular paste that was collected amounted to 193 grams. Supernatant clarity was excellent, with all samples containing less than our visual detection limit of 0.1 % solids.

Introduction

The CEPA LE Model, a tubular-bowl continuous centrifuge, is one of a family of separation instruments characterized by their ability to process many times the capacity of their bowl total volume without interruption. This characteristic results from a design that allows continuous feeding of a solid-liquid mixture, while simultaneously expelling the liquid component. The solids, in this application, are the cell mass, and are retained in the bowl.

Clarified liquid is obtained from an exit port while the machine is running. Cell mass is taken from the tubular bowl after the machine is stopped. A removable plastic bowl liner is often used to simplify cell paste removal.

Figure 1: The CEPA LE is a benchtop laboratory centrifuge, featuring variable speed contol as standard and a wide array of optional bowls for research, scaleup experiments, and small volume production. The LE is typically used with 2 to 15 liter cultures. Maximum throughput is 30 liters/hour.

Materials and Methods

Fermentation

A five liter fermentation was carried out in a New Brunswick BioFlo® benchtop fermentor for the purpose of evaluating the CEPA LE High Speed Centrifuge in a typical *E. coli* separation. The fermentation broth was determined to contain approximately 6 % wet solids by volume by spinning down a small sample in a laboratory batch centrifuge operated at 2500 rpm for 10 minutes. Dry cell weight was 11.48 grams per liter.

Setup and Operation

When the fermentation was completed, a Watson-Marlow® peristaltic pump was used to transfer the broth from the fermentor to the centrifuge. A length of silicone flexible tubing was attached to a dip tube in the fermentor vessel, fed through the pump head, and connected to the centrifuge's inlet nozzle. A second length of tubing was run from the centrifuge's supernatant outlet port into a collection vessel.

The fermentor was set to maintain temperature at 19 °C. After starting the centrifuge and waiting for it to attain full speed, broth was pumped to the CEPA LE at a rate of 190 mL/min (11.4 L/hr). This value was arbitrarily selected and is near the low end of the system's range — the CEPA LE has throughput capability up to 30 L/hr. The fermentor agitation was set to a low speed during the transfer to prevent settling and to help maintain temperature uniformity.

The centrifuge was configured with a type clarifying cylinder, and a 2 mm inlet nozzle. It was operating at full speed (40,000 rpm) which produces a radial acceleration or G-force of 45,000. The centrifuge and pump operation continued until the liquid in the fermentor fell below the dip tube level.

Clarity Measurements

Six 10 mL samples of supernatant were taken at 4 minute intervals during the separation process, and the 600 nanometer optical density was measured off-line. The 10 mL samples were spun down in a laboratory centrifuge for 10 minutes at 2500 rpm to get a visual measure of residual cell mass.

Results and Discussion

A total volume of 4.2 L was processed through the centrifuge in 22 minutes yielding 193 grams of wet cellular paste in the CEPA bowl. The 250 mL bowl was approximately 75 % full of paste at the point the processing was complete.

Sample	OD	Visual
1	0.162	< 0.1%
2	0.203	< 0.1%
3	0.226	< 0.1%
4	0.249	< 0.1%
5	0.300	< 0.1%
6	0.392	< 0.1%

Table 1: Supernatant clarity as indicated by optical density (600 nm) and visual observation of sediment samples taken at four minute intervals.

In addition to separation efficiency, we noted that the time required to carry out the procedures was very short, and handling the system during operation was obvious.

The separation itself took approximately 22 minutes. The entire process from setup through cleaning took less than an hour.

CEPA LE Processing Time in Minutes	
Setup	5 min
Accelerate	2 min
Process 4.2 L	22 min
Shut down and allocate paste	15 min
Clean and reassemble	10 min

Table 2: Time taken for different processing steps for the CEPA® LE in minutes.

We determined the LE model to be easy to use, as depicted from the short times for setup and clean up. The ease of handling is partly due to its small size, and partly because of its accessible design.

Predictably, the supernatant OD increased as the separation progressed, but even the last sample showed less than 0.1 % wet cell volume. Visually, this was a barely perceptible amount of cells in the supernatant, which could have been reduced further, either by feeding more slowly, or by exchanging the partially filled rotor for an empty one during the harvesting process.

Tests under various operating conditions could be used to develop a protocol that results in the optimum compromise between process time and supernatant clarity for a specific application. Acceptable residual cell mass depends on several factors, including whether the desired product is in the supernatant or the cells, as well as the post-centrifuge filtration and downstream purification processes, if any. Certainly, this particular process could have been run more quickly or more slowly with a change in clarity. Although not explored here, more complex protocols could be established to optimize the process for the user. One example would be to discharge a high feed rate initially, and then decrease it as the bowl fills to take advantage of the initially higher separation efficiency to improve either speed or clarity with no penalty in the harvest process.

This test showed that the smallest CEPA centrifuge efficiently and conveniently harvested and clarified *E. coli* broth, making it a highly effective instrument for fermentation applications.

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APPLICATION NOTE No. 290 I March 2013

Amino Acid Fermentation: Evaluation of Scale-Down Capabilities Using DASbox® Mini Bioreactors

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Abstract

Optimization of bioprocesses needs accurate monitoring and control while small working volumes are saving media and other resources. Evonik® has established a 2 L process for production of a nutritionally relevant amino acid in *E. coli*. The following application note descripes how this process was adapted to the smaller working volume of a DASbox Mini Bioreactor System.

Multiple runs were performed with close monitoring of all relevant process parameters and comprehensive evaluation of data to prove reliable and reproducible results. The scale-down capabilities of the DASbox system were evaluated by comparing the fermentation results to the data collected at 2 L scale.

Introduction

Rising cost and time pressures in bioprocess development together with rapidly evolving regulatory requirements make process development efforts a special challenge these days. Optimizing every step of the total development workflow is crucial for maintaining a competitive business. Advanced miniaturized benchtop bioreactor systems can harmonize operations between development and production groups while supporting the aims of Quality by Design (QbD). To meet today's demands of process development these mini bioreactor systems need to mimic all aspects of large-scale fermentation, and offer comprehensive data and information management tools to support regulatory requirements for both filing support and QbD-driven process development. *In situ* sensors as well as an integrated supervisory control and data acquisition (SCADA) are used to control, monitor, and record critical process parameters such as temperature, pH, dissolved oxygen, and agitation. As in production-scale bioreactors, gassing and feeding proceed according to defined settings. DASGIP® Parallel Bioreactor Systems have the potential to address process consistency and harmonization of operations between development and production.

The following application note illustrates how the DASbox Mini Bioreactor System with its working volume range of 60 - 250 mL supports bioprocess development in microbial applications. Scale-down capabilities were investigated by transferring a 2 L fermentation process to a 10x smaller working volume in the DASbox system.

Figure 1: DASbox® Mini Bioreactor System for Microbial Applications

Evonik Industries AG (headquartered in Essen, Germany) is one of the world's leading specialty chemicals companies. Its Health & Nutrition Business Unit produces and markets essential amino acids, mainly for animal nutrition and for specialties for the pharmaceuticals industry.

Materials and Methods

To evaluate the reproducibility and scale-down capabilities of the DASbox Mini Bioreactor System (Figure 1) experimental series with two different systems were carried out and compared.
Fed-batch fermentation of the amino acid-producing *E. coli* strain was performed in a standard benchtop bioreactor. The corresponding small-scale approaches were carried out in a DASbox System equipped with four DASGIP Mini Bioreactors.
The *E. coli* strain was cultivated at 36 °C. During fermentation glucose was added according to predefined feed profiles. Both systems used comparable feeding profiles, the one of the DASbox system being adapted to the smaller working volumes. The DO set-point was maintained by adjusted agitation speed. The bioreactors were equipped with two Rushton impellers each. The pH value was regulated to a constant value throughout the fermentation process.
The critical process parameters were monitored, controlled and visualized online while additionally, optical density (OD_{600}) and glucose concentration were entered manually for collective analysis and storage in a joint database. Product concentration was measured at the end of each run.

Results and Discussion

All critical process parameters such as feeding profiles and impeller tip speed as well as pH, DO, and temperature set-points were successfully transferred from the 2 L scale to the DASbox Mini Bioreactor System. The two systems showed similar growth characteristics. Online measured Oxygen Transfer Rates (OTR) resulted in highly comparable curves indicating a successful scale-down (data not shown). Comparison of parallel fermentation runs performed with the DASbox prove the results to be highly reproducible. OTR values of all four runs again followed highly similar curves. Same was observed for online parameters such as temperature, dissolved oxygen concentration and pH.
Fermentation using the DASbox system resulted in product yields comparable to the ones achieved with the larger benchtop system (Figure 2). Again, data obtained from the four individual runs performed with the DASbox Mini Bioreactor System strongly resembled each other and thus prove its reproducibility.

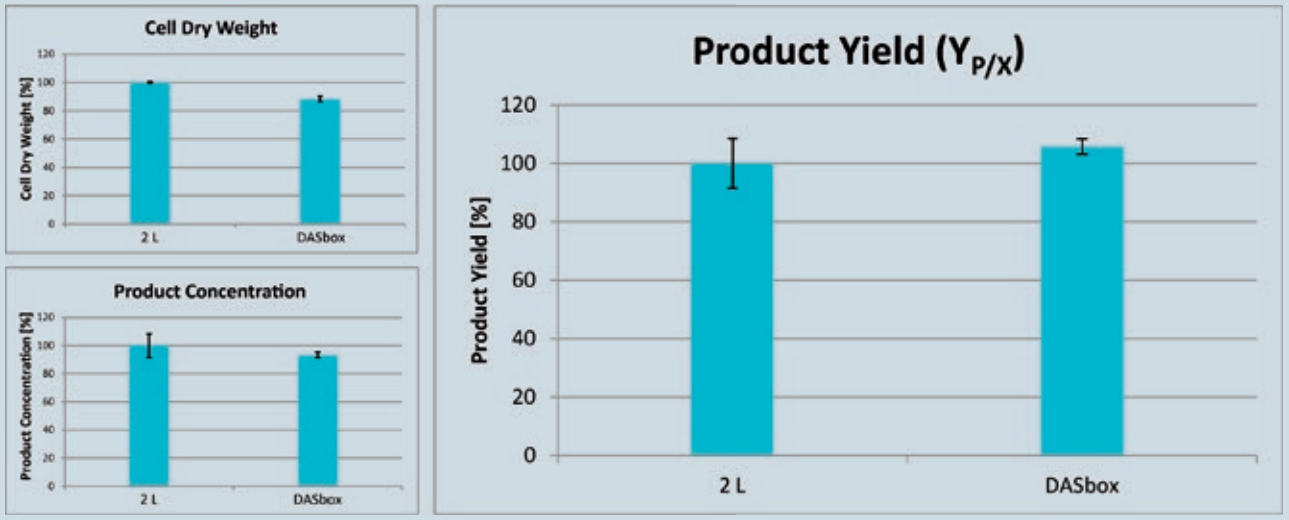

Figure 2: Cell dry weight (X), product concentration (P) and specific product yield ($Y_{P/X}$), each normalized to the 2 L system.

Conclusion

The results presented in this application note give direct evidence to the scale-down capability of the DASbox Mini Bioreactor System. This proves the DASbox to be an excellent tool for microbial process development. With its small working volumes it helps saving resources without cutting back the comprehensive process control of advanced large-scale bioreactor systems. Summarized, the DASbox is a truly parallel mini bioreactor system that provides reliable and reproducible results.

Ordering information	Order no.
DASbox® Mini Bioreactor System for Microbial Applications	
with 4 vessels, max. 25 sL/h gassing, for microbial applications	76DX04MB
with 8 vessels, max. 25 sL/h gassing, for microbial applications	76DX08MB

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APPLICATION NOTE No. 293 I October 2013

Scalability of Parallel *E. coli* Fermentations in BioBLU® f Single-use Bioreactors

Claudia M. Huether-Franken[1*], Anne Niehus[1] and Sebastian Kleebank[2]
[1] Eppendorf AG Bioprocess Center, Rudolf-Schulten-Str. 5, 52428 Juelich, Germany; [2] DASGIP Information and Process Technology GmbH, Juelich, Germany; * Corresponding author: huether.c@eppendorf.com

Abstract

Single-use bioreactor solutions have been successfully established in animal and human cell culture in the last years. Now this technology is going to make its way for microbial applications. In the following case study reproducible process control was achieved with single-use mini bioreactors and 1 L single-use vessels running in parallel. Fermentation of *E. coli* K12 led to highly reproduclible results thus proving the tested rigid wall single-use stirred-tank vessels to be an appropriate tool to accelerate microbial process development and shorten time-to-market in biopharmaceutical industry.

Introduction

Regardless if in cell culture or in microbial applications, single-use bioreactors provide a couple of advantages for time- and cost-effective bioprocessing. Minimal setup times, no need for cleaning procedures and therefore reduced labor time can accelerate bioprocess development rigorously. Compared to the use of single-use bioreactors in cell culture, microbial applications make specific demands on bioreactor design and functionality. Fermentation processes need much higher k_La values for proper mass transfer as well as suitable heating and cooling options.

Figure 1: BioBLU 0.3f and BioBLU 1f Single-use Vessels for Microbial Applications

Materials and Methods

To evaluate the reliability of microbial fermentation processes using single-use technology *E. coli* K12 (DSM 498) was cultivated in a fully instrumented Eppendorf BioBLU 0.3f single-use mini bioreactor and compared to fermentations in BioBLU 1f single-use bioreactors. This rigid wall stirred-tank single-use bioreactors were specifically designed for microbial applications and are equipped with two (BioBLU 0.3f) and three Rushton-type (BioBLU 1f) impellers, respectively. The BioBLU 1f vessel

carries baffles as well. Both vessel types include a liquid-free Peltier exhaust condensation and magnetic-coupled overhead drive for high performance agitation.
A 4-fold parallel Eppendorf DASbox® Mini Bioreactor System was used with BioBLU 0.3f Single-use Vessels and the BioBLU 1f fermentations were carried out using a 4-fold DASGIP® Parallel Bioreactor System with DASGIP Bioblock. Both Systems feature active heating and cooling capacities. DASGIP Control Software was used for precise process control.

The cultures were grown for 24 h in PAN media with an initial glucose concentration of 40 g/L and fed with 50 % glucose solution in the fed batch phase. The processes were started with working volumes of 0.1 L in BioBLU 0.3f and 0.7 L in BioBLU 1f Single-use Vessels, respectively. The temperature was controlled at 37 °C.
When using the BioBLU 0.3f vessels the pH was adjusted to 6.8 via 4 % ammonia solution. The cultures were submerged aerated through dip tubes with a constant rate of 6 sL/h (1 vvm). Dissolved oxygen was maintained at 30 % whereas the stirrer speeds ranged from 600 rpm to 2000 rpm which equals to tip speeds of 0.94 m/s to 3.14 m/s. When using the BioBLU 1f vessels the pH was adjusted to 6.8 via 25% ammonia solution. The cultures were submerged aerated through dip tubes with a constant rate of 42 sL/h (1 vvm). Dissolved oxygen was maintained at 30 % whereas the stirrer speeds ranged from 600 rpm to 1600 rpm which equals to tip speeds of 1.35 m/s to 3.59 m/s. Oxygen transfer rates (OTR) were automatically calculated via a DASGIP exhaust analyzer GA4.

Results and Discussion

A two-phase cultivation with automatic feed-start was sucessfully carried out. As shown by the dissolved oxygen the utilization of BioBLU single-use vessels in combination with Eppendorf DASbox or DASGIP Bioblock allows highly parallel and reproducible fermentation (figure 2). Comparing the BioBLU 0.3f and the BioBLU 1f processes proves the capability for seamless scale-up from single-use mini bioreactors to 1 L single-use vessels. k_La values of up to 2500 h^{-1} in BioBLU 0.3f and up to 4000 h^{-1} when using

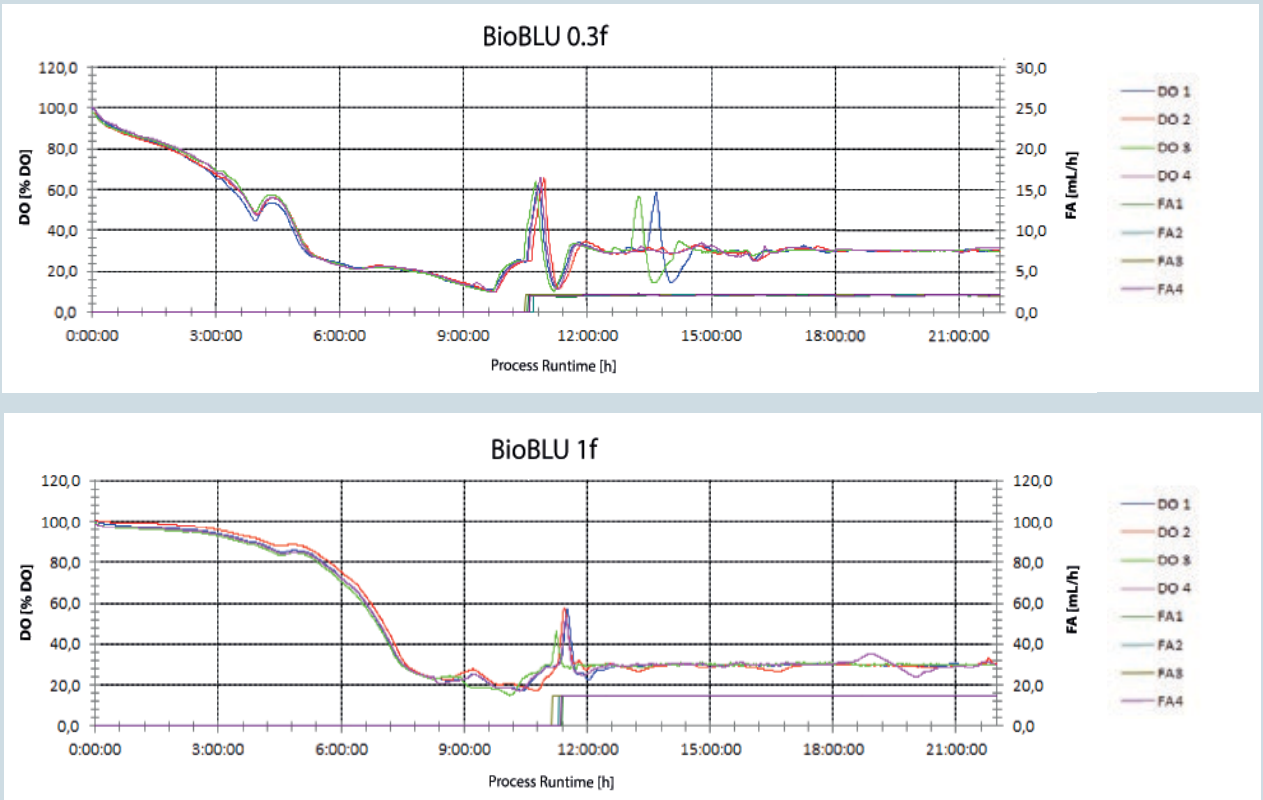

Figure 2: Parallel fermentation in BioBLU 0.3f and BioBLU 1f Single-use Vessels. The automatic feed-start was triggered by the glucose depletion induced DO peak in all four vessels in parallel. BioBLU 0.3f: 10.54 h ± 0.09 h after inoculation, BioBLU 1f: 11.31 h ± 0.11 h after inoculation. DO = dissolved oxygen concentration, FA = glucose pump rate.

BioBLU 1f vessels were determined by static sulfite depletion method (data not shown) and demonstrate that these single-use bioreactor designs perfectly match the demands of microbial applications. The biomass production (figure 3) was determined offline as cell wet weight and revealed comparable growth characteristics in both single-use bioreactors. The maximal biomasses of about 160 g/L gained in the fermentation runs correspond to an OD_{600} of about 100 (data not shown).

Conclusion

This case study shows that the BioBLU f single-use bioreactors address the specific needs of *E. coli* fermentations especially in regard to mass and heat transfer. The specifically adapted single-use design, featuring Rushton-type impellers, active heating and cooling, and overhead drive enabling high performance agitation, supports the high demands of microbial applications. Currently, single-use bioreactor technology is mainly used in cell culture. With the introduction of the Eppendorf

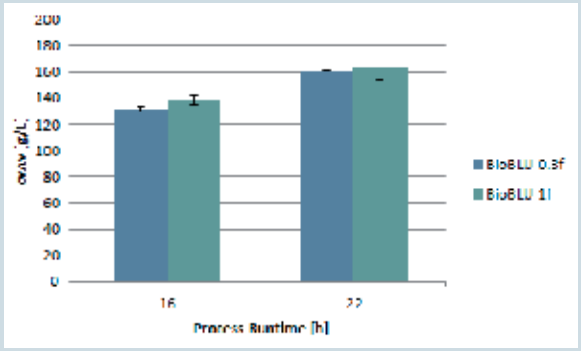

Figure 3: Biomass production. Cell wet weight (cww) of fermentations carried out in BioBLU 0.3f and BioBLU 1f Single-use Vessels, respectively.

BioBLU f Single-use Vessels adequate tools to accelerate bioprocess development in microbial applications, even high cell density fermentation, are available now.

Ordering information	Order no.
DASbox® Mini Bioreactor System for Microbial Applications with BioBLU® 0.3f Single-use Vessels, max 25 sL/h gassing	
with 4 Single-use Vessels	76DX04MBSU
with 8 Single-use Vessels	76DX08MBSU
DASGIP® Parallel Bioreactor System for Microbial Applications, TMFC gassing, variable speed pumps	
Bioblock with 4 vessels	76DG04MBBB
Bioblock with 8 vessels	76DG08MBBB
BioBLU® f Single-use Vessels	
4 x BioBLU® 0.3f Single-use Vessel for Microbial Application	78903503
4 x BioBLU® 1f Single-use Vessel for Microbial Application	78903505

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APPLICATION NOTE No. 294 I November 2013

High Cell Density E. coli Fermentation using DASGIP® Parallel Bioreactor Systems

Claudia M. Huether[1]* and Ute Ehringer[2]

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Abstract

In contract manufacturing one of the most challenging needs is the adaption of proprietary production pro-cesses, their optimization as well as process improve-ments after a transfer from one Contract Manufacturing Organization (CMO) to another. Each single process modification has to be verified with comparable data. The following application note gives an example for such an adapting procedure achieved by the use of the DASGIP Parallel Bioreactor System. The established process of one CMO included a high cell density fed-batch process, controlled by the key process parameters pH, agitation, temperature and growth dependent glucose feeding. This process was adapted and optimized for the use with another CMO.

Introduction

Biopharm GmbH, a GLP certified company offering research and development services for CMOs is using a derivative of the *E. coli* K-12 strain W3110 as their expression platform for prokaryotic production of recombinant, therapeutically proteins. The modified W3110BP strain is a property of Biopharm and is optimized for increased plasmid retention compared to the wild type *E. coli* W3110 as well as other conventional strains like BL21. Additionally, the Biopharm W3110BP strain is outstandingly capable of fermentation with high cell densities.

Aiming to reduce operation time parallel fermentation processes were used. The main item was the comparability of two or more parallel processes to show continuous process development by bridging results from one development round to another one.

The most important process parameter in the Biopharm's fermentation procedure is the dissolved oxygen (DO) concentration since the DO level determines the set-point from which additional feeding of the culture is needed. Thus, the precise observation and control of the DO level is the crucial factor for efficient fermentation procedures.

Scientists in the Biopharm laboratories are using the Eppendorf DASGIP Parallel Bioreactor System for microbial small-scale process development to get flexibility for their changing needs combined with highest precision and reliability.

Materials and Methods

All experiments were carried out using the cytokine producing recombinant strain *E. coli* K-12 W3110BP in complex

Figure 1: DASGIP Parallel Bioreactors System for Microbial Applications

media supplemented with vitamins, trace elements and other additives.
Initial small-scale experiments were performed with the DASGIP Parallel Bioreactor System in 250 mL fermentation vessels which were afterwards replaced by 500 mL vessels to increase biomass production. All key process parameters like pH, agitation and temperature were controlled online as well as the critical DO. The online DO level was used as trigger for automated activation of a glucose feeding profile.
To proof the scalability properties of the DASGIP Parallel Bioreactor System additional cultivations were run in a 5 L glass bioreactor (3rd party supplier). Temperature, pH and DO were measured online whereas the agitation was manually adapted to the current DO levels. The applied glucose feeding profile was similar to the profile in the 500 mL fermentation approaches.
The average cultivation time for all high cell density fermentations described in this application note was approximately 28 h.

Results and Discussion

High cell density fermentation was performed successfully. As shown in table 1 all fermentation results were similar with regard to the final biomass production and product formation. The different working volumes of 500 mL in contrast to 5 L did not influence the course of the process, demonstrating the easy scalability of test results gained with the DASGIP Parallel Bioreactor System.

Taking the online measured DO levels into account (figure 2) the vantages of an online controlled agitation as offered by the DASGIP Parallel Bioreactor System are displayed: Constant and precise regulation of the DO by automatically controlled stirring.
All recent improvements which were achieved for the described fermentation processes were successfully implemented into a large scale manufacturing process by a CMO (confidential data, not shown).

	Unit 1	Unit 2	5 L
Final optical density	230	240	268
Final bio dry mass [g/L]	56	54	65
Final bio wet mass [g/L]	260	242	275
Final cell number [cells/mL]	9.0×10^{10}	7.0×10^{10}	8.8×10^{10}
Final product level [g/L]	2.8	3.0	2.5

Table 1: Comparison of fermentation results. High cell density fermentations of E. coli K-12 W3110BP were performed in a DASGIP Parallel Bioreactor System (500 mL, units 1 and 2) as well as in a 5 L bioreactor.

The parallel set-up and control of independent fermentations guarantees an easy comparability of different approaches. "When comparing e.g. different bacterial host/

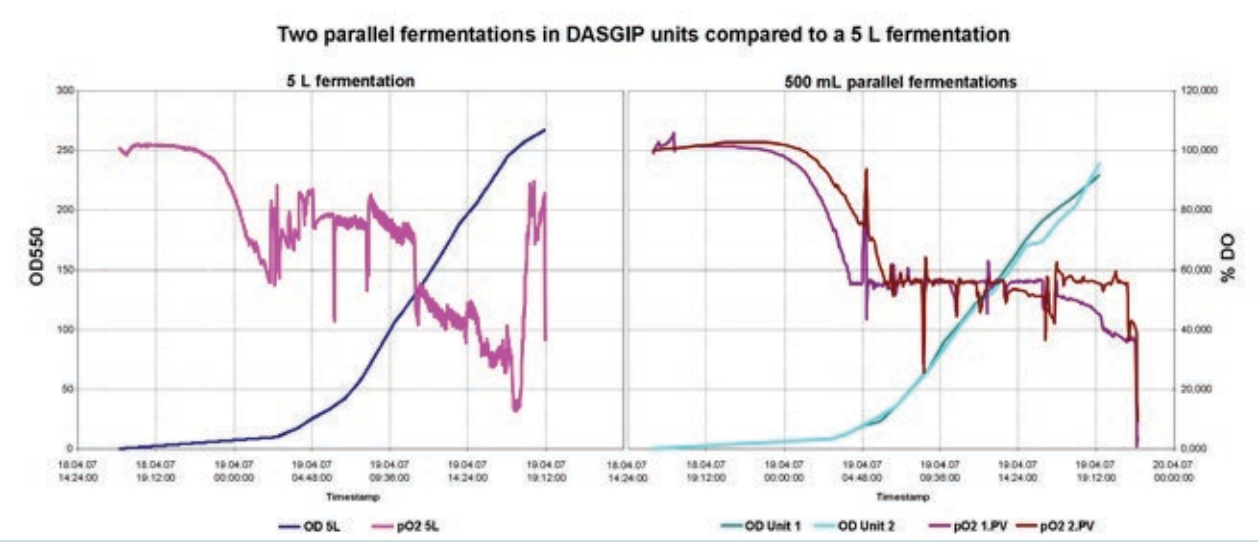

Figure 2: Two parallel fermentations in DASGIP units (500 mL) compared to a 5 L fermentation

plasmid combinations for protein production it is advantageous to use parallel approaches to avoid environmental influences. Thus, the experimental outcomes can be compared directly.", points out Ute Ehringer, Head of Development at Biopharm.
The Eppendorf DASGIP Parallel Bioreactor System for microbiology was also used for several other projects at Biopharm to accelerate the process development. When searching for suitable host/plasmid combinations for new products, advanced fermentation processes could be established with short development cycles by the time-saving parallel fermentation approaches.

Ordering information	Order no.
DASGIP® Parallel Bioreactor System for Microbial Applications	
4-fold system, benchtop, max. 250 sL/h gassing	76DG04MB
8-fold system, benchtop, max. 250 sL/h gassing	76DG08MB
4-fold system with Bioblock, max. 250 sL/h gassing	76DG04MBBB
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APPLICATION NOTE No. 318 I November 2013

Fed-Batch Biofuel Production Process Using a New Brunswick™ BioFlo® 115

Rivera, Yamakawa, Garcia, Geraldo, Rossell, Bonomi, Brazilian Bioethanol Science and Technology Laboratory, Campinas, SP, Brazil. **Filho,** School of Chemical Engineering, State University of Campinas, Campinas, SP, Brazil. **Capone, Sierra, Sha,** Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

In Brazil, it is common in the biofuel industry to utilize a biochemical process in which glucose, fructose and sucrose (derived from sugar cane juice and sugar cane molasses) are used to produce biofuel through a fed-batch fermentation process. In this experiment, *Saccharomyces cerevisiae* is used for biofuel production from sugar cane juice. The fermentation process metabolizes glucose into ethanol, and is used to produce many biofuel products in large production volumes. This strain of yeast is also widely used in other industrial applications to manufacture enzymes and proteins for beer, wine and bread. In this application note, we show that *Saccharomyces cerevisiae* can successfully be cultivated in high densities to convert sugar cane into biofuel using a BioFlo 115.

Introduction

The BioFlo 115 features a versatile and easy-to-use control station with color touchscreen monitor and built-in capability to operate in either fermentation or cell-culture mode. Switching between the operation modes automatically adjusts the control settings. Three fixed-speed pumps, temperature control, agitation control, and a rotameter with choice of gas flow ranges are available in BioFlo 115 systems. Pre-packaged kits for Basic or Advanced Fermentation and Advanced Cell Culture simplify the ordering process. Various kits can include options for direct-drive or magnetic-drive agitation, as well as water-jacketed or heat-blanketed vessels in 1 - 10 L range (approximate working volume). Ancillary equipment such as pH/DO and foam/level sensors are either included in kits, or can be added separately as options.

Materials and methods

Fermentation and cell recycling

For this application, a BioFlo 115 controller with advanced fermentation kit, direct drive and 2 L water jacketed vessel was used (Figure 1). The total fermentation process consisted of two distinct phases: An initial cell propagation and growth phase using complex medium (yeast extract) followed by sugar cane juice (growth medium) under aerobic conditions and the biofuel production phase using sugar cane juice (alcohol fermentation medium) under anaerobic conditions. The initial cell propagation phase utilized 20.0 g/L of medium substrate (dry mass) under aerobic condition, after that, the sugar cane growth media was added. Cells were recovered for use in the biofuel production phase operated under anaerobic conditions. Cell recycling was conducted through centrifugation. The cells were recovered and diluted with 500 mL of sterile water and transferred back to the bioreactor aseptically via an addition port in the headplate. Sugar cane juice feeding was performed over a four-hour period using peristaltic pump 3 (fixed flow of 6.25 mL/min) up to a final volume of 1.5 L and was maintained for two more hours to ensure uptake of accumulated sugar. Cells were recycled for three consecutive alcoholic fermentations. The cell propagation

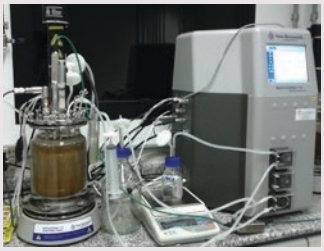

Figure 1: BioFlo 115 system used for biofuel production

phase and the biofuel production phase were both operated under fed-batch mode.

Medium

The initial cell propagation phase used complex medium as follows (per liter of de-mineralized water):

Initial complex medium composition	Concentration
K_2SO_4	6.60 g/L
KH_2PO_4	3.00 g/L
$MgSO_4$	0.50 g/L
$CaCl_2 \cdot 2H_2O$	1.00 g/L
Yeast extract	5.00 g/L

After autoclaving at 121 °C for 15 min, the medium was cooled to room temperature. The carbon source and additional supplements passed through a sterile filter were also added according to the following concentrations:

Filter-sterilized elements	Concentration
Urea	2.30 g/L
Thiamine	3.00 g/L
EDTA	15.00 mg/L
$ZnSO_4 \cdot 7H_2O$	4.50 mg/L
$CoCl_2 \cdot 6H_2O$	0.30 mg/L
$MnCl_2 \cdot 4H_2O$	0.84 mg/L
$CuSO_4 \cdot 5H_2O$	0.30 mg/L
$FeSO_4 \cdot 7H_2O$	3.00 mg/L
$NaMoO_4 \cdot 2H_2O$	0.40 mg/L
H_3BO_3	1.00 mg/L
KI	0.1 mg/L

The carbon source for growth medium and alcoholic fermentation medium were both formulated with sugar cane juice and sterilized separately at 121 °C for 15 min.

The growth medium contained 129 g/L of total reducing sugar (TRS). The sugar content is shown in the table below:

Sugar cane juice composition	Concentration
Sucrose	102.51 g/L
Glucose	10.99 g/L
Fructose	10.01 g/L

The alcoholic fermentation medium contained 171.65 g/L of total reducing sugar (TRS):

Sugar cane juice composition	Concentration
Sucrose	133.01 g/L
Glucose	16.79 g/L
Fructose	14.85 g/L

Inoculum

The *Saccharomyces cerevisiae* strain used in this work was an unnamed strain cultivated at the Brazilian Bioethanol Science and Technology Laboratory. It was originated from the department of Food Engineering, State University of Campinas, Brazil. The strain was maintained on agar plates prepared as follows (per liter of de-mineralized water):

Inoculum composition	Concentration
Yeast extract	10.00 g/L
Peptone	20.00 g/L
Glucose	20.00 g/L
Agar	20.00 g/L

Before the inoculation, the strain was transferred from agar plate to a liquid complex medium containing the following (per liter of de-mineralized water):

Liquid complex medium composition	Concentration
Yeast extract	10.00 g/L
Peptone	20.00 g/L
Glucose	20.00 g/L

The inoculum was cultured in shake flask for 24 hours using a shaker set to 33 °C at 250 rpm.

Fermentor control conditions for cell propagation phase

During the cell propagation phase, the Dissolved Oxygen (DO) was controlled to 60 % using the cascade feature through agitation and air control. Temperature was controlled to 33 °C throughout the run and pH was controlled to 5.0 via the acid and base assigned to peristaltic pumps 1 and 2. These parameters were maintained until the biofuel production phase (alcohol fermentation phase) was initiated. When the cell propagation phase was completed, the medium was transferred to a 2 L flask using an external pump (Watson Marlow®). The medium was then placed inside the laminar flow cabinet and transferred into a centrifuge (Beckman Coulter® centrifuge with JLA-9.100 rotor) and was spun down at 8.000 rpm/4 °C for 10 minutes. Setpoints are listed below:

BioFlo® 115 setpoints	
Agitation	Cascaded range at 250 – 600 rpm
Temperature	33 °C
pH	5.0
DO	> 60 %
Gas (supplied by sparge)	Cascaded range at 0.5 – 1.0 SLPM (Air)

Fermentor control conditions for biofuel production phase

During the biofuel production phase, the process was changed from aerobic to anaerobic fermentation, and DO was monitored between 0 % and 2.3 %, but not controlled. Agitation was set to auto mode and held at 100 rpm. Temperature controlled to 33 °C throughout the run. pH was controlled to 5.0. These parameters were maintained until the alcoholic fermentation/biofuel production phase was initiated. Gas flow was shut-off and the alcohol fermentation medium was introduced so that the *Saccharomyces cerevisiae* could start anaerobic fermentation and produce ethanol. Setpoints are listed below:

BioFlo® 115 setpoints	
Agitation	100 rpm
Temperature	33 °C
pH	5.0
DO (monitored only)	0 – 2.3
Gas	None

DO calibration

The DO sensor was calibrated using a standard two-point calibration method: 0 % (often referred to as the zero point) and 100 % (often referred to as span). The electronic zero method was performed by disconnecting the sensor from the cabinet, allowing the value to stabilize, and then reconnecting the sensor to the cable. The span was achieved by bringing the vessel filled with medium to all of the operational setpoints to a stable value and then spanning the DO sensor. DO should be calibrated post-autoclave as part of the pre-inoculation setup. The sensor was allowed to polarize for a 6 hour period. After calibration, DO should remain around 100 % until after inoculation.

pH calibration

The pH sensor was calibrated prior to the autoclave cycle outside the vessel using a two-point calibration method with two standard pH buffers. The pH 7.0 buffer was used to zero the sensor and the pH 4.0 was used as the span.

pH control

The pH parameters were maintained by adding a sulfuric acid solution (H_2SO_4) via pump 1 (assigned as "acid") to lower the pH and adding potassium hydroxide solution (KOH) via pump 2 (assigned as "base") to raise the pH. The dead-band implemented for pH control was 0.02.

Fed-batch

Filter-sterilized media were fed to the vessel through pump 3.

Monitoring

Sucrose, glucose and fructose concentrations were detected by high-performance liquid chromatography (HPLC) using an Agilent® 1260 Infinity with RI detector through an Aminex® column (HPX-87P, 300 mm x 7.8 mm) at 60 °C. EMD Millipore® Milli-Q® water was used (column flow rate 0.5 mL/min) for the eluent phase. Ethanol concentration was determined by HPLC as well, using a Thermo Fisher Scientific® Dionex® UltiMate® 3000 with RI detector (Shodex RI-101) via an Aminex column (HPX-87H 300 mm x 7.8 mm) at 50 °C. Sulfuric acid, 5 mM at a rate of 0.5 mL/min, was used for the eluent phase. Dry weight mass measurements were carried out in triplicate using an analytical balance. Cell propagation was monitored on line through an Aber® capacitance probe as well as by taking samples for optical density measurements at 600 nm using a spectrophotometer.

Results and discussion

Figure 2, right, shows plots of the three key process parameters monitored during the biofuel production phase: total cell mass, X (kg/m³); substrate, S (kg/m³); and ethanol, P (kg/m³). All three are important factors in monitoring ethanol production from metabolized sugar cane juice. The cell concentration profile presented in Figure 2 (A) and the substrate profile presented in Figure 2 (B) illustrate the typical results in a fed-batch configuration. For the first 3 hours of the biofuel production phase, the initial density of yeast cells (44 kg/m³) decreased due to the dilution factor by sugar feeding. In the meantime, the sugar concentration (S) started to accumulate. After necessary sugar feeding, the cell concentration stabilized and the sugar concentration started to decrease and was completely consumed over time. The ethanol, as illustrated in Figure 2 (C), was produced according to available sugars in the medium and the production increased over time to reach a final concentration of approximately 56 kg/m³.

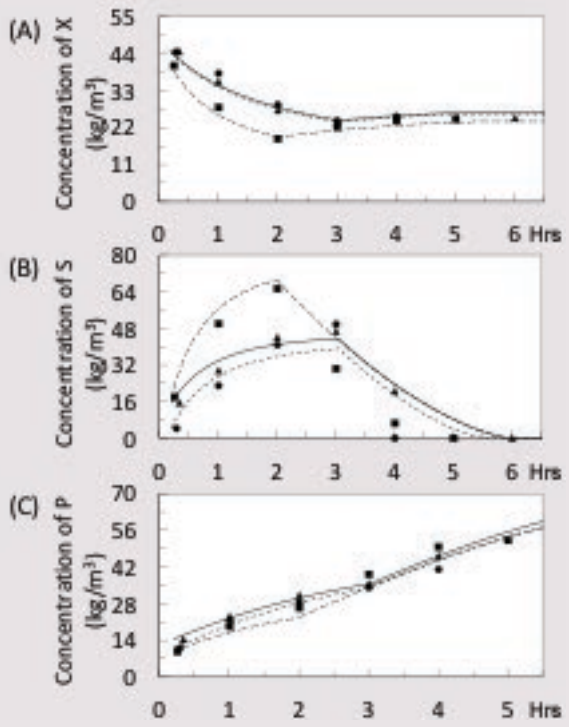

Figure 2: Experimental (Ferm. 1 (■); Ferm. 2 (▲) and Ferm. 3 (●) for concentration of (A) Cell, (B) Substrate and (C) Ethanol

Ordering information

Description	N. America Order no.	International Order no.
New Brunswick™ BioFlo® 115 Master Control Station w/Thermal Mass Flow Controller (TMFC)	Call	Call
Add-a-Vessel Advanced Fermentation Kit 3 L (Water Jacketed)	M1369-1612	M1369-1612

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APPLICATION NOTE No. 311 I December 2008

Anaerobic Yeast Fermentation for the Production of Ethanol in a New Brunswick™ BioFlo® 310 Fermentor

Yinliang Chen, Jeff Krol, Weimin Huang, Rich Mirro and Vikram Gossain, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

Whether used for research or production, the versatile New Brunswick BioFlo 310 fermentor from Eppendorf allows growth of a wide variety of aerobic and anaerobic microorganisms, including bacteria, plant, algae, fungi and yeast. Its advanced controller can regulate up to four vessels simultaneously, 120 process loops in all. Here we demonstrate one facet of its versatility—a technique for inducing ethanol production in yeast, by switching from an aerobic growth phase to an anaerobic steady-state culture.

Introduction

Saccharomyces cerevisiae is a model eukaryotic organism, often used in research because it is easy to manipulate and culture, and is comparatively similar in structure to human cells. This yeast is also widely used in industrial applications to manufacture enzymes and proteins for beer, wine and bread, and because it metabolizes glucose to ethanol, is also used to produce many biofuel products. We produced ethanol from a *S. cerevisiae* (American Type Culture Collection® strain 20602) in a 7.5 liter BioFlo 310 fermentor, to demonstrate the flexibility of this advanced fermentation system. In the first phase, we grew the yeast in an aerobic environment, using a dissolved oxygen cascade control strategy to produce a sufficient cell density. Then we pumped in nitrogen gas to create an anaerobic environment for inducing ethanol production, and used reduction and oxidation (redox) potential measurements to monitor any increase in dissolved oxygen levels, which signaled a slowdown in cell growth. Redox potential activates ethanol production and changes the total soluble protein pattern of *S. cerevisiae*[1]. We used redox potentials to control the oxidation-reduction level by adding feed medium to the vessel when the redox potential value in the fermentor vessel rose above 130 mV.

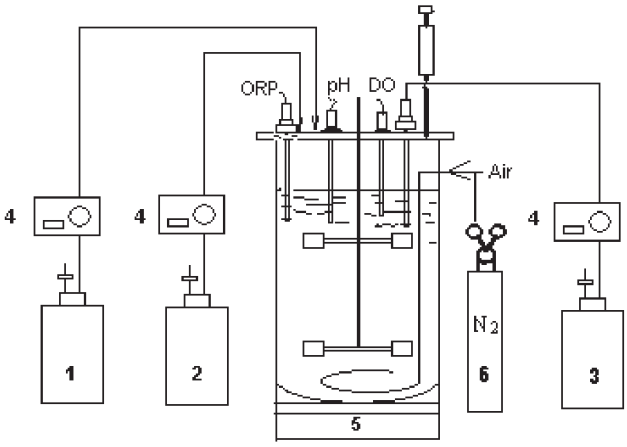

Figure 1. BioFlo 310 setup for anaerobic growth. Shown are containers for base solution (1) and feed medium (2), a collection reservoir (3), three pumps (4), the fermentor vessel with pH, dissolved oxygen (DO) and redox (OPR) probes (5), and a nitrogen gas tank (6).

Materials and Methods

Advanced Control in a Compact Package

The BioFlo 310 fermentor (Fig. 1) consists of a master control station with built-in controller, 15-inch color touchscreen display, three built-in pumps and 4-gas mixing with a thermal mass flow controller for gas flow control. The

BioFlo 310 is available with choice of four interchangeable autoclavable vessels, 2.5, 5.0, 7.5 and 14.0 liter total volume, and includes a pH probe, dissolved oxygen probe and level or foam probe, as well as hoses, sterile sampler and more. The system can be operated in batch, fed-batch and continuous modes for handling a variety of applications, and it meets current good manufacturing practice (cGMP) requirements. Here we used a 7.5 L vessel and operated the system in fed-batch mode. We added a Mettler-Toledo® redox sensor to the vessel and directly connected it to the fermentor controller's optional redox input. We used New Brunswick's optional BioCommand® Plus with OPC Control software to automatically control the process and log data throughout the run. Ethanol production and glucose concentrations were measured off-line using a YSI® 2700 Select™ Biochemistry Analyzer, and we used a Beckman Coulter® Vi-Cell® XR Cell Viability Analyzer to measure the cell viability and concentration during the entire process.

Inoculum Preparation and Growth Phase

We prepared a seed culture in a one-liter Erlenmeyer flask containing 250 mL of Difco™ YM growth medium (Becton Dickinson), using a 1.0 mL frozen suspension. The culture was incubated at 29 °C for 18 hours at 240 rpm in an orbital shaker (New Brunswick model Innova® 43R). Then we transferred the inoculum to the BioFlo 310 vessel containing 4.75 liters of fermentation medium. The fermentor was controlled at 30 °C and pH 5.0. Aeration rate was set at 2.5 L/min[1] (0.5 vessel volumes per minute), and agitation speed was 200 - 800 rpm. We used a 29 % NH_4OH base solution to control pH and 50 % glucose as feed medium. Dissolved oxygen and redox potential were measured during the entire process (Fig. 2). Dissolved oxygen was cascade-controlled at 30 % via agitation. The optical

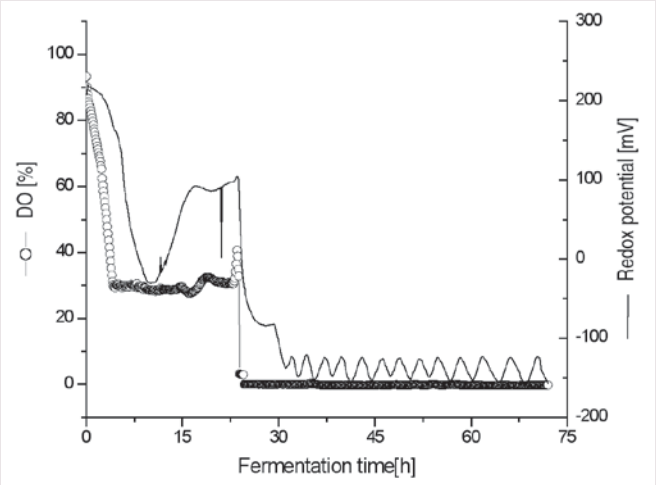

Figure 2. Time profiles of dissolved oxygen and redox measurements during *S. cerevisiae* fermentation. Dissolved oxygen was controlled at 30% during the cell growth phase followed by an anaerobic production phase in nitrogen gas.

density of the fermentation broth was measured at 600 nm to monitor cell growth. Glucose feed started at 7 hours of elapsed fermentation time (EFT), after the glucose was close to 1 g/L[1]. To determine concentrations of glucose, ethanol and the dry cell mass, we centrifuged the samples and collected the supernatant and biomass separately. Biomass samples were dried at 80 °C for 48 hours. The cell concentration reached 3.26×10^8 cells/mL[1] in 24 hours, and cell viability remained above 96 %. The feed volume was 1,200 mL in 17 hours of fed-batch growth phase, with the feeding rate ramping from 0 to 16 mL/L/H[1]. Although cells were in an aerated fermentation condition, a small quantity of ethanol (20 g/L[1]) was produced before the beginning of anaerobicity.

Anaerobic Ethanol Production Phase

After 24 hours, we exposed aerobic, glucose-limited cultures grown at a moderate specific growth rate to two simultaneous perturbations: a rapid depletion of oxygen and glucose feeding regulated by redox potential measurement. Nitrogen, instead of air, was used to sparge the fermentor. The gas flow rate was kept at 0.5 VVM (2.5 L/min[1]). The medium feed was controlled by BioCommand OPC software using online redox potential measurement as an on-line input. Redox potential readings of 130 mV triggered a medium feed pump to add carbon source medium to maintain the oxidation-reduction level for the ethanol production (Fig. 3). Cell viability was monitored and measured, validating that cells remained healthy in the anaerobic production phase. Optical density values were maintained around 80 and viability at 88 % at 72 hours elapsed fermentation time. Culture broth pH was well maintained at 5.0.

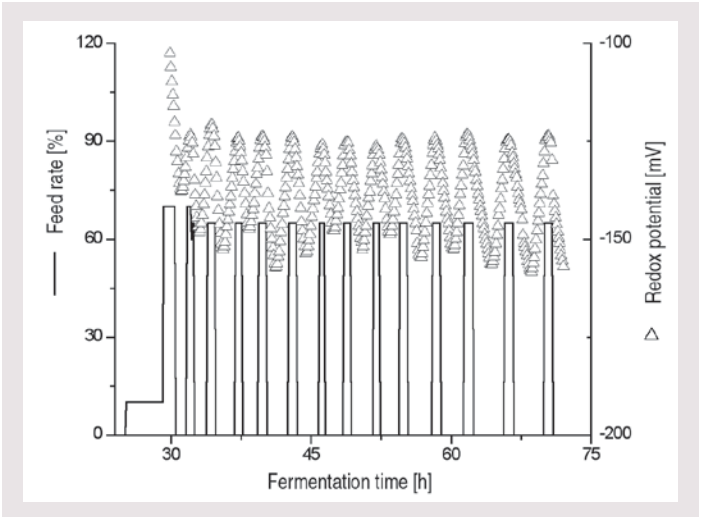

Figure 3. Use of redox potential to control the medium feed.

Conclusion

We cultured yeast in an aerobic fermentation and then switched to an anaerobic process to induce ethanol production, demonstrating how versatile the BioFlo 310 fermentor can be. We produced 85 g/L[1] of ethanol in 70 hours, while cell viability was maintained at levels as high as 88 %, proving this fermentor to be a very powerful and capable research or production instrument.

In addition to being used for a wide variety of fermentation processes, the BioFlo 310 can also be adapted for mammalian or insect culture with the use of optional accessories. Multiple connections are provided for integrating data from ancillary sensors, analyzers, scales or other devices for optimized process control. Eppendorf offers optional validation and training packages, as well as the services of an in-house lab to assist with process development and scale-up. For more information on this system or on our full range of advanced fermentors and bioreactors for research through production, see www.eppendorf.com or write to us at newbrunswick@eppendorf.com.

References

[1] **Effect of electrochemical redox reaction on growth and metabolism of Saccharomyces cerevisiae as an environmental factor.** Kwan, N.B., Hwang, T.S., Lee, S.H., Ahn, D.H. & Park, D.H. *J. Microbiol. Biotechnol.* 17, 445–453 (2007).

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APPLICATION NOTE No. 317 I November 2008

Using Redox Measurements to Control Anaerobic Yeast Fermentation in a New Brunswick™ BioFlo® 310 Fermentor

R & D Laboratory, Eppendorf Inc., Enfield, CT, U.S.A.

Abstract

In this study, we used *Saccharomyces cerevisiae* to produce ethanol under anaerobic conditions in a BioFlo 310 fermentor. Initially, the yeast culture was conducted under aerobic conditions; after 24 hours, the culture was switched to anaerobic growth for the production of ethanol. During the fermentation process, redox potential was maintained at -180 mV using a redox sensor and gas flow control. Toward the end of fermentation, a significant amount of ethanol was produced and the cells retained maximum viability. Our study indicated that with a redox sensor, the BioFlo 310 fermentor was capable of maintaining anaerobic conditions, extremely low oxygen level, during the entire fermentation process.

Introduction

Metabolic activity of microorganisms depends on many factors, including oxidation and reduction reactions, or the "redox potential" of the culture environment. Redox reactions govern metabolism of biologically important nutrients such as carbon, hydrogen, oxygen, nitrogen and sulfur. Measuring their redox potential allows the fermentor operator to monitor the addition of reducing or oxidation agents, while ensuring that the potential is in the proper range for cell growth, especially when the DO level is very low.

Since free electrons never exist in any noteworthy concentration, reduction and oxidation reactions are always coupled together, and can be considered a measure of the ease with which a substance either absorbs or releases electrons. The determination of redox potential is a potentiometric measurement, expressed as millivolts (mV). Practically, however, no electrical current flows through the sample solution during this potential measurement.

Redox sensors are most commonly used to maintain anaerobic conditions in a fermentation broth. They can be used to measure trace amounts (< 1 ppm) of dissolved oxygen, at levels that are too low for the DO sensors in various anaerobic fermentation processes. Glucose-containing feed medium can be treated as a reducing source in oxidation-reduction of the culture medium. When the oxidation capacity is increased, the redox potential level will elevate to a higher value. On the flip side, its value will become lower when the culture broth has a higher reducing capacity.

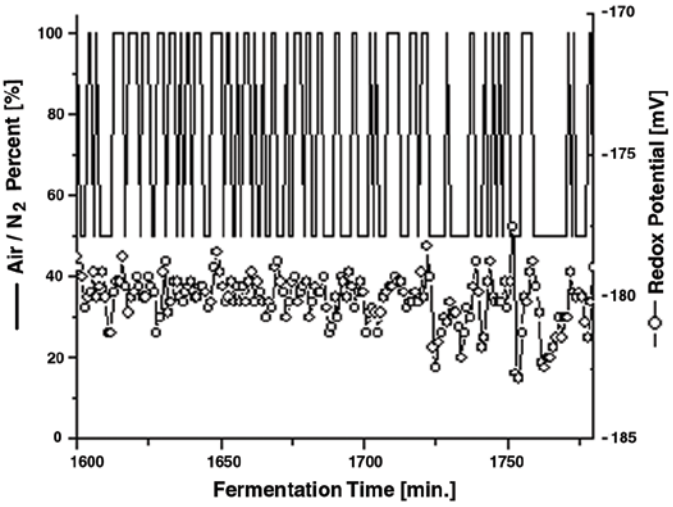

Figure 1: Redox potential used in the gas mix control to maintain redox potential level at -180 mV.

Our study used *Saccharomyces cerevisiae* yeast (ATCC® 20602), because Saccharomyces is widely used in industry (e.g. beer, bread and wine fermentation and ethanol production), as well as in the lab due to its ease of manipulation and growth. Additionally, yeasts are eukaryotic and comparatively similar in structure to human cells. *S. cerevisiae* metabolizes glucose to ethanol primarily

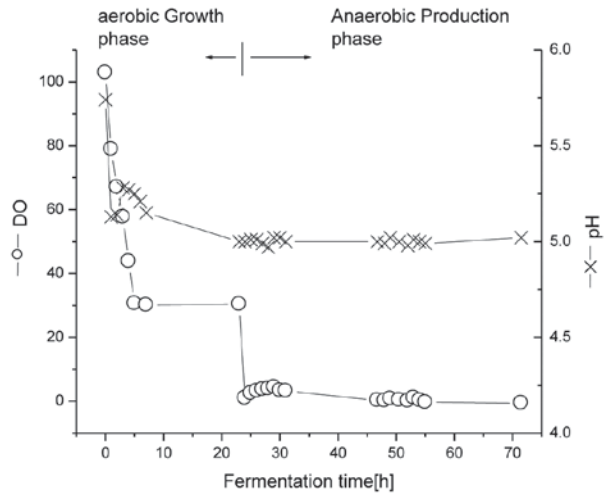

Figure 2: DO and pH controls in the cell growth and production phases

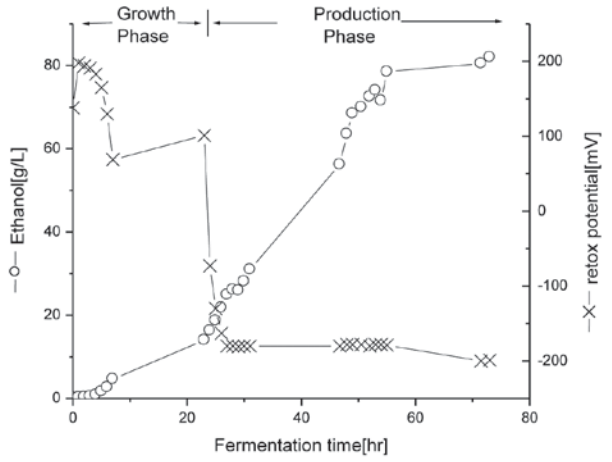

Figure 3: Ethanol production in the fermentation of *S. cerevisiae*

by way of the Embden-Meyerhof pathway. However, a small concentration of oxygen can be provided to the fermenting yeast, as it is a component in the biosynthesis of polyunsaturated fats and lipids. We used redox potential measurements to maintain these special anaerobic fermentation conditions. A trace of air (oxygen) was introduced as an oxidation agent to raise the redox potential level. A short pulse of air was introduced into the vessel when redox potential fell below -180 mV (Figure 2).

Materials and Methods

S. cerevisiae strain ATCC 20602 was grown in a 5 L working volume benchtop Eppendorf New Brunswick BioFlo 310 fermentor. Ethanol production and glucose concentrations were measured with a YSI® 2700 Select™. A Mettler-Toledo® redox sensor was directly connected to the BioFlo 310 controller to track redox potential. A Vi-CELL® XR Cell Viability Analyzer was used to measure cell viability and concentration throughout the entire process.

A seed culture using a 1.0 mL frozen suspension was prepared in a 1 L Erlenmeyer flask containing 250 mL of Becton Dickinson Difco™ YM growth medium. The culture was incubated at 29 °C for 18 hours in an orbital shaker (New Brunswick Innova® 43R) at 240 rpm. The entire inoculum was transferred to the BioFlo 310 fermentor vessel containing 4.75 liters of medium. Medium composition was as follows:

Medium Composition	Concentration
Glucose	10.00 g/L
$MgSO_4 \cdot 7H_2O$	0.60 g/L
$(NH_4)_2SO_4$	3.00 g/L
KH_2PO_4	10.00 g/L
$CaCl_2 \cdot 2H_2O$	0.14 g/L
Yeast extract	18.00 g/L
Soy Peptone	18.00 g/L
Na_2HPO_4	1.00 g/L
Thiamine	0.01 g/L
Trace metal solution	1.00 mL/L
Antifoam	0.5 – 1.00 mL/L

Setpoints were as follows:

Medium Composition	Concentration
Temperature	30.0 °C
pH	5.0
Aeration rate	2.5 L/min 0.5 VVM
Agitation speed	200 – 800 rpm

pH was controlled with a 29 % NH_4OH base solution. 50 % glucose was used as feed medium. DO and redox potential were measured during the entire process. DO was cascade-controlled at 30 % via agitation in the growth phase. Glucose feed started at 7 hours of elapsed fermentation time (EFT) after the glucose was close to 1 g/L. New Brunswick BioCommand® software was used to control and log the entire process. The optical density of fermentation broth was measured at 600 nm to monitor cell growth. To

determine concentrations of glucose, ethanol and the dry cell mass, samples were centrifuged and the supernatant and biomass were collected separately. Biomass samples were dried at 80 °C for 48 hours.

Anaerobic Ethanol Production Phase
After 24 hours of cell growth, the fermentation process was switched to an anaerobic condition by exposing culture to two simultaneous perturbations: a rapid depletion of oxygen and glucose feeding regulated by redox potential measurement. Nitrogen, instead of air, was used to sparge the fermentor vessel. Gas flow rate was kept at 0.5 VVM (2.5 L/min). On-line redox potential readings of -180 mV triggered a solenoid valve of air supply to maintain the oxidation-reduction level for the ethanol production (Shown in Figure 1). pH was well controlled at 5.0. Cells remained healthy; OD values were maintained around 80 and viability was 85 % at 72 hours EFT. 85 g/L of ethanol were produced in 70 hours as shown in Figure 3.

Conclusion

S. cerevisiae was cultured in an aerobic fermentation, and then switched to an anaerobic process using on-line redox measurements to maintain oxidation-reduction levels for ethanol production. Our study produced 85 g/L of ethanol in 70 hours, while cell viability was maintained at levels as high as 88 %. The study provides a new technique for using redox potentials to monitor and control ethanol production from yeast, but also demonstrates the BioFlo 310 fermentor as a versatile fermentor for aerobic and anaerobic fermentations.

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BioProcessing JOURNAL

Trends & Developments in BioProcess Technology

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

APPLICATION NOTE

Hybridoma and CHO Cell Culture Using the New Brunswick™ S41i, An Environmentally-Friendly, "Low Emission" Incubator Shaker

Introduction

Automobiles contribute significantly to CO_2 emissions in the atmosphere. However, laboratory equipment such as CO_2 incubators can potentially release over 120,000 liters of CO_2 gas per year.[1] The "epGreen initiative" established by Eppendorf® will ensure that their products are developed with the mindset of reducing environmental impact.

Most of the CO_2 gas consumed by incubators is released into the environment. However, Eppendorf's new incubator shaker, the New Brunswick S41i, releases extremely low amounts of CO_2 under normal cell culture conditions without sacrificing performance. This study evaluates the product's performance in culturing hybridoma and Chinese hamster ovary (CHO) cells and compares CO_2 gas consumption to comparable products from other companies. The CO_2 consumption data reveals that the New Brunswick S41i consumes 5–10 times less CO_2 gas versus those competitors studied—a significant carbon footprint reduction.

Superior engineering helps to minimize incubator gas leakage by tightly sealing the: (1) inner glass door, protected by a sturdy outer door; (2) motor drive boots; and (3) incubation chamber. Based on our cell culture growth rate comparisons for cell density and viabilities, the performance evaluation clearly demonstrates the impressive performance of the New Brunswick S41i. This new CO_2 incubator includes a robust quadruple eccentric drive shaker to provide the accurate and stable parameters required for growth of non-adherent cells. The shaker drive is optimized for high performance within a humid and carbon dioxide-rich environment.

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Materials and Methods

Equipment

- New Brunswick S41i incubator shaker equipped with high-temperature disinfection capability
- CO_2 incubator shaker from competitor 1
- CO_2 incubator shaker from competitor 2
- Vi-CELL® Cell Viability Analyzer (Beckman Coulter, Inc.)
- YSI 2700 SELECT™ Biochemistry Analyzer (YSI Inc.)
- New Brunswick GALAXY® S series CO_2 incubator
- Omega® FMA-1608A thermal mass flowmeter (Omega Engineering, Inc.)
- Eppendorf consumables:
  - › Research® plus single-channel pipettes
  - › epT.I.P.S.® pipetting tips
  - › Easypet® pipetting dispensers

Media and Cells

- DG44 CHO cells (Invitrogen)
- EX-CELL® CD CHO serum-free medium (SFM) for CHO cells (Sigma-Aldrich Co. LLC)
- Hybridoma cell DA4-4 (ATCC® Number HB-57™)
- Dulbecco's Modified Eagle's Medium (DMEM) (ATCC)
- Fetal bovine serum (FBS) 5% (Gibco)
- Penicillin Streptomycin (Pen Strep) 100× (Gibco)

Gas Consumption Determinations

Prior to cell culture, all incubator shakers were programmed at 37 °C, 95 rpm and 5% CO_2 and were allowed to equilibrate for at least 12 hours. Inline CO_2 gas pressures were set at the lowest values recommended by each manufacturer. An offline gas analyzer was used to verify the CO_2 levels within each incubator. Thermal mass flowmeters were used to record volumetric gas consumption over a time period of up to 48 hours on each unit. Tests were repeated multiple times and the average values are reported below.

CHO Culture Protocol

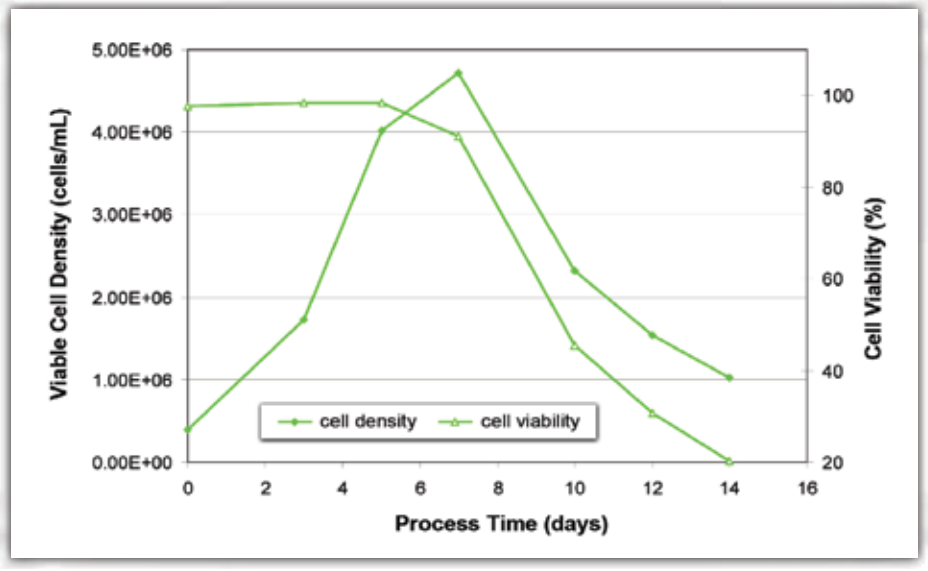
CHO cells were grown in SFM supplemented with 1% Pen Strep. Six 250 mL Erlenmeyer flasks were inoculated with 60 mL of the same stock culture at a concentration of 0.3×10^6 cells/mL. Two flasks were placed on each of the three incubator shaker platforms (New Brunswick S41i, and competitors 1 and 2). The flasks were incubated at 37 °C in a mixture of 5% CO_2, 95% air, and agitated at 130 rpm (4.69 rcf).

CHO cells were grown for a period of 14 days. Samples of 1.5 mL were taken on days 3, 5, 7, 10, 12, and 14, and analyzed with the YSI 2700 SELECT instrument for glucose concentrations. The Vi-Cell determined cell concentrations and viability. Six flasks were cultured per incubator and the results were averaged out.

Hybridoma Culture Protocol

Hybridoma cells were grown in DMEM medium supplemented with 5% FBS and 1% Pen Strep. Six 250 mL Erlenmeyer flasks were each inoculated with 45 mL of stock culture at a concentration of 0.2×10^6 cells/mL. Six flasks were placed on each of the three incubator shaker platforms (New Brunswick S41i, and competitors 1 and 2). The flasks were incubated at 37 °C in a mixture of 5% CO_2, 95% air, and agitated at 95 rpm (2.52 rcf).

Hybridoma cells were subcultured on days 2 and 4 to a concentration of approximately 0.2×10^6 cells/mL. A sample of 0.6 mL was taken every day from each of the flasks and analyzed with the YSI 2700 SELECT instrument for glucose concentrations. The Vi-Cell determined cell concentrations and viability. Six flasks were cultured per incubator and the results were averaged out.



Results and Discussion

Growth Assessment of CHO and Hybridoma Cells

Viable CHO cell density reached a maximum of 4.72×10^6 cells/mL by day 7. The cell viability was maintained at approximately 98% up to day 5 and dropped steadily thereafter (Figure 1).

FIGURE 1. Average viable cell density and viability of CHO culture in the New Brunswick S41i.

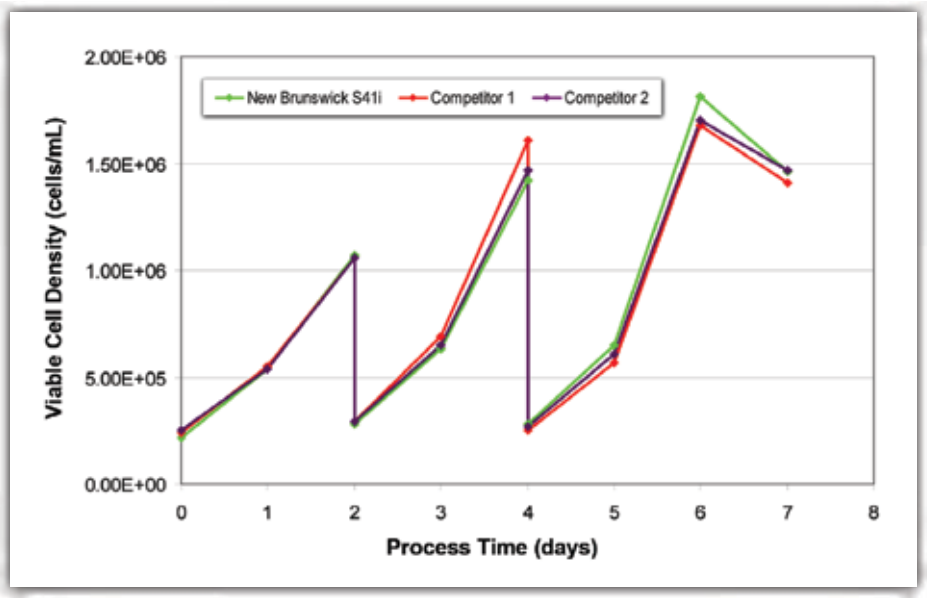


FIGURE 2. Comparison of average viable cell densities of hybridoma cultures grown in New Brunswick S41i and competitors 1 and 2.

In comparison to CHO cells, hybridoma cultures maintained a high average viability of approximately 95% through day 6 due to the subculturing of cells during log phase of growth on days 2 and 4. A maximum viable cell density of 1.81×10^6 cells/mL was achieved on day 6 (Figures 2 and 3).

Measurements of Gas Consumption

The measurement of CO_2 gas consumption at a 5% CO_2 setpoint revealed that the competitive units evaluated consume much higher levels of CO_2 gas over the same period as compared to the New Brunswick S41i (Figure 4).

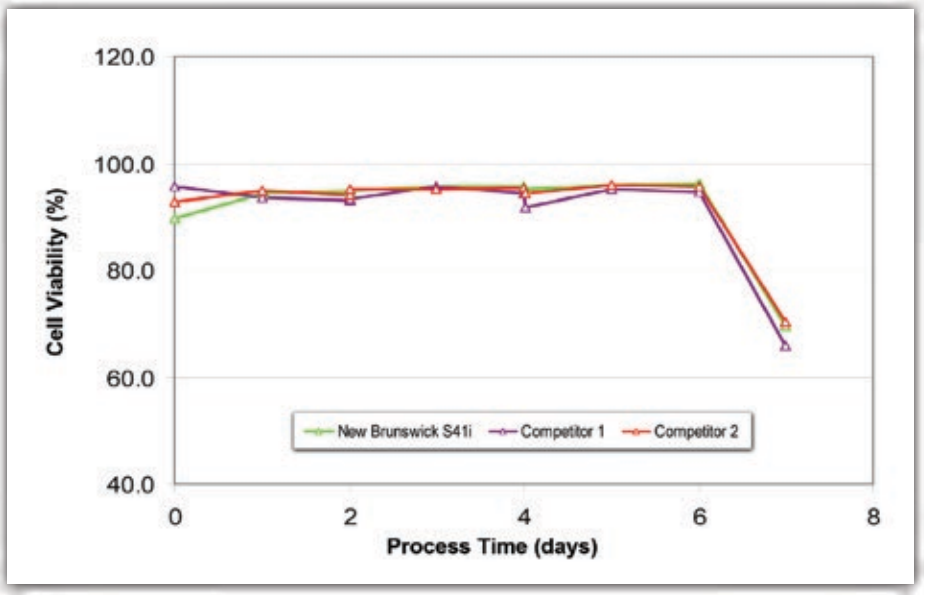


FIGURE 3. Comparison of average percentage viabilities of hybridoma cultures grown in New Brunswick S41i and competitors 1 and 2.

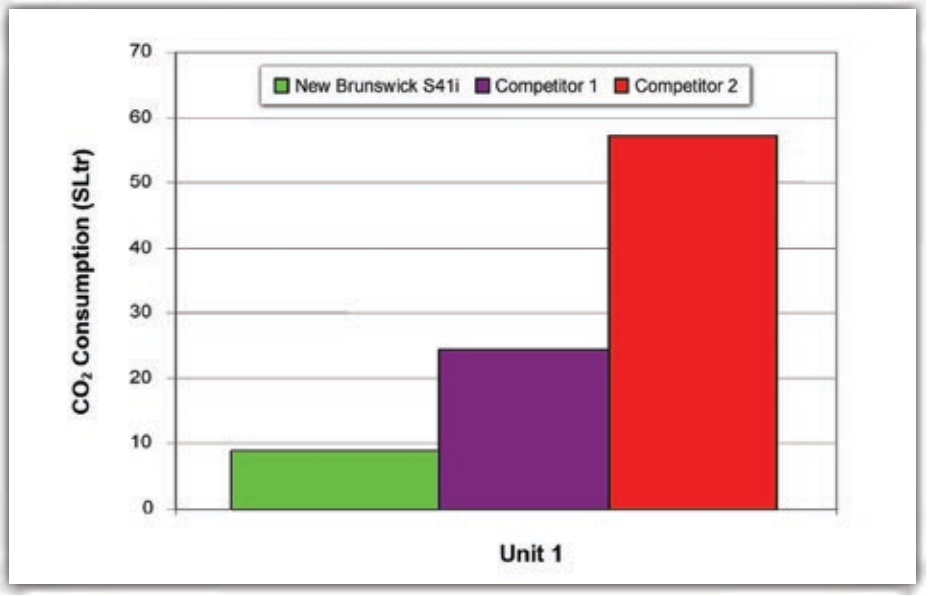


FIGURE 4. Average CO_2 gas consumption in standard liter (SLtr) of tested units over a 24-hour period.

REFERENCE

[1] Based on calculation of competitor 2 data in Figure 4 (~57 L/24-hr day, 365 days/yr = 20,805 L/yr). Most CO_2 are not really "consumed" by cells — they are released into the environment.

NOTES

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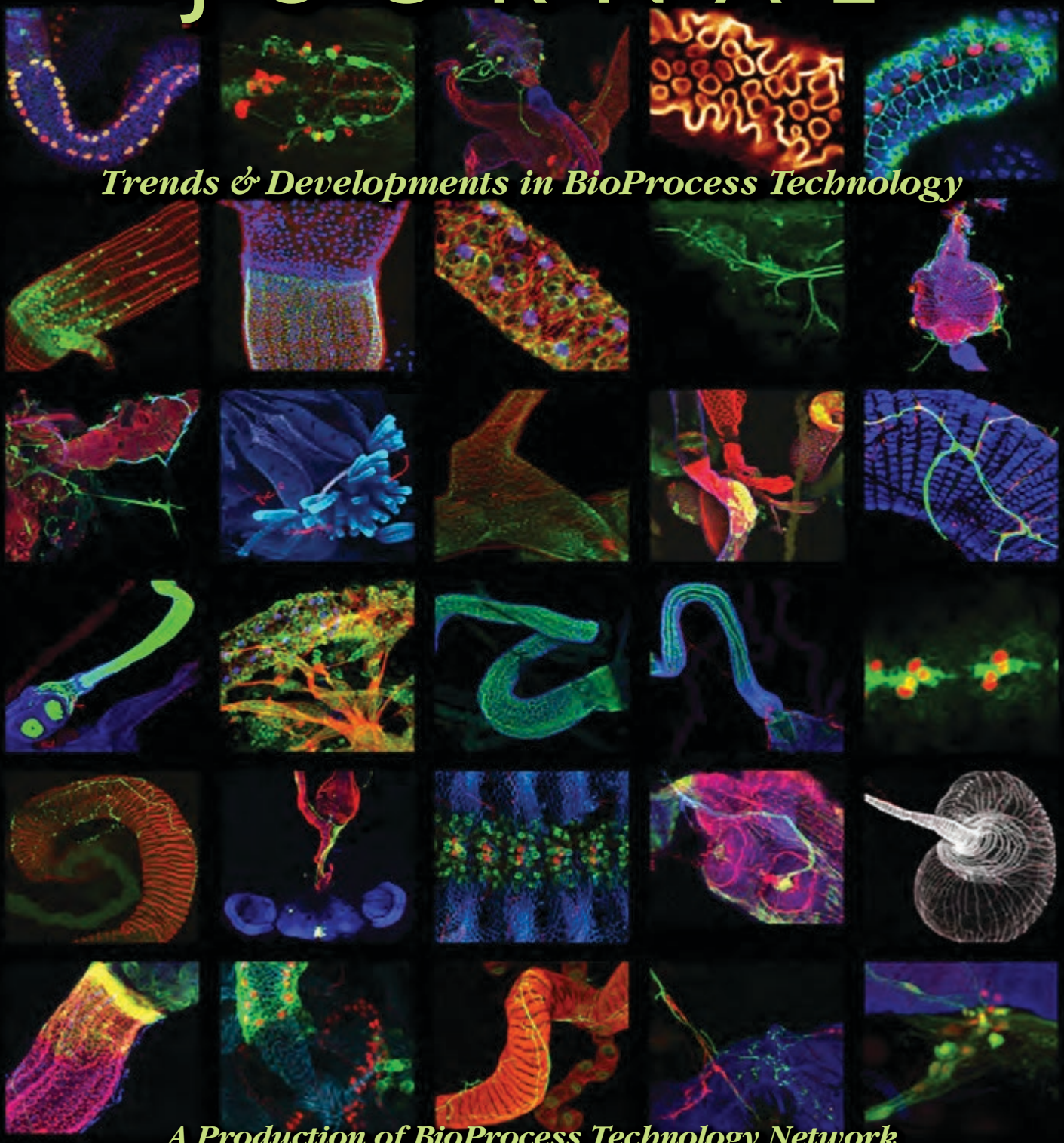

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A Comparative Bioreactor Vessel Study: Conventional Reusable Glass and Single-Use Disposables for the Production of Alkaline Phosphatase

By TAYLOR HATTON, SHAUN BARNETT, MA SHA, and KAMAL RASHID

Abstract

Single-use, stirred-tank bioreactor systems have been used in large-scale production for a number of years. Bench-scale, stirred-tank bioreactors have not been commercially-available for single-use until recently. The New Brunswick™ CelliGen® BLU pitched-blade bioreactor was introduced in 2009, and the CelliGen BLU packed-bed bioreactor, in 2012.

Little information is currently available on the utility of these bioreactors for bench-scale production of recombinant products. Thus, we designed this study to perform multiple comparisons with these single-use bioreactors and their traditional glass vessel counterparts. The data comparisons included: (1) CelliGen BLU pitched-blade vs. glass pitched-blade; and (2) CelliGen BLU pitched blade in batch mode vs. CelliGen BLU packed-bed in perfusion mode. Chinese hamster ovary (CHO) cells were used to measure alkaline phosphatase (ALKP) production in each bioreactor. The final measured concentration of ALKP, after eight days of batch-mode culture in the single-use, pitched-blade bioreactor, was 1.6 U/mL compared to 2.1 U/mL in the reusable bioreactor. After six perfusion harvests in the single-use, packed-bed bioreactor, the combined ALKP production was 16.2 U/mL compared to 17.4 U/mL in the reusable bioreactor in batch mode. Multiple batch culture runs in the pitched-blade bioreactor would be required to match the output of a single run in the packed-bed bioreactor in perfusion mode.

Results demonstrate that there are no significant differences between the reusable and single-use systems for bench-scale production of recombinant proteins. Our results also suggest that the CelliGen BLU packed-bed bioreactor, when operated in perfusion mode, is superior to the CelliGen BLU pitched-blade bioreactor when operated in batch mode, confirming our studies from 2012.[1]

Introduction

Stainless steel, stirred-tank bioreactors have been the trusted and dominant design for decades in scale-up of animal cells. Along with proven reproducibility of these bioreactors come some minor disadvantages, namely, cleaning and maintenance.[2-4] When reusable bioreactors are used for production of biopharmaceuticals, the cleaning process needs to be validated which increases cost in economic terms. In a survey conducted by BioPlan Associates, the primary reason for biopharmaceutical developers increasing their utilization of single-use systems was to eliminate cleaning requirements (90.2 % of respondents).[5]

The New Brunswick CelliGen BLU single-use bioreactor line, available in the pitched-blade and now in packed-bed design, has helped to make single-use, stirred-tank bioreactors readily available commercially to the bench-scale community.

The CelliGen BLU pitched-blade bioreactor incorporates one large plastic pitched-blade impeller which efficiently mixes the media while disrupting the sparger bubbles. The CelliGen BLU packed-bed bioreactor incorporates two horizontal perforated plastic sheets which entrap Fibra-Cel® disks, creating a bed for cells.[6] This design allows for media exchange while eliminating the need for filtration, sedimentation, or centrifugation of the cells, as is the case with other bioreactor types.[7] The packed-bed bioreactor, combined with a perfusion mode of operation, is a very useful means of increasing cell growth and productivity of recombinant proteins. Cells cultured in

packed-bed bioreactors are not exposed to hydrodynamic forces which allows for maximum cell growth and protein expression.[8]

Reusable packed-bed bioreactors in perfusion mode have been compared to reusable pitched-blade bioreactors in batch mode[1] and perfusion systems have been compared to fed-batch systems.[9] However, to date, no published studies have compared the productivity of protein-secreting cells in reusable packed-bed and pitched-blade bioreactors to single-use, packed-bed and pitched-blade bioreactors. Therefore, the objective of this study was to perform a multi-comparative study between single-use and reusable bioreactors. We expect the results from these experiments will help aid in the introduction of the single-use (CelliGen BLU) bioreactors as an alternative to reusable packed-bed bioreactors while also highlighting the advantages of the CelliGen BLU packed-bed bioreactor operated in perfusion mode.

Perfusion modes of operation offer many advantages over batch or even fed-batch modes of operation. Systems operated under perfusion mode do not accumulate toxic byproducts, as seen in the batch operations, because the media is removed on a regular basis. Perfusion systems can often be operated at smaller scales. These systems have the ability to increase cell concentrations up to 30 × more than batch systems. Long run times allowed with the packed-bed bioreactor decrease the constant need for re-seeding cells and re-establishing seed cultures. This dramatically reduces setup time and labor over batch systems, advantages that will lead to reduced cost of operation[10, 11] A study performed by Biopharm Services showed that conventional stainless steel bioreactor facilities were the slowest to become cash positive. This was due to the increased capital investment required up front and the longer process of building a stainless steel bioreactor facility.[12]

Materials and Methods

Culture Procedures

In order to evaluate the impact of these bioreactor systems on protein secretion by cultured cells, we employed a recombinant ALKP-secreting CHO cell line (rCHO), generously provided by CDI Bioscience, Inc. The rCHO cells were engineered with their IPTG-regulated RP Shift® vector so that the rCHO cells stop replicating and shift to protein production when induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). CD-CHO medium (Gibco, Life Technologies) was used throughout these experiments. The media contained 6.3 g/L glucose and was supplemented with 8 mM L-glutamine and 100 µg/mL of an antibiotic/antimycotic solution (Gibco, Life Technologies). Frozen rCHO cells were thawed and transferred to T-75 flasks with CD-CHO serum-free medium and allowed to expand. Once a sufficient number of cells were achieved, sterile disposable spinner flasks were utilized to further expand the cells. Cell subculturing continued until a sufficient number of viable cells was achieved for use as a seed culture at a density of approximately 5×10^5 cells/mL. Two New Brunswick CelliGen 310 advanced bench-top, stirred-tank bioreactors incorporating two single-use CelliGen BLU bioreactors were utilized to grow the rCHO cells. A solution of $NaHCO_3$, 8 % w/v (8 g/100 mL), was used to help control the pH inside the bioreactor systems.

Pitched-Blade Impeller Operated in Batch Mode

The first bioreactor system utilized the pitched-blade impeller. The CelliGen BLU pitched-blade bioreactor comes pre-packaged and sterilized with the pitched-blade impeller, tubing, sparger, and filters for easy setup. The vessels are made of materials that meet USP Class VI standards and have been tested for leachables.[13] The blades on the pitched-blade impeller are flat and set at a 45° angle. This blade orientation provides good axial and radial mixture of the media while also increasing the oxygen mass transfer rate and disruption of bubbles released from the sparger. The pitched-blade impeller is designed to minimize the stress of mixing on shear-sensitive cells.[5]

Two experimental trials were performed utilizing the pitched-blade bioreactor. One trial was performed in a reusable (glass) 2.2 L total volume vessel (1.75 L working volume) and a second trial was performed utilizing a single-use CelliGen BLU 5.0 L total volume vessel (3.5 L working volume). For each trial, the bioreactor was allowed to operate until the cell concentration reached approximately 2×10^6 cells/mL at which time the cells were induced with IPTG. Both experimental trials had the following parameters, as shown in Table 1.

Packed-Bed Basket Impeller Operated in Perfusion Mode

The second bioreactor system utilized the packed-bed basket impeller. The CelliGen BLU packed-bed bioreactor

TABLE 1. Bioreactor parameters.

Parameter	Setpoint	
	Glass 2.2 L	CelliGen BLU 5.0 L
Temperature	37 °C (± 0.1°C)	37 °C (± 0.1°C)
Agitation	120 rpm (± 5 rpm)	120 rpm (± 5 rpm)
Dissolved O_2	35 % (± 1%)	35 % (± 1%)
pH	7.1 (± 0.01)	7.1 (± 0.01)
Gas flow	0.5 slpm	1.5 slpm

comes pre-assembled and sterilized with the impeller pre-packed with 150 g of Fibra-Cel disks. The head-plate is equipped with all necessary tubing, filters, sparger, and connectors for easy setup. The single-use packed-bed bioreactors meet the same USP Class VI standards as the pitched-blade bioreactor. This bioreactor system is suitable for both anchorage-dependent and suspension cells, and this system does not require the adaptation of anchorage-dependent cells to suspension culture. The packed-bed basket impeller is commonly used in the production and collection of extracellular proteins.[14] This system incorporates a basket with two horizontally positioned perforated screens. Fibra-Cel disks are placed in between the screens creating a bed to entrap suspension cells or provide a surface for attachment of anchorage-dependent cells. The Fibra-Cel disk bed provides a culture environment that allows freshly oxygenated media to slowly pass over the cells while also providing protection from external shear forces.[1] The rotation of the impeller creates a negative pressure that draws media up through the hollow center shaft where the sparger introduces oxygen to the media. The packed-bed bioreactor is the ideal system to use when a product is secreted out of the cell. Because cells are immobilized in the Fibra-Cel bed, samples of media can easily be removed without cell loss or culture disruption.

Two experimental trials were performed utilizing the packed-bed bioreactor. One trial was performed in a 2.2 L reusable (glass) vessel and a second trial was performed utilizing a single-use CelliGen BLU 5.0 L vessel. Both vessels were equipped with a basket (as described above) containing 85 g of Fibra-Cel disks in the reusable bioreactor and 150 g in the single-use bioreactor. The perfusion process was initiated once the cells reached the exponential growth phase as shown in Table 2. All experimental trials had the same growth conditions (temperature, oxygen, and pH) as the batch process (Table 1).

Biomarkers of Cell Growth and Productivity

Cell productivity was assessed by measuring activity of the secreted ALKP protein using an enzyme assay (AnaSpec, Inc.), according to the manufacturer's protocol.

TABLE 2. Comparison of perfusion volumes.

Perfusion	Volume	
	Glass 2.2 L	CelliGen BLU 5.0 L
Day 1	0.5 L	1.0 L
Day 2	1.0 L	2.0 L
Days 3–15*	2.0 L	4.0 L
**NOTE: Perfusion occurred every other day.*		

For simplicity, unit measurements were used in this study. A unit (U) of ALKP activity was defined as the amount of enzyme that hydrolyzes 1 µmol of p-nitrophenylphosphate to p-nitrophenol in a total reaction volume of 1 mL in one minute at 37 °C. The YSI 2700 SELECT™ biochemistry analyzer (YSI, Inc.) was utilized to monitor the glucose and lactate levels in the culture media every 24 hours for the duration of each trial.

Results

Pitched-Blade Bioreactor Operated in Batch Mode

Cell Density and Viability

Figure 1 shows the cell growth and viability of two independent experimental trials in the pitched-blade bioreactor. The seeding density in the single-use (CelliGen BLU) bioreactor was 6.1×10^5 cells/mL (Figure 1A) while the seeding density in the reusable (glass) bioreactor was 5.7×10^5 cells/mL (Figure 1B) as calculated by trypan

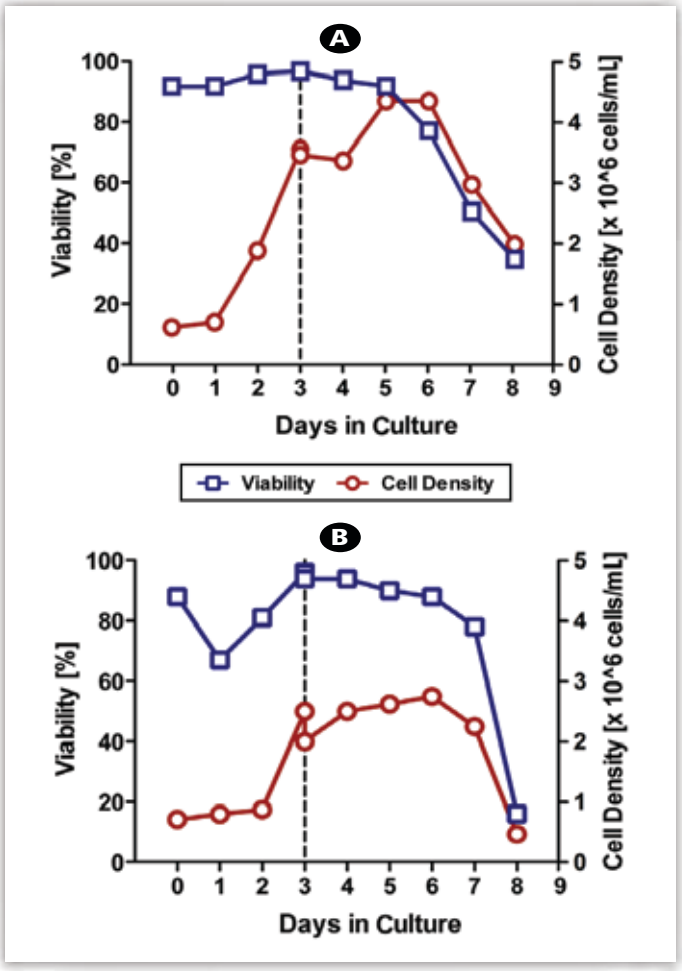

FIGURE 1. Growth of rCHO cells in the pitched-blade bioreactor system. Values shown are the cell density and viability on each day of culture. Each panel represents an independent experimental trial: **(A)** single-use [CelliGen BLU]; and **(B)** reusable [glass]. The dashed line indicates the time of induction of ALKP production by IPTG.

blue staining utilizing the Countess® cell counter. The maximum cell density observed in the single-use bioreactor (4.4×10^6 cells/mL) was significantly higher than in the reusable bioreactor (2.2×10^6 cells/mL). Cell viability was greater than 90 % for the majority of the experimental trial in the single-use bioreactor while the viability in the reusable bioreactor recovered to greater than 90 % after a few days in culture.

Glucose Utilization and Lactate Production

Glucose is the main energy source for cell proliferation and ALKP production. Thus, glucose levels were expected to directly correlate with ALKP production in each experiment. Because lactate is a secondary energy source, lactate levels were expected to decline following this initial increase and the utilization of glucose in the media. Lactate metabolism is beneficial to the system by reducing a major metabolic by-product from the system.[15, 16] Glucose levels measured at the time of induction (day 3) were nearly 0 g/L in both experiments (Figure 2). Media lactate concentrations increased in response to decreasing glucose availability. The observed gradual decrease of lactate near the end of each trial indicates its use as a secondary energy source.

ALKP Production

Figure 3 shows the concentrations of ALKP measured daily in two independent experimental trials in the pitched-blade bioreactor. ALKP concentrations increased over the six culture days post-induction. However, a decrease in ALKP activity was observed at the time of harvest in both trials, possibly due to degradation of the ALKP protein at the end of the experiment.[17] Serum-free media was utilized for growth of rCHO cells in this study. Thus, ALKP was susceptible to the action of proteases made by the rCHO cells.[18] As reported above, cell density in the single-use bioreactor was higher than the reusable bioreactor, suggesting that cell proliferation was dominant over ALKP production. This observation likely accounts for the slightly lower amount of ALKP detected in the single-use bioreactor.

Packed-Bed Bioreactor Operated in Perfusion Mode

Cell Density and Viability: The packed-bed bioreactors in both experimental trials were seeded with 5.0×10^5 cells/mL. However, because of the presence of the Fibra-Cel discs, it was not possible to sample the cells directly during culture to determine cell yield and viability. Therefore, cell density and viability were not monitored on a daily basis; rather, the rate of glucose consumption was used as a surrogate to approximate changes in cell density.[19] The growth of cells in the packed-bed bioreactor was estimated using the average glucose consumption

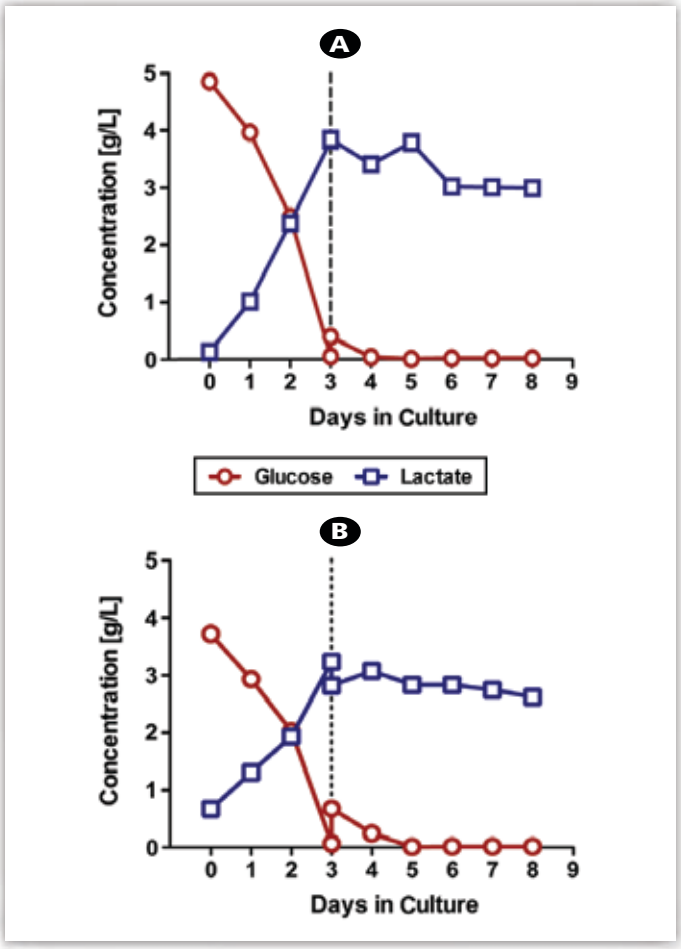

FIGURE 2. Glucose consumption and lactate production by rCHO cells cultured in the pitched-blade bioreactor system. Values shown are the concentrations of glucose and lactate in the culture media measured daily. Each panel represents an independent experimental trial: **(A)** single-use [CelliGen BLU]; and **(B)** reusable [glass]. The dashed line indicates the time of induction of ALKP production by IPTG.

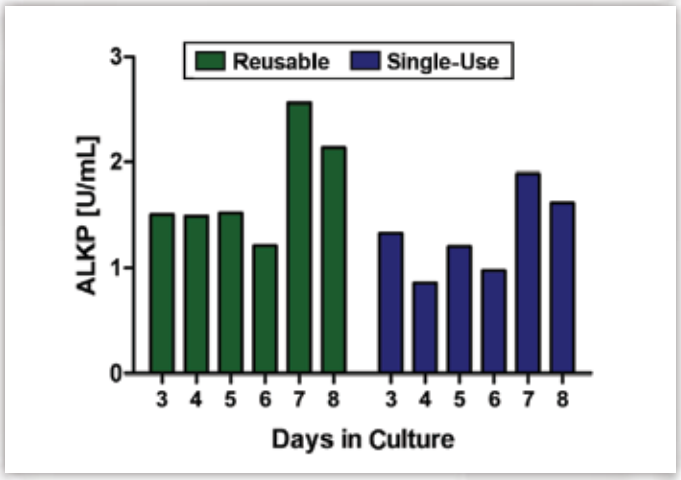

FIGURE 3. ALKP production by rCHO cells cultured in the pitched-blade bioreactor system. ALKP concentration in the culture media was measured each day after induction. IPTG induction of ALKP occurred on culture day 3.

rate. Glucose consumption rates were similar in both the pitched-blade and packed-bed bioreactor systems up to day four (Figure 4). However, after day four of culture, glucose utilization in the packed-bed bioreactor continued to increase exponentially, while the trend for glucose consumption in the pitched-blade bioreactor increased linearly. Increased glucose consumption observed on culture day five suggests that cell density in the packed-bed bioreactor had likely increased.

Glucose Utilization and Lactate Production: Glucose consumption was very similar in both the single-use (Figure 5A) and reusable (Figure 5B) experimental trials. As previously observed with the pitched-blade bioreactor system, media lactate concentrations increased in response to decreasing glucose availability in both trials. The use of lactate as a secondary energy source can also be observed as lactate levels decrease at each 2 and 4 L perfusion.

ALKP Production: Concentrations of ALKP in the two

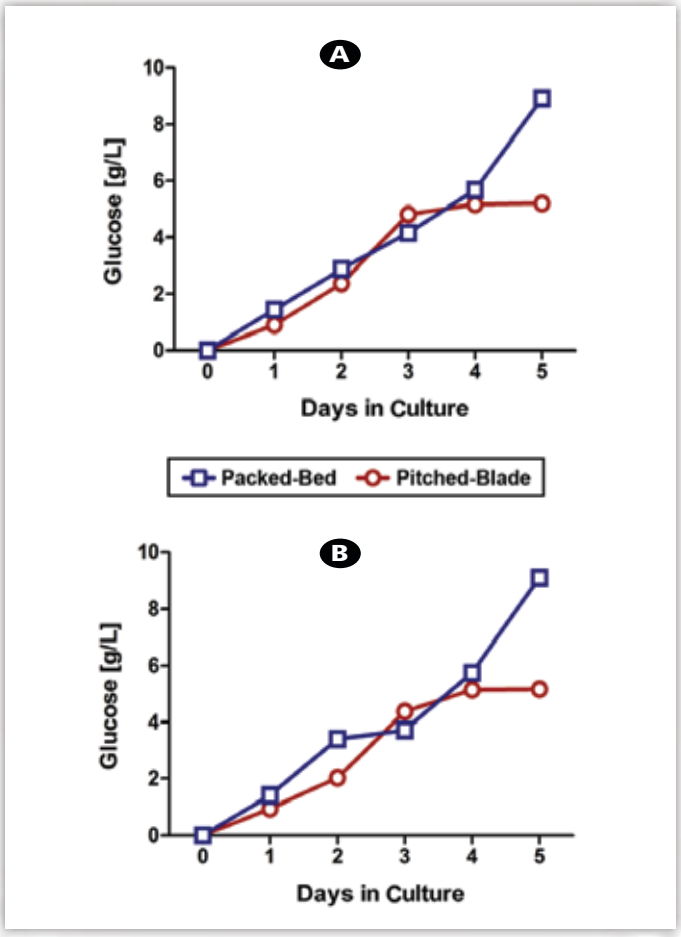

FIGURE 4 (right). Comparison of glucose uptake by rCHO cells in the pitched-blade and packed-bed bioreactor systems. Values shown are the daily glucose media concentrations consumed in two independent experimental trials: **(A)** single-use [CelliGen BLU]; and **(B)** reusable [glass].

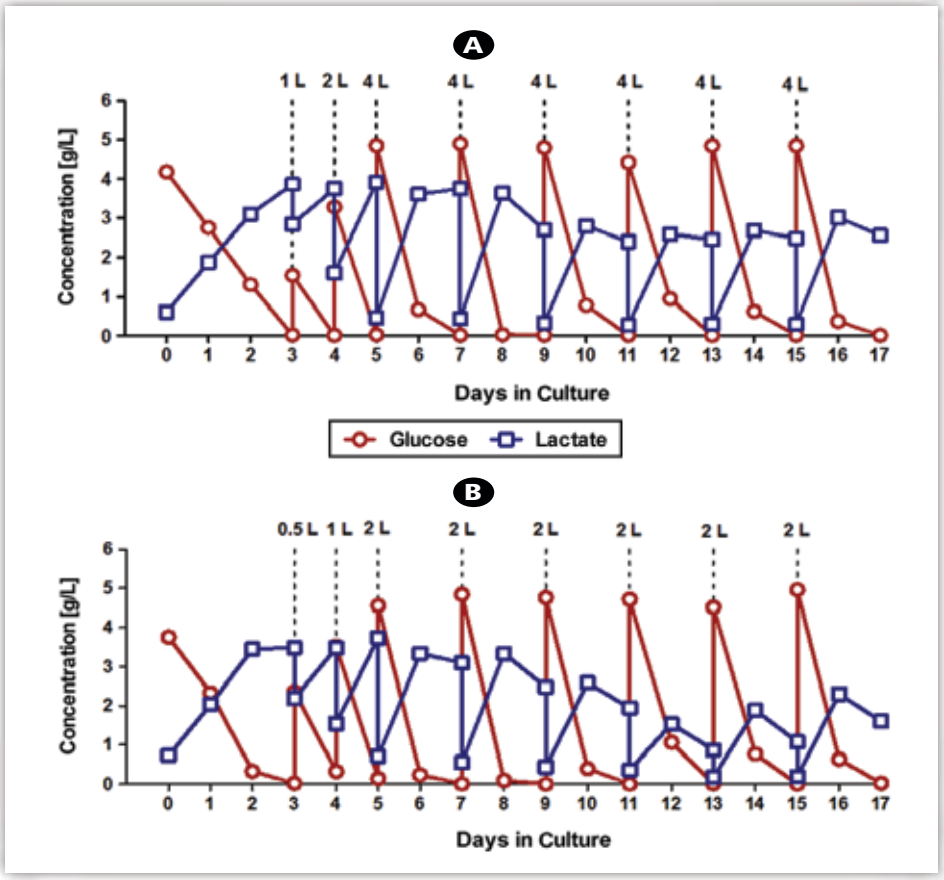

FIGURE 5 (left). Glucose consumption and lactate production by rCHO cells cultured in the: **(A)** pitched-blade; and **(B)** packed-bed bioreactor systems. Values shown are the amounts of glucose and lactate measured in the culture media at each media exchange. The time and volume of the media exchange is indicated at each dashed line. Induction of ALKP activity by IPTG began on culture day 5 and continued every two days throughout the remainder of the experiment.

independent experiments utilizing the packed-bed bioreactor are shown in Figure 6. Following initial expansion culture of rCHO cells for five days, ALKP production was induced every two days with a media exchange containing IPTG. We determined previously that continuous culture with IPTG in the media yielded greater production of ALKP as compared to a transient exposure to the inducing agent (data not shown). A modest increase in ALKP production was observed at each media exchange, although the level of ALKP varied by induction day and by experiment trial (Figure 6). The correlation between glucose utilization and ALKP production, as previously observed, supports the inference that the rate of glucose consumption in both experiments was conducive to the production of large amounts of ALKP. The rapid exhaustion of glucose and a presumed high cell density were likely contributing factors to the large amounts of ALKP observed.

Comparison of the Reusable and Single-Use Bioreactor Systems for ALKP Production

A major objective of this study was to compare the single-use to the reusable pitched-blade bioreactor, the single-use to the reusable packed-bed bioreactor, and finally the pitched-blade bioreactor to the packed-bed bioreactor in both vessel types. The total ALKP production per experimental trial is shown in Figure 7. Figure 7A shows the comparison between the two trials performed in the pitched-blade bioreactor. Final ALKP concentrations are very similar in both trials. Figure 7B shows the comparison between the two trials performed in the packed-bed bioreactor. Resembling the pitched-blade bioreactor, final ALKP concentrations in both packed-bed bioreactor types were markedly similar. The total ALKP production in the CelliGen BLU pitched-blade vs. the CelliGen BLU packed-bed bioreactor is shown in Figure 7C. Overall, the packed-bed bioreactor system produced ALKP to a much greater extent (nearly 9-fold greater) compared to the pitched-blade system.

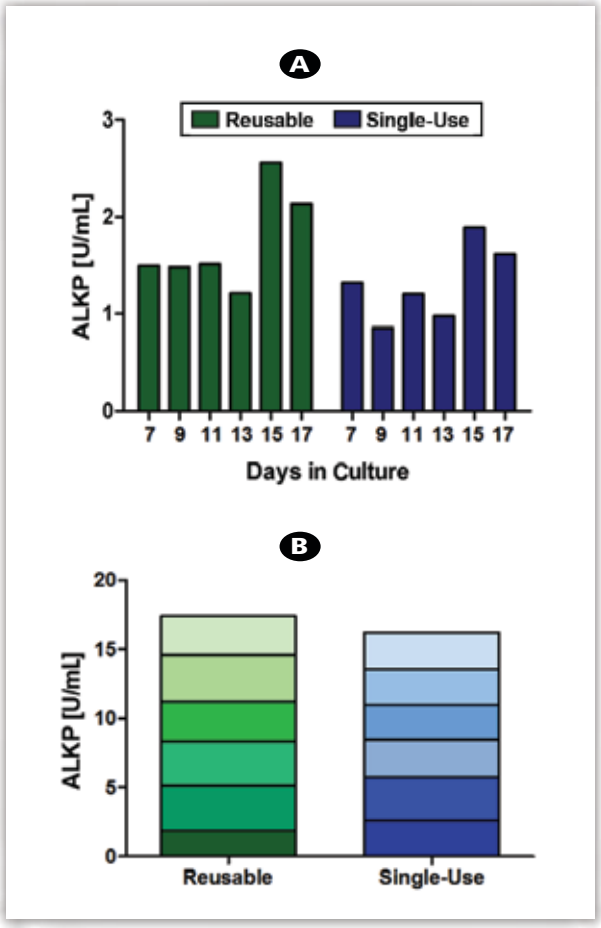

FIGURE 6. ALKP production by rCHO cells cultured in the packed-bed bioreactor system: **(A)** ALKP concentrations in culture media measured each day in two independent experimental trials using the packed-bed bioreactor. IPTG induction of ALKP began on culture day 5 and continued every two days for the remainder of each experiment; and **(B)** cumulative production of ALKP throughout each experiment with each bar representing a perfusion.

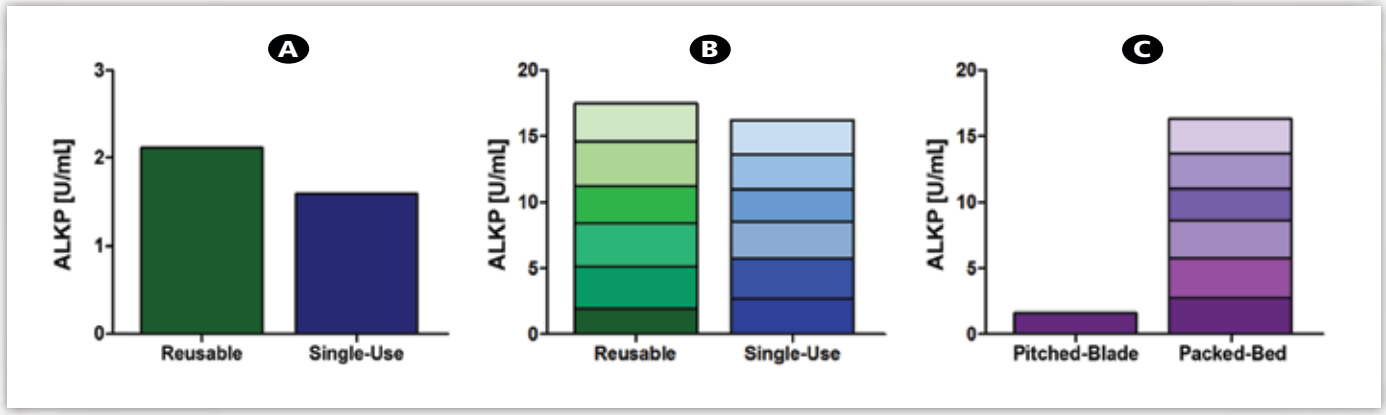

FIGURE 7. Comparison of ALKP production by rCHO cells cultured in the pitched-blade and packed-bed bioreactor systems: **(A)** final ALKP concentrations in the culture media measured in two independent experimental trials using the pitched-blade bioreactor; **(B)** cumulative ALKP concentrations in the culture media measured in two independent experimental trials using the packed-bed bioreactor; and **(C)** ALKP concentrations for two independent experimental trials using either the CelliGen BLU pitched-blade or packed-bed bioreactor systems.

Discussion

Small-scale bioprocessing has been regularly performed in reusable, stirred-tank bioreactors. Cleaning and sterilization of reusable bioreactors is mandatory between runs. Regular maintenance, careful handling, and storage of the glass vessel are also required. A significant monetary investment in sterilization equipment and manpower is required to maintain and clean reusable bioreactors. Cleaning and maintenance of single-use (disposable) bioreactors is not necessary and therefore implementation of these bioreactors can potentially lower required initial investments. Internal analysis conducted by Eppendorf Inc. indicates that the overall cost of using CelliGen 310 vs. CelliGen BLU is nearly identical (data not shown). The cost of operating the CelliGen 310 is driven by upfront investments including control cabinet, autoclavable vessel(s), probes, and autoclave sterilization hardware, as well as the additional labor costs in vessel set-up, break-down, cleaning, and validation. Conversely, the cost of operating the CelliGen BLU is mostly driven by the investment of the control cabinet and the single-use consumable vessels. The reduction in labor hours, cleaning, and validation are major driving forces behind single-use products in bioprocessing.

Process performance by each impeller type were similar in both the reusable and single-use bioreactors, indicating that single-use bioreactors can perform all manufacturing operations analogous to reusable bioreactors.

By virtue of its design, when operated in perfusion mode, the packed-bed bioreactor may be used continuously for months. Cell line maintenance is reduced since preparation of new seed cultures is not required. Moreover, initial setup efforts associated with the packed-bed bioreactor is substantially less than would be required using the pitched-blade system which requires multiple, shorter cultures to equal the production of a single culture with the packed-bed system. Although the pitched-blade bioreactor requires less monitoring over the duration of an experiment, as cells are cultured until nutrient depletion has occurred, this approach does require significant initial input in terms of labor and resources. Multiple culture runs, and thus, multiple seed cultures and system preparations, are required to match the output of the packed-bed system.

In summary, the single-use CelliGen BLU bench-top bioreactor performance was markedly similar to their respective reusable bioreactors. Therefore, significant consideration of single-use bioreactors should be taken into account when small-scale manufacturing of biologics is needed. In both vessel types the packed-bed bioreactor operated in perfusion mode is superior to the pitched-blade bioreactor operated in batch mode for growth of rCHO cells secreting ALKP. However, if a simple batch process is preferred then use of the CelliGen BLU pitched-blade bioreactor should be considered.

Acknowledgements

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Three additional articles published in *BioProcessing Journal* by USU's Center for Integrated Biosystems incorporating Eppendorf's New Brunswick CelliGen products:

Hatton T, Barnett S, Rashid K. CHO cell culture with New Brunswick CelliGen BLU single-use packed-bed Fibra-Cel basket. *BioProcess J*, 2012; 11(2): 50-52.

Hatton T, Barnett S, Benninghoff AD, Rashid K. Productivity studies utilizing recombinant CHO cells in stirred-tank bioreactors: a comparative study between pitched-blade and packed-bed bioreactor systems. *BioProcess J*, 2012; 11(2): 29-36.

Parasar P, Barnett S, Wilhelm A, Rashid K, Davies CJ. Large-scale growth of mouse P815 cells expressing a bovine non-classical major histocompatibility complex class I protein utilizing a pitched-blade bioreactor. *BioProcess J*, 2012; 11(3): 27-34.

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SOLOHILL APPLICATION NOTE APP-006B

Expansion of MRC-5 Cells on Microcarriers via Serial Passage in Stirred Vessels

SOLOHILL Microcarriers for MRC-5 Cell Expansion

INTRODUCTION

Common production platforms employed for manufacture of vaccines, biologics, and cell therapeutics include 2-dimensional culture systems such as roller bottles or cell cubes/factories. These systems are typically used for expansion of cells to seed large bioreactors. Although well-established, these formats occupy a large footprint, are labor intensive and are susceptible to frequent contamination problems due to numerous open handling steps.

Closed-impeller bioreactors provide logical alternatives to 2-dimensional culture systems. Advantages of bioreactors include the ability to precisely control and optimize cell growth conditions, ease of use and avoidance of contamination due "closed" nature of the system.

Microcarriers offer a large surface area for growth of anchorage-dependent cell types. Because specific cell types have different requirements for attachment and growth, the optimal microcarrier should be selected experimentally. This can be accomplished in small-scale studies with multiple microcarrier types to identify the best microcarrier for a particular application. In order to facilitate and streamline manufacturing processes, consideration should be given to the physical characteristics of the microcarriers. For example, durable and rigid microcarriers facilitate efficient harvest of cells from bioreactors. Additionally, some microcarrier types require minimal preparation steps prior to use.

MRC-5 cells are a human-derived lung fibroblast diploid cell line, commonly used for production of vaccines such as rubella, hepatitis A, varicella, rabies, and smallpox.. Preliminary studies performed using SoloHill's microcarriers and other microcarrier brands indicated that MRC-5 cells exhibit excellent growth characteristics on Collagen (SoloHill C102-1521), FACT III (SoloHill F102-1521), ProNectin®F (PF102-1521), and Plastic Plus (SoloHill PP102-1521) microcarriers with potential growth on Hillex® II microcarriers (SoloHill H112-170). In this application note we characterize MRC-5 cell growth on multiple SoloHill and GE's Cytodex 1 microcarriers in stirred vessels and present data demonstrating feasibility of serial passage and scale-up into bioreactor expansion of MRC-5 cells (ATCC CCL17).

MATERIALS AND METHODS

MRC-5 cells purchased from ATCC (CCL-171; population doubling level 22) were expanded to create working cell banks. Cells from the working cell bank were used for these experiments. Corning™ brand 250 mL spinner vessels (Fisher Scientific 10-203B) containing 200 mL of Minimal Essential Medium (MEM, ATCC, Cellgro, or Sigma M3024) supplemented with 5% fetal bovine serum (Thermo Scientific HyClone SH30071.03) and pen/strep (ATCC 30-2300) were used for MRC-5 cell

propagation. Microcarrier concentrations equivalent to a total surface area of 1030 cm^2 per vessel were employed and all microcarriers were prepared according to manufacturer's instructions.

Cytodex 1 was prepared by hydrating in Dulbecco's Phosphate Buffered Saline (DPBS; HyClone SH30028.03) for 3 hours followed by two PBS washes and autoclaving at 121°C in 15 mL of PBS for 30 minutes. All SoloHill microcarriers were prepared by autoclaving at 121°C for 30 minutes in 15 mL of deionized water.

Spinner cultures were essentially performed as described in technical briefs (http://www.solohill.com/Solohill/Microcarrier/Protocols/).

For first passage (P1), MRC-5 cells were harvested from roller bottles (Fisher 09-761-113) using 1X trypsin EDTA (Sigma T3924) after 2 washes with DPBS. Cell counts were performed using standard assays to assess cell numbers and viability. Spinners were seeded (Ni) at either 1.5 x 10^4 cells/cm^2 or 2.0 x 10^4 cells/cm^2 (approximately 18 to 19 cells per microcarrier). Hillex II spinners were maintained at 60 rpms, whereas, all other microcarrier spinners were kept at 40 rpms for the first 48 hrs and increased to 60 rpms for remainder of the culture. Incubation conditions were 37°C ± 0.5 with 5% CO_2. Media exchanges of 100 mL (50%) were performed on Days 3 and 5 of the culture.

For subsequent passages (P2 and P3), cells were harvested from microcarriers and passaged to new spinners. To harvest from Hillex II microcarriers, spinners were transferred to a biological safety cabinet and microcarriers were allowed to settle to the bottom of the spinner. The higher relative density of Hillex II microcarriers (1.1) compared to other microcarrier types, decreases settling time and expedites process steps. The supernatant was carefully decanted through the side arm of the vessel to a waste container and microcarriers were washed for 10 minutes in 100 mL dPBS with constant stirring at 40 rpm. The spinner was removed from the stir plate, microcarriers were allowed to settle, dPBS was decanted from the vessel and 20 mL of 1X trypsin EDTA was added. The spinner was then incubated for 30 minutes at room temperature with occasional swirling of the spinner by hand. Following trypsinization, the microcarrier/cell/trypsin slurry was pipetted to aid removal of cells from microcarriers. To further increase cell yield during harvest, the microcarrier/media slurry was filtered into a sterile 50 mL conical tube through a 100 μM cell strainer (removing microcarriers from the flow through). The microcarriers were washed with equivalent volumes of media (20ml). Cells were used to seed spinner vessels containing surface area equivalents (1030 cm^2) of microcarriers.

The trypsinization of the lower specific gravity microcarriers (all carriers except Hillex II) followed a similar procedure. The only variation from the above protocol was replacement of one spinner arm cap with a 100μM mesh filter. Due to the lower settling velocity and easily suspended characteristic of these carriers, media removal and washes were done by pipetting off as much volume as possible and subsequently decanting the remaining volume through the mesh filter.

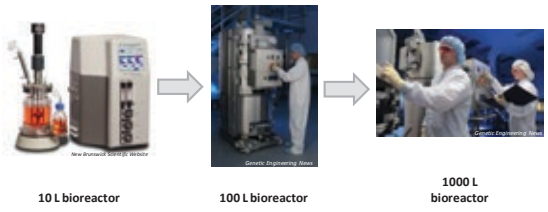
During culture, samples were retrieved daily for nuclei counts using the citric acid/crystal violet method. Nuclei were counted using a Nexcelom counter and software and the number of nuclei per cm^2 surface area was calculated for each sample.

To seed the bioreactor (5L New Brunswick Celligen 310 with a working volume of 3.75L), MRC-5 passage 5 cells were trypsinized off of roller bottles and 5150 cm^2/L Collagen microcarriers (14 g/L; 2.5L total) were seeded at 1.5 x 10^4 cells/cm^2 in MEM (Sigma M3024; supplemented with sodium pyruvate). Due to previous spinners being grown in MEM (from ATCC or Cellgro), two spinner controls were seeded. Spinner #1 contained cells/microcarriers in MEM from Cellgro, and Spinner #2 contained cells/microcarriers in MEM from Sigma. An additional spinner control (Spinner #3) was created by taking 200 mL from the bioreactor two hours after seeding.

RESULTS

The goal of these studies was to demonstrate feasibility of performing serial passage of MRC-5 cells on SoloHill microcarriers and to demonstrate the development parameters for efficient cell growth in bioreactors. The study was designed to establish a reproducible method for expansion of these anchorage-dependent cells in stirred tank reactors. To this end, we performed independent experiments in spinner flasks where MRC-5 cells were passaged

on SoloHill microcarriers or GE Cytodex 1 for three passages. The design for these experiments was chosen to provide feasibility data for a scenario in which 1 to 10 scale-up ratio between bioreactors would culminate in the seeding of a 1000 L bioreactor. Using this scenario, the train would proceed from a 10 L reactor, to a 100 L vessel, to a final seeding of a 1000 L production bioreactor seeded with 2 e^4 cells per cm^2 surface area of microcarriers (Figure 1).



Bioreactor Size (L)	10	100	1000
Grams Hillex II	100	1,000	10,000
Surface Area (cm^2)	51,500	515,000	5,150,000
Roller Bottles (850 cm^2)	60.5	605	n/a

Figure 1. *Diagrammatic representation of a microcarrier-based scale up manufacturing and roller bottle surface area equivalents.*

The significance of this proposed bioreactor process is highlighted by the fact that a 10 L bioreactor is equivalent to approximately 61 x 850 cm^2 roller bottles and an 100 L bioreactor replaces 605 x 850 cm^2 roller bottles given equivalent confluent cell densities are reached. Outside of direct material cost savings for large scale production, each bottle manipulation represents an "open" step with a potential for introduction of contaminants and the need for extensive labor to be eliminated.

To successfully implement a production scheme as proposed it is important to obtain sufficient data to warrant transition into larger bioreactor formats. The logical and most cost-effective path toward this goal is to obtain data in small-scale static and spinner-based feasibility studies prior to transitioning into costly studies in large bioreactors. In order to establish a baseline for cell growth and to establish a gauge to evaluate the performance of our stirred tank system, we first characterized growth of the MRC-5 cell line in static culture. MRC-5 cells were seeded into T25 flasks at 2 x 10^4 cells/cm^2 and a growth curve was generated (Figure 2).

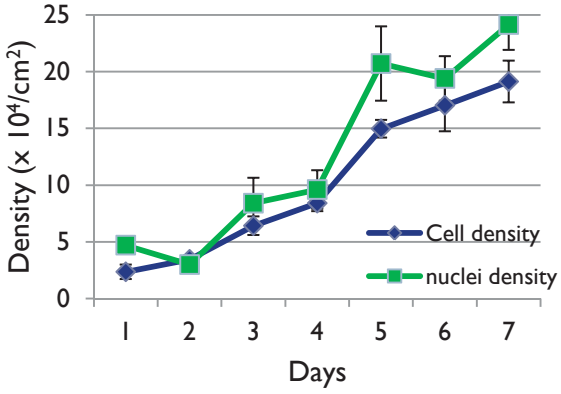


Figure 2. *Growth curve of MRC-5 cells in static culture establishes baseline for comparison of growth in stirred tank vessels. Data is presented as means ± SEm (n = 3).*

These cells grew with a population doubling time (PDT) of ~48 hours throughout the 7 day growth experiment. The average cell density was 19.1 ± 1.8 x 10^4 whereas the nuclei counts were 24.1 ± 2.2 x 10^4 nuclei per cm^2. The slight differences in counts between methods may reflect protocol differences or it is possible that a subset of the cell population exhibits synchronized growth and may be multinucleated at the time of harvest for counting.

Figure 3 demonstrates the ability to expand MRC-5 cells on SoloHill microcarriers in stirred vessels for three passages with satisfactory growth on Collagen, FACT III, and Plastic Plus microcarriers. Under the conditions tested in this study suboptimal growth was observed on Hillex II and Cytodex 1 microcarriers. Both of these microcarrier types are animal product free and contain a positively charged surface.

Whereas, growth on Hillex II could be maintained over the passage regimen, cell numbers on Cytodex 1 steadily decreased over the three passages suggesting that scale-up on Cytodex 1 would not be feasible without further optimization.

Cells reached a maximum confluent cell density of 20.0 ± 5.5 x 10^4 nuclei/cm^2, 14.3 ± 4.2 x 10^4 nuclei/cm^2, 14.4 ± 5.8 x 10^4 nuclei/cm^2, 18.8 ± 2.8 x 10^4 nuclei/cm^2 in Collagen, FACT III, Plastic Plus and ProNectinF spinner vessels, respectively, containing MEM media supplemented with 5 % FBS (n = 3). These nuclei counts were slightly lower than those obtained in static T flask culture which yielded 24.1 ± 2.2 x 10^4 nuclei/cm^2 on day 7 of culture (n = 3; cell density of 19.1 ± 1.8 x 10^4 cell/cm^2).

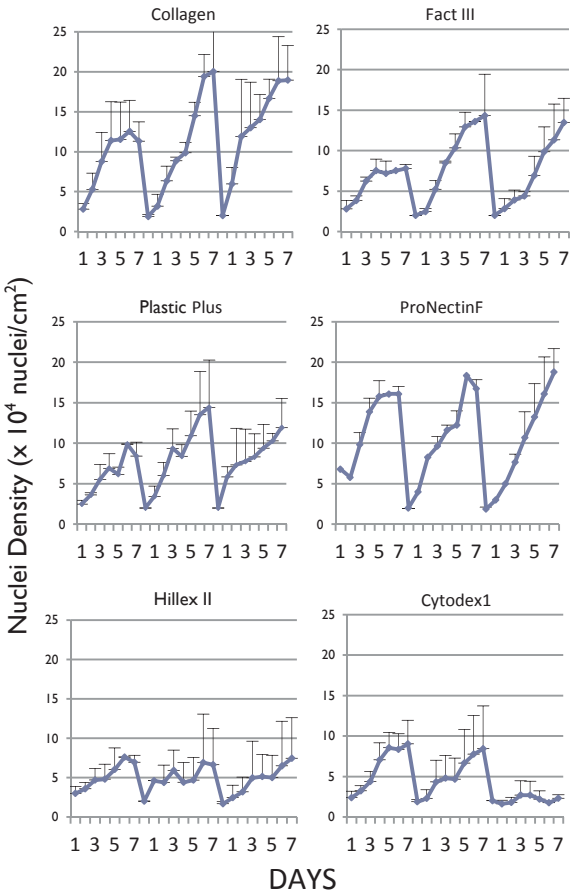


Figure 3. *MRC-5 serial passages on SoloHill microcarriers and Cytodex 1 suggest potential for scale-up on Collagen, Fact III, and Plastic Plus and possibly with Hillex II after further optimization. Data represents means ± SD (n = 3).*

All SoloHill microcarriers outperformed Cytodex 1 under the conditions used in these studies. Cytodex 1 reached a maximum confluent cell density of 9.1 ± 2.9 x 10^4 nuclei/cm^2 in the first pass on microcarriers.

Not only is scale-up on Cytodex 1 not feasible due to a decrease in growth over several passages, but the lower maximum confluent cell density translates into a 37-55% lower cell yield per spinner when compared with Collagen, FACT III, Plastic Plus, and ProNectin F microcarriers.

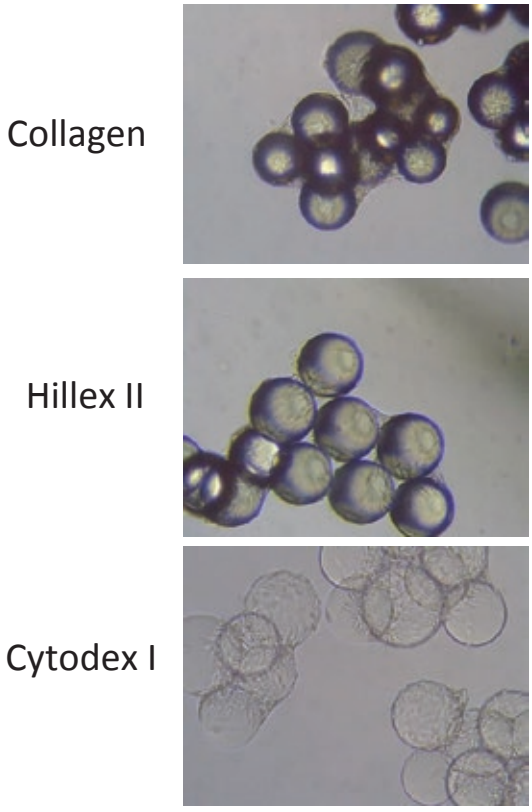


Figure 4. *Sample pictures of Day 7 expansion on SoloHill or cytodex 1 microcarriers. Note presence of clumping on confluent microcarriers as a function of cell density, i.e. more clumping as cells grow to higher densities.*

Visual observations of cells on microcarriers were consistent with these data as well as published data on MRC-5 (Figure 4). Cells and microcarriers began to clump as early as Day 3 in spinner cultures. Clumping was more noticeable in spinner cultures with higher cell densities but did not appear to affect cell growth.

To determine feasibility of scale-up production with the MRC-5 cell line, MRC-5 cells were expanded in roller bottles and used to seed Collagen microcarriers in a 2.3 L bioreactor. Figure 5 shows that MRC-5 cells grew to a density of 19.1 ± 0.78 x 10^4 nuclei/cm^2 which is similar to the densities obtained from multiple Collagen spinner experiments (shown in Figure 3).

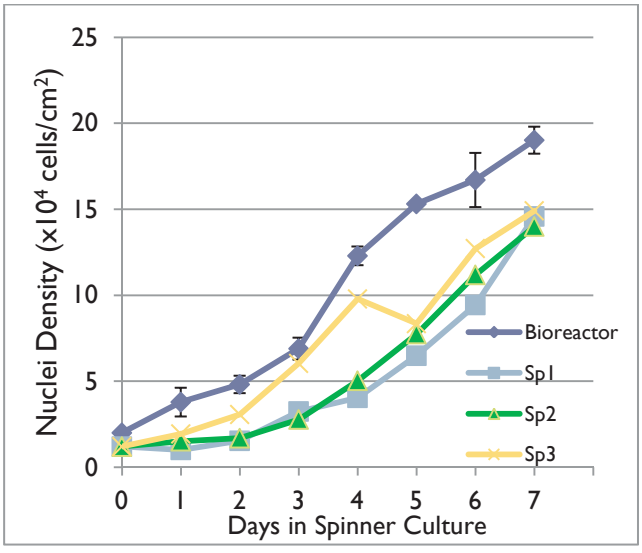
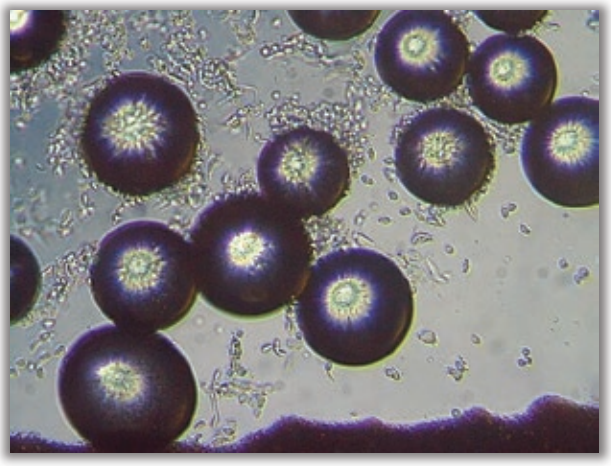


Figure 5. *MRC-5 grown on Collagen microcarriers in a 2.3L bioreactor.*

A critical feature of SoloHill microcarriers is the ability to easily and efficiently harvest cells from microcarriers. Standard enzymatic techniques can be used to readily harvest MRC-5 cells in a single-cell suspension. Figure 6 shows MRC-5 cells after a 15 minute trypsinization incubation. Once these cells and microcarriers are pipetted, a single cell suspension is achieved that can be used to seed new spinners.

Figure 6. *MRC-5 cells after a 15 minute trypsinization step. Pipetting creates a single cell suspension that can be used to seed new spinners.*



The rigid core of all SoloHill microcarriers not only imparts stability and durability but promotes cell removal by preventing fouling of screens or filters used to separate cells from microcarriers. .

Another benefit of SoloHill microcarriers is that they do not require lengthy microcarrier preparation steps. In this regard, microcarriers suspended evenly in deionized water can be autoclaved and used immediately after sterilization. SoloHill microcarriers can also be gamma irradiated without adverse effects on performance. Cytodex microcarriers require multiple long processing steps and if bead preparation is not performed properly and consistently, major difficulties with cell release from microcarriers and subsequent detrimental effects on cell yield are encountered.

CONCLUSIONS

Results presented here demonstrate the feasibility of using several SoloHill microcarriers for expansion of MRC-5 cells via microcarrier-based serial passage in stirred vessels. The data indicated that growth of MRC-5 cells on microcarriers at a bioreactor level is also achievable. In these studies, minimal effort was expended to optimize growth of MRC-5 cells in the spinner and bioreactor formats so it is likely that higher confluent cell densities can be achieved with further experimentation. Although the counts from microcarrier spinner cultures were slightly lower than static cultures, the number of 850 cm^2 roller bottles required to produce equivalent cell yields is still significant in that 45 or 450 roller bottles would be required to achieve the same cell yield as a single 10 L or 100 L bioreactor, respectively. For SoloHill microcarriers, the cell numbers were sufficient for serial passage.

All SoloHill microcarriers supported serial passaging between spinners with no decrease in confluent cell densities, as opposed to Cytodex 1 serial passage which demonstrated a continual decrease in maximal cell densities over multiple passages.

These feasibility studies demonstrate that SoloHill's microcarriers provide an ideal substrate for expansion of MRC-5 cells in a closed stirred vessel systems and lay the groundwork for subsequent developmental studies in larger bioreactor formats.

For additional information and technical assistance, please visit our web site at http://www.solohill.com , or call our technical hotline: **(734) 973-2956.**

REFERENCES

[1] http://www.solohill.com/Solohill/Microcarrier/Protocols/

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SOLOHILL APPLICATION NOTE APP-003B

Expansion of Vero Cells on SoloHill Hillex II Microcarriers via Serial Passage in Stirred Vessels

SOLOHILL Microcarriers for Vero Cell Expansion

INTRODUCTION

Bioreactor systems provide a logical platform for expansion of the large numbers of cells required for production of therapeutic molecules or for cellular therapies. Arguments for employing bioreactors for these purposes include the ability to precisely control and optimize cell growth conditions, ease of use, and avoidance of contamination due to the "closed" nature of the system along with the recent emergence of disposable bioreactor technology. In addition, large numbers of cells can be produced under current good manufacturing practices (cGMP) in a small space.

For anchorage-dependent cells, microcarriers provide a large surface area for propagation and growth. Because specific cell types possess unique requirements for attachment and spreading, the optimal microcarrier should be selected empirically. It is practical to perform small-scale studies with multiple microcarrier types to identify the best microcarrier for the application and to minimize costs during development.

To streamline manufacturing processes, consideration should be given to the physical characteristics of the microcarriers. For example, durable and rigid microcarriers are amenable to long production runs and facilitate efficient harvesting of cells from bioreactors.

Additionally, production processes can be simplified by employing microcarriers that require minimal preparation steps prior to use.

Vero cells (ATCC CCL-81) are commonly used in veterinary and human medicine. Preliminary studies performed using SoloHill's microcarriers with Vero cells in spinner culture demonstrated

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that Hillex® II microcarriers (SoloHill H112-170) support excellent cell growth[1].

In this application note we present data demonstrating feasibility of serial passaging Vero cells on SoloHill Hillex II microcarriers in bioreactors. Three serial passages were performed proceeding from a 0.2 L spinner through a 10 L culture in a bioreactor (Figure 1).

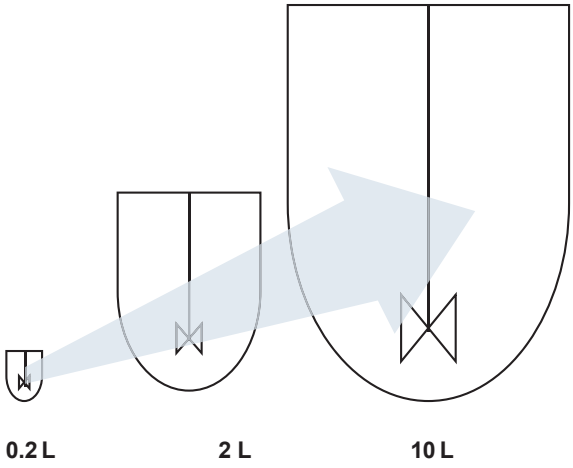

Figure 1. Diagram detailing bioreactor-based expansion scheme used in these studies.

MATERIALS AND METHODS

Vero cells purchased from ATCC (CCL-81; P121) were expanded to create master (P125) and working cell banks (P127). Cells from the working cell bank were used for these experiments. Cell suspensions at Passage 136 were used to seed initial spinner cultures for scale up.

Spinner Culture

For the first passage on Hillex II (HP1), Vero cells were harvested from vented T150 tissue culture flasks (Corning 430825) with trypsin (0.05% 1X Sigma T 3924) after two washes with Dulbecco's phosphate buffered saline (DPBS) (HyClone SH30028.03).

Corning™ brand 250 mL spinner vessels (Fisher Scientific 10-203B) containing 200 mL of DMEM media (HyClone SH30585.02) supplemented with 5% fetal bovine serum (HyClone SH30071.03), 1% Non Essential Amino Acids (HyClone SH30238.01), 2mM L-Glutamine (HyClone SH30034.01) and 1% antibiotic/antimycotic (Mediatech 30-004-C1) were used for Vero cell propagation. Microcarrier concentrations equivalent to a total surface area of 5150 cm^2/L (10 g/L) were used and all microcarriers were prepared according to manufacturer's instructions by autoclaving at 121°C in deionized water for 30 minutes. Spinner cultures were essentially performed as described in SoloHill's microcarrier technical briefs[2].

A 200 mL spinner containing 2 g of Hillex II (1,030 cm^2) was seeded (Ni) at a density of 2.0 x 10^4 cells/cm^2 (18 to 19 cells per microcarrier). Incubation conditions were 37°C with 5% CO_2 and batch volume exchanges of 150 mL media were performed on Days 3 and 4. One mL of a 2 mg/mL stock solution of folic acid (Sigma F8758-5g) was added daily to Hillex II cultures to promote growth during expansion and after passage to fresh substrate. In the biological safety hood, with the spinner on the stir plate and the impeller mode set at 60-70 rpm, the single-cell suspension was added to seed the spinner and warmed media was added to bring the final spinner volume to 200 mL. The spinner flask was then immediately transferred to an incubator equipped with a stir plate set at 55-60 rpm.

The cell attachment rate to Hillex II can be faster than other SoloHill microcarriers depending upon conditions used for cell seeding. We have

observed rapid attachment to Hillex II within one hour. The cell attachment rate can vary based on the enzyme brand and type used for cell dissociation.

For subsequent passages on Hillex II (HP2 and HP3), cells were harvested from the microcarriers and passaged to fresh Hillex II microcarriers. For cell harvest, the 200 mL spinner was transferred to a biological safety hood, microcarriers were allowed to settle and the media was removed from the settled microcarrier pack by pouring through the side-arm of the vessel. Few microcarriers are discarded along with the media because of the higher specific gravity of Hillex II.

The cell-laden microcarriers were thoroughly washed by adding 50 mL DPBS to the vessel taking care not to dispense the liquid directly onto the microcarriers to avoid dislodging cells. This wash solution was decanted; an additional 50 mL of DBPS and the culture was then stirred at 40 rpm at room temperature for 10 minutes. After removing the final rinse, 22.5 mL of trypsin solution was added and the culture was incubated for 10 minutes. When cells were rounded and on the verge of dislodging, the microcarrier-cell suspension was triturated up and down to generate a single cell suspension. Cell counts were performed using standard assays to assess cell numbers and viability and this cell suspension was used to seed a 2 L bioreactor containing 10 g/L of Hillex II.

Bioreactor Cultures

A New Brunswick CelliGen bioreactor Model 310 with a 5 L vessel was used for the second passage. The final working volume chosen for these experiments was 2 L. The vessel was equipped with a ring sparger, spin filter, 3-segmented pitched blade impeller (up-pumping), and 4-gas control at 100 mL/min (Air, N_2, CO_2, and O_2). Twenty grams of Hillex II (10 g/L) was combined with 125 mL of deionized water, and the slurry was added to the vessel. The reactor was autoclaved for 20 minutes at 100ºC to warm the vessel and contents and then immediately autoclaved a second time for 40 minutes at 125ºC. After autoclaving, 2 L media was added to the reactor. The vessel was maintained at 37ºC while stirring at 60 rpm (impeller tip speed ~0.25 m/s) for 18 hours. Prior to cell addition, the pH was adjusted to 7.2, and the dissolved oxygen (DO) set to 30% air saturation. Trypsinized cells from the 200 mL spinner were added to the vessel via a peristaltic pump. The agitation rate was increased to 70 rpm during the cell addition, and returned to 60 rpm for the remainder of the run. Throughout the culture, pH was maintained with an air overlay and 1N sodium hydroxide (NaOH). DO was maintained with an oxygen overlay and sparging. Media perfusion at a rate of three quarter volumes per day began on the second day of culture and ended on Day 4 so that a total of 3 L of media was used for perfusion. Folic acid additions (10 mL of a 2 mg/mL stock solution) were performed daily. During culture, samples were retrieved daily and nuclei counts were performed using the citric acid/crystal violet method. Nuclei were enumerated using a hemocytometer and the nuclei per cm^2 surface area for each sample were calculated. On the fifth day of culture, cells were harvested for passage to the next vessel. The harvest step began with turning off agitation and allowing the Hillex II microcarriers to quickly settle. The media was pumped out of the reactor via a dip tube and 1 L of DPBS was the added to wash the cells. Microcarriers were re-suspended at an agitation rate of 55 rpm and the entire contents of the vessel were transferred to a sterile 1 L spinner vessel using air pressure.

Further processing took place in a biological safety hood. Microcarriers were allowed to settle and the DPBS was removed. An additional liter of DPBS was added and the

microcarrier suspension was stirred at 40 rpm for 10 minutes. The microcarriers were then allowed to settle, wash solution was removed, 300 mL of trypsin was added and the vessel was incubated at room temperature for 10 minutes with occasional swirling by hand. The entire contents of the vessel were then stirred at 50 to 60 rpm for 5 minutes. The microcarriers and cells were then triturated up and down with a pipette and the resulting slurry was filtered through a 150 micron screen to separate cells from microcarriers.

A Sartorius BioStat C 15 L bioreactor containing a final working volume of 10 L media was used for the third and final passage. The bioreactor was equipped with a ring sparger, spin filter, paddle impeller (up-pumping), and 4-gas controller, which was set at 100 mL/min (Air, N_2, CO_2, and O_2). One hundred grams of Hillex II was combined with 700 mL of deionized water and added to the vessel.

The vessel and contents were then in-situ steam sterilized for 40 minutes at 125ºC and after sterilization, nine liters of media was added to the vessel. Media temperature was maintained at 37ºC, pH set to 7.2, DO set to 30% of air saturation, and agitation set to 35 rpm (impeller tip speed ~0.29 m/s). The agitation rate was increased to 45 rpm in preparation for cell addition and the single-cell suspension from the 2 L bioreactor was added to the bioreactor vessel via a peristaltic pump. After cell addition, media was added to bring the total working culture volume to 10 L. Agitation rate was maintained at 35 rpm for the remainder of the run. Throughout the culture, pH was maintained with an air overlay and addition of a 1N NaOH solution. DO was maintained with oxygen sparging. A 1.5 volume per day media perfusion began on Day 2 and ended on Day 4. During culture, samples were retrieved daily at a stir speed of 45 rpm and subsequently processed for nuclei counts using the citric acid/crystal violet method. Nuclei were counted using a hemocytometer and the number of nuclei per cm^2 surface area was calculated for each sample. Folic acid additions of 49 mL were performed daily.

RESULTS

To successfully implement a production scheme using microcarrier-based scale-up in large bioreactors it is important to obtain sufficient data to warrant transition into larger formats. The logical and most cost-effective path toward this goal is to obtain data in small-scale static and spinner-based feasibility studies prior to transitioning into costly studies in large bioreactors.

The maximum cell density achieved in the bioreactors was 2.75 x 10^6 nuclei/mL and 53.7 x 10^4 cells/cm^2 after seven days of culture. These results demonstrate the ability to expand Vero cells on Hillex II microcarriers in stirred vessels for three passages. (Figure 2.)

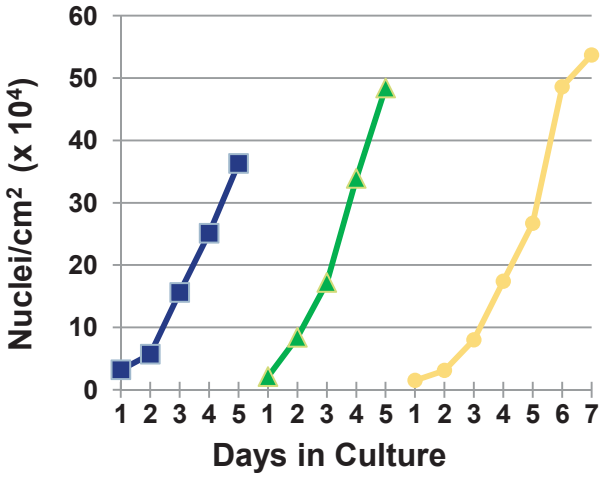

Figure 2. Vero cells serial passaged on Hillex II microcarriers. Passage 1 0.2L Spinner (squares), Passage 2, 2L bioreactor (triangles) Passage 3, 10 L bioreactor (circles) microcarriers.

Microscopic visualization of cells on microcarriers was consistent with these data (Figure 3).

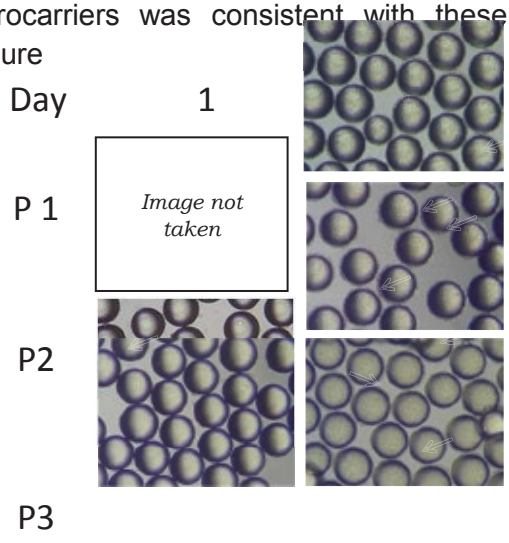

Figure 3. Images of Vero cells on Hillex II microcarriers (Passage 1 through 3) were captured after one and five days of culture. Photo of P3 cells taken after seven days of culture.

A critical feature of SoloHill microcarriers is the ability to easily and efficiently harvest cells from microcarriers. Standard enzymatic techniques can be used to readily harvest Vero cells in a single-cell suspension (Figure 4).

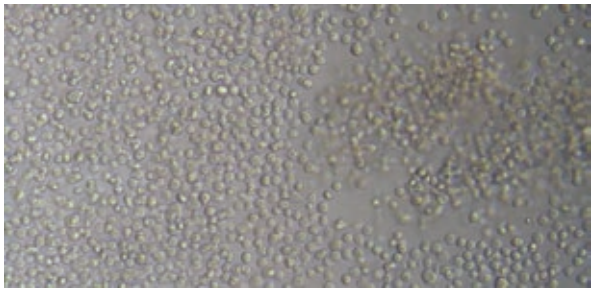

Figure 4. Single-cell suspension of cells trypsinized from a 2 L culture used to seed a 10 L culture.

When cells from these microcarriers are seeded into new vessels with fresh microcarriers the cells attached very quickly and evenly as seen in Figure 5.

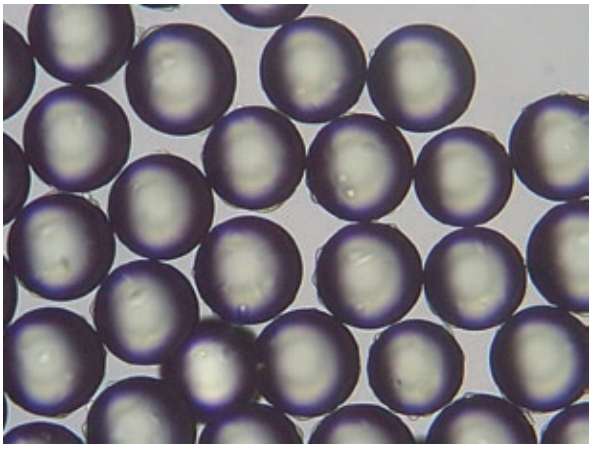

Figure 5. *Initial cell attachment to Hillex II in a 2 L bioreactor (5 hours after seeding).*

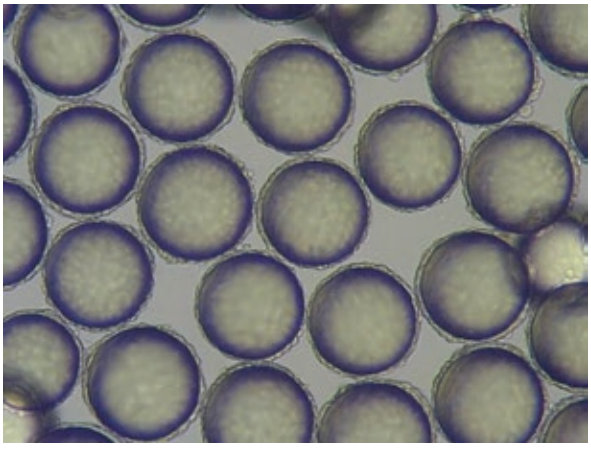

Figure 6. *Confluent Vero cells grown on Hillex II microcarriers in a 15 L bioreactor.*

Subsequent culture reproducibly produced confluent layers of cells on the surface of the entire population of microcarriers which are clearly identified by bright field images shown in Figure 6.

CONCLUSIONS

Results presented here demonstrate the feasibility of using SoloHill's Hillex II microcarriers for expansion of Vero cells via microcarrier-based serial passage in stirred vessels.

The number of 850 cm^2 roller bottles required to produce equivalent cell yields is significant in that 45 or 450 roller bottles would be required to achieve the same cell yield as a single 10 L or 100 L bioreactor, respectively.

These feasibility studies demonstrate that SoloHill's Hillex II microcarriers provide an ideal substrate for expansion of Vero cells in closed stirred vessels and lay the groundwork for subsequent developmental studies in even larger bioreactor formats.

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[2] http://www.solohill.com/Solohill/Microcarrier/Protocols/

For additional information and technical assistance, please visit our web site at http://www.solohill.com , or call our technical hotline: **(734) 973-2956.**

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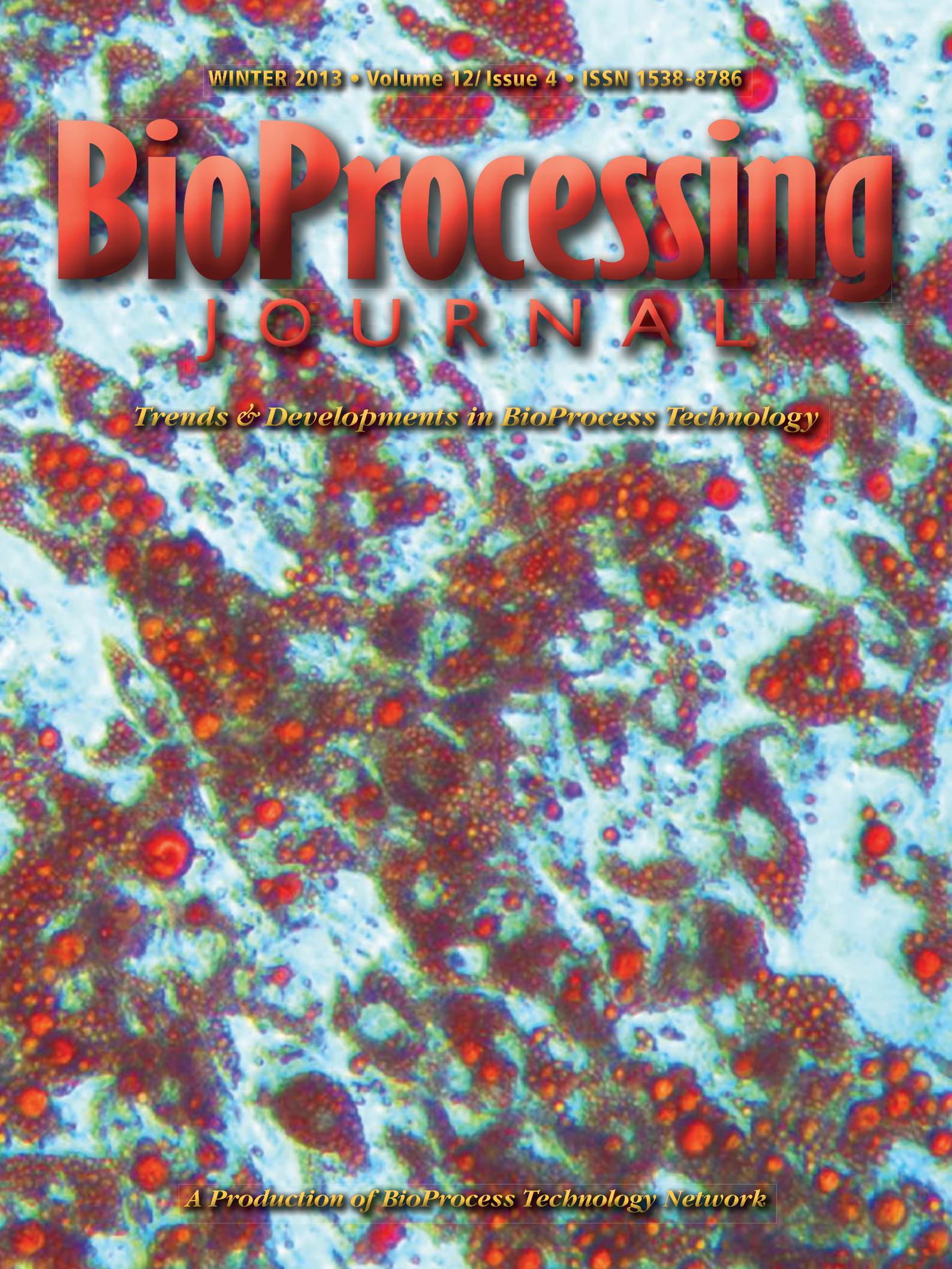

A Production of BioProcess Technology Network

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TRENDS & DEVELOPMENTS IN BIOPROCESS TECHNOLOGY

Microcarrier-Based Expansion of Adipose-Derived Mesenchymal Stem Cells in Shake Flasks

By KHANDAKER SIDDIQUEE and MA SHA

Abstract

The expansion of stem cells, including mesenchymal stem cells (MSCs), has been successfully demonstrated using microcarrier-based small bioreactors such as spinner flasks. In this study, we explored a simple alternative for microcarrier-based MSC expansion using conventional shake flasks. This method relies on a new type of shaker with built-in CO_2 gas control capability, the New Brunswick™ S41i incubator shaker. The expansion of adipose-derived mesenchymal stem cells (AdMSCs) was compared between shake and spinner flasks containing microcarriers. The AdMSCs were seeded at a density of 3×10^3 cells/cm^2 in both setups, each containing 0.5 g of plastic microcarriers and 50 mL of stem cell growth medium.

The cell culture experiments were conducted over 12 days with samples collected daily for cell growth, biochemistry, and metabolite analysis. The study revealed that AdMSCs cultured under shake flask conditions achieved excellent growth under 12-day batch-culture conditions.

Finally, the AdMSCs expanded using the shake flask method retained high quality stem cell characteristics, as indicated by CD44 and CD90 stem cell marker assays, and the ability of these cells to differentiate into either adipocytes or osteocytes.

Introduction

Stem cells are undifferentiated cells that have the capability of self-renewal and the potential to divide for a long period of time. They have the ability to differentiate into various specialized cells when appropriate growth factors and conditions are present. Stem cells can be broadly classified as either embryonic, adult, or induced pluripotent stem cells (iPSCs). Adult stem cells can be further characterized by their tissue of origin, for example, hematopoietic, mammary, intestinal, mesenchymal, endothelial, neural, and hair follicle stem cells.[1,2] Many recent studies performed using adult stem cells utilized either hematopoietic or adipose-derived mesenchymal stem cells (AdMSCs). Like other adult stem cells, AdMSCs express all of the common stem cell markers and can be differentiated into various types of specialized cells under appropriate growth conditions. AdMSCs have a unique advantage over hematopoietic stem cells since they can be isolated in large quantities from fat tissue and are resistant to apoptosis.[2-7]

MSCs have enormous potential for regenerative medicine, drug screening, and drug discovery, but their applications are limited by the large quantities of cells required for industrial or clinical applications.[4] In an effort toward addressing the cell availability issue, we demonstrated a simple shake flask technique to culture AdMSCs on microcarrier beads. The method can be quickly expanded into liter-scale, thus allowing potential scale-up into large-scale industrial bioreactors. We expanded the AdMSCs on microcarrier beads using shake flasks and, at the same time, we also cultivated them on identical microcarrier beads using spinner flasks. After expansion, we performed stem cell marker immunoassays and differentiation assays on AdMSCs obtained via the shake flask method. Our study identified several advantages of the shake flask method over the spinner flask for the growth of AdMSCs.

Materials and Methods

Initial Cell Culture in T-Flasks

AdMSCs were obtained from ATCC® (PCS-500-011™) at passage 2 and cells were seeded at a density of 5×10^3 cells/cm^2 into a T-75 cm^2 flask (Eppendorf®) using 15 mL of MSC basal medium (ATCC PCS-500-030™). The medium was supplemented with ATCC products: 2% fetal bovine serum (FBS); 5 ng/mL each: rh FGF-basic, rh FGF-acidic, and rh EGF; and 2.4 mM L-alanyl-L-glutamine.

Cultivation of Cells on Microcarriers

Prior to the start of the experiment, 0.5 g of 125–212 µm SoloHill® Engineering polystyrene microcarriers (180 cm^2 for a 50 mL culture) were transferred into siliconized Corning® 250 mL glass spinner flasks (Sigma-Aldrich) and 250 mL glass baffled shake flasks (DURAN®) along with 25–30 mL of phosphate-buffered saline (PBS). The flasks were then autoclaved at 121°C for 30 minutes. Microcarriers were allowed to settle to the bottom of each flask (shake and spinner) and the autoclaved PBS buffer was carefully aspirated using an Easypet® electronic pipette controller (Eppendorf) equipped with 25 or 50 mL pipettes. The AdMSCs were initially seeded at a density of 3×10^3 cells/cm^2 into both types of flasks, each containing 40 mL of MSC basal medium.

For the initial cell attachment incubation, the agitation speed of the New Brunswick™ S41i incubator shaker and rotation speed of the spinner (HI 313 autospeed magnetic stirrer, HANNA instruments housed inside of a New Brunswick Galaxy® 170 R CO_2 incubator) were both kept at 50 rpm for two hours at 37°C with 5% CO_2. After incubation, the cell culture volumes were adjusted to 50 mL each with 10 mL of serum-containing medium to reach a final FBS concentration of 4%. The targeted concentrations of growth supplementation were finalized at 10 ng/mL each: rh FGF-basic, rh FGF-acidic, and rh EGF; and 4.8 mM L-alanyl-L-glutamine. Following the addition of FBS and growth supplements, the rpm for both setups were raised to 70 rpm. Each day of incubation, a 1 mL sample (homogenous mix of microcarriers in solution) were collected for microscopic observations, cell counting, and biochemistry analysis.

Cell Counting

Following supernatant removal, the microcarrier beads with attached cells were resuspended in a citric acid solution (0.1 M) containing crystal violet (0.1%). The contents of the tube were incubated overnight at 37°C and vortexed for a few seconds to release the cells and stained nuclei from the beads. The nuclei were counted using a Bright-Line Hemacytometer (Hausser Scientific).

Biochemistry and Metabolite Analysis

The supernatants collected during cell counting were used for biochemistry and metabolite measurements using the YSI® 2950 Biochemistry Analyzer (YSI Inc. Life Sciences).

Stem Cell Surface Marker Assays

To assess the quality of AdMSCs after expansion and to confirm that the stem cell markers were retained during the microcarrier-based shake flask culture, CD44 and CD90-specific fluorescent immunoassays were performed. Samples were collected from the shake flasks (5 mL) on day 12. After the microcarriers settled to the bottom of the tubes, supernatants were removed and the microcarrier beads were gently washed three times with PBS at room temperature. Cells on the microcarrier beads were then fixed with 4% paraformaldehyde for 30 minutes and washed three more times in PBS. Cell-containing microcarrier beads were blocked with 5% FBS at room temperature for one hour and immunostained with BioLegend® FITC-conjugated antihuman CD44 and APC-conjugated antihuman CD90 antibody solutions for one hour, also at room temperature. The beads were washed five times (for five minutes each) with room-temperature PBS and visualized using an EVOS® FL LED-based fluorescence microscope (Life Technologies™).

Stem Cell Differentiation Assays

AdMSCs were harvested from shake flasks into 50 mL conical tubes (USA Scientific). Once the beads had settled at the bottoms of the tubes, supernatant was transferred to separate tubes. The microcarrier beads with attached cells were washed with Dulbecco's phosphate buffered saline (DPBS) and then treated with 5 mL of pre-warmed trypsin-EDTA solution at 37°C for ten minutes. During incubation, the tubes were vortexed for two seconds intermittently to aid in cell release. Following incubation, equal volumes (5 mL) of trypsin-neutralizing solution (ATCC PCS-999-004™) were added to each tube. Once the microcarrier beads had settled to the bottom of the tubes, supernatants were again collected. Microcarrier beads were washed three times with DPBS. Supernatants were collected each time and combined with the initial supernatant. AdMSCs were pelleted in the tube bottoms by centrifugation at 120× g for five minutes and then resuspended with 5 mL of MSC basal medium.

Cells were seeded at a density of 1.8×10^4 cells/cm^2 into a 24-well plate. Differentiations were induced with Adipocyte (ATCC PCS-500-050™) and Osteocyte (ATCC PCS-500-052™) Differentiation Toolkits. Following manufacturer's instructions, differentiated adipocytes were identified by oil red O staining (ScienCell™) and osteocytes were identified with alizarin red S staining (ScienCell). Both were visualized using an Olympus® CK40 inverted microscope.

Results and Discussion

Our cell culture study using the shake flask method as well as the spinner flask method was conducted over a 12-day period. The study revealed that AdMSCs cultured under shake flask conditions achieved excellent growth during the 12-day batch culture (Figure 1A).

Biochemistry analysis revealed that glucose concentrations decreased from 1.09 g/L to 0.548 g/L for shake flask culture and to 0.798 g/L for spinner culture. In contrast, lactate concentrations increased from 0.042 g/L to 0.396 g/L for shake flask culture and 0.259 g/L for spinner culture after 12 days (Figure 1, B and C). The higher glucose consumption and lactate production rate seen in the shake flask culture supports the finding that the stem cells grew at a faster rate than the spinner cultures.

Furthermore, during early growth phase (day 4), the amount of ammonium accumulating in spinner flask culture (2.4 mM) was 1.8-fold higher than with the shake flask culture (1.3 mM) (Figure 1D). It has been shown that even low levels of ammonium (1.9 mM) inhibit MSC growth.[8] The spinner culture has shown ammonium levels exceeding 1.9 mM early and throughout the culture process. This finding indicates that the slower growth exhibited by the spinner method could be a result of ammonium toxicity-induced growth inhibition. The fact that the spinner culture had elevated ammonium levels early may also indicate possible stem cell damage due to shear force by the spinner rod. The spinner rod displayed a "stop and go" motion at low speeds— precise speed control may not be possible with certain laboratory spinner devices, especially at low rotation speeds. However, our observations were based on the specific spinner device utilized by our research facility. Our results may not represent typical or average

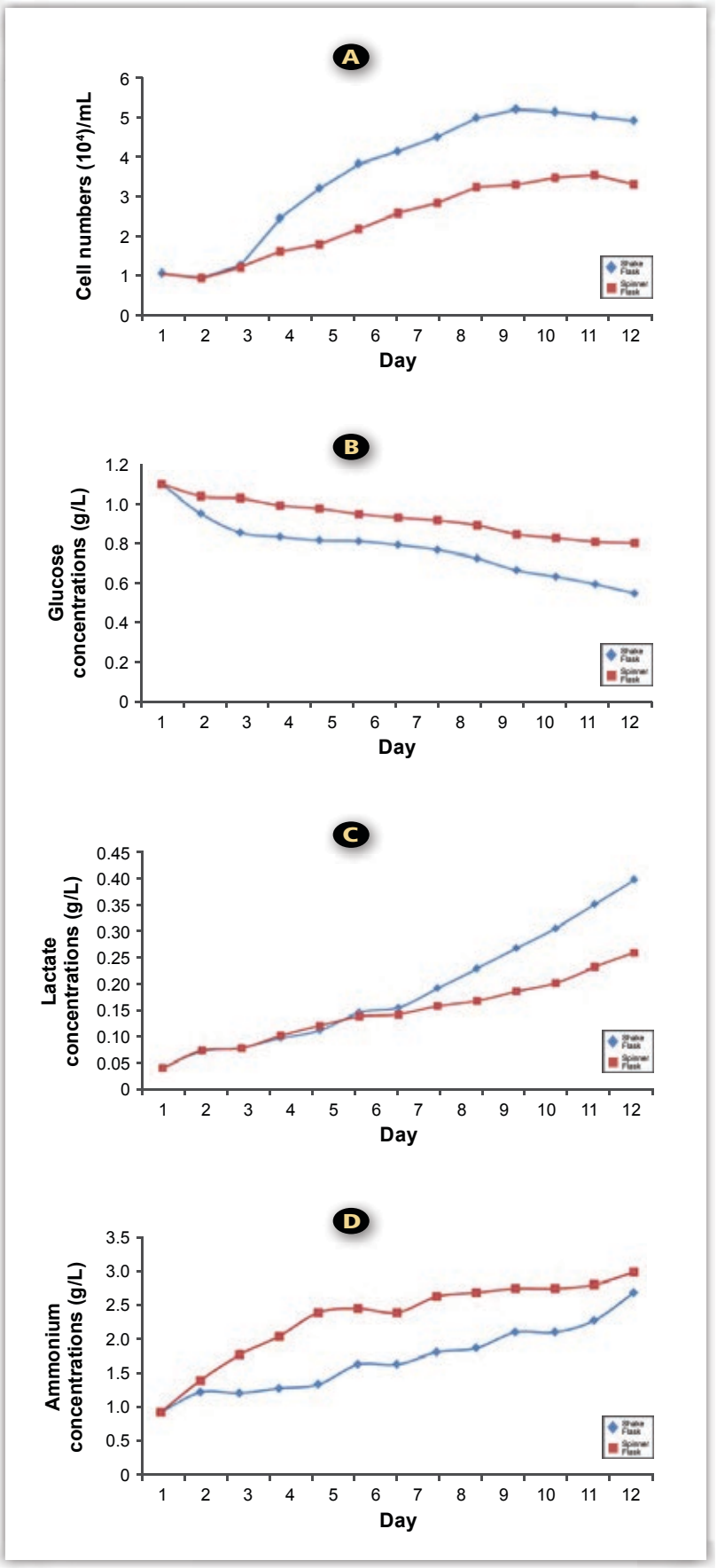

FIGURE 1. Analysis of AdMSCs growth and metabolism in shake flask and spinner flask culture conditions. **(A)** growth; **(B)** glucose utilization; **(C)** lactate production; and **(D)** ammonium production.

performance from the various spinner devices available in the marketplace.

MSCs express various cell surface markers such as CD73, CD90, CD105, and CD44.[1, 2, 9] To determine whether or not AdMSCs retained their stem cell properties during growth under shake flask conditions, immunostaining of stem cell surface markers and differentiation assays were performed. Microcarrier beads containing AdMSCs were immunostained with FITC-conjugated antihuman CD44 and APC-conjugated anti-human CD90 stem cell surface marker antibodies. The results revealed that AdMSCs retained stem cell surface markers during growth under shake flask culture conditions (Figure 2, A and B). To evaluate if the AdMSCs grown under the shake flask method retained their pluripotency capacity, adipocyte and osteocyte differentiation assays were performed. For

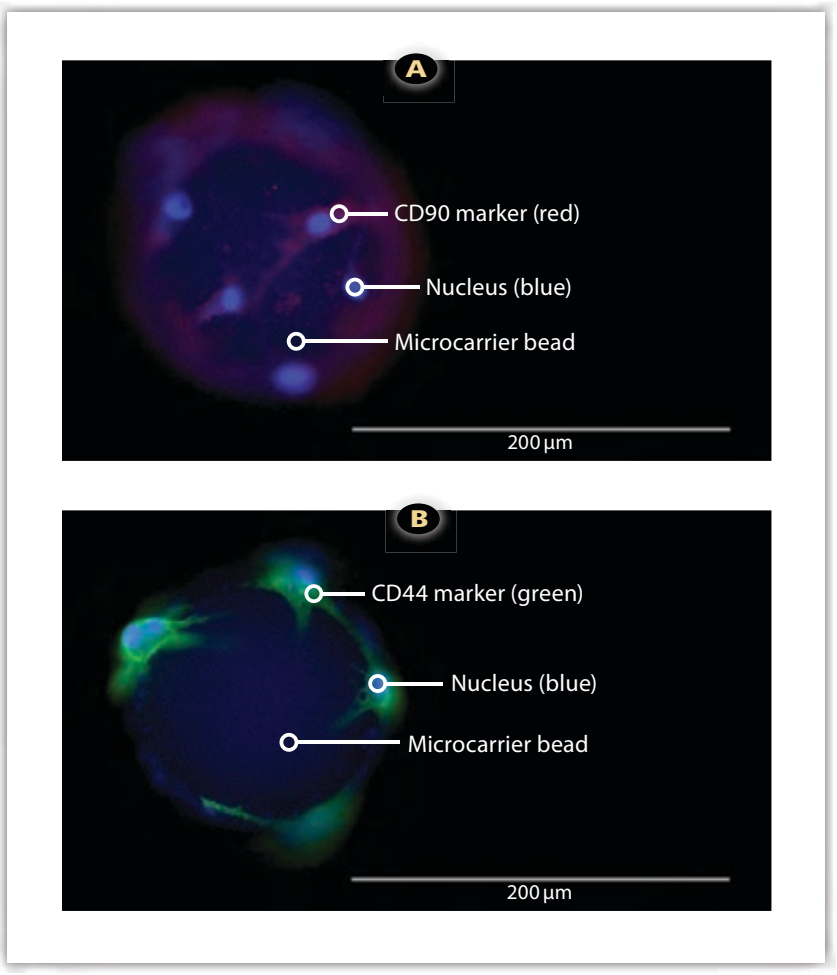

FIGURE 2. Stem cell marker identification assay for AdMSCs expanded on microcarriers in shake flasks. **(A)** AdMSCs on microcarrier beads are positive for CD90 stem cell marker, as indicated in red by fluorescence imaging. **(B)** AdMSCs on microcarrier beads are positive for CD44 stem cell marker, as indicated in green by fluorescence imaging. The blue color indicates stem cell nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI).

these two differentiation assays, AdMSCs were collected from the microcarrier beads and seeded into 24-well plates that either contained adipocyte or osteocyte differentiation media. During the treatment of cells with osteocyte differentiation medium, cells transformed into long polygonal shaped osteocytes and produced calcium deposition onto the extracellular matrix. And then when cells were treated with adipocyte differentiation medium, cells became oval shaped and accumulated lipid droplets. Non-treated AdMSCs did not change their phenotypic properties, verified by their resistance to staining (Figure 3).

After 17 days of 24-well plate culture, adipocyte-

differentiated wells were stained with oil red O and osteocyte-differentiated wells were stained with alizarin red S solution. Microscopic observation indicated that most of the AdMSCs from the shake flask culture were successfully differentiated either into adipocytes or osteocytes (Figure 3, A and B).

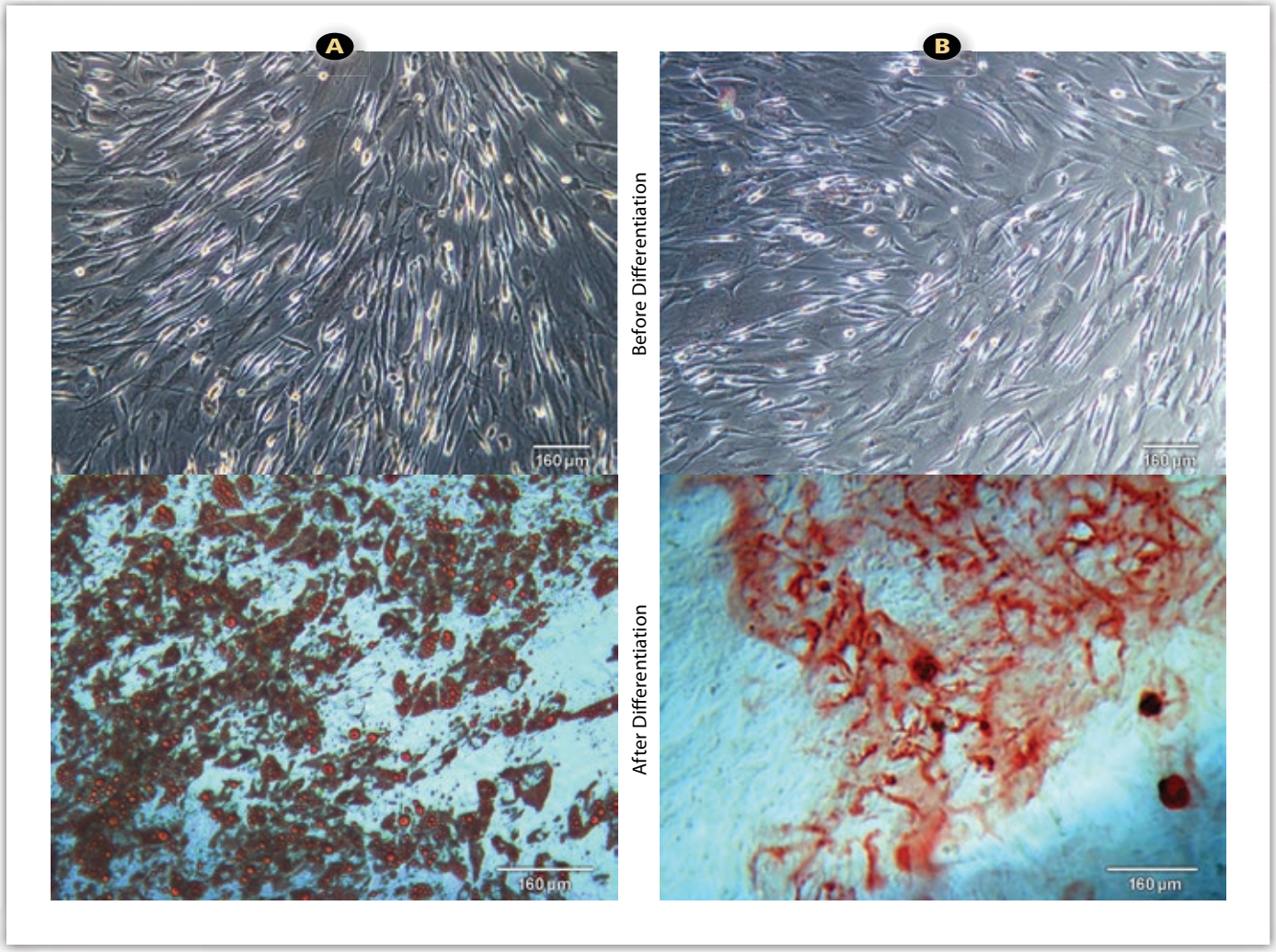

FIGURE 3. Differentiation assays for AdMSCs expanded on microcarriers in shake flasks. **(A)** Adipogenic differentiation formed lipid droplets, as indicated by oil red O-positive staining. Undifferentiated cells (top) could not retain the oil red O stain (red color) due to a lack of fatty lipids, whereas differentiated cells (bottom) retained the oil red O stain due to the presence of adipocytes with an abundance of fatty lipids. **(B)** Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by alizarin red S positive staining. Undifferentiated cells (top) could not retain the alizarin red S stain (red color) due to a lack of calcium deposition in the cells, whereas differentiated cells (bottom) retained the alizarin red S stain due to the abundance of calcium deposition in the cells after differentiation into osteocytes.

Conclusion

In this study, we successfully utilized the incubator shaker to develop a shake flask technique to expand AdMSCs on microcarrier beads. We demonstrated that the expansion of adherent AdMSCs using shake flasks appears to be a simple yet effective alternative to the spinner flask method. This technique reduces experimental complexity and decreases the risk of contamination associated with inserting a non-sterile magnetic stirrer base into an incubator. The shake flask method also reduces the risk of cell damage by the spinner rod. This is especially important for culturing stem cells, as stem cells are sensitive cell lines, which are more prone to shearing and mechanical damage than robust industrial cell lines such as CHO or Vero cells.

In addition, the growth of MSCs under shake flask conditions did not alter their stem cell properties. This was evident by their ability to differentiate into adipocytes and

osteocytes. Furthermore, we found that the S41i incubator shaker was able to accommodate more shake flasks than the standard CO_2 incubator equipped with a stir plate for spinner flasks. In the case of the spinner flask, the use of multiple or large magnetic stirrer bases can potentially generate excessive heat. This could lead to temperature control issues, especially for incubators that do not have refrigeration capabilities. We believe that the shake flask method provided an important improvement for the future scale-up potential of stem cells into large-scale industrial bioreactors.

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Notes

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Other

APPLICATION NOTE No. 295 I December 2013

Isobutanol from Renewable Feedstock - Process Optimization by Integration of Mass Spectrometry to two 8-fold DASGIP® Parallel Bioreactor Systems

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Abstract

This application note describes the integration of a Thermo Scientific Prima dB Mass Spectrometer (MS) with DASGIP Parallel Bioreactor Control Systems implemented at Gevo, Inc. in Englewood, Colorado. The availability of real-time MS data will aid in maximizing cell growth and isobutanol production.

Introduction

Isobutanol has broad market applications as a solvent and a gasoline blendstock that can help refiners meet their renewable fuel and clean air obligations. It can also be further processed using well-known chemical processes into jet fuel and feedstocks for the production of synthetic rubber, plastics, and polyesters. Isobutanol is an ideal platform molecule that can be made inexpensively using fermentation. The ability to automate the data analysis would increase production and reduce costs.

Gevo, a leading renewable chemicals and advanced biofuels company is developing biobased alternatives to petroleum-based products using a combination of synthetic biology and chemistry. Gevo plans to produce isobutanol, a versatile platform chemical for the liquid fuels and petrochemical market.

The main objective of implementing OPC communication

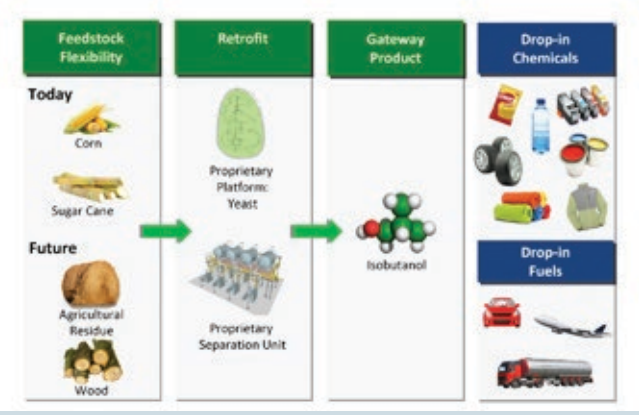

Figure 1: Isobutanol - A versatile platform Chemical

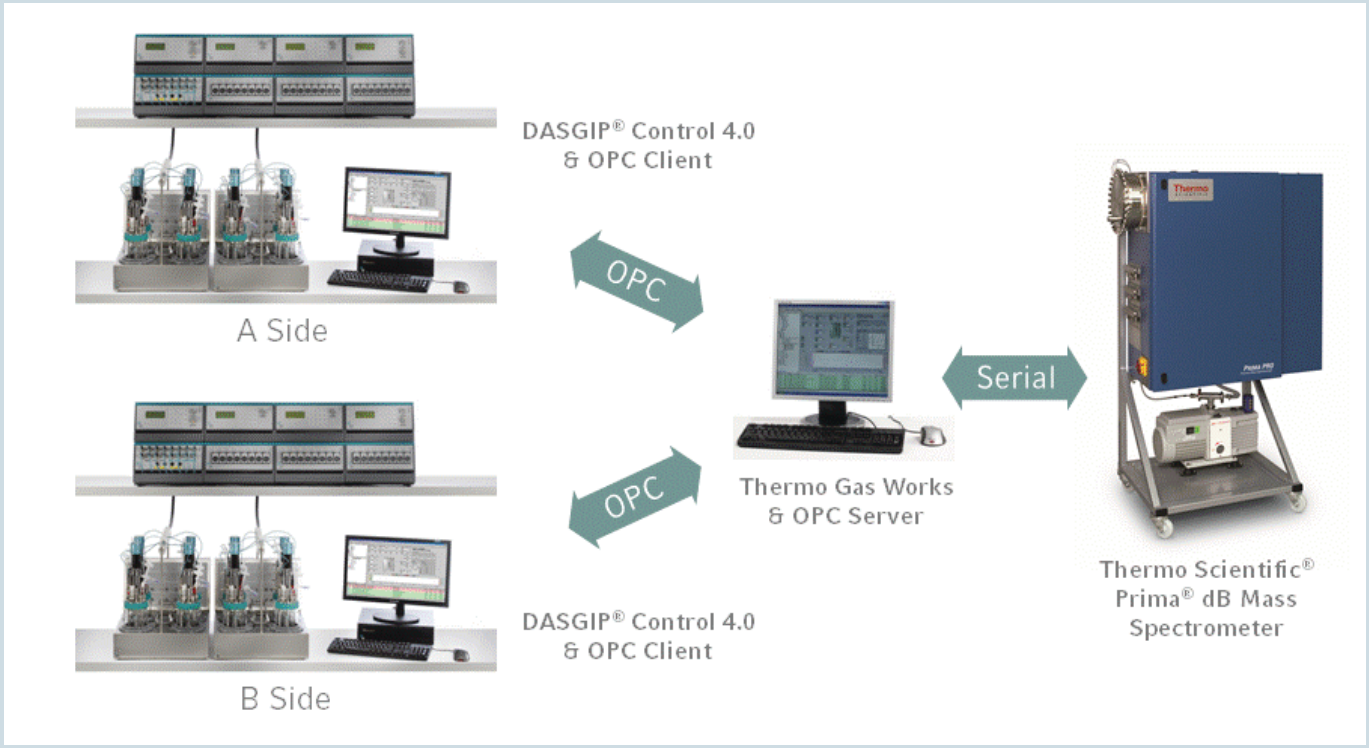

Figure 2: MS Integration: Data Display and Charts. The DASGIP Bioreactor view shows online bioreactor runtime data including real time MS results. Editable scripting allows for online calculation of production rate and graphic display in defined charts.

between the Thermo Scientific Mass Spectrometer and the DASGIP Parallel Bioreactor Control System during a fermentation run was to optimize growth and isobutanol production through automation. The system previously in place at Gevo required that manual data calculations had to be performed by merging the bioreactor runtime data with the MS data to assess the fermentation performance.

Materials and Methods

Corn mash was used as a substrate for the production of isobutanol by fermentation. The fermentation process was carried out using two DASGIP Parallel Bioreactor Systems with eight vessels each. The working volume in all 16 bioreactors was 1L, respectively.

OPC communication was implemented between the Thermo Scientific Prima dB Mass Spectrometer and the DASGIP Control Software to provide real-time off-gas results. Script calculations were used to take the MS data as inputs and generate meaningful metrics to automatically analyze key fermentation operating values and quickly make process control changes.

Results and Discussion

By integrating the Thermo Scientific Prima dB Mass Spectrometer with the DASGIP Parallel Bioreactor Control System the calculation of key fermentation operating values was successfully automated. This automation streamlined the workflow and allowed for data-driven control decisions using the real-time off-gas based analytical results.

Before Automation:
Without integration of the MS and the DASGIP Parallel Bioreactor Control System using OPC, calculation of key fermentation operating values was time-consuming and labor-intensive.

Optimized by MS Integration:
Using OPC communication the real-time MS results were sent to the two DASGIP Control Systems. Within the control system, the fermentation runtime data and the MS results were charted and transferred to the data historian with synchronized time stamps.

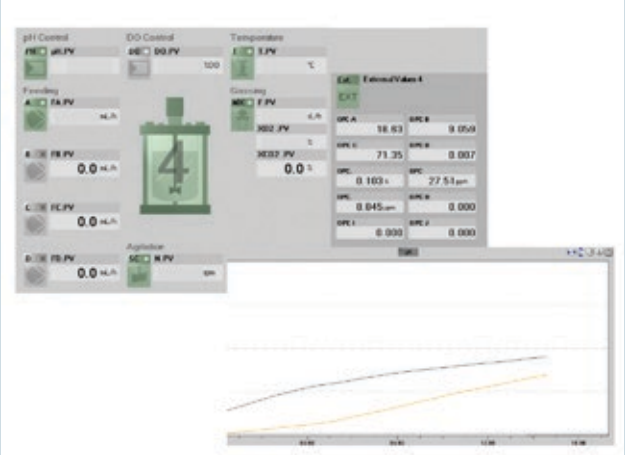

Figure 1: MS Integration: Data Display and Charts. The DASGIP Bioreactor view shows online bioreactor runtime data including real time MS results. Editable scripting allows for online calculation of production rate and graphic display in defined charts.

Key fermentation operating values were calculated online from combined fermentation and MS runtime data, charted and sent to the data historian and were then available for data-driven control decisions. Set-up and script calculations were stored in a user-editable recipe.

Conclusion

With its comprehensive data management functions the DASGIP Parallel Bioreactor System allowed the seamless integration of the Thermo Prima dB Mass Spectrometer.

The most important success criterion was the ability to calculate isobutanol production rates in real-time giving instant feedback on the quality of run. The availability of the off-gas-based analytical results made data-driven control decisions possible. A secondary success criterion was the fermentation runtime data and MS data was logged with synchronized time stamps to allow for post-run analysis if needed.

Ordering information	Order no.
DASGIP® Parallel Bioreactor System for Microbial Applications	
4-fold system, benchtop, max. 250 sL/h gassing	76DG04MB
8-fold system, benchtop, max. 250 sL/h gassing	76DG08MB
4-fold system with Bioblock, max. 250 sL/h gassing	76DG04MBBB
8-fold system with Bioblock, max. 250 sL/h gassing	76DG08MBBB
DASGIP® Control System	
incl. PC, OS, DASGIP Control and licenses for 4 vessels	76DGCS4
incl. PC, OS, DASGIP Control and licenses for 8 vessels	76DGCS8
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Vero Cell-based Vaccine Production: Rabies and Influenza Cell lines, Media and Bioreactor Options

Review Article, Dec. 2013

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Table of Contents

1. Abstract

We review strategies for optimizing vaccine production with examples given for rabies and influenza using cell culture systems. The Vero cell line is one of the most satisfactory based on its stability and well-documented performance in quality and quantity of viral yield. It has received FDA approval and is used throughout the world. Cell culture media technology has advanced dramatically in recent years, and a number of serum free and protein free options are available through commercial suppliers. Because serum tends to bind toxins and contaminants, its elimination calls for careful monitoring of culture conditions in order to achieve optimal performance.

Improvements in microcarriers have been important additions to the range of possible choices for optimizing in vitro production systems. With a series of bioreactor options available, we can foresee the elimination of hens eggs for virus production.

2. Introduction

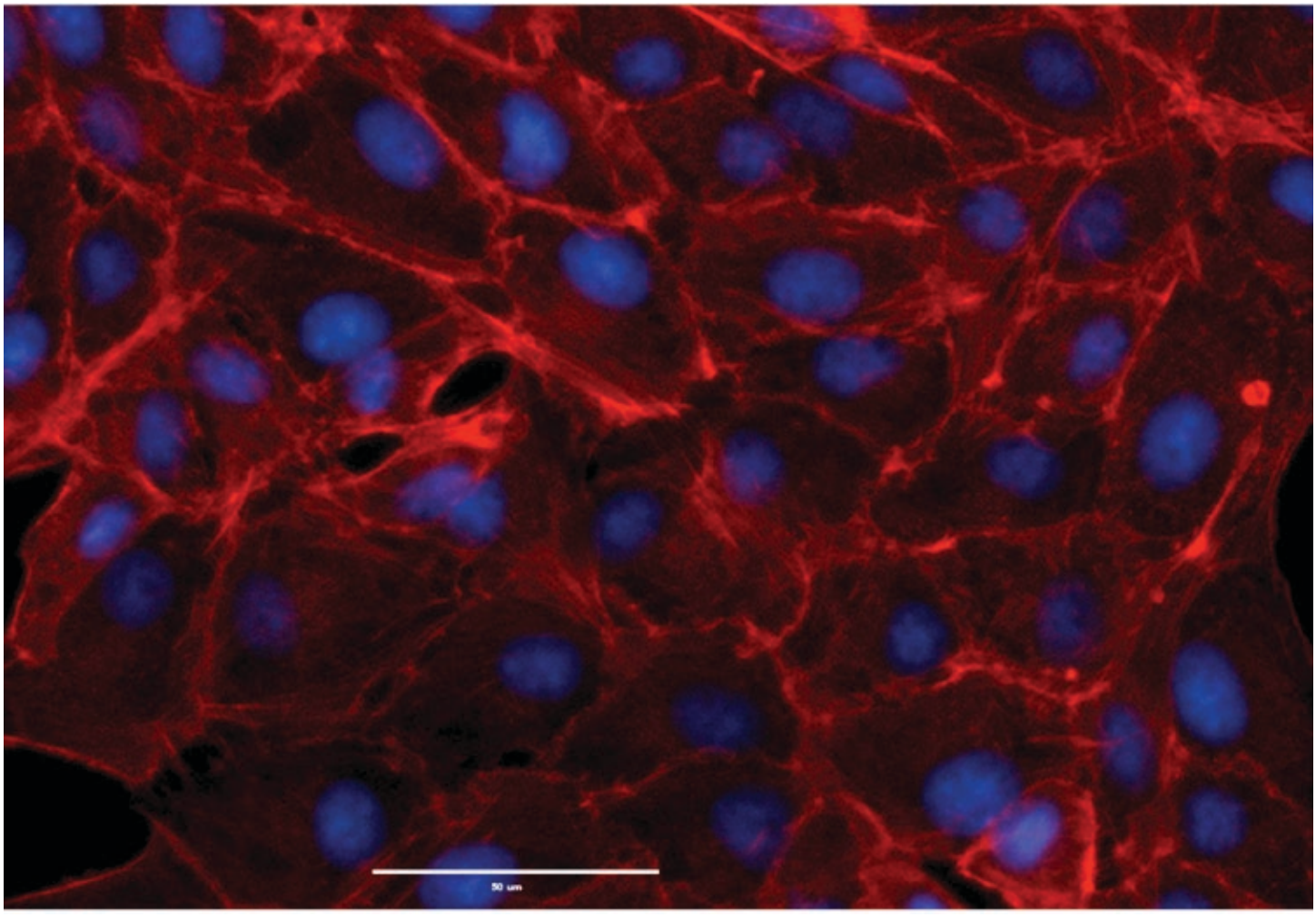

Figure 1. Fluorescent image of confluent Vero cells; DAPI-stained nuclei appear blue, and actin filaments stained with rhodamine-conjugated phalloidin appear red (Eppendorf Inc.).

Viral diseases, including rabies and influenza, are worldwide challenges to the international biomedical community. WHO notes that in 1998 over 32,000 deaths due to rabies were reported, while influenza has been responsible for millions of deaths worldwide over the course of the last century.[1]

Rabies is often transmitted to humans from infected domestic animals. Dogs infected with rabies can become extremely aggressive and attacks on humans are widespread, especially in certain Asia countries where using unleashed dogs for home security is common. The virus is spread through their saliva and bites by infected animals can be fatal. In China, the disease is referred to as “Kuang Quan Bing” (狂犬病 in Chinese), i.e. “Mad Dog Disease”. The annual number of deaths worldwide caused by rabies had grown to 55,000 by 2006.[2]

Influenza is a second worldwide scourge. The CDC Influenza Division reported an estimated range of deaths between 151,700 and 575,400 individuals resulting from the 2009 H1N1 virus infection during the first year the virus circulated.[3] These figures, however pale in comparison to reports of a half a million deaths *every year* throughout the world due to influenza.[3] Annual deaths in the United States top 36,000 with 114,000 hospitalizations accompanied by a staggering cost of $600 million in health care and an additional $1 billion in economic costs.[4] Anti-viral drugs are employed for acute treatment, but vaccination remains far and away the most effective approach for combating viral illnesses.

Moreover, there is a constant, underlying concern regarding the possibility of the emergence of a truly deadly influenza strain, on a level with the 1918 influenza outbreak, the “Spanish Flu" that caused ~50 million deaths worldwide. For this reason, existing technologies are being relentlessly evaluated and upgraded with the aim of avoiding a devastating pandemic.[5]

Since the 18th century, vaccination has proven to be the most successful (and perhaps the only) route to the total elimination of viral diseases. The history of smallpox is well known, as is the introduction of the use of cowpox virus from lesions in infected animals by Jenner in 1796.[6] Despite his work and that of others, smallpox epidemics continued throughout the 19th century, due to improperly applied or non-existent vaccination regimes. The work of Pasteur and others toward the end of the 19th century put vaccination on a sound scientific footing.[7]

3. Biological Systems for Viral Cultivation

Today throughout the world there is a rapidly expanding demand for vaccine products. These growing requirements have necessitated the development of a range of techniques for growing large quantities of antigenic proteins. Traditionally, viruses have been grown in embryonated hen’s eggs, but numerous shortcomings compromise their utility. These include a bottleneck in the availability of high quality, pathogen-free eggs, as well as low titers of emerging viruses.[8] A major concern is the fact that when viruses are cultivated through extended passages in hens eggs, there is an evolutionary process in the amnion or allantoic cavity of the egg resulting in the selection of a virus subpopulation, antigenically and biochemically distinct from the original innoculum.[9] Because of these and other factors, permanent cell lines are coming to dominate the field.

As an alternative to egg-based vaccine production, the advantages of mammalian cell culture systems have been widely recognized. Cultured cells provide much shorter lead times, a more controlled production process that takes advantage of closed-system bioreactors, a reduced risk of microbial contamination, and the opportunity to cultivate viral stocks without significant egg passage-dependent antigenic changes.[10]

A WHO conference some years ago enunciated concern regarding the rapid emergence of pandemic viral strains. It was concluded that insufficient time would be available to generate the large quantity of high quality, fertile hens’ eggs that would be required to the demands of a worldwide pandemic.[11] In the intervening years, the situation has only exacerbated. Thus the cell culture alternative provides a flexible and scalable platform that can make use of existing biopharmaceutical infrastructure for Influenza vaccine production. Indeed, Montomoli et al[12] argue that because of these inherent limitations, cell culture will replace egg-based vaccines within the foreseeable future.

4. Cell Line Options

In the past few years, several continuous cell lines have been approved by regulatory authorities for influenza virus production, such as the *Spodoptera frugiperda* insect cell line (Protein Sciences[13]), the Madin-Darby canine kidney (MDCK) and the Vero line, one of the most widely used. A fourth alternative is the PER.C6® cell line,[14] designed for growth to high densities. This property means that much more biological product can be harvested from much smaller bioreactors. The manufacturers claim that the PER.C6 cells, infected with virus for manufacturing purposes, produce at

least 10 times more virus per ml than other FDA approved cell lines. It should be noted that PER.C6 is a proprietary cell line, and licensing costs, obtained from Crucell, may be substantial.

It is important to be aware that certain cell lines may provide an environment favoring selection of viral subpopulations, and these types may be inappropriate for vaccine production. Anez et al attempted[15] production of Dengue virus vaccine candidates using FRhL-2 diploid fetal rhesus monkey lung cells. However, passage in this cell line resulted in the accumulation of a mutational variant which was responsible for reduced infectivity and immunity in Rhesus monkeys. This phenomenon was not observed in viruses passaged in the Vero cell line. Other lines of investigation support the Vero cell line as the candidate of choice for viral vaccine production, including: efficiency of primary virus isolation and replication to high infectivity titers; genetic stability of the hemagglutinin molecule, while maintaining the antigenic properties of human-derived viruses; and similarities in the pattern of protein synthesis and morphological changes between virus-infected Vero and MDCK cells.[16] Vero is the only cell line that has received worldwide regulatory acceptance.[17]

There are claims that head to head comparison of growth performance in lab-scale bioreactors (stirred tank, wave bioreactor) resulted in lower yield for Vero cells as opposed to the MDCK line, although both displayed comparable productivity in small scale systems. However, this observation is applicable only under the specific conditions and specific cell lines employed in this study. Given the regulatory acceptance of the Vero cell line as well as the abundance of vaccines already successfully developed (Table 1), Vero remains one of the most attractive platforms for cell based viral vaccine production.

5. Media Alternatives

There are a variety of different Vero isolates available from commercial suppliers (Vero, Vero 76, Vero E6, Vero B4), but all are quite similar, and their nutritional needs are comparable.[18] The search for the ideal mammalian cell culture medium began in the 1950s, with the holy grail being an economical, protein-free, serum-free medium that would provide strong growth support and have the property of scalability to large volumes, up to thousands of liters, while coming in at an affordable price.

Anti-viral Vero Cell-Based Vaccines

Study (year)	Disease	Vaccine Type	Genus
Wang et al (2008)	Chikungunya Fever	Live attentuated	Alphavirus
Howard et al (2008)	Chikungunya Fever	Inactivated	Alphavirus
Guirakhoo et al (2004) Blaney et al (2007) Blaney et al (2008)	Dengue Fever	Live attenuated or live chimeric	Flavivirus
Tauber et al (2007) Tauber et al (2008) Srivastava et al (2001)	Japanese encephalitis	Inactivated	Flavivirus
Kuzuhar et al (2003) Guirakhoo et al (1999) Monath et al (2003)	Japanese encephalitis	Live attenuated or live chimeric	Flavivirus
Vesikari et al (2006) Ruis-Palacios et al (2006)	Viral gastroenteritis	Live attenuated	Rotavirus
Montagnon (1989) Montagnon (1989)	Polio	Live attenuated Inactivated	Picornovirus
Montagnon (1989)	Rabies	Inactivated	Lyssavirus
Aycardi E (2002)	Rabies	Inactivated	Lyssavirus
Kistner et al (2007)[19]	Ross River fever	Inactivated	Alphavirus
Spruth et al (2006) Qu et al (2005) Qin et al (2006)	Severe acute respiratory syndrome	Inactivated	Cornovirus
Monath et al (2004)	Smallpox	Live attenuated	Orthopoxvirus

Lim et al (2008)	West Nile Encephalitis	Inactivated	Flavivirus
Monath et al (2006)	West Nile Encephalitis	Live attenuated	Flavivirus
Kistner et al (1998) Ehrlich et al (2008)	Influenza	Inactivated	Orthomyxovirus
Bonnie and William (2009)	Influenza	Inactivated	Orthomyxovirus
Chan and Tambyah (2012)	Influenza	Inactivated	Orthomyxovirus

Table 1. Anti-viral vaccines using Vero cell culture production technologies. Modified from Barrett et al[23].

Serum provides a protective function to cultured cells and binds toxins and other contaminating materials. Thus serum-free [20] media must be extremely carefully formulated. Albumin can be substituted for serum, but it may impede the downstream steps of purification.[21]

Chen et al[21] have tested five different serum free media, combined with Cytodex 1 microcarriers. The following were evaluated: OptiPro SFM (Invitrogen®), VPSFM (Invitrogen), EX-CELL™ Vero SFM (SAFC Biosciences®), Provero-1 (Lonza®) and HyQ SFM4MegaVir (HyClone®). The EX-CELL Vero SFM gave one of the highest cell densities, demonstrating that the use of serum free media has become routine for Vero cell cultivation. Comparable results were observed with a commercial serum-free medium MDSS2N (manufactured under the name AXCEVIR-VeroTM by Axcell Biotechnologies). In this case, Vero cells were compared with MDCK cells grown in T-flasks and microcarrier cultures.

6. Rabies Virus Cultivation Strategies

The Brazilian group led by Frazatti-Gallina[22] has been active in the field of Rabies vaccine production. Using Vero cells adhered to microcarriers, and cultivated in a bioreactor with serum-free medium, they generated an effective rabies vaccine. With the aid of tangential filtration, they purified the Rabies virus by chromatography and inactivated it using beta-propiolactone.

Their protocol states that 350 cm^2 T-flasks were harvested and inoculated into a 3.7 liter New Brunswick™ CelliGen® bioreactor, at a proportion of 16 cells per microcarrier (Cytodex® 3-GE), yielding an initial seeding of 2.5×10^5 cell/ml. The cells were grown in serum-free MDSS2 medium (Axcell Biotechnologies).

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and CHO cell growth. This medium drives the adherence of the Vero cells to the microcarriers. After 4 days of cultivation in VP-SFM medium, the cells were infected with PV rabies virus (multiplicity of infection = 0.08). The harvests of the culture supernatant were carried out 3 days after the virus inoculation and four times thereafter at intervals of 24 h. During this period, culture conditions were maintained at 60 rpm at a pH of 7.15 and 5% dissolved oxygen. Only the temperature varied from 36.5 °C in the cellular growth phase of the culture to 34°C after virus inoculation. In the course of the program, seven batches of virus suspensions were produced in the bioreactor (16L per cycle) at a mean viral titer of 104. FFD50/0.05 ml.

The effectiveness of the preparation was demonstrated by immunizing mice with three doses of the new vaccine (seven batches), comparing it with the commercial Verorab and HDCV (Rabies vaccine). Mean titers of neutralizing antibodies of 10.3-34.6, 6.54 and 9.36 IU/ml were found, respectively.

The choice of the serum-free medium was fortunate. In this case the amount of contaminating DNA was very low, and tolerable, less than 22.8 pg per dose of vaccine. The authors argue that this protocol is especially applicable in the developing world, where rabies is a constant hazard and a major public health problem.

Yu et al[23] sought methods to increase yield in Vero cell culture systems over that obtained using roller bottles. In a recent review, they summarized the production technology developed over the course of the last seven years. They have adopted the 30 L New Brunswick BioFlo® 4500 Fermentor/Bioreactor. The cells were cultivated in media containing 10% serum, first grown as a monolayer, and when the cell density reached $1.0–1.2 \times 10^6$ cells/ml, they were transferred to the bioreactor containing 25 g/L of Cytodex-1 for perfusion culture. The virus preparations, also cultured in roller bottles, were infected with the PV2061 virus strain, harvested and transferred to the bioreactors.

Wang[24] et al have described a purified Vero cell rabies vaccine that has been widely produced in China, which currently is responsible for almost two-thirds of the total rabies vaccines used in Asia. The most successful offering used in China is a purified Vero cell vaccine, referred to as ChengDa (Liaoning ChengDa Biological Co., Ltd., Shengyang, China[25]). It is grown on a Vero cell line utilizing the L. Pasteur 2061 strain of rabies virus, inactivated with β-propiolactone, lyophilized, and reconstituted in 0.5 ml of physiological saline. It fulfills the WHO recommendations for potency.

The process used at ChengDa was developed by Aycardi.[26] A single New Brunswick bioreactor was capable of producing one million dose of rabies vaccine per year. The method uses ultra-high density microcarrier cell cultures adapted to a 30 L New Brunswick CelliGen bioreactor equipped with a patented Cell Lift Impeller (Figure 2), specifically configured for a perfusion system to feed the growth media into the bioreactor. A specially designed decanting column (New Brunswick Scientific) was used to prevent perfusion loss of microcarrier and keep the cells in high concentration. The system delivers high oxygen transfer, high nutrient level and low shear stress, thus allowing cell growth up to 1.2 x 10^7 million cells/ml) under continuous perfusion for up to 20 days.

ChengDa was licensed by the Health Ministry of China and the State Food and Drug Administration of China (SFDA) in 2002 and has been marketed throughout the country since that time. Although not approved for sale in the United States, purified Vero cell rabies vaccine is permitted for use by US citizens if available in a destination country, according to the CDC[24].

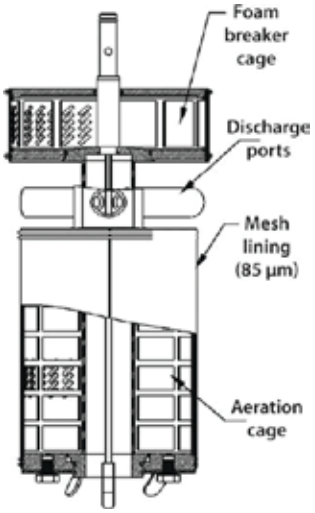

Figure 2. New Brunswick Cell Lift Impeller (Eppendorf Inc). Patented design consists of three discharge ports located on the impeller shaft to provide uniform circulation without traditional spinning blades for conducing microcarrier cultures under ultralow-shear conditions. The flow is driven by centrifugal force, the rotation of the three ports creates a low-differential pressure at the base of the impeller shaft, lifting microcarriers up through the hollow shaft and expelling them out through its ports (The discharge ports must be submerged during operation). Bubble-sheer is eliminated by the Cell Lift impeller, which utilizes a ring sparger generating bubbles only within the aeration cage, so that the oxygenation works without any bubbles coming into contact with the cells.

7. Influenza Virus Cultivation Strategies

The application of Vero cells for the propagation of influenza virus in animal-derived component free (ADCF) media was extensively described by Wallace et al in their US patent[27] (no. 7,534,596 B2). The patent application includes the steps of attaching ADCF-adapted cells to a microcarrier (SoloHill® Engineering Inc.) and infecting the cells with vaccine media, producing virus within the cells and harvesting of the virus. The influenza viruses produced by this method achieved higher titer than that of the egg produced vaccine (Table 2.).

Production System	Panama H1N1 Titers (log_{10} $TCID_{50}$/mL)
Egg	7.8
Vero: Serum-containing	7.9
Vero: Serum-free ADCF	8.0

Table 2. Comparing egg-based influenza production with Vero-cell–based production using Hillex II microcarriers (SoloHill Engineering).

A method for microcarrier-based expansion of cells from a 0.2 L spinner culture to a 2L and 10 L bioreactor culture was developed (Figure 3). A New Brunswick CelliGen 310 bioreactor with a 5 L vessel was used for the 2 L culture stage. The vessel was equipped with a ring sparger, spin filter, 3-segmented pitched blade impeller (up-pumping), and 4-gas control

at 100 mL/min (Air, N_2, CO_2, and O_2). This expansion strategy couple with the demonstration of viral productivity represents an ideal closed system platform for vaccine production.

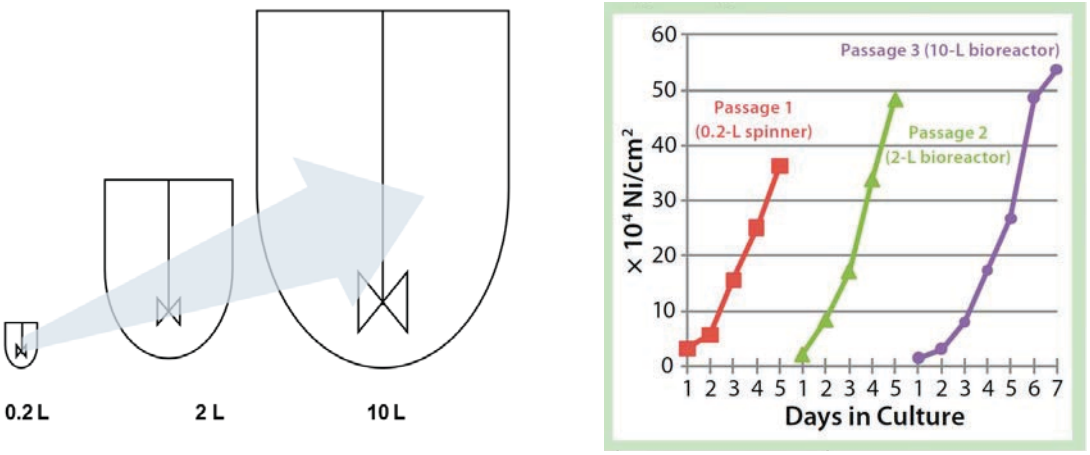

Figure 3. Vero-based expansion on microcarriers; seed train of Vero cells cultured on Hillex II micocarrier beads (SoloHill Engineering). Left: Diagram detailing bioreactor based expansion scheme; Right: Scale-up from Spinner flask to industrial bioreactors.

A similar method using Vero cell line for influenza vaccine production was demonstrated by Chen et al[4]. Using Cytodex 1 microcarrier beads, these investigators were able to achieve cell densities of 2.6 x 10^6/ml in serum free, protein free medium. These findings were obtained using a 250 ml Bellco microcarrier spinner flask equipped with a paddle impeller, inoculated with 2.5 10^5/ml Vero cells in 5% CO_2 atmosphere. In a subsequent expansion phase, starting from an initial number of 5 x 10^5 /ml, the cells were expanded in a 3L bioreactor. After 24 hours the cells had adhered to the microcarriers and the virus was added together with fresh medium. Using these procedures, the authors were able to obtain high virus titers up to 10 Log10 $TCID_{50}$/ml. They conclude that their approach could serve as a basis for large scale commercial production of influenza virus.

In 2011, Baxter International Inc. announced the approval for PREFLUCEL, the first Vero Cell based seasonal influenza vaccine, available for 13 participating European Union countries, including Germany, Spain, UK and the Scandinavian countries. Preflucel is comprised of purified, inactivated split influenza virions, manufactured using Baxter's adaptation of the Vero cell platform.

Although not approved for sale in the United States, data from a U.S. Phase III study with over 7,200 healthy individuals has shown that Preflucel provided 78.5% protective efficacy against subsequent culture-confirmed influenza infection, and robust immune responses against the three viral strains contained in the vaccine.

8. Conclusions

The combination of advances reviewed here provides strong support for the use of cell culture systems for virus production for vaccines. The fact that Vero cells have been approved for clinical products represents an important step on the road to technologies that do not rely upon hen's eggs for generation of adequate quantities of viruses. Advances in culture media enable the elimination of serum, thus driving the rapid and efficient purification of proteins. Whereas serum-containing media may continue to occupy a default position, it is now generally recognized that serum-free media are now the optimal choice. The use of carrier beads adds to the efficiency of culture technology, allowing greatly increased cell densities to be reached. Finally, improvements in bioreactor design combined with these various technological advances results in a greatly improved and more functional production train.

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