Evaluation of Disposable Bioreactors

## Rapid Production of Recombinant Proteins By Several Animal Cell Lines

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During the past decade, the pharmaceutical and biotechnology industries have experienced increasing pressures on new drug-development timelines. In addition to securing a "first to market" commercial advantage, the ability to make rapid go/no-go decisions during the development cycle of new molecules is crucial to reaching greater effectiveness (**1**). Timely allocation/reallocation of resources toward the most promising clinical candidates helps keep organizations agile and efficient. Compounding the increasing pressure on bioprocess development teams to speed production of required quantities of their molecules of interest (**2**) are economic mandates to efficiently use human and material resources. Because bioprocesses often require extensive use of sophisticated equipment, novel technologies that translate into faster and simpler operations while reducing the impact on resources appear very attractive.

Generally, the use of disposable equipment for biotechnology applications offers a wealth of advantages including reduction of preparation time, elimination of cleaning and sterilization steps, and a greater ease of use (**3**). These benefits are likely to contribute to significant cost savings in time and capital. The upward trend toward cultivating animal cells for production of recombinant proteins (including monoclonal antibodies) is poised to continue for the foreseeable future (**4**),

often requiring manufacture of hundreds of milligrams to gram quantities of recombinant proteins to support early evaluations. Generally, support activities rely on cultivating animal cells in stirred laboratory-scale bioreactors. Although the reactors are highly reliable and flexible, their preparation, operation, and cleaning are time consuming activities. The recent commercialization of disposable and easy-to-use animal-cell cultivation devices such as the Wave bioreactor (WBR, www.wavebiotech.com) and Applikon's AppliFlex (www.single-use-bioreactor.com) offer the prospect of reducing the use of small-scale stirred bioreactors. In addition to their simplicity of use, the costs of rocking bioreactors and their ancillary accessories may be lower than for sterilizable-in-place bioreactors.

Briefly, a Wave-style apparatus consists of a sterile disposable plastic bag that is half filled with cultivation medium, and the head space is filled with the desired gas mixture. Bags are placed on a rocking platform that delivers a wave-like motion to the liquid, thereby delivering adequate mixing and gas transfer to the culture while preventing formation of damaging gas bubbles (**5**). WBRs offer the possibility for continuous gassing and are available in nominal volumes ranging from two to 1000 L. Since their market introduction, WBRs have been used for cultivation of suspension (**5, 6**) and anchorage-dependent mammalian cells (**7, 8**) as well as
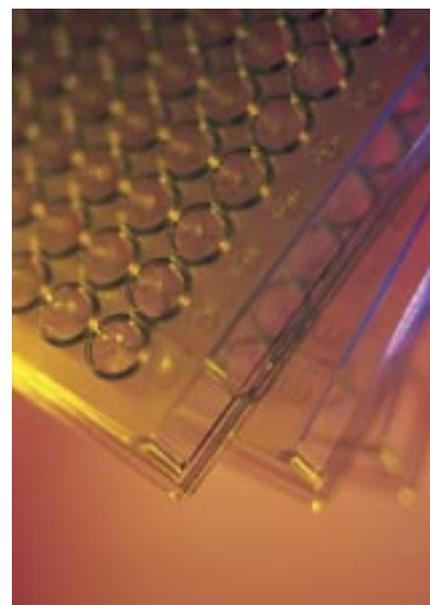
insect cells (**9**). Recently, Hami et al. reported the use of WBRs for clinical production of activated autologous T-cells used in the treatment of various forms of cancers (**10**). A WBR has also been fitted with a floating filter and successfully used as a perfusion reactor, supporting cell concentrations of up to 3×10^7 cells/mL, a sixfold increase over concentrations routinely achieved in batch cultures (**11**).

Because WBRs offer the advantage of reducing investments in both capital and time, we evaluated their use in routine cultivation of various animal cells in support of material deliveries. We determined that acceptable and reproducible recombinant protein production was achieved using three

industrially relevant cell lines. The data presented here support implementation of disposable bioreactors for routine small-scale cultivation of animal cells in support of production of hundreds of milligram to gram quantities of recombinant proteins.

CELL LINES AND CULTURE MAINTENANCE

GS-NS0 Cell Line: A GS-NS0 cell line secreting a recombinant monoclonal antibody was obtained from Lonza Biologics (Slough, UK, www.lonza.com). This glutamine synthetase (GS) deficient mouse cell line has been developed to be used in conjunction with a plasmid carrying both genes for GS and the recombinant protein of interest. Cultivation in a glutamine-free medium allows for efficient posttransfection selection of cells carrying the GS and protein of interest genes (**12, 13**). Although NS0 cells are usually cultivated with exogenous cholesterol added to the medium (**13–15**), under gradual removal of cholesterol from the medium they can be made to fully reexpress their cholesterol synthesis pathway (**16–18**).

After adaptation, the cell line used in these studies was routinely cultivated in the absence of cholesterol in a glutamine-free proprietary animal protein-free medium. A rolling seed was maintained in 500-mL vented shake flasks (Corning; Corning, NY, www.corning.com) containing 100 mL of cultivation medium. The flasks were incubated at 36.5 °C in a 5% CO_2 incubator on an orbital shaker (both from Thermo Forma, Marietta, OH; www.thermoforma.com) and operated at 100 rpm. Every three or four days, once the viable cell (vc) concentration reached 0.8 to 1.2×10^6 vc/mL, the cells were subcultured by dilution to 1.0 to 2.5×10^5 vc/mL into a fresh flask.

dhfr⁻ CHO Cell Line: A dihydrofolate reductase deficient (dhfr⁻)–CHO (Chinese hamster ovary) cell line (**19, 20**) was obtained from Dr. Chasin at Columbia University (New York City). When this cell line is cotransfected with plasmids carrying genes for dhfr and the recombinant protein of interest, efficient posttransfection selection is achieved in the presence of methotrexate (MTX), a dhfr inhibitor. The cells used in this study were cultivated in a proprietary animal-protein–free medium containing 300 nM of MTX to maintain selective pressure. A rolling seed was maintained as described above. Every three or four days, once the viable cell concentration reached 0.9 to 1.5×10^6 vc/mL, the cells were subcultured by dilution to 1.0 to 1.5×10^5 vc/mL into a fresh flask.

***Drosophila* S2 Cell Line:** Schneider's *Drosophila* S2 line was obtained from ATCC (accession number: ATCC CRL-1963; www.atcc.org) and transformed with a plasmid coding for a recombinant interleukin. A pool of cells that secreted promising amounts of the desired protein was selected for further studies. The cells were routinely cultivated in Excell 420 + glutamine medium (JRH Biosciences, Lenexa, KS; www.jrhbio.com). A rolling seed was maintained in 2-L vented shake flasks (Corning) containing 500 mL of cultivation medium. The flasks were incubated at 26.5 °C in a non-CO_2 incubator (Thermo Forma) on an orbital shaker operated at 100 rpm (Cole-Parmer Instrument Company, Vernon Hills, IL; www.coleparmer.com). Every three to four days, once the viable cell population reached 7 to 15×10^6 vc/mL, the cells were subcultured by dilution to 1.5 to 2.0×10^6 vc/mL into a fresh flask. Expression of the recombinant protein was under control of a copper-inducible promoter, similar in principle to that described by Lim et al. (**21**). Induction was achieved by adding 10 mL of 100-mM copper sulfate (Sigma, St Louis, MO; www.sigma-aldrich.com) per liter of culture.

ANALYTICAL PROCEDURES

Measuring Cell Concentration: Viable cell concentrations were determined by the trypan blue exclusion method (**22**). Mammalian viable cell concentrations (NS0 and dhfr⁻ CHO) were determined using a Cedex automated image analysis system (Cedex Innovatix, Frazer, PA; www.innovatix.com) (**23**). Because the insect cells were too small to measure with the Cedex, S2 viable cell concentrations were obtained using a microscope and a hemocytometer counting chamber (**24**).

Measuring Environmental Parameters: Culture pH, dissolved CO_2, and dissolved O_2 were measured using a blood gas radiometer APL5 (Radiometer Medical A/S, Denmark; www.radiometer.com) immediately following sampling. Samples were then centrifuged for 10 min at 200 *g*. Glucose in the supernatant was measured using an immobilized glucose oxidase assay by a NOVA Bioprofile Analyzer 100 (Waltham, Mass; www.novabiomedical.com).

Measuring Product Concentration: Assays for the quantification of recombinant protein production were performed on supernatant samples prepared as described above.

Measuring MAb Production: The monoclonal antibody secreted by the GS-NS0 cells was quantified by measuring affinity to immobilized protein A using surface plasmon resonance by using a BIAcore 1000 instrument (Piscataway, NJ; www.biacore.com) (**25, 26**). A capture antibody, goat antihuman IgG Fc fragment (Jackson ImmunoResearch, West Grove, PA; www.jacksonimmuno.com), was immobilized onto a Sensor Chip CM5 surface (BIAcore). The analyte solution (either antibody standard or culture supernatants) was passed over the immobilized capture antibody at a continuous flow of 5 μL/min. The change in refractive index caused by binding of the antibody present in culture supernatants to the immobilized capture antibody in the liquid/solid interface was monitored in real-time, and the signal (measured in resonance units, RU) was directly related to antibody concentration in the culture broth. A calibration curve (RU vs. concentration) was prepared using a commercial human IgG4/κ as the antibody standard. This surface plasmon resonance-based immunoassay showed intraassay variability below 5% and interassay variability below 11%.

The monoclonal antibody secreted by the dhfr⁻ CHO cell line was measured by affinity chromatography using an HPLC comprising a gradient pump, a thermostated autosampler, and a UV detector (Perkin Elmer Instruments; Shelton, CT; www.perkinelmer.com) and fitted with a Bio Separations Protein A HLD disk

Table 1: Operating parameters for animal cell cultivation in 3-L stirred-tank bioreactors and 20-L Wave bioreactors

	3-L Stirred Bioreactor			20-L Wave Bioreactor		
	GS-NS0	DHFR$^-$ CHO	*Drosophila* S2	GS-NS0	DHFR$^-$ CHO	*Drosophila* S2
Inoculation Density (viable cells/mL)	1.5×10^5	1.5×10^5	1.5×10^6	1.5×10^5	1.5×10^5	1.5×10^6
Temperature (°C)	36.5	36.5	26.5	36.5	36.5	26.5
DO (%)	30	30	50	30	30	50
pH	7.1	7.1	6.3	7.1	7.1	6.5
Agitation (rpm)	75	125	75	14–17	18–20	15–22
Rocking Angle (°)	NA	NA	NA	8	8	8

Table 2: Comparison of cell growth, metabolism, and protein production during animal cell cultivation in 3-L stirred bioreactors (STBR) and 20-L Wave bioreactors (WBR)

	GS-NS0		DHFR$^-$ CHO Bioreactor		*Drosophila* S2	
	3-L STBR (n = 4)	20-L WBR (n = 3)	3-L STBR (n = 3)	20L WBR (n = 2)	3-L STBR (n = 2)	20-L WBR (n = 2)
Growth Rate (hr^{-1})	0.020 ±0.05	0.022 ±0.01	0.023 ±0.0	0.021 ±0.0	0.016 ±0.01	0.024 ±0.02
$Q_{glucose}$ (pmol/cell-hr)	0.09 ±.04	0.11 ±.02	0.18 ±0.03	0.12 ±0.024	0.01 ±0	0.01 ±0
Maximum Cell Concentration (10^6 viable cells/mL)	0.92±0.1	1.0±0.2	3.1±0.2	1.42±1	10.2±1.4	10.6±2.4
Qp (pg/cell-day)	15.5±3.1	20.4±3	2.0±0.5	9.3±0.1	11.1±0.2	6.7±1.0
Final Titer (mg/L)	66±9	83±12	26±1	47±18	166±59	156±42

column (IRIS technologies, Lawrence, KS; www.iristechnologies.net). The column was operated at a constant temperature of 30 °C, with UV detection at 280 nm. The buffers were 20 M Tris-HCl at pH 7.4 (load buffer), 500 mM acetic acid at pH 2.5 (elution buffer), and 100 mM Tris-HCl + 1 M NaCl (regeneration buffer). The gradient program for this assay first flows load buffer at 3 mL/min for 0.5 minutes followed by the elution buffer at 13 mL/min for one minute. That cycle is followed by first flowing the regeneration buffer at 1.5 mL/min for two minutes and finally returning to the load buffer at 3 mL/min for an additional one minute. There is a 0.5-min equilibration between samples. Under these conditions, the antibody eluted at 1.47 minutes. This method showed an intraassay variability of about 10% and an interassay variability of about 20%.

Recombinant protein secreted by the *Drosophila* S2 cell line was measured using an immunoassay, using time-resolved fluorescent detection.

Dynatech Immulon 4 plates (www.dynatech.com; cat# 011-010-3855) were first coated with 50 μL per well of a mouse antiinterleukin capture antibody and incubated overnight at 4 °C. Following overnight incubation, 200 μL of blocking solution (1 × Tris buffer saline (TBS), 0.01% azide, 1% bovine serum albumin, and 0.05% Tween 20) was added to each well. Following incubation at room temperature for one hour, the plates were washed three times with washing buffer (TBS 0.05% Tween 20), and 50 μL of culture supernatant sample to test was added to each well. Following a two-hour incubation at room temperature, the plates were washed three times with washing buffer. Then 75 μL of biotinylated mouse antiinterleukin detection antibody was added to each well, and the plates were incubated at room temperature for one hour, followed by three washes with washing buffer. Next, 100 μL of the detection agent, streptavidin-Europium, was added to each well, and the plates were incubated at room temperature for

20 minutes, followed by three final washes with washing buffer. Finally, 150 μL of enhancement solution (Perkin Elmer cat. 1244-105) was added to each well, and the plates were incubated for an additional one hour at room temperature. Time-resolved fluorescence was determined using a Delfia plate reader (Perkin Elmer). This method showed an intraassay variability of about 20% and an interassay variability of about two to three fold.

THE BIOREACTORS

3-L Stirred-Tank Bioreactor: The 3-L stirred-tank bioreactors (STBR) (B. Braun Biotech; Bethlehem, PA) were operated with a 2-L working volume. Temperature was controlled at the desired set point using an electric heating blanket. The pH was controlled by a cascade of CO_2 and sodium bicarbonate additions. During the initial days of the culture, CO_2 was required to lower the culture pH to the desired set point. As the cell concentrations increased and the amount of CO_2 and lactate produced by the culture increased, sodium bicarbonate was used to maintain the pH at the desired set point. Dissolved oxygen (DO) was controlled by intermittent oxygen sparging, constant air flow in the headspace, and constant agitation. DO was measured as percent air saturation at 1 atm pressure. Table 1 lists the specific parameters for the cultivation of the three cell lines in 3-L STBRs.

Wave Bioreactor Operation: 20-L Wave bioreactors (WBR, www.wavebiotech.com) were operated with a 10-L working volume, and the temperature was controlled using an electric heating pad installed beneath the bag. The rocking rate and angle were automatically controlled to the desired set points. DO and pH were manually adjusted by manipulating the ratio of CO_2 and air and the flow-rate of the gas mixture delivered to the cultures. When required, based on the off-line DO measurements, the rocking rate of the platform was increased to allow higher oxygen transfer. DO was measured as percent air saturation at 1 atm pressure. Table 1 lists the specific parameters for the cultivation of the three cell lines in 20-L WBRs.

RESULTS AND DISCUSSION

The two major differences in operation of the WBR and the STBR were the pH control and the agitation. In a WBR, the pH was controlled manually by adjusting the flow rate and ratio of CO_2/air in the gassing mixture; whereas in the STBR, the pH was automatically controlled with either CO_2 blending in the gassing mixture or by the addition of $NaHCO_3$. Agitation in the WBR is delivered by a rocking motion that can be adjusted by varying either or both the rocking angle and the rocking rate, whereas mixing in the STBR is delivered by a pitched blade impeller that operates at variable speed. The pictoral outline in Figure 1 presents the differences between the two systems. Because the design and operation of WBR and STBR are significantly different, we evaluated the recombinant protein production performance of three cell lines (GS-NS0, dhfr⁻ CHO, and *Drosophila* S2) in WBRs and compared it against a baseline performance achieved in conventional STBRs.

Cultivation of GS-NS0 Cells: The cell line used in this work is an antibody-secreting GS-NS0 cell line. Figure 2 presents growth, pH, glucose, and antibody evolution kinetics achieved in 20-L WBRs and 3-L STBRs. Panel A demonstrates comparable growth and viability in both vessels. Exponential growth was exhibited from inoculation time until day four, with an average growth rate of 0.022 hr⁻¹ in the WBRs and 0.020 hr⁻¹ in the STBRs. Average maximum cell concentrations of 1.0×10^6 vc/mL in the WBRs and 0.9×10^6 vc/mL in the STBR were reached after four days of cultivation.

Following exponential growth in the WBRs, a drop in pH from 7 to 6.5 was observed on day five (panel B), which was triggered by elevated CO_2 concentrations in the culture. The partial pressure of dissolved CO_2 was 30–70 mmHg during the first four days of culture but increased to 140–145 mmHg on day five. The CO_2 accumulation was addressed by gassing the culture with air, and the pH value returned close to neutral by the end of the cultivation period. The elevated CO_2 concentration and decreased pH level did not appear to negatively affect

Figure 1: Schematic representation of animal cell cultivation vessels; Panel A = classical stirred bioreactor; Panel B = typical Wave bioreactor
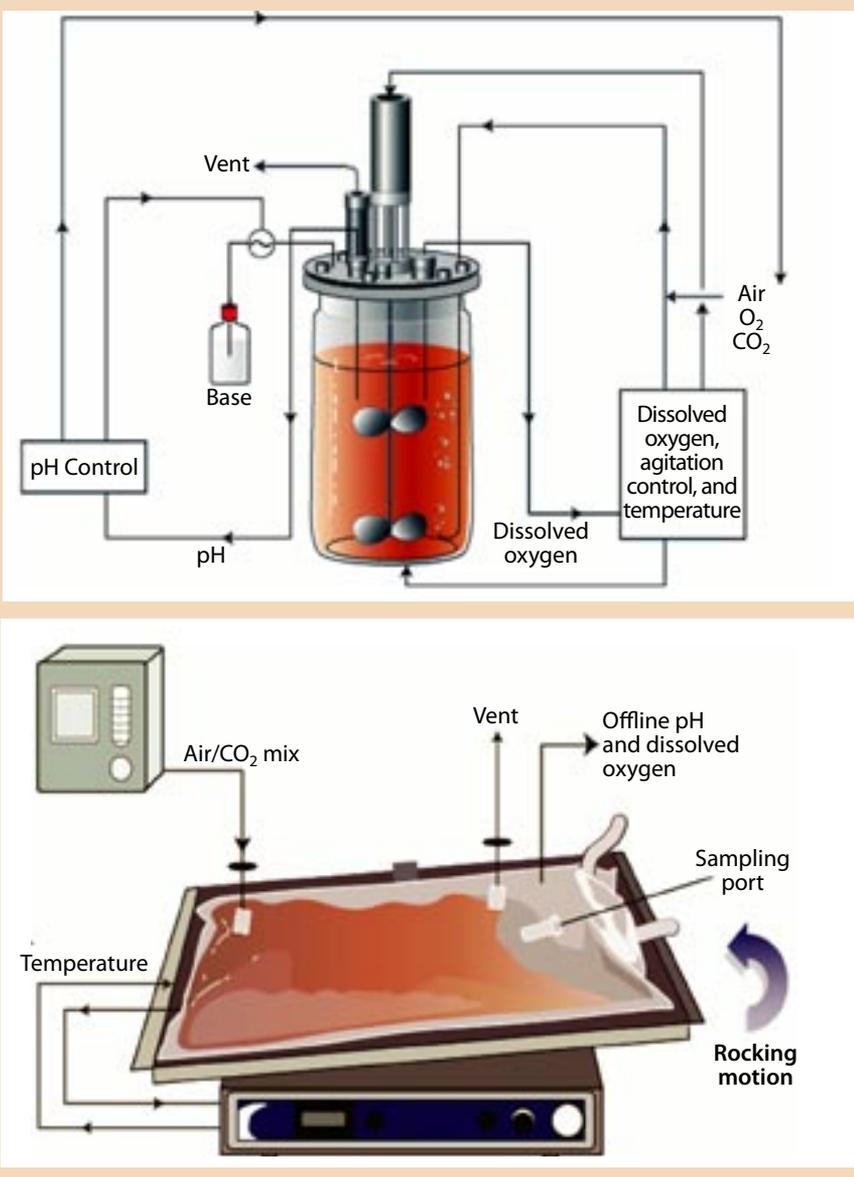
the culture viability compared with that of the STBRs (panel A).

Off-line analysis indicate that the dissolved oxygen levels were maintained between 30% and 80% saturation in the WBRs. The dissolved oxygen was controlled at 30 ± 10% saturation in the STBRs. Panel C shows that less than half of the glucose was consumed by the cells at average specific consumption rates of 0.11 pmol/cell-hr in the WBRs and 0.09 pmol/cell-hr in the STBRs. Panel D presents the antibody production profiles and shows the average maximum antibody concentrations of 83 mg/L and 64 mg/L in the WBRs and STBRs, respectively.

The data presented in Figure 2 and compiled in Table 2 were from three

independent WBR batches and four independent STBR batches. These data demonstrate the reproducible biomass ($1.0 \pm 0.2 \times 10^6$ vc/mL) and antibody production (83 ± 15 mg/L) were routinely achieved in 20-L WBRs. And although there were some disparities in the pH and dissolved oxygen levels in the WBRs compared with the STBRs, as a result of the mode of operation, those differences did not affect the growth kinetics or glucose metabolism and resulted in relatively similar final antibody titers in both types of reactors.

Cultivation of dhfr⁻ CHO Cells: Figure 3 presents growth, pH, glucose, and antibody production profiles of the dhfr⁻ CHO cell line secreting a

Figure 2: Kinetics of GS-NS0 cells cultivated in 20-L Wave bioreactors (black line) and 3-L stirred bioreactors (blue line); Panel A = cell growth and viability kinetics; Panel B = kinetics of pH evolution; Panel C = kinetics of glucose consumption; Panel D = kinetics of antibody production
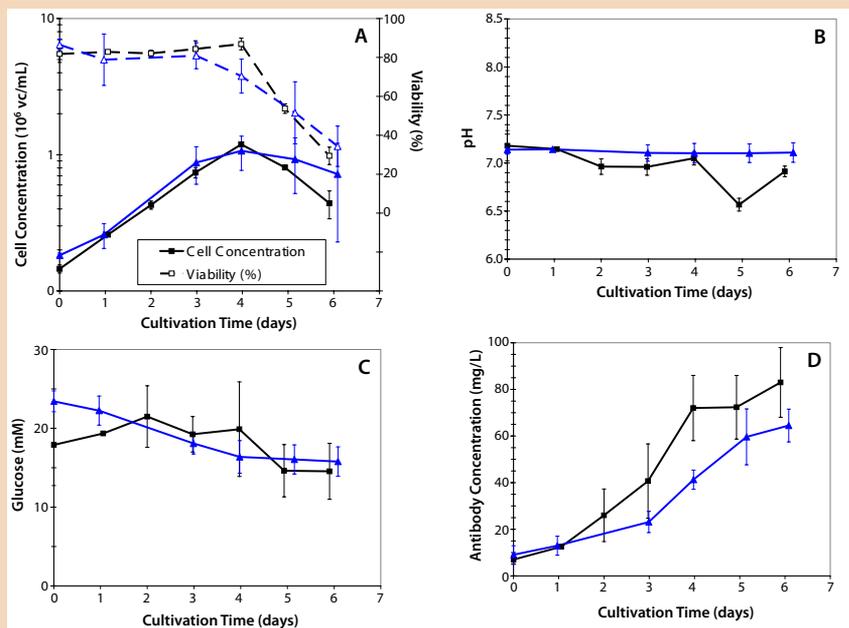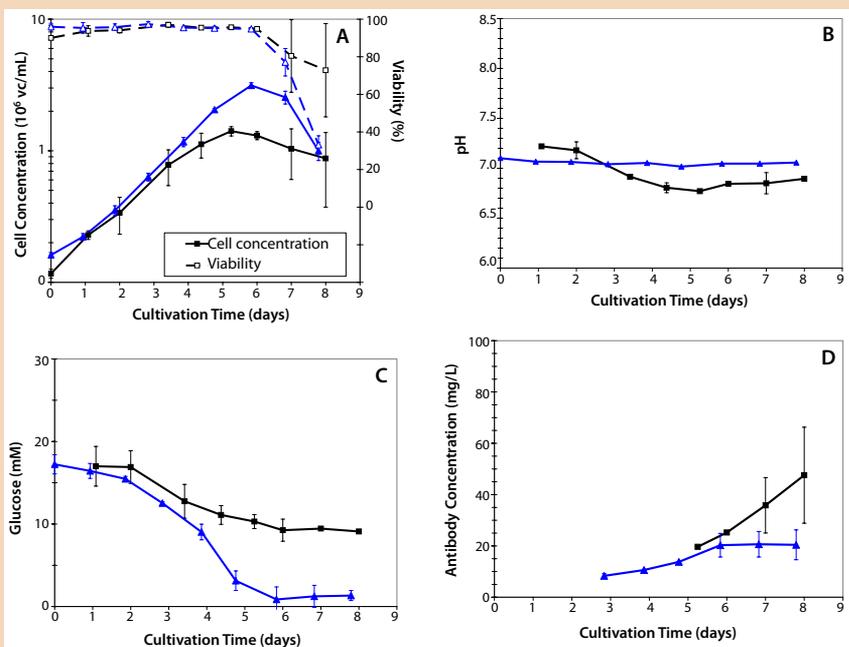

Figure 3: Kinetics of dhfr- CHO cells cultivated in 20-L Wave Bioreactors (black line) and 3-L stirred bioreactors (blue line); Panel A = cell growth and viability kinetics; Panel B = kinetics of pH evolution; Panel C= kinetics of glucose consumption; Panel D = kinetics of antibody production

exponential growth phase. Data presented in panel B show that as observed in the previous example, a fluctuating pH profile was observed in the WBRs. The partial pressure of dissolved CO_2 in the culture oscillated between 14 and 30 mmHg and is probably responsible for the fluctuation observed in the pH. Off-line analysis indicates that the dissolved oxygen was maintained between 80% and 100% saturation in the WBRs and 30 ± 10% saturation in the STBRs.

Panel C compares the glucose profiles and shows that although only half the available glucose was consumed in the WBR cultures, all the available glucose was consumed in the STBRs. The increase in glucose consumption was the result of the increased biomass achieved in the STBRs (panel A) and not the result of a significant increase in consumption rate, which was 0.12 pmol/cell-hr in the WBRs and 0.18 pmol/cell-hr in the STBRs. The average maximum antibody concentrations were 47 and 26 mg/L, with specific productivities of 9.3 and 2.0 pg/cell-day in the WBRs and STBRs, respectively (panel D).

What is important for this comparison study is that the data from the two independent experiments, presented in Table 2, show that very good reproducibility in antibody production (58 ± 5 mg/L) and biomass (1.4 ± 0.1 × 10^6 vc/mL) were routinely achieved in WBRs. That was despite the differences observed in the extent of biomass production, metabolic patterns, and antibody formation observed when these dhfr⁻ CHO cells were cultivated in WBRs rather than in STBRs.

Cultivation of *Drosophila* S2 Cells: Figure 4 presents the growth, pH, glucose, and recombinant protein evolution kinetics of a *drosophila* S2 cell line secreting a recombinant protein achieved in two independent 20-L WBRs and two independent 3-L STBRs. Panel A demonstrates comparable growth and viability in both vessels. Exponential growth was exhibited following a one-day lag until day four, with an average growth rate of 0.024 hr⁻¹ in the WBRs and 0.016 hr⁻¹ in the STBRs. Average maximum cell concentrations of 10.6 × 10^6

recombinant antibody achieved in two independent 20-L WBRs and three independent 3-L STBRs. Data presented in panel A demonstrate that exponential growth occurred from inoculation until day five, with an average growth rate of 0.021 hr⁻¹ and average maximum cell concentration of 1.4 × 10^6 vc/mL in the WBRs.

Those trends differ slightly from the growth trends observed in the STBRs, in which exponential growth occurred until day six, with an average growth rate of 0.023 hr⁻¹ and average maximum cell concentration of 3.1 × 10^6 vc/mL.

In both vessels, cell viabilities dropped rapidly following the

vc/mL in the WBRs and 10.2×10^6 vc/mL in the STBR were reached after four days of cultivation.

Because of the high cell mass in the culture, constant aeration was needed in the WBRs to maintain the dissolved oxygen between 60% and 85% saturation. This resulted in the stripping of dissolved CO_2, which ranged in partial pressure from 6 to 10 mmHg throughout the culture, probably accounting for the observed increase in pH (panel B). Dissolved oxygen was maintained at $30 \pm 10\%$ saturation in the STBRs.

Panel C shows that only about one third of the available glucose was used during cultivation, with consumption rates of 0.01 pmol/cell-hr in both types of bioreactors. Once the cells reached late exponential phase, the cells were induced, and a rapid decline in cell viability was observed (panel A). Following six days of culture, final secreted protein concentrations of 156 mg/L and 166 mg/L were achieved in the WBRs and STBRs, respectively.

As in the two previous examples, the data presented in Table 2 demonstrate that good batch-to batch reproducibility in biomass ($10.6 \pm 2.4 \times 10^6$ vc/mL) and recombinant protein production (156 ± 42 mg/L) can be achieved when using 20-L WBRs. As observed with the two previous cell lines, the data presented in Table 2 also show that although differences were observed in the behavior of the cells when cultivated in WBRs and STBRs, comparable recombinant protein production was achieved.

SUCCESSFUL CULTIVATIONS

The data presented in this work have shown that all three suspension cell lines evaluated here can be successfully cultivated in 20-L WBRs. These observations support the conclusion that WBRs are suitable for rapid and simple production of recombinant monoclonal antibodies and proteins. More important was the good reproducibility of batch-to-batch biomass formation and product secretion observed for each case (Table 2).

As expected from the wide differences in the geometry and the mode of operation of the two types of reactors, differences in growth, metabolism, and pH trends were

Figure 4: Kinetics of S2 cells cultivated in a 20-L Wave bioreactors (black line) and 3-L stirred bioreactors (blue line); Panel A = cell growth and viability kinetics; Panel B = kinetics of pH evolution; Panel C = kinetics of glucose consumption; Panel D = kinetics of recombinant protein production
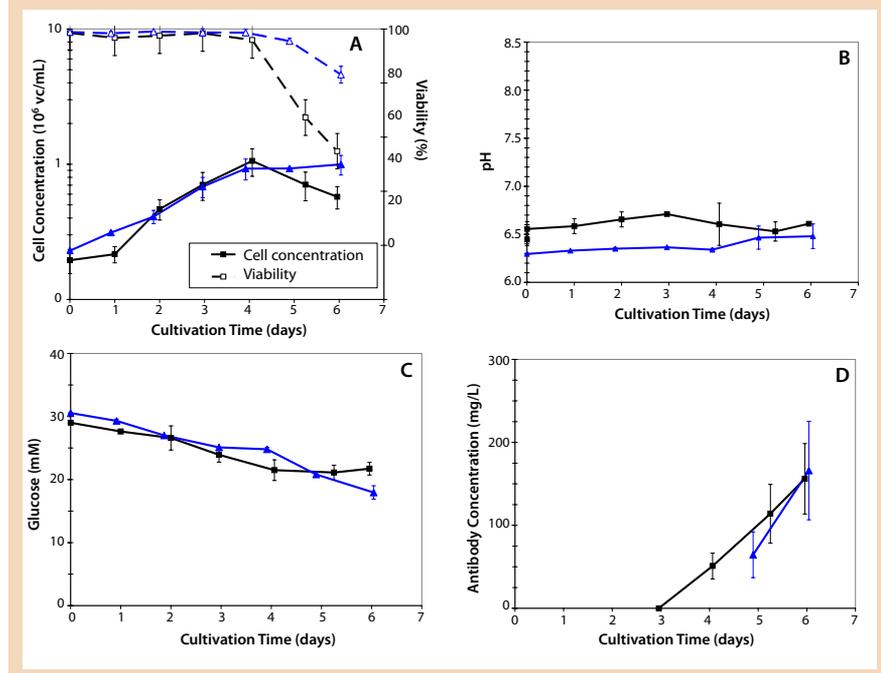
observed when each of the three cell lines evaluated were cultivated in both WBR and STBR. However, and despite these observed behavioral differences, volumetric product formation was found to be quite comparable between the two types of bioreactors for all cell lines evaluated (Table 2). In addition, the quality of the recombinant proteins produced, as measured by SDS PAGE analyses, was found to closely match expectations. Taken all together, these data support the use of disposable bioreactors such as WBRs for routine production of small to medium quantities of recombinant proteins produced by mammalian cells.

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