Disposable bioreactor for cell culture using wave-induced agitation

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Received 31 March 1998; accepted 22 November 1998

Key words: bioreactor, cell culture, disposable, wave agitation

Abstract

This work describes a novel bioreactor system for the cultivation of animal, insect, and plant cells using wave agitation induced by a rocking motion. This agitation system provides good nutrient distribution, off-bottom suspension, and excellent oxygen transfer without damaging fluid shear or gas bubbles. Unlike other cell culture systems, such as spinners, hollow-fiber bioreactors, and roller bottles, scale-up is simple, and has been demonstrated up to 100 L of culture volume. The bioreactor is disposable, and therefore requires no cleaning or sterilization. Additions and sampling are possible without the need for a laminar flow cabinet. The unit can be placed in an incubator requiring minimal instrumentation. These features dramatically lower the purchase cost, and operating expenses of this laboratory/pilot scale cell cultivation system. Results are presented for various model systems: 1) recombinant NS0 cells in suspension; 2) adenovirus production using human 293 cells in suspension; 3) Sf9 insect cell/baculovirus system; and 4) human 293 cells on microcarrier. These examples show the general suitability of the system for cells in suspension, anchorage-dependent culture, and virus production in research and GMP applications.

Introduction

Suitable cultivation technology for the culture of animal, plant and insect cells is dependent on the batch volume required. In the laboratory, devices such as spinner flasks, roller bottles, T-flasks, and similar systems are typically used. However, these devices can only produce 1 to 2 L of culture per batch due to their inherently limited oxygen transfer capabilities. For example, spinner flasks, though very popular for suspension culture, as well for anchorage-dependent systems on microcarriers, are only useful for volumes less than 1 L (Hu et al., 1986). For larger volumes, it is usually necessary to use stirred-tank bioreactors that are modified to reduce shear forces. These bioreactors are complex and expensive devices, yet they do not provide an ideal environment for cell growth due to high local fluid shear and the use of bubble aeration.

There is no simple, commercially available system for the production of 1 to 100 L of cell culture, which is a typical volume required for protein characterization, inoculum propagation, and pilot-scale production.

Adapting stirred tank technology to cell culture is a futile exercise because this design has intrinsically high local shear rates making scale-up very difficult. Instead, it is critical to recognize the special demands of cell culture and design a new device to directly satisfy these needs. These demands include shear sensitivity, use of bubble-free aeration and pre-sterilized media, and small oxygen uptake rates. For practical use it is also essential that any new cultivation system be simple to operate. Many otherwise excellent devices, such as fluidized bed bioreactors and hollow-fiber systems, have turned out to be too complex to displace the spinner flask as the workhorse of cell cultivation.

The objectives of this work were to develop a new cell culture bioreactor that:

1. *Is scalable, and can be used to cultivate up to 100 L of cell culture.*
2. *Reduces damage due to gas bubbles by eliminating sparging of air.*
3. *Reduces shear damage by eliminating mechanical mixers along with associated complex rotating seals and gears.*

4. *Is presterile, disposable, and does not require cleaning or sterilization.*
5. *Provides closed-system containment without the need for a laminar flow cabinet.*
6. *Does not require complex controls to operate.*
7. *Has lower cost due to the elimination of mixers, sterilization piping, complex instrumentation, and stainless steel construction.*
8. *Is easy to operate and maintain.*

A bioreactor system was developed that meets all these objectives. It is low cost, simple to operate, and provides an optimal environment for cell and virus culture. This paper summarizes the oxygen transfer capabilities, mixing characteristics, and cell cultivation potential of this technology based on wave-induced agitation.

Materials and Methods

Bioreactor system and operation

The bioreactor consists of a presterilized, flexible plastic chamber that is partially filled with media and inoculated with cells. The remainder of the chamber is inflated with air. The air is continuously passaged through the headspace during the cultivation. Mixing and mass transfer are achieved by rocking the chamber back and forth. This rocking motion generates waves at the liquid-air interface, greatly enhancing oxygen transfer. The wave motion also promotes bulk mixing, and off-bottom suspension of cells and particles. This concept of using rocking for agitation is used extensively for the agitation of liquids in laboratory assay plates and gels. The use of a similar idea for small-scale cell culture has been reported (Kybal and Sikyta, 1985).

In this disposable bioreactor, the cultivation chamber is discarded after harvest, eliminating any need for cleaning or sterilization since this is the only component in contact with the cells. The chambers are made of FDA approved biocompatible polyethylene. These materials are commonly used for blood collection and biological fluid handling and are sterilized by gamma radiation. Special ports were developed to allow sterile additions, and to withdraw samples, without the need to place the bioreactor inside a laminar flow cabinet. The rocking device developed by the author is shown in Figure 1 and consists of a platform that rotates in one axis through an angle that can be adjusted between 5 to 10°. Pneumatic bellows are used to rock

the platform at a rocking rate that can be adjusted from 5 to 40 rocks/minute (rpm). The pneumatic system ensures that unit does not generate any heat during operation, enabling it to be placed inside an incubator. A pre-sterilized disposable cell culture chamber is placed on the platform; partially filled with media and then inflated using the integral sterile inlet filter. Air is continuously passaged through the headspace of the chamber. This airflow provides oxygenation and gas exchange for pH control and CO₂ removal. Exhaust air passes through a sterilizing filter and a backpressure control valve. The backpressure control valve ensures that the chamber is always fully inflated at any airflow. The valve also prevents overinflation and potential bursting of the chamber. Temperature and pH control can be achieved by placing the entire unit inside a conventional cell culture CO₂ incubator. Alternatively, temperature control can be achieved by heating the underside of the culture chamber. The wave motion promotes excellent bulk liquid movement and minimizes any temperature gradients.

The rocking platform systems: WaveBioreactor™, and specially designed cell culture chambers (Cellbag™) can be obtained from Panacea Solutions, Inc, Bedminster, New Jersey, U.S.A. (info@panasol.com).

Oxygen transfer measurement

The oxygen transfer capabilities of the system were determined by measurement of the volumetric oxygen transfer coefficient k_La at various rocking speeds, liquid volumes, and aeration rates. The classic dynamic method was used for k_La measurement. Here, the liquid in the bioreactor chamber was deoxygenated by passing nitrogen through the headspace. Once the dissolved oxygen (DO) concentration was near zero, the headspace was quickly evacuated and then air was introduced into the headspace. The resulting rise in DO was recorded and the k_La was calculated from the slope of the following mass balance equation:

$$\ln \frac{(C^* - C_1)}{(C^* - C_2)} = k_La(t_2 - t_1) \quad (1)$$

where

- C^* = the saturation DO;
- C_1 = the DO at time t_1 ;
- C_2 = the DO at time t_2 .

For the oxygen transfer studies, an oxygen sensitive dye (porphine coupled to albumin) was added to water in the bag. This enabled non-invasive dissolved

Figure 1. Bioreactor with wave-induced agitation.

oxygen measurements using special optical equipment to determine the phosphorescence quenching lifetime (Oxyspot[®], Medical Systems Corporation, Greenvale, NY, U.S.A.). This technique provided the fast response necessary for the dynamic method. Absolute dissolved oxygen concentrations were confirmed using conventional polarographic DO probes inserted into the chamber.

Mixing time measurement

Mixing times in the bioreactors at various rocking rates were determined by injecting a fluorescent tracer dye and videotaping the dispersion of the dye. UV light was used to enhance the contrast. Time-tagged images were captured from the videotape and the mixing time was determined visually from the images. Mixing time was defined, as the time required after injection to achieve complete homogeneity.

Dissolved oxygen and carbon dioxide

Off-line measurement of dissolved oxygen and dissolved CO₂ were done by sampling the chamber using a syringe and analyzing it using a blood gas analyzer (Radiometer ABL5, Radiometer, Copenhagen, Denmark). Cell counts were done by hemocytometer,

and product assays were done by appropriate methods such as ELISA.

Cell culture procedures

Many different cell lines were tested. Since different procedures were used for each system they are, for clarity, summarized in the respective results section.

Results

Oxygen transfer

The wave motion of liquid in the bioreactor chamber generates a larger mass transfer surface than static culture resulting in a much greater volumetric transfer coefficient (k_La). Figures 2 and 3 show the k_La measured at various rocking and aeration rates in 2 and 20 L systems.

Oxygen transfer data was obtained in spinner flasks for comparison using the same noninvasive dissolved oxygen measurement technique described earlier. Table 1 shows the k_La for various size spinners. This data is in good agreement with published values. Aunins et al. (1989) report a k_La of around 1 hr⁻¹ for 500 mL liquid in a 1000 mL spinner. Dorrestijn et al. (1994) reported a value around 2 hr⁻¹ for 300 mL

Figure 2. Oxygen transfer in 2 L Wave Bioreactor.

Figure 3. Oxygen transfer in 20 L Wave Bioreactor.

liquid in a 600 mL spinner system. Comparing the oxygen transfer into 1000 mL of liquid shows that a 2 L *Wave Bioreactor* can provide a k_La of 3 hr^{-1} (Table 1) which is three-fold higher than the k_La for 1000 mL of liquid in a comparable 3 L spinner. Therefore, if oxygen transfer were limiting, then this could potentially result in three-fold higher maximal cell density in the *Wave Bioreactor* compared to a spinner.

With 10 L of liquid in a 20 L *Wave Bioreactor*, the maximum k_La measured was around 4 hr^{-1} . There is no comparable spinner system since the surface to volume ratio decreases substantially as the spinner volume is increased. For example, a 10 L spinner would have a calculated k_La less than 0.4 hr^{-1} , making it essentially useless for cell cultivation.

With 100 L of liquid volume in a 200 L *Wave Bioreactor*, the k_La remained at 4 hr^{-1} indicating a minimal reduction in oxygen transfer capacity on scale up to this volume.

With a k_La of 3 to 4 hr^{-1} , and assuming a typical oxygen demand of $0.1 \text{ mMO}_2/10^6 \text{ cell-hr}$ (Singh,

1996), it is possible to grow up to $7 \times 10^6 \text{ cells mL}^{-1}$ while maintaining dissolved oxygen concentrations above 10% saturation (Fleischaker and Sinsky, 1981).

With this knowledge of oxygen transfer it is possible to guarantee against oxygen depletion at any desired cell density by selecting the appropriate rocking/aeration rate. Unlike stirred tank bioreactors, there is no bubble damage or danger of 'overaeration'. Adjusting the CO_2 concentration of the inlet air can control CO_2 stripping. This effectively eliminates the need for a dissolved oxygen control system. Samples may be taken and assayed periodically on a blood gas analyzer, off-line, to verify the DO and pCO_2 levels.

Mixing studies

Mixing times were estimated by injecting a fluorescent dye and videotaping the dispersion of the dye under various conditions. These experiments showed that the wave-induced motion was very effective in mixing the liquid in the bag. Mixing time (defined as time for complete homogeneity) ranged from 5 to 10 sec for 10 L (in 20 L bag) to 60 sec for 100 L (in 200 L bag). Subsequent studies with microcarrier cultivation showed good off-bottom suspension with some particle gradients observed in the liquid. However, no significant settling of microcarriers or cells was observed. This was verified by taking samples at various rocking rates. Particle counts were performed on these samples. There was no change in particle count in samples taken at rock rates above 6 rpm indicating that no significant settling took place at these agitation rates. Much lower particle counts were obtained at rock rates below 6 rpm.

Biological systems

Monoclonal antibody production – 1, 10 and 100 L scale

An important application for cell culture is the production of monoclonal antibodies *in vitro*. Spinner or shake flasks can be used for 1 to 3 L of culture, however oxygen transfer limitations in these systems preclude further scale-up. For larger volumes, it is necessary to use complex bioreactors based on stirred tanks, hollow-fiber, or immobilized technology.

The suitability of the *Wave Bioreactor* system for lab-scale (10 L) culture was evaluated. The cells used were a NS0 cell line expressing a humanized monoclonal antibody. Culture was performed in serum-free media commonly used for this cell line. Cultivation

Table 1. k_{La} in Wave Bioreactor versus Spinner Flasks

Liquid (ml)	k_{La} in Wave Bioreactor	k_{La} in spinner
100	1.61 hr ⁻¹ (in 2 L bag)	1.94 hr ⁻¹
500	3.5 hr ⁻¹ (in 2 L bag)	1.15 hr ⁻¹
1000	3 hr ⁻¹ (in 2 L bag)	0.9 hr ⁻¹
10000	4 hr ⁻¹ (in 20 L bag)	0.4 hr ⁻¹ (estimated)
100000	4 hr ⁻¹ (in 200 L bag)	–

was started in a 1 L culture volume (in 20 L bag) with 250 mL of inoculum generated in a 500 mL spinner flask. After 96 hr the cell density was 2.3×10^6 viable cells mL⁻¹. Two Ls of fresh media were added to the bag and the cultivation continued. After an additional 96 hr the cell density again reached 2.1×10^6 cells mL⁻¹ and 7 L of fresh media were added to the bag to bring it to the final culture volume of 10 L, and cultivation was continued until zero viability (another 300 hr). Figure 4 shows the cell counts and monoclonal antibody production. The stepwise dilution technique used demonstrates how the large turndown ratio (maximal volume/minimum volume) of the bag enables large culture volumes without the need for transfers as would be the case using spinner flasks.

Culture profile was very consistent with smaller scale spinners. Cell densities exceeded 5×10^6 cells mL⁻¹. Dissolved oxygen levels remained above 50% saturation. Antibody expression was normal at over 600 mg L⁻¹. The system is clearly well suited for suspension cell cultivation and monoclonal production.

A NS0 cell line producing a different antibody was evaluated at pilot scale in 100 L of culture in a 200 L *Wave Bioreactor*. Figure 5 shows the cell growth and antibody production profile compared to a conventional 160 L stirred tank bioreactor run with the same cell inoculum. Despite different seeding densities, the growth patterns were very similar and the final antibody titer was almost identical at around 170 mg L⁻¹. The maximal viable cell density achieved was 3×10^6 cells mL⁻¹. Dissolved oxygen levels remained above 50% saturation indicating adequate oxygen transfer. This experiment clearly demonstrates comparable performance to stirred tank bioreactors at a 100 L culture volume.

293 cells/adenovirus production

The potential of the *Wave Bioreactor* for virus production was tested using a recombinant adenovirus system. A human embryonic kidney cell line (293) grown

in suspension was used as the host. Initial seeding density in a 2 L *Wave Bioreactor* containing 1000 mL of serum-free media was 0.3×10^6 viable cells mL⁻¹. The unit was placed in a 37 °C incubator with a 5% CO₂ atmosphere. The culture was grown for 5 days at a rocking rate of 10 rpm and 0.1 vvm aeration. At this stage, the viable cell count was 2.7×10^6 cells mL⁻¹. 500 mL of the culture was removed and replaced with 500 mL of fresh media. After an additional 24 hr, the culture was infected with a multiplicity-of-infection (MOI) of 30 virus particles/cell. The culture was harvested four days post-infection at which point the viable count was essentially zero (Figure 6).

The final virus titer was 10000 particles/cell, which was very similar to the titer obtained in conventional cultivation systems.

From Figure 6, it is apparent that the system is quite capable of maintaining adequate dissolved oxygen levels at these cell densities. The pH remains very constant due to the excellent gas exchange as evidenced by the constant pCO₂ until late in the infection when the pH drops rapidly beyond the capability of the buffer system as lactate builds up to high concentrations.

The system is very easy to use. All additions and sampling were done in the incubator itself. No laminar flow cabinet was required. The standard 0.1 micron exhaust filter was supplemented by a Pall DFA cartridge filter. The integrity of the exhaust filter system was tested by passing the exhaust gases into an uninfected 293 culture. Absence of any infection in the second culture confirmed the effectiveness of the exhaust filtration in containing the adenovirus.

Baculovirus production – 10 L scale

The Sf9/Baculovirus system is an excellent method for the rapid expression of large amounts of recombinant proteins. However, the oxygen requirements of Sf9 cells are higher than mammalian cells and this restricts the volume of spinner or flask culture to around 1 L.

Figure 4. Growth and antibody production in a 10 L Wave Bioreactor.

Figure 5. Comparison of 100 L Wave Bioreactor and 160 L stirred tank with respect to cell growth and antibody production.

Typically, larger volumes are required to produce sufficient protein for isolation. This requires the use of more complex bioreactors.

Experiments were performed to demonstrate that the simple *Wave Bioreactor* system could be used to

scale-up Sf9 cells, and produce baculovirus in 10 L culture volume. The gene sequence for a recombinant protein was cloned into the pAcHLT-B baculovirus expression vector; transfected into Sf9 cells, and used to generate a high titer virus stock. For this experiment

Figure 6. Adenovirus production in the Wave Bioreactor

Figure 7. Insect cell cultivation in the Wave Bioreactor.

Typical sequence of spinners required to make 1 liter of culture

Figure 8. The Wave Bioreactor simplifies inoculum propagation.

1 L of Bio-Whittaker X-Press Insect Cell Media was added to a 20 L *Wave Bioreactor*. This was inoculated with 0.5×10^6 Sf9 cells mL^{-1} . Rocking rate was set at 20 rpm in a humidified incubator maintained at 27 °C. After 5 days of cultivation the cell count reached 4.75×10^6 cells mL^{-1} . At this point 4 L of fresh media were added to the bag. After an additional 3 days, the cell count reached 3.0×10^6 cells mL^{-1} in this increased volume. Then another 4 L of fresh media were added along with the virus (2 mL) at a MOI of 0.5 bringing the system to a culture volume of 9 L. Harvest was done 2 days post infection.

This system produced a titer comparable to 100 mL shake flasks. It demonstrated the ability of the *Wave Bioreactor* to increase cell mass by simply adding fresh media to the system. No conventional series of flasks or splits were required, reducing labor and potential for contamination. All additions and transfers into the bag were done in the incubator. Figure 7 shows that oxygen transfer and CO_2 desorption were not limiting.

Microcarrier culture

The experiments reported so far were all performed with cells in suspension. In order to demonstrate that the *Wave Bioreactor* could also be used for anchorage-dependent cells, an experiment was performed with cells growing on microcarriers.

Figure 9. Containment features of the Wave Bioreactor

Human 293 cells were cultivated on Cytodex 3 microcarriers. Growth media was DMEM with 10% bovine calf serum. Inoculum was collected by trypsinizing microcarrier cultures grown in spinner flasks. Sixty ml of inoculum was added to 3 g Cytodex 3 in 900 mL of media and then transferred to a 2 L *Wave Bioreactor*. Ten mL of the inoculum was added to a spinner flask with 0.45 g Cytodex 3 and 140 mL media (250 mL Bellco) that was run in parallel at 40 rpm.

Growth in the *Wave Bioreactor* was very similar to the spinner run in parallel. Cells grew to confluency (>50 cells/bead) in both systems. A total cell count of 3×10^6 cells mL^{-1} was estimated in either system. Some aggregation of microcarriers was observed in

the *Wave Bioreactor*. Glucose consumption and lactate production rates were equivalent with the exception of slightly higher cell counts in the supernatant from the *Wave Bioreactor*.

Discussion

The disposable *Wave Bioreactor* has many potential applications:

Replace spinner flasks, roller bottles and conventional bioreactors

Spinner flasks cannot be used for liquid volumes greater than 1 L because of insufficient oxygen transfer. The *Wave Bioreactor* generates much more mass transfer surface because of the unique wave action. Operation to 100 L is possible with cell densities routinely over 5×10^6 cells mL⁻¹. This high oxygen transfer is accomplished without damaging bubbles or high shear forces.

The *Wave Bioreactor* has a greater range of operating volume. This reduces the number of transfers that need to be made, minimizing handling, and potential for contamination as shown in Figure 8.

The *Wave Bioreactor* is an ideal way to make a few liters of cell culture for lab purification, inoculum propagation and cell bank generation.

Low cost bioreactor

Traditionally the only way to produce 5 to 100 L of cell or virus culture was to use expensive and complex bioreactors. The *Wave Bioreactor* is 1/10 the price of a comparable bioreactor and does not require substantial training. No cleaning or sterilization is necessary which saves time and reduces risk of contamination. No utilities such as steam, cooling water or process gases are required. No complex instrumentation is necessary. The unit can be set up in a conventional cell culture incubator. A CO₂ controlled incubator can be used if pH control is desired. This makes it possible, even in small labs, hospitals, and universities, to perform lab/pilot scale cell and virus cultivation.

High containment/GMP systems

The bioreactor is completely closed. The cultivation chamber is certified sterile by gamma radiation and meets USP Class VI specifications. Additions and samples can be taken using a hospital-type needleless

syringe connector or tube-fuser. These operations do not require a laminar flow cabinet and can be done with the unit inside the incubator or on the bench. Vent gases from the bioreactor can be sterilized by filters appropriate to the required containment level. These features are illustrated in Figure 9. At the end of cultivation, the bioreactor chamber can be removed to another area for processing. The disposable nature of the system makes it very suitable for virus production and BL2/BL3 operations. There is also no danger of improper cleaning or product cross-contamination.

Cell therapy and gene screening

The *Wave Bioreactor* allows even the smallest lab or clinic to perform cell culture. The simplicity of the system enables the scientist to concentrate on the use of the cells rather than learning how to operate a complex bioreactor system. The large cultivation volumes and the ability to grow attachment-dependent lines make it useful for primary cell cultivation for cell therapy applications and rapid expression/screening of hundreds of genes needed for gene therapy and human genome elucidation.

Conclusions

A novel bioreactor for cell culture has been developed. The system utilizes an inflated disposable plastic bag as the cell cultivation chamber. These specially designed chambers are made of biocompatible materials and are delivered sterile, eliminating the need for cleaning and sterilization. Oxygen transfer and mixing are accomplished by wave-induced agitation caused by rocking the chamber back and forth. The rocking mechanism has been optimized in terms of rocking angle, rocking rate, aeration, and mechanical design to provide a k_La for oxygen transfer of 2 to 4 hr⁻¹. Based on typical cell respiration rates, this transfer rate is sufficient to grow up to 7×10^6 cells mL⁻¹. The bioreactor system, unlike spinner flasks, is not limited by gas-liquid transfer surface, and scale-up to 100 L operating volume has been demonstrated.

This simple, low cost, *Wave Bioreactor* system can be used for animal, insect, and plant cell culture. The closed design is very suitable for virus production or other applications requiring high containment. The system requires minimal instrumentation and can be operated inside a laboratory incubator. All handling can be done in the open, eliminating the need for a laminar flow cabinet.

Acknowledgments

I would like to thank Mr. John McDowall, Ms. Nancy Connelly, Ms. Colleen Brolly, Mr. Jeff Hutter, Mr. Benjamin Gill, and Ms. Daniella Kranjac for their assistance in conducting the experiments.

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