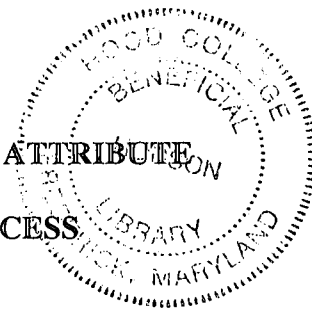


STUDIES OF PROTEIN OXIDATION AS A PRODUCT QUALITY ATTRIBUTE  
IN A SCALE-DOWN MODEL FOR CELL CULTURE PROCESS  
DEVELOPMENT



by

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B.A. (College of Notre Dame of Maryland) 1997

THESIS

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE


in the


GRADUATE SCHOOL

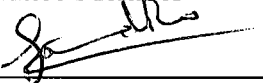
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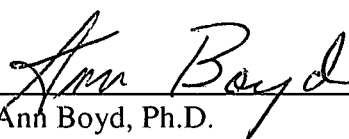
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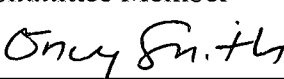
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## ABSTRACT

High throughput mini-bioreactors (HTBRs), 50-mL bioreactors employing non-invasive optical sensor technology were previously validated and compared to bench-scale bioreactors on the basis of dissolved oxygen (DO), pH and viable cell density (VCD) profiles (Xudong *et al.* 2006). To establish HTBRs as a valid scale-down model for cell culture process development and optimization, the ability to preserve product quality attributes across scales is essential. Experiments to evaluate and compare the effect of varying DO levels on product quality in HTBRs, traditional lab-scale systems and bench-scale bioreactors were conducted.

Relative protein carbonyl concentration was measured as an indicator of oxidative damage. The effect of which is experimentally significant for the conditions tested. Although a trend of increasing protein carbonyl content in response to increasing DO was conserved across scales, observed differences in measured levels of protein carbonyl content between scales was evident.

## DEDICATION

This work is dedicated to my family. Thank you for your support, patience and understanding.

## ACKNOWLEDGEMENTS

Research reported in this thesis was sponsored by the University of Maryland Baltimore County (UMBC) under the direction of Doctor Govind Rao in the Department of Chemical, Biochemical and Environmental Engineering and submitted to The Graduate School of Hood College in Department of Biomedical Science.

The guidance and assistance of Ph.D. candidates Shaunak Uplekar, Jose Vallejos, and Bhargavi Kondragunta in the Department of Chemical, Biochemical and Environmental Engineering, UMBC is also acknowledged.

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## LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
DO	dissolved oxygen
DNP	dinitrophenyl
DNPH	dinitrophenylhydrazine
ELISA	enzyme linked immunosorbent assay
HPLC	high-performance liquid chromatography
HTBR	high throughput bioreactor
HTCB	high throughput controlled bioreactor
HRP	horseradish peroxidase
LED	light-emitting diode
nmol	nanomole
OTR	oxygen transfer rate
PBS	phosphate buffered saline
QBD	quality by design
ROS	reactive oxygen species
rpm	revolutions per minute
T-flasks	tissue culture flasks
VCD	viable cell density
V/V	volume per volume

## INTRODUCTION

Production of high titer cell lines and high quality antibody products has become one of the primary objectives for many process development scientists and engineers. To achieve these objectives with reduced development timelines, the necessity to utilize high throughput methods is becoming more evident. Meeting the increasing demand of the biopharmaceutical industry for antibody based therapeutics; development of a high throughput approach to evaluate and develop robust processes should be established. HTBRs (high throughput mini-bioreactors) may provide a high throughput method to develop and optimize cell culture processes, as well as understand how variation in process conditions can impact product quality.

Development of optimized cell culture processes have heavy resource requirements and often employ simplified systems such as micro-well plates and shake flasks which offers minimal to no control over critical process parameters. HTBRs offer control and monitoring of process conditions critical for cell culture process development and optimization. Real-time monitoring and control of pH and dissolved oxygen in the HTBR system is achieved through the use of optical sensors. Real-time monitoring of pH and DO (dissolved oxygen) allows for study of cells and condition of the bioreactor without removing samples or risk of damage to the cells as well as provide information on cellular growth and metabolism (Naciri *et al.* 2008). Optical sensors developed for use with the HTBR system are low-cost and function on properties of differential quenching of the fluorescence lifetime of a fluorophore in response to the partial pressure of oxygen (Eichhorn *et al.* 1996). In addition to low-cost, the sensors are autoclavable and maintenance-free which in comparison to standard polarographic electrodes does not

have issues with long term stability, is not flow dependent (consumes oxygen), and has a rapid response time (Eichhorn *et al.* 1996). This low-cost HTBR system along with the use of non-invasive sensor technology for real-time monitoring of process conditions on a small-scale is a valuable tool for bioprocess optimization studies, and can provide early insight into understanding the impact of variations in process conditions on product quality.

There has been an increasing interest in evaluating how process conditions influence post-translational modifications (Goochee *et al.* 1990). Of importance to the biological properties of products produced in cell culture systems are the complex post-translational modifications that can be impacted by variations in process conditions (Goochee *et al.* 1990; Sharfstein 2008). Protein oxidation, oxidative modification to intracellular proteins which can involve cleavage of the polypeptide chain and modifications of the amino acid side chains can be impacted by such process variations (Stadman 2006). Studies have suggested that DO levels, the amount of oxygen that is dissolved or carried in a liquid medium, in cell culture systems, can induce oxidative damage (carbonylation) to proteins (Konz *et al.* 1998; Quiang *et al.* 2008). DO is a parameter of increasing interest, and studies have shown that despite the necessity of oxygen for respiration, there may also be some detrimental effects of oxygen to the cell (Dhir *et al.* 2000). Production and accumulation of reactive oxygen species (ROS) can cause damage as a result of oxidation of the cell and cellular components (Dhir *et al.* 2000). Oxidation of proteins can result in loss of activity and structural modifications. Defense mechanisms employed by cells under normal conditions which include expression of low molecular weight compounds and enzymes that have antioxidant

properties cannot be sustained when oxidative stress levels are too high (Dhir *et al.* 2000). There is also evidence to suggest that the response of cells to DO levels can vary depending on the cell line and the antioxidant defenses employed (Quiang *et al.* 2008).

Since variation in process parameters can impact post-translational modifications of cell culture products, it is imperative to monitor product quality during cell culture process development (Li *et al.* 2005). Variation, or changes to cell culture products can impact function, clearance rate, immunogenicity, and specific activity which translates into clinical implications (Goochee *et al.* 1990; Konz *et al.* 1998; Quiang *et al.* 2008; Yang *et al.* 2007). Although cell culture process changes are common in the development lifecycle, to ensure that the impact of process changes are minimal, it is common practice to compare product quality before and after process changes are made (Brorson *et al.* 2002).

The impact of process parameters on final product quality has been studied. The impact however is not predictable for a given cell line without performing time consuming empirical experiments. The concept of Quality by Design (QbD) is transforming the approach to process development and is driving the need to have a comprehensive understanding of the impact of variability in process parameters on process robustness and product quality. Having access to a validated high throughput scale-down model in which these experiments can be performed will provide a higher degree of confidence on how process conditions impact final product quality. Identification of critical process parameters in the early stages of development will minimize impact of process change efforts and shorten development timelines (Meuwly *et al.* 2006).

This study evaluates HTBRs as a scale-down model for cell culture process development and optimization through comparison of relative IgG3 protein carbonyl levels produced in shake flasks, tissue culture flasks (T-flasks), bench-scale bioreactors (5 L) and HTBRs. The suitability of HTBRs as a scale-down model for cell culture process development will be further assessed by measuring oxidation levels as a function of percent DO. In addition to evaluating the sensitivity of protein oxidation to changes in percent DO, comparison of HTBRs to traditional lab-scale systems and bench-scale bioreactors on the basis of performance and product quality will assess the utility of HTBRs as a tool for cell culture process development and optimization.

Traditionally, small-scale cell culture development is carried out in shake flasks or T-flasks in which critical factors such as pH and temperature are monitored and DO is not maintained under tight control. This method of development will be used as a baseline study to evaluate impact to product quality without DO control. Additionally, the HTBR (50 mL) and 5-L bench-scale system will be operated at varying DO levels and compared to determine if trends in measured relative protein carbonyl concentration predicted by HTBR will be maintained across scales from 50 mL to 5 L (100-fold scale increase). Similar trends of carbonyl oxidation in response to variations in DO will confirm that the HTBR is able to maintain a homogeneous environment similar to the impeller-agitated 5-L bioreactor system, and hence is an acceptable small-scale model system.

## Protein Carbonyls

Commonly used biomarkers for protein oxidation are protein carbonyls which are usually introduced to proline, arginine, lysine and threonine by oxidation (Quiang *et al.* 2008). Reactive oxygen species (ROS) resulting from oxygenation during cell culture modifies amino acid side chains resulting in the generation of free carbonyls (Quiang *et al.* 2008). The use of protein carbonyls as an indicator for oxidative damage has advantages over other biomarkers of oxidation due to early formation and relative stability of carbonylated proteins (Quiang *et al.* 2008). Carbonylation is irreversible and un-repairable (Dasari *et al.* 2008).

Various methods to detect protein carbonyls have been developed. These methods include liquid chromatography tandem mass spectrophotometric analysis, 2D electrophoresis coupled with immunodetection and mass spectrometry, and various available assay kits for spectrophotometric analysis, and immunodetection (Cell Biolabs, San Diego, CA; Magi *et al.* 2004; Shacter 2000; Soreghan *et al.* 2003). Some electrophoretic methods to detect protein carbonyls are highly specific for purified protein samples; however the assay becomes complicated for cell culture supernatants due to high background and poor sensitivity. Detection of protein carbonyls by immunoblotting after (1D or 2D) gel electrophoresis has more sensitivity and specificity than other methods of measuring protein carbonyls, however this method is only semi quantitative (Dalle-Donne *et al.* 2003). Spectrophotometric detection is quantitative and can be performed on a mixture of proteins, but this method has a lack of specificity and is therefore unreliable in samples that contain other contaminants that absorb at 370 nm or additionally those that react with DNPH (dinitrophenylhydrazine) (Dalle-Donne *et al.*

2003). Spectrophotometric detection however, has proven to be a sensitive and reliable means to determine protein carbonyl content in purified protein samples. The enzyme-linked immunosorbent assay (ELISA) method developed by Buss *et al.* (2007) is highly sensitive and reproducible. Standard curve generation used for this method allows for greater accuracy and sensitivity at lower ranges as compared to the spectrophotometric method. Additional benefits of the ELISA method is its ease of use and requirement for minimal protein sample (Dalle-Donne *et al.* 2003). Variability of sample analysis in this method results from the possibility that some proteins adhere better than others to the ELISA plate and is therefore only useful for comparing samples (Dalle-Donne *et al.* 2003).

Various types of protein oxidative modifications can occur thus various methods to analyze these samples exist. The most common oxidative modification involves the formation of carbonyl derivatives (Cell Biolabs, San Diego, CA). Many assays involve derivatization of the carbonyl group with dinitrophenylhydrazine (DNPH) and immunoblotting with an anti DNPH antibody (Cell Biolabs, San Diego CA). This method is highly sensitive and has become one of the most widely used methods for detection of protein carbonyls. For this study, Cell Biolabs Inc. OxiSelect™ Protein Carbonyl ELISA Kit was employed for analysis of purified protein samples.

## MATERIALS AND METHODS

### Baseline Study

A baseline study using an IgG3 producing SP2/0 mouse hybridoma cell line was conducted to compare trends in oxidation levels of products produced in HTBRs to those produced in shake flasks and T-flasks.

### *T-Flask Set-up*

T75-Flasks (Corning) containing CD Hybridoma medium (Invitrogen) (adjusted to an initial pH of 7.2 ( $\pm 0.1$ ) supplemented with 3 mM L-Glutamine (Hyclone Laboratories) and  $1.4 \times 10^{-4}$  % beta-mercaptoethanol (v/v), and D-Glucose concentration of 3.6 g/L) were seeded to approximately 0.2 million cells/mL at 20 mL working volume. T-flasks were incubated in a CO<sub>2</sub> incubator set to 5% CO<sub>2</sub> at 37°C. pH and DO profiles of the T-flasks were not monitored.

### *HTBR set-up*

Calibrated optical DO and pH patches were placed diagonally in the bottom of the HTBR (Fluorometrix, Stow, MA) for autoclaving. Prior to autoclaving, pH and DO patches were aligned with the pH and DO sensors located in the bottom of the HTBR console. Markings were placed on the bioreactors to aid with realignment of patches and sensors after autoclaving. Assembled bioreactors containing DO and pH patches were filled to a 35-mL working volume with deionized water (DI) and autoclaved at 121°C for 15 minutes.

After autoclaving, HTBRs were allowed to cool to room temperature before removing the DI water and replacing it with 35 mL of CD Hybridoma medium adjusted to an initial pH of 7.2 ( $\pm 0.1$ ) (Invitrogen) supplemented with 3 mM L-Glutamine



(Hyclone Laboratories) and  $1.4 \times 10^{-4}$  % beta-mercaptoethanol (v/v), and D-Glucose concentration of 3.6 g/L. HTBRs were seeded to approximately 0.2 million cells/mL using a working volume of 35 mL. Temperature, pH, DO set points were 37°C, 7.2 and 30% DO respectively (Fig. 1).

The HTBR system offers closed feedback control of pH, temperature and DO. Generally control of pH is achieved by base addition to increase pH and CO<sub>2</sub> to decrease pH when pH trends higher than the set-point. DO control is achieved with a combination of nitrogen supplementation to decrease DO in the bioreactor, while oxygen supplementation and agitation are used to increase DO. For this study, pH and DO control was one-sided and was not configured to compensate for pH readings below pH set-point and DO levels above the DO set-point. This approach was taken to minimize dilution of the culture with base addition and to provide an oxygen rich environment at the initiation of the culture to minimize time in the lag phase.

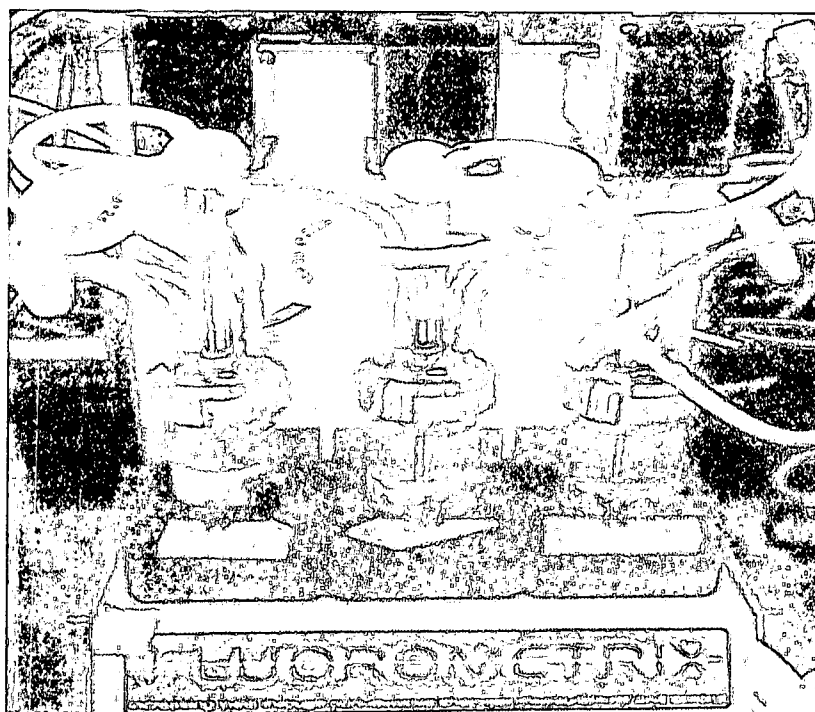


Figure 1. Assembled 50-mL stirred HTBR system run under baseline conditions (30% DO, pH  $7.2 \pm 0.1$  at  $37^{\circ}\text{C}$ ).

### *Shake flask set-up*

DO and pH patches were placed at the bottom of a glass beaker filled with DI water and weighted down to prevent warping. The beaker containing pH and DO patches was autoclaved at 121°C for 25 minutes. After autoclaving, the patches were allowed to cool to room temperature prior to placement of patches onto the bottom surface of the shake flasks. Autoclaved pH and DO patches were placed approximately 1mm apart at the bottom of one disposable 250-mL shake flask (Corning). Fifty milliliters of CD Hybridoma medium adjusted to an initial pH of 7.2 ( $\pm 0.1$ ) (Invitrogen) supplemented with 3mM L-Glutamine (Hyclone Laboratories) and  $1.4 \times 10^{-4}$  % beta-mercaptoethanol (v/v), and D-Glucose concentration of 3.6 g/L was added to 3 x 250-mL shake flasks. Shake flasks were incubated in CO<sub>2</sub> incubator set to 5% CO<sub>2</sub>, 37°C, and transferred to an orbital shaker set to 100 rpm. One shake flask containing DO and pH patches was placed atop a light-emitting diode (LED) coaster containing the pH and DO sensors (Fig. 2).

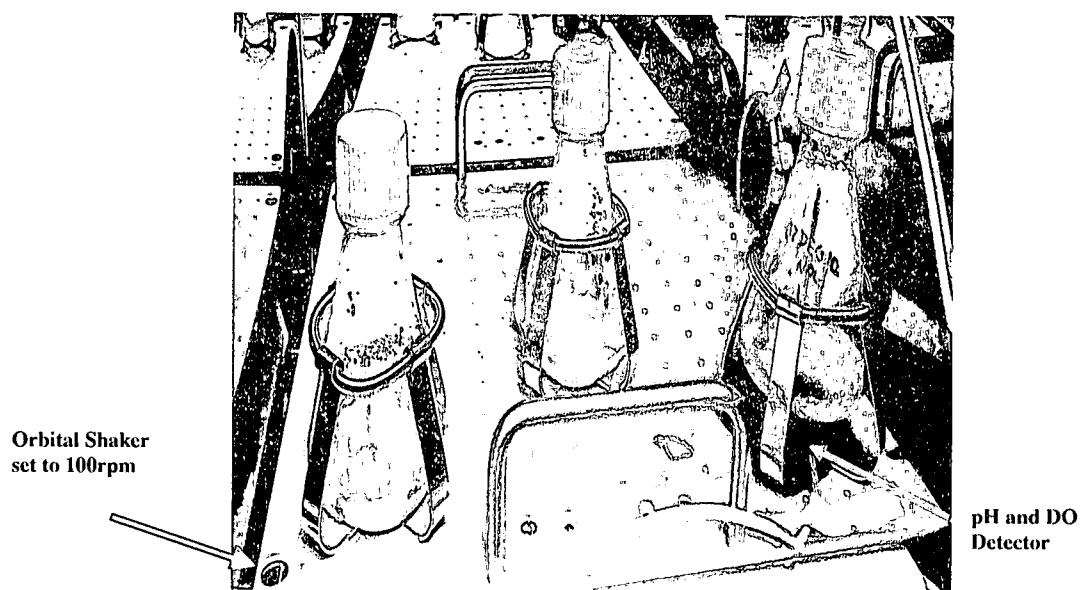


Figure 2. Cells were cultured in 250-mL disposable shake flasks at 5% CO<sub>2</sub>, pH 7.2 ± 0.1 and 37°C. Shake flasks were agitated at 100 rpm for the duration of the experiment.

## HTBR and 5-L Bench-scale Bioreactor Comparison

In subsequent experiments comparability of HTBRs and a 5-L bench-scale bioreactor system were assessed on the basis of relative protein carbonyl concentration. DO set points of 10%, 40%, 60% and 80% were evaluated in HTBRs and 10% and 60% in the 5-L bench-scale bioreactor system. A third run with a DO set-point of 10% was attempted, but due to operational issues, the resulting average DO was 31.9%. Data from this run was used as a center-point for the evaluation of the 5-L bioreactor. Process conditions ( $\text{pH } 7.2 \pm 0.1$ ,  $37^\circ\text{C}$ ) were held nominally constant at both scales. Relative protein carbonyl content of the purified antibody product (IgG3) was measured and compared. ANOVA of carbonyl content in antibody products produced in HTBRs was conducted to determine if the response of increasing protein carbonyl content in response to increasing DO % is experimentally significant.

## Sample Preparation and Analysis

Cell culture devices were sampled 1-2 times daily and % viability and cell density were determined. Cell density and % viability determination was made by dilution of 10- $\mu$ L cells with an equal volume trypan blue (Sigma). Ten micro-liters of diluted cells were transferred onto a hemocytometer and counted using an Olympus BH2 series microscope.

Supernatant from daily samples were also analyzed for glucose consumption and lactate production using the YSI 2700 Select Biochemistry Analyzer. Remaining cell culture broth was harvested via centrifugation at 200 x g for 7 minutes. Clarified supernatant was decanted and stored at -80°C for future purification and analysis.

Prior to the start of purification, supernatant samples were thawed and 0.2  $\mu$ m filtered and purified using MabSelect affinity chromatography. Purified sample concentration was measured using A280 nm. Protein carbonyl content was measured using the Cell Biolabs Inc. OxiSelect™ Protein Carbonyl ELISA Kit.

## Protein Carbonyl ELISA Assay Evaluation

Standards and protein samples were adsorbed to 96 well plates. Carbonyl present in samples was derivatized to DNPH and probed with anti-DNP antibody followed by a secondary antibody (Cell Biolabs, San Diego, CA). Carbonyl content was determined by comparing the absorbance of the sample to a standard curve generated using a reduced/oxidized BSA standard (Cell Biolabs, San Diego, CA).

A standard curve was run as described by Cell Biolabs protocol for the Protein Carbonyl ELISA Assay. Standards were prepared as described in Table 1. Primary and secondary antibody was diluted 1000 fold. ELISA results were evaluated on the basis of specificity, accuracy, precision, correlation, linearity, and range (Table 2).

Table 1. Standard curve preparation for the protein carbonyl ELISA assay. Reduced and oxidized BSA standards were mixed as shown in the table below. The protein carbonyl assay was used to determine the protein carbonyl in purified cell culture samples exposed to increasing levels of dissolved oxygen.

Tube Label	Volume Oxidized BSA added ( $\mu$ L)	Volume Reduced BSA added ( $\mu$ L)	Protein Carbonyl Concentration (nmol/mg)
1	400	0	7.5
2	320	80	6.0
3	240	160	4.5
4	160	240	3.0
5	80	320	1.5
6	40	360	0.75
7	20	380	0.375

## Protein Carbonyl Measurements

Relative protein carbonyl content of purified IgG3 samples was measured using a Protein Carbonyl ELISA kit (Cell Biolabs Inc.). A standard curve was generated by diluting 1 mg/mL bovine serum albumin (BSA) standards, oxidized and reduced to 10  $\mu\text{g/mL}$  in 1X phosphate buffered saline (PBS). BSA standards were prepared according to Cell Biolabs Inc. ELISA protocol. Diluted oxidized and reduced BSA standards were mixed in known ratios to produce standard curve (Table 1).

Purified IgG3 samples were diluted to 10  $\mu\text{g/mL}$  using 1X PBS Phosphate Buffer Saline (PBS). One hundred micro-liters of standards and purified IgG3 samples were added to a 96-well plate in duplicate and incubated at 37°C for 2 hours for binding. Wells were washed 3 times with 250- $\mu\text{L}$  of 1X PBS. Samples were derivatized by addition of 100  $\mu\text{L}$  of DNPH working solution to each well and incubation in the dark at room temperature for 45 minutes. Wells were washed 5 times with 1:1 v/v solution of 1X PBS and Ethanol, incubating 5 minutes between each wash. Wells were washed with 250  $\mu\text{L}$  of 1X PBS to remove Ethanol solution. Wells were blocked with 200  $\mu\text{L}$  of Blocking Solution per well and incubated for 1 hour at room temperature on an orbital shaker. Wells were washed 3 times with 250  $\mu\text{L}$  1X wash buffer. One thousand fold diluted anti-DNP antibody (100  $\mu\text{L}$ ) was added to each well and incubated for 1 hour at room temperature on an orbital shaker. Each well was washed 3 times with 250  $\mu\text{L}$  of 1X wash buffer. One thousand fold diluted horseradish peroxidase (HRP) conjugated secondary antibody (100  $\mu\text{L}$ ) was added to each well and incubated at room temperature on an orbital shaker for 1 hour. Room temperature Substrate Solution (100  $\mu\text{L}$ ) was added to each well and incubated at room temperature for 10 minutes or until developed. Enzyme



reaction was stopped with the addition of 100  $\mu$ L of Stop Solution to each well. Absorbance was read at 450 nm using a Spectra Max plate reader (Molecular Devices, CA). Absorbance data was analyzed using SoftMax Pro v5 and Microsoft Excel.

## RESULTS

### Evaluation of Cell Biolabs Protein Carbonyl ELISA Assay

Cell Biolabs Protein Carbonyl ELISA assay was evaluated for use as the primary method for assessing comparability of the bioreactor systems and the impact of %DO on product quality. Initial test of this analytical method resulted in saturated standard curves and was believed to be attributed to reagent preparation. Standard curves were generated using 1000-fold and 2000-fold dilutions of primary and secondary antibody. A linear response was observed at both 1000-fold and 2000-fold dilutions.  $R^2$  values for both dilutions were acceptable as  $\geq 0.96$ . Accuracy and precision of the analytical method was assessed as reported in Table 2.

Table 2. Protein carbonyl assay evaluation results for 1000-fold and 2000-fold primary and secondary antibody dilutions.

Assay Criteria	1000-fold Dilution	2000-fold Dilution
Accuracy (average % error)	7.28	7.21
Precision (standard deviation)	$\pm 0.07$	$\pm 0.03$
Correlation Coefficient	0.96	0.97

### Baseline Study

Shake flasks, T-flasks and HTBRs were tested under baseline conditions pH  $7.2 \pm 0.1$  at 37°C (Table 3). An average DO level of 82% was recorded using optical sensor technology in the shake flasks while the average %DO measured in the three HTBRs were 44%, 36% and 49% respectively (Table 3). Dissolved oxygen levels were not

monitored in T-flasks but under static conditions they typically reach DO levels near 0% (Randers-Eichhorn *et al.* 1996; Vallejos *et al.* 2009).

Table 3. Experimental conditions for each cell culture system tested in the baseline study. % DO was not measured in T-flasks.

Process Conditions	Culture Conditions		
	Shake Flask*	T-Flask	HTBR*
Initial pH		7.2 ± 0.1	
Temperature (°C)		37	N/A
CO <sub>2</sub> (%)	5	5	
Average Measured DO (%)	82	Not measured	R1:44 R2:36 R3:49

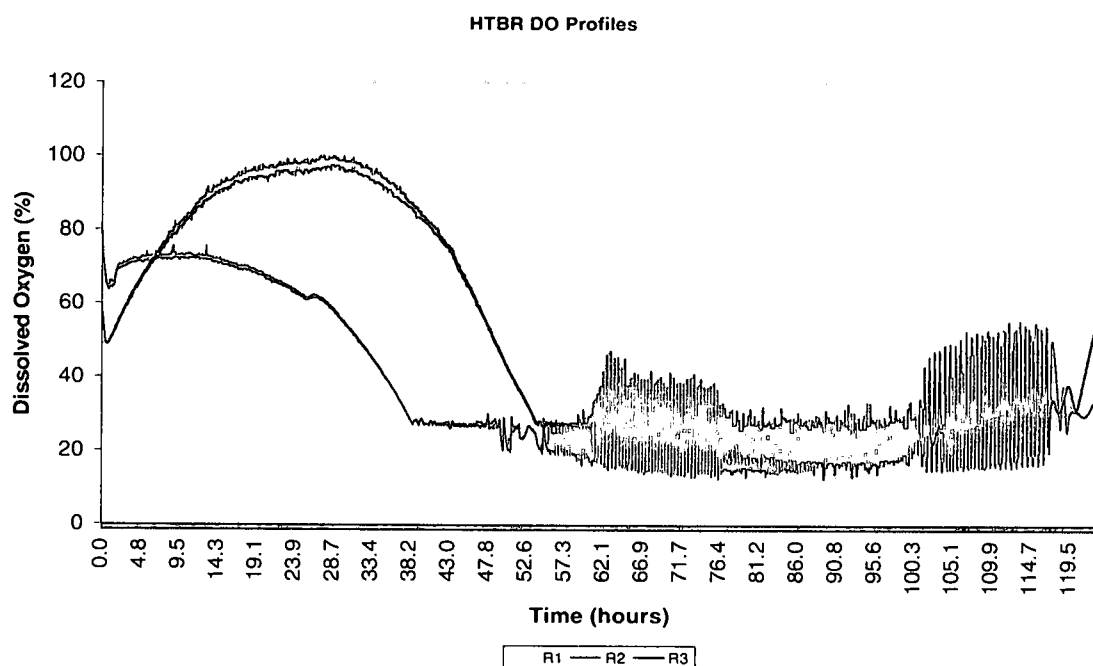
\* Denotes use of optical sensors for pH and DO monitoring/control.

#### *pH and DO profiles*

Fig. 3 and Fig. 4 show dissolved oxygen and pH profiles of HTBRs and 250-mL disposable shake flasks generated from data collected using non-invasive pH and DO sensor technology. Both systems were operated under the conditions described in Table 3. DO levels in the shake flask containing 50-mL of culture broth and incubated at 37° C on an orbital shaker at 100 rpm was measured at 82% DO, while DO levels in the HTBR with 30% DO set-point was maintained at an average of 43% over the three culture vessels. pH and DO was not monitored in T-flasks, but based on previous experiments conducted by Eichhorn *et al.* 1996, DO levels typically reach near 0% without agitation.

Measured %DO in the HTBR system did not achieve the DO set-point of 30% however overlay of pH and DO profiles in Fig. 3 show that measurements across the three HTBR vessels were consistent. pH and DO profiles measured in 250-mL disposable shake flasks are shown in Fig. 4. Dissolved oxygen % was measured at approximately 82% while pH profile is consistent with target pH of  $7.2 \pm 0.1$ . DO levels of 82% in the shake flask indicated a high oxygen transfer rate (OTR) in the shake flask for the conditions used in the baseline study. To confirm this hypothesis, studies to determine  $k_La$  in the disposable 250-mL shake flask was executed (Table 4).

A.



B.

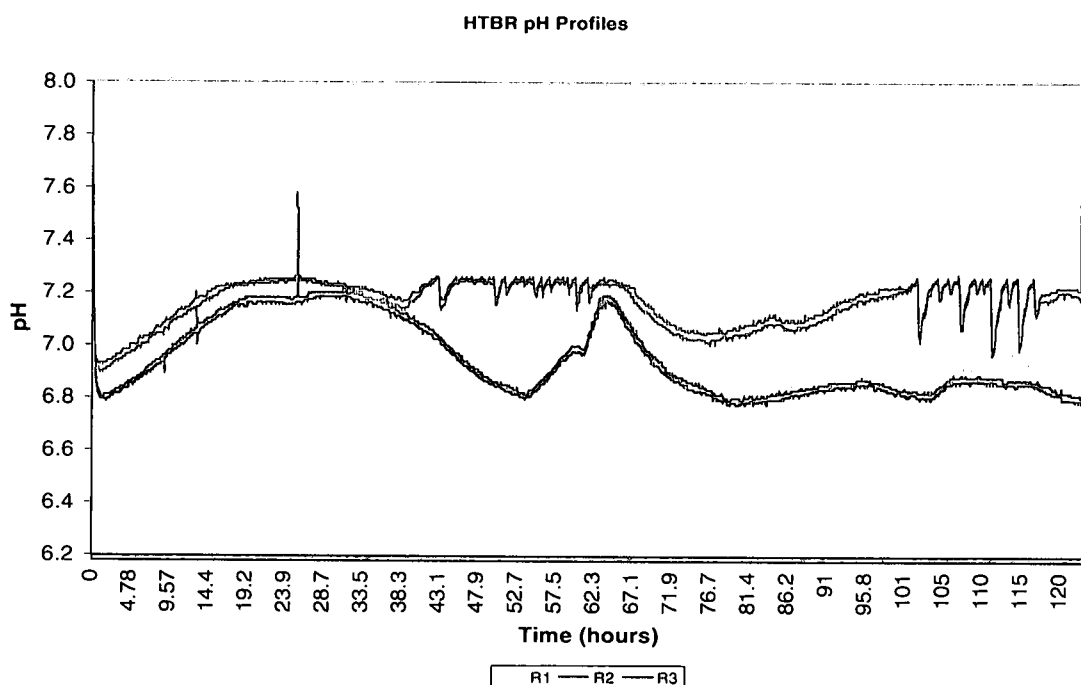
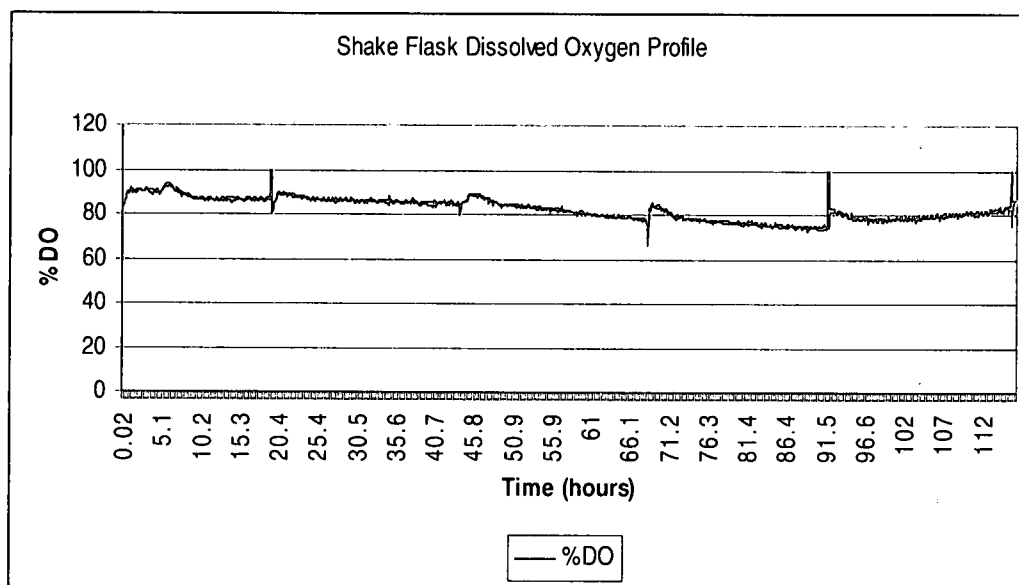


Figure 3. HTBR data collected using non-invasive sensor technology at 30% DO set-point run in triplicate. (A) DO profiles of HTBR. (B) pH profiles of HTBR.

A.



B.

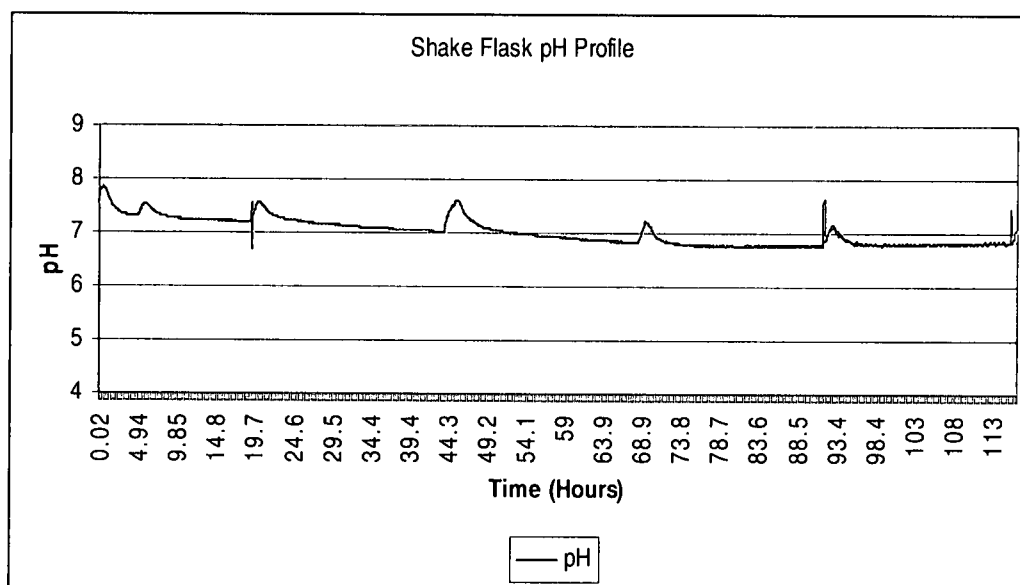
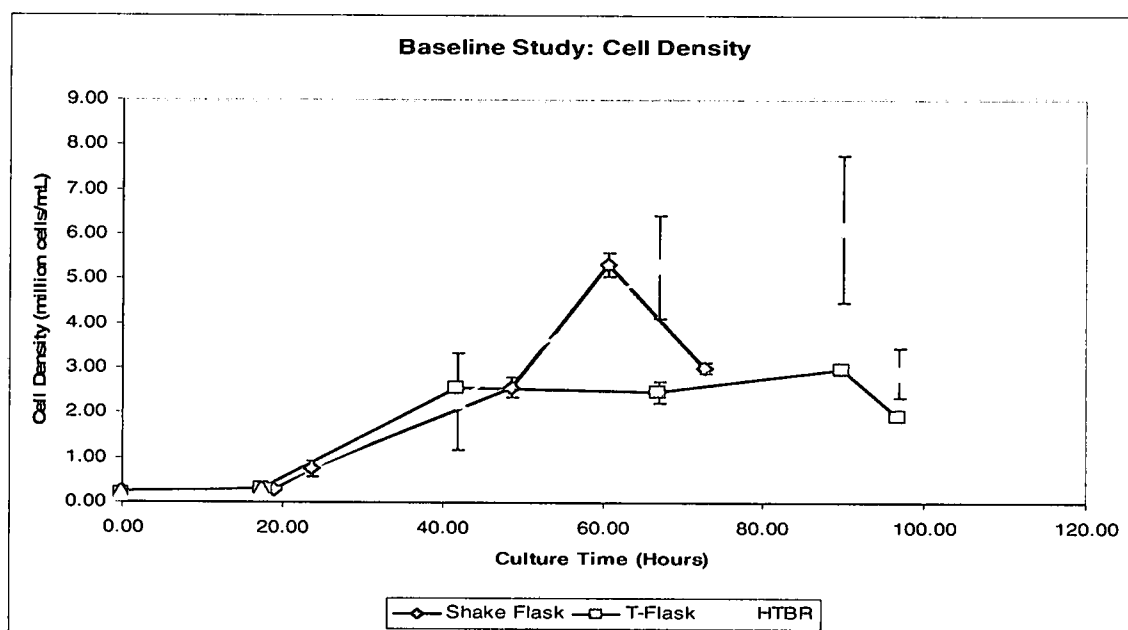


Figure 4. (A) Dissolved oxygen and (B) pH profiles measured in 250-mL disposable shake flasks using optical sensors. Cells were incubated under 5% CO<sub>2</sub> at 37°C. DO and pH profiles were only monitored in one shake flask throughout the study.

### *Cell Density and Cell Viability Profiles*

Cell density and cell viability profiles of HTBRs, T-flasks and shake flasks are compared. Peak cell densities achieved in HTBRs were comparable to those achieved in shake flasks (Fig. 5). Replicate peak cell densities achieved in all systems were consistent with the most variability observed in the HTBR system (Fig. 5). Peak cell densities achieved in T-flasks were considerably lower than cell densities achieved in HTBRs and shake flasks. Cell viability profiles were consistent in each of the three systems tested (Fig. 5). Additionally, total culture time observed in shake flask was shorter than total culture time observed in HTBRs and T-Flasks by at least 24 hours.

A.



B.

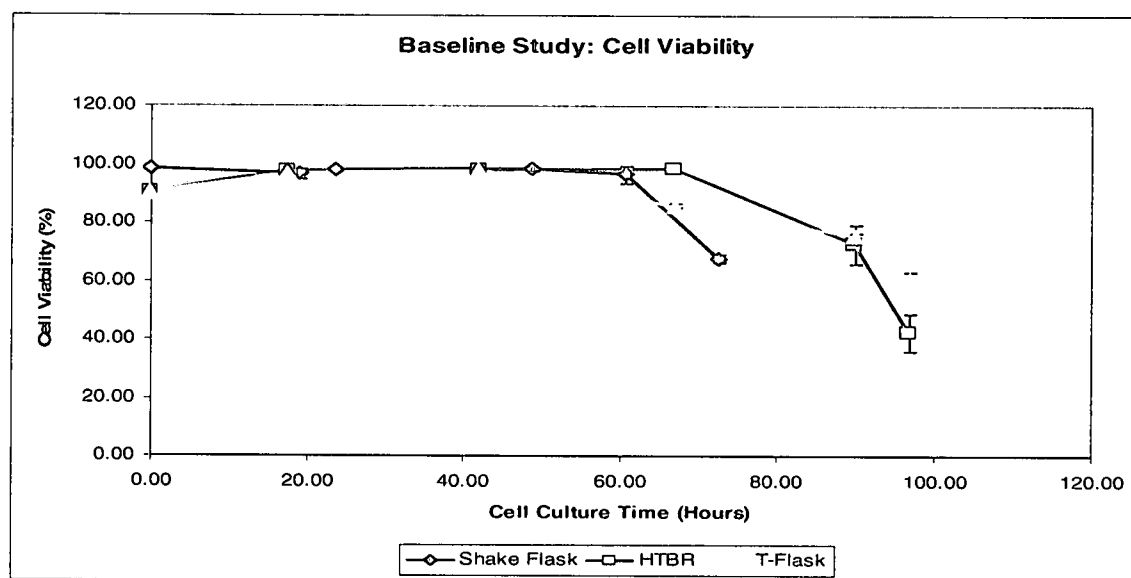


Figure 5. (A) Average cell density and (B) average cell viability profiles of small-scale cell culture devices (Shake flasks, T-flasks and HTBRs) tested for the baseline study. Each small-scale device was tested in triplicate. Error bars represent one standard deviation.



### Shake Flask $k_La$ Determination

Dissolved oxygen and pH profiles of a 250-mL shake flask were monitored and logged using non-invasive sensor technology (Fig. 4). Under the conditions tested (Table 3), the measured DO level during the course of the shake flask run was approximately 82%. This indicated that the OTR under these conditions was relatively high as compared to OTR's observed in other cell culture systems under active DO control.  $k_La$  of 250-mL disposable shake flasks was derived using the unsteady-state method or "gassing out" method.  $k_La$  was determined for fill volumes of 25-100 mL and agitation speeds ranging from 50-150 rpm at 37°C (Riet 1979).

Disposable 250-mL shake flasks were filled with deionized (DI) water and agitated. The DO in the shake flask was depleted with a nitrogen overlay until measured DO level in the shake flask was zero. Shake flasks containing a known volume of DI water was agitated while monitoring DO until oxygen saturation was observed. Results are shown in Table 4. Measured  $k_La$  ranged from 4.4-91.5  $\text{h}^{-1}$ .

Table 4. Standard deviation of experimentally determined  $k_{La}$  in 250-mL disposable shake flasks.  $k_{La}$  was determined using the gassing out method at volumes ranging from 25-100 mL and agitation speeds ranging from 50-150 rpm at 37°C.

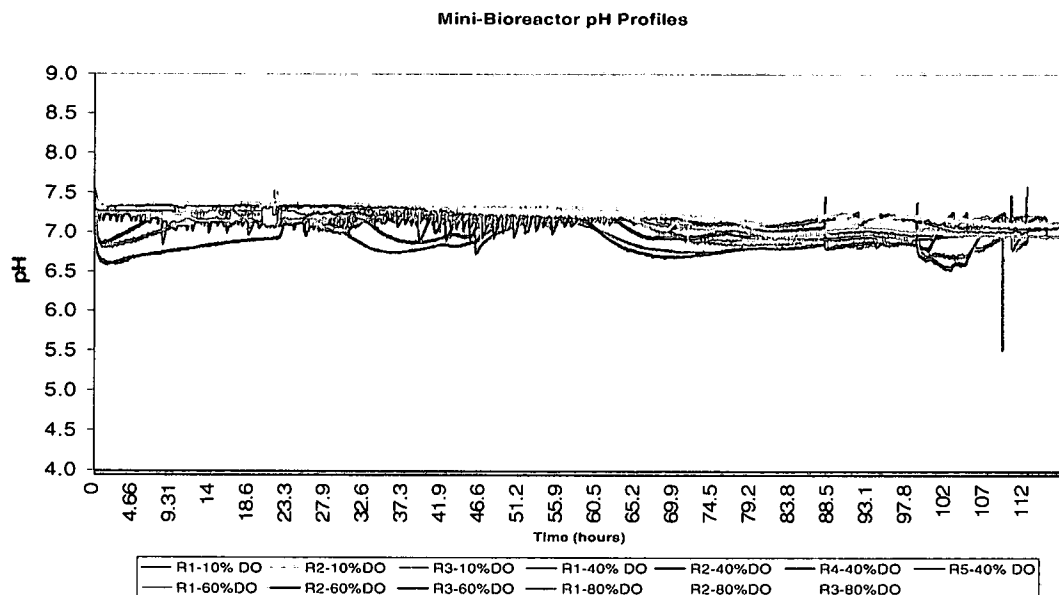
Volume (mL)	Agitation Speed (rpm)	Experimental $k_{La}$ (Trial 1)	Experimental $k_{La}$ (Trial 2)	Standard Deviation
25	50	41.29	30.10	7.91
	80	58.28	59.22	0.67
	100	76.22	75.39	0.59
	125	66.87	71.65	3.38
	150	91.16	92.00	0.60
50	50	5.28	4.47	0.57
	80	10.36	10.36	0.00
	100	11.15	11.97	0.58
	125	16.31	16.31	0.00
	150	17.38	21.13	2.65
75	50	7.84	8.22	0.27
	80	9.56	9.60	0.03
	100	10.39	10.69	0.21
	125	15.93	16.33	0.28
	150	16.70	15.37	0.94
100	50	4.44	4.50	0.04
	80	5.03	5.50	0.33
	100	6.67	7.09	0.30
	125	10.18	10.15	0.02
	150	13.78	13.46	0.22

## HTBR Evaluation

### *pH and DO profiles*

HTBRs were evaluated at various DO set-points for comparison to 5-L bench-scale bioreactor systems on the basis of relative protein carbonyl content trends as a result of exposure to increasing levels of dissolved oxygen. pH and DO profiles of the HTBR system at varying DO levels show consistent performance and control at DO set-points tested (10% 40%, 60% and 80%) (Fig. 6). Standard deviation of the average DO achieved using the HTBR system ranged from 0.6 to 4.8% (Table 5). Tighter control of DO levels was achieved at DO levels less than 80%. Average DO levels achieved were within 5% of the DO set point at 10%, 40%, 60% and 80% DO (Table 5).

A.



B.

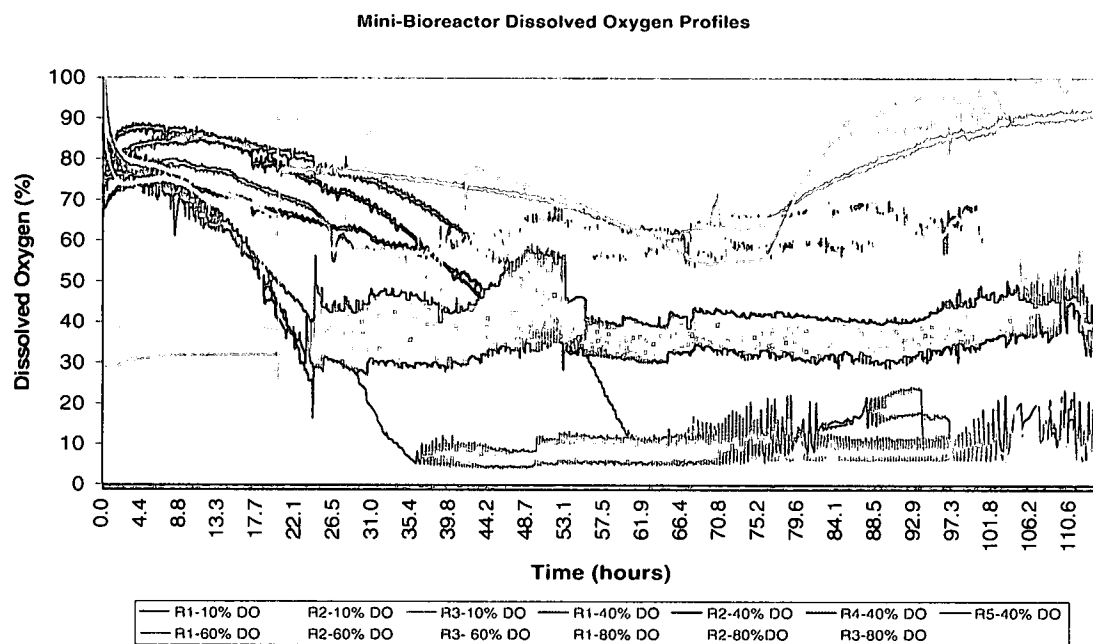


Figure 6. pH and DO profiles of HTBRs at varying DO set-points collected using non invasive sensor technology. (A) pH profiles of mini-bioreactor at 10%, 40%, 60%, and 80% DO set-points. (B) DO profiles of min-bioreactor at 10%, 40%, 60% and 80% DO set-points.

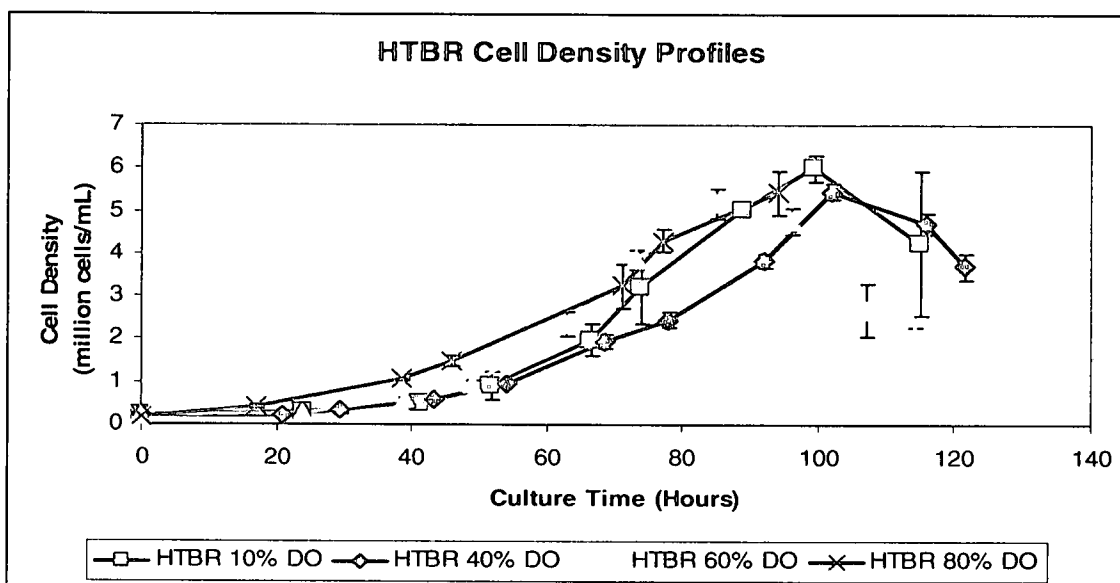
Table 5. Average %DO achieved in the stirred HTBR system in controlled region at 10%, 40%, 60% and 80% DO set-points.

HTBR DO Set-point (%)	HTBR 1	HTBR 2	HTBR 3	Std. Dev.
10	10.1	9.1	12.7	1.9
40	37.9	38.8	-----	0.6
60	62.3	61.2	62.7	0.8
80	86.3	76.9	80.2	4.8

### *Cell Density and Cell Viability Profiles*

Cell density and cell viability profiles of HTBRs are shown in Fig. 7 at 10%, 40%, 60% and 80% DO set-points. Cell density and cell viability was consistent across the HTBR vessels at the various DO set-points tested. Maximum peak cell density of 6.0 million cells/mL was achieved at 10% DO, and the lowest peak cell density of 5.4 million cells/mL was achieved at 80% DO (Table 6).

A.



B

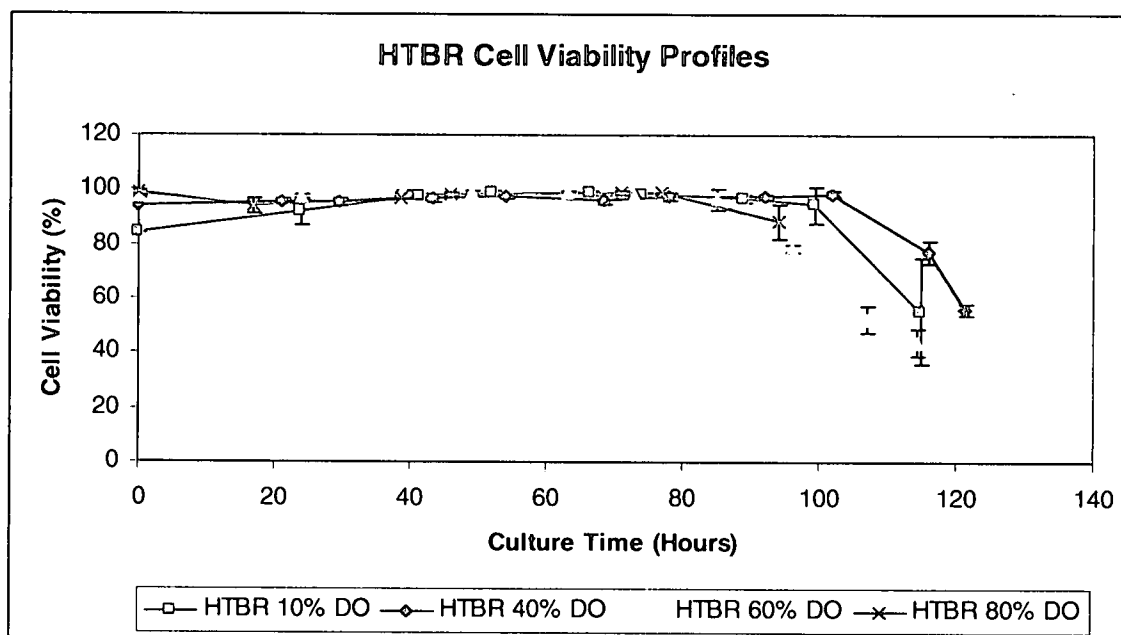


Figure 7. Cell density and cell viability profiles of 10% dissolved oxygen concentration in mini-bioreactors. Cells were cultured in 35-mL working volume at pH 7.2 ( $\pm 0.1$ ), at 37°C. Error bars represent standard deviation of cell density (A) and cell viability profiles (B).

Table 6. Peak cell densities observed in HTBRs at 10%, 40%, 60% and 80% DO set-points. Cells were grown in 35-mL working volume at pH  $7.2 \pm 0.1$  and 37° C.

HTBR DO Set-point (%)	Average DO (%)	Average peak Cell Density (million cells/mL)
10	10.1	6.0
40	37.9	5.6
60	62.3	5.5
80	86.3	5.4

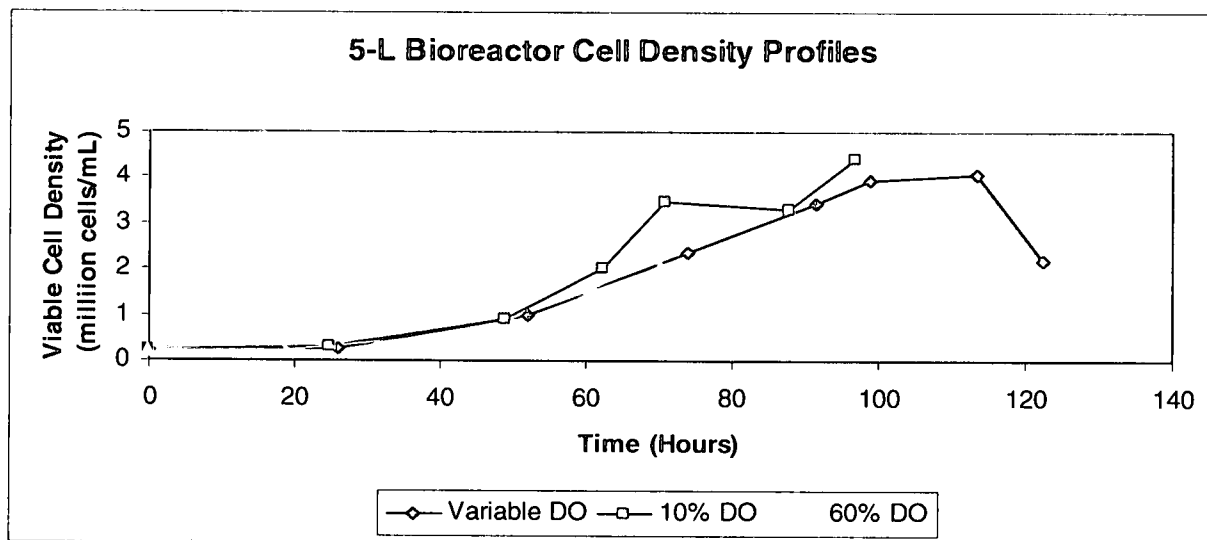
As shown in previous studies evaluating HTBRs on the basis of DO, pH and VCD profiles (Kostov *et al.* 2001), consistent performance and control of process conditions in the HTBR system is demonstrated in this study.



### **Five-Liter Bioreactor Evaluation**

A 5-L bench-scale bioreactor system was evaluated at various DO set-points for comparison to the HTBR system. Cell density and cell viability profiles of the 5-L bench-scale bioreactor system are shown in Fig. 8 at 10%, 31.9% and 60% dissolved oxygen concentrations. The maximum peak cell density of 4.4 million cells/mL was achieved at 10% DO, and the lowest peak cell density of 2.5 million cells/mL was achieved at 60% DO (Table 7). Overall the cell densities achieved were lower in the 5-L bioreactor system than cell densities observed in the HTBR system. The trend of decreasing cell density in response to increase % DO is observed in both bioreactor systems.

A.



B.

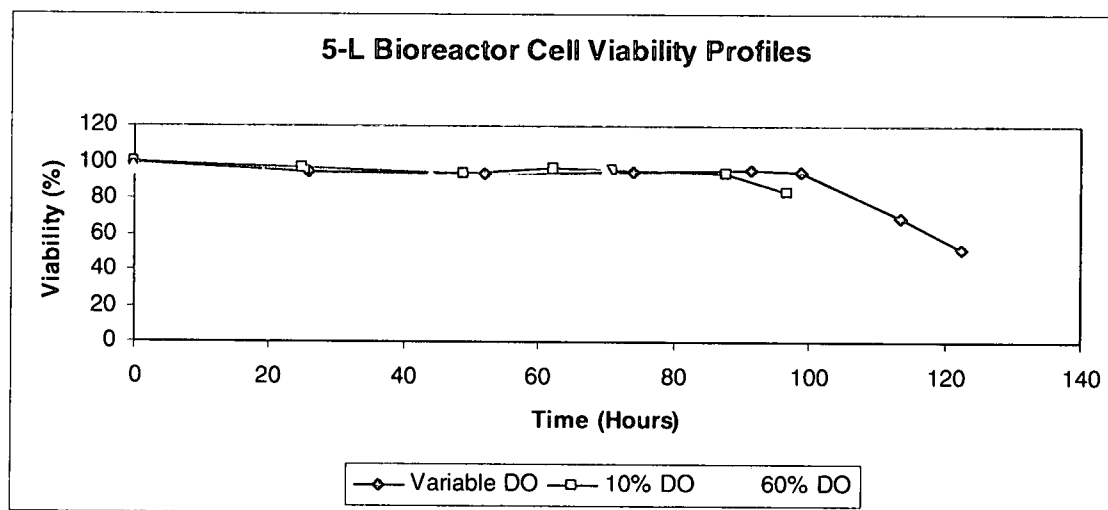


Figure 8. (A) Cell density and (B) cell viability profiles of 5-L bioreactors at various dissolved oxygen set-points. Average DO levels recorded were 17.9%, 31.9% and 60.6% respectively. Cells were cultured in a 4.5-L working volume at pH  $7.2 \pm 0.1$  at  $37^{\circ}\text{C}$ .

Table 7. Peak cell densities and average DO% achieved in 5-L bioreactor. Cells were grown in a 4.5 L working volume at pH  $7.2 \pm 0.1$  at 37° C.

5-L Bioreactor DO Set-point (%)	Average DO (%)	Peak Cell Density (million cells/mL)
10%	17.9	4.4
-----*	31.9	4.0
60%	60.6	2.5

\*Bioreactor did not maintain set-point of 10% DO. Average DO achieved for this bioreactor was 31.9%.

## Relative Protein Carbonyl Content Trends

### Baseline Study

Comparison of relative protein carbonyl content measured in T-flasks, HTBRs, and 250-mL disposable shake flasks are shown in Fig. 9. Traditional cell culture systems, T-flasks and shake flasks were compared to the mini-bioreactor system under baseline conditions (37°C, pH  $7.2 \pm 0.1$ ). Relative protein carbonyl content of IgG3 produced in T-flasks was  $0.24 \pm 0.12$  nmol/mg,  $0.66 \pm 0.06$  nmol/mg in shake flasks, and  $0.58 \pm 0.02$  nmol/mg in HTBRs (Fig. 9). The trend of increasing protein carbonyl content in response to increasing levels of DO can be identified (Fig. 12).

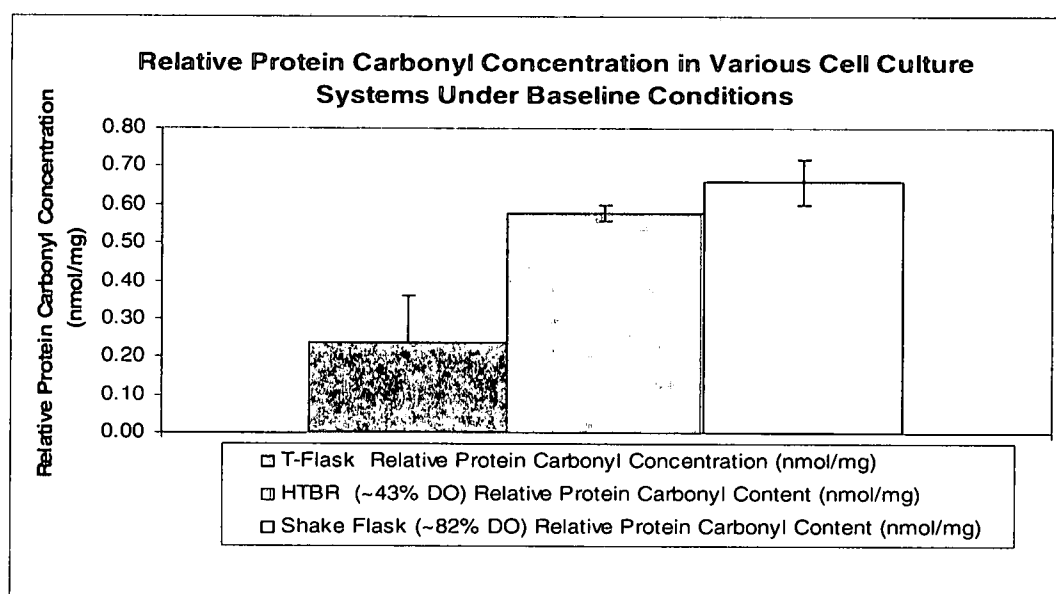


Figure 9. Averaged relative protein carbonyl concentration measured in purified protein samples from various cell culture systems under baseline conditions. Relative protein carbonyl concentrations were measured using the Cell Biolabs OxiSelect ELISA assay. Error bars represent standard deviation of protein carbonyl content.

### *Relative Protein Carbonyl Content in HTBRs*

Comparison of relative protein carbonyl content in HTBRs at various DO levels (10%, 40%, 60% and 80%) is shown in Fig. 10. Average relative protein carbonyl content at various DO levels in HTBRs was measured at  $2.04 \pm 0.11$  nmol/mg,  $2.69 \pm 0.22$  nmol/mg,  $3.50 \pm 0.16$  nmol/mg, and  $3.23 \pm 0.00$  nmol/mg respectively. The trend of increasing protein carbonyl content in response to increasing levels of DO can be identified ( $R^2 = 0.79$  (10-80% DO),  $R^2 = 0.99$  (10-60% DO)) (Fig. 12).

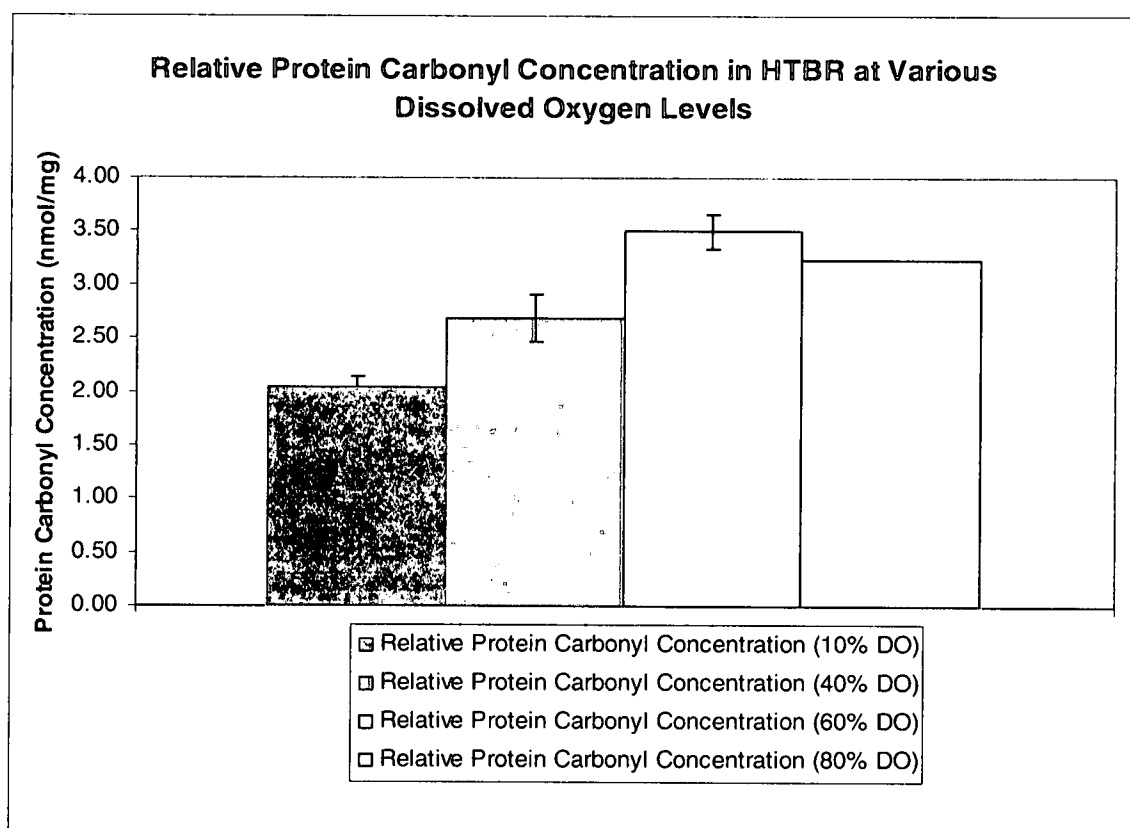


Figure 10. Comparison of averaged relative protein carbonyl concentration measured by ELISA in HTBRs at various %DO (10%, 40%, 60% and 80%). Purified protein samples were assayed using the Cell Biolabs OxiSelect ELISA assay. Error bars represent standard deviation of protein carbonyl content.

### *Relative Protein Carbonyl Content in 5-L Bioreactor*

Comparison of relative protein carbonyl content in a 5-L bioreactor at various DO levels (10%, variable, and 60%) is shown in Fig. 11. Actual measured DO levels in the 5-L bioreactor system was 17.9%, 31.9% and 60.6 % respectively. Average relative protein carbonyl content at various DO levels in the 5-L bioreactor was measured at  $0.39 \pm 0.0021$  nmol/mg,  $0.43 \pm 0.0021$  nmol/mg, and  $1.2 \pm 0.10$  nmol/mg respectively.

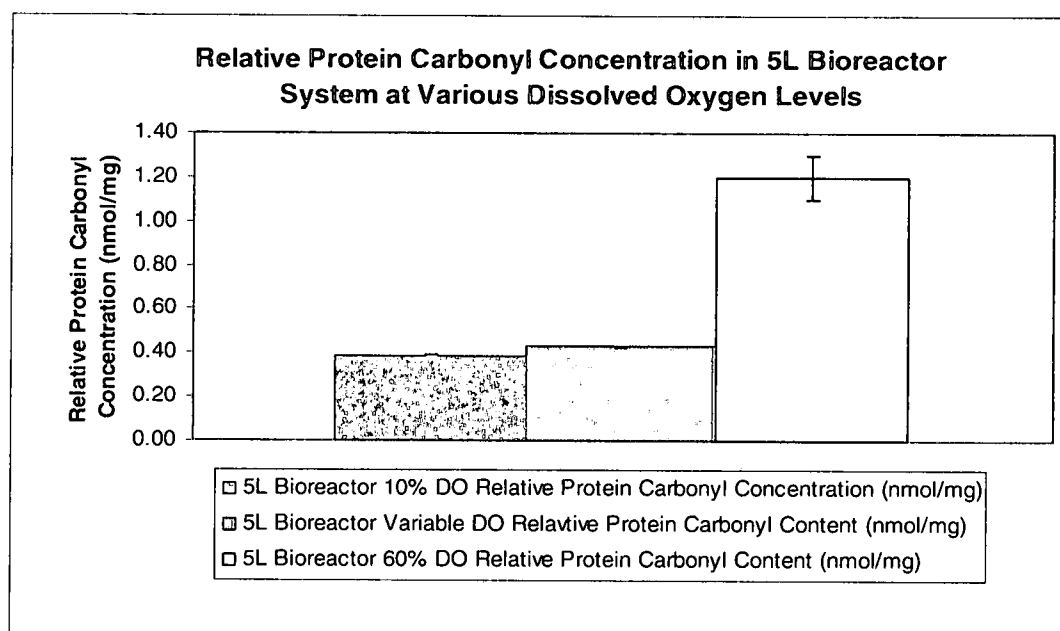


Figure 11. Average relative protein carbonyl concentration in 5-L bioreactors at varying dissolved oxygen levels (between 10-60% DO). Purified protein samples were analyzed using the Cell Biolabs OxiSelect ELISA assay. Error bars represent standard deviation of protein carbonyl content.

Correlation of protein carbonyl content to increasing levels of DO is shown below in Fig. 12. Relative protein carbonyl content is plotted against DO from 10-60% for direct comparison of HTBRs and 5-L bioreactor.  $R^2$  values of 0.99 and 0.92 were observed in HTBRs and the 5-L bioreactor systems respectively at DO levels between 10% and 60%, indicating that the increase in protein carbonyl content in response to increasing DO levels is linear. When HTBR data is plotted from 10-80% DO the correlation of increasing protein carbonyl content to increasing DO is not as evident (Fig. 12). The  $R^2$  value decreases to 0.79 and is likely due to marginal increases of protein carbonyl content observed at DO levels higher than 40% DO.

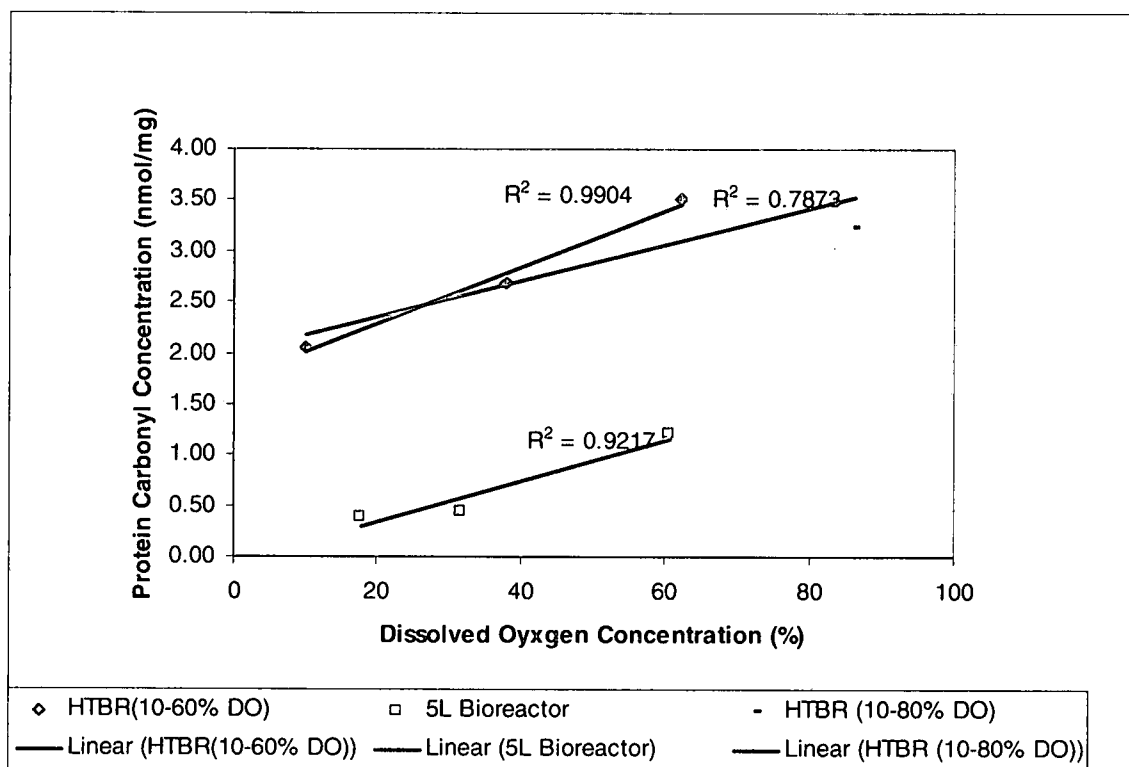


Figure 12. Correlation of averaged relative protein carbonyl concentration to dissolved oxygen level (%). Cells cultured in HTBRs and 5-L bioreactor systems were exposed increasing levels of dissolved oxygen 10-80% and 10-60% respectively.

## **Statistical Analysis of Relative Protein Carbonyl Data**

### *Baseline Study*

ANOVA analysis of relative protein carbonyl content of samples generated in the baseline study is shown in Table 8. Average protein carbonyl content of shake flasks, T-flasks and HTBRs are shown (Table 8). Variance and standard deviation of protein carbonyl content measurements for each bioreactor system are also shown (Table 8 and 9). ANOVA of relative protein carbonyl measurements of the three bioreactor systems resulted in a p-value of 0.0035 (Table 8). This data confirms that the relative protein carbonyl content of the products produced is affected by the resulting DO levels achieved in the three cell culture systems tested. Higher levels of protein carbonyls was observed in the shake flask which exhibited the highest measured DO level, while lowest levels of protein carbonyls was observed in T-flasks in which DO is observed to reach near zero.



Table 8. ANOVA output for relative protein carbonyl concentration in various cell culture systems under baseline conditions; HTBR (pH  $7.2 \pm 0.1$ ,  $37^{\circ}\text{C}$ , 30% DO set-point), T-flask (pH  $7.2 \pm 0.1$ ,  $37^{\circ}\text{C}$ , no DO control or monitoring), shake flask (pH  $7.2 \pm 0.1$ ,  $37^{\circ}\text{C}$ , no DO control, w/ monitoring using non-invasive sensor technology).

#### SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Shake Flask	3	1.9920	0.6640	0.0035
T-Flask	3	0.7170	0.2390	0.0134
HTBR	2	1.1580	0.5790	0.0005

#### ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	0.2953	2	0.1477	21.6005	0.0035	5.7861
Within Groups	0.0342	5	0.0068			
Total	0.3295	7				

## *HTBR*

ANOVA analysis of relative protein carbonyl content of samples generated in HTBRs is shown in Table 9. Average protein carbonyl content at various DO levels (10%, 40%, 60% and 80%) is shown (Table 9). Variance and standard deviation of protein carbonyl content measurements for each bioreactor system are also shown (Table 9). ANOVA of relative protein carbonyl measurements of the three bioreactor systems resulted in a p-value of 0.002 (Table 9), demonstrating the affect of DO level on protein carbonyl content. As shown in Fig. 12, the relative protein carbonyl content increases in response to increasing levels of DO.

Table 9. ANOVA output for relative protein carbonyl concentrations in HTBRs at varying dissolved oxygen levels (10%, 40%, 60% and 80%). HTBR conditions (pH 7.2  $\pm$  0.1, 37°C at specified DO set-point).

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
HTBR 10%	2	4.078	2.039	0.012
HTBR 40%	2	5.385	2.692	0.047
HTBR 60%	2	7.000	3.500	0.027
HTBR 80%	2	6.461	3.231	0.000

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	2.499	3	0.833	38.851	0.002	6.591
Within Groups	0.086	4	0.021			
Total	2.584	7				

## DISCUSSION

To further validate HTBRs as a scale-down model for process development and optimization, the ability to preserve the quality of products produced in HTBR at the 50 mL scale and those produced in bench-scale systems is essential. Comparability of HTBRs to traditional small-scale cell culture systems and a 5-L bench-scale bioreactor system was assessed on the basis of relative protein carbonyl concentration in response to increasing dissolved oxygen levels. HTBRs performed consistently on the basis of cell growth, DO, pH and cell viability profiles (Fig. 6 and Fig. 7) at various DO levels thus demonstrating control of process set points and confirming results of previous studies that evaluated HTBRs (Kostov *et al.* 2001).

The utility of a small-scale device that offer real-time monitoring and control of critical process parameters has been demonstrated. HTBRs with optical sensor technology offer real-time monitoring and closed feedback control of critical process parameters and thus provide a benefit over traditional small-scale cell culture devices; eliminating the black box approach to cell culture process development. Important to note is that DO levels achieved in each of the traditional cell culture systems (T-flasks and shake flasks) in the baseline study was an indirect and unpredictable response to the baseline conditions tested (Table 3), and minimal to no control of process conditions was possible. Resulting DO levels achieved in each of these systems resulted from various inherent factors of the cell culture device and the conditions to which the device was subjected. Resulting DO in the disposable 250-mL shake flask was attributed to the volume in the vessel and agitation speed, while DO level in the T-Flask resulted from lack of agitation and consumption of oxygen in the vessel during cell growth.  $k_{La}$

determination of the 250-mL disposable shake flask can lend more insight into which conditions (volume, rpm) should be chosen to achieve desired cell culture conditions in early stages of development such as clone selection and cell banking or in early stages of a seed train. Additional time consuming studies to generate data in which an experimentally determined  $k_La$  in the disposable shake flask can be correlated to DO% would be necessary to model the behavior of this cell culture vessel. Since no control of DO can be achieved in this system, a constant DO level will not be achieved throughout the culture duration, however some knowledge of your starting DO conditions may minimize exposure of cells to high DO levels when choosing initial culture volume and agitation speeds.

Data suggests that exposure of cells to high DO % during cell growth does impact product quality in terms of oxidative damage (Fig. 9, Fig. 10 and Fig. 11), and that the response to changes in %DO is linear for the cell line examined. An  $R^2$  value correlating the response of increasing protein carbonyl content to increasing DO% was 0.79 for DO levels between 10-80% and 0.99 for 10-60% DO (Fig. 12) in HTBRs. A decrease in linearity of the response of increasing protein carbonyl content to DO between 10-80% DO is likely a result of the observed minimal to no increase in protein carbonyl content above 60% DO. An  $R^2$  value correlating an increase in protein carbonyl content to an increase in DO % was 0.92 in the 5-L bioreactor from 10-60% DO. As shown, the response of increasing protein carbonyl content to increasing DO level is conserved across scales. The protein carbonyl concentration measured in the other cell culture systems evaluated also indicated a response to DO level. This suggests that HTBRs can

serve as a good tool for predicting responses resulting from changes to critical process parameters.

The observed response of increasing protein carbonyl content to increasing DO is an expected outcome. This response to oxidative stress is also common in studies that implicate the presence of relatively high levels of protein carbonyls in aging and the on set of some age related diseases (Stadtman 2004). However, marginal increases observed in protein carbonyl content between 60% and 80% DO were not expected. Additional studies to understand this observation are needed. Marginal increases in relative protein carbonyl content above 60% DO may have resulted from exposure of cells to a critical DO level at which all product residues susceptible to oxidation have been oxidized. This response to increasing levels of DO may not apply to other cell lines. Studies conducted to evaluate the impact of dissolved oxygen levels in other protein expression systems support the idea that oxidative stress resulting in the production of protein carbonyls does occur in response to a highly oxidative environment, but that response is highly dependent upon the cell line (Saarinem *et al.* 2003). This cell line specific response to increasing levels of DO could be due to the ability of some organisms to employ defense mechanisms which involve low molecular weight molecules with antioxidant properties (Saarinem *et al.* 2003).

Although a trend of increasing protein carbonyl content in response to increasing DO was conserved across scales, observed differences in measured levels of protein carbonyl content between scales was evident. Higher levels of protein carbonyl content was measured in the small-scale HTBR system compared to that measured in the 5-L bioreactor system. Known challenges to scaling cell culture processes may have

impacted these results. Process scale-up is challenging and involves many factors (Yang *et al.* 2007) that were not evaluated in this study. Factors such as shear sensitivity of the cells, bioreactor design, mixing speed and probe placement in the bioreactor can influence bioreactor performance at different scales. One concern during scale-up from small-scale devices to larger scale bioreactor systems is homogeneity of critical process parameters such as DO and pH. Localized differences in DO concentration in the liquid medium at the larger scale may have contributed to observed differences in measured protein carbonyl content between scales. Also note that the cell densities achieved in the 5-L bioreactor were also lower than those achieved in the HTBR system which may also be indicative of scaling challenges or a result of sampling. Elevated shear interaction with bubbles at the larger scale may cause suppression of cell growth (Yang *et al.* 2007). This highlights the importance of understanding what factors are critical for scaling bioreactor processes.

Also critical for scaling comparisons is the selection of appropriate characterization methods (Yang *et al.* 2007). Additional product quality profiles such as purity, specific protein production and peptide mapping in addition to the protein carbonyl measurements may have provided more information from which to make a comparison. Since measurement of protein carbonyl content is an indirect method for measuring oxidative damage, additional analysis of samples produced in the two bioreactor systems may provide a more comprehensive data set from which a conclusion can be drawn.

ANOVA of protein carbonyl content in HTBRs suggests that changes in protein carbonyl content in response to increasing DO levels is experimentally significant (p-value = 0.002) for conditions evaluated (10-80% DO). Control of pH and DO levels in cell culture experiments are critical process parameters and should be monitored and controlled to minimize the impact of these conditions on product quality. Optimal pH and DO parameters will vary from culture to culture depending upon growth requirements for the cell line, but conditions resulting in suboptimal DO levels whether low or high can lead to an adverse impact to product quality. Since oxidative stress also impact other cellular components, such as DNA control of critical process parameters in early stages of process development such as cell line selection, cell banking and seed trains, attention to critical quality attributes and their impact on product quality is paramount.



## Conclusions

This research demonstrates the utility of a small-scale cell culture device that offer real-time monitoring and control of critical process parameters. Studies comparing HTBRs, 50-mL stirred mini-bioreactors that employ non-invasive sensor technology to traditional small-scale cell culture systems (shake flasks and T-flasks) and 5-L bench-scale bioreactor system on the basis of pH, DO, and VCD profiles have been completed (Kostov *et al.* 2001). Although a direct comparison of protein carbonyl concentration across scales was not afforded in this study due to observed differences in measured protein carbonyl content between scales, consistent trends of increasing protein carbonyl concentration in response to varying levels of DO across scales show that HTBRs serve as a good predictor for assessing the response of product quality attributes namely oxidation in response to changes in cell culture process conditions. This work also highlights the importance of monitoring critical process parameters on the small-scale. Advantages of HTBRs over traditional bioreactor systems include real-time monitoring and control of critical process conditions while offering a low cost option to large scale cell culture studies.

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