Disposable rocking bioreactors for recombinant protein production in *Escherichia coli*: Physical characterization and assessment of therapeutic protein expression

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Applied Science

in

Chemical Engineering

Waterloo, Ontario, Canada, 2013

Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Disposable technology has gained increasing acceptance in the biopharmaceutical industry over the last decade, and provides many advantages over conventional stainless steel equipment. Disposable rocking bioreactors (RBs) are widely employed for cultivation of recombinant mammalian and insect cell lines, although the perception of inadequate mass transfer has prevented their application to bioprocesses based on microbial platforms. In an effort to thoroughly evaluate the suitability of disposable RBs for cultivation of aerobic microorganisms, a comparative study of one-dimensional (1D) and two-dimensional (2D) disposable RBs, and the conventional stirred tank reactor (STR) was performed. The comparison involved: 1) physical characterization of oxygen mass transfer efficiency and mixing intensity, 2) batch cultivation of *Escherichia coli* BL21 for comparison of growth characteristics, and 3) batch cultivation of recombinant *E. coli* BL21 expressing a clinical therapeutic, hCD83ext (the extracytoplasmic domain of human CD83). Oxygen mass transfer (evaluated as the mass transfer coefficient, k_La) was comparable between the 1D RB and STR (approximately 150 h^{-1}) at low working volume (WV), declining linearly with increasing WV, while k_La was highest in the 2D RB for all tested WVs, providing the maximum k_La (394 h^{-1}) at 3 L WV. Fast mixing (t_{95} of 8-20 s) was observed in all three systems for water and aqueous carboxymethylcellulose (CMC) solutions. Batch growth characteristics of *E. coli* BL21 were similar in each system, although acetate accumulation was significant in the 1D RB. Batch production of GST-hCD83ext (glutathione S-transferase-hCD83ext fusion protein) resulted in similar soluble protein yields and inclusion body formation between bioreactors. Although cell growth and protein expression were comparable between all bioreactors, the 1D RB is not considered a suitable cultivation system for *E. coli* under experimental conditions given the significant acetate accumulation observed

and high supplemental oxygen requirement for low cell density cultures. On the other hand, considering its formidable mass transfer capacity and overall performance in batch cultivations, the CELL-tainer® is an attractive alternative to the STR for cultivation of recombinant *E. coli* expressing high value therapeutic proteins.

Acknowledgements

I wish to begin by acknowledging the guidance and support provided by my supervisors Dr. C. P. Chou and Dr. M. Moo-Young. Your encouragement and scientific expertise was critical for the successful completion of this project and my professional development.

I sincerely thank the late Dr. J. Scharer & Dr. A. Chan for serving on my reading committee. My condolences go to Dr. J. Scharer's family; he was a very kind and well respected man and will be deeply missed by all that knew him. I would also like to thank Dr. W. Anderson for serving as my committee member on such short notice while on sabbatical. I would also like to thank Dr. N. Oosterhuis for his invaluable consultations throughout the course of the project, and the technical staff at CELLution Biotech for their assistance with the CELL-tainer® installation and training.

I appreciate the financial support for this project supplied by the Natural Sciences and Engineering Research Council (NSERC), the Canadian Research Chair (CRC) program, and CELLution Biotech BV.

I would also like to thank all of my colleagues in Dr. C. P. Chou's lab for their insightful technical discussions and camaraderie.

Last but certainly not least, I would not be where I am today without the love and support of my mother, father, and sister. You all made this possible and never gave up on me, thank you! Finally, to my beloved Rocky, it's not the same without you here.

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List of Abbreviations

1D	One-dimensional
2D	Two-dimensional
AA	Amino acid
cDNA	Complementary deoxyribonucleic acid
CFD	Computational fluid dynamics
CIP	Clean-in-place
CMA	Compartment model approach
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
Fab	Antigen binding fragment
FHL	Formate hydrogenylase
GST	Glutathione-*S*-transferase
HCD	High cell density
hHG	Human growth hormone
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
LB	Lysogeny broth
mAB	Monoclonal antibody
MAO	Monoamine oxidase
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PBS	Phosphate buffered saline
PGA	Penicillin G acylase

ROS	Reactive oxygen species
RB	Rocking bioreactor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIP	Steam-in-place
VD	Vertical displacement

List of Symbols

a	Interfacial area
C	Wave speed
C_0	Wave speed in deep water
C^*	Saturation concentration of oxygen in bulk liquid
C_L	Time dependent concentration of oxygen in bulk liquid
D	Diffusivity of oxygen in water
D_T	Tank diameter
E	Total wave energy
E_z	Axial dispersion coefficient
g	Gravitational acceleration constant
H	Wave height
H_0	Wave height in deep water
H_b	Critical height for wave breakage
h	Liquid height beneath wave
h_b	Critical liquid height for wave breakage
K	Rocking rate
k_L	Local liquid-phase mass transfer coefficient
k_La	Volumetric liquid-phase mass transfer coefficient
L	Wave length
N	Stirrer speed
N_c	Critical stirrer speed
T	Stirrer diameter
t	Time
t_e	Exposure time for mass transfer

t_m	Mixing time
u	Fluctuation velocity of turbulent eddies
V_s	Superficial gas velocity
ε	Energy dissipation rate of turbulence
η	Kolmogorov length scale
θ	Angle of incidence of wave
θ_0	Angle of incidence of wave in deep water
κ	Shore slope constant
μ_e	Effective dynamic liquid viscosity
ν	Kinematic viscosity
ρ	Fluid density
σ	Interfacial tension
τ	Wave period

Chapter 1- Overview

1.1 Research background

Biopharmaceuticals are the fastest growing segment of the pharmaceutical industry, accounting for approximately one-sixth of the $600 billion global market by 2007 [1]. Due to innovations in recombinant protein technology over the last 10 years, the number of available therapeutics has dramatically increased along with product quality and titer [2, 3]. Biopharmaceuticals are categorized according to biological activity: blood factors (thrombolytics, anticoagulants), hormones, growth factors, cytokines, monoclonal antibodies (mAbs), and enzymes [4]. mAbs-based products, accounting for 40% of total annual sales, are the most lucrative biological therapeutics on the market [1]. However, the rapidly increasing presence of 'biosimilars' is driving down large premiums once associated with new therapeutics [1, 5]. Additionally, high failure rates associated with biopharmaceuticals impart great risk to the considerable investment required for product development [3, 5]. The push to reduce capital investment in conjunction with dramatic increases in titer, pressure to reduce health care costs, preference (in some cases, requirement) to manufacture locally, and increased focus on multiproduct capability and niche markets drives the need for small, flexible, and cost effective manufacturing facilities [5, 6].

Implementing disposable technology (i.e., bioreactors, depth filters, filtration cassettes, chromatography columns, piping, etc.) can address many of the concerns associated with biopharmaceutical process development [5-7]. Disposable components provide additional benefits beyond reduced initial capital investment and increased production flexibility, i.e., reduced occurrence of contamination (especially for multiproduct facilities) and labor costs associated with validation and cleaning, faster market entry due to expedited facility

construction, and shorter turnover times between production runs. On the other hand, disposable unit operations have potential drawbacks such as increased risk of failure, increased waste disposal costs, and additional operating costs due to regular component replacement which must be considered. The first widely adopted disposable bioreactor, the WAVE Bioreactor [8], was introduced in the late 1990s and was later acquired by GE Healthcare Life Sciences. The cultivation chamber is a disposable bag mounted on a rocking platform, facilitating enhanced surface aeration via wave propagation at the gas-liquid interface. Agitation intensity in rocking bioreactors (RBs) is controlled through adjustment of rocking rate (*K*), expressed in rocks per min (rpm) and vertical displacement (VD). Subsequent disposable formulations include rotary oscillating [9], orbitally shaken, pneumatically driven, and stirred cultivation systems [10]. Modern versions of these bioreactors are equipped with disposable or multiuse pH and dissolved oxygen (DO) probes, enabling sophisticated control schemes already employed in traditional stirred-tank reactors (STR).

Initially, disposables were intended for mammalian cell culture prone to contamination (due to slow growth characteristics) and shear stress [11, 12]. The relatively low oxygen transfer required for mammalian and insect cell culture is easily achieved by disposable systems [13-16], although application to microbial cultivation has generally been avoided due to mass transfer limitations. Disposable RBs have been evaluated for cultivation of *Saccharomyces cerevisiae* [17] and *Escherichia coli* [18, 19] demonstrating their capability in generating low to moderate cell densities in batch and fed-batch mode. The latest development in disposable RBs, the two-dimensional (2D) CELL-tainer® (CELLution Biotech), is differentiated by simultaneous rocking and horizontal movement, facilitating significantly higher oxygen mass transfer efficiency compared to one-dimensional (1D) RBs. High cell density cultivation of *E. coli* was achieved

during fed-batch operation at 12 and 120 L scales, and mass transfer efficiency (represented as the volumetric mass transfer coefficient, k_La) was comparable to conventional STRs [20].

As of 2011, approximately one-third of biopharmaceuticals having received regulatory approval in the United States and European Union are produced in *Escherichia coli* [4]. Demonstration of acceptable growth characteristics in disposable RBs was an important step towards establishing their suitability for biopharmaceutical production in microbial hosts. However, studies of recombinant protein expression extend only to relatively low value, prokaryotic proteins of no pharmaceutical interest [19, 20]. Given the considerable cost of disposable cultivation systems, their application is most appropriate for high value products such as recombinant therapeutics. Considering *E. coli* is the preferred recombinant host for production of many biopharmaceuticals, characterization of disposable RBs for eukaryotic therapeutic protein expression in *E. coli* was undertaken to provide valuable insight for future biopharmaceutical process development.

1.2 Research objectives

The overall objectives of this thesis were to:

1) characterize 1D and 2D disposable RBs in terms of oxygen mass transfer efficiency (k_La) and mixing intensity (represented as the mixing time, t_m);
2) compare batch growth characteristics, and glucose and metabolite profiles of nonrecombinant *E. coli* in 1D and 2D disposable RBs to elucidate suitability for aerobic cultivation under typical conditions; and
3) evaluate eukaryotic therapeutic protein expression in recombinant *E. coli*, in terms of titer and inclusion body formation, relative to the conventional STR.

1.3 Outline of thesis

Chapter 2 is a review of economic comparison studies of stainless steel, hybrid (stainless steel and disposable), and fully disposable biopharmaceutical production schemes; measurements of oxygen mass transfer coefficients (k_La) and approaches to modelling mass transfer in bioreactors; measurements of mixing time (t_m) and approaches to modelling mixing time in bioreactors; and typical cultivation conditions for recombinant *E. coli* and strategies to enhance recombinant protein expression. In Chapter 3, 1D and 2D disposable RBs are assessed and compared with conventional stirred tank reactor (STR) for recombinant therapeutic protein production in Escherichia coli. The comparison involves: 1) physical characterization of oxygen mass transfer efficiency and mixing intensity, 2) batch cultivation of non-recombinant BL21 for comparison of growth characteristics, and 3) batch cultivation of recombinant BL21 expressing a clinical therapeutic, hCD83ext (the extracytoplasmic domain of human CD83). Finally, Chapter 4 summarizes the results from the investigation presented in Chapter 3 and implications to the biopharmaceutical industry, and proposes future studies to adequately characterize mass transfer, mixing, and recombinant protein production in disposable RBs.

Chapter 2 - Literature Review

The considerable capital investment and high risk associated with product success and timing of market entry are critical factors under consideration in the planning stage of biopharmaceutical process development. The majority of biopharmaceutical processes are based on stainless steel components due to reliability and passive interaction with process media, buffers, etc. However, the considerable costs associated with stainless steel construction and large footprint of fixed unit operations ensures that even the smallest production schemes require investments well above $10 million [3, 5-7]. For example, a small scale commercial facility operating a single 1,000 L stainless steel bioreactor would cost $25-40 million to construct [6, 7], while a higher capacity facility (≥ 10,000 L) can easily exceed $100 million [3, 6]. Additionally, novel biopharmaceutical life cycles are often short due to patent expiration and biosimilar introduction requiring jump investments every 5-10 years [1, 3, 5]. Integration of disposable components can significantly reduce capital investment and time-to-market, while enabling flexibility and increased productivity via reduced downtime. That aside, aspects such as production scale, expression host, and process conditions must also be carefully considered when selecting bioprocess unit operations.

2.1 Cost effectiveness of disposable technology

As previously discussed, disposable technology may afford key benefits for biopharmaceutical manufacturing in the appropriate context. Critical factors addressed early on in the development process are capital availability, time-to-market, and flexibility for future expansion (either for increased volume or additional products) [5]. Existing data indicates that significant reduction in capital investment is achievable by replacing stainless steel equipment with disposables, enabling

even distribution of capital costs over the project lifecycle. A case study of a 100 kg capacity mAb plant in which stainless steel and hybrid (stainless steel and disposable components) production platforms were compared, projected total capital cost reduction of 54% for hybrid versus stainless steel options [21]. This estimate is similar to that obtained in an earlier study in which a 42% reduction in fixed capital investment was projected for a fully disposable plant [5]. Variation in capital investment estimates can be significant given the dependence on Lang factor estimation, the ratio of total installed cost to equipment cost alone.

Analysis of annual operating costs is a more complicated issue, particularly when evaluating hybrid production schemes. Relative to the conventional option, a fully disposable plant was projected to incur 70% greater running costs, primarily due to increased materials and waste disposal costs [5]. Downstream separation and purification equipment (i.e., membranes and chromatography columns) represented the largest expense for disposables replacement. This trend was observed in a subsequent cost analysis study in which the author pointed out that some companies use chromatography matrices in a product-specific manner, reducing the need for validation of performance in the absence of cross-contamination [22]. Interestingly, in this study operating costs were 30 and 19% lower for fully disposable and hybrid production facilities, respectively, compared to the stainless steel option. This is largely due to the higher number of projects completed annually in fully disposable and hybrid plants, owing to reduced (or absence of) downtime for clean-in-place/steam-in-place (CIP/SIP). This issue was not formally considered in the earlier study although the authors speculated that increased productivity via reduced turnaround time was a critical factor to address in future work [5]. Returning to the mAb production case study, annual operating costs were estimated to be 16% lower for the hybrid facility. However, certain limitations of this study must be considered: 1) disposable components

were mainly limited to storage bags for media, buffers, culture, etc. (disposable filters and membranes were employed but did not contribute significantly to cost adjustments); and 2) adjustments in productivity were not considered due to reduced downtime for CIP/SIP [21]. The first item is important as the use of disposable bioreactors, piping, ultrafiltration and diafiltration equipment, etc. could significantly increase waste disposal costs while, at the same time, further reduce utility and labor expenditures associated with CIP/SIP. The latter effect could outweigh the former as CIP/SIP was the greatest running expense for the stainless steel scenario [22]. In accordance with this study, increased productivity resulting from diminished downtime could further enhance cost savings estimated for the hybrid mAb production scheme.

Another point to consider is the impact of expression host and reactor configuration, and inclusion of disposable chromatography matrices on yield. The preceding studies assumed equal yield for each scenario although sensitivity analyses indicated significant reductions in net present value (NPV) [5], or annual cost of goods (per gram of product) [22] for corresponding decrease in fermentation titer. Accordingly, selecting the appropriate bioreactor for cultivation of microbial expression hosts is a critical step in the development process. For example, opting for a disposable bioreactor to cultivate recombinant *E. coli* may reduce operating costs and time-to-market while increasing annual productivity, if oxygen mass transfer is sufficient to support the required cell density and physiology for target protein titer. Table 2-1 presents a comparison of cost and performance metrics for stainless steel, hybrid, and fully disposable production facilities. Currently, fully disposable facilities are not necessarily a practical option, particularly when considering the substantial cost of disposable chromatography hardware for capturing and polishing therapeutics [6, 22]. Furthermore, disposable bioreactors are currently limited to 2000 L or less such that higher production volumes will require multiple units in parallel, or

conventional stainless steel fermenters [6]. Consequently, the integration of disposable technology and biopharmaceutical development is an attractive option of increasing interest in industry, although factors such as expression host, production volume, and recovery costs must be carefully considered when deciding between disposable and stainless steel components.

Table 2-1 - Comparison of cost and performance metrics between stainless steel, hybrid, and fully disposable production facilities [5, 21, 22].

Component	**Stainless steel**	**Hybrid**	**Fully disposable**
Fixed capital investment	H	M	L
Consumables/raw materials	L	M	H
Waste disposal	L	M	H
Utilities	H	M	L
Labor	H	M	L
Fermentation titer	H	M/H*	M/H*
Overall yield	H	M/H**	M
Production runs	L	M	H

H ≡ highest; M ≡ moderate; L ≡ lowest
*dependent on choice of expression host and bioreactor
**dependent on chromatography hardware

2.2 Oxygen mass transfer in bioreactors

Bioreactor selection is a critical step in the development of any commercially feasible bioprocess, particularly when microbial expression hosts are employed due to the high oxygen demand and viscous nature of fully developed cultures. The STR has been the default choice for cultivation of aerobic bacteria due to superior mass transfer and flexibility in terms of operating conditions (i.e., air flow, agitation, and pressure). The limiting factor for application of disposable bioreactors to microbial cultivation is assumed to be mass transfer. On the other hand, fed-batch operation at low growth rate along with supplemental oxygen has proven effective in

generating moderate to high cell densities in disposable RBs [19, 20]. Understanding the nature of oxygen transfer and response to fermentation parameters ensures appropriate bioreactor selection to achieve target yields.

In general, the oxygen transfer rate (OTR) is derived from the simple two film model in which resistance occurs across the thin film occurring on either side of the gas-liquid interface. Equation 1 is obtained by assuming mass transfer resistance is negligible on the gas side of the interface:

$$OTR = \frac{dC_L}{dt} = k_L a(C^* - C_L) \quad (1)$$

where $k_L a$, the volumetric mass transfer coefficient, is the product of the local mass transfer coefficient (k_L) and interfacial area (a); C^* is the bulk liquid saturation concentration of oxygen; and C_L is the bulk liquid concentration of oxygen versus time (t) [23]. Mass transfer is driven by the concentration gradient between the interface and bulk liquid. The concentration gradient is affected by cellular oxygen uptake and solubility, the latter being a function of temperature, salinity, and pressure [24]. For the STR, spargers generate gas bubbles which are reduced in size (increasing interfacial area for mass transfer) and distributed by mechanically driven stirrers (e.g., Rushton turbine, curved blade turbine, curved blade paddle, flat blade paddle, etc.). Considerable effort has been made to develop empirical correlations for $k_L a$ based on parameters such as power input per unit volume (P/V), liquid effective viscosity (μ_e), and superficial gas velocity (V_s) [24]:

$$k_L a = A V_s^a (P/V)^b \mu_e^c \quad (2)$$

The exponents found in Equation 2 vary significantly between studies, for example, [25-27], owing largely to experimental techniques used to measure $k_L a$ [27] and inherent difficulty in obtaining reproducible estimates. Alternatively, some correlations replace P/V with stirrer speed,

N [28-30], while others make use of dimensionless variables such as the Reynolds ($\rho NT/\mu_e$), Schmidt ($\mu_e/\rho D$), and Weber ($\rho N^2T^3/\sigma$) numbers to evaluate k_La via the Sherwood number, k_LaT^2/D, where T is the stirrer diameter, ρ is fluid density, D is the oxygen diffusivity in liquid, and σ is the interfacial tension [31-33].

A theoretical approach has also been used to determine k_La based on Higbie's penetration theory [34], which estimates k_L via the exposure time (t_e) for mass transfer represented by the ratio of the Kolmogorov length scale, $\eta = (\nu^3/\varepsilon)^{0.25}$, and fluctuation velocity, $u = (\nu\varepsilon)^{0.25}$, of turbulent eddies [35] where ν is the kinematic viscosity and ε is the energy dissipation rate of turbulence:

$$k_L = 2\sqrt{\frac{D}{\pi t_e}} = 2\left(\frac{D}{\pi}\right)^{1/2}\left(\frac{\varepsilon}{\nu}\right)^{1/4} \quad (3)$$

A theoretical basis for k_La determination is attractive due to inconsistency between empirical correlations obtained under similar conditions, and the simplified manner in which dependencies on reactor geometrical parameters are lumped together (i.e., constant A in Equation 2). Theoretical models will provide a better understanding of the relationships between process parameters and k_La, reducing the difficulty inherent to bioreactor scale-up, a complicated process which is highly dependent on maintaining acceptable mass transfer. For example, referring to Equation 2, dependence on V_s varies widely ($0.3 \leq a \leq 0.7$) although an increase in k_La with air flow rate is anticipated (over the range of conditions tested). On the other hand, ε and, in turn, k_L have an inverse dependence on V_s (aeration reduces power consumption relative to non-aerated systems), while interfacial area (a) increases with V_s [34]. Sensitivity analyses and optimization could enhance bioreactor performance and process efficiency, particularly when interactions between V_s and N are considered. Qualitatively, k_La is controlled by mechanical agitation for $N>N_c$ (agitation controlled), where N_c is the critical impeller speed, negating the impact of

aeration [29, 36]. Similarly, under vigorous aeration and $N<N_c$, agitation has a negligible effect on k_La (bubbling controlled) [29]. A previous study demonstrated that constants associated with V_s and N in standard correlations vary significantly across the intermediate regime. [29]. Consequently, operation in the intermediate regime (the most common situation) would be most efficient under optimized conditions based on consideration of expression host, sensitivity of product to oxidation, relative utility and process gas costs, etc. Obviously, this approach could be extended to disposable bioreactors as models become available. k_La values from select studies are presented in Table 2-2.

Table 2-2 - k_La data from selected studies of STRs

Reference	V_s **x10^3 (m/s)**	P/V **x10^{-3} (W/m^3)**	N **(s^{-1})**	D_T **(m)**	T **(m)**	V **(L)**	**Liquid**	μ_e **(mPa·s)**	k_La **(h^{-1})**
[25]	6.3	0.6-4.8	4.2-8.3	0.9	0.27	600	water	1.0	170-1613
	12.7	0.4-3.7							218-1228
[27]	5.0	1.0-6.0	---	---	---	9	water	1.0	125-220
	14.0	0.5-6.0							150-360
[29]	4.2	---	1.7-16.7	0.15	0.05	2.7	water	1.0	23-250
	11.3								60-360
	0.9-11.3		5.0						13-65
			13.3						100-200
[32]	3.8	---	5-10	0.25	0.1	12.3	glycerol-water	9.1	70-230
	2.0-8.0		6.7						100-150
	3.8		5.0-10.0				CMC (0.4 %)	---	15-180
[28]	0.6-2.1	---	5.0	0.12	0.05	2	xanthan gum	2.7	13-18
	2.1		5.0-6.7						20-30

As previously discussed, oxygen transfer in disposable RBs occurs through surface aeration via wave propagation at the gas-liquid interface. Although empirical correlations relating k_La to rocking rate, K (expressed as rocks/min, rpm), VD, working volume (WV), air flow rate, and P/V have not been formulated, general trends have been established for 1D and 2D RBs. During the introduction of the 1D RB, k_La was reported to depend weakly on K at low air flow rate ($\leq$ 0.05 vvm), while increasing significantly with rpm at 0.1 vvm [8]. The k_La increased moderately from 0.01 to 0.04 vvm, but increased dramatically from 0.04 to 0.1 vvm (2.7-fold). In another study of a modified GE Wave bioreactor fitted with a frit sparger, k_La increased marginally with K without headspace aeration, although for agitation below 25 rpm the effect of headspace aeration was less significant [17]. Similarly, increasing the gas flow rate from 0.25 to 0.5 vvm corresponded to a small increase in k_La (14%) at moderate rocking speeds [37]. Accordingly, the low gas flow capacity of 1D systems is not a limiting factor during cultivation of mammalian, plant, and insect cells at low to moderate K. However, increased gas flow capacity may provide significant improvements in oxygen transfer at high agitation, bolstering the potential of 1D RBs for aerobic microbial fermentation. As anticipated, k_La increased with VD, although K and VD were relatively low as mAB production in a myeloma/mouse hybridoma cell line was under investigation [38].

Similar trends in k_La were observed for K and VD in the CELL-tainer® compared to Wave bioreactor. k_La increased with K up to 35 rpm (maximum rpm tested) for 5, 10, and 15 L WV (20 L cultivation bag), exceeding values of 500 h^{-1} [20], and no significant increase in k_La was observed beyond 40 rpm for 15 L WV except under maximum VD [39]. Two key advantages of the CELL-tainer® relative to 1D RBs are the capacity to 1) process up to 75% of total bag volume (compared to 50% in 1D RB) while maintaining formidable k_La, and 2) handle

significantly higher gas flow rates, although k_La is not dependent on air flow rate [39]. Due to the complex nature of fluid flow in the CELL-tainer®, optimization of VD and *K* for a given WV may be prudent. During 10-fold scale-up of the CELL-tainer®, k_La maxima were observed for different combinations of rpm and WV [20]. It follows that selecting extremes of *K* for any WV may inhibit the k_La, while maximizing energy consumption.

To better understand the dynamics of fluid flow in RBs, one must consider the nature of wave development in shallow water.

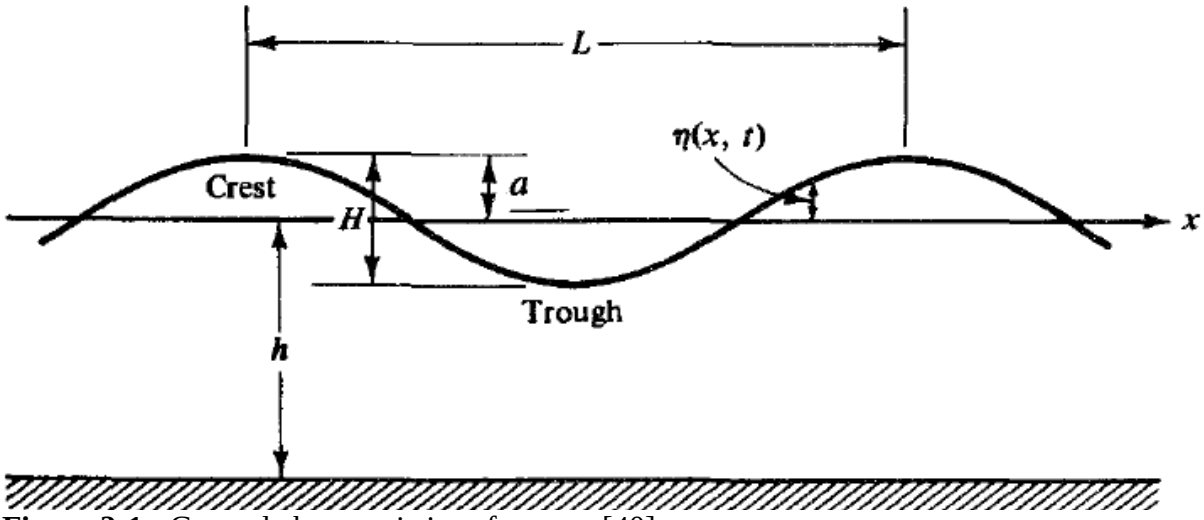


Figure 2-1 - General characteristics of a wave [40]

Referring to Figure 2-1, a wave of period τ, height *H*, and length *L* initially travels at speed $C = L/\tau$. As the wave travels into shore, the water depth beneath it (*h*) decreases, until the wave reaches shallow water based on the criterion, $h/L \approx 1/20$ [40]. In shallow water, wave speed is described by Equation 4:

$$C = \sqrt{g(h - mx)} \tag{4}$$

where *x* is positive in the direction of the shore and m is the slope of the shore [41]. *H* is inversely proportional to *C*, and as the wave slows down (i.e. as h decreases), *H* begins to

increase (a process known as shoaling) until the critical height is achieved. At this point, wave breakage occurs dissipating energy in the form of turbulence. The water depth at which breakage occurs (h_b) is approximated by Equation 5 [40]:

$$h_b = \frac{1}{g^{1/5}\kappa^{4/5}}\left(\frac{H_0^2 C_0 cos\theta_0}{2}\right)^{2/5} \quad (5)$$

where κ is a constant dependent on the slope of the shore (m), θ is the angle of incidence of the wave, and the zero subscript denotes properties in deep water. The breaking height (H_b) is simply, $H_b = \kappa h_b$ [40]. In general, H_0 and C_0 increase with τ [42], C and κ decrease with increasing m [40, 41], and τ depends on the total energy of the wave [42]. In the context of a disposable RB, C and κ decrease with increasing VD, and τ depends upon P/V which is dictated by WV, K, and VD. The bag geometry is fixed, and as a result, H cannot exceed an upper limit. Due to the increase in h across the length of the cultivation bag, and, in part, a restriction on H, C will tend to decrease more gradually as WV increases. Consequently, as WV increases the wave travels farther across the bag before breaking, approaching the end of the bag opposite inception for large WV. Delayed breaking of the wave reduces the extent of turbulent air entrainment, in turn, reducing mass transfer (and the k_La). This logic favors smaller WV to obtain the maximum k_La. A final point to consider is the total wave energy (per unit width), E, taken as the sum of a wave's potential and kinetic energies as per Equation 6 [40]:

$$E = \frac{1}{8}\rho g H^2 L \quad (6)$$

E could be used to estimate ε for a breaking wave which, in turn, could be used to determine k_L via Equation 3. This approach could be used in subsequent studies to obtain theoretical models of mass transfer in disposable RBs in the same way it has been applied to STRs [34]. Note that Equation 6 provides only an approximation of E as its derivation assumes the wave to be a non-dissipative system, and is based on linear wave theory which is most accurate in water of

intermediate depth [40]. In any case, it is clear that deeper investigation is required to elucidate the effects of operational parameters on mass transfer in RBs. While empirical correlations describing k_La would be a step in the right direction, the development of theoretical models or application of advanced computational methods (i.e. computational fluid dynamics, CFD) will greatly enhance process optimization and scale-up. Table 2-3 contains k_La values for disposable RBs obtained under different operating conditions.

Table 2-3 - k_La data from studies of disposable RBs

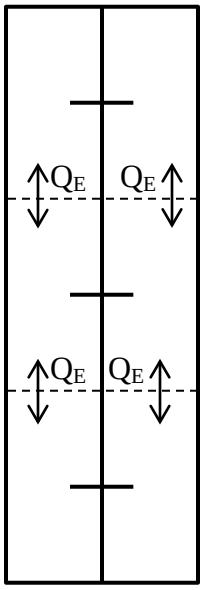
Reference	Type	Flow rate (vvm)	VD (°)	Rocking rate (rpm)	WV(L)	Bag volume (L)	Liquid	k_La (h^{-1})
[8]	1D	0.01	---	5-30	10	20	Water	0.6
		0.04						0.5-1.5
		0.10						0.7-4.0
[17]	1D	0.10	10	40	5	10	PBS, antifoam	38.0
							PBS	68.0
[38]	1D	0.05	4.5	8-13	2	10	---	1.6-3.0
			6	8				3.2
			5	8-10	3.5			2.3
[20]	2D	---	---	35	5-15	20	---	50-500
[39]	2D	---	8	24-48	15	20	water	40-200
			12	30-48				120-240
				24-48				60-295

2.3 Mixing intensity in bioreactors

The quality of bulk mixing in STRs can be expressed in terms of the axial dispersion coefficient (E_z), or the mixing time (t_m). [43]. E_z is an important design characteristic describing the extent of mixing, although t_m, representing the time required to reach a specified degree of homogeneity after pulse introduction, is considered more relevant in the field of biotechnology [44]. The distribution of substrate (i.e. oxygen, glucose, etc.), metabolites (i.e., lactate, acetate, ethanol,

etc.), and acid and base for pH control can significantly impact culture performance [45, 46]. Accordingly, in combination with k_La, t_m is a critical parameter used in the scale-up of industrial fermenters. As in the determination of k_La, Kolmogorov's theory of isotropic turbulence has been applied to models describing E_z in STRs [43, 44]. A relationship between E_z and t_m was established leading to the conclusion that, in the turbulent flow regime, the dimensionless mixing time (t_mN) is essentially constant for fixed reactor configurations [44], substantiating the results of previous studies [47]. However, relatively simple theoretical models of this nature do not adequately address the influence of aeration on t_m, as only an indirect dependence of ε on gas flow rate exists (as previously discussed in Section 2.3).

Empirical correlations have been developed for dimensionless mixing time in single stage (i.e. single agitator) unaerated systems, and for aerated systems operating above and below N_c [36]. In each case, agreement with experimental data was within 20-25%. More recently, the use of multi-impeller STRs of reduced diameter/height (D_T/H_T) ratios has become common practice, reducing the footprint of larger fermenters. Multi-stage agitation requires new approaches to hydrodynamic characterization given the increased complexity of fluid and gas dispersion. Currently, the Compartment Model Approach (CMA), an effective tool demonstrated in earlier studies of bioreactor scale-up [48, 49], is commonly used to evaluate mixing time in multi-stage STRs [50-55]. Studies in which CMA is applied vary in the assignment of reactor compartments. The simplest configuration consists of a single compartment per agitator stage [51, 52], where each compartment is treated as an ideally mixed vessel in series as per Equation 7:



$$V_k \frac{dC_k}{dt} + \sum_{j=k-1}^{k+1} (C_k - C_j) Q_E = 0 \quad (7)$$

Figure 2-2 - Schematic representation of single compartment (per stage) model for STR with three agitators (stages).

where V_k is the volume of compartment k; C_k is the tracer concentration in compartment k; and the exchange flow rate (Q_E) between compartments is the unknown parameter obtained through regression analysis [51, 52]. Although single compartment/stage models were considered adequate (as long as agitator spacing is $\geq D_T$), increasing the number of compartments (up to four/stage) improved model accuracy in another study of an unaerated system [50]. Additionally, other investigators have employed as many as 15 compartments per stage, plus an additional 10 compartments for the secondary circulation loop (induced by aeration) in the reactor top, for axisymmetric STR modelling. Sensitivity analysis indicated this configuration to be suitable for modeling overshoot response curves, and model estimates of t_m were in good agreement with experimental data (±5-15%) [54, 55]. As expected, more sophisticated models require estimation of an increasing number of flow parameters, e.g. the division of exchange flow to components of

circulation flow (Q_{CF}) due to mechanical power, axial turbulent exchange (Q_{TE}), and gas induced flow (Q_{GI}) resulting from density gradients due to gas hold-up [54].

As previously discussed, the accuracy of empirical models is often unsatisfactory for moderate variations in operating conditions and reactor configurations. While CMA is a useful tool for bioreactor design, it suffers from reliance on regression analysis of empirical correlations to obtain flow parameter estimates. A fundamental approach to reactor characterization is computational fluid dynamics (CFD), an advanced technique recently applied to modelling of aerated STRs [56-58] and disposable RBs [59]. CFD relies on the numerical solution of continuity equations for mass, momentum, and energy to simulate multi-phase flow systems [56]. In a recent CFD simulation of a single-stage aerated STR, excellent agreement was observed between experimental and predicted t_m (±5-10%) [58], demonstrating the utility of the theoretical approach to bioreactor modelling. It is anticipated that CFD will be the dominant method of bioreactor scale-up in the years to come.

A reduction in t_m with increasing N is intuitive and has been well established [36, 51, 55]. A pronounced effect on t_m was observed between impeller types in the absence of aeration. Axial flow impellers (Scaba type) were found to reduce t_m by half compared to Rushton impellers (radial flow) for the same specific power consumption [55], which was attributed to reduced zoning in the flow field of the axial impeller facilitating enhanced circulation and Q_E. When aeration is present, two opposing effects dictate power distribution in STRs: 1) reduced mechanical power from the agitator(s) due to gas hold-up (ρ declines), and 2) induced axial flow from rising air bubbles [53]. Earlier work demonstrated that below N_c, an increase in V_s reduces t_m to a point at which it levels off as flow rate increases [36]. For operation near N_c, t_m initially increases upon aeration, however, further increase in V_s reduces t_m until it eventually stabilizes.

Similar trends were observed in a later study in which drastic reductions in t_m were observed during aerated operation at low N (<150 rpm), relative to no aeration, after which t_m increased gradually with N approaching the unaerated condition [51]. The decrease in t_m at high V_s in the critical agitation region observed in the former study was most likely due to impeller flooding. A slight increase in t_m with aeration rate was observed prior to a sharp drop as flooding commenced [54]. Table 2-4 contains selected t_m values for STRs obtained under different operating conditions.

Table 2-4 - t_m data from selected studies of STRs

Reference	V_s **x10^3 (m/s)**	N **(s^{-1})**	D_T **(m)**	T **(m)**	V **(L)**	**Liquid**	**Agitator type**	t_{95} **(s)**
[36]	0-30	5	0.3	0.10	21.2	Water	FBDT	10.3-4.0
		10.6						5.5-7.5
		6.4					PBT	10.3-7.8
		14.3						5.0-6.5
[51]	0	2-6	0.29	0.1	76.6	Water	RT x 4	195-70
	3.8							30-75
	10.1	0-6						15-75
	0	2-6					PBT x 4	235-80
	3.8							20-70
	10.1	0-6						15-40
[60]	3.7	5-10	0.24	0.08	10.9	Water	RT	16-8
	0-7.4	6.7						10-13
		8.3						8-9.5
[53]	0-8.7	2.7	0.29	0.146	58.7	Water	RT x 3	82-12
		5.0						43-48
		10.0						21-28

FBDT: flat bladed disc turbine
PBT: pitched blade turbine
RT: Rushton turbine

As is the case for mass transfer studies, investigations of mixing characteristics in disposable RBs are limited in number. Upon introduction of the Wave Bioreactor, t_m of 5-10 s was reported for 20 L cultivations bags (10 L WV), while 200 L bags (100 L WV) could achieve

homogeneity in 60 s [8]. However, operating conditions (i.e. *K* and VD) were not fully specified providing only a rough estimate of expected performance. More detailed analysis of mixing in 1D RBs was performed in a later study of the GE Wave bioreactor [37]. A modified Reynolds number ($Re_{mod,1D}$) was derived by implementing correction factors *B*, accounting for the effects of bag geometry and VD on the volumetric flow rate (*Q*) of fluid, and *F*, relating $Re_{mod,1D}$ to the modified Reynolds number obtained for stirred reactors ($Re_{mod,str}$):

$$Re_{mod,1D} = \frac{QKBF}{15\upsilon(2y+W)} \tag{8}$$

where *y* is the liquid level in the bag and *W* is the bag width. The critical value of $Re_{mod,1D}$, i.e. for which turbulent flow ensues, was found to vary significantly with cultivation bag size, ranging from 400 to 1000. Application of moderate to high *K* (≥18 rpm) and VD (≥6°) was sufficient to ensure operation in the turbulent flow regime. Accordingly, t_m was independent of WV during operation under the specified conditions in an analogous manner to the STR, for which dimensionless mixing time was constant (with increasing $Re_{mod,str}$) in the turbulent flow regime for a particular reactor configuration. These findings indicate that adequate mixing intensity is achievable for moderate *P*/*V*; however, mass transfer remains the limiting factor for cultures of high oxygen demand. As anticipated, t_m was a strong function of *K* and VD [37].

Mixing characteristics were thoroughly evaluated in both the BIOSTAT® Cultibag RM (1D RB) and CELL-tainer® via response surface modelling of t_m as a function of *K*, VD, and WV [61]. Equations 9 and 10 were proposed for the BIOSTAT® Cultibag RM and CELL-tainer®, respectively:

$$t_m = -125.82K - 42.89VD + 135.65K^2 + 42.08K \cdot VD \tag{9}$$

$$t_m = -0.308K - 0.345VD + 0.179WV + 0.219VD^2 - 0.112K \cdot \mathrm{VD} \tag{10}$$

Adherent Vero cells were cultivated in the 1D RB first to establish conditions (corresponding to measured t_m) for which microcarrier suspension and homogeneity were acceptable. Process transfer to the CELL-tainer® was achieved using Equation 10 to establish *K* and VD (for 3 L WV) corresponding to t_m established in the 1D system. This approach was successful as cell density, microcarrier distribution, metabolite profiles, and virus production were comparable between 1D and 2D RBs. As in the previous study, WV did not significantly impact t_m in the 1D RB, as per the proposed model (Equation 9), although this parameter was included in the model for the CELL-tainer® (Equation 10). However, the full range of *K* was not tested in the latter case such that the CELL-tainer® may not have been operating in the fully turbulent flow regime. In a previously mentioned study, t_m estimation was conducted in both 20 and 200 L cultivation bags for different WV [20]. As in the previous study, t_m decreased with increasing *K* and a minor dependence on WV appeared to exist, although the latter observation is inconclusive based on the negligible difference observed between many of the data points and lack of available standard errors. The authors pointed out that the modified Reynolds number derived for the 1D RB ($Re_{mod,1D}$) may not be adequate in describing the hydrodynamics of the CELL-tainer® as the fluid is completely passing the point of rotation due to horizontal platform movement. In any case, further investigation of wave propagation, velocity distribution, and shear rates via advanced modelling techniques such as CFD would greatly improve our understanding of the complex hydrodynamics in 2D RBs. CFD has been successfully employed for this purpose in 1D RBs [59], and is proving to be an indispensable tool for bioreactor scale-up. Table 2-5 contains t_m data for disposable RBs:

Table 2-5 - t_m data for disposable RBs

Reference	Type	VD (°)	Rocking rate (rpm)	WV (L)	Bag volume (L)	Liquid	t_{95} (s)
[37]	1D	5	6-24	80	200	water	540-120
				100			880-120
		8	6-20	80			320-90
				100			390-90
		10		80			200-70
				100			240-70
[61]	1D	4	6-30	1	2	PBS	300-30*
		8	10, 16				200, 95*
	2D	8.5	15	5	20		65*
		17	5, 25				125, 20*
		8.5	5-25	10			200-50*
		17	15				70*
		8.5	15	15			150*
		17	5, 25				270, 45*
[20]	2D	---	15-35	5	20	water	15-9
				10			16-14
				15			20-14
			8-30	75	200		138-100
				115			140-66
			15, 30	150			114, 84

*t_{85}

2.4 Cultivation of E. coli for recombinant protein expression

E. coli is the organism of choice for industrial recombinant protein production due to 1) its superior growth characteristics in inexpensive media, 2) high productivity, 3) the extensive knowledge of its physiology, and 4) the availability of advanced techniques for genetic manipulation [4, 62, 63]. Obtaining high level protein expression in *E. coli* is a challenging endeavor, one that demands compromise between cell growth and functional protein expression. On the one hand, cell densities exceeding 200 g/L dry cell weight (DCW) have been obtained during dialysis fermentation of *E. coli* [64], while on the other, high level expression can result in yields of up to 50% of total cellular protein via strong promoters [65]. Cultivation conditions

facilitating optimal growth rarely favor high functional protein yields [63] such that process optimization is required to identify satisfactory conditions for both.

Process development for recombinant protein expression in *E. coli* requires optimization of media components, temperature, oxygen saturation, agitation, and induction conditions. Media tailoring is an essential step in bioprocess development as an appropriate formulation facilitates control of growth rate (μ), cell density, and metabolite formation [62, 66, 67], protein folding and solubility [68], and downstream purification of recombinant proteins [69]. Defined media containing essential nutrients such as ammonium, phosphorus, zinc, iron, and magnesium can significantly improve biomass yields relative to complex media (i.e. lysogeny broth) [62], while glucose limiting feeding strategies are used to moderate growth rate, in turn, reducing respiration and metabolite formation [62, 70]. Inclusion of the trace elements selenium, nickel, and molybdenum was shown to reduce formate accumulation in oxygen limited fed-batch cultures of strain W3110 through activation of the formate hydrogenylase (FHL) complex [67]. Additionally, higher growth rates were achievable prior to the onset of significant acetate accumulation in glucose-mineral salts media as compared to complex media (0.35 versus 0.2 h^{-1}), and the maximum specific acetate production rate was two-fold greater in complex media [71]. Inhibitory acetate levels seem to vary significantly between strains and in the presence of different carbon sources. One study of strain K12 found acetate to be growth inhibiting at concentrations as low as 0.5 g/L in glucose minimal media, while growth inhibition occurred above 1 g/L with glycerol as sole carbon source [72]. On the other hand, growth inhibition of recombinant MC1061 (K12 derivative) expressing human growth hormone (hGH) was observed once acetate reached 6 g/L in semi-defined glucose media [73]. Specific hGH production

decreased when acetate exceeded 2.4 g/L. As a general rule of thumb, however, acetate should be maintained below 2 g/L to avoid inhibition of growth and protein expression [62].

Cultivation of *E.* coli for recombinant protein expression is often conducted at suboptimal growth temperatures to facilitate protein folding [68], reduce proteolytic degradation [74], and enhance chaperone activity [75]. Penicillin acylase (PGA), a common industrial enzyme used in the production of semisynthetic penicillins and cephalosporins, is produced via fermentation of recombinant *E. coli* at temperatures of 25-30 °C to enhance translation and enable proper folding [76]. Particularly low optimal temperature (21°C) has been reported for functional expression of the κ-chain and Fd fragment of an antigen-binding fragment (Fab) possessing antibody activity against carcinoembryonic antigen [77]. While the growth rate of *E. coli* is significantly diminished at temperatures below 30 °C, expression of heterologous chaperones originating from a psychrophilic organism (*Oleispira Antarctica*) was shown to significantly increase growth rate at low temperature (< 15°C) [75]. Tailoring the temperature dependency of growth rate based on optimal protein expression conditions could be an attractive genetic strategy in the future.

Critical DO in *E. coli* fermentations, based on the criterion $\mu = 0.97\mu_{max}$, was reported as relatively low (0.12 mg/L) [78], although in practice DO is maintained well above critical levels to minimize metabolite formation [64, 79]. DO is controlled through a cascade of agitation rate and gas flow composition (oxygen/air) and volume, and has been used as feed indicator in fed-batch cultivations [80]. Mass transfer in high cell density (HCD) cultures is a limiting factor due to the poor solubility of oxygen in aqueous media and high oxygen demand of fully developed cultures. Fed-batch strategies in which the carbon source remains low to reduce oxygen requirement are commonly employed to offset inadequate oxygen transfer [81]. While average

oxygen levels in a bioreactor are typically not excessive, local concentrations can be markedly elevated in oxygen-enriched cultures, particularly in the vicinity of the sparger(s) [82]. Oxidative stress from exposure to reactive oxygen species (ROS) such as H_2O_2, O_2^-, and $OH^\cdot$ can result in DNA instability, and protein and lipid denaturation [83]. Previous studies have demonstrated that the effects of excess DO on the growth of *E. coli* are strain dependent. Marginal reductions in the growth rates of strains TB-1 [84], W3110 [85], and MG1655 [86] were observed in oxygen-enriched culture, while the growth rate of JM101 declined significantly [84]. Moreover, excess DO can reduce the yields of certain recombinant proteins via oxidation of Met and Cys residues [83], resulting in misfolding, loss of activity, and protease degradation [87, 88]. Reduced yields have been observed for recombinant hGH [85], p24Gag (human immunodeficiency virus-1 protein) [89], and monoamine oxidase (MAO) [90] in oxygen-enriched *E. coli* fermentations.

Selection of induction conditions (i.e. inducer concentration and induction timing) depends on the structure of recombinant DNA, expression host, and type of desired product. For inducible expression systems, e.g. those derived from the *lac* operon of *E. coli*, promoter strength, translational efficiency, and posttranslational processing capacity dictate optimal inducer concentration [91, 92]. Plasmid copy number is usually complementary to promoter strength [91], although copy number can be manipulated to tailor gene dosage [93]. A strong promoter is most beneficial when structural components such as ribosome binding site (RBS), start codon, and spacer between RBS and start codon are optimized [94] reducing bottlenecks associated with translation. Strong promoters coupled with excessive inducer levels may cause severe insoluble protein aggregation (inclusion bodies) resulting in reduced soluble protein production and cell lysis [92]. Moreover, optimal inducer concentrations vary widely for different expression systems (i.e. strain-vector-promoter combinations) intended for the same

protein, such that inducer optimization is required upon modification of expression vector or strain. For example, in strain JM109, specific PGA activity was highest upon induction of the *trc* promoter with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), while specific activity peaked at 0.2 mM IPTG for T7 driven expression (peak specific activity was within 7%) [95]. Differences in posttranslational processing efficiency (i.e. translocation, proteolysis, and folding) between strains also influences extent of induction [92], although coexpression of cytoplasmic chaperones may improve posttranslational maturation, in turn, reducing inclusion body formation [96]. In general, for *lac* derived promoters, it is generally accepted that IPTG need not exceed 1 mM for full induction of protein expression [97]. Induction timing depends on the type of recombinant protein expressed (i.e. growth, mixed-growth, or non-growth associated) [76, 98] and the extent of growth arrest upon induction [97]. Induction during exponential phase (i.e. at high growth rate) is optimal for expression of growth-associated proteins [98], although for mixed-growth associated products induction could be delayed until early stationary phase, potentially improving yields via increased biomass accumulation during uninduced exponential growth [76]. Accordingly, induction during early stationary phase at maximum cell density is ideal for non-growth associated protein production.

Chapter 3 - Application of two-dimensional disposable rocking bioreactors for recombinant protein production in *Escherichia coli*

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The research presented in this chapter was submitted for publication to the Biochemical Engineering Journal. Declaration: I initiated and conducted all experiments presented herein under the supervision of Dr. C. P. Chou, Dr. J. Scharer and Dr. M. Moo-Young. Dr. Oosterhuis was consulted as an expert on fermentation, and bioreactor characterization and development.

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3.1 Introduction

Due to the highly competitive nature of today's biopharmaceutical market and the high failure rates associated with biopharmaceuticals [5], flexible and cost-effective manufacturing facilities are prerequisite for survival of biopharmaceutical producers. Over the last decade, disposable bioreactors have become integral components in the production of many high-value biopharmaceuticals [6]. Advantages of disposable bioreactors compared to conventional tank reactors include high flexibility, reduced occurrence of cross-contamination, lower capital investment, reduced labor costs associated with validation and cleaning, and shorter turnover times between production runs [5, 10]. Disposable bioreactors are classified by mode of power input as mechanically driven (wave-mixed, stirred, orbitally shaken or vertically oscillating), pneumatically driven, or hybrid systems [10].

Wave-mixed RBs were introduced in the late 1990s [8], and are commonly employed in the production of biopharmaceutical products (e.g. monoclonal antibodies, vaccines, therapeutic and diagnostic proteins, etc.) in mammalian [13, 15], plant [99], insect [16], and human cell cultures [100]. These cultivation systems typically consist of an oscillating or sectional platform, supporting one or more pre-sterilized cultivation bags made of biologically inert polymers such as polyethylene, polypropylene, or polytetrafluoroethylene. Temperature is controlled through the moving platform or external cabinet. Bags are equipped with disposable or multiuse online pH and DO sensors. Gas-liquid mass transfer occurs through surface aeration and turbulent air entrainment via wave propagation and is controlled by adjustment of VD and rocking rate. While reports of cultivation of microorganisms in disposable RBs [17-20] exist, it is commonly assumed that these disposable systems are not capable of meeting the high oxygen demand of microbial cultures. Towards the end of 2011, 66 out of 211 biopharmaceuticals receiving

regulatory approval in the United States and European Union are produced in *Escherichia coli* while mammalian cells represent the most common host system for biopharmaceutical production [1]. However, few reports exist on the application of disposable RBs to recombinant therapeutic protein production in *E. coli*.

The oscillation trajectory for most RB platforms is 1D, limiting the extent of wave development and, in turn, the efficiency of mass transfer. The CELL-tainer®, a RB with an innovative design of 2D oscillation trajectory, moves around the axis of rotation in a closed loop facilitating simultaneous vertical and horizontal displacement [61]. 1D systems tilt along a central pivot axis which only a portion of the fluid is able to pass with each oscillation [59]. In the CELL-tainer®, the fluid completely passes the center of the bag due to the additional horizontal movement, which may partially explain increased mass transfer due to additional film formation along the bottom of the bag [20]. Additionally, the CELL-tainer® provides more efficient mass transfer with respect to specific power input, and is capable of processing larger WVs and higher gas flow rates compared to 1D RBs [39]. Previous studies of oxygen transfer efficiency in 1D RBs report k_La values in range of 38 to 55 h^{-1} [17, 19], while k_La exceeded 500 h^{-1} in the CELL-tainer® at maximum WV [20]. In this study, the CELL-tainer® was used for recombinant therapeutic protein production in *E. coli* and the culture performance was compared with the traditional stirred-tank reactor (STR) and 1D RB. The target protein of GST-hCD83ext is a protein fusion of glutathione *S*-transferase and the extracytoplasmic domain of human CD83 (hCD83ext), and the bioprocess for its expression and purification was previously developed [63]. We specifically investigated oxygen transfer efficiency and mixing intensity of 1D and 2D disposable RBs to evaluate performance relative to the STR. RBs are believed to provide an environment suitable for shear-sensitive and fragile microbial recombinant cells. Batch growth

characteristics, glucose consumption, and metabolite profiles of non-recombinant *E. coli* were compared to elucidate suitability of RBs for cultivation under typical culture conditions. Also, culture performance for recombinant GST-hCD83ext expression was evaluated under previously optimized culture conditions, and soluble and insoluble fractions of cell lysates were analyzed to assess titer and inclusion body formation.

3.2 Materials and methods

3.2.1 Physical characterization and OUR estimation

10 L and 20 L cultivation bags were used for physical characterization and cultivation in the 1D and 2D (CELL-tainer®, CELLution Biotech, The Netherlands) disposable RBs, respectively. Different WVs were tested under conditions providing maximum oxygen transfer, i.e. 40 rpm and 12° VD (1D RB), and 40 rpm and 17° VD (CELL-tainer®). Trials were conducted at 28 °C and 0.4 vessel volume per minute (vvm) aeration rate. Measurements of k_La were performed using the dynamic "gas out-gas in" method [101]. The cultivation chamber was filled with an appropriate volume of deionized water, which was subsequently stripped of oxygen by nitrogen purging until the DO level fell below 5% of air saturation. The headspace was evacuated with a vacuum pump (GAST, Michigan, USA) and then filled with air. Once the headspace was full, agitation resumed and DO measurements were recorded at appropriate time intervals. An optical DO sensor was used without the oxygen permeable membrane resulting in a time constant of < 6 s. k_La estimates were obtained from the mass balance equation:

$$ln\left(\frac{DO^*-DO(t)}{DO^*-DO(t_0)}\right) = -k_La(t-t_0) \quad (11)$$

where DO^* is the saturation reading of the probe. The volumetric oxygen uptake rate (OUR) was measured during exponential growth by temporarily stopping the supply of gas to the cultivation

chamber, evacuating the headspace, and recording DO measurements upon resuming platform movement. The following mass balance equation was used to estimate the volumetric OUR:

$$C_{O_2,l}(t_0) - C_{O_2,l}(t) = OUR(t - t_0) \quad (12)$$

where $C_{O_2,l}$ is oxygen concentration in the liquid phase (approximated using solubility data and DO measurements). t_m was evaluated by adding an acidic tracer (HCl) to the system at a steady-state pH, and estimating the time required to achieve 95% of the pH set-point change (t_{95}). Experimental conditions were identical for k_La and t_m estimation. k_La, OUR, and t_m measurements in the 1 L STR (Omni-Culture, VirTis, NY, USA) were performed similarly, except that headspace evacuation and filling was not required, enabling continuous operation. For the STR, tank diameter = 10 cm, stirrer diameter = 5 cm (2 × 6 flat blade paddle), liquid height = 12 cm, and agitation was maintained at 600 revolutions per minute (rpm). All other conditions were maintained the same as those of the disposable RBs.

3.2.2 Bacterial strains and plasmids

E. coli BL21 (F^- *ompT gal* [*dcm*] [*lon*] *hsdS*$_B$ (r_B^- m_B^-) λ(DE3)) was used to investigate growth characteristics. Plasmid pGEX2ThCD83ext containing hCD83ext cDNA fused to the *gst* gene, served as expression vector for production of recombinant GST-hCD83ext under control of the strong *tac* promoter. BL21 containing plasmid pLysS harboring the gene encoding T7 lysozyme was used as the expression host to minimize leaky expression [102].

3.2.3 Cultivation

Cells were maintained as glycerol stocks at -80 °C and revived on LB agar plates (10 g/L NaCl, 5 g/L Bacto yeast extract, 10 g/L Bacto Tryptone, 15 g/L Bacto Agar) supplemented with

ampicillin (50 mg/L) and chloramphenicol (17 mg/L) as needed. Selected colonies were transferred to LB shake-flasks and grown for 16 h at 225 rpm and 37 °C in an orbital shaker (New Brunswick Scientific, New Jersey, USA) to generate the seed culture. *E. coli* BL21 was cultivated in LB broth supplemented with 20 g/L glucose and 15 µL/L Antifoam 204 (Sigma Aldrich). Cultivations were performed at 30 °C, 0.4 vvm aeration rate, 40 vol% O_2, 40 rpm and 12° VD (1D RB), 40 rpm and 17° VD (CELL-tainer®), and 600 rpm (STR). pH was maintained at 6.8 ± 0.1. Recombinant BL21(pGEX2ThCD83ext, pLysS) was cultivated in modified LB broth (5 g/L NaCl, 20 g/L Bacto yeast extract, 20 g/L Bacto tryptone) supplemented with 5 g/L glucose, 15 µL/L Antifoam 204, and 50 mg/L ampicillin. The same cultivation conditions were used as for non-recombinant BL21 except for small variations in temperature (28 °C) and pH (7 ± 0.1). When the cell density reached 1.5-1.8 OD_{600}, isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM for induction of protein expression.

3.2.4 Sample preparation and analysis

To obtain cell extract, cells in the amount of 30 OD_{600}-units (defined as the product of OD_{600} and sample volume in mL) were centrifuged at 8,000g and 2 °C for 10 min. The supernatant was collected and filter sterilized for analysis of glucose and metabolites using a Shimadzu LC-10AT HPLC with RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA). The cell pellet was resuspended in 1.5 mL phosphate-buffered saline (PBS) and sonicated intermittently (0.5/0.5 s on/off) for 2 min with an ultrasonic liquid processor and microtip (Sonicator 3000, Misonix, NY, USA). The lysate was centrifuged at 18,000g for 15 min at 2 °C for cell debris removal and the supernatant containing soluble GST-hCD83ext was used for GST assay and SDS-PAGE. The pellet containing insoluble

fraction and cell debris was washed once with PBS, and resuspended in TE/SDS buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS) upon boiling for 5-10 min. The solubilized pellet was analyzed using SDS-PAGE as the insoluble fraction. GST assay and SDS-PAGE were performed as previously described [63].

3.3 Results and discussion

3.3.1 Physical characterization

Operating conditions providing maximum oxygen transfer (i.e. rocking rate and VD) were established for the 1D RB and CELL-tainer®, and subsequently used to evaluate k_La, t_m, nonrecombinant growth characteristics, and recombinant protein expression. A comparison of k_La values over a range of WVs is presented in Figure 3-1. Oxygen transfer efficiency was a linear function of WV in the 1D RB, presenting similar k_La values to the STR for 1 L WV. Under these conditions the immersion depth of the DO probe was minimal as the platform tilted away from the side of the bag in which the probe was inserted. However, excellent linearity was obtained when applying Equation 1 to obtain k_La estimates (R^2>0.99). Similar k_La values were obtained previously for 1D RBs operating at maximum WV [17, 19]. An inherent limitation of the 1D RB was the low gas flow capacity due to the type of outlet filter provided, and the inability of the heated filter jacket to adequately dry the filter [17]. Enhanced gas flow capacity may improve oxygen transfer efficiency in 1D RBs under high agitation. Increasing the gas flow rate from 0.25 to 0.5 vvm corresponded to a 14% increase in k_La at moderate rocking speeds [37]. However, k_La was weakly dependent on headspace oxygen transfer at low to moderate rocking rates while the opposite effect was observed at maximum agitation [17].

An optimal WV of 3 L was observed for the CELL-tainer®, reaching k_La values of nearly 400 h^{-1}, while maintaining formidable oxygen transfer capacity over all tested conditions (Figure 3-1). Our data is in range of recently published k_La measurements [20], although discrepancies exist with regard to optimal WV. A reduction of k_La with increasing WV is intuitive considering the nature of wave development in shallow water. The wave propagates from the corner of the bag, shoaling across the length of the bag until the critical height is achieved, resulting in wave breakage [40]. As water depth increases with WV, the wave travels farther across the bag before slowing down and breaking. Consequently, wave breakage occurs increasingly near the end of the bag opposite inception, in turn, reducing the extent of turbulent air entrainment. This rationale supports our observations of low optimal WV for both disposable systems. Moreover, an earlier study of mass transfer in the CELL-tainer® reported k_La values at 15 L WV that were approximately 60% lower compared to the recently published data [39]. Finally, reduction in k_La with increasing WV cannot be solely attributed to reduced specific power input given the sharp reduction in k_La observed at maximum rocking rate in the CELL-tainer® (Figure 3-2). Optimal rocking rates resulting in the maximum k_La at different WVs has been reported elsewhere [20].

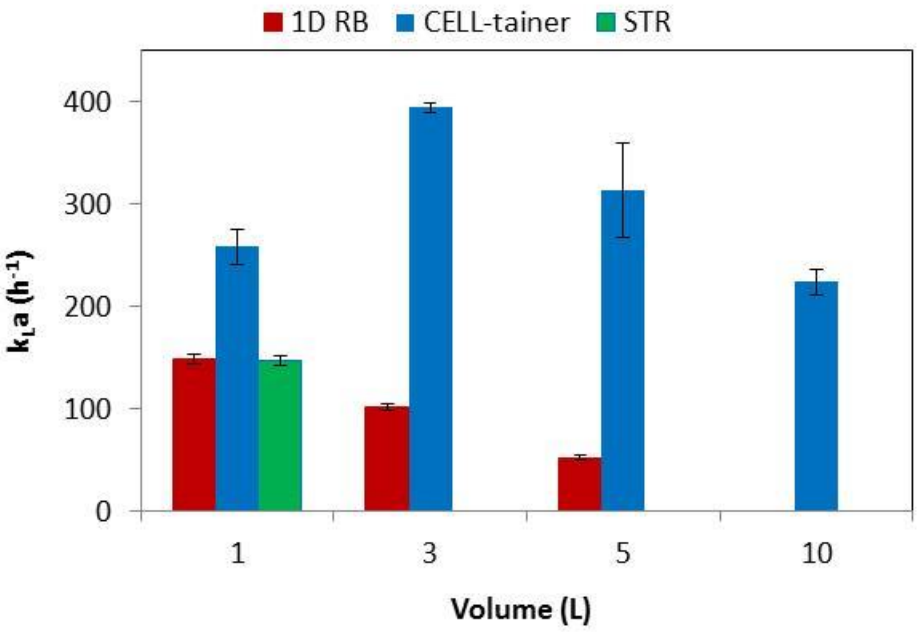

Figure 3-1 - Results for comparative k_La analysis. Experimental conditions described in M&M. Standard deviations of three experiments are included.

k_La values increased with increasing rocking rate in the 1D RB up to the maximum setting of 40 rpm (Figure 3-2). In contrast, a marginal difference was observed between 40 and 45 rpm in the CELL-tainer® after which k_La values decreased at the maximum setting of 50 rpm (Figure 3-2). Maximum rocking rates may prove beneficial for WVs exceeding 10 L as an increase in k_La was obtained beyond 40 rpm at 15 L WV [39]. An advantage of 2D RBs, such as the CELL-tainer®, over 1D systems is the capacity to process WVs of up to 75% of the total bag volume while maintaining high mass transfer [39]. Finally, k_La values were consistent with those previously reported for lab-scale STRs [27, 29, 32]. Although atypically low air flow rates were employed due to the limitations of the 1D RB, a significant increase in k_La is not expected at higher air flow rates as the STR was operating well above the critical impeller speed (approximately 290 rpm for this system) [36].

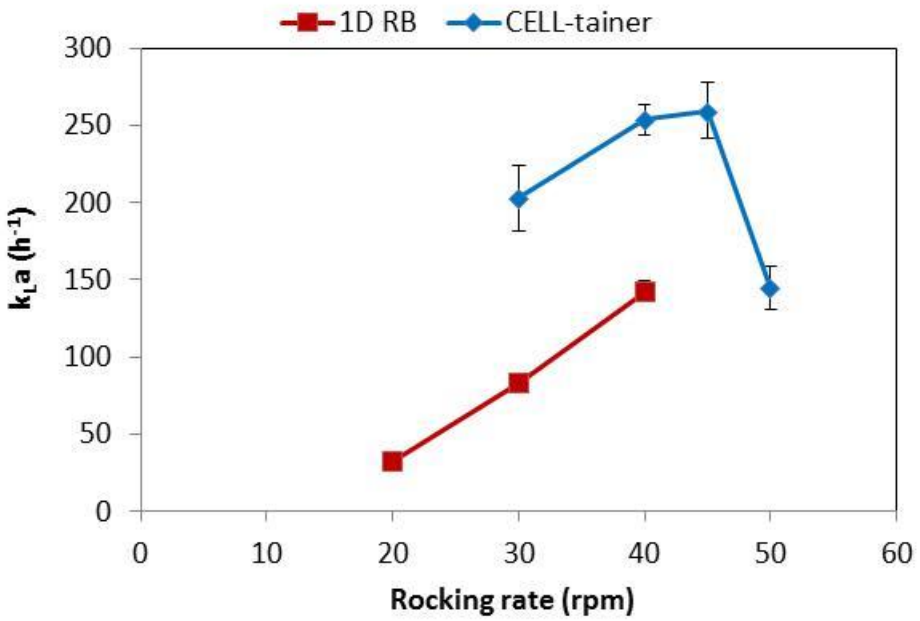

Figure 3-2 - Results for comparative k_La analysis. 5 L and 2.5 L WV in 1D RB and CELL-tainer®, respectively. Experimental conditions for 1D RB: 20 L cultivation bag, 35 °C, 0.1 vvm air, and 12° VD. Experimental conditions for CELL-tainer® described in M&M. Standard deviations of two experiments are included.

Effective mixing was observed in all three systems investigated using water and CMC solution (Figure 3-3). t_m estimates were consistent with previously reported data using 1D RBs [8, 37]. A strong dependence on WV was observed at low rocking rates, although, consistent with our observation, t_m was independent of WV as rocking rate increased [37]. For the CELL-tainer®, our t_m results were in good agreement with recent studies [20, 61], in which minimal dependence of t_m on WV was observed. In addition, our t_m estimates were in good agreement with previous studies of mixing intensity in lab-scale STRs [36, 60]. Comparison of t_m associated with water and aqueous CMC allows qualitative comparison of the relative shear stress applied

in a reactor due to the pseudoplastic nature of CMC. t_m of water was shorter than that of CMC solution for the 1D RB, while the opposite trend was observed in the CELL-tainer® (Figure 3-3). This suggests that greater shear stress may be applied to the fluid in the CELL-tainer® relative to the 1D RB under experimental conditions, as t_m is often a function of liquid viscosity [103, 104]. For STRs, it is well known that local shear stress at the impeller tip is significantly higher than the average value [105], and therefore is expected to exceed that of disposable RBs. Shear stress is typically not a concern when cultivating *E. coli* [106], although it has been implicated in the reduction of product formation in recombinant *E. coli* [107, 108]. In such a case, one may benefit from reducing the rocking rate without sacrificing adequate oxygen transfer efficiency in RBs.

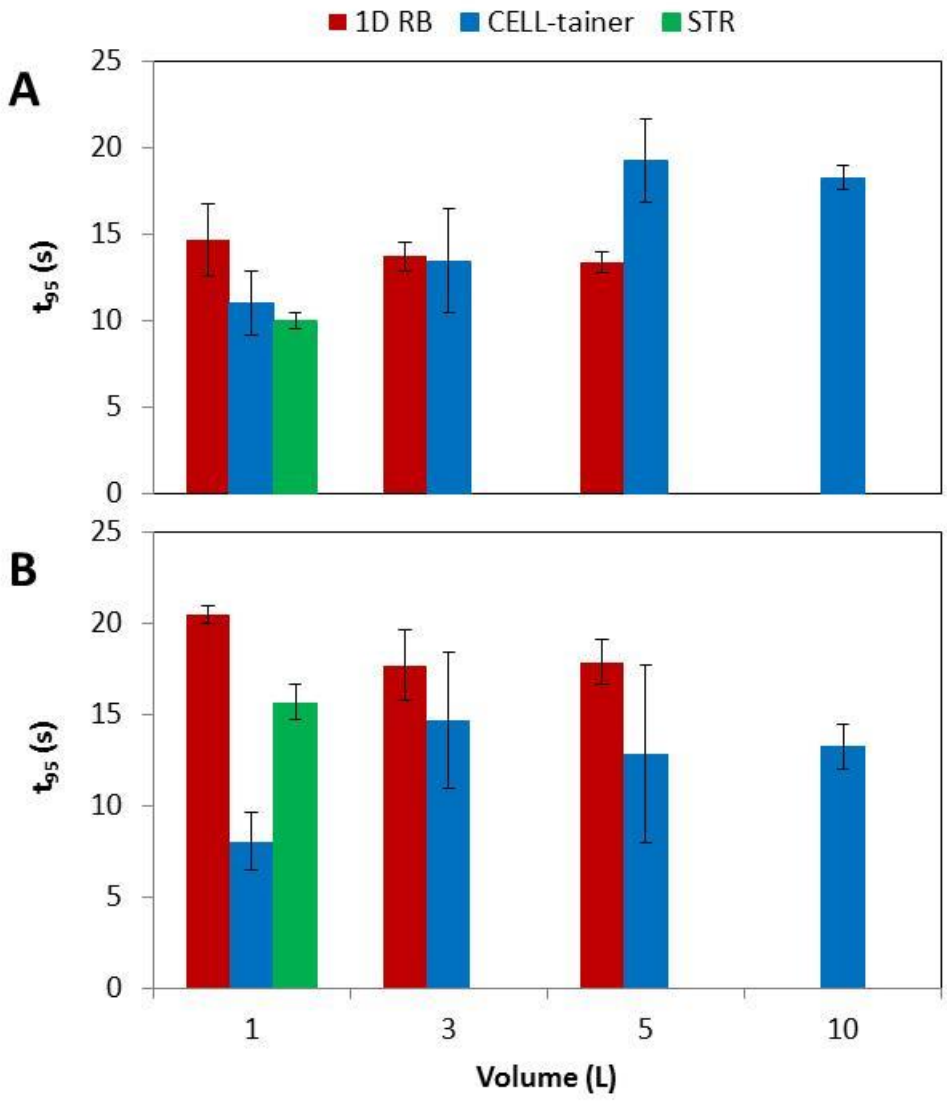

Figure 3-3 - Results for comparative t_m analysis. Experimental conditions described in M&M. DI water (Panel A) and 0.5% CMC (Panel B). Standard deviations of three experiments are included.

3.3.2 BL21cultivation and GST-hCD83ext expression

In addition to evaluating cultivation systems under typical operating conditions and large WV, it was interesting to compare culture performance between the STR and 1D RB under comparable k_La values (Figure 3-4). A steep decline in DO during early exponential phase resulted in oxygen limitation which, in turn, caused significant undesired metabolite accumulation and retarded cell growth. Interestingly, the maximum specific growth rate (μ_{max}) for STR cultivation was 18.5% higher than that for 1D RB (0.88 vs. 0.74 h^{-1}) although, as shown in Table 3-1, oxygen uptake was typically more effective in the 1D RB in the higher DO range (i.e. 50-100% saturation). The lower specific OUR measured in the STR in this range can be partially due to surface aeration from residual oxygen in the headspace, which can contribute significantly to oxygen transfer in smaller vessels [109].

Note that the influence of antifoam addition on culture performance should be considered (Figure 3-4). The k_La value decreased by 45% upon addition of antifoam in a recent study of yeast cultivation in the GE WAVE Bioreactor® [17], while a smaller reduction (30%) was observed in an STR [110]. These findings are not surprising considering the mode of oxygen transfer in RBs, and explain, in part, the difference in DO profiles observed in Figure 3-4. Increasing culture viscosity was shown to inhibit wave development in the BIOSTAT® CultiBag RM [111], although viscosity likely had minor influence on oxygen transfer at low cell density. Consequently, the reliability of k_La estimation at low WV due to superficial probe immersion depth was, once again, called into question. Based on our observations, and lack of available data for comparison of k_La at minimum WV, further investigation is required to identify the cause(s) of severe oxygen limitation under experimental conditions.

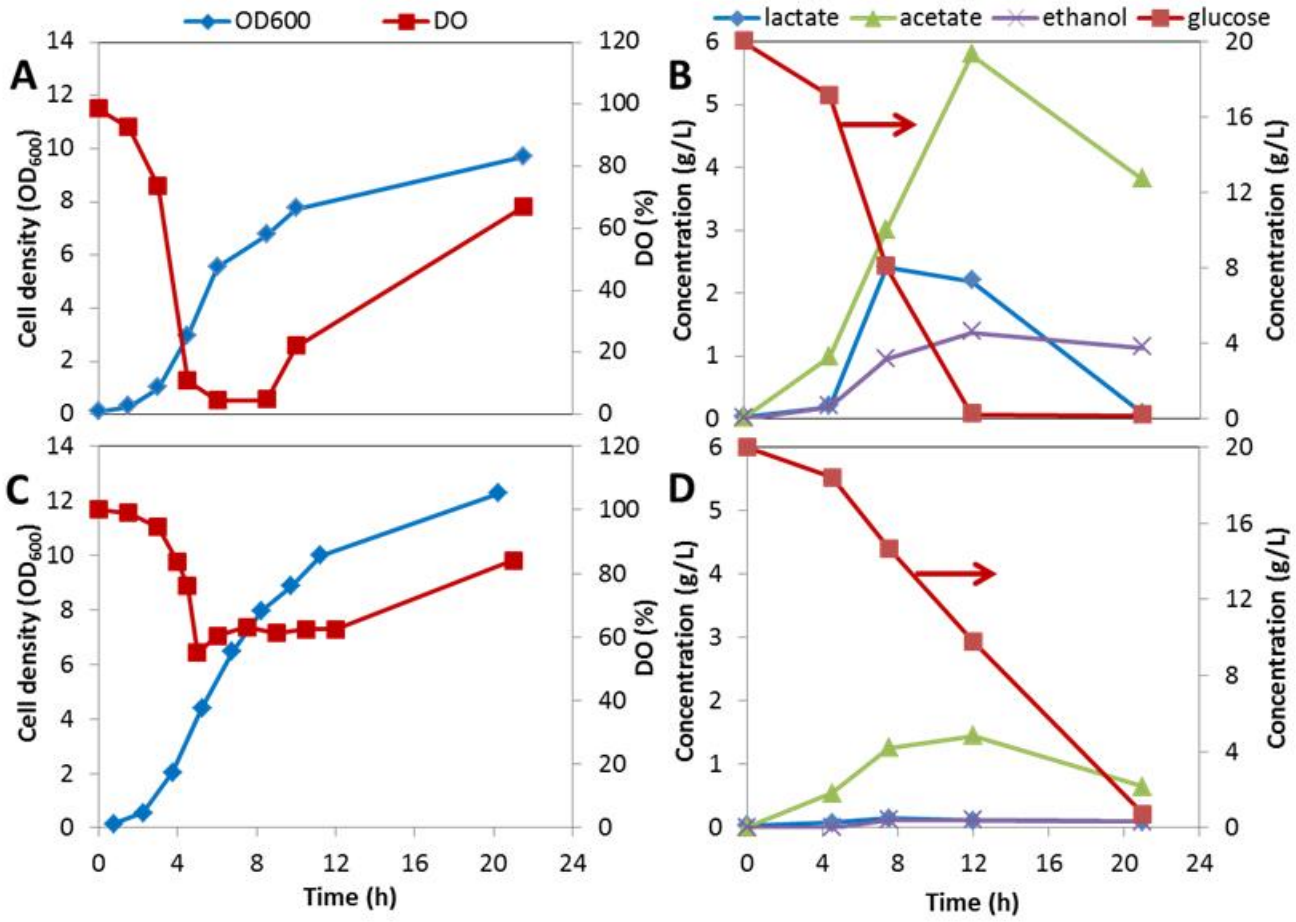

Figure 3-4 - Typical results for cultivation of *E. coli* BL21 in the 1D RB (Panels A and B) and STR (Panels C and D). Cell density and dissolved oxygen (Panels A and C), and metabolite and glucose profiles (Panels B and D). 1 L WV in the 1D RB and 0.4 vvm air. All other cultivation conditions described in M&M.

Table 3-1 - Typical results for cultivation of *E. coli* BL21 in the 1D RB, CELL-tainer®, and STR. 5 L and 10 L WV in 1D RB and CELL-tainer®, respectively. All other cultivation conditions described in M&M. Maximum specific growth rate (μ_{max}) was observed during the first 5-6 h of cultivation. Minimum and maximum specific OUR measured over DO range of 20-50% and 50-100% saturation, respectively. Linear regression errors are included.

System	μ_{max} (h^{-1})	Minimum specific OUR ($mg\ O_2 \cdot L^{-1} \cdot min^{-1} \cdot OD_{600}^{-1}$)	Maximum specific OUR ($mg\ O_2 \cdot L^{-1} \cdot min^{-1} \cdot OD_{600}^{-1}$)
1D	0.66 ± 0.01	0.93 ± 0.02	1.95 ± 0.02
CELL-tainer®	0.67 ± 0.02	1.64 ± 0.05	1.64 ± 0.05
STR	0.70 ± 0.02	1.00 ± 0.02	1.35 ± 0.04

The results of full-scale non-recombinant *E. coli* cultivation are presented in Figure 3-5. As expected, oxygen limitation ensues quickly in the 1D RB after which DO is rescued temporarily upon increasing inlet oxygen supply to 60 vol% at 8.5 h. Cell density was not significantly affected under oxygen limitation during which acetate titer reached 6.9 g/L after only 11 h. Comparing Figures 3-4b and 3-5b, markedly different glucose consumption patterns were evident, indicating improved oxygen availability for 5 L cultivation. This is not unexpected as a 2.5-fold increase in available oxygen could offset the 65% decrease in the k_La from 1 to 5 L WV [17]. Moreover, the accumulation of various metabolite byproducts (including acetate, lactate, and ethanol) was higher after 12 h cultivation at 1 L WV as compared to the 5 L batch. Cultivation in the CELL-tainer® produced completely different DO and metabolite profiles, although only slight differences in μ_{max}, specific OUR, and cell density were observed. Acetate production was merely 2 g/L and all other metabolites were essentially undetectable, corresponding to DO exceeding 110% saturation for the entire culture. Comparing Figures 3-5d

and 3-5f, glucose consumption occurred at a slower rate during cultivation in the CELL-tainer® and faster biomass accumulation was observed in the STR, potentially due to higher oxygen availability in the CELL-tainer®. Previous studies have demonstrated that the effects of excess DO on the growth of *E. coli* are strain-dependent. For examples, strains TB-1 [84], W3110 [85], and MG1655 [86] exhibited minimal change in growth rate in oxygen-enriched culture. In contrast, in accordance with our observations of BL21, the growth rate of JM101 declined at high DO [84]. Up to approximately 7 h of cultivation, the growth was similar in the STR and CELL-tainer®, after which biomass accumulation slowed as DO increased in the CELL-tainer®, while growth rate was maintained with decreasing DO in the STR.

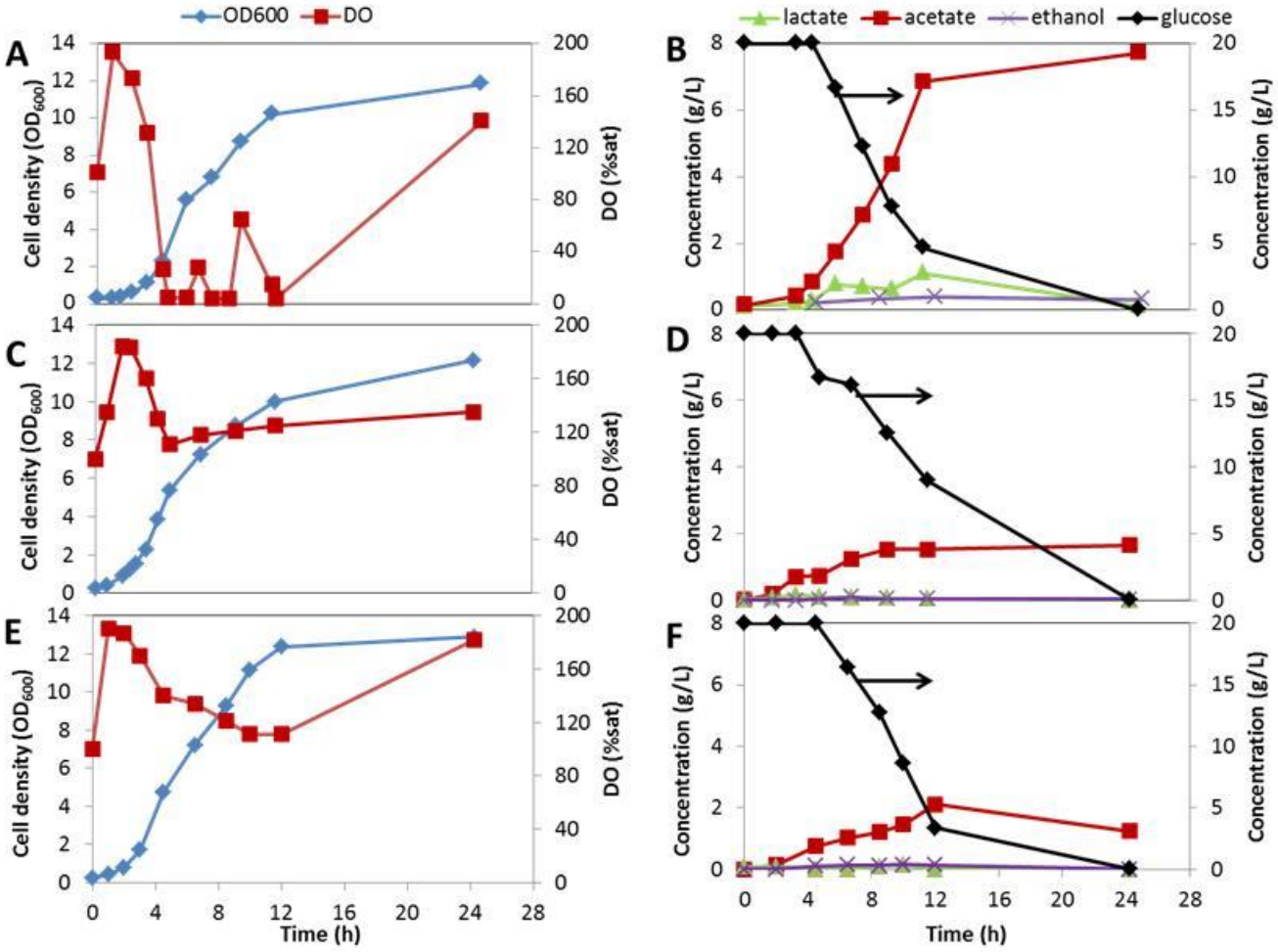

Figure 3-5 - Typical results for cultivation of non-recombinant BL21 in the 1D RB (Panels A and B), CELL-tainer® (Panels C and D), and STR (Panels E and F). Cell density and dissolved oxygen (Panels A, C, and E), and metabolite and glucose profiles (Panels B, D, and F). 5 L and 10 L WV in the 1D RB and CELL-tainer®, respectively. All other cultivation conditions described in M&M.

Our investigation reveals that the CELL-tainer® is suitable for batch operation at a relatively high growth rate, while the mass transfer capacity of the 1D RB was inadequate under current experimental conditions. Although growth characteristics were quite similar, acetate accumulation in the 1D RB exceeded levels shown to significantly reduce growth rate [72],

potentially compromising cell physiology. The use of the GE WAVE Bioreactor® in generating seed culture for a production fermenter was demonstrated at reduced growth rate (0.42 h^{-1}), resulting in delayed oxygen depletion relative to our study [18]. Additionally, fed-batch cultivation has proven feasible in the BIOSTAT® CultiBag RM, yielding a moderate cell density of 20 g/L dry cell weight (dcw) at 5 L WV [19]. A higher biomass yield (42.8 g/L dcw) was obtained in 12 L fed-batch cultivation in the CELL-tainer® and 10-fold scale-up resulted in similar cell density (45 g/L dcw) even though gas flow was only 10% of that employed in 12 L culture due to prototype limitations [20].

As shown in Figures 3-6 and 3-7, culture performance was rather comparable in all bioreactors. SDS-PAGE confirmed the presence of the GST-hCD83ext fusion (Figure 3-7) and corroborated the trends observed for specific GST activity (Figure 3-6). Previously, it was reported that specific GST activity peaked at approximately 6 h post-induction coinciding with accumulation of the insoluble GST-hCD83ext [63]. Our current results generally agree with these observations, although the formation of insoluble GST-hCD83ext appeared less significant. The increase in specific GST activity and decrease of insoluble protein could be, in part, due to the efficacy of cell lysis at reduced sample concentration. Interestingly, during CELL-tainer® cultivation specific GST activity increased slightly from 6 to 8 h post-induction, resulting in a 16% increase in volumetric GST activity. Specific and volumetric GST activities were similar between disposable RBs, although the specific GST activity peaked earlier in the 1D RB.

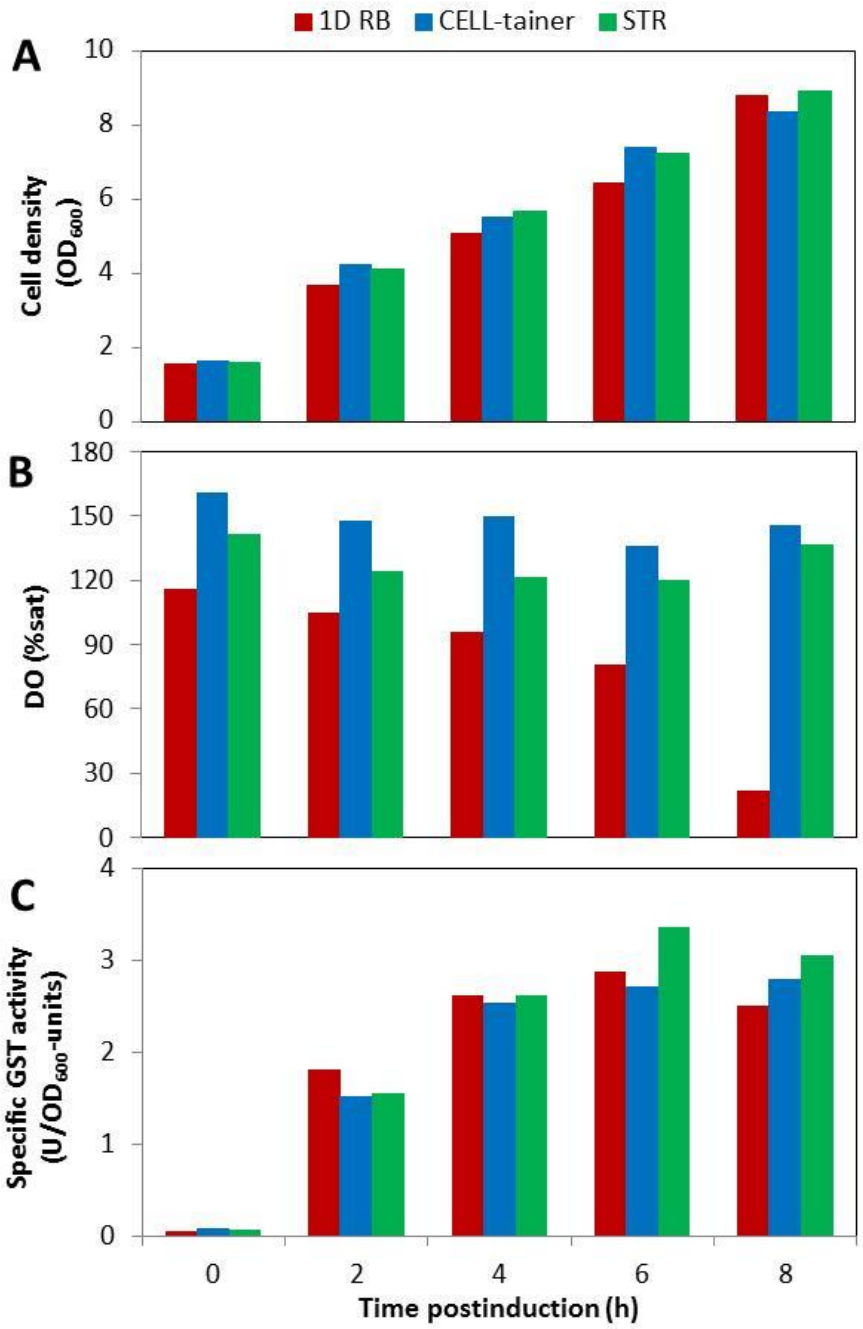

Figure 3-6 - Typical results for cultivation of BL21(pGEX2ThCD83ext,pLysS). 5 L and 10 L WV in the 1D RB and CELL-tainer®, respectively. All other cultivation conditions described in M&M. Cell density (Panel A), DO (Panel B), and specific GST activity (Panel C).

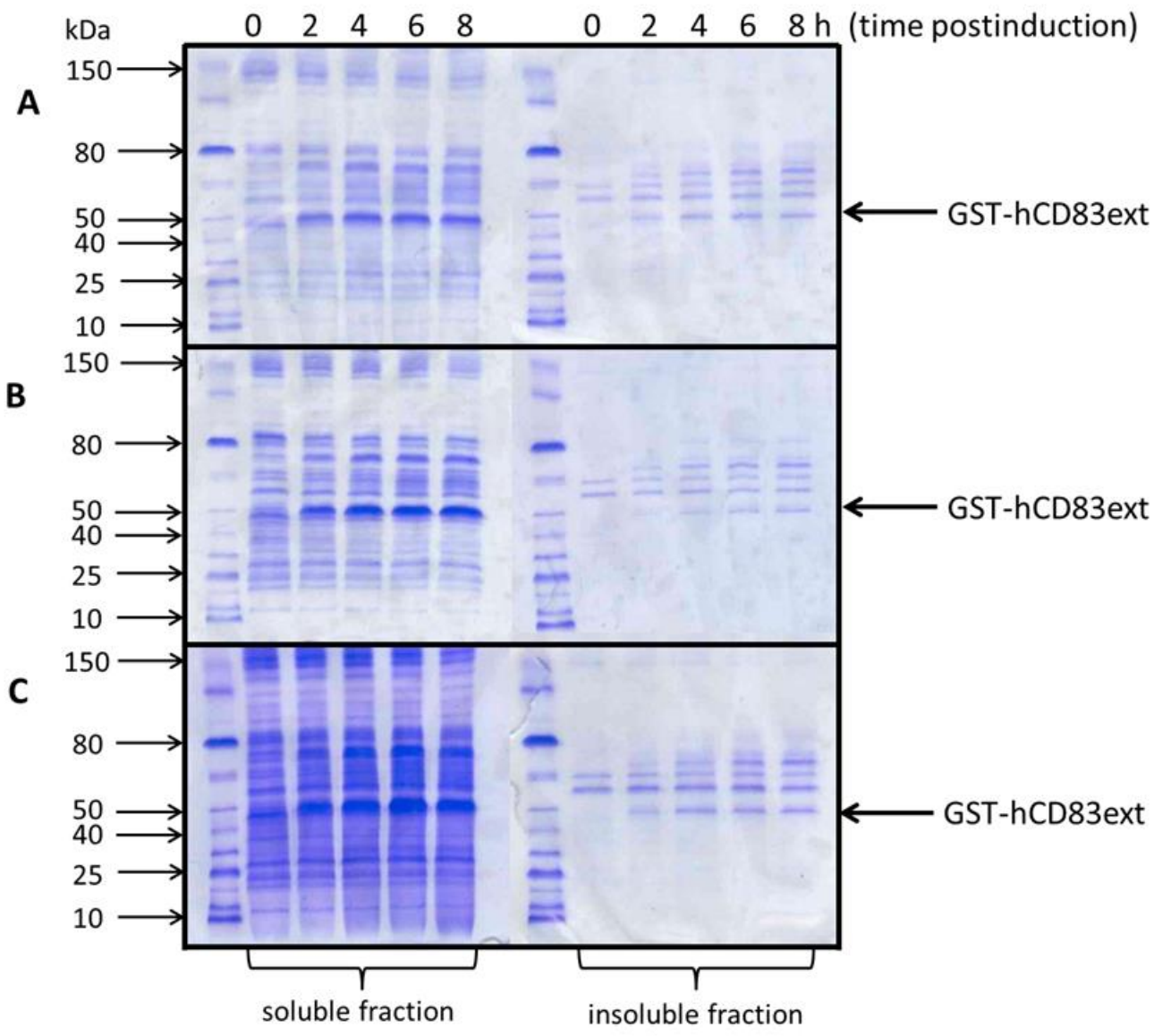

Figure 3-7 - Typical results for cultivation of BL21(pGEX2ThCD83ext,pLysS) in the 1D RB (Panel A), CELL-tainer™ (Panel B), and STR (Panel C). 5 L and 10 L WV in the 1D RB and CELL-tainer®, respectively. All other cultivation conditions described in M&M. SDS-PAGE analysis of soluble and insoluble intracellular protein fractions postinduction.

Compared to a previous bioprocess development study [63], a 36% increase in specific GST activity with similar cell growth characteristics was observed in the current study. It was previously hypothesized that a critical balance exists between GST-hCD83ext expression and DO. Recombinant protein expression in oxygen-enriched cultures has been studied extensively [84, 85, 89, 90], and excess DO is often associated with lower specific protein yields. Under conditions of severe oxidative stress, *E. coli* may be unable to reduce oxygen radicals (i.e., O_2^-, H_2O_2, etc.) sufficiently to prevent oxidation of Met and Cys residues [83], potentially resulting in protein misfolding, loss of activity, and protease degradation [87, 88]. Considering that hCD83ext contains five Cys residues known to cause protein structural variability and instability under oxidative conditions [112], varying levels of oxidative stress could partially explain differences in protein expression between cultivations. However, the recently observed increase in specific GST activity relative to previous studies without oxygen enrichment suggests other factors could be at play. A side note to oxidative stress in our cultivations concerns a natural function of GST, which reduces organic hydroperoxides using glutathione as electron donor [113], a process natively facilitated by alkylhydroperoxide reductase (Ahp) in *E. coli* [83]. Hence, oxidative stress response could be potentially bolstered in *E. coli* upon GST overexpression under our cultivation conditions.

3.4 Conclusions

In this study, we explored the application of RBs for bacterial cultivation. The results suggest that shear stress and DO mildly affects recombinant GST-hCD83ext expression during *E. coli* cultivation in the CELL-tainer®, consistent with our earlier studies using STR. Note that the accumulation of undesirable metabolites such as acetate was minimal for cultivations using

enriched media with low glucose concentrations. While both 1D and 2D disposable RBs proved feasible for *E. coli* cultivation for recombinant GST-hCD83ext production, the CELL-tainer® afforded greater flexibility in terms of operating conditions. However, the use of moderate oxygen with extreme agitation (i.e. VD and rocking rate corresponding to maximum k_La) was excessive for recombinant protein production. Accordingly, potential oxidative and shear stresses could be significantly alleviated under optimized conditions of oxygen supply and agitation. Such bioreactor operation and control strategies can be critical, particularly for the production of therapeutics, which are often unstable under oxidative conditions. In summary, disposable RBs present great opportunity for microbial cultivation compared to conventional STRs, particularly for production of high value therapeutics requiring strenuous validation and our study provides valuable insight for future biopharmaceutical bioprocess development.

Chapter 4 – Conclusions and Recommendations

4.1 Conclusions

The comparative analysis presented herein demonstrates the utility of disposable RBs in obtaining typical biomass yields and high level recombinant protein expression in batch cultures. k_La values in the CELL-tainer® were 2.7 and 4.5-fold higher for optimal and maximum WV, respectively, compared to the 1D RB; the k_La was 1.5-fold higher at maximum WV compared to the STR. k_La at maximum WV (5 L) in the 1D RB was consistent with earlier studies. Maximum k_La in the CELL-tainer® was in range of recently reported data, although discrepancies exist with regard to optimal WV (12 L in the previous study). Based on large discrepancies in k_La values for 15 L WV between the aforementioned study and an earlier investigation, and upon consideration of the nature of wave development in shallow water, it is believed that our observations of low optimal WV corresponding to maximum k_La are more reliable. Fast mixing was observed in all three systems, each providing t_{95} values of less than 20 s in water and aqueous CMC solution under experimental conditions. Comparison of t_m in water and aqueous CMC solution indicated that shear stress was greater in the CELL-tainer® relative to the 1D RB. The 1D RB and CELL-tainer® produced comparable cell densities and recombinant hCD83ext yields relative to the conventional STR. In the 1D RB, cell growth was severely hindered without supplemental oxygen in 1 L culture, and acetate accumulation was excessive for 5 L nonrecombinant cultivation regardless of inlet oxygen concentration. Biomass accumulation and glucose consumption occurred at a faster rate in the STR relative to the CELL-tainer®, possibly due to enhanced mass transfer given that the growth rate of BL21 was inhibited by elevated DO. Variations in hCD83ext expression between bioreactors were attributed to significant differences

in DO profiles, and possibly shear stress. In the CELL-tainer®, reduced oxygen and/or agitation could potentially enhance growth characteristics and protein expression, and improve process efficiency via reduced oxygen and power consumption. While hCD83ext expression and cell growth in the 1D RB were comparable to other systems, the use of oxygen for low cell density cultures is not desirable in the context of process scale-up given that other cultivation systems are capable of meeting the relatively low oxygen demand without oxygen enrichment. On the other hand, the CELL-tainer® provides mass transfer capacity similar to the STR, and improved efficiency with respect to specific power input compared to 1D RBs. Accordingly, the CELL-tainer® presents new opportunities for cost reduction in biopharmaceutical manufacturing processes based on microbial platforms, and provides versatility to multiproduct facilities utilizing prokaryotic and shear sensitive eukaryotic expression hosts.

4.2 Recommendations

The utility of disposable RBs for cultivation of *E. coli*, a robust aerobic organism, to achieve high level expression of a eukaryotic therapeutic protein was successfully demonstrated. While protein expression and cell density were comparable between the 1D RB, CELL-tainer®, and conventional STR, the low mass transfer capacity of the 1D RB barely met the relatively low oxygen demand of batch cultures, even with supplemental oxygen. 1D RBs are, therefore, not recommended for cultivation of fast growing aerobic microorganisms given the current limitations of these systems. Fed-batch cultivation in 1D RBs has been investigated in an earlier study [19], although oxygen demand at moderate cell density (20 g/L dcw) and low growth rate ($< 0.15\ h^{-1}$) exceeded mass transfer capacity. As previously discussed, higher gas flow capacity may significantly increase the k_La at high agitation rates in 1D RBs. Therefore, it is

recommended that 1D RBs evolve with improved gas flow capacity if biopharmaceutical production in microbial expression hosts is a target application.

The CELL-tainer®, on the other hand, shows promise as a cultivation system for the full range of recombinant expression hosts, demonstrating comparable performance to the STR with regard to cell growth, DO profiles, and hCD83ext expression in batch cultures of *E. coli*. Furthermore, the $k_L a$ exceeded that of the STR and t_m was comparable for all WV. Although atypically low air flow rates were employed due to the limitations of the 1D RB, a significant increase in $k_L a$ is not expected at higher air flow rates as the STR was operating well above the critical impeller speed. Subsequent investigations needed to fully characterize the CELL-tainer®'s performance are outlined:

1. Fed-batch cultivation of nonrecombinant BL21 (or other robust *E. coli* strain) under typical operating conditions for recombinant protein expression (i.e. 25-30 °C, 30-35 rpm, 1-1.5 vvm) to establish limits on achievable cell density, and corresponding undesirable metabolite profiles and oxygen consumption. Fed-batch cultivation of nonrecombinant BL21 has been investigated in the CELL-tainer® , however, cultivation temperature (37 °C) greatly exceeded temperatures typically employed during recombinant protein production [20].
2. Fed-batch cultivation of BL21(pGEX2ThCD83ext, pLysS) to assess the CELL-tainer®'s potential in achieving production scale titers of a recombinant therapeutic. hCD83ext is an ideal candidate for this type of study due to its sensitivity to oxidative conditions and propensity for inclusion body formation. Under the appropriate conditions of oxygen enrichment and agitation, the CELL-tainer® may provide certain advantages over STRs

for production of oxygen sensitive proteins due to the lack of oxygen hot spots found in the vicinity of the sparger(s).

3. Theoretical modelling of mass transfer and CFD simulations to characterize k_La, t_m, velocity distribution, wave properties (i.e. H, C, T, L, and θ), liquid height profiles, and shear rates. As previously discussed, ε can be approximated via Equation 6 which, in turn, can be used to determine k_L from Equation 3. The difficulty in this approach is obtaining reliable measurements of a, H, and L. In the absence of reliable experimental data, CFD modeling could provide predictions of these parameters for a given set of operating conditions (i.e. K, VD, and WV). This study would entail a more thorough assessment of k_La and t_m over a broad range of operating conditions to better characterize mass transfer and mixing, and to verify the theoretical model.

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