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Journal

Biotechnology and Bioengineering, 83(1)

ISSN

0006-3592

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Publication Date

2003-07-05

DOI

10.1002/bit.10647

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The Use of Dinoflagellate Bioluminescence to Characterize Cell Stimulation in Bioreactors

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Received 11 July 2002; accepted 11 December 2002

DOI: 10.1002/bit.10647

Abstract: Bioluminescent dinoflagellates are flow-sensitive marine organisms that produce light emission almost instantaneously upon stimulation by fluid shear in a shear stress dose-dependent manner. In the present study we tested the hypothesis that monitoring bioluminescence by suspended dinoflagellates can be used as a tool to characterize cellular response to hydrodynamic forces in agitated bioreactors. Specific studies were performed to determine: (1) impeller configurations with minimum cell activation, (2) correlations of cellular response and an integrated shear factor, and (3) the effect of rapid acceleration in agitation. Results indicated that (1) at a volumetric mass transfer coefficient of $3 \times 10^{-4} \text{ s}^{-1}$, marine impeller configurations were less stimulatory than Rushton configurations, (2) bioluminescence response and a modified volumetric integrated shear factor had an excellent correlation, and (3) rapid acceleration in agitation was highly stimulatory, suggesting a profound effect of temporal gradients in shear in increasing cell stimulation. By using bioluminescence stimulation as an indicator of agitation-induced cell stimulation and/or damage in microcarrier cultures, the present study allows for the verification of hypotheses and development of novel mechanisms of cell damage in bioreactors. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* 83: 93–103, 2003.

Keywords: bioluminescence; bioreactor; dinoflagellate; *Pyrocystis*; shear; temporal gradients

INTRODUCTION

Agitation in bioreactors is required for effective transport of oxygen and nutrients, and for providing a homogenous environment for cell growth. However, hydrodynamic forces generated during agitation may damage cultured cells. Animal cells adhering on microcarriers are especially susceptible to damage from hydrodynamic forces. In agitated mi-

crocarrier cultures, cell damage has been observed to occur at agitation rates between 100 and 250 rpm, where the Kolmogorov eddy length scale approximates the size of typical microcarriers (100–400 μm) (Cherry and Papoutsakis, 1986, 1988, 1989; Croughan and Wang, 1989; Croughan et al., 1987; Lakhotia and Papoutsakis, 1992; O'Connor and Papoutsakis, 1992). The effects of hydrodynamic stimuli include inhibition of growth and mortality. Because many viral vaccines and recombinant proteins are produced only by anchorage-dependent cell lines, devising strategies to reduce the detrimental effects of hydrodynamic forces in microcarrier cell cultures is of critical importance.

Local flow measurements are essential in understanding cell damage in bioreactors because they can identify those regions in the complex and transient flow field where cells are damaged. Unfortunately, the flow field in agitated bioreactors is very difficult to characterize because of the difficulty in quantifying the magnitude of shear present in the complex, transient flow field. Examples of currently available flow measuring techniques include hot wire and hot film anemometry, laser doppler velocimetry, and 3D particle tracking velocimetry. While recent studies have demonstrated the applicability of these techniques in spinner flasks (Venkat et al. 1996) and agitated bioreactors (Nagata, 1975; Riet and Smith, 1975; Venkat and Chalmers, 1996; Wu and Patterson, 1989; Yianneskis et al. 1987), these techniques are known to have limited accuracy, high expense, and tedious setup (refer to Peters and Redondo, 1997 for an extensive review).

While studies of the lethal and nonlethal effects of shear on cultured cells in laminar flow systems have established a link between cell stimulation and shear (Augenstein et al., 1971; Dewey, 1984; Frangos et al., 1985; McIntire et al., 1987; Rhee and McIntire, 1986), the results are not necessarily compatible with the effects of shear in turbulent flows. Although flow measurements can provide information about the levels of shear stresses in bioreactors, it is

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Contract grant sponsor: National Science Foundation

Contract grant number: BES-9730782

extremely difficult to characterize the effects of turbulence in the vicinity of cells. To date, much understanding of the cellular effects of hydrodynamics has been gained through correlation studies that can only partially explain how and why cell damage occurs. The issues at hand become even more difficult to address when other factors (including impeller design, bioreactor geometry, aeration techniques, media additives, operation strategies) affecting cell damage are also considered. As for design and optimization purposes, it is necessary to determine the most relevant factors critical to cell stimulation in agitated systems. This requires a link between cell stimulation and damage and the shear stresses they experience in turbulent flow.

To address these challenges, we propose the novel use of dinoflagellate bioluminescence as a flow visualization tool to characterize shear-induced cell stimulation in a bioreactor. Dinoflagellates are eukaryotic protists that are important members of the phytoplankton of aquatic communities. Some marine dinoflagellates are luminescent, responding to flow stimuli with flashes approximately 100 ms in duration (Latz and Lee, 1995; Widder and Case, 1981b). Light emission is thought to occur as a result of mechanical deformation of the cell which, via an unknown transduction pathway, results in a flash-triggering action potential propagated along the vacuole membrane (Eckert, 1965; Widder and Case, 1981a) resulting in acidification of the cytoplasm and activation of the luminescent chemistry (Wilson and Hastings, 1998). Dinoflagellate bioluminescence is a promising tool for examining flow stimulation in bioreactors because (1) cells are nearly neutrally buoyant and have limited motility relative to bioreactor flow fields, making them ideal flow tracers; (2) they are of similar size (40–900 μm) to microcarrier beads; (3) they respond essentially instantaneously to above threshold flow stimuli, with only a 20 ms response latency (Eckert, 1965; Widder and Case, 1981a); and (4) their shear sensitivity is similar to that of endothelial cells. Stimulus thresholds for flow-induced bioluminescence occurs at shear stresses of 0.1–0.3 N m^{-2} (Latz and Rohr, 1999; Latz et al., 1994), the physiological level present in venous flow. In fully developed pipe flow, the maximum cellular response of dinoflagellates occurs at shear stress levels of 1–2 N m^{-2} (Latz and Rohr, 1999), similar to in vivo arterial levels, while flash duration does not change with the level of shear stress (Latz and Rohr, 1999). Thus, dinoflagellate bioluminescence serves as a useful alternative model to study the effect of physical forces on mammalian cells (Mallipattu et al., 2002).

Although dinoflagellate bioluminescence has not yet been used as a flow visualization tool in biotechnology, it has provided unique opportunities for visualizing flow fields associated with moving objects (Bityukov, 1971; Hardy, 1956; Morin, 1983; Rohr et al., 1998) and has the potential for visualizing some geophysical flow fields (Rohr et al., 2002). In an agitated bioreactor where fluid dynamics is turbulent and complex, the shear sensitivity of dinoflagellates may be utilized to give a qualitative and semi-quantitative flow analysis.

In this study, stimulation of bioluminescence was used to simulate and characterize animal cell damage/stimulation of microcarrier cultures due to bioreactor agitation. The objectives were to: (1) determine impeller and bioreactor configurations which lead to the least bioluminescence stimulation at a given level of oxygen mass transfer, (2) correlate bioluminescence with integrated shear factors, to assess its use as an index of shear, and (3) determine the effect of rapid changes in agitation speed in agitated bioreactors based on the stimulation of bioluminescence.

MATERIALS AND METHODS

A 6.6-L capacity bioreactor (Bioflow 3, New Brunswick Scientific) with glass vessel and stainless steel components containing 3-L working volume of local filtered seawater was used. The vessel inner diameter was 17 cm. The aeration system within the bioreactor consisted of the headspace air in contact with the liquid surface (no sparging). A single impeller was mounted 5.8 cm above the bottom of the agitator shaft to provide mixing in the liquid phase. The four impellers investigated were three Rushton impellers of similar design with diameters of 5.08 cm (2 inches), 7.62 cm (3 inches), 10.16 cm (4 inches), and one Marine impeller 7.62 cm in diameter.

This study used the dinoflagellate *Pyrocystis fusiformis*, expected to generate approximately 20–30 flashes cell^{-1} at the rates of stimulation encountered in this study (Widder and Case, 1981b). This brightly luminescent species was chosen because its large luminescent capacity (Sweeney, 1981) allowed experimental testing before depletion of luminescent substrate occurred.

Cultures of dinoflagellates *Pyrocystis fusiformis* were grown in f/4 growth medium (Guillard and Ryther, 1962) minus silicate in a 20°C growth chamber on a 12:12 h light dark cycle. Bioluminescence by dinoflagellates exhibits a circadian rhythm, with light emission 1–2 orders of magnitude brighter at night than during the day (Sweeney, 1981). Experiments were performed 2.5–6 h into the dark phase, when maximal levels of stimulated bioluminescence occur (Biggley et al., 1969). Aliquots were prepared at the end of the light phase when cells are minimally excitable. Immediately before each experiment, the appropriate amount of seawater and cells were carefully transferred into the bioreactor to obtain the final concentration of 1 cell mL^{-1} . All experiments were performed in a completely dark room maintained at 20°C.

Bioluminescence was measured using a photon-counting photomultiplier detector (Electron Tubes Model P10232) positioned 40 cm from the bioreactor vessel. The field of view of the detector encompassed the entire width of the vessel. The light signal was attenuated to 6% transmission with a 1.2 neutral density filter to prevent saturation of the detector. Light readings were acquired every 10 ms and stored on a computer. Prior to each experiment, background light levels in the absence of stimulation were measured. Imaging was performed using a low-light intensified CCD

video camera (Dage MTI model 72 fitted with a 25 mm Fujinon lens operated at f/1.4) positioned 60 cm from the reactor vessel, near the detector. The gain of the intensifier was set at 125 for all recordings. Video was recorded at 30 frames s⁻¹ with a digital video recorder (Sony model GV-D900). To show the spatial pattern of the bioluminescence response, composites of 9–10 sequential frames were made. The positions of the bioreactor, PMT, and CCD camera were invariant between experiments.

The bioreactor motor was controlled by a computer to perform a variety of agitation protocols; each protocol started with an 8-s delay followed by an initial agitation speed of 30 rpm for 27 s to homogeneously suspend the cells with minimal stimulation of bioluminescence. Motor speed was recorded approximately every 0.35 s on a laptop computer.

The bioreactor was tested with a 7.62-cm diameter Rushton or 7.62-cm diameter Marine impeller, with or without wall baffles, to give four bioreactor configurations: Rushton, Baffled Rushton, Marine, and Baffled Marine. The Rushton impeller had six blades; each blade was 1.5 cm (height) by 1.8 cm (width) by 0.5 cm (thickness) in dimension. The Marine impeller had three inclined curved blades of standard configurations. Each of four wall baffles (quantity 4) was 20.3 cm (h) by 1.6 cm (w) by 0.1 cm (thickness) in dimension.

Bioluminescence responses subject to a range of agitation speeds were obtained for each bioreactor design. The bioreactor motor control was programmed to perform the following protocols: (1) For the Rushton and the Baffled Rushton bioreactors, a smooth 3 rpm/s ramp increase of agitation speed from 30 to 175 rpm; and (2) For the Marine and the Baffled Marine bioreactors, a smooth 3 rpm/s ramp increase of agitation speed from 30 to 300 rpm. The estimated temperature increase due to viscous heating during an experiment was <10⁻⁴°C. Noticeable bioluminescence depletion occurred approximately after the maximum agitation speed had been reached in protocol (1). Five independent replicates, each using separate cell cultures, were used for each bioreactor configuration. For each replicate, data were processed with a 0.5 s moving average filter, a standard signal processing method to reduce noise in continuous data. Tests using lower filter settings of 0.1 and 0.3 s had no appreciable effect on values of calculated integrated shear factors. The filtered data were then combined point-by-point to generate one mean response curve for each configuration. The two-tailed *t*-test was employed to compare the mean response curves at a 0.5-s interval against the null-hypothesis that the difference between the mean response curves was due to random noise. Significant difference was taken at the *P* < 0.05 level.

Volumetric oxygen mass transfer coefficient (*k_La*) was measured using the standard dynamic gassing out method (Riet, 1979). Percent dissolved oxygen (%DO) in the liquid volume was measured with an oxygen electrode (polarographic probe, Mettler Toledo) and recorded with standard data acquisition software. *k_La* was measured at impeller

speeds of 150, 175, 200, 225, 250, 300, 350, 375, 425, 475, and 500 rpm for each bioreactor, with three replicates at each speed. The relationship between *k_La* and agitation speed was modeled with a power law least-squares regression (Schmidtke and Horvath, 1977). Overall test of coincidence of the slope and intercept was used to compare regression lines. Significant difference was taken at the *P* < 0.05 level. Typical *k_La* values under normal culturing conditions for stirred liquid surface aeration range from 10⁻⁶ to 10⁻³ s⁻¹ (Bliem and Katinger, 1988a; 1988b). A *k_La* value of 3 × 10⁻⁴ s⁻¹, a value that was generated in all four bioreactors, was selected as the standard aeration efficiency. The impeller speed required to generate this *k_La* value was calculated from the regression for each bioreactor design.

For the 3 rpm/s ramp increase of agitation speed, the bioluminescence response on each filtered response curve was matched with the agitation speed required to generate the desired *k_La* value (3 × 10⁻⁴ s⁻¹). The mean and standard deviation were calculated over the five corresponding bioluminescence responses for each design. Statistics were performed using one-way ANOVA with posthoc testing of pairwise comparisons using Fischer's Protected Least Significant Difference. Significant difference was taken at the *P* < 0.05 level.

Integrated Shear Factors

The extent of cell growth on microcarriers under different shear conditions has been correlated with an integrated shear factor (ISF) in small-scaled (0.1 to 1 L) agitated bioreactors. (Hu, 1984; Sinskey et al., 1981). Integrated shear factor is related to the impeller angular speed (*N*), the impeller diameter (*D_I*) and the vessel diameter (*D_T*) as follows:

$$ISF = \frac{2\pi ND_I}{D_T - D_I} \quad (1)$$

where πND_I represents the impeller tip speed and $(D_T - D_I)/2$ the distance between the impeller tip and the vessel wall (Hu, 1984). Integrated shear factor is derived from laminar flow between two parallel plates, with one plate moving at the impeller tip speed and the other fixed, and provides a measure of the magnitude of shear rate between impeller tips and vessel wall (Hu, 1984).

Our observations of bioluminescence stimulation in Rushton bioreactors suggest that cell stimulation occurred primarily in the volume between the impeller tips and the wall. Therefore, a modified integrated shear factor, referred to as the volumetric integrated shear factor (VISF), was modeled as a function of impeller diameter, impeller speed, and the shear volume between the impeller tip and the vessel wall as follows:

$$VISF = \left(\frac{V_S}{V_T} \right) \left(\frac{2\pi ND_I}{D_T - D_I} \right) \quad (2)$$

where *V_S* represents the shear volume (the circumferential volume between the impeller tip and the wall) and *V_T* the

total working volume of the vessel (3 L in all experiments) (Fig. 1). By multiplying the dimensionless volume scale (V_S/V_T) by the original ISF, VISF distinguishes the volume around the impeller regions (where cell stimulation was apparent) from the bulk fluid (where cell stimulation was less). While both integrated shear factors are derived from laminar shear principles (which is not the case in turbulent flow bioreactors), VISF was hypothesized to be a more suitable shear parameter. In the present study, the two models of integrated shear factor were compared to measured bioluminescence responses.

The bioreactor was tested with 5.08-cm, 7.62-cm, and 10.16-cm Rushton impellers of the same number of blades and blade dimensions. Dinoflagellates in the bioreactors were subjected to a smooth 3 rpm/s ramp increase of agitation speed from 30 to 165 or 175 rpm. Noticeable depletion was observed at 165 rpm for the 10.16-cm Rushton impeller. From 4–5 replicates, response curves were obtained for each impeller size. Data were processed with a 0.5-s moving average filter and mean filtered bioluminescence was calculated for each impeller configuration. A two-tailed *t*-test was used to compare between the mean response curves at a 0.5-s interval. Significant difference was taken at the $P < 0.05$ level.

Bioluminescence responses on the mean response curve were matched with the corresponding agitation speeds for

each bioreactor. Responses generated at higher agitation rates, when bioluminescence stimulation occurred at the free surface vortex, were not considered. The ranges of agitation speeds used to calculate both integrated shear factors were between 89 to 165 rpm for the 5.08-cm Rushton, 40 to 149 rpm for the 7.62-cm Rushton, and 40 to 115 rpm for the 10.16-cm Rushton. The impeller Reynolds number ($Re = ND_I^2/\nu$) calculated at these agitation speeds confirmed that the fluid flow was turbulent ($Re > 1000$) (Papoutsakis and Michaels, 1993).

Rapid Acceleration Versus Ramp Acceleration in Agitation

In this experiment, effects of rapid acceleration vs. slow ramp acceleration on the stimulation of bioluminescence were determined using the 7.62-cm Rushton bioreactor. The bioreactor was programmed to perform the following agitation protocols: (1) repeated impulse protocol, a sequence of 1-s impulses every 6 s, with baseline agitation of 30 rpm in between and a 5-rpm increment in agitation speed for each impulse; (2) ramp protocol, a smooth 100-s ramp increase of agitation speed from 30 to 100 rpm at 1.4 rpm/s. The period of acceleration (from 30 rpm to a new set point) in each impulse of the actual repeated impulse protocol was approximately 0.35 s. Five independent replicates were performed for each protocol. Data were processed with a 0.5-s moving average filter. The repeated impulse response was calculated by averaging the bioluminescence response over the 0.35-s period of acceleration in each impulse. The smooth ramp response was calculated by averaging the bioluminescence response over a 0.35-s time interval while approaching the same agitation speed as that of each impulse. Comparisons between responses were made at the same agitation speeds in both protocols. Statistical comparisons were performed using Student's *t*-test. Significant difference was taken at the $P < 0.05$ level.

RESULTS

Bioreactor Evaluation

Bioluminescence increased with increasing agitation speed for each bioreactor configuration (Fig. 2). Over the agitation range of 30 to 175 rpm, the Baffled Rushton bioreactor generated the most bioluminescence, the Rushton bioreactor generated the second most bioluminescence, and Marine and Baffled Marine bioreactors both generated lower but similar levels of bioluminescence. Agitation speeds from 175 and 300 rpm were tested only for the Marine and Baffled Marine bioreactors. For these configurations, the bioluminescence responses were similar. All configurations were significantly different from each other ($P < 0.0001$) except between the Marine and Baffled Marine bioreactors.

The value of $k_L a$ increased with increasing agitation speed between 150 and 300 rpm for each bioreactor con-

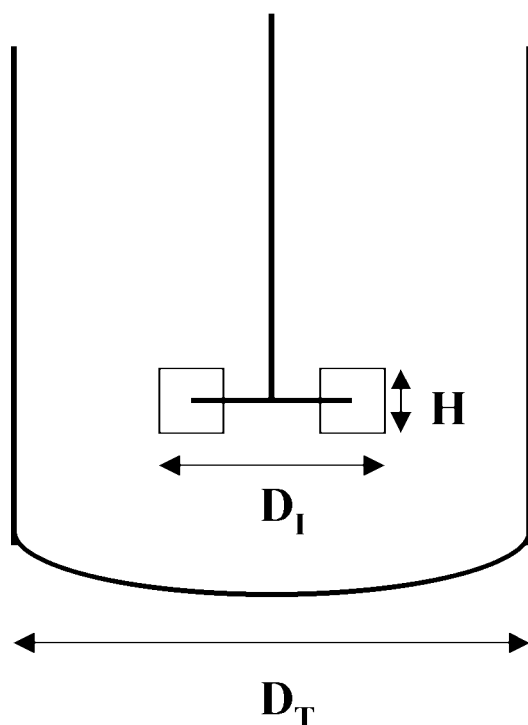


Figure 1. Principle behind volumetric integrated shear factor, using a Rushton impeller as the model. A side cross-sectional view shows the distance between impeller and the tank diameter and the height of the impeller tip. The shear volume is obtained by multiplying the impeller tip height (H) by the circumferential area ($(\pi/4)(D_T^2 - D_I^2)$) enclosed between the impeller tip and the wall. Not drawn to scale; bioreactor internals are not shown.

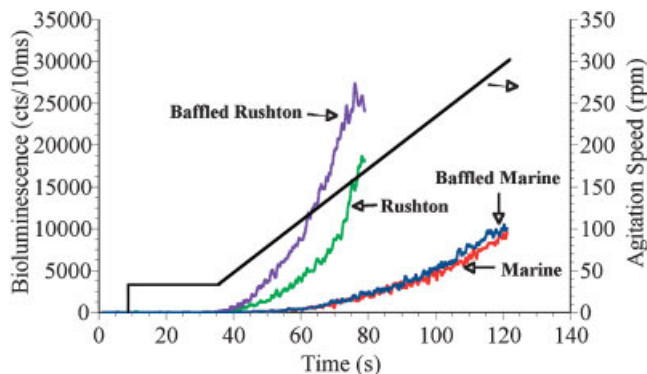


Figure 2. Mean stimulated bioluminescence as a function of agitation rate and time for the four bioreactors: Rushton, Baffled Rushton, Marine, and Baffled Marine configurations. The black solid line indicates the programmed agitation speed. Subthreshold agitation began at 8 s; at 35 s agitation speed increased by 3 rpm/s.

figuration (Fig. 3). On average, baffled configurations generated higher $k_L a$ values than unbaffled configurations, and Rushton configurations generated higher $k_L a$ values than marine configurations. Significant differences in the slope and the intercept of the regression lines were found between all bioreactor configurations ($P < 0.01$). Agitation speeds required to generate a $k_L a$ of $3 \times 10^{-4} \text{ s}^{-1}$ for the bioreactor configurations were determined to be 150 rpm for the Baffled Rushton, 170 rpm for the Rushton, 220 rpm for the Marine, and 170 rpm for the Baffled Marine configurations. A higher $k_L a$, corresponding to higher agitation speeds, was not chosen due to bioluminescence depletion.

The four bioreactor designs were further evaluated based on the bioluminescence response when tested at the respective agitation speeds giving a $k_L a$ value of $3 \times 10^{-4} \text{ s}^{-1}$. Baffled Rushton (at 150 rpm) and Rushton (at 170 rpm) bioreactors generated the most and the second most biolu-

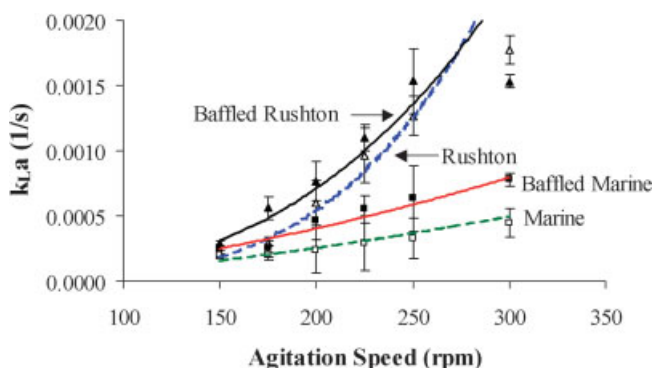


Figure 3. Volumetric oxygen mass transfer coefficient ($k_L a$) as a function of agitation rates for each bioreactor design. Only data between 150 and 300 rpm are shown. Symbols represent the mean and standard deviations of three measurements. Lines represent the least-squares exponential regressions of $k_L a$ on agitation speed: Rushton (open triangle), $y = 1.124 \times 10^{-12} x^{3.772}$ ($r^2 = 0.976$); Baffled Rushton (closed triangle), $y = 1.542 \times 10^{-10} x^{2.896}$ ($r^2 = 0.973$); Marine (open square), $y = 3.769 \times 10^{-8} x^{1.663}$ ($r^2 = 0.972$); and Baffled Marine (closed square), $y = 5.946 \times 10^{-8} x^{1.665}$ ($r^2 = 0.978$).

minescence response, respectively, while Marine (at 220 rpm) and Baffled Marine (at 170 rpm) bioreactors both generated the least bioluminescence response (Fig. 4). The differences in bioluminescence response between bioreactors was significant (ANOVA, $F = 62.388$, $P < 0.0001$). The Rushton configurations were significantly different from each other and from the Marine configurations ($P < 0.0001$). On average, the Baffled Rushton bioreactor generated 34% more bioluminescence than the Rushton bioreactor, which generated 400% more bioluminescence than the Marine bioreactor. There was no significant difference in responses between the Marine and Baffled Marine bioreactors ($P = 0.342$).

At an agitation speed of 170 rpm for the Rushton bioreactor, bioluminescence stimulation was apparent in the space between impeller tips and the wall, and the free surface (air–water interface) vortex around the agitator shaft (Fig. 5B). Cell stimulation at the vortex was roughly 30% of the total stimulation. At agitation speeds < 140 rpm, cell stimulation at the free surface around the shaft was not observed (results not shown). At an agitation speed of 150 rpm for the Baffled Rushton bioreactor, bioluminescence stimulation was apparent between impeller tips and the wall, the free surface vortex, and near the baffles (Fig. 5C). There was less cell stimulation at the free surface around the shaft compared to the Rushton bioreactor, possibly due to the presence of wall baffles that impeded the formation of free surface vortex. At 220 rpm for the Marine bioreactor and 170 rpm for the Baffled Marine bioreactor, bioluminescence stimulation was observed at the impeller tips and in the upward flow generated by the impeller (Fig. 5D and E). Stimulation of bioluminescence was not observed at the baffles and the free surface.

Integrated Shear Factors

Bioluminescence increased with increasing agitation speed for the three Rushton configurations (Fig. 6). For agitation speeds of 30 to 165 rpm, the level of bioluminescence was related to impeller size, with the 10.16-cm Rushton impeller

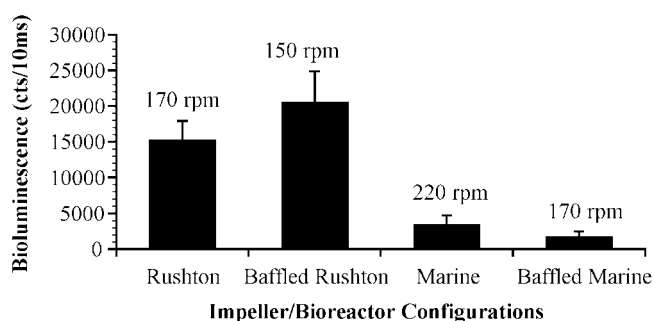


Figure 4. Bioluminescence responses at the agitation speeds corresponding to the volumetric oxygen mass transfer coefficient, $k_L a$, of $3 \times 10^{-4} \text{ s}^{-1}$. For each bioreactor configuration the corresponding agitation speed is shown. Values represent the mean and the standard deviation of five replicate experiments.

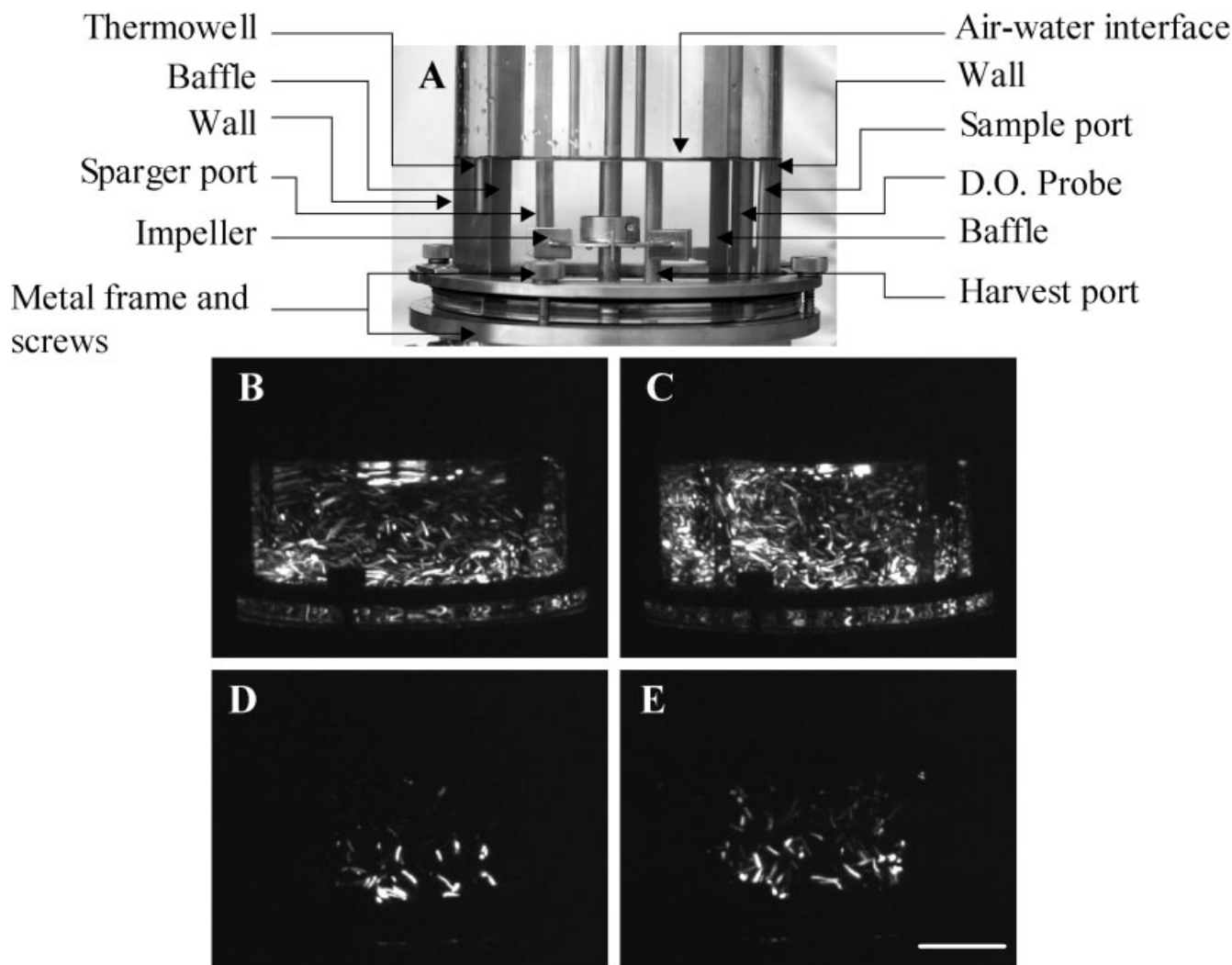


Figure 5. Stimulated bioluminescence in various bioreactor designs for oxygen mass transfer coefficient ($k_L a$) of $3 \times 10^{-4} \text{ s}^{-1}$. (A) Set-up of the Baffled Rushton bioreactor, showing the positions of the impeller, probes, baffles, and wall. (B) Rushton impeller, (C) Baffled Rushton impeller, (D) Marine impeller, and (E) Baffled Marine impeller. Scale bar in (E) = 5 cm.

generating the greatest bioluminescence response. Significant differences in bioluminescence responses ($P < 0.0001$) were found between all impeller sizes.

Spatial patterns of bioluminescence generated by the three Rushton impellers were similar. Bioluminescence was primarily observed in the space between the impeller tips and the vessel wall at increasing agitation speeds, before stimulation at the free surface vortex became noticeable. Stimulation at the free surface vortex occurred at an agitation speed of 115 rpm for the 10.16-cm Rushton impeller, 150 rpm for the 7.62-cm Rushton impeller, and 165 rpm for the 5.08-cm Rushton impeller.

Based on a least-squares power law relationship of bioluminescence vs. shear stress for Couette flow (Latz et al., 1994), bioluminescence responses generated by the three Rushton impellers correlated well with both integrated shear factors (Fig. 7). VISF, however, correlated better ($r^2 = 0.963$) than ISF ($r^2 = 0.862$). The F values indicated that the variability about the regression line was less for VISF (F

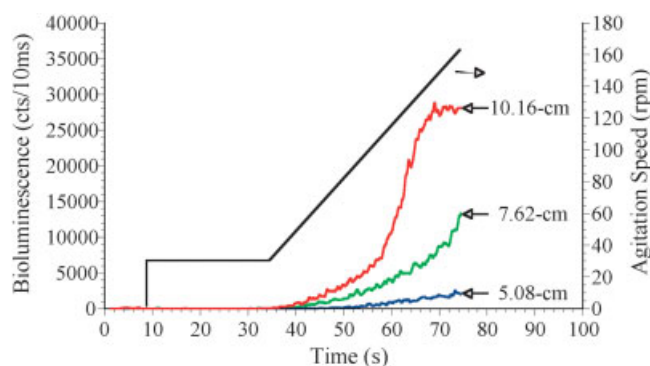


Figure 6. Effect of impeller size on bioluminescence stimulation. Mean bioluminescence response is shown as a function of agitation rates and time for Rushton impeller of three different sizes. The black solid line indicates the programmed agitation speed. Subthreshold agitation began at 8 s; at 35 s agitation speed increased by 3 rpm/s. Results are the average of five independent experiments for the 10.16-cm and 7.62-cm impellers, and four replicate experiments for the 5.08-cm impeller.

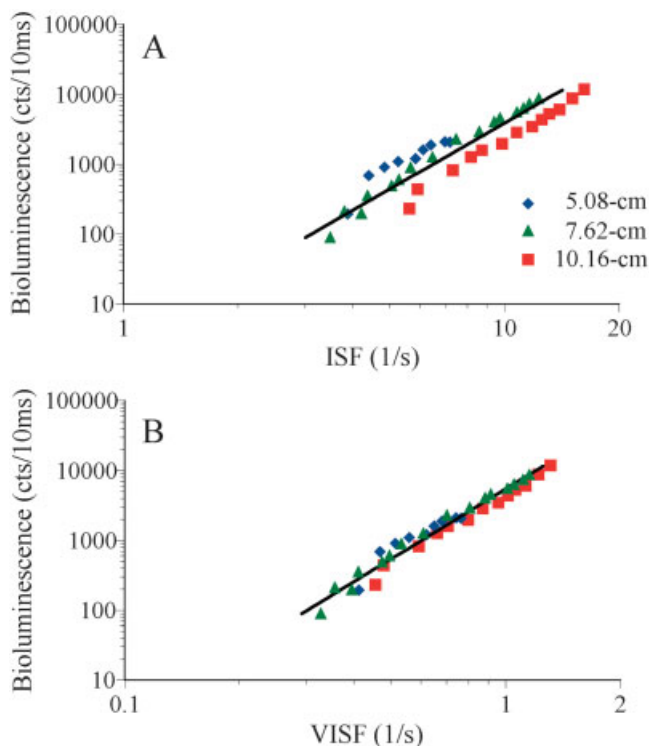


Figure 7. Relationship between bioluminescence response and integrated shear factors. Data from Figure 6 were used in both cases. The line represents the least-squares power regression of bioluminescence on integrated shear factor. (A) Integrated shear factor (ISF) (Sinskey et al., 1981). The regression equation was $y = 6.7174 x^{2.698}$ ($r^2 = 0.862$). (B) Volumetric integrated shear factor (VISF); $y = 5176.3 \times 10x^{3.212}$ ($r^2 = 0.963$).

= 939.3) than for ISF ($F = 225.1$). Overall, bioluminescence was an excellent index of integrated shear.

Effect of Rapid Acceleration in Agitation

Although the motor controller had difficulty in providing consistent 1-s impulses (Fig. 8A), presumably due to limitations of the motor speed feedback loop, the bioluminescence response to impulses was very different from that to the ramp protocol. For both agitation protocols, the bioluminescence response increased with increasing agitation speeds (Fig. 8B). More bioluminescence was generated by each impulse than the corresponding ramp agitation. There were significant differences in average bioluminescence responses between the two protocols (Fig. 9). Rapid acceleration stimulated more bioluminescence than ramp acceleration for agitation rates >60 rpm, and the difference in bioluminescence response between the protocols became more pronounced with increasing agitation speed. For example, a rapid acceleration to 90 rpm stimulated nearly 200% more bioluminescence than smooth ramp acceleration to that speed.

There was a difference in the spatial pattern of bioluminescence responses generated by both protocols (Fig. 10). Stimulation of bioluminescence in both cases was mainly

observed in the space between the impeller tips and the wall. Rapid acceleration, however, generated more bioluminescence than smooth ramp acceleration.

DISCUSSION

Bioreactor Evaluations

Critical to the design and optimization of agitated bioreactor processes is understanding and assessing the effect of hydrodynamics on cultured cells. In this investigation, flow-stimulated bioluminescence of dinoflagellates was used as a tool to characterize cell stimulation in various bioreactor configurations. Over the range of 30 to 175 rpm, Rushton impellers were much more stimulatory than Marine impellers. This explains why Marine impellers, having high pumping capacity and low shear characteristics, are often used in culturing shear-sensitive mammalian cells.

While the presence of wall baffles has been shown to increase cell stimulation (Leckie et al., 1991), our results indicated that wall baffles significantly increased bioluminescence emissions in the Rushton bioreactor but not in the Marine bioreactor. This may reflect the force and frequency with which cells encounter the baffles. Presumably, the in-

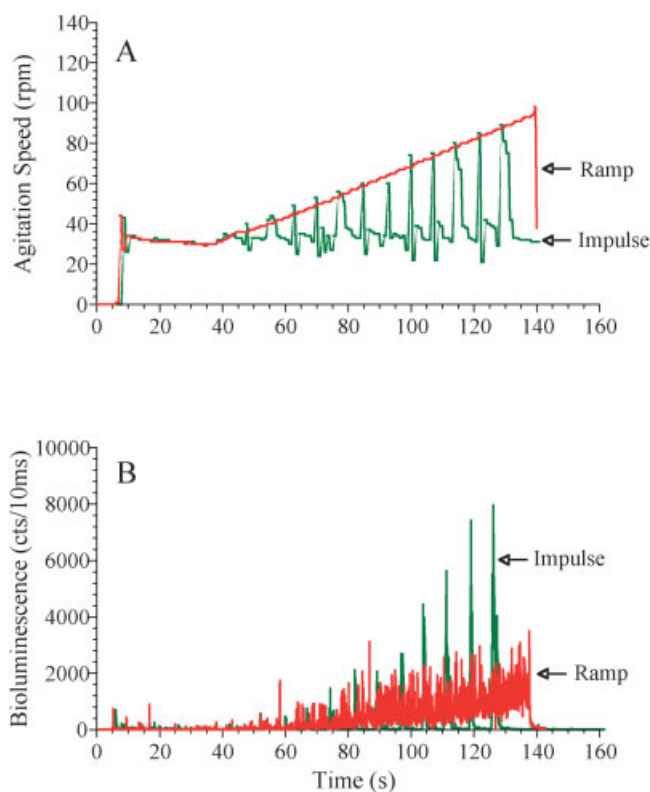


Figure 8. Effect of rapid changes in agitation speed on the bioluminescence response. Results of two representative experiments are shown. (A) Agitation speed for the two protocols. Both protocols started at a sub-threshold agitation speed of 30 rpm at 8 s; at 35 s the agitation speed increased at 1.4 rpm/s for the ramp protocol while 1-s duration pulses in 5-rpm increments were given every 5 s for the impulse protocol. (B) Unfiltered bioluminescence response to each protocol in (A). The impulse protocol was much more stimulatory than impeller speed at agitation speeds >75 rpm.

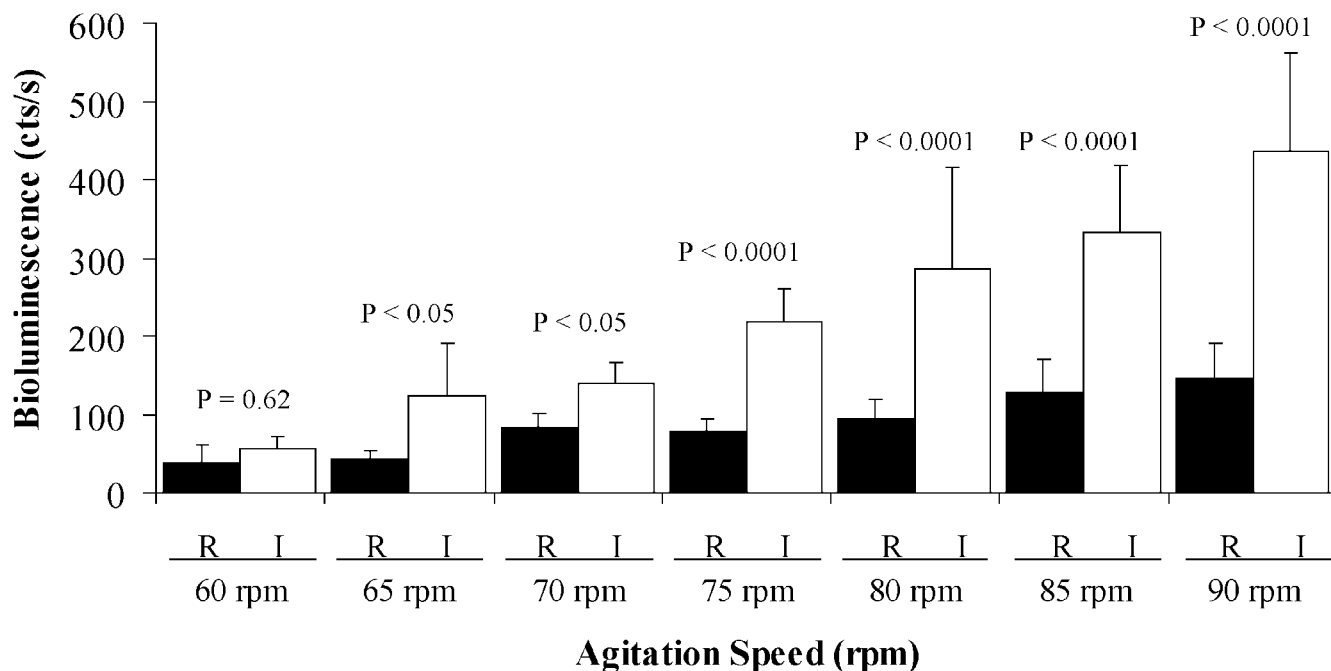


Figure 9. Average bioluminescence responses to impulse (I, open bars) and ramp (R, closed bars) protocols described in Figure 8. Primary data were processed using a 0.5-s weighted moving average filter. Values represent means and standard deviations for five measurements. At agitation speeds of 60 rpm there were significant differences in bioluminescence between the two protocols. See Methods for a description of data processing.

interactions between cells and wall baffles were less frequent in the Baffled Marine than in the Baffled Rushton bioreactors. Wall baffles were positioned against the vessel wall and oriented axially in the meridional direction. Cells in the Baffled Rushton bioreactor constantly encountered the baffles, which were positioned perpendicularly to the radial flow, when being advected radially and circumferentially from the impeller to the wall. In the Baffled Marine bioreactor, cells were first advected axially from the impeller to the fluid above, and then circulated downward as more fluid

and cells were pumped upward. As a result, interaction between the cells and wall baffles in the Marine bioreactor may have been of lower frequency and impact.

Based on the bioluminescence responses at a $k_L a$ value of $3 \times 10^{-4} \text{ s}^{-1}$, Marine and Baffled Marine configurations provided the least cell-stimulatory environment compared to the Rushton configurations. For the range of agitation speeds from 150–300 rpm, the Baffled Marine bioreactor is preferred over the Marine bioreactor because it generated higher levels of oxygen mass transfer for similar levels of

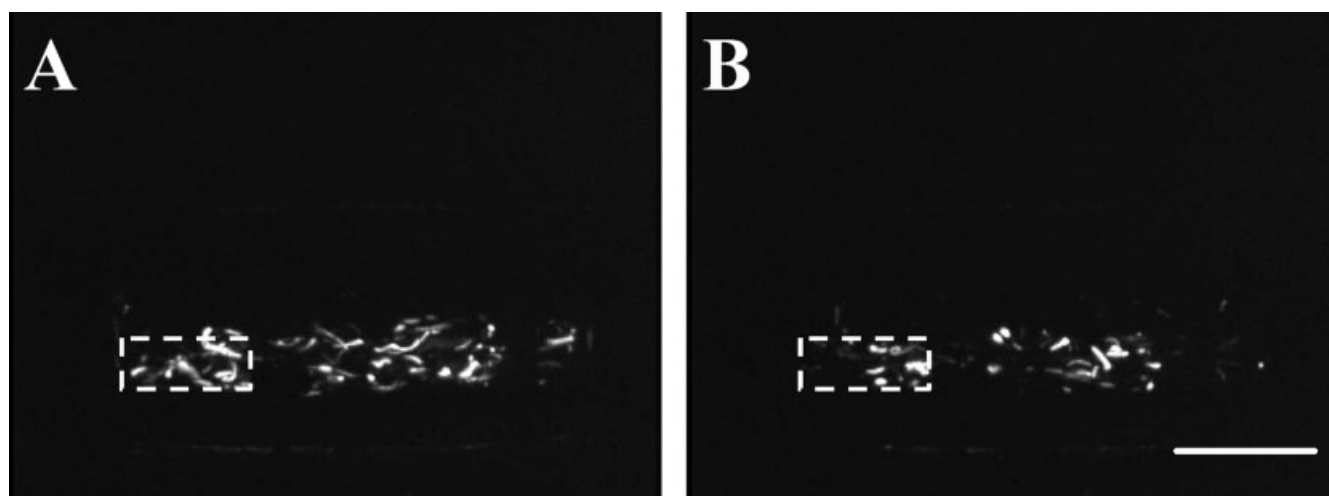


Figure 10. Effect of agitation protocol on the spatial pattern of bioluminescence. (A) Response during single 1-s impulse acceleration from 30 to 90 rpm. (B) Response at agitation speed of 90 rpm during 1.4 rpm/s ramp. Highlighted in the white dash lines is the space between impeller tips and wall. Greater stimulated bioluminescence was observed in this region during the impulse stimulus. Scale bar in (B) = 5 cm.

cell stimulation. Therefore, the Baffled Marine bioreactor should be used for culturing shear-sensitive cells.

Correlations Between Bioluminescence and Integrated Shear Factors

Hydrodynamic stresses can be generated in a variety of ways in an agitated bioreactor. Bubble entrainment and breakup (Kunas and Papoutsakis, 1990), interactions between cells, (Cherry and Papoutsakis, 1988) and between cell and turbulent eddies (Aloi and Cherry, 1996; Cherry and Papoutsakis, 1986, 1989; Papoutsakis, 1991), rapid extension flow (Gregoriades et al., 2000), and free surface vortex (Kunasand Papoutsakis, 1990; Venkat et al., 1996), have all been reported to cause animal cell damage. To quantify the effect of hydrodynamic forces on dinoflagellates, the volumetric integrated shear factor was proposed.

The inverse relationship between impeller speed and the impeller diameter in stimulating bioluminescence formed the basis for correlating bioluminescence with both ISF and VISF. In developing the correlations, only bioluminescence data acquired before stimulation became noticeable at the free surface vortex were considered. Our observation that cell stimulation was primarily in the flow stream between impeller and the wall was consistent with the findings of Wu and Patterson (1989), who reported that the most energy dissipation occurred around the impeller and in the impeller stream of a Rushton impeller. Based on the better correlation provided by VISF, it is potentially suitable for scale-up of bioreactor processes. VISF characterizes the effective shear volume critical to cell stimulation in bioreactors. In contrast to previous studies involving rigorous modeling (Cherry and Papoutsakis, 1988; Gregoriades et al., 2000) or difficult flow measurements (Nagata, 1975; Riet and Smith, 1975; Venkat et al., 1996; Wu and Patterson, 1989; Yianeskis et al., 1987), this study presents the first work of deriving a correlation based only on the observed cellular responses in bioreactors. Bioluminescence in these bioreactor configurations provided an excellent index of volumetric integrated shear.

Temporal Gradient Studies

Laminar fluid shear presents two distinct mechanical stimuli to anchorage-dependent cells: the rate of change of shear (temporal gradients in shear) and the shear stress itself (Bao et al., 1999, 2000, 2001; Frangos et al., 1996; White et al., 2001). Dinoflagellates are shear-sensitive cells that are evolutionarily and structurally very distant from animal cells. While dinoflagellates have a protective layer of polysaccharide plates or a cell wall, making them more robust than animal cells, they are not necessarily less shear-sensitive. Using a variety of independent flow fields, it has been shown that the threshold stimuli for bioluminescence of dinoflagellates are similar to those present in venous and arterial blood flow (Latz and Rohr, 1999; Latz et al., 1994). The highly stimulatory effect of volumetric integrated shear

during rapid acceleration in agitation suggests that, like the shear sensitivity of endothelial cells, dinoflagellates have the ability to sense and distinguish not only steady levels of shear but also the rate of change of shear. It has been suggested that dinoflagellates and endothelial cells experience shear stimulation by similar mechanisms (Mallipattu et al., 2002), and that there may be a general mechanism underlying flow-sensitive mechanotransduction in eukaryotic cells.

To date there have been no reports describing the effects of rapid acceleration in agitation on cellular damage in bioreactors. The stimulatory effects of rapid changes in agitation speed on bioluminescence suggested that temporal gradients in shear may promote cell damage. This may be particularly important for animal cells growing in lag and stationary phases of a batch culture, which are more susceptible to shear damage than in exponential phase (Papoutsakis et al., 1991; Peterson et al., 1988, 1990). A desirable operational strategy is to minimize cell stimulation during the transient start-up of agitation for newly inoculated cultures by slowly ramping up the agitation speed to the desired value.

CONCLUSION

This is, to our knowledge, the first study to explore the use of dinoflagellate bioluminescence to characterize cell stimulation in agitated bioreactors. The almost instantaneous bioluminescence response to fluid shear allows for a dynamic assessment of the effects of turbulent mixing on cells. In this study, dinoflagellate bioluminescence was an effective tool for selecting a bioreactor configuration with minimal cell stimulation. In addition, dinoflagellate bioluminescence proved to be an excellent index of integrated shear. Furthermore, it revealed that rapid accelerations in agitation, such as during start-up, can be highly stimulatory. In conclusion, the use of dinoflagellate bioluminescence is a novel method to characterize and optimize the design and operation of bioreactor processes.

The authors wish to thank Oliver Bell for his valuable assistance in bioluminescence data acquisition, cell preparation, and his useful comments; Dr. Mark Haidekker for his technical support; James Su and Jennifer Snell for their effort in reviewing this manuscript; and members of Dr. John Frangos' lab for their useful comments.

NOMENCLATURE

D_I	impeller diameter	(cm)
D_T	vessel diameter	(cm)
H	impeller tip height	(cm)
ISF	integrated shear factor	(s^{-1})
k_{La}	volumetric oxygen mass transfer coefficient	(s^{-1})
Re	Reynolds number	
VISF	volumetric integrated shear factor	(s^{-1})
V_S	shear volume enclosed between impeller tip and vessel wall	(L)
V_T	total liquid volume in bioreactor	(L)

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