

Optical Analysis of Liquid Mixing in a Minibioreactor

Jose R. Vallejos,¹ Yordan Kostov,¹ Arun Ram,² Joseph A. French,³ Mark R. Marten,¹ Govind Rao¹

¹Center for Advanced Sensor Technology, Department of Chemical and Biochemical Engineering, University of Maryland at Baltimore County, Baltimore, Maryland 21250; telephone: (410)-455-3400; fax: (410) 455-1049; e-mail: grao@umbc.edu

²Department of Computer Science and Electrical Engineering, University of Maryland at Baltimore County, Baltimore, Maryland

³Department of Physics, University of Maryland at Baltimore County, Baltimore, Maryland

Received 27 June 2005; accepted 24 October 2005

Published online 29 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20785

Abstract: A novel optical sensor was used to study mixing and mean circulation time in a model minibioreactor (12.5 mL stirred vessel, equipped with a paddle impeller). Rotational rates in the range of 10–1,000 rpm corresponding to Reynolds number between 14 and 1,350 were studied. Results suggest that depending on the impeller rotational speed, mixing times up to 214 ± 87 s can be reproducibly achieved. The minibioreactor was operated in the transitional regime, and it was determined that the non-dimensional form for mixing time, $N\theta_M$ was linearly dependent on Reynolds number. A linear correlation between mean circulation time and the inverse of rotational speed was also determined. The mean circulation time dependence on rotational speed in the 12.5 mL stirred vessel is similar to those found in large-scale stirred vessels. These results suggest that mixing and circulation times found in large-scale reactors can be replicated in minibioreactors. © 2005 Wiley Periodicals, Inc.

Keywords: mixing; minibioreactor; high throughput; bioprocessing; scale up

INTRODUCTION

Minibioreactors designed for use in the pharmaceutical industry have the potential to both increase process development rates and to reduce costs (Lye et al., 2003). If these benefits are achieved in the early stages of drug discovery (i.e., during screening and development phases), they have the potential to significantly reduce overall product development cost. To date, a number of mini- and micro-bioreactors have been described in the literature. For example, Kostov et al. (2001) described a 2 mL working volume cuvette-based micro-bioreactor equipped with pH, dissolved oxygen, optical density sensors for continuous measurement of process variables. Maharbiz et al. (2004) described an array of eight 250 μ L reactors capable of

temperature and pH control and as well as optical density measurement. Zanzotto et al. (2004) used a micro-bioreactor with volumes in the range of 5–50 μ L with integrated sensors for online measurement of optical density, dissolved oxygen, and pH during growth of *Escherichia coli*. Elmahdi et al. (2003) measured pH during microscale fermentation by inserting a micro-pH probe into a 7 mL well in a microtiter plate. Finally, Doig et al. (2005) used three different microplates (24, 96, and 384 wells) with working volumes between 65 and 1,182 μ L to measure $k_{L,a}$ during growth of *Bacillus subtilis*.

With the development of these miniaturized bioreactors, numerous questions regarding mixing at small scales emerge. For example, is mixing behavior in small-scale stirred vessels similar to that in large-scale stirred vessels? What roles do convection, diffusion, and eddy scales play during mixing processes in micro-bioreactors? Is it reasonable to expect large circulation times in small-scale stirred vessels? To date, the study of mixing and flow in minibioreactors has primarily been conducted using computational fluid dynamic (CFD) simulations (Lamping et al., 2003), and relatively few experimental studies have been carried out.

In contrast, mixing and hydrodynamics have been studied extensively at lab and large scale (Barneveld et al., 1987; Bittorf and Kresta, 2000; Hall et al., 2004; Nere et al., 2003). As a measure of overall degree of bioreactor agitation, a number of authors have used mixing time (Marten et al., 1997; Papagianni et al., 2003; Vasconcelos et al., 1995) or the time required to reach homogeneity after addition of a soluble tracer. Most of the current mixing operations in bioreactor applications are turbulent but laminar and transitional scenarios are becoming industrially more relevant (Alvarez et al., 2005).

The aim in this study was to begin to characterize agitation in a minibioreactor, operated in the transitional regime, by studying both mixing time (θ_M) and mean circulation time

Correspondence to: Govind Rao or Mark Marten

Contract grant sponsors: Fulbright; NIH; Merck & Co.

Contract grant numbers: U104336; RR018608

($\bar{\theta}_C$). To accomplish this, a 12.5 mL stirred vessel for high throughput cell culture fermentation was used as a model system. Mixing time is defined as the time required for the system to reach 95% of its final value after addition of a miscible tracer. Circulation time is defined as the time interval between successive passes of a fluid element through a fixed point in the vessel. A novel optical sensor is used to assess both mixing and circulation times. Then, correlations between mixing time and Reynolds number and between mean circulation time and impeller rotational are compared with correlations determined in large-scale stirred vessels.

MATERIALS AND METHODS

The vessel used was a glass vial with a footprint identical to that of a single well in a 24-well plate. In addition, it consists of a stainless steel cap, shaft with impeller, coupling and driving motor (See Fig. 1). In all our experiments, florescein disodium salt was used as a tracer. The vessel geometry and impeller position for the bioreactor are presented in Table I. Since standard dimensions do not exist for bioreactors, our studies were done in normalized conditions of H to D ratios. The Reynolds number defined as $Re = ND^2\rho/\mu$ was varied ranging from 14 to 1,350, where N is the rotational speed (s^{-1}), D is the impeller diameter (m), ρ is liquid density (kg/m^3) and μ is the viscosity (Pa.s).

A drop of dye (i.e., 10 μL) was injected in the vessel without disturbing the system. The drop of tracer was gently placed on the surface of the liquid (i.e., water) at approximately 3 mm near the impeller shaft. The time at which the tracer is added to the vessel is considered as the initial time ($t=0$). A confocal optical sensor that was previously validated (Vallejos et al., 2005) was used during the experiments. The optical sensor transmits a signal to an Analog-to-Digital Converter (LabJack, www.labjack.com),

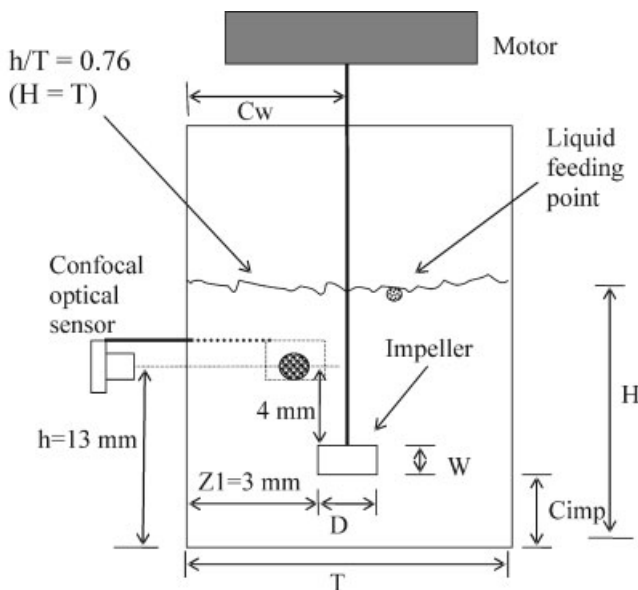


Figure 1. Vessel geometrical parameters. The most important geometrical parameters of the 12.5 mL stirred vessel are indicated.

Table I. Vessel geometrical parameters.

Reactor size	T (mm)	D (mm)	D/T	W/D	C_{imp} (mm)	C_w/T
12.5mL	17	9	0.53	0.44	8.8	0.5

Table I presents the most important geometry parameters of the 12.5 mL stirred vessel.

which finally transmits the digital signal to a PC equipped with LabView software. On the PC, the signal is displayed in millivolts versus time.

The signal (X_t) was normalized by dividing it by its final value (X_f) using the following equation (Khang and Levenspiel, 1976):

$$E = \frac{X_t}{X_f} \quad (1)$$

where X_t is the actual signal transmitted by the confocal sensor (mV) and X_f is the final value of the signal transmitted by the confocal sensor (mV).

A total of 20 runs for each rotational speed were performed. For each run, mixing time was determined as the time needed for the amplitude of the oscillations to become less than 5% of steady state value. Following this, the mean value was computed. Mean circulation time was calculated using the following equation (Roberts et al., 1995):

$$\bar{\theta}_C = \frac{\sum_{i=1}^n t_i}{n} \quad (2)$$

where t_i is the time between two consecutive circulation peaks (sec) and n is the number of circulation peaks (t_i) detected.

The stirred vessel was equipped with a single paddle impeller. A 31.5 mm model 2111-13-02 motor (Lin Engineering) drove the impeller.

Mixing Time Studies

A ratio of $H/T=1$ was used and the working volume was 3.9 mL, this geometrical ratio (i.e., $H/T=1$) is commonly used in industrial fermentors or stirred tanks. In order to achieve the ratio, the vessel was filled up with liquid to 1/3 of the total height. Mixing time was studied at a fixed location with coordinates ($h, Z1, R1$) (13, 3, 9) in mm. See Figure 1 for more details.

Circulation Time Studies

Circulation time studies were performed at a fixed location and only the liquid height (H) was changed. Circulation time at two different H/T ratios (i.e., $H/T=1$ and $H/T=2$) was studied. In the case where $H=T$, the conditions employed are as described above. In the second hydrodynamic case $H=2T$

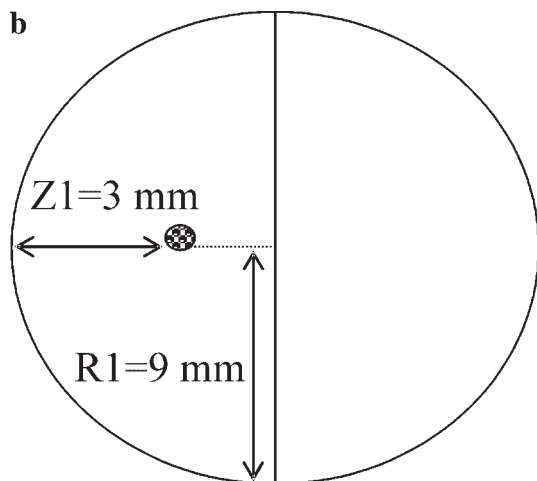
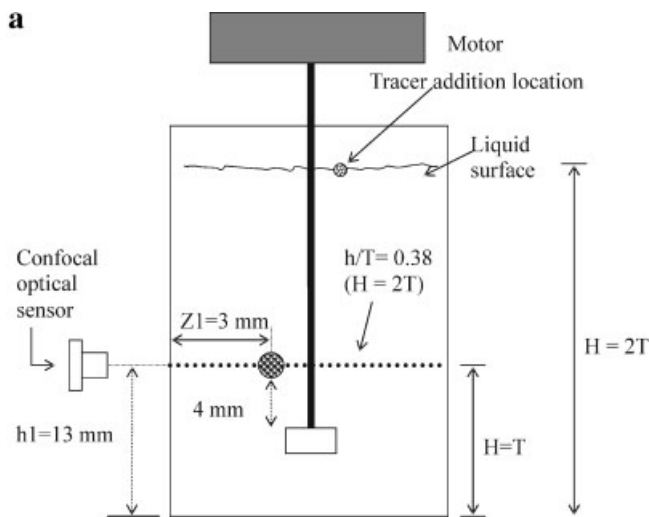


Figure 2. Circulation time measurement location. Figure shows the locations in the stirred vessel where circulation time was measured. (a) Cross-sectional view; (b) top view.

(i.e., 7.8 mL working volume), the sensor was kept at the same position as in $H = T$ (Fig. 2).

RESULTS AND DISCUSSION

Mixing Time

The 12.5 mL stirred vessel was primarily operated in the transitional regime (i.e., $10 < Re < 10^4$). It was observed that the vessel was perfectly mixed (i.e., after mixing time was reached, no stagnant zones were seen in the minibioreactor) over the range of $14 < Re < 1,350$. However, in the range $14 < Re < 41$ the presence of doughnut-like segregated regions (i.e., Poincaré sections or isolated mixing regions (IMR)) were visually observed in the 12.5 mL stirred vessel. These segregated regions eventually disappeared as mixing progressed. It is well known that Poincaré sections can create barriers to efficient mixing. Zalc et al. (2002) reported the presence of Poincaré sections for a 0.24 m diameter stirred tank for $20 < Re < 40$. Poincaré sections may also negatively

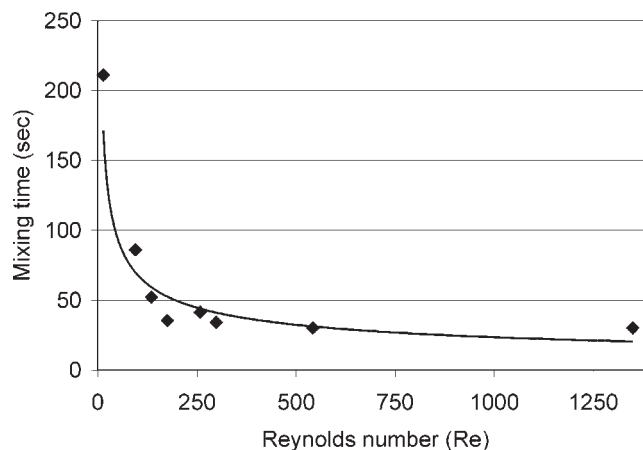


Figure 3. Mixing in the 12.5 mL stirred vessel as a function of Re . (◆) Experimental data. (—) Power correlation from experimental data. Figure shows how mixing in the stirred vessel is dependent on Reynolds number vessel. The experimental data fits a power curve of the form $Y = aX^b$. For the 12.5 mL model bioreactor $a = 568$, $b = -0.46$ with an $r^2 = 0.86$.

affect the mixing efficiency in the 12.5 mL stirred vessel. As these isolated mixing regions are known not to exchange significant fluid with the outside active mixing region (Ohmura et al., 2003), this may be responsible for the relative large mixing time of 214 ± 87 s determined in the minibioreactor at $Re = 13.5$. For $Re > 41$, no Poincaré sections were observed.

Figures 3 and 4 show experimentally measured mixing time values and non-dimensional mixing time as a function of Reynolds numbers, respectively. After $Re = 176$ (i.e., 130 rpm), mixing time in the 12.5 mL vial seems to reach a constant value of approximately 34 s. It appears that at $Re = 176$, the maximum pumping capacity of the impeller has been achieved such that additional increases in rotational speed have little effect on mixing time. Figure 4 shows a linear (i.e., $r^2 = 0.99$) dependency of the non-dimensional ($N\Theta_M$) form of mixing time on Reynolds number. This implies that the product, $N\Theta_M$, in the small-scale 12.5 mL stirred vessel is not constant in the transition regime. Nienow

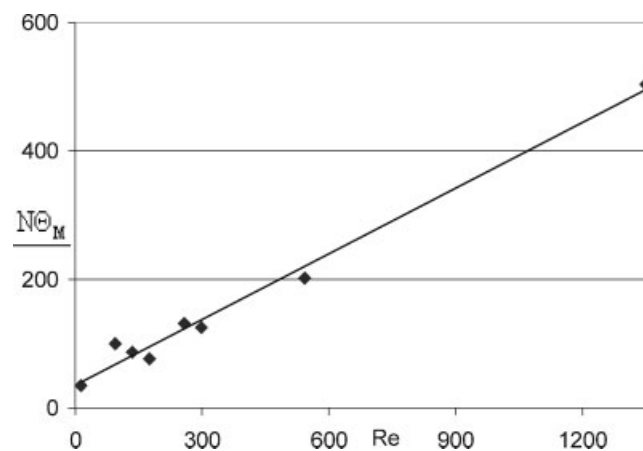


Figure 4. Non-dimensional form of mixing as a function of Re . Figure shows the dependence of the non-dimensional mixing form on Reynolds number.

(1997) has observed that when convection is controlling, the product $N\theta_M$ is constant under turbulent regime. Sano and Usui (1985) experimentally found that $N\theta_M$ is independent of Re in the turbulent regime (i.e., $Re > 5 \times 10^3$), and only depends on the geometrical shape of the impeller. Since a linear dependency of the non-dimensional form of mixing ($N\theta_M$) on Re was determined in the 12.5 mL stirred vessel, it may suggest convection is not always controlling the mixing process. In the transition regime, both laminar and turbulent flow elements exist. In the laminar regime, turbulence is not relevant but chaos is the only mechanism for mixing (Alvarez et al., 2005). The presence of chaotic mixing may also be responsible not only for the relative large mixing time values but also for the segregated regions observed in the minibioreactor for $Re < 41$. Since chaotic mixing is very sensitive to initial conditions, the tracer injection point may have a significant effect on the mixing time when laminar flow is predominant. Studies of mixing with different tracer injection location are under way to study its influence.

Mean Circulation Time

Case 1. $H = T$

Circulation peaks at two agitation speeds are shown in Figure 5. It was determined that as rotational speed is increased, mean circulation time is decreased. For example, at lower rotational speed (i.e., 10 rpm), the circulation peaks are more separated between each other than at 30 rpm. This leads to larger mixing time values and circulation time (i.e., 41 s).

Interestingly, the number of peaks before homogenization in the vessel is reached at 10 rpm, is similar to the number reported in the literature for large-scale stirred vessels. For example, Nienow (1997) when describing the bulk flow model reported that the system was homogenized after about five circulation peaks. Similarly, at 10 rpm in a 12.5-mL-stirred vessel five circulation peaks are required before homogenization is achieved. At 30 rpm, 24 circulation peaks were seen in the region near the impeller. As the rotational speed is increased, the number of circulation peaks is increased and the mean circulation time decreases. Above 130 rpm (data not shown), no more circulation peaks were observed.

Case 2. $H = 2T$

As it was for the first case (i.e., $H = T$), the results for $H = 2T$ show that mean circulation time is inversely proportional to increasing rotational speed. The results for the case when $H = 2T$ also show that for a given rotational speed mean circulation time increases by increasing the liquid height. Results are shown in Figure 6.

It has been previously demonstrated in moderately large stirred tanks that θ_C is inversely proportional to the impeller

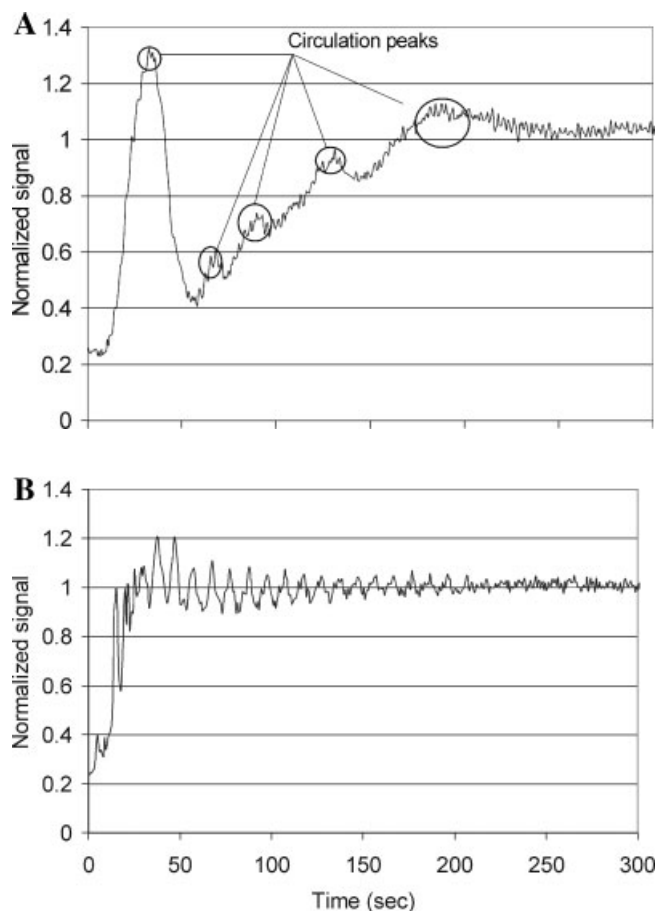


Figure 5. Mixing and circulation at 10 and 30 rpm. Figure shows how mixing and circulation time is achieved in the stirred vessel at a rotational speed of (A) 10 and (B) 30 RPM.

speed (Holmes et al., 1964; Middleton, 1979; Roberts et al., 1995). Figure 6 shows the same type of dependence of mean circulation time on rotational speed for the 12.5 mL vessel as it was for the 8–785 L vessels used by Holmes et al. (1964). Our results show that as the rotational speed is increased, the circulation time decreases in the 12.5 mL stirred vessel.

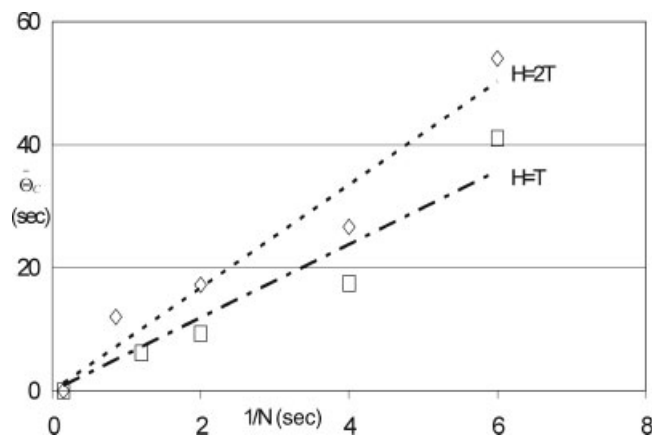


Figure 6. Influence of impeller speed and liquid height on circulation time. Figure shows the influence of impeller speed and liquid height on circulation time. (---◇) $H = 2T$. (—□) $H = T$.

For the case where $H = T$, Holmes et al. (1964) suggested that:

$$\bar{\theta}_C \sim N^{-1}(T/D)^2 \quad (3)$$

or

$$\bar{\theta}_C = k(T/D)^2(1/N) \quad (4)$$

In the particular case of the 12.5 mL vessel equipped with a single paddle impeller, for $H = T$, k was found to be 1.67 with a correlation coefficient (r^2) of 0.9244. For the case $H = 2T$, k was found to be 2.35 with a correlation coefficient (r^2) of 0.9476. Roberts et al. (1995) present values of k for Rushton and pitched blade turbines of 0.64 and 0.88, respectively. These results are in some extent unexpected as the flow regimes in our small scale minibioreactor differ considerably from the large scale bioreactor (Roberts et al., 1995). In our case, the flow varies from predominantly laminar to predominately turbulent, while in the big reactors the flow is fully turbulent. Still, results at small scale are comparable with those at large scales where the same pattern for circulation time as a function of rotational speed has been observed.

CONCLUSIONS

In summary, the 12.5 mL stirred vessel was operated in the Re range of $14 < \text{Re} < 1,350$. The stirred vessel is completely mixed for the entire range of Re. Isolated mixing regions were seen in the minibioreactor for $\text{Re} < 41$ but disappeared before mixing time were reached. The non-dimensional form of mixing time, $N\theta_M$, for the 12.5 mL stirred vessel operated in the transition regime is linearly dependent on the Reynolds number. Mean circulation time in the stirred vessel is inversely proportional to the impeller rotational speed. This dependency of mean circulation time on impeller rotational speed is similar to those previously reported (Holmes et al., 1964; Middleton, 1979; Roberts et al., 1995). Depending on the rotational speed and the liquid height chosen, mean circulation times of 54 and less than 1 s were found in the stirred vessel. Finally, it was verified experimentally that mean circulation time is proportional to the liquid height.

The results suggest that the mixing behavior of large tanks can be replicated at the small-scale bioreactors although the hydrodynamic conditions at both scales are different. The implications are that non-ideal cultivation conditions existing in large-scale bioreactors may be conveniently studied in minibioreactors and large numbers of experiments may be readily conducted.

NOMENCLATURE

C_{imp}	Clearance (mm)
C_w	Impeller clearance from vessel sidewall (mm)
D	Diameter of the agitator (mm)
E	Normalized signal dimensionless
Fl	Impeller flow number dimensionless

H	Liquid height (mm)
n	Number of t_i detected
N	Impeller speed (rpm)
Re	Reynolds Number dimensionless
T	Internal diameter of the vessel (mm)
t_i	Time between two consecutive circulations peaks (sec)
X_t	Actual signal transmitted by the confocal sensor (mV)
X_f	Final value of the signal transmitted by the confocal sensor (mV)
W	Width of the impeller (mm)

Greek Letters

$\bar{\theta}_C$	Mean circulation time (sec)
θ_M	Mixing time (sec)

References

- Alvarez MM, Guzmán A, Elías M. 2005. Experimental visualization of Mixing pathologies in laminar stirred tank bioreactors. *Chem Eng Sci* 60:2449–2457.
- Barneveld JV, Smit W, Oosterhuis NMG, Pragt HJ. 1987. Measuring the liquid circulation time in a large gas-liquid contactor by means of a radio pill. 2. Circulation time distribution. *Ind Eng Chem Res* 26:2192–2195.
- Bittorf KJ, Kresta SM. 2000. Active volume of mean circulation for stirred tanks agitated with axial impellers. *Chem Eng Sci* 55:1325–1335.
- Doig S, Pickering SCR, Lye GJ, Baganz F. 2005. Modelling surface aeration rates in shaken microtitre plates using dimensionless groups. *Chem Eng Sci* 60:2741–2750.
- Elmahdi I, Baganz F, Dixon K, Harrop T, Sugden D, Lye GJ. 2003. pH control in microwell fermentations of *S. erythrae* CA340: Influence on biomass growth kinetics and erythromycin biosynthesis. *Biochem Eng J* 16:299–310.
- Hall JF, Barigou M, Simmons MJH, Stitt EH. 2004. Mixing in unbaffled high-throughput experimentation reactors. *Ind Eng Res* 43:4149–4158.
- Holmes DB, Voncken RM, Dekker JA. 1964. Fluid flow in turbine-stirred, baffled tanks-I. Circulation time. *Chem Eng Sci* 19:201–208.
- Khang SJ, Levenspiel O. 1976. New scale-up and design method for stirrer agitated batch mixing vessels. *Chem Eng Sci* 31:569–577.
- Kostov Y, Harms P, Randers-Eichhorn L, Rao G. 2001. Low-cost microbioreactor for high-throughput bioprocessing. *Biotechnol Bioeng* 72(3):346–352.
- Lamping SR, Zhang W, Allen B, Shamlou PA. 2003. Design of a prototype miniature bioreactor for high throughput automated bioprocessing. *Chem Eng Sci* 58:747–758.
- Lye GJ, Ayazi-Shamlou P, Baganz F, Dalby PA, Woodley JM. 2003. Accelerated Design of Bioconversion Process Using Automated Microscale Processing Technique. *Trends Biotechnol* 21(1):29–37.
- Maharbiz MM, Holtz WJ, Howe RT, Keasling JD. 2004. Microbioreactor Arrays with Parametric Control for High-Throughput Experimentation. *Biotechnol Bioeng* 85(4):376–381.
- Marten MR, Wenger KS, Khan SA. 1997. In: Nienow AW, editor. 4th International Conference on Bioreactor & Bioprocess Fluid Dynamics. Bury St Edmonds, UK: Mechanical Engineering Publications.
- Middleton JC. 1979. Measurement of Circulation within Large Mixing Vessels. Third European Conference on Mixing. April 4th–6th.
- Nere NK, Patwardhan AW, Joshi JB. 2003. Liquid-phase mixing in stirred vessels. Turbulent flow regime. *Ind Eng Chem Res* 42:2661–2698.
- Nienow AW. 1997. On impeller circulation and mixing effectiveness in the turbulent flow regime. *Chem Eng Sci* 52:2557–2565.
- Ohmura N, Makino T, Kaise T, Kataoka K. 2003. Transition of organized flow structure in a stirred vessel at low Reynolds Numbers. *J Chem Eng J* 36(12):1458–1463.

- Papagianni M, Matthey M, Kristiansen B. 2003. Design of a tubular loop bioreactor for scale-up and scale-down of fermentation processes. *Biotechnol Prog* 19:1498–1504.
- Roberts RM, Gray MR, Thompson B, Kresta SM. 1995. The effect of impeller and tank geometry on circulation time distribution in stirred tanks. *Trans IChem* 73 Part A:78–86.
- Sano Y, Usui H. 1985. Interrelations among mixing time, power number and discharge flow rate number in baffled mixing vessels. *J Chem Eng J* 18:47–52.
- Vallejos JR, Kostov Y, Marten MR, Rao G. 2005. A novel noninvasive confocal optical sensor to study mixing. *Biotechnol Prog* 21:1531–1536.
- Vasconcelos JMT, Alves SS, Barata JM. 1995. Mixing in Gas-Liquid Contactors agitated by multiple turbines. *Chem Eng Sci* 50(14):2343–2354.
- Zalc JM, Szalai ES, Alvarez MM, Muzzio FJ. 2002. Using CFD to understand chaotic mixing in laminar stirred tanks. *AIChE J* 48(10): 2124–2134.
- Zanzotto A, Szita N, Boccazzi P, Lessard P, Sinskey AJ, Jensen KF. 2004. Membrane-aerated microbioreactor for high-throughput bioprocessing. *Biotechnol Bioeng* 87(2):243–254.