

Authors

Miss Yi Li¹, Dr Andrea Ducci², Dr Martina Micheletti^{1*}

Title

Mixing time in intermediate-sized orbitally shaken reactors for small orbital diameters

Abstract

Orbitally Shaken Reactors (OSRs) are widely used in bioprocess development, however, a scaling law between large reactors, usually shaken in incubators at an orbital diameter of $d_o=1.5-5$ cm, and microwell plates, shaken in benchtop ThermoMixers at $d_o=3$ mm, is still missing. In this work, mixing time was measured in two reactors (157 mL) of cylindrical and square geometries for $d_o=3$ mm. For such a small d_o , the acceleration mode to reach the final speed in the cylinder was found to greatly affect the free surface oscillations and thus the mixing time. The stepwise mode resulted in mixing time approximately six times smaller than the direct mode. The natural frequency of the reactor, which is independent of d_o , was found to be an effective scaling parameter for systems with small d_o .

Keywords

Dual indicator method; Microwell plates; Mixing time; Orbitally shaken; Scaling.

Author Affiliations

¹University College London, Department of Biochemical Engineering, Torrington Place, London, WC1E 7JE, United Kingdom.

²University College London, Department of Mechanical Engineering, Torrington Place, London, WC1E 7JE, United Kingdom.

*Correspondence: Martina Micheletti (Email: m.micheletti@ucl.ac.uk), University College London, Department of Biochemical Engineering, Torrington Place, London, WC1E 7JE, United Kingdom.

1. Introduction

Bioreactors are widely used in the biopharmaceutical and regenerative medicine industries for the production of a broad range of therapeutics [1]. There are numerous types of bioreactors with different agitation mechanisms and scales varying from mL to thousands of litres. In the early stages of bioprocess development, orbitally shaken microscale multi-well plates are commonly employed for parallel experimentation. Microwell plates come in different cross-sectional shapes, either with a square or cylindrical geometry, while lab-scale reactors consist mainly in shaken flasks, either baffled or unbaffled, or cylindrical vessels. In recent years, shaken reactors up to 1000 L have been manufactured, but most production scale reactors still heavily rely on impellers to enhance flow and mass transfer across the entire bioreactor.

The flow and mixing dynamics of Stirred Tank Reactors (STRs) are thoroughly characterized in the literature due to their wide use in the chemical industry [2-6]. On the contrary, microwell plates are commonly used by biologists and microbiologists, who are mainly focused on the biological outcome of the process, while engineering aspects associated to mixing and flow dynamics have been investigated to a lesser extent [7].

Gardner and Tatterson [8] was the first study to characterize the flow dynamics and mixing time in shaken systems. By adopting a dye dispersion approach, they found the presence of toroidal vortices in the laminar regime, while a sharp flow transition, promoting splashing of the free surface, occurred as Re was increased, resulting in very short mixing time. Since the seminal work of Gardner and Tatterson [8], the fluid dynamics and mixing of shaken bioreactors have been investigated in several studies, both numerically and experimentally. Computational Fluid Dynamics (CFD) in shaken flasks and 24- and 96-well microtitre plates were carried out by Zhang *et al* [9] and Zhang *et al* [10], respectively, while Kim and Kizito [11] performed both flow visualisations and numerical simulations of the flow in shaken cylinders for fluids of viscosity higher than water. In order to address the lack of understanding of mixing in shaken reactors and identify scaling flow parameters, Ducci and Weheliye [12] and Weheliye *et al* [13] carried out phase resolved PIV measurements in cylindrical OSRs for a wide range of operating conditions, including orbital diameter, d_o , reactor diameter, d_i , fill volume, V_f , rotational speed, N , and fluid viscosity, ν . They identified a mean flow transition where the toroidal vortex present below the free surface is replaced by an axial precessional vortex at sufficiently high shaker rotational speed. A flow scaling law based on the Froude number, $Fr = \frac{2\pi^2 N^2 d_o}{g}$, was derived by Weheliye *et al* [13] to predict the onset of the transition and depending on the operating parameters, the critical Froude number, Fr_{cr} , can be estimated from the following set of equations.

$$Fr_{cr} = \begin{cases} \frac{1}{a_w} \left(\frac{h}{d_i}\right) \left(\frac{d_o}{d_i}\right)^{0.5} & \text{for } \left(\frac{h}{d_i}\right) < \left(\frac{d_o}{d_i}\right)^{0.5} \quad (1) \\ \frac{1}{a_w} & \text{for } \left(\frac{h}{d_i}\right) > \left(\frac{d_o}{d_i}\right)^{0.5} \quad (2) \end{cases}$$

Where h is the fluid height and a_w is a coefficient proportional to the free surface wave amplitude, which depends on the fluid viscosity (1.4 for water, see Ducci and Weheliye [12]).

This flow scaling law was successfully employed by Rodriguez *et al* [14] to scale mixing time measurements obtained across different reactor sizes, $d_i=8-13$ cm, by means of the Dual Indicator System for Mixing Time (DISMT) technique. Direct comparison with the mixing time data obtained by Tissot *et al* [15] for larger reactors ($d_i=10-125$ cm), indicated that the Froude number ratio, Fr/Fr_{cr} , is an effective scaling parameter for mixing time measurements in shaken cylinder under a wide range

of operating conditions. The flow transition identified by the Froude number ratio, Fr/Fr_{cr} , was also found to accurately predict the full suspension speed of microcarriers across different bioreactor geometries (cylindrical vessels and flasks) and scales [16, 17]. The two-phase PIV study of Pieralisi *et al* [17] demonstrated that the flow dynamics in the presence of GE Cytodex 3 microcarriers ($\approx 400 \mu\text{m}$ after swelling) is only marginally affected by the solid phase with minimal slip velocity ($\approx 0.02 \pi N d_o$).

The current study builds upon the flow and mixing dynamics studies of Weheliye *et al* [13] and Rodriguez *et al* [14] and aims at assessing the validity of the established scaling rules for smaller reactors sizes ($d_i=35.4$ and 40.0 mm) and significantly reduced orbital diameters ($d_o=3$ mm). From this perspective, mixing time in two intermediate-sized reactors with cylindrical and square geometries were quantified by the DISMT methodology. The reactor sizes are smaller than the cylindrical OSRs used by Rodriguez *et al* [13, 14, 18] but larger than those commercially-available microtiter plates, thus bridging the gap between litre-scale and mL-scale OSRs.

2. Material and methods

2.1 Experimental set up

The cylindrical and square reactors had the same cross-sectional area with internal and hydraulic diameters of 40.0 and 35.4 mm, respectively. The effects on mixing time of three different fill volumes were assessed in each reactor, $V_f=25.1$, 37.7 and 50.3 mL, corresponding to $h/d_i=0.5$, 0.75 and 1 . The majority of the mixing time experiments were carried out in an Eppendorf ThermoMixer C, a shaker commonly used with microwell plates and characterised by a fixed orbital diameter ($d_o=3$ mm). The range of speed available in the ThermoMixer is between 300 to 3000 rpm, with an interval of 50 rpm. Due to the practical limitations of rigidly fixing the camera on the shaker at very high shaken speeds, the maximum speed investigated was 1000 rpm. The mixing time obtained in the ThermoMixer were compared against those from selected experiments obtained in a Kuhner LS-X bench top shaker table with adjustable orbital diameters ($d_o=1.5$ - 5 cm). Full details of the reactors and operating conditions examined are provided in Tab. 1.

(Table 1)

2.1 Mixing time quantification

The pH-colour based technique, Dual Indicator System for Mixing Time, was adopted in this work to measure mixing time. The technique was firstly developed by Melton *et al* [19] and successfully adapted to quantify mixing time in shaken systems by Rodriguez *et al* [14] and Tissot *et al* [15]. Two pH indicator solutions – Thymol Blue (Fisher Scientific) and Methyl Red (Fisher Scientific), were prepared in 70% ethanol and added to de-ionised water to form the working reagent of the experiment. A small amount of sodium hydroxide was added to the working reagent prior to the start of the experiment to change the colour of the solution from red to bright yellow. The desired fill volume was subsequently measured and adjusted by adding small amount of 0.075 M HCl. When the flow induced by the orbital shaking motion was fully established in the reactor (at least 20 revolutions were complete), the reaction between the base and acid was initiated by the insertion of the same amount of 0.075 M NaOH and the time evolution of the fluid colour variation was recorded by a NET iCube camera. To minimise statistical error each set of measurements comprised of 10

experiments and the average value was used to estimate the mixing time. The image analysis routine and standard deviation method already employed in Rodriguez *et al* [18] were used to obtain an objective measurement of the mixing time. This allowed direct comparison with the results of Rodriguez *et al* [18]. The method is based on the standard deviation of the normalised value of the green channel, $G_{i,j}^*$, defined in Eq. 3.

$$G_{i,j}^* = \frac{G_{i,j}(t) - G_{i,j}(t_0)}{G_{i,j}(t_\infty) - G_{i,j}(t_0)} \quad (3)$$

Where indices i and j are associated to a pixel and $G_{i,j}(t_0)$, $G_{i,j}(t_\infty)$ denote the green intensity of a pixel at the beginning and end of the experiment, respectively. Fully mixed conditions were reached when the standard deviation, σ_G^* , had reach the desired degree of homogeneity. The final mixing time was estimated as the average time required to achieve 13 degrees of homogeneity (in the range of 92% to 98%), as indicated in Micheletti *et al* [20]. Fig. 1 (a, b) show the typical variation in time of the standard deviation with the number of reactor revolutions for two different speeds, $N=300$ rpm and 500 rpm, respectively. Each plot includes three repeats of the same experimental condition. Notable differences between low- and high-speed conditions can be identified. At 300 rpm, the peak is skewed towards higher Nt values, indicating a relatively slow dispersion after insertion. On the contrary the curve for 500 rpm is initially characterized by a sharper increase of σ_G^* , followed by a slower mixing rate part after maximum dispersion is achieved (point of maximum σ_G^*). These general patterns occur consistently across the three repeats for each set of experiments.

(Fig 1)

3. Results and discussion

3.1 Mixing time measurements in a shaken cylinder

The variation of mixing time with respect to rotational speed ($N=100 - 1000$ rpm), fill volume ($V_f=25.1, 37.7$ and 50.3 mL) and orbital diameter ($d_o=3, 15$ and 50 mm) was initially assessed in a bioreactor of cylindrical geometry. The final rotational speed of the shaker was reached with two different acceleration modes, namely stepwise and direct modes.

Fig. 2(a) shows the mixing time variation with shaken speeds for three different fill volumes when the speed was increased gradually (stepwise mode). The plot is characterised by two distinct behaviours, above and below 700 rpm (indicated by the dotted line). For $N < 700$ rpm the plot shows the expected trend with the mixing time gradually decreasing as the speed is increased. This is clear from the instantaneous phase-locked snapshots of Fig. 2(b), where the free surface exhibits a continuous increase in maximum inclination with increasing speed ($N=300$ and 450 rpm), thus resulting in a faster mixing process. It is interesting to note that in this range of speed the fill volume does not significantly affect the mixing time. However, for $N > 700$ rpm the mixing time becomes counterintuitively longer despite the increase in speed, and reaches a peak at 800 rpm, thereafter showing the normal decreasing behaviour. Larger fill volumes determine a greater increase in mixing time. From the phase-locked images of Fig. 2(b) it is evident that at 700 rpm the free surface does not exhibit a clear inclination, resulting in reduced oscillations and therefore longer mixing. This behaviour is more accentuated at 800 rpm (last snapshot in Fig2 b), where the flow is mainly characterised by an axial vortex which trapped the acid solution (red) at its center and prevented mass transfer with the surrounding base solution (yellow). These findings are in agreement with those of Rodriguez *et al* [14], who also documented slow mixing zones inside vortical structures in larger shaken cylinders.

If the direct mode (i.e. the final speed was achieved directly from zero) was adopted, the mixing time variation showed a different behaviour. As shown in Fig. 3(a), an additional and more pronounced peak in mixing time occurs at a speed of 450 rpm. This is mainly related to the small orbital diameter of the ThermoMixer which, when combined with the sudden acceleration of direct mode and the large rotational speed, does not give enough time for the free surface to develop a coherent oscillation, hence resulting in poor mixing. This is well captured in the corresponding phase-lock snapshot in Fig. 3(b), where the free surface does not show any clear inclination in contrast to the large wave amplitude present at 300 rpm. When the speed is further increased the behaviour already outlined in Fig 2(a) with a secondary mixing time peak at 800 rpm still persists in direct mode. Also in this case the intensity of the peak correlates to the fill volume, and it is related to the reduced mass transfer across the axial vortex.

To further understand the impact of the two acceleration modes on the mixing time, the non-dimensional wave amplitude of the free surface was measured for increasing Froude number (see Fig 4). Consistently with Figs 2 and 3, also this set of results shows that the two acceleration modes have a significant impact on the free surface oscillations, and therefore on the overall flow occurring within the reactor. This difference is particularly pronounced in the lower end of shaken speeds investigated ($N < 650$ rpm), where the wave amplitude of the stepwise mode can reach values nearly 6 times greater than those obtained for direct mode ($Fr = 0.4$). The wave amplitudes of the two acceleration modes are closer for $Fr > 0.7$ (i.e. $N > 650$ rpm) where $\Delta h/d_i$ is decreasing with increasing speed. This is related to the formation of an axial vortex within the reactor, which is mainly characterised by solid body rotation. It should be noted that this behaviour is different to that found by Weheliye *et al* [13] for larger reactor sizes and orbital diameters, where the wave amplitude exhibited a constant increase with speed even after flow transition. This difference has to be related to the small orbital diameter combined with the large rotational speed.

To assess whether the peaks identified in the ThermoMixer were related to the small orbital diameter ($d_o = 3$ mm), further experiments were carried out in the intermediate-sized cylindrical reactor for orbital diameters of 15 and 50 mm. The corresponding mixing times are plotted in Fig. 5 for three different fill volumes. Both set of experiments display the standard decrease of mixing time with increasing rotational speed as found by Rodriguez *et al* [14], and, as expected, their values are proportional to the fill volume. However, in this case, the mixing time peaks identified in Figs 2 and 3 for the smaller orbital diameter are not present. Moreover, in this set of results the acceleration mode, either stepwise or direct, did not affect the final mixing time. Therefore, it can be concluded that the small orbital diameter combined with the large rotational speeds and sudden acceleration mode can have a major impact on the flow and mixing dynamics within the reactor, and operating conditions should be carefully selected to obtain representative and reliable results when a ThermoMixer is being used.

(Fig 2)

(Fig 3)

(Fig 4)

(Fig 5)

3.2 Mixing time measurement in the square reactor

Similar to Sec 3.1, mixing time measurements with a small orbital diameter, $d_o=3$ mm, were obtained in the square reactor for three fill volumes, $V_f = 25.1, 37.7$ and 50.3 mL, corresponding to the same fluid height, h , as in the cylindrical reactor and different acceleration modes. However, in this case, the difference in mixing time between the stepwise and direct modes was found to be within the statistical error associated to multiple experiment repeats, and therefore the mixing time data presented in Fig. 6 are an average of the two sets of results.

The mixing time variation with shaken speeds of Fig.6 is characterised by a two-peak curve, which is similar to that obtained in the cylindrical vessel in direct mode (cf. Fig. 3). Both peaks occur at a slightly lower rotational speed, 400 rpm and 750 rpm, when compared against the corresponding peaks of Figs. 3 (450 rpm and 800 rpm). Also in this case the first peak is associated to suppressed oscillations of the free surface, while the second one is related to the presence of an axial vortex inducing solid body rotation, and preventing mass transfer with the surrounding fluid.

(Fig 6)

The variation of the free surface wave amplitude with Froude number for the square reactor is shown in Fig. 7. The large inclination of the free surface at $N=300$ rpm is consistent with the fast mixing occurring at the lowest speeds considered in Fig. 6. Similar to Fig. 5, there is a range of rotational speeds centered around $Fr=0.4$ (i.e. $N=500$ rpm), where the system is denoted by marginal free surface oscillations and does not develop a significant wave amplitude. In this case the free surface is mainly vibrating due to the combination of small orbital diameter and large rotational speed. The presence of the corners in the square reactor also contributes to reduced free surface oscillations, even when a stepwise acceleration mode is employed.

(Fig 7)

3.3 Scaling in shaken bioreactors

The mixing time results obtained in this work in a ThermoMixer are compared against those reported by Rodriguez *et al* [18] for larger reactor sizes and greater orbital diameters in an attempt to identify a scaling law that is valid for OSRs of various scales and geometries. In their study, Rodriguez *et al* [18] demonstrated that the mixing number (Nt_m) correlates well with the Froude number ratio, Fr/Fr_{cr} , and the power law $Nt_m = 100.7(Fr/Fr_{cr})^{-1.245} + 25$ was found to best fit data over a broad range of operating conditions, including different reactor sizes, $d_i=8-13$ cm, and orbital diameter, $d_o=1.5-5$ cm. It should be noted that the same definition of $Fr = \frac{2\pi^2 N^2 d_o}{g}$, as mentioned in Sec. 1, was used throughout the analyses. The ratio Fr/Fr_{cr} takes the flow transition identified by Weheliye *et al* [13] into account, as Fr_{cr} is estimated by d_o and the critical speed which is obtained from either Eq. 1 or Eq. 2 depending on the satisfying condition. The power law proposed by Rodriguez *et al* [18] is also reported in Fig. 8 and compared against the mixing number curves for the intermediate-sized square and cylindrical reactors which are already discussed in the previous sections. From Fig. 8, it is evident that the power law found by Rodriguez *et al* [18] tend to overpredict the mixing number obtained in this work, and the ratio Fr/Fr_{cr} is not effective at scaling when small orbital diameters, such as in the ThermoMixer, are employed. The overprediction is most likely due to the scaling parameter Fr_{cr} , which is obtained based on d_i , d_o and h , in the range of $d_i=8-13$ cm, $d_o=1.5-5$ cm and $h=3-4$ cm, respectively. Nevertheless, both of the reactor dimensions and operating conditions investigated in this work are outside those ranges, suggesting the scaling law may not be valid for smaller shaken systems.

The natural frequency of the free-liquid surface has a direct effect on the performance and dynamic stability of moving reactors [21]. Therefore, as an alternative, the natural frequency of the liquid present in the reactor was considered as a possible scaling parameter. It is independent of the orbital diameter and can be found from Eq. 4 and Eqs. 5 & 6 for cylindrical and square containers, respectively, as described by Ibrahim 2005 [21].

$$\omega_{11}^2 = \frac{2g\varepsilon_{11}}{d_i} \tanh\left(\frac{2\varepsilon_{11}h}{d_i}\right) \quad (4)$$

$$\omega_{11}^2 = gk_{11} \tanh(k_{11}h) \quad (5)$$

$$k_{11} = \pi \sqrt{\frac{8}{d_i^2}} \quad (6)$$

Where ε_{11} is the first root of the derivative of the Bessel's function of the first kind and first order ($\varepsilon_{11}=1.841$).

It is evident from Fig. 8 that the mixing numbers obtained in this study correlate better with those found by Rodriguez *et al* [18], when the angular velocity to natural frequency ratio, ω/ω_{11} , is used for scaling. Despite the different peaks present in the intermediate reactor sizes for small orbital diameters, the two sets of results display closer mixing number magnitudes over the range of ω/ω_{11} investigated.

(Fig 8)

(Fig 9)

4. Conclusions

In this study, mixing time measurements were obtained in two intermediate-sized reactors with cylindrical and square geometries by means of a pH colour-based technique (DIMST). When a cylindrical reactor was shaken in the ThermoMixer ($d_o=3$ mm), the acceleration mode to reach the desired rotational speed had a significant impact on the mixing time, with stepwise acceleration inducing faster mixing than direct acceleration mode. Depending on the acceleration mode employed, single- or double-peak mixing curves were found. The first peak only occurred in direct acceleration mode, and it is related to the inability of the fluid to react to the sudden high speed motion of the system. This resulted in marginal free surface oscillations and poor mixing. The second peak occurring for both acceleration modes was found to be related to the transition to a vertical vortex flow, which prevented mass transfer with the surrounding fluid.

A double peak mixing curve was also observed for the square reactor, but in this case the acceleration mode did not affect the mixing time. Direct comparison between current results and the mixing time measurements reported in larger OSRs by Rodriguez *et al* [18], showed that the angular velocity to natural frequency ratio can offer a viable solution to scale mixing time measurements across reactor sizes in the order of the mL to L scales and orbital diameter ranging from 3 mm to 5 cm. The mixing time results presented in this work indicate that operating conditions and acceleration modes should be carefully selected to obtain representative and reliable results when a ThermoMixer with small orbitally shaken parameter is being used. Future work could be carried out in even smaller OSRs, such as microwell plates, to assess the validity of the newly-identified scaling parameter.

Symbols used

a_w	[-]	Water coefficient
d_i	[m]	Reactor internal diameter
d_o	[m]	Orbital diameter
Fr	[-]	Froude number
g	[ms ⁻²]	Gravity of Earth
h	[m]	Fluid height
N	[rpm]	Rotational speed
Nt_m	[-]	Mixing number
Re	[-]	Reynolds number
t_m	[s]	Mixing time
V_f	[mL]	Fill volume

Greek letters

ε	[-]	Derivative of Bessel's function
σ^*	[-]	Standard deviation of the normalized channel output
ω	[rad s ⁻¹]	Angular velocity
ω_{11}	[rad s ⁻¹]	Natural frequency

Abbreviations

CFD	Computational Fluid Dynamics
DISMT	Dual Indicator System for Mixing Time
OSR	Orbitally Shaken Reactor
STR	Stirred Tank Reactor

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Table

Table 1. Summary of the reactor sizes and operating conditions investigated.

Geometry	Reactor internal diameter, d_i [mm]	Reactor height, [mm]	Orbital diameter, d_o [mm]	Fluid height, h [mm]	Fill volume, V_f [mL]	Rotational speed range, N [rpm]
Cylinder	40.0	125	3, 15, 50	20.0, 30.0, 40.0	25.1, 37.7, 50.3	100 - 1000
Square	35.4	125	3	17.7, 26.6, 35.4	22.2, 33.3, 44.4	300 - 1000

Collection of figure legends

Fig. 1 Variation in time of the standard deviation of the normalized green channel, σ_G^* , for three repetitions at two different shaken speeds (Cylindrical reactor, $d_o=3$ mm, stepwise acceleration mode): (a) 300 rpm. (b) 500 rpm.

Fig. 1 (a) Variation of mixing time with rotational speeds for three different fill volumes, $V_f=25.1$, 37.7 and 50.3 mL, with stepwise acceleration mode (cylindrical reactor). (b) Phase-locked images showing different free surface behaviours for increasing speeds.

Fig. 2 (a) Variation of mixing time with rotational speeds for three different fill volumes with direct acceleration mode (cylindrical reactor). (b) Phase-locked images showing different free surface behaviours at increasing speeds.

Fig. 3 Non-dimensional free surface amplitude versus Froude number for the two different acceleration modes (cylindrical reactor).

Fig. 4 Variation of mixing time with shaken speed for large orbital diameters ($d_o=15$ and 50 mm).

Fig. 5 Variation of mixing time with rotational speeds for three different fill volumes in a square reactor ($d_o=3$ mm).

Fig. 6 Variation of the dimensionless wave amplitude versus Fr for the square reactor.

Fig. 7 Scaling between current data and those reported in Rodriguez et al [18] for large cylindrical OSRs using Nt_m and Fr_{d_o}/Fr_{cr} .

Fig. 8 Scaling between current data and those reported in Rodriguez et al [18] for large cylindrical OSRs using Nt_m and ω/ω_{11} .

Table of Contents: Graphical Abstracts

Research Article: The mixing time in two intermediate-sized shaken reactors were quantified by the DISMT technique for small orbital diameter shakers. The natural frequency of a filled container, which only depends on the reactor size, fill volume and geometry was found to be an effective mixing time scaling parameter for shaken systems of different reactor sizes (mL to L) and orbital diameters (3-50 mm).

Mixing Time Quantification in Intermediate-sized Orbitally Shaken Reactors.

Y. Li, A. Ducci, M. Micheletti*

Chem. Eng. Technol. **20XX**, *XX* (X),
[XXXX...XXXX](#)

