Microcarriers’ suspension and flow dynamics in orbitally shaken bioreactors

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Abstract

In the present work an effort is made to determine the suspension speed of microcarriers in an orbitally shaken bioreactor of cylindrical geometry, and to assess the associated two-phase flow by means of Particle Image Velocimetry (PIV). Microcarrier technologies are commonly used in the bioprocess industry to culture adherent-dependent cells in three dimensional flow. Commercial GE Cytodex microcarriers were employed throughout this study to best mimic the flow conditions occurring in a bioreactor under standard operating conditions. Suspension speed measurements were obtained at different solid concentrations, that are typical for cell cultures, and for different combinations of orbital to cylinder diameters’ ratio, \(d_o/d_i\) (\(c = 2.5 - 12.5\) g/L; \(d_o/d_i = 0.2 - 0.7; N = 0 - 200\) RPM). The current two-phase PIV results show that mean flow dynamics occurring in the cylindrical bioreactor are not significantly affected by the presence of the microcarriers, and that their suspension is directly associated to the flow transition reported by Weheliye et al. (2013). The flow scaling law included in their study can be successfully employed to predict the full suspension speed across bioreactors of different scales and working under different operating conditions (i.e. inner diameter of the cylinder, \(d_i\), orbital diameter, \(d_o\), and filling volume, \(V_f\)).

Keywords: Orbitally shaken bioreactor, microcarriers’ suspension speed, PIV, two-phase flow.

1. Introduction

Stem cells represent attractive therapeutic agents for a wide range of diseases due to their capacity to differentiate into a specialized cell type. The large number of cells required for clinical trials (up to millions cells/kg of body weight) demands a fast and reproducible expansion protocol. Stem cells are adherent-dependent cells, as they are able to grow and differentiate only if attached to an appropriate support. Two-dimensional (2D) static culture methods rely on the use of disposable multi-layer vessels and have rapidly become the most common route for stem cells expansion (Simaria et al., 2014). However, these methods do not seem appropriate for stem cell large scale production because of the limited cell productivity, labor intense handling procedures and long cultivation times. For example, recent studies proved that commercial requirements would be satisfied only with the production of up to \(10^{13}\) cells per batch, and the use of \(10^5\) layered vessels per lot, which is not a feasible process (Simaria et al., 2014). In
addition, these systems are not able to supply reproducible batch culture conditions (Mohamet et al., 2010). A cost-effective approach which has demonstrated to overcome many of the limitations of 2D cultures is represented by three-dimensional (3D) dynamic culture methods based on microcarriers suspension technologies (Frauenschuh et al., 2007; Sart et al., 2009; Storm et al., 2010). Microcarriers are generally spherical beads with an ideal size of 100-300 µm, and can be made of different materials (plastics, glass, silica dextran, collagen). Cell attachment is promoted through electrical charges or collagen coating. In microcarriers culture cells grow as monolayers on the surface of the beads or as multilayers in the pores of macroporous structures, that are usually suspended in culture medium by gentle stirring (GE Healthcare Life Sciences, 2013). With this technique the physiological microenvironment of stem cells can be easily monitored and reproduced, with significant advantages towards large scale production (King and Miller, 2007; Liu et al., 2014). The use of microcarriers in cell cultures allows an increase in the surface area (SA) per unit volume (cm²/mL), improving product consistency and decreasing costs (Frauenschuh et al., 2007; Sart et al., 2009; Schop et al., 2008, 2009; Ferrari et al., 2012).

Most studies have focused on investigating the optimal medium components, the microcarrier type and concentration, however only a few considered the engineering aspects, the quality of the microcarriers suspension and their impact on the liquid phase flow and turbulence levels. Conditions that promote efficient attachment and uniform distribution of the cells over the microcarriers population must be sought and optimized, and from this point of view, the flow and mixing dynamics occurring in the bioreactor must be thoroughly investigated and carefully selected. Efficient flow dynamics is crucial to achieve complete suspension of the microcarriers, thus preventing particle agglomeration and enhancing the available adherence area for the cells, while mixing is essential to promote mass transfer within the environment and to avoid spatial gradients in culture parameters (e.g. dissolved gases, nutrient concentration, pH), that can directly affect cell growth (Lara et al., 2006). At laboratory scale, adherent-dependent cell cultures are often grown on microcarriers in orbitally shaken reactors (OSRs), which offer an effective solution in the early stages of bioprocess development. Once the process is optimized, it is then scaled-up to traditional stirred tank reactors (STRs), where the velocity characteristics and turbulence levels are different from those found in shaken cultures. To overcome the scaling up/down limitations due to the different types of bioreactor, current bioprocess strategies have seen the development of miniature stirred tanks (for example the Ambr15 cell culture, 10-15 mL), to be employed in bioprocess development, while large scale shaken systems up to a scale of 1000 L have recently become available in the market, and studies have demonstrated their mixing effectiveness and oxygen transfer capabilities (Zhang et al., 2009).

Recently a few studies have focused on the mixing and fluid dynamics of shaken bioreactors. The works of Weheliye et al. (2013) and Ducci and Weheliye (2014) have provided a detailed understanding of the single-phase flow generated in an orbitally shaken bioreactor at different operating conditions (e.g. shaker rotational speed, N, and medium height inside the tank, h), geometrical characteristics (e.g. cylinder inner diameter, d_i, and orbital shaking diameter, d_o) and fluid viscosity, ν. A Fr-Re flow transition map was derived, where four types of mean
flow were identified depending on the combination of Froude and Reynolds numbers selected. A transition from a toroidal to a precessional vortex configuration was detected with increasing Froude number, $Fr$, for fluids of water-like viscosity close to those employed in cell culture (high $Re$ range). At low $Fr$ the free surface exhibited an elliptic shape in phase with the shaker table orbital movement, while an increasing degree of out-of-phase and a highly three-dimensional free surface characterised the high end of shaker speeds investigated (Weheliye et al., 2013). A flow scaling law was derived to predict the occurrence of this flow transition based on the Froude number, $Fr$, the fluid non-dimensional height, $h/d_i$, and the orbital to cylinder diameter ratio, $d_o/d_i$. More specifically it was found that for $h/d_i \leq \sqrt{d_o/d_i}$ the critical Froude number can be obtained from Equation 1, and it is associated to the toroidal vortex reaching the bottom of the cylindrical bioreactor before transition occurs, while for $h/d_i \geq \sqrt{d_o/d_i}$ transition takes place without the toroidal vortex expanding all the way to the reactor bottom, and the critical speed/Froude number can be found from Equation 2.

$$Fr_{d_o} = \frac{1}{a_{ow}} \frac{h}{d_i} \left( \frac{d_o}{d_i} \right)^{0.5} \quad (1)$$

$$Fr_{d_i} = \frac{1}{a_{ow}} \quad (2)$$

Where $a_{ow}$ is a constant depending on the fluid employed (1.4 for water), and the Froude number is defined as the ratio of the centrifugal to the gravitational accelerations, $Fr_d = 2\pi^2 N^2 d/g$, with $d$ being either the orbital ($d = d_o$, Equation 1) or cylinder ($d = d_i$, Equation 2) diameters.

The flow scaling law of Weheliye et al. (2013) was successfully applied to the mixing time experiments of Rodriguez et al. (2013, 2014) obtained by means of a base-acid colorisation technique in shaken bioreactors of cylindrical geometry. Rodriguez et al. (2014) compared their data to those obtained by Tissot et al. (2010) for very different operating conditions ($d_o$, $V_f$) and bioreactor sizes ($d_i$), and found out that the two sets of data scaled well when the mixing number was plotted against the ratio of $Fr/Fr_{cr}$, and achieved a constant value after flow transition occurred ($Fr > Fr_{cr}$).

Recently Mancilla et al. (2015) compared the mean flow and turbulence levels in orbitally shaken flasks with conventional, coiled, 1 and 3 baffle geometries. The 2D-PIV results obtained on a horizontal plane of measurements for increasing rotational speed, $N$, indicate that the configuration with a single baffle is characterised by turbulence levels 25% higher than in the other configurations investigated, and should be employed for production of bacterial cultures. Numerical simulation studies of the flow dynamics in shaken systems have been carried out by Zhang et al. (2005) and Zhang et al. (2008) for 250-ml Erlenmeyer flasks and for 24-well and 96-well bioreactors with water-like viscous fluids, respectively, while Kim and Kizito (2009) simulated the flow in a cylindrical shaken bioreactor for different fluid viscosity. Discacciati et al. (2012) developed a pressure correction method to best capture the free surface deformation and assess the shear stress levels in an orbitally shaken cylindrical container for a high viscous fluid, while Reclari et al. (2014) compared the free surface wave measurements in a shaken cylinder against those predicted by a potential sloshing model, and identified the presence of different
modal responses inducing different flow regimes.

Little information can be found in the literature regarding the flow and mixing dynamics taking place in bioreactors when microcarriers suspensions are considered. Collignon et al. (2010) investigated the suspension of microcarriers for TTP Mixel, A325-A320 Lightnin, three streamed-blades VMI-Rayneri, and Elephant Ear Applikon impellers in a stirred tank reactor, and compared the flow characteristics, shear rate and power consumptions of the different impellers at the corresponding just suspended speed, \( N_{js} \). Their results indicated that the TTP Mixel and the Ear Elephant Applikon impellers produced the lowest mechanical constraints at their just suspended speed. PIV measurements in a spinner flask were carried out by Ismadi et al. (2014) to assess to what extent flow shear stresses can affect cell culture of mouse induced pluripotent stem cells (iPSC) attached to microcarriers. They show that optimum number of cells was achieved over 7 days in 25 RPM suspension culture, corresponding to a maximum shear of 0.0984 Pa. Nienow et al. (2014) developed a new method for the harvesting of human mesenchymal stem cell (hMSC) in a spinner flask. The cells were cultured in dimple-bottomed spinner flasks equipped with a magnetic horizontal stir bar and a vertical paddle at a working volume of 100 mL and at 30 RPM (\( N_{JS} \)). After expansion, harvesting was implemented by adding trypsin-EDTA and agitating the microcarriers suspension for 7 mins at 150 RPM. Their study indicates that intense agitation for a short period (7 mins) under the presence of a suitable enzyme can promote cell detachment without damaging the cells or affecting their attributes. The overall harvesting efficiency was above 95 %.

Recently Olmos et al. (2015) determined the critical agitation speed for microcarriers’ suspension in orbitally shaken Erlenmeyer flasks and cylindrical reactors. They stained the microcarriers with Trypan blue and used a camera rigidly moving with the shaker table to assess their suspension at increasing speed. The Vachy-Buckingham theorem was employed to obtain the non-dimensional model of Equation 3.

\[
\frac{N_s}{\sqrt{g/d_o}} = \sqrt{\frac{Fr_s}{2\pi^2}} = A \left( \frac{h}{d_i} \right)^{0.5} \left( \frac{d_o}{d_i} \right)^{0.25} (\rho^* \frac{d_p}{d_i})^{-0.07}
\]

Where \( A \) is a constant depending on the type of geometry used (1.39 for cylinder, 0.12 for Erlenmeyer flask), and \( \rho^* \) and \( d_p \) are the relative density and diameter of the microcarriers, respectively. It should be noted that in Equation 3 they considered a Froude number which is defined as a velocity ratio, and it is related to the one defined in this work by the square root of \( Fr \). Direct comparison of Equations 1 and 3 shows that the critical Froude number, \( Fr_{cr} \), associated to the flow transition reported by Weheliye et al. (2013), is related to the suspension Froude number, \( Fr_s \), obtained from the model of Olmos et al. (2015), with the non-dimensional fluid height, \( h/d_i \) and orbital to cylinder diameter ratio, \( d_o/d_i \), terms having the same exponents. It is interesting to point out that their model showed a very good agreement also for Erlenmeyer flasks, implying that a similar flow transition to the one reported by Weheliye et al. (2013) could take place also in this geometry.
In the present study a different approach has been developed, where the “just-suspended” speed is estimated from the light scattered by the microcarriers on a laser plane parallel to the bottom of the cylindrical bioreactor, while vertical plane measurements were obtained to assess the homogeneity of microcarriers across the tank volume. Furthermore, two-phase Particle Image Velocimetry experiments were carried out to better comprehend the flow and mixing dynamics in the presence of microcarriers, and to assess how their concentration affects the mean flow characteristics.

2. Materials and methods

Depending on the measurements being carried out, two different experimental rigs were employed. Figure 1 (a) shows the experimental set-up used to obtain the “just suspended speed”, where a 300 mW continuous diode laser, a mirror, a Net iCube camera with Macro Lens, and a cylindrical bioreactor with a flat bottom, were all rigidly mounted on a Lab LS-X Kühner shaker table. The laser-light was directed horizontally in order to illuminate the plane located immediately over the vessel bottom, while a camera gained optical access to the measurement plane through a mirror located underneath the bioreactor. The camera was equipped with a macro lens with a shallow depth-of-field, that allowed to capture any small variation of the image brightness, which was directly related to the light scattered by the microcarriers sitting at the bottom of the bioreactor, as the shaking speed was varied. For each orbital speed investigated, 50 images were captured, and analysed by home-built Matlab routines to obtain a quantitative average result of the suspension conditions of the system. Before capturing a set of images a sufficient time was given to ensure steady-state condition was achieved at each speed investigated. Experiments were carried out in a borosilicate glass cylindrical bioreactor of size $d_i = 7$ cm, for different ranges of orbital diameters, $d_o = 1.5 - 5$ cm, and shaker speeds, $N = 60 - 140$ RPM. The working liquid was distilled water with a fluid height $h = 3$ and 5 cm ($V_f = 115.5, 192.5$ mL). Commercial microcarriers, GE Cytodex 1 ($\rho = 1.03$ kg/L, $d_{50} = 190$ µm) and GE Cytodex 3 ($\rho = 1.04$ kg/L, $d_{50} = 175$ µm), were employed at concentrations typically adopted for stem cell cultures: 2.5, 7.5, 12.5 g/L (0.25, 0.75, 1.25 wt%). Their settling velocity was approximately 0.6 mm/s. More information on the characteristics of the microcarriers employed can be obtained in GE Healthcare Life Sciences (2013).

The two-phase PIV system is shown in Figure 1 (b), where a larger Kühner shaker table (1 $\times$ 1 m², SR200-X shaker) is used to hold two cameras sharing the same field of view by a 50 % transmission/50 % reflection mirror and an optical guiding arm shining the laser onto a mirror positioned underneath the reactor. Contrary to the suspension speed experiments, in this case the measurement region consisted on the vertical plane bisecting the bioreactor into two halves. Each camera was equipped with a different light filter (either green, $\lambda = 532$ nm, or orange $\lambda = 570$ nm) to distinguish between the solid and liquid phases. To improve the image quality of the solid phase, fluorescent Rhodamine B isothiocyanate was employed to stain GE Cytodex 3
microcarriers, by exploiting the strong bond occurring between the dye and the thin collagen layer that coats the microcarriers’ surface. The staining protocol consisted in mixing 2 mg of Rhodamine in 50 ml of deionized water for a 200 mg sample of GE Cytodex 3. Staining was done at room temperature for 12 hrs and a 45 µm sieve was used to filter the stained particles. After this procedure the two-phase measurements could be carried out up to a solid concentration of 0.75 g/L (0.075 wt%). Above this threshold the image quality decreased due to the laser attenuation across the measurement plane induced by the presence of the microcarriers. Distilled water seeded with 1-40 µm flakes of painting was used as the continuous phase. Experiments were performed in a glass cylindrical bioreactor of size \( d_i = 10 \) cm, with an orbital diameter, \( d_o = 5 \) cm, and a fluid height \( h = 5 \) cm (\( V_f = 392 \) mL) for different shaker speeds, \( N = 80 – 130 \) RPM.

Phase-locked measurements were obtained by a magnetic encoder coupled to the Kühner shaker table. The origin of the angular coordinate, \( \phi \), was set when the system reaches its position furthest to the left as the clockwise orbit is viewed from above. To fully resolve the large scale flow structures the measurement spatial resolutions of the liquid and solid phases were \( \Delta x_i = 1.66 \) mm and 1.84 mm, respectively, while the time interval between PIV image pairs was \( \Delta t = 1-2 \) ms. The time interval, \( \Delta t \), was selected according to the optimisation protocol developed by Gomez et al. (2010). In the rest of the article a cylindrical coordinate system \( r, \phi, z \) is employed with the origin positioned on the cylinder axis at the bioreactor base. As mentioned in the introduction the Froude number based on the orbital diameter is an essential parameter to control the flow dynamics inside the bioreactor, and will be referred to here after either as \( Fr_{d_o} \) or, to simplify, as \( Fr \). A comprehensive list of the operating conditions investigated for the suspension speed and PIV experiments is provided in Table 1.

<table>
<thead>
<tr>
<th>SUSPENDED SPEED</th>
<th>SOLID-LIQUID PIV</th>
</tr>
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<tbody>
<tr>
<td>( d_i = 7 ) cm</td>
<td>( d_i = 10 ) cm</td>
</tr>
<tr>
<td>( d_o = 1.5, 2, 2.5, 3, 4, 5 ) cm</td>
<td>( d_o = 5 ) cm</td>
</tr>
<tr>
<td>( N = 0 - 200 ) RPM</td>
<td>( N = 80, 90, 96, 110, 130 ) RPM</td>
</tr>
<tr>
<td>( h = 2, 3, 4, 5 ) cm (( V_f = 76.9 - 192.5 ) mL)</td>
<td>( h = 5 ) cm (( V_f = 392.5 ) mL)</td>
</tr>
<tr>
<td>( c = 2.5, 7.5, 12.5 ) g/L (0.25, 0.75, 1.25 wt%)</td>
<td>( c = 0.25, 0.5, 0.75 ) g/L (0.025, 0.05, 0.075 wt%)</td>
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Table 1: Geometrical details of the shaken systems and operational conditions investigated for the two-phase measurements.

3. Results and discussion

In the following sub-sections the three parts of the investigation, that is, microcarriers’ suspension speed (§ 3.1), microcarriers’ dispersion (§ 3.2), and two-phase flow dynamics (§ 3.3), are
discussed in sequence. In brief, the rationale for the selection of these three parts of the work was to identify the range of speeds over which suspension occurs for different operating conditions, to assess the microcarriers’ suspension and dispersion mechanisms as the shaker speed is increased, and to determine the flow dynamics and transition of the two-phase system as well as compare them against those obtained for a single-phase (Weheliye et al., 2013).

3.1. Microcarriers suspension speed

The just suspended speed was estimated from the brightness of the images taken on the horizontal measurement plane, which is directly proportional to the amount of particles sitting at the bottom of the reactor. The image brightness, $I_B(N)$, at a given shaking speed, $N$, is defined in Equation 4 by adding the pixel greyscale, $p_{ij}$, across the area delimited by the bioreactor walls on the horizontal plane of measurement:

$$ I_B = \sum_{N_{tot}} p_{ij} $$

where $N_{tot}$ is the total number of pixels across the area.

The microcarriers’ suspension process and its correlation to the brightness percentage index, $I_B(N)/I_B(0)$, for increasing shaking speed, $N$, can be gained from Figure 2, where steady-state images of the microcarriers’ concentration over horizontal planes are coupled to the $I_B(N)/I_B(0)$ curve at key speeds. This set of experiments was carried out for an orbital diameter $d_o = 2.5$ cm and a microcarriers’ concentration $c = 2.5$ g/L. At low shaking speeds the microcarriers are uniformly distributed over the vessel bottom, and the brightness index is approximately constant up to a speed of 110 RPM, when the particles start being arranged in a spiral pattern on the bioreactor base and a drop of $I_B(N)/I_B(0)$ occurs. As the orbital speed is further increased a nearly constant value of the brightness index is attained above 150 RPM, implying that the “just-suspended” condition is achieved.

To better compare the results obtained for the different conditions analysed, the normalised brightness index, $I^*$, of Equation 5, which is scaled with the zero-speed, $I_B(0)$, and final-speed, $I_B(\infty)$, brightnesses, is used in the rest of the work.

$$ I^* = \frac{I_B(N) - I_B(\infty)}{I_B(0) - I_B(\infty)} $$

The suspended speed is associated to a 95 % decrease of the brightness index with respect to the zero-speed condition, and it is identified as the speed at which $I^* = 5\%$. Based on the statistical error of the brightness index, $\approx 3\%$, and the non-linear regression method used to fit the data points, the uncertainty affecting the just suspended speed was found to be $\approx 5\%$.

A video showing the particle suspension dynamics is also provided in the supplementary materials (JS-Video.avi). In this case however the shaker table was started from still conditions and, similarly to standard operating procedures, was gradually accelerated to a final speed of 140 rpm by the controller mounted on the shaker system (i.e. steady-state conditions were not achieved.
at intermediate speeds). As a consequence the instantaneous velocity associated to each frame is unknown, and the following discussion is made in terms of number of revolutions of the shaker tray (i.e. the encoder was used to acquire a frame per revolution). In agreement with the data reported in Figure 2, darker zones start appearing at the periphery of the bioreactor ($t = 3 - 5$ s of the video), with microcarriers being more concentrated at the centre for increasing speed. This is well captured in Figure 3 (a), where the radial profiles of the normalised brightness index, $I^*$, are shown for selected time instants, counted in number of revolutions, $n$, of the shaker tray, and corresponding to increasing shaking speed. After 100 revolutions, the shaker table has not gained a speed high enough to lift the particles, and the index $I^*$ is nearly constant across the bioreactor diameter and close to unity. As the shaker table is accelerated, a drop of $I^*$ occurs after 110 revolutions, with the microcarriers being suspended for $r/R \geq 0.6$, while the center of the bioreactor, $r/R \leq 0.3$, is still unaffected after 130 revolutions. It is worth noticing that also the rate of suspension is lower in proximity of the bioreactor axis. For example, a 10 revolutions increment ($n = 120 - 130$) for $r/R \geq 0.6$ determines a variation of the normalised brightness index of $\Delta I^* \approx 0.45$, while a similar drop ($\approx 0.5$) occurs at $r/R = 0.3$ over a larger range of shaker revolutions, $\Delta n = 30$ ($n = 140 - 170$).

The spiral pattern, described in Figure 2 and shown in the supplementary video, is further analysed in Figure 3 (b), where the azimuthal profiles of $I^*$ are plotted at $r/R = 0.8$ for an increasing number of shaker table revolutions ($n = 100 - 135$). It is evident that for $n = 110 - 122$ the profiles show a cyclic variation in the azimuthal direction, with 5 peaks over the range of $\theta$ considered. As expected the intensity of the profiles is decreasing as more microcarriers are lifted with increasing speed (i.e. number of revolutions), and the profiles are randomly shifted with respect to each other along $\theta$, because the instants considered were taken far apart in time, and the spiral structure might have rotated with respect to the bioreactor. However an estimate of the spiral inclination can be gained from Figure 4 (a), where a single cycle of $I^*$ has been obtained through a phase-average, $\langle \rangle$, along the azimuthal direction with a period $\Delta \theta = 20^\circ$. This analysis was performed at different radii for a single frame, $n = 117$. The phase-averaged profiles were normalised by their maximum variation $\langle \Delta I_B \rangle$, so that the final brightness parameter assumed a maximum absolute intensity of $\approx 1$ for all the radii considered ($r/R = 0.6 - 0.9$). It should be noted that in Figure 4 (a) the flow direction is from right to left and opposite to that of $\theta$. The peak shifts to the right as the radius increases, which means that the spiral is oriented towards the center in the direction of motion. The variation of the peak azimuthal coordinate, $\theta_{max}$, against the radius is shown in Figure 4 (b) for two time instants, $n = 117$ and 120. The peak azimuthal coordinate, $\theta_{max}$, shows a linear increase with $r/R$ and the slope magnitude is nearly the same for both instants considered (i.e. $18.57^\circ$ vs $18.86^\circ$). A visualisation of the spiral locus is provided in the inset diagram, where the arrow points in the flow direction.

The variation of $I^*$ against the shaker tray speed is plotted in Figures 5 (a) and (b) for two orbital diameters, $d_o = 1.5$ and 2.5, respectively. Three different microcarriers’ concentrations are considered, $c = 2.5, 7.5$ and $12.5$ g/L, while the fluid height and vessel size are kept constant.
(h = 5 cm, di = 7 cm). It should be noted that by definition the index, I*, can assume only
values between 0 and 1 at high and low shaking speeds, respectively. Data points are fitted with
the model of equation 6, where in the remainder part of the work the variable x can either be
the shaker speed, N, or the Froude number ratio, Fr/Fr_cr.

\[ I^*(x) = \frac{1}{1 + e^{a(x-x_0)}} \]  

(6)
The parameters x0 and a position the curve along the x coordinate, and control its rate of decay,
respectively. The plots of Figure 5 (a) cross the 5 % reference line within a relative small range
of suspension speeds, N_s = 153 – 160 RPM, and a correlation between the concentration and
the suspension speed seems to be present (i.e. lower suspension speeds occur for lower concen-
trations). However this correlation is not present in the data of Figure 5 (b) for do = 2.5 cm,
where an opposite behaviour is observed (i.e. lowest suspension speed for greatest concentration
considered). Also in this case the range of variation of the suspended speed is relatively small,
N = 145 – 152 RPM, and it is within the error of the measurement technique employed. Based
on this consideration it was concluded that the concentration should not affect to a large extent
the suspension of the microcarriers, at least within the range of concentration considered in this
study, which includes those commonly employed in the bioprocess industry.

On the contrary the variation of the suspension speed with the orbital diameter is significant.
This is evident in Figure 6 (a) where the normalised brightness index, I*, is plotted against the
shaker speed for different orbital diameter, do = 1.5, 2.5 and 5 cm. As expected the suspension
speed, N_s, increases with decreasing orbital diameter, and assumes values of 120 RPM, 144 RPM
and 153 RPM for do = 5 cm 2.5 cm and 1.5 cm, respectively. In Figure 6 (b) an attempt was
made to assess whether the suspension mechanism would scale with the critical Froude number
ratio, Fr/Fr_cr. In fact the three systems are associated to different do/di and therefore reach
the flow transition at different speeds (Weheliye et al., 2013). However the plot of Figure 6 (b)
does not support this scaling procedure with the lowest (highest) orbital diameter still being
associated to the greatest (lowest) critical Froude number ratio. This was explained by consid-
ering that the fluid height (h = 5 cm) of two, do = 1.5 cm and 2.5 cm, out of the three systems
investigated is too large for the flow to fully develop to the cylinder bottom before transition
occurs. In both cases h/di > \sqrt{do/di} (0.71 > 0.46 for do = 1.5 cm and 0.71 > 0.59 for do = 2.5
cm) and Equation 2 shall be used to determine the critical Froude number, Fr_cr.

Based on these considerations a second set of measurements was carried out to assess the sus-
pension process when h/di ≤ \sqrt{do/di}, and a critical speed exists for the flow to extend to the
bottom of the reactor. The variation of I* with do is provided in Figures 7 (a) and (b) for
increasing speed and critical Froude number ratio, respectively. In agreement with Figure 6
(a) the plots of Figure 7 (a) intercept the 5% reference line at increasing suspension speed for
decreasing orbital diameter. In this case however when the brightness index is plotted against
the critical Froude number ratio (see Figure 7 b) the data tend to collapse on a single curve,
indicating that the parameter Fr/Fr_cr can be successfully used for scaling across different con-
figurations (i.e. do/di), provided that the fluid height satisfies the condition h/di ≤ \sqrt{do/di}.
The data presented in Figures 6 and 7 are summarised in Figure 8, where the suspended to critical Froude number ratio is plotted against the parameter $h/d_i/\sqrt{d_o/d_i}$. As indicated by the inset schematics values of $h/d_i/\sqrt{d_o/d_i} < 1$ identify those configurations for which the toroidal vortices extend to the bottom of the bioreactor when the critical speed is achieved, while this does not occur for $h/d_i/\sqrt{d_o/d_i} > 1$, and flow transition takes place without the flow developing to the reactor base. The error bars in Figure 8 are supposed to provide a reference, and correspond to a 2 RPM variation in the suspension speed $N_s$ (i.e. $d_Fr_s/Fr_{cr} = 2 \times (N_s/N_{cr}^2) dN_s$). From Figure 8, the 95% suspension condition is achieved for $Fr_s/Fr_{cr} \leq 1$ when $h/d_i/\sqrt{d_o/d_i} < 1$, while the suspended to critical Froude number ratio tends to drift further away from the dashed reference line at $Fr_s/Fr_{cr} = 1.1$ as $h/d_i/\sqrt{d_o/d_i}$ increases above 1. It is interesting to note that the suspension speed data obtained by Olmos et al. (2015) in Erlenmeyer flasks showed a good scaling with the critical speed, $N_{cr}$, also for $h/d_i/\sqrt{d_o/d_i} > 1$.

The coefficients $a$ and $x_0$ of Equation 6, used to determine the suspended to critical Froude number ratio (i.e. $Fr_s/Fr_{cr} = \log(19)/a + x_0$ for 95% suspension), are provided in Table 2. It is worth pointing that the range of variation of the decay coefficient for data associated to $h/d_i/\sqrt{d_o/d_i} > 1$ ($7 < a < 14.3$) is lower than that for $h/d_i/\sqrt{d_o/d_i} < 1$ ($14 < a < 17.8$). This implies that for $h/d_i/\sqrt{d_o/d_i} < 1$ suspension occurs more sharply with increasing speed.

<table>
<thead>
<tr>
<th>$h/d_i/\sqrt{d_o/d_i} &gt; 1$</th>
<th>$h/d_i/\sqrt{d_o/d_i} &lt; 1$</th>
</tr>
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<tbody>
<tr>
<td>$a$</td>
<td>$a$</td>
</tr>
<tr>
<td>$x_0$</td>
<td>$x_0$</td>
</tr>
</tbody>
</table>

Table 2: Coefficients $a$ and $x_0$ obtained for all the sets of data analysed in this work.

### 3.2. Microcarriers’ dispersion

A similar analysis to that employed in the previous section was carried out over vertical planes of measurement to assess the dispersion across the bioreactor of the microcarriers’ suspension. In this case the normalisation of the brightness index was done according to Equation 7, where the coefficient varies from 0 (low concentration of microcarriers’ over the volume) to 1 (homogenous concentration across the bioreactor volume).

$$I^* = \frac{I_B(N) - I_B(0)}{I_B(\infty) - I_B(0)}$$  \hspace{1cm} (7)

The variation of $I^*$ with the critical Froude number ratio, $Fr/Fr_{cr}$, is provided in Figure 9, where inset snapshots provide a visual reference of the degree of dispersion. Data refer to a
system with \( d_i = 13 \text{ cm}, d_o = 5 \text{ cm} \) and \( h = 6.5 \text{ cm} \) \( (h/d_i/\sqrt{d_o/d_i} < 1) \). The vertical and horizontal lines provide a reference of the suspended to critical Froude number ratio, \( Fr_s/Fr_{cr} = 1.1 \), found in the previous section, and of the 95 \% degree of homogeneity, respectively. From Figure 9 it can be concluded that complete dispersion is achieved at a speed slightly higher than the suspended one, \( \approx 1.2 \times Fr_{cr} \) (95 \% threshold).

A closer view at the dispersion of microcarriers across the tank can be gained from the axial and radial cumulative brightness profiles of Figures 10 (a) and (b), respectively \( (d_i = 10 \text{ cm}, d_o = h = 5 \text{ cm}) \). The axial (radial) cumulative brightness was obtained by adding the image brightness along the radial (axial) direction. Before proceeding with the discussion, it is worth mentioning that a limitation of adopting the brightness index as a reference for microcarriers’ concentration is that in the vertical plane of measurements the laser enters the bioreactor from the base, and therefore complete brightness homogeneity is impossible to achieve due to reflections. This explains why brightness maxima are always located at \( z = 0 \), even at the higher speed investigated, when microcarriers’ suspension has certainly occurred. Despite this the current data provide a reliable description of the suspension over a vertical plane for increasing speed.

Bearing this in mind, the plot of Figure 10 (a) shows that the axial distribution of microcarriers is poor for \( N \leq 100 \) with the normalised brightness index, \( I_B(z, N)/I_B(0, N) \), being relatively low for \( z/d_i \leq 0.04 \), while, in agreement with the higher decay coefficients observed in Table 2 for \( h/d_i/\sqrt{d_o/d_i} < 1 \), a sharp change in \( I_B(z, N)/I_B(0, N) \) occurs over a relatively small range of shaker speeds, \( N = 100 – 105 \text{ RPM} \). The curves of \( N = 105 \text{ RPM} \) and \( N = 130 \text{ RPM} \) are nearly parallel for \( z/d_i \geq 0.06 \) indicating that a similar degree of dispersion along the axial direction has been achieved for both, while the lower intensity of \( I_B(z, N)/I_B(0, N) \) indicates that fewer microcarriers are suspended for the lower speed considered.

Similarly to the axial profiles, the radial profiles of the cumulative brightness index, \( (I_B(z, N) – I_B(0, N))/I_B(0, N) \), Figure 10 (b), show little suspension for \( N < 102 \), while at greater speeds the radial distribution is characterised by double crested profiles, where the peaks capture the higher microcarriers’ concentration already present in the top-right inset of Figure 9. The peaks are located close to the reactor axis and they occur in the region swept by the precessional vortex once flow transition has occurred. Based on these results and those in the previous section it can be concluded that microcarriers are pushed from the periphery towards the centre of the reactor base, and they are then sucked into the bulk flow by the depression created close to the axis of the bioreactor by the two-counter rotating and precessional vortices, before and after flow transition, respectively.

3.3. Two-phase flow dynamics

Two-phase Particle Image Velocimetry experiments were carried out to better understand the influence of the solid phase on the mean characteristics of the flow, and to assess whether the flow transition reported by Weheliye et al. (2013) can be extended to the two-phase system. A preliminary analysis was carried out to assess whether the free surface wave, which is the flow
driving mechanism, is affected by the microcarriers’ concentration. The study of Weheliye et al. (2013) showed that for a single-phase system the nondimensional wave amplitude, $\Delta h/d_i$, is proportional to the Froude number, meaning that for selected combinations of $N$ and $d_o$, the free surface will assume a fixed inclination, which is independent of the fluid height $h$ and vessel diameter, $d_i$. The constant of proportionality, $a_o$, depends on the fluid considered, and is equal to 1.4 in the case of water, and decreases with increasing fluid viscosity (Ducci and Weheliye, 2014). The variation of $\Delta h/d_i$ against $Fr$ ($0.25 < Fr < 0.5$) for different microcarriers’ concentrations at $h/d_i = 0.5$, and $d_o/d_i = 0.5$ is provided in Figure 11. The data points are all located close to the reference line, which corresponds to a single-phase system with water as the working fluid ($a_{ow} = 1.4$). A small decrease of the slope might be seen for increasing microcarriers’ concentrations, that is consistent with the behaviour reported by Ducci and Weheliye (2014) for increasing viscosity. This means that the flow dynamics of the two-phase system is not remarkably affected by the presence of microcarriers at the concentration considered, and that the applicability of the relation found by Weheliye et al. (2013) can be extended to the two-phase system. Lower values of the slope coefficient, $a_o$, might imply that the critical Froude number for the two-phase system is slightly higher than that of the single-phase (see Equation 1), and therefore the suspended speed data points of Figure 8 might get closer to the horizontal reference line of $Fr/Fr_{cr} = 1$.

The phase-resolved velocity vector fields and tangential vorticity, $\omega \theta / (\pi N)$, contour maps of the liquid and solid phases are shown in Figure 12 (a-b) and (c-d) for in-phase, prior to flow transition, and out-of-phase conditions, respectively. For both flow conditions the phase angle was $\phi = 0$ and the microcarriers’ concentration, $c = 0.5$ g/L. The velocity fields of the liquid and solid phases for in-phase flow (Figures 12 a and b) are qualitatively similar to each other, and are characterised by the two vortical cell configuration already identified by Weheliye et al. (2013) at the same speed for single-phase flow. However, in the toroidal vortex region, the vorticity of the solid phase assumes values slightly higher than for the liquid one (mainly on the left hand side vortex), indicating that a slip velocity is present between the two phases. Similar conclusions can be drawn when comparing the velocity fields for the out of phase flow (Figures 12 c and d). In this case the axial slip velocity, $|u_{zS} - u_{zL}| < 0.02 \times \pi Nd_o$ (0-6 mm/s). It is worth mentioning that this range of values is comparable to the average and maximum velocities of the liquid phase over the plane of measurement, 0.033 and 0.10 $\times \pi Nd_o$, respectively.

4. Conclusions

This study is the first one to provide insight on the two-phase flow dynamics occurring in an orbitally shaken bioreactor when microcarriers are used in suspension under real process conditions. The suspension dynamics of the two-phase system was investigated using a visualization approach, which allowed to estimate the “just - suspended” shaking speed from the light scattered by the microcarriers on a laser plane parallel to the bottom of the cylindrical bioreactor. The shaking system was studied varying solid concentration and orbital diameter, and the results highlightened the correlation between the microcarriers suspension and the critical Froude
number corresponding to the occurrence of the flow transition identified by Weheliye et al. (2013) for a single-phase system. It was found that for bioreactor configurations corresponding to $h/d_i/\sqrt{d_o/d_i} < 1$ the suspended Froude number, $Fr_s$, is nearly constant and equal to $1.1 \times Fr_{cr}$, while for $h/d_i/\sqrt{d_o/d_i} > 1$ the suspended speed tends to increase, and suspension is delayed to higher speeds after flow transition. From this point of view the first type of configuration should be sought because it achieves full suspension and at the same time minimises power consumption and shear rates.

An analysis of the suspension mechanisms highlighted that microcarriers are pushed from the periphery towards the centre of the reactor base along a spiral pattern, and then they are sucked into the bulk flow by the depression created close to the axis of the bioreactor by the two-counter rotating and precessional vortices, before and after flow transition, respectively. Vertical plane measurements were used to assess the homogeneity of the microcarriers across the reactor volume, and it was found that full dispersion is achieved at $\approx 1.2 \times Fr_{cr}$. A model was developed to fit the suspension data, and showed that suspension dynamics are faster and occur over a narrower range of speeds for $h/d_i/\sqrt{d_o/d_i} < 1$. The free surface experiments validated the relation found by Weheliye et al. (2013) between the non-dimensional wave amplitude of the cylindrical bioreactor, $\Delta h/d_i$, and the Froude number, and it was found that the presence of the microcarriers might reduce the constant of proportionality between the two parameters, and result in slightly higher critical Froude number, $Fr_{cr}$. The velocity fields of the liquid and solid phases were simultaneously measured over a vertical plane bisecting the vessel, and their mean flows were found to be very similar both for in-phase and out-of-phase conditions. This is in agreement with previous studies on stirred tank reactors where low solid concentrations are employed. The range of variation of the axial slip velocity, $|u_{zS} - u_{zL}| < 0.02 \times \pi N d_o$ (0-6 mm/s), was comparable in magnitude to the average and maximum velocities of the liquid phase over the plane of measurement, 0.033 and $0.10 \times \pi N d_o$, respectively.

Further studies are called for to investigate the suspension dynamics of the next generation of microcarriers. Biodegradable materials are increasingly used to make microcarriers for cell adherent applications in order to avoid the need for the cell detachment and recovery steps. However the materials used are often characterised by densities much heavier than water, thus requiring considerable energy to be suspended. The flow visualisation methodology established in this work, as well as the simultaneous measurement of the two-phase flow characteristics, could be implemented for other microcarriers’ types to assess the quality of suspension, and its dependence on the bioreactor geometry and operating conditions.
Nomenclature

Abbreviation

2D Two-Dimensional
OSB Orbitally shaken bioreactor
STR Stirred Tank Reactor
PIV Particle Image Velocimetry
3D Three-Dimensional

Greek Symbols

ν Kinematic viscosity, m^2/s
ρ Microcarriers’ density kg/m^3
ρ* Microcarriers’ relative density, –
ϕ Phase angle of the table, °
ωi Vorticity component in the ith direction, s^-1

Roman Symbols

a Decay coefficient of Equation 6, –
a_ow Constant of proportionality for water, –
di Inner diameter of the cylinder, m
do Orbital diameter, m
dp, d50 Microcarriers’ diameter, m
Fr Froude number, –
Fr_cr Critical/transitional Froude number, –
Fr_s Suspended Froude number, –
g Gravitational acceleration, m/s^2
h Fluid height at rest, m
Δh Free surface height, m
I* Normalised brightness index, –
IB Brightness index, –
n Number of shaker revolution, –
N Shaking frequency, s^-1
N_cr Critical shaking frequency, s^-1
N_s Suspension shaking frequency, s^-1
R Inner radius of the cylinder, m
Re Reynolds number, –
u_i Velocity in the ith direction, m/s
Vf Fluid filling volume, m^3
x0 Position coefficient of Equation 6, –
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\[ \frac{\langle I_B(\theta) \rangle - \langle I_B \rangle}{0.5 \Delta I_B} \]

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