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# The flow inside shaking flasks and its implication for mycelial cultures

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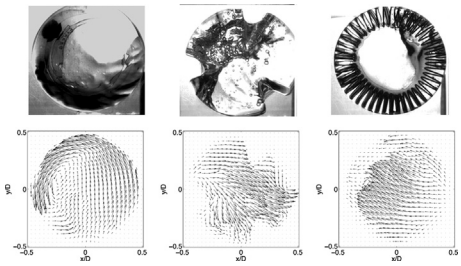
## HIGHLIGHTS

- A hydrodynamic analysis for three shaking flask geometries is presented.
- Velocity fields were experimentally measured by Particle Image Velocimetry technique.
- Strain rate is the hydrodynamic variable which strongly influences the cultures.
- This work contributes to understand more the physics of the flow inside shaken flasks.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Several parameters such as mixing time, power consumption and deformation rates have been commonly reported in the literature for the hydrodynamic characterization of shaken flasks. In the present work, flow fields of orbital shaken flasks having different geometries have been experimentally obtained. Conventional, baffled and coiled flasks were tested at constant shaking speed of 150 rpm at which the cultures are grown. Flow fields in terms of turbulence intensity and deformation rate were both determined by means of the Particle Image Velocimetry (PIV) technique. Velocity fields are strongly dependent on the flask geometry; in particular, the main flow is confined near the wall for the conventional geometry. In general, large velocity fluctuations are found in the whole flask for the baffled and coiled geometries, while the turbulence intensity is virtually zero at the center region for the conventional flask. The measurement of the average deformation rate indicates that flow obstacles, such as indentations and coiled springs, generate regions with high hydrodynamic stresses promoting the elongation and breakup of bubbles and biomass. Results from this study have been compared with previous studies finding good agreement for the same flask configurations at similar experimental conditions.



## 1. Introduction

The shake flask method is the most widely used technique for bioprocess optimization and culture research, because of its simplicity, low cost and easy handling (Büchs, 2001). Flasks are available in different sizes and materials, baffled, non-baffled and with other additions such as spring coils inserted at the bottom of the flask (Hopwood et al., 1985). These designs are used for different bioprocess development projects (Suresh et al., 2009), and therefore, many studies have been conducted on flasks since this shaking process was introduced into the biotechnology field at the beginning of the previous century. Different aspects of shaken

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flasks have been investigated (Freedman, 1970; Büchs et al., 2000a, b; Hansen et al., 2011; Klöckner and Büchs, 2012); however, only few studies provide complete information of the inside flow. Kurata and Mita (1996) reported a qualitative description of the shaking process in plant cell cultures using a flow visualization technique. Zhang et al. (2005) and Li et al. (2013) studied the mixing and gas-liquid mass transfer rate on baffled and unbaffled flasks using computational fluid dynamics (CFD). From a similar approach, Kim and Kizito (2009) also performed numerical simulations to study the flow in a shaken system taking into account the deforming free surface and comparing their numerical results with flow visualizations at 60 rpm finding them in good agreement. Discacciati et al. (2013) developed a numerical method to study the velocity field and the interface discontinuities to describe the flow behavior in orbitally shaken bioreactors at different agitation rates. Recently, Weheliye et al. (2013) and Rodríguez et al. (2013) provided a thorough description of the dynamics of the flow occurring inside a cylindrical shaken bioreactor using phase-resolved particle image velocimetry (PIV) as well as the mixing time data obtained with a colorimetric pH technique. Several parameters are used for the hydrodynamic characterization of stirred vessels and shaken flasks, namely: power consumption, mixing times and flow fields. Giese et al. (2014) developed an empirical correlation to determine the effective shear rate of fermentation broths in shaken flasks. For that purpose, the specific power drawn of flasks placed in an orbital shaker was measured. Although the obtained correlation is valid for a limited shake flask volume range, it could be applied for scaling up purposes. In regard to mixing times, Tan et al. (2011) used the colorimetry technique to measure the dispersing time of a tracer into the rotating bulk of a shaken flask; they observed that mixing time was similar irrespective of the shaking diameters. On the other hand, the specific power consumption in shaken flasks was measured by Peter et al. (2006) finding that more power is needed when increasing the shaking frequency and decreasing filling volume.

Nevertheless, there is still limited understanding of the flow physics and lack of engineering studies for shaken vessels, which would be required to assure reproducible and meaningful scaling-up to bioreactors (Suresh et al., 2009). In particular, the geometrical arrangement may play an important role on the quality of the mixing process in biotechnological applications. For instance, Gamboa-Suasnavart et al. (2011) reported a significant influence of the flask configuration on the production of the recombinant Ala-Pro-rich O-protein (rAPA) from *Mycobacterium tuberculosis* produced by *Streptomyces lividans*. This process has been proposed to generate part of a new tuberculosis vaccine and for diagnosis kits. They performed measurements in three shake flask configurations (conventional, baffled and with coiled spring) and evaluated the production of rAPA. Some of the differences in production were the aggregation morphology, productivity and the glycosylation properties of the recombinant protein. It was observed that the quality of the rAPA (measured as the amount of mannose residues attached to the C-terminal of the protein) increased in coiled and baffled flasks in comparison with the conventional ones. The authors firstly attributed these significant differences to the aeration/hydrodynamic stresses generated by the flask configuration. Moreover, dissimilar volumetric power inputs for the three configurations were observed for the same shaking velocity (Marín-Palacio et al., 2014). More recently, Mancilla et al. (2015) studied the flow behavior in shaken flasks using the PIV technique based on Gamboa-Suasnavart et al. (2011) flask configurations. They performed experiments at different shaking velocities ranging from 25 to 250 rpm. They found that at high shaking rates the turbulent distribution increased for all flask configurations; however, the highest turbulent production for all geometric conditions occurred at a shaking speed of about 150 rpm which is the frequency used by different authors (Gamboa-Suasnavart et al., 2011, 2013; Marín-Palacio et al., 2014). Rodríguez et al. (2015) reported a study on the effect of a conical bottom flask in an orbital shaker. They found that similar flow dynamics are obtained at lower shaking rates compared to flat bottom flasks, resulting in lower shear rates. The results reported in this paper are a continuation of Mancilla et al. (2015). In contrast to what was previously reported, we focus the investigation on a single shaking speed and only three flask geometries, searching for the dominant hydrodynamic parameters that explain the influence of the flask geometry on the production of the recombinant glycoprotein rAPA. Particular attention was paid to the flow deformation rate and turbulence intensity inside the flasks. We show that these parameters greatly influence the performance of the system in bio-culture growth. It is important to remark that the term *turbulence intensity* used in the present study, represents the turbulence of the global shaking cycle and includes the contribution from both the velocity fluctuations and the cyclic nature of the flow. In that case, this kind of turbulence is commonly known as *pseudo-turbulence*. The latter term was used throughout the text in order to be in line with common papers in chemical and biochemical engineering (Ducci and Yianneskis, 2006).

### 1.1. Background: culture conditions and biomass properties

In this section we discuss some results obtained by Gamboa-Suasnavart et al. (2011) and Marín-Palacio et al. (2014) which are summarized in Table 1. These authors used the wild type *Streptomyces lividans* 66 strain 1326 (Kieser et al., 2000) transformed with plasmid pIJ6021MT-45 which carries the *M. tuberculosis apa* gene under a thiostrepton-inducible promoter, conferring also resistance to kanamycin (Lara et al., 2004). Flask cultures were made in triplicates using the conventional, three baffled and coiled flask configurations of 250 ml (filled with 50 ml) Erlenmeyer flasks (Pyrex, Mexico). The flasks were shaken at 30 °C and 150 rpm during 60 h using a New Brunswick Scientific lab shaker. Analytical determinations such as biomass concentration, total protein, electrophoresis and Western blots were used for quantification, purification and identification of rAPA production as well as to obtain the morphological measurements (average diameter and roundness) which were described by Gamboa-Suasnavart et al. (2011) and Marín-Palacio et al. (2014). Also, the characterization of O-linked glycans at the C-terminal region of rAPA was made by MALDI-TOF analysis as reported by Marín-Palacio et al. (2014). The measurement of the volumetric power input (P/V) was obtained

**Table 1**
Morphological measurements and analytical determinations during the cultivation of *Streptomyces lividans*. Data from Gamboa-Suasnavart et al. (2011) and Marín-Palacio et al. (2014).

| Shake Flask | Conventional | Baffled | Coiled |
|---|---|---|---|
| Volumetric power input ($kW/m^3$) | 0.20 | 0.51 | 0.44 |
| Final biomass concentration (g/L) | $2.1 \pm 1.2$ | $5.6 \pm 0.2$ | $5.2 \pm 0.1$ |
| Average diameter (μm) | $370 \pm 82$ up to 700 | $150 \pm 37$ | $170 \pm 42$ |
| Morphology of *S. lividans* | Larger pellets Rounded shape compacted | Small clumps Less compact | Smaller clumps Less compact |
| Final concentration of rAPA (μg/μL) | 0.3 | 0.5 | 0.7 |
| O-mannosylation pattern in the C-terminal | Up to 2 mannoses | Up to 5 mannoses | Up to 6 mannoses |