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| Tips and Tools

A procedure to harmonize the hydrodynamic force during microbial cultivation in shaking flasks

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ABSTRACT Shake flask cultivation is a routine technique in microbiology and biotechnology laboratories where cell growth can be affected by the hydrodynamic conditions, which depend on the agitation velocity, shaking diameter, and shake flask size. Liquid agitation is implemented inherently to increase aeration, substrate transfer to the cells, and prevent sedimentation, disregarding the role of hydrodynamics in microbial growth and metabolism. Here, we present a simple approach to help standardize the hydrodynamic forces in orbital shakers to increase the experimental accuracy and reproducibility and give students a better knowledge of the significance of the agitation process in microbial growth.

KEYWORDS agitation, hydrodynamic force, microbial growth, shake flasks, standardization

The cultivation of microbial cells under controlled conditions is a key aspect for accurately characterizing their growth, physiology, and metabolism. The standardization of experiments is a critical laborious process to obtain replicable and reproducible results (within a lab and among labs) from responsive and rugged microbial cultivation conditions (1). Diverse parameters need to be considered when cultivating a microorganism: the reactor design, geometry and mode of operation (batch, fed-batch, or continuous), type of microorganism(s), nutrients and their concentrations in the liquid phase, pH, temperature, the composition and concentrations at the gas phase, pressure, and hydrodynamics (2). The effects of temperature, pH, type, and concentration of nutrients are well-known to affect microbial growth and are already comprehensively described and transposed to teaching and learning content (3, 4). In a bioreactor operating under optimal process conditions, the nutrients are transported swiftly to the cells that will consume them. This happens if the hydrodynamic conditions are adequate to cause the desired degree of mixture (5). The design of microbial cultivation experiments for laboratory classes typically disregards the role of the relative dimension of the hydrodynamic forces, i.e., experiments are carried out typically with microorganisms cultivated under static or (unspecific) shaking conditions. However, the hydrodynamic conditions under which microorganisms are exposed influence microbial dynamics and behavior, namely motility, morphology, growth kinetics, metabolic activity, and biofilm formation (6–9).

Shake flask cultivation is a routine technique for screening and early process development in industry and academic research, where orbital shakers provide the energy input to agitate the liquid in the shaking bioreactors via rotating centrifugal force. Orbital shaking exclusively focused on a fixed shaker agitation rate, or rotation speed does not ensure a comparable hydrodynamic condition when moving from shakers with different orbital diameters. The size of the orbit will affect how the bacterial cells circulate and the degree of mixture. The same rotation speed in distinct shakers can originate different values of the hydrodynamic force. In addition, while knowing the

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rotation speed *per se* has limited translational relevance, the information on the Reynolds (Re, dimensionless) number has the potential to allow the cultivation of microorganisms under conditions that mimic a specific scenario of microbiological or biotechnological relevance (Table 1). The Re is determined by the ratio of inertial forces (momentum per unit volume) to the viscous forces of a fluid. Whenever a microorganism moves in a liquid medium, it changes the conditions of the surroundings and is therefore subject to a force generated as a result of this change (10).

Here, we describe how to ensure that the hydrodynamics force can be easily maintained when using orbital shakers with distinct orbital diameters or operating under different agitation speeds and how to determine the Re number in a shaking unbaffled flask (the conventional conical Erlenmeyer shake flasks), operating with a Newtonian fluid, for which the viscosity is not affected by the agitation speed—representative of most of the conventional culture media.

The fundamental understanding of process conditions in microbial growth kinetics, metabolism, and physiology is a learning objective of courses in applied microbiology, biotechnology, and biochemical engineering. Our teaching experience has shown that undergraduate students have benefited from microbial cultivation with higher replicability, making the implementation of the approach proposed useful in a laboratory setting. As an example, undergraduate students performed an experiment to assess the growth profile and kinetics of *Pseudomonas fluorescens* ATCC 13525^T (standard microorganism for food microbiology experiments as per NSF/ANSI Standard 12). The experimental work was performed as part of the Integrated Project in Biological Engineering course to third-year students of the Integrated Masters in Biological Engineering. Microbial growth was performed under 120 rpm of agitation using two distinct orbital shakers—ISF4-X, Kuhner (orbital diameter, d = 12.5 mm); CERTOMAT BS-1, Sartorius AG (d = 50 mm). Students have been organized in laboratory sessions of four groups, with three students per group. The students performed the experiment autonomously, under the supervision of an instructor. The overall results were shared by all students for analysis and to deliver a final report. By the time the work was concluded, the students were able to report that using the distinct orbital shakers, operating under the same agitation speed, provided statistically significant ($P < 0.05$—for a 95% statistical confidence) different growth rate (μ) values: $\mu = 0.268 \pm 0.018$ h^{-1} (d = 12.5 mm); $\mu = 0.332 \pm 0.013$ h^{-1} (d = 50 mm). The use of the overall data from using both shakers, considering exclusively a constant agitation speed, provided $\mu = 0.301 \pm 0.11$ h^{-1}, with a considerably higher standard deviation value. It was further assessed that the students were able to achieve the intended learning outcome of critically analyzing that replicability in microbial growth experiments using orbital shakers is influenced by the equipment characteristics. The ability to ensure the use of a constant agitation speed in shakers with different orbital diameters does not provide conditions for replicability (obtain an identical result when an experiment is performed under precisely identical conditions). The use of distinct orbital shakers further allowed the students to describe the role of distinct hydrodynamic conditions in microbial growth kinetics.

PROCEDURE

How to maintain an equivalent hydrodynamic force in orbital shakers with different orbital diameters

Newton's second law of motion (Force = Mass × Acceleration) allows the establishment of a new agitation speed in a shaker that will create the same force and resulting liquid movement in the shaking flask as that of the original shaker. Ensuring that the rate of change of momentum is equal in the original and the new orbital shaker means that if the mass is the same on each shaker (the same platform and same volume of liquid are used), the acceleration for each shaker is given by the ratio: Velocity^2/Radius^6. The velocity equals the agitation speed (in rotation per minute or second) multiplied by the circumference of the orbit. The radius is half the orbital diameter of the shaker. Therefore,

TABLE 1 Examples of ranges of Re values observed in diverse biotechnological processes [obtained from reference (11)]

Biotechnological process	Reynolds number
Animal cell culture	7,350–22,161
Bacterial adhesion	5,000–72,000
Bioethanol production by recombinant *Escherichia coli*	478–675
Microalgae cultivation in tubular photobioreactors	12,677–25,354
Synthesis of clavulanic acid by *Streptomyces clavuligerus*	15,896

to maintain the agitation force, the new agitation speed for the new shaker can be obtained by equation 1.

$$n_2 = \sqrt{(n_1^2 \frac{d_1}{d_2})} \quad (1)$$

where n_2 is the agitation speed, shaking frequency, or the number of revolutions (rotation per minute or second) for the new shaker; n_1 is the agitation speed for the old shaker; d_1 is the orbital diameter or shaking diameter of the old shaker; d_2 is the orbital diameter of the new shaker.

How to determine the Reynolds number in the shaking flask

The Re number of agitation of a shaking unbaffled flask can be determined from equation 2.

$$Re = \frac{\rho n d^2}{\mu} \quad (2)$$

While equation 2 provides a simplified approach to assess the Re number for Newtonian culture media, the extended Re number equation presented by Büchs et al. (13) should be used for high-viscosity media. However, the use of computational fluid dynamics coupled with particle image velocimetry should be implemented if higher accuracy is needed to characterize the fluid dynamics (14).

No safety issues exist from the implementation of the approach here presented. The specifications of the equipment provided by the manufacturer need to be considered to obtain the orbital diameter and for the selection of the agitation speed, which should follow the equipment specifications.

CONCLUSION

The hydrodynamic conditions under which microorganisms are cultivated influence the growth dynamics. The use of different shakers operating under the same agitation speed does not ensure that microorganisms will be subjected to equivalent hydrodynamic forces. However, the hydrodynamic conditions under which microorganisms are cultivated influence the growth dynamics. The implementation of the approach here described has the potential to increase the replicability of hydrodynamic conditions for microbial cultivation in shaking flasks.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (jmbe00099-23-s0001.xlsx). Excel file for agitation speed and Reynolds number calculation.

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