Vol. 18, No. 3 (2019) 1085-1099
Revista Mexicana de Ingeniería Química

VOLUMETRIC POWER INPUT AS A RELIABLE PARAMETER FOR SCALE-UP FROM SHAKE FLASK TO STIRRED-TANK BIOREACTOR: PRODUCTION OF A RECOMBINANT GLYCOPROTEIN BY *Streptomyces lividans*

LA POTENCIA VOLUMÉTRICA COMO UN PARÁMETRO CONFIABLE PARA EL ESCALAMIENTO DE MATRACES AGITADOS A BIORREACTOR DE TANQUE AGITADO: PRODUCCIÓN DE UNA GLICOPROTEINA RECOMBINANTE POR *Streptomyces lividans*

R.A. Gamboa-Suasnavart[1†], L.D. Marín-Palacio[1,2†], L. López-Griego[1], M.S. Córdova-Aguilar[3], N.A. Valdez-Cruz[4], M.A. Trujillo-Roldán[1*]

[1] *Programa de Investigación de Producción de Biomoléculas, Unidad de Bioprocesos, Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. AP. 70228, Ciudad de México, CP. 04510, México.*

[2] *Departamento de Ingeniería de Procesos, Universidad EAFIT, Medellín, Colombia.*

[3] *Instituto Ciencias Aplicadas y Tecnología, Universidad Nacional Autónoma de México, Ciudad de México, México.*

[4] *Programa de Investigación de Producción de Biomoléculas, Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. AP. 70228, Ciudad de México, CP. 04510, México.*

Received: October 23, 2018; Accepted: April 17, 2019

Abstract

The filamentous morphology of *Streptomyces lividans* depends on the culture conditions, affecting the production, secretion and post-translational modifications of recombinant glycoproteins. In this work, the previously reported volumetric power input (P/V) in conventional (NF) and coiled (CF) shake flasks were scaled-up to a stirred bioreactor. The effects on the growth and morphology of *S. lividans* were analyzed, as well as, the production and *O*-mannosylation of the recombinant APA glycoprotein from *Mycobacterium tuberculosis*. Specific growth rates of *S. lividans* and similar recombinant glycoprotein (rAPA) yields were observed between NF and bioreactor cultures. In addition, we have found up to seven mannose residues attached to the C-terminal of the rAPA in bioreactor cultures, one more than in NF and CF. However, at similar P/V values, morphological and kinetic differences were found. Our data indicate that P/V as scale-up criteria in the production of recombinant glycoproteins in *S. lividans* can be successful in some, but not all the kinetic and stoichiometric parameters, suggesting that the metabolic cell responses can be affected by aeration/hydrodynamics between bioreactor and shake flasks.

Keywords: *S. lividans*, power input, morphology, rheology, scale-up, shake flasks.

Resumen

La morfología filamentosa de *Streptomyces lividans* depende de las condiciones de cultivo, afectando la producción, secreción y modificaciones post-traduccionales de glicoproteínas recombinantes. En este trabajo, la potencia por unidad de volumen (P/V) previamente reportada en matraces convencionales (NF) y con resorte (CF) se escaló a un biorreactor agitado. Se analizaron los efectos del escalamiento en el crecimiento y la morfología de *S. lividans*, como también en la producción y *O*-manosilación de la glicoproteína recombinante APA de *Mycobacterium tuberculosis*. Se observaron velocidades específicas de crecimiento de *S. lividans* y rendimientos de la glicoproteína recombinante (rAPA) similares entre NF y biorreactores. Además, se encontraron hasta siete residuos de manosa adheridos al C-terminal de la rAPA en los cultivos en biorreactor, uno más que en NF y CF. Sin embargo, a valores similares de P/V, se encontraron diferencias morfologicas y cinéticas en el crecimiento de *S. lividans*. Nuestros datos indican que el escalamiento en la producción de glicoproteínas recombinantes en *S. lividans* usando la P/V puede ser exitoso en terminos de algunos, pero no todos los parámetros cinéticos y estequeométricos, sugiriendo que las respuestas celulares pueden estar afectadas por las diferencias en hidrodinámica/aireación entre matraces biorreactores.

Palabras clave: *S. lividans*, potencia, morfología, reología, escalamiento, matraces agitados.

* *Corresponding author. E-mail*: maurotru@gmail.com; † both authors contributed equally to this work

https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n3/Gamboa
issn-e: 2395-8472

1 Introduction

Shake flasks are inexpensive bioreactors that are widely used to evaluate multiple operational conditions, to screen several microbial strains simultaneously, and to optimize culture media (Freedman, 1970; Freyer *et al*. 2004; Büchs, 2001; Orozco-Sanchez and Rodríguez-Monroy 2007; Giese *et al*. 2014; Villegas *et al*. 2016). However, shake flasks have several limitations, including the inability to control environmental conditions (Büchs, 2001; Orozco-Sanchez *et al*. 2011), such as pH and dissolved oxygen tension (DOT). In addition, for commercial production, many bioprocesses need to occur on a larger scale than in shake flasks; therefore, it is necessary to scale-up these to bioreactors, in which, it is possible to monitor and control several variables of the process independently (Tajsoleiman *et al*. 2019). Various criteria have been proposed for scaling-up biotechnological processes, such as the volumetric mass transfer coefficient (k_La) (Humphrey, 1998; Garcia-Ochoa and Gomez, 2009; Trujillo-Roldán *et al*. 2013), the oxygen transfer rate (Garcia-Ochoa and Gomez, 2009; Raval *et al*. 2017), impeller tip speed (Junker *et al*. 2009), mixing/circulation time (Junker, 2004), and volumetric power input (P/V) (Pérez *et al*. 2006; Rocha-Valadez *et al*. 2006, Gamboa-Suasnavart *et al*. 2013; Xu *et al*. 2017). P/V is a global parameter defined as the amount of energy required to maintain the fluid motion within a vessel for a given period of time (Marques *et al*. 2010), and it is associated with the physical phenomena that occur in the bioreactor, such as oxygen transfer, removal of carbon dioxide, macro and micro mixing, turbulence intensity, uniformity of the liquid phase, and hydrodynamic stress (Lara 2011, Olmos *et al*. 2013).

Streptomyces is a genus of actinomycetes, a group of Gram-positive bacteria with high GC content (65-75%) that are commonly used to produce a wide range of secondary metabolites (Jakimowicz and van Wezel 2012; Olmos *et al*. 2013; Xia *et al*. 2014; van Dissel and van Wezel 2017). Additionally, some strains, such as *S. lividans*, are used for the expression of heterologous proteins and glycoproteins (Lee and Lee 1996; Vrancken and Anné 2009; Anné *et al*. 2012; Lara *et al*. 2004; Gamboa-Suasnavart *et al*. 2011). In submerged culture, *S. lividans* shows a filamentous morphology of branched and non-branched hyphae, clumps, and pellets (Giudici *et al*. 2004; Gamboa-Suasnavart *et al*. 2011; Anné *et al*. 2012; Marín-Palacio *et al*. 2014; Gamboa-Suasnavart *et al*. 2018; Zacchetti *et al*. 2018). The filamentous morphology in *S. lividans* plays an important role in the production, secretion, and post-translational modification of recombinant proteins (Tamura *et al*. 1997; Yun *et al*. 2001; Lara *et al*. 2004; Gamboa-Suasnavart *et al*. 2011; Gamboa-Suasnavart *et al*. 2013; Marín-Palacio *et al*. 2014).

Understanding the effect of the P/V on pellets size (influencing product yields) is of great interest for industry-scale production (van Veluw *et al*. 2012; van Dissel and van Wezel 2017; Koepff *et al*. 2017). For example, large pellets are optimal for the production of antibiotics (Wardell *et al*. 2002), while for the production of enzymes or novel products (such as anticancer agents) small pellets are preferred (van Wezel *et al*. 2006).

For the production of the recombinant glycoprotein APA (a 45/47-kDa *O*-mannosylated immuno-dominant antigen from *Mycobacterium tuberculosis*) by *S. lividans*, higher yields and an increased degree of *O*-mannosylation (up to five residues of mannose attached to the C-terminal of rAPA) were observed when small aggregated and dispersed mycelia were present in cultures. These were found in baffled (BF) and coiled (CF) orbital shake flasks cultures. However, when *S. lividans* were grown in normal (conventional) Erlenmeyer orbital shake flasks (NF) lower yields and just two residues of mannose were found (Gamboa-Suasnavart *et al*. 2011).

On the other hand, Marín-Palacio *et al*. (2014) measured the P/V in NF, BF and CF using the method described by Büchs *et al*. (2000). During the exponential growth phase, the highest P/V was observed in BF (~0.51 kW/m^3), followed by CF (~0.44 kW/m^3), and NF (~0.20 kW/m^3). All cultures were carried out at the same agitation rate of 150 rpm (Marín-Palacio *et al*. 2014). In this work, the measured P/V in NF and CF were used as a criterion to scale-up the cultures to stirred-tank bioreactors. We measure and compare *S. lividans* growth and morphology, rheological parameters, the production of rAPA and its post-transcriptional modifications in shake flasks and bioreactor cultures to determine if P/V is an appropriate parameter to scale-up recombinant filamentous bacterial cultures.

2 Materials and methods

2.1 Microorganisms and culture conditions

We used a transformed strain of *Streptomyces lividans* 66 (strain 1326) harboring the plasmid pIJ6021MT-45, which carries the *Mycobacterium tuberculosis apa* gene (*Rv*1860) under the thiostrepton-inducible promoter (P_{tipA}) (kindly donated by Dr. Luis Servín-Gonzalez). YT medium was used for the pre-germination of *S. lividans* spores at 37 °C, 150 rpm and 8 h in CF (coiled flasks consist of conventional normal flasks with an inserted 30 cm stainless steel spring of 1.3 cm diameter, 19 sw gauge), as previously described Gamboa-Suasnavart *et al*. (2011).

All shake flasks cultures were carried out at 30 °C, at 150 rpm with an orbital diameter of 2.5 cm (C25 Incubator Shaker, New Brunswick Scientific, Edison, NJ, USA) in 250-mL flasks containing 50 mL of Luria-Bertani broth (LB: 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl), modified with 34% w/v sucrose with kanamycin (50 μg/mL).

2.2 Bioreactor culture conditions and scaling up strategy

All cultures were run at least in triplicate in 1.0-liter bioreactor (Applikon Biotechnology, Delft, The Netherlands) containing 750 mL of LB broth supplemented with 34% sucrose (Gamboa-Suasnavart *et al*. 2011). The bioreactor has a height:diameter ratio of 2.0 and is equipped with two Rushton impellers (diameter, 0.045 m) and two baffles (1/10 of tank diameter). The bioreactor was inoculated with 50 mL from CF over-night cultures. Bioreactor cultures were carried out without dissolved oxygen tension (DOT) or pH control, maintained at 30 °C and a constant total gas flow rate of 0.5 L/min and agitated at 260 or 340 rpm. Production of rAPA was induced by the addition of thiostrepton (10 μg/mL; Merck-Sigma-Aldrich St. Louis, MO, USA) at 20 h of culture.

The unaerated power number (Np), defined by dimensional analysis since 1950´s (relating the resistance force to the inertia force), given by Equation 1, was used to set the stirrer speed of the impeller (Rushton *et al*. 1950; Rushton 1952).

$$N_p = \frac{P}{\rho N^3 D_i^5} \quad (1)$$

The gassed power input (P_g) was calculated by Equation 2 (Nagata 1975).

$$\log_1 0 \frac{P_g}{P} = -192\left(\frac{D_i}{D_t}\right)^{4.38} Re^{0.115} Fr^{1.96 D_i/D_t} A_e \quad (2)$$

In equation 2 the P_g/P ratio is a function of Aeration number (A_e), Froude number (Fr) and Reynolds number (Re). This correlation is applicable for stirred tank bioreactors equipped with six bladed Rushton type turbines in various diameters ($D_t/4$, $D_t/3$, $D_t/2$ and $3D_t/4$) with two helices heights ($D_i/5$ and $D_i/4$), four baffles ($D_t/10$), and in a wide range of air flow rate. Air was introduced from a ring sparger or from a single nozzle at the center of the vessel (Nagata 1975). The design of the bioreactor used in this work fulfills the conditions of equation 2.

Moreover, this correlation is applicable for a range of A_e between 0.0 and 0.13 (Amanullah *et al*. 2004), in our case A_e was 0.023 and 0.032 for 260 and 340 rpm, respectively. Also, this equation has been used at a stirring speed of up to 600 rpm in laboratory bioreactors with low viscosity fluids and densities close to the water (Nagata 1975; Ni *et al*. 1995; Amanullah *et al*. 2004). This would allow us to use this equation reliably since the conditions of this work were in the range of Re and Fr previously used (Nagata 1975; Ni *et al*. 1995; Amanullah *et al*. 2004).

It is important to consider that the equation 2 is based on a bioreactor configuration with four baffles. To obtain a better approximation of the real P_g/V, a correction proposed by Dickey *et al*. (2004) was used to account for the number of baffles. When two baffles are used, the calculated P_g/V must be multiplied by 0.8. This correction has been published and is available online: (https://www.chemicalprocessing.com/assets/Media/MediaManager/Baffle_Effect.pdf).

2.3 Analytical determinations

Biomass was determined as dry weight as previously reported by Gamboa-Suasnavart *et al*. (2011). Extracellular protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay Kit; Bio-Rad, Hercules, CA, USA), and a calibration curve was constructed using bovine serum albumin (BSA) as a standard. Culture filtrate was obtained by removing the mycelia by centrifugation at 8,000 × g for 30 min at 4 °C and filtration through a 0.45 μm membrane filter (Merck-Millipore, Billerica, MA, USA). The supernatant was precipitated with ammonium sulfate (75 %), and the precipitated proteins were recovered by centrifugation at 8,000

× g for 30 min at 4 °C, suspended in PBS (pH 7.4), and dialyzed against distilled water overnight at 4 °C. Electrophoresis in 12 % SDS-PAGE and subsequent immunoblotting were carried out as previously reported (Gamboa-Suasnavart *et al*. 2011; Gamboa-Suasnavart *et al*. 2013; Marín-Palacio *et al*. 2014). Briefly, the membranes were blocked by incubation with skim milk (5 %w/v) in PBS containing Tween 20 (0.05 %v/v; PBS-T) for 1 h, washed with PBS-T (0.05 %), and then incubated with the primary antibody (mAb6A3; donated by Dr. Clara Espitia) at a 1:1000 dilution overnight at 4 °C. The membranes were incubated with peroxidase-conjugated anti-mouse IgG (Merck-Sigma-Aldrich, St. Louis, MO, USA) at a 1:2000 dilution for 1 h. Also, membranes were incubated with anti-concanavalin A (ConA)- peroxidase conjugate (Concanavalin A from *Canavalia ensiformis* (Merck, Sigma-Aldrich, USA) for 1 h at room temperature. After incubation, the blots were stained with SuperSignal West Pico Chemiluminescent Substrate (Thermo-Pierce Chemical, USA), and images were taken using the C-DiGit Chemiluminescent Western Blot Scanner (Li-Cor, USA).

2.4 Rheological measurements

A stress-controlled rheometer (Physica MCR 101; Anton Paar, Graz, Austria), equipped with a plate-and-plate measuring system (diameter, 50 mm; Anton Paar) and a gap of 1 mm was used for the determination of the shear viscosity curves of the complete culture broths at 30 °C in a shear rate range of 0.1-100 s^{-1} (Marín-Palacio *et al*. 2014). The flow curves of each sample were measured in triplicate and fitted to the Ostwald-de Waele model (equation 3), and the rheological parameters were calculated with correlation coefficients ($R^2 > 0.99$).

$$\eta = K\gamma^{n-1} \tag{3}$$

For turbulent flows ($Re > 10^4$) in a stirred tank of standard geometry ($H = D_t$, and $D_t = 3D_i$) in non-Newtonian fluids, Equation 4 can be used to obtain the average shear rate (γ) (Sánchez-Pérez *et al*., 2006).

$$\gamma = \left(\frac{4N_p\rho D_i^2}{\pi 3^3 K}\right)^{\frac{1}{1+n}} N^{\frac{3}{1+n}} = mN^{3/(1+n)} \tag{4}$$

Where m is a constant due to Np is a constant in turbulent flows (5.5 for Rushton turbines). Equation 4 is quite general and applies also to Newtonian fluids ($n = 1$ and $K = \eta$) (Sánchez-Pérez *et al*. 2006).

To calculate the size of the *eddie* of the microscale and compare it with the sizes of the cellular aggregates, the Kolmogorov equation was used (Kolmogorov 1941; Rocha-Valadez *et al*. 2007).

$$\lambda = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4} \tag{5}$$

2.5 S. lividans aggregate morphology measurements

The morphology of *S. lividans* was measured as previously reported (Gamboa-Suasnavart *et al*. 2011; Marín-Palacio *et al*. 2014). Briefly, to avoid actual morphology losses, 20 μL of culture broth was fixed with formalin solution (10% v/v), placed on a slide, and covered with a cover slip (Marín-Palacio *et al*. 2014). A minimum of 300 objects in each sample were analyzed for each determination. The image was captured with a digital camera (Coolpix 4300; Nikon, Tokyo, Japan) mounted on a microscope (Nikon Optiphot-2) at 4× magnification. Image analysis was performed with ImageJ (National Institutes of Health, Bethesda, MD, USA; http://imagejnihgov/ij/index.html). The average diameter was measured to characterize the size of aggregates. The average diameter is defined as the diameter of a perfect circle, having the same area as the aggregated to be measured.

Also, the average fractal dimension (D_B) and lacunarity (L) were measured for samples taken at the end of each culture. The first step of procedure was conversion of color images to grayscale images using the auto-threshold selection (ImageJ -> Image -> Adjust -> auto-threshold). Fractal dimension (D_B) and lacunarity (L) was calculated by using FracLac pluging (ImageJ -> plugins -> Fractal Analysis -> FracLac) for ImageJ, and the program was allowed to convert the images to binary images (Karperien 2013). A minimum of 50 objects in each sample were analyzed for each experimental condition.

2.6 Protein digestion and MALDI-TOF analysis

To characterize the *O*-linked glycans in the C-terminal region of rAPA, 50 μg of protein was digested with LysC to obtain eight peptides. The C-terminal peptide (P8) was analyzed by MALDI-TOF mass spectrometry on a Bruker Microflex time-of-flight mass spectrometer equipped with a nitrogen laser at l = 337 nm. Spectra were recorded in reflector or

linear positive mode as previously reported (Gamboa-Suasnavart *et al*. 2011; Gamboa-Suasnavart *et al*. 2013; Marín-Palacio *et al*. 2014).

2.7 *Statistical treatment*

One-way ANOVA and pair-wise comparisons using Tukey HSD (Test for Post-ANOVA) were carried out for independent samples. Those were done to assess the morphological statistical differences between shake flask and bioreactor cultures. Analysis were done using Excel® (2007) and the "VassarStats: Website for Statistical Computation" available on-line of Vassar College, NY-USA (http://vassarstats.net/).

3 Results

3.1 *Streptomyces lividans growth kinetics in an agitated bioreactor at the same volumetric power input observed in shake flasks*

To determine if the volumetric power input (P/V) is a critical parameter for scaling-up recombinant filamentous bacterial cultures, we compared the growth and morphology of *S. lividans*, as well as, the production and *O*-mannosylation of rAPA between shake flasks and mechanically agitated bioreactors at two similar P/V values. These two values were equaled by calculating the agitation rate (by using Equations 1 and 2) in a 1.0 L bioreactor in which the initial gassed volumetric power input (P_g/V) was similar to the P/V previously measured in NF and CF (Marín-Palacio *et al*. 2014).

Table 1. Operational, stoichiometric and kinetic parameters of *S. lividans* growth and rAPA production. Cultures were carried out in conventional normal (NF) and coiled (CF) shake flasks. Moreover, cultures were carried out in 1.0 L bioreactors at similar volumetric power inputs than NF and CF. The average and standard deviation for at least three biological replicates per condition are shown.

Parameter	**Erlenmeyer flasks**[a]		**Bioreactor**[b]	
	Normal	**Coiled**	**260 rpm**	**340 rpm**
P/V (kW/m^3)[c]	~0.20	~0.44	~0.20	~0.40
k_La (h^{-1})	41.4±5.2^A	87.4±0.6^B	35.1±0.7^A	67.3±0.6^C
μ (h^{-1})	0.08±0.01^A	0.14±0.01^B	0.09±0.01^A	0.12±0.02^{A,B}
X_{max} (g)	2.1±0.2^B	5.2±0.1^C	3.7±0.2^A	4.0±0.2^A
Lag phase (h)	~16	~16	~12	~12
Maximum pellet size (μm)	370±82^A	160±40^B	245±6^C	214±4^D
Fractal dimension (D_B)	1.391±0.298^A	1.151±0.207^A	1.485±0.202^A	1.340±0.133^A
Fractal lacunarity (L)	2.285±1.599^A	2.552±0.578^A	3.481±1.041^B	3.363±1.258^B
K (mPas^n)	0.11±0.02^B	0.05±0.01^C	0.08±0.0^A	0.08±0.01^A
Fluid index n (-)	0.40±0.06^A	0.63±0.01^B	0.46±0.08^A	0.46±0.08^A
rPLA2 (%)[d]	25±3^A	36±3^B	27±5^A	25±2^A
Maximum mannose residues at C-terminal	6	6	7	7

a. Data from shake flasks was taken with permission from Marín-Palacio *et al*. (2014).

b. This work

c. In shake flasks P/V was measured by RAMOS device (Marín-Palacio *et al*. 2014) and in bioreactor initial P_g/V was calculated by equations 1 and 2.

d. The percentage corresponding to the recombinant protein was calculated based on gel densitometry (Figure 3A). The results with the same capital letter in superscript are statistically similar (P> 0.01).

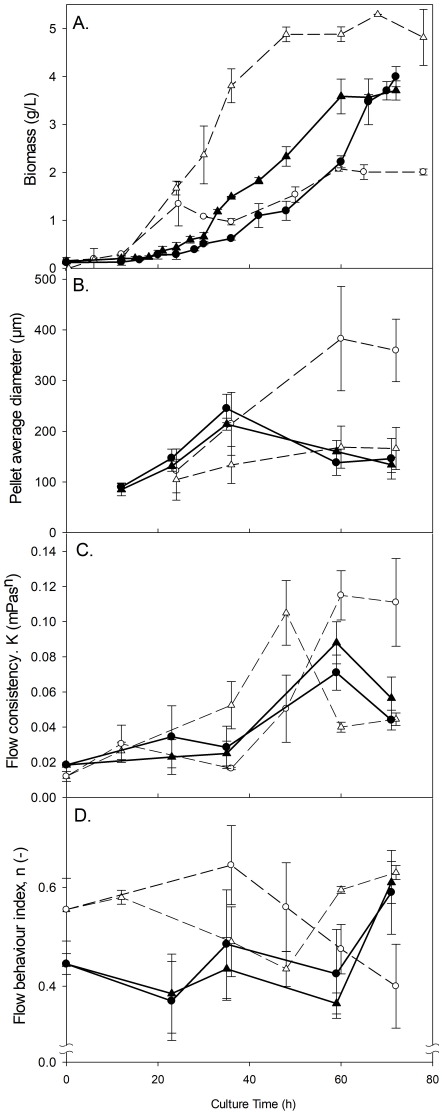

Fig. 1. (A) Kinetics of biomass growth of *S. lividans* producing rAPA from M. tuberculosis, in 1L bioreactor carried out at initial 0.20 kW/m^3 and 0.40 kW/m^3 and in conventional normal flasks at 0.20 kW/m^3 (NF) and coiled shake flasks at 0.44 kW/m^3. (B) Average diameter and standard deviation of *S. lividans* pellets. (C) Evolution of the flow consistency index K (mPas^n). (D) flow behavior index n (-). Cultures were carried out at initial 0.20 kW/m^3 (260 rpm, black triangles, solid line) and at initial and 0.40 kW/m^3 (340 rpm, black dots, solid line). For comparison data of bacterial growth in coiled (0.44 kW/m^3, open triangles, dotted line) and conventional (0.20 kW/m^3, open dots, dotted line) shaken flask carried out at 150 rpm (Previously reported by Marín-Palacio *et al*. 2014, with permission).

Figure 1A shows *S. lividans* growth kinetics in an agitated bioreactor at 260 rpm (P_g/V = 0.20 kW/m^3) and 340 rpm (P_g/V = 0.40 kW/m^3) in comparison to those cultures carried out in NF (P_g/V = 0.20 kW/m^3) and CF (P_g/V = 0.44 kW/m^3). In both cases, the lag phase took between 12 and 16 h, similar to that observed (~12 h) in shake flasks (Marín-Palacio *et al*. 2014). A slightly higher specific growth rate (μ) was observed in bioreactor cultures at 340 rpm (0.12±0.02 h^{-1}) than at 260 rpm (0.09±001 h^{-1}). Similar specific growth rates were observed between CF (014±0.01 h^{-1}) and 340 rpm, as also, between NF (0.08±0.01 h^{-1}) and 260 rpm cultures (Table 1). However, similar maximum biomass was obtained in the bioreactors at both P_g/V values (3.7±0.2 and 4.0±0.2 g/L for 260 and 340 rpm, respectively), whereas lower biomass was obtained in NF (2.1±0.2 g/L), and higher final biomass was obtained in CF (5.2±0.1 g/L; Figure 1, Table 1).

3.2 Morphological parameters of S. lividans in the bioreactors

The kinetics of the pellet size (average diameter) was evaluated in triplicate cultures (samplings at 24, 36, 60, and 72 h) and analyzing at least 300 objects per sample (Figures 1B and 2). An increase in pellet size was observed in mid-exponential phase (up to 245±6 μm at 260 rpm and 214±4 μm at 340 rpm), but a decrease, to approximately 140 μm at both P_g/V values, was observed at the end of culture (Figure 2). This reduction in size could be a result of erosion or dispersion of surface aggregates (Rocha-Valadez *et al*. 2006). At the same power input in the NF, the pellets increased to an average up to 370±82 μm; however, a wider range of diameters was observed, including pellets of up to 700 μm in diameter (Marín-Palacio *et al*. 2014), while in the CF cultures, the average diameter was 160±40 μm, which was similar to those obtained in the bioreactors. These morphological differences in size may occur in response to the different mixing patterns present in shake flasks and bioreactors with mechanical agitation, which may hinder scaling-up from shake flasks to bioreactors (Gerson and Kole, 2001).

To quantify the complexity of *S. lividans* mycelial morphology, the fractal dimension (D_B) and the lacunarity (L) were also measured. D_B is in essence, a scaling rule comparing how a pattern's detail changes with the scale at which it is considered (i.e. complexity). The term lacunarity (L) is defined as gappiness, visual texture or inhomogeneity of the analyzed structure (Smith *et al*. 1996; Karperien,

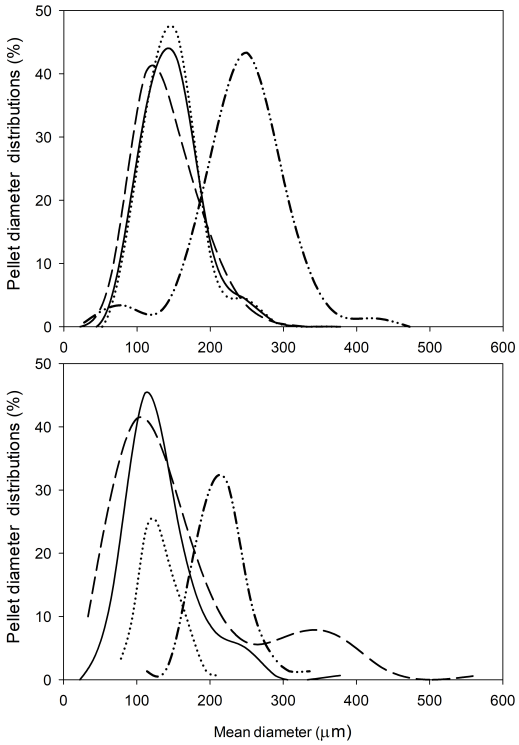

Fig. 2. *S. lividans* pellet diameter distributions of in 1L bioreactor cultures carried out at initial 0.20 kW/m^3 (A) and 0.40 kW/m^3 (B). Samples were taken at 24 h (dotted), 36 h (dash-dot-dot), 60 h (long-dash), and 72 h (solid) of culture.

2013). In lacunarity the calculation is based on pixel masses if the image instead of box counts as in D_B (Karperien, 2013). Here, no significant differences were found in terms of D_B at the end of shake flask and bioreactor cultures (Table 1). However, greater lacunarity was found in both bioreactor cultures than in shake flask cultures (Table 1). This allows us to determine that there is a greater number of gaps (and homogeneity) in bioreactor cultures (no significant difference between 260 rpm and 340 rpm), than in those cultures in NF and CF (no significant difference between CF and NF).

3.3 Rheological behavior of S. lividans in the bioreactors

The data obtained from the flow curves of the *S. lividans* cell suspensions fitted the Ostwald-de Waele model. The rheological behavior of the cultures was non-Newtonian, acting as a pseudoplastic fluid (shear thinning, n < 1). The fluid index (n) was 0.46±0.08 in both bioreactor cultures (Figure 1D), and it remained nearly constant during cultivation. It is important to mention that the cultures in this study developed a similar rheological behavior to those previously reported for CF and NF (Marín-Palacio *et al*. 2014). We also observed no significant difference in the flow consistency index (K) between the cultures agitated at 260 and 340 rpm, even though the initial P_g/V values of 0.20 kW/m^3 and 0.40 kW/m^3, respectively. The flow consistency index was essentially the same for the first 30 h of culture (0.03±0.01 mPas^n), and then increased to 0.08±0.01 mPas^n at 50 h (Figure 1C).

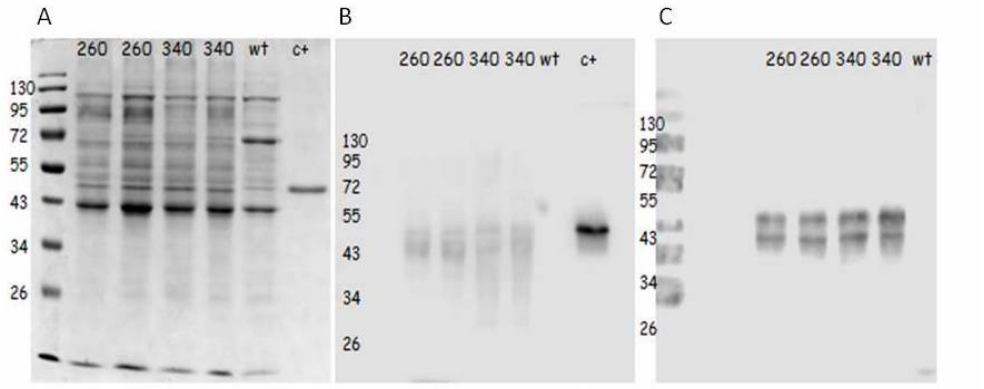

Fig. 3. SDS-PAGE (A) of total secreted proteins by *S lividans*. Western blot (B) of rAPA production by *S. lividans* using mAB 6A3 (B) and using ConA-peroxidase (C). Lane 1: Page ruler; Lanes 2 and 3 are two independent bioreactor cultures carried out 260 rpm; Lanes 4 and 5 are two independent bioreactor culture carried out 340 rpm; Lane 6 (wt): negative control, *Streptomyces lividans* 66 strain 1326, without transformation; Lanes 7: positive control, recombinant APA produced in shake flasks cultures of *E coli*.

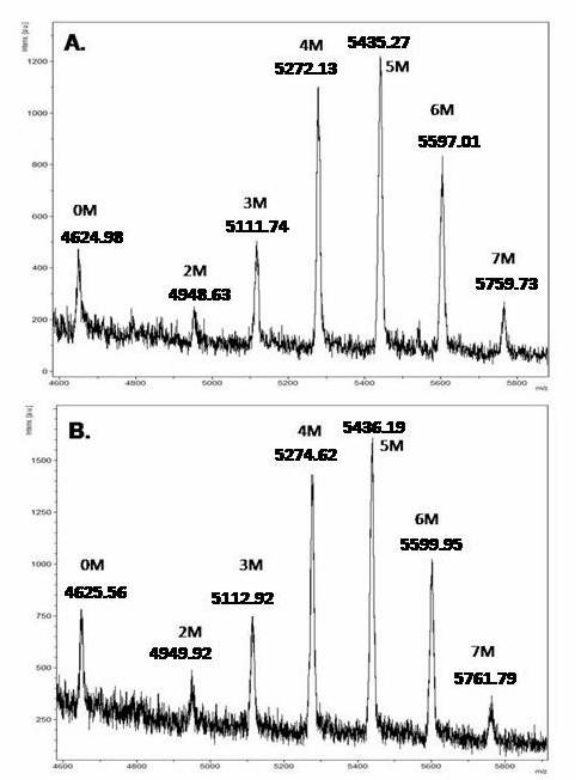

Fig. 4. MALDI-TOF analysis for the carboxy-terminal peptide of the recombinant APA protein by LysC digestion and obtained in (A) Bioreactor culture carried out 260 rpm (B) Bioreactor culture carried out 340 rpm. Numbers above each peak mean the number of mannose units linked to the peptide. At least three MALDI-TOF analyses were done at the end of each independent culture.

A higher flow consistency index was obtained in NF (0.11±0.02 mPas^n), whereas a lower K (0.045±0.003 mPas^n) was obtained in CF (Marín-Palacio *et al*. 2014).

3.4 Production of recombinant APA and O-mannosylation in shake flasks and bioreactors at the same P/V

Densitometric analysis of the protein gels showed that rAPA comprised 27±5 % of the total extracellular protein in bioreactor cultures at 260 rpm (Figure 3A), which was similar to that obtained in the NF (25±3 %) (Marín-Palacio *et al*. 2014). In contrast, in bioreactor cultures at 340 rpm, which has a P/V similar to that in CF, rAPA comprised 25±2 % (Figure 3A), which is ~10 % less than that obtained in the CF (36±3 %). It is worth to note that no significant differences were observed in rAPA in bioreactor cultures at 260 and 340 rpm. In addition, rAPA production levels were confirmed by Western blotting with mAB6A3 and ConA-peroxidase (Figures 3B and 3C).

We also characterized the *O*-linked glycans attached to the C-terminal region of rAPA. Here, up to seven mannose residues in cultures at 260 and 340 rpm were detected (Figures 4A and B, Table 1), which is one residue more than those observed in CF and NF cultures (Marín-Palacio *et al*. 2014). However, in all cultures, peptides with just one mannose residue were not detected.

4 Discussion

In this study, the P/V of *S. lividans* cultures in NF and CF was scale-up to a stirred tank bioreactor to determine if the P/V has a direct effect on cell growth, morphology, production and *O*-mannosylation of a recombinant glycoprotein (rAPA). A comparison of the final biomass concentration did not show significant differences in bioreactor cultures, although the values were lower from the maximum biomass obtained from CF and higher than those from NF (Table 1), showing that P/V cannot be used as a scale-up parameter for biomass growth in *S. lividans*. However, when the specific growth rate (μ) was compared at the same P/V similar values were observed (between shake flasks and bioreactors), meaning that this parameter is directly related to P/V (Table 1). This may be due to the fact that other factors in addition to P/V are important when scaling up a shaken-flask culture to a bioreactor, such as the hydrodynamic profiles in the cultivation systems (Mancilla *et al*. 2015; Palacios-Morales *et al*. 2016). These results are in agreement with those obtained by Kelly *et al*. (2006) and Lin *et al*. (2010) who found that P/V favored a similar specific growth rate but decreased biomass concentrations in cultures of Aspergillus niger.

The lower biomass in the NF cultures (2.1±0.2 g/L) compared to the bioreactor cultures agitated at 260 rpm (3.7±0.2 g/L) may be due to the difference in gas induction, which is only provided by surface aeration in the shake flasks compared to sparging and stirring in the bioreactors (Shukla *et al*. 2001). The modifications of conventional flasks, such as baffles (Freedman 1970) or coiled springs (Hopwood *et al*. 1985; Gamboa-Suasnavart *et al*. 2018), are

often used to increase the oxygen transfer rate and prevent the formation of large pellets by filamentous microorganisms (Mancilla *et al*. 2015; Palacios-Morales *et al*. 2016). As shown in Table 1, when the P/V values obtained in the shake flasks were scale-up to bioreactors, higher k_La values were obtained in CF and NF than in the bioreactor cultures (Table 1). It is already proven that the gas-liquid mass transfer coefficient (k_La) in any culture is a function of the mode of energy dissipation and the physico-chemical properties of the liquid media (Shukla *et al*. 2001).

Additionally, larger average pellet diameters were obtained in the NF cultures than in the bioreactor cultures at the same P/V. These morphological differences may be the result of the different mixing patterns present in the NF and bioreactor (Gerson and Kole 2001). It has been shown that the morphology of *S. lividans* is closely related not only to the hydrodynamic stress, but also to the time that the stress is applied to the microorganism (Zacchetti *et al*. 2018). Büchs and Zoels (2001) demonstrated that the power consumption is more uniformly distributed in orbital agitation processes using NF than in stirred tanks. Therefore, the hydrodynamic stress is lower in NF. According to Peter *et al*. (2006), at the same power consumption, the maximum rate of energy dissipation in a stirred-tank bioreactor is ten times higher than that in a NF.

The decrease observed in the average diameter of the pellets after mid-exponential growth phase in both bioreactor cultures (Figure 1B) is consistent with results of Rocha-Valadez *et al*. (2007), who assessed the influence of the frequency of movement on the morphology of *Trichoderma harzianum*. In addition, Jüsten *et al*. (1996) showed that power input is a parameter that is weakly correlated with damage to fungal hyphae when different scales of operation are used, indicating that the changes in morphology at any given P/V depend significantly on the geometry of the impeller. In fact, power dissipation/circulation time (EDCF) showed a better correlation with mycelial morphology (Jüsten *et al*. 1998), as hyphae fragmentation is caused by the intermittent passage of the mycelium through the impeller region under high-energy dissipation (Jüsten *et al*. 1998). Moreover, the architecture of *S. lividans* pellets changes when cultures enter the stationary phase and the fragmentation coincides with nutrient exhaustion in submerged cultures (Zacchetti *et al*. 2018). The fact that a greater number of gaps (and homogeneity), measure as lacunarity, was found in bioreactor cultures (Table 1), than in those cultures in shake flasks is an indicator that the hydrodynamics and aeration in bioreactors are surely different from that which occurs in shake flasks. It has been reported that lacunarity (L) and fractal dimension (D_B) correlate strongly with branching frequency, being lacunarity a robust estimator of branching behavior (Barry 2013).

No significant differences were observed in the flow consistency and behavior indexes (K and n) in bioreactor cultures (at both P/V values), which indicate that both the biomass concentration and morphology act similarly, and that the differences between the cultures are not sufficiently large to affect the rheological parameters (Marín-Palacio *et al*. 2014). Equations 3 to 5 were used to calculate the average shear rate, the apparent viscosity and the microscale size of Kolmogorov (Kolmogorov 1941; Rocha-Valadez *et al*. 2007). With the change of the rheological parameters of the culture in bioreactor it is observed that the size of the *eddie* of Kolmogorov is in the interval between 50 and 280 micrometers. This value is in the range of the average diameters of the cellular aggregates (Table 2). Those data indicate that the hydrodynamics in the bioreactors are determining the pellet size and that these eddies may be different in the NF and CF (Rocha-Valadez *et al*. 2007; Olmos *et al*. 2013; Palacios-Morales *et al*. 2016).

Table 2. Average shear rate, apparent viscosity, Kolmogorov microscale size and average pellet diameter for different culture times in agitated bioreactors.

Parameter	**Bioreactor (260 rpm)**				**Bioreactor (340 rpm)**			
Culture time (h)	γ (s^{-1})	η (mPa.s)	λ (μm)	Pellet diameter (μm)	γ (s^{-1})	η (mPa.s)	λ (μm)	Pellet diameter (μm)
0	184.5	0.89±0.1	43.6	–	322.1	0.66±0.1	29.1	–
24	193.8	0.72±0.2	40.5	147±4	274.8	0.81±0.4	36.9	131±3
36	152.2	1.3±0.1	58.2	245±6	202.3	1.4±0.8	58.4	214±4
60	78.3	4.6±0.6	157.6	138±4	133.3	3.7±1.7	109.2	160±10
72	53.1	10.8±0.5	282.3	146±43	108.4	6.0±2.1	148.9	134±5

At the end of bioreactor and NF cultures, the consistency index (i.e., the "thickness") increased substantially as the biomass concentration increased, even with the reduction in pellet size. This suggests that the consistency index of small pellets (e.g., <200 μm in diameter) is not particularly sensitive to an increase in the concentration of the pellets, as was previously reported (Casas-López *et al*., 2005). However, we cannot determine whether these changes in both, the consistency index and behavior index, are due to morphology, cell biomass concentration, or composition of the culture medium. Finally, the rheological similarity between the bioreactor and NF cultures may be associated with an increase in the P_g/V of the bioreactor culture medium at the end of culturing, as was observed in NF (Marín-Palacio *et al* 2014). It will be very interesting to determine the behavior of P_g/V in bioreactors as has been previously reported (Reséndiz *et al*., 1991; Kaiser *et al*., 2017).

In terms of productivity, previous reports suggest that recombinant protein production is only affected when the pellet size is greater than 2.0 mm (Gamboa-Suasnavart *et al*., 2011). However, there was an increase in the amount of extracellular protein in CF, compared to those cultures in NF and bioreactor (Table 1). This even allows us to suppose that smaller pellets or dispersed mycelium (as in CF) can have higher specific productivity of recombinant proteins than larger pellets, as in NF and bioreactors (van Wezel *et al*., 2006; Willemse *et al*., 2018).

Finally, native APA from *M. tuberculosis* contains 0-3 mannose residues in the C-terminal region (Dobos *et al*. 1996; Dobos *et al*. 1995). In contrast, rAPA produced in *S. lividans* contained up to four mannose residues in 1.0 L NF cultures (Lara *et al*. 2004), up to five or six mannoses in 250 mL baffled/coiled shake flask or bioreactor cultures at 150 rpm (Gamboa-Suasnavart *et al*. 2011; Gamboa-Suasnavart *et al*. 2013), and up to six mannose residues in NF cultures containing 25 and 15 mL of culture medium (250 mL, flask volume), shaken at 168 and 150 rpm, respectively (Marín-Palacio *et al*. 2014). In contrast, up to seven mannose residues were attached to the C-terminal peptide in both bioreactor cultures (at 0.20 and 0.40 kW/m^3), which was one more mannose than obtained in the NF and CF (Marín-Palacio *et al*. 2014). Previously, we suggested that the limiting factor for post-translational modifications, such as *O*-mannosylation, is mass transfer phenomena, especially oxygen transfer, rather than hydrodynamic factors. In this study, the DOT in the bioreactors was above 10% allowed us to avoid oxygen as a limiting factor inside large pellets, for this reason, we were able to detect one additional mannose residue. Moreover, we detected up to five more mannose residues than that reported by Gamboa-Suasnavart *et al*. (2011) in NF, where the pellet sized was four times higher than observed here (1570±410 μm).

Conclusions

Our results show that volumetric power input can successfully be used for scaling up some, but not all, kinetic, stoichiometric and productivity parameters. We propose that some cellular responses, even the morphology, could be affected by the differences in patterns of hydrodynamics/aeration between shake flasks and bioreactors. This is clearly an indication that the actual mechanisms governing the production of recombinant *O*-glycoproteins in filamentous bacterial cultures, as well as the effects of other important process variables, such as pH and dissolved oxygen tension, remain to be established, and should be taken into account in future process development.

Acknowledgement

RAGS is grateful for the scholarship from the National Council for Science and Technology, Mexico (CONACYT, Consejo Nacional de Ciencia y Tecnología, México, 312169). LDMP thanks the scholarship Francisco José de Caldas from Colciencias-Colombia and EAFIT University Colombia for support her PhD studies at the "Programa de Doctorado en Ciencias Bioquímicas, Universidad Nacional Autónoma de México". We also thank Dr. Miguel Tapia Rodríguez, Eng. Abel Blancas-Cabrera, for his technical assistance. We thank Dra. Clara Espitia for the donation of the primary antibody mAb6A3, and Dr. Luis Servín for the donation of the transformed strain of *S. lividans* and the plasmid pIJ6021MT-45, which carries the *M tuberculosis apa* gene-Rv1860. We thank Editage (www.editage.com) for English Language editing. This project was developed under the Institutional Program of the Institute of Biomedical Research, National Autonomous University of Mexico: "The production of biomolecules of biomedical interest in bacteria and fungi (Instituto de Investigaciones Biomédicas-UNAM: La producción de biomoléculas de interés biomédico en bacterias y hongos".

Financial support

This work was partially financed by the "Consejo Nacional de Ciencia y Tecnología" (CONACYT 247473, 220795), and "Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica, Universidad Nacional Autónoma de México" (PAPIIT-UNAM IT-200719, IN-208415).

Nomenclature

Ae	aeration number (dimensionless: $Q/(D_i^3\ N)$)
BF	baffled Erlenmeyer shake flasks
CF	coiled Erlenmeyer shake flasks
D_B	fractal dimension (mean)
D_i	impeller diameter (m)
DOT	dissolved oxygen tension (% air saturation)
D_t	bioreactor diameter (m)
Fr	Froude number (dimensionless: $D_i\ N^2/g$)
g	gravitational acceleration (9.8 m/s^2)
K	flow consistency index ($Pa.s^n$)
k_La	volumetric oxygen transfer coefficient (h^{-1})
L	fractal lacunarity
N	agitation speed (rpm: 1/min, rps: 1/s)
n	flow behavior index (dimensionless)
NF	conventional normal Erlenmeyer shake flask
Np	power number (dimensionless: $P/[N^3D_i^5\rho]$)
P	power input (W)
P/V	volumetric power input (kW/m^3)
P_g/V	volumetric gassed power input (kW/m^3)
Q	gas flow rate (m^3/sec)
rAPA	recombinant alanine and proline rich antigen, or 45/47 kDa protein from *Mycobacterium tuberculosis*
Re	impeller Reynolds number (dimensionless: $D_i\ N^2\ \rho/\eta$)
t	culture time (h)
V	volume (m^3)
X	biomass (g/L)
Y	yield (g/g)

Greek symbols

γ	average shear rate (s^{-1})
ε	energy dissipating rate (m^2/s^3)
η	apparent broth viscosity (Pa.s)
λ	Kolmogorov microscale (μm)
ν	kinematic viscosity (μ/ρ: m^2/s)
μ	specific growth rate (h^{-1})
π	Pi number
ρ	density (kg/m^3)

References

Amanullah, A., Buckland, B.C., and Nienow, A.W. (2004). Mixing in the fermentation and cell culture industries. *Handbook of Industrial Mixing: Science and Practice*. Chapter 18:1071-1170. doi:10.1002/0471451452.ch18

Anné, J., Maldonado, B., Van Impe, J., Van Mellaert, L., and Bernaerts K. (2012) Recombinant protein production and streptomycetes. *Journal of Biotechnology 158*, 159-167. doi: 10.1016/j.jbiotec.2011.06.028.

Barry, D. J. (2013). Quantifying the branching frequency of virtual filamentous microbes using fractal analysis. *Biotechnology and Bioengineering 110*, 437-447. doi: 10.1002/bit.24709

Büchs, J, and Zoels B. (2001). Evaluation of maximum to specific power consumption ratio in shaking bioreactors. *Journal of Chemical Engineering Japan 34*, 647-653. doi: 10.1252/jcej.34.647

Büchs, J. (2001). Introduction of advantages and problems of shaken cultures. *Biochemical Engineering Journal 7*, 91-98. doi: 10.1016/S1369-703X(00)00106-6.

Büchs, J., Maier, U., Milbradt, C., and Zoels B. (2000). Power consumption in shaking flasks on rotary shaking machines: I Power consumption measurement in unbaffled flasks at low liquid viscosity. *Biotechnology and Bioengineering 68*, 589-593. doi: 10.1002/(SICI)1097-0290(20000620)68:6<589::AID-BIT1>3.0.CO;2-J.

Casas-López, J.L., Sánchez-Pérez, J.A., Fernández-Sevilla, J.M., Rodríguez-Porcel, E.M., and Chisti Y. (2005) Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus. Journal of Biotechnology 116*, 61-77. doi: 10.1016/j.jbiotec.2004.10.005

Dickey, D. S., K. J. Bittorf, C. J. Ramsey, and K. E. Johnson (2004). Understand flow patterns in glass-lined reactors. *Chemical Engineering Progress 100*, 21-25.

Dobos, K., Khoo, K., Swiderek, K., Brennan, P., and Belisle J. (1996). Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. *Journal of Bacteriology 178*, 2498-2506. doi: 10.1128/jb.178.9.2498-2506.1996.

Dobos, K., Swiderek, K., Khoo, K., Brennan, P.J., and Belisle J.T. (1995). Evidence for glycosylation sites on the 45-Kilodalton glycoprotein of *Mycobacteria tuberculosis. Infect Immunology 63*, 2846-2853.

Freedman, D. (1970). The shaker in bioengineering. In Norris, J.R., Ribbons, D.W., eds *Methods in Microbiology* (pp 175-185) London: Academic Press.

Freyer, S. A., M. König, and A. Künkel (2004). Validating shaking flasks as representative screening systems. *Biochemical Engineering Journal 17*, 169-173. 10.1016/S1369-703X(03)00175-X.

Gamboa-Suasnavart, R.A., Marín-Palacio, L.D., Martínez-Sotelo, J., Espitia, C., Servín-González, L., Valdez-Cruz N.A., and Trujillo-Roldán M.A. (2013) Scale-up from shake flasks to bioreactor, based on power input and *Streptomyces lividans* morphology, for the production of recombinant APA (45/47 kDa protein) from *Mycobacterium tuberculosis. World Journal of Microbiology and Biotechnology 29*, 1421-1429. doi: 10.1007/s11274-013-1305-5.

Gamboa-Suasnavart, R.A., Valdez-Cruz, N.A., Cordova-Davalos, L.E., Martinez-Sotelo, J.A., Servin-Gonzalez, L., Espitia, C. and Trujillo-Roldán M.A. (2011). The *O*-mannosylation and production of recombinant APA (45/47 KDa) protein from *Mycobacterium tuberculosis* in *Streptomyces lividans* is affected by culture conditions in shake flasks. *Microbiology Cell Fact 10*, 110. doi: 10.1186/1475-2859-10-110.

Gamboa-Suasnavart, R.A., Valdez-Cruz, N.A., Gaytan-Ortega, G., Reynoso-Cereceda, G.I., Cabrera-Santos, D., López-Griego, L., Klöckner W., Büchs J., Trujillo-Roldán, M.A. (2018). The metabolic switch can be activated in a recombinant strain of *Streptomyces lividans* by a low oxygen transfer rate in shake flasks. *Microbiology Cell Fact 17*, 189.

Garcia-Ochoa, F, and Gomez E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnology Advances 27*, 153-176. doi: 10.1016/j.biotechadv.2008.10.006.

Gerson, D.F., and Kole M.M. (2001). Quantitative measurements of mixing intensity in shake-flasks and stirred tank reactors: use of the Mixmeter, a mixing process analyzer. *Biochemical Engineering Journal 7*, 153-156. doi: 10.1016/S1369-703X(00)00115-7.

Giese, H., Klöckner, W., Peña, C., Galindo, E., Lotter, S., Wetzel, K., Meissner, L., Peter, C.P., and Büchs J. (2014). Effective shear rates in shake flasks. *Chemical Engineering Science 118*, 102-113. doi: 10.1016/j.ces.2014.07.037.

Giudici, R., Pamboukian, C.R. and Facciotti M.C. (2004). Morphologically structured model for antitumoral retamycin production during batch and fed-batch cultivations of *Streptomyces olindensis. Biotechnology and Bioengineering 86*, 414-424. doi: 10.1002/bit.20055.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Bruton, K.T., Kieser, C.J., Lydiate, H.M., Smith, D.J., Ward, C.P., and Schrempf H. (1985). *Genetic Manipulation of Streptomyces: A Laboratory Manual*. Norwich, UK: The John Innes Foundation.

Humphrey, A. (1998). Shake flask to fermentor: what have we learned? *Biotechnology Progress 14*, 3-7. doi: 10.1021/bp970130k

Jakimowicz, D., and van Wezel G.P. (2012) Cell division and DNA segregation in Streptomyces: how to build a septum in the middle of nowhere? *Molecular Microbiology 85*, 393-404. doi: 10.1111/j.1365-2958.2012.08107.x.

Junker, B.H. (2004). Scale-up methodologies for Escherichia coli and yeast fermentation processes. *Journal of Bioscience and Bioengineering 97*, 347-364. doi: 10.1016/S1389-1723(04)70218-2

Junker, B.H., Walker, A., Hesse, M., Lester, M., Christensen, J., and Connors N. (2009). Actinomycetes scale-up for the production of the antibacterial, nocathiacin. *Biotechnology Progress 25*, 176-88. doi: 10.1002/btpr.122.

Jüsten, P., Paul, C.G., Nienow, A.W. and Thomas C.R. (1996). Dependence of mycelial morphology on impeller type and agitation intensity. *Biotechnology and Bioengineering 52*, 672-684. doi:10.1002/(SICI)1097-0290(19961220)52:6<672::AID-BIT5>3.0.CO;2-L

Jüsten, P., Paul, G.C., Nienow, A.W., and Thomas C.R. (1998). Dependence of *Penicillium chrysogenum* growth, morphology, vacuolation, and productivity in fed-batch fermentations on impeller type and agitation intensity. *Biotechnology and Bioengineering 59*, 762-775. doi: 10.1002/(SICI)1097-0290(19980920)59:6<762::AID-BIT13>3.0.CO;2-7

Kaiser, S.C., Werner, S., Jossen, V., Kraume, M. and Eibl D. (2017). Development of a method for reliable power input measurements in conventional and single-use stirred bioreactors at laboratory scale. *Engineering for Life Sciences 17*, 500-511. doi: 10.1002/elsc.201600096

Karperien, A. (2013). FracLac for ImageJ. Charles Sturt University. (https://imagej.nih.gov/ij/plugins/fraclac/fraclac.html)

Kelly, S., Grimm, L.H., Bendig, C., Hempel, D.C., and Krull R. (2006). Effects of fluid dynamic induced shear stress on fungal growth and morphology. *Process Biochemistry 41*, 2113-2117. doi: 10.1016/j.procbio.2006.06.007.

Koepff, J., Keller, M., Tsolis, K.C., Busche, T., Rückert, C., Hamed, M.B., Anné, J., Kalinowski, J., Wiechert, W., Economou, A., and Oldiges M. (2017). Fast and reliable strain characterization of *Streptomyces lividans* through micro-scale cultivation. *Biotechnology and Bioengineering 114*, 2011-2022. doi:10.1002/bit.26321 Kolmogorov A.N. (1941). Dissipation of energy in locally isotropic turbulence. *Doklady Akademii Nauk 32*, 16-18.

Lara, A. (2011). Recombinant protein production in *Escherichia coli. Revista Mexicana de Ingeniería Química 10*, 209-223.

Lara, M., Servín-González, L., Singh, M., Moreno, C., Cohen, I., Nimtz, M. and Espitia C. (2004). Expression, secretion, and glycosylation of the 45-and 47-kDa glycoprotein of *Mycobacterium tuberculosis* in *Streptomyces lividans. Applied Environmental Microbiology 70*, 679-685. doi: 10.1128/AEM.70.2.679-685.2004.

Lee, J.H., and Lee K.J. (1996). Analysis of expression rate of cloned beta-lactamase gene in a recombinant of *Streptomyces lividans. Journal of Biotechnology 52*, 161-165. doi: 10.1016/S0168-1656(96)01633-1

Lin, P.J., Scholz, A. and Rainer K. (2010). Effect of volumetric power input by aeration and agitation on pellet morphology and product formation of *Aspergillus niger. Biochemical Engineering Journal 49*, 213-220. doi: 10.1016/j.bej.2009.12.016.

Mancilla, E., Palacios-Morales, C.A., Cordova-Aguilar, M.S., Trujillo-Roldán, M.A., Ascanio, G. and Zenit R. (2015). A hydrodynamic description of the flow behavior in shaken flasks. *Biochemical Engineering Journal 99*, 61-66. 10.1016/j.bej.2015.03.003.

Marín-Palacio, L.D., Gamboa-Suasnavart, R.A., Valdez-Cruz, N.A., Servín-González, L., Córdova-Aguilar, M.A., Soto, E., Klöckner, W., Büchs, J. and Trujillo-Roldán M.A. (2014). The role of volumetric power input in the growth, morphology, and production of a recombinant glycoprotein by *Streptomyces lividans* in shake flasks. *Biochemical Engineering Journal 90*, 224-233. doi: 10.1016/j.bej.2014.06.010.

Marques, M.P., Cabral, J. and Fernandes P. (2010). Bioprocess scale-up: quest for the parameters to be used as criterion to move from microreactors to lab-scale. *Journal of Chemical Technology and Biotechnology 85*, 1184-1198. doi: 10.1002/jctb.2387

Nagata, S. (1975) Mixing: Principles and application. Pp 5562 John Wiley New York, NY.

Ni, X., Gao, S., Cumming, R.H., and Pritchard, D.W. (1995). A comparative study of mass transfer in yeast for a batch pulsed baffled bioreactor and

a stirred tank fermenter. *Chemical Engineering Science 50*, 2127-2136.

Olmos, E., Mehmood, N., Haj Husein, L., Goergen, J.L., Fick, M. and Delaunay S. (2013). Effects of bioreactor hydrodynamics on the physiology of *Streptomyces. Bioprocess and Biosystem Engineering 36*, 259-72. doi: 10.1007/s00449-012-0794-1.

Orozco-Sánchez, F., Rodríguez-Monroy, M. (2007). Cell suspension culture of *Azadirachta indica* for production of a bioinsecticide. *Revista Mexicana de Ingeniería Química 6*, 251-258.

Orozco-Sánchez, F., Sepúlveda-Jiménez, G., Trejo-Tapia, G., Zamilpa, A., Rodríguez-Monroy, M. (2011). Oxygen limitations to grow *Azadirachta indica* cell culture in shake flasks. *Revista Mexicana de Ingeniería Química 10*, 343-352.

Palacios-Morales, C., Aguayo-Vallejo, J.P., Trujillo-Roldán, M.A., Zenit, R., Ascanio, G., and Córdova-Aguilar M.S. (2016). The flow inside shaking flasks and its implication for mycelial cultures. *Chemical Engineering Science 152*, 163-171. doi: 10.1016/j.ces.2016.06.016

Pérez, R.E., Lasa, A.M., Rodríguez, R.S., Menéndez, E.C., Suárez, J.G. and Balaguer H.D. (2006). Scale-up of recombinant Opc protein production in Escherichia coli for a meningococcal vaccine. *Journal of Biotechnology 127*, 109-114. doi: 10.1016/j.jbiotec.2006.06.005.

Peter, C.P., Suzuki, Y. Rachinskiy, K., Lotter, S. and Büchs J. (2006). Volumetric power consumption in baffled shake flasks. *Chemical Engineering Science 61*, 3771-3779. doi: 10.1016/j.ces.2005.12.020.

Raval, K., Gehlot, K. and Prassana B.D. (2017). Scale-up of naringinase production process based on the constant oxygen transfer rate for a novel strain of *Bacillus methylotrophicus. Preparative Biochemistry and Biotechnology 47*, 192-198. doi: 10.1080/10826068.2016.1201680.

Reséndiz, R, Martínez, A., Ascanio, G. and Galindo E. (1991). A new pneumatic bearing dynamometer for power input measurement in stirred tanks. *Chemical Engineering Technology 14*, 105-108.

Rocha-Valadez, J.A., Galindo, E. and Serrano-Carreón L. (2007). The influence of circulation frequency on fungal morphology: A case study considering Kolmogorov microscale in constant specific energy dissipation rate cultures of *Trichoderma harzianum. Journal of Biotechnology 130*, 394-401.

Rocha-Valadez, J.A., Estrada, M., Galindo, E., and Serrano-Carreón L. (2006). From shake flasks to stirred fermentors: Scale-up of an extractive fermentation process for 6-pentyl-α-pyrone production by *Trichoderma harzianum* using volumetric power input. *Process Biochemistry 41*, 1347-1352.

Rushton, J.H., Costich, E.W. and Everett, H.J. (1950). Power characteristics of mixing impellers. *Chemical Engineering Progress 46*, 395-476.

Rushton, J.H. (1952). Mixing of liquids in chemical processing. *Industrial and Engineering Chemistry 44*, 2931-2936. doi:10.1021/ie50516a045.

Sánchez-Pérez, J.A., Rodríguez-Porcel, E.M., Casas-López, J.L., Fernández-Sevilla, J.M., and Chisti Y. (2006). Shear rate in stirred tank and bubble column bioreactors. *Chemical Engineering Journal 124*, 1-5

Shukla, V.B., Parasu Veera, U., Kulkarni, P.R., and Pandit A.B. (2001). Scale-up of biotransformation process in stirred tank reactor using dual impeller bioreactor. *Biochemical Engineering Journal 8*, 19-29. doi: 10.1016/S1369-703X(00)00130-3.

Smith Jr, T.G., Lange, G.D., and Marks, W.B. (1996). Fractal methods and results in cellular morphology-dimensions, lacunarity and multifractals. *Journal of Neuroscience Methods 69*, 123-136. doi: 10.1016/S0165-0270(96)00080-5.

Tajsoleiman, T., Mears, L., Krühne, U., Gernaey, K.V., & Cornelissen, S. (2019) An industrial perspective on scale-down challenges using miniaturized bioreactors. *Trends in Biotechnology* In Press. Doi: 10.1016/j.tibtech.2019.01.002.

Tamura, S., Park, Y., Toriyama, M., and Okabe M. (1997). Change of mycelial

morphology in tylosin production by batch culture of *Streptomyces fradiae* under various shear conditions. *Journal of Fermentation Bioengineering 83*, 523-528. doi: 10.1016/S0922-338X(97)81131-2

Trujillo-Roldán, M.A., Valdez-Cruz, N.A., Gonzalez-Monterrubio, C.F., Acevedo-Sánchez, E.V., Martínez-Salinas, C., García-Cabrera, R.I., Gamboa-Suasnavart, R.A., Marín-Palacio, L.D., Villegas, J., and Blancas-Cabrera A. (2013). Scale-up from shake flasks to pilot-scale production of the plant growth-promoting bacterium *Azospirillum brasilense* for preparing a liquid inoculant formulation. *Applied Microbiology and Biotechnology 97*, 9665-9674. doi: 10.1007/s00253-013-5199-9.

van Dissel, D., and van Wezel, G. (2017). Morphology-driven downscaling of *Streptomyces lividans* to micro-cultivation. *bioRxiv*, 159509. doi: https://doi.org/10.1101/159509

van Veluw, G.J., Petrus, M.L., Gubbens, J., de Graaf, R., de Jong, I.P., van Wezel, G.P., Wösten, H.A. and Claessen D. (2012). Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach. *Applied Microbiology and Biotechnology 96*, 1301-1312. doi: 10.1007/s00253-012-4490-5.

van Wezel, G.P., Krabben, P., Traag, B.A., Keijser, B.J.F., Kerste, R., Vijgenboom, E., Heijnen, J.J., and Kraal B. (2006). Unlocking *Streptomyces* spp for use as sustainable industrial production platforms by morphological engineering. *Applied Environmental Microbiology 72*, 5283-5288. doi: 10.1128/AEM.00808-06. Villegas, E., Téllez-Téllez, M., Rodríguez, A., Carreón-Palacios, A.E., Acosta-Urdapilleta, M.L., Kumar-Gupta, V., and Díaz-Godínez, G. (2016). Laccase activity of Pycnoporus cinnabarinus grown in different culture systems. *Revista Mexicana de Ingeniería Química 15*, 703-710.

Vrancken, K., and Anné J. (2009). Secretory production of recombinant proteins by *Streptomyces. Future Microbiology 4*, 181-188. doi: 10.2217/17460913.4.2.181. Wardell, J.N., Stocks, S.M., Thomas, C.R., and Bushell M.E. (2002). Decreasing the hyphal branching rate of *Saccharopolyspora erythraea* NRRL 2338 leads to increased resistance to breakage and increased antibiotic production. *Biotechnology and Bioengineering 78*, 141-146.

Willemse, J., Büke, F., van Dissel, D., Grevink, S., Claessen, D., and van Wezel, G.P. (2018). SParticle, an algorithm for the analysis of filamentous microorganisms in submerged cultures. *Antonie Van Leeuwenhoek 111*, 171-182. doi: /10.1002/bit.26321

Xia, X., Lin, S., Xia, X.X., Cong, F.S., and Zhong J.J. (2014). Significance of agitation-induced shear stress on mycelium morphology and lavendamycin production by engineered *Streptomyces flocculus. Applied Microbiology and Biotechnology 98*, 4399-4407. doi: 10.1007/s00253-014-5555-4.

Xu, S., Hoshan, L., Jiang, R., Gupta, B., Brodean, E., O'Neill, K., Seamans, T.C., Bowers, J., and Chen H. (2017). A practical approach in bioreactor scale-up and process transfer using a combination of constant P/V and vvm as the criterion. *Biotechnology Progress 33*, 1146-1159. doi: 10.1002/btpr.2489.

Yun, S.I., Yahya, A.R., Malten, M., Cossar, D., Anderson, W.A., Scharer, J.M., and Moo-Young M. (2001) Peptidases affecting recombinant protein production by *Streptomyces lividans. Canadian Journal of Microbiolgy 47*, 1137-1140.

Zacchetti, B., Smits, P., and Claessen, D. (2018). Dynamics of pellet fragmentation and aggregation in liquid-grown cultures of *Streptomyces lividans. Frontiers in Microbiology 9*, 943. doi: 10.3389/fmicb.2018.00943.