World J Microbiol Biotechnol (2013) 29:1421–1429
DOI 10.1007/s11274-013-1305-5

ORIGINAL PAPER

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*

Ramsés A. Gamboa-Suasnavart · Luz D. Marín-Palacio · José A. Martínez-Sotelo · Clara Espitia · Luis Servín-González · Norma A. Valdez-Cruz · Mauricio A. Trujillo-Roldán

Received: 25 November 2012 / Accepted: 25 February 2013 / Published online: 10 March 2013
© Springer Science+Business Media Dordrecht 2013

Abstract Culture conditions in shake flasks affect filamentous *Streptomyces lividans* morphology, as well the productivity and *O*-mannosylation of recombinant Ala-Pro-rich O-glycoprotein (known as the 45/47 kDa or APA antigen) from *Mycobacterium tuberculosis*. In order to scale up from previous reported shake flasks to bioreactor, data from the literature on the effect of agitation on morphology of *Streptomyces* strains were used to obtain gassed volumetric power input values that can be used to obtain a morphology of *S. lividans* in bioreactor similar to the morphology previously reported in coiled/baffled shake flasks by our group. Morphology of *S. lividans* was successfully scaled-up, obtaining similar mycelial sizes in both scales with diameters of 0.21 ± 0.09 mm in baffled and coiled shake flasks, and 0.15 ± 0.01 mm in the bioreactor. Moreover, the specific growth rate was successfully scaled up (0.09 ± 0.02 and 0.12 ± 0.01 h^{-1}, for bioreactors and flasks, respectively), and the recombinant protein productivity measured by densitometry, as well. More interestingly, the quality of the recombinant glycoprotein measured as the amount of mannoses attached to the C-terminal of APA was also scaled- up; with up to five mannose residues in cultures carried out in shake flasks; and six in the bioreactor. However, final biomass concentration was not similar, indicating that although the process can be scaled-up using the power input, others factors like oxygen transfer rate, tip speed or energy dissipation/circulation function can be an influence on bacterial metabolism.

Keywords Scale-up · Power input · APA 45/47 kDa · *O*-mannosylation · *Mycobacterium tuberculosis* · *Streptomyces lividans*

Abbreviations

Di	Impeller diameter (m)
Fg	Volumetric gas flow rate (m^3/min)
g	Gravitational acceleration (g/m^2)
N	Agitation speed (rev/min)
Np	Power number (Dimensionless)
P	Power input by agitation (W)
Pg	Gassed power input (W)
*Pg**	Corrected gassed power input by bioreactor geometry (W)
Re	Reynolds number (Dimensionless)
V	Operation volume (m^3)
W	Impeller blade width (m)
μ	Fluid viscosity (mPa.s)
ρ	Density (kg/m^3)

Introduction

Shake flasks have been widely used in the study and optimization of biotechnology processes, allowing to the

R. A. Gamboa-Suasnavart · L. D. Marín-Palacio · M. A. Trujillo-Roldán (✉)
Unidad de Bioprocesos, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, AP. 70228, Mexico, DF CP. 04510, Mexico
e-mail: maurotru@gmail.com; maurotru@biomedicas.unam.mx

R. A. Gamboa-Suasnavart · L. D. Marín-Palacio · L. Servín-González · N. A. Valdez-Cruz · M. A. Trujillo-Roldán
Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, AP. 70228, Mexico, DF CP. 04510, Mexico

J. A. Martínez-Sotelo · C. Espitia
Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, AP. 70228, Mexico, DF CP. 04510, Mexico

performance of experiments with minimal costs and material (Buchs et al. 2000a, b). However, shake flasks have several limitations, one of them, and probably the most important, is the complex understanding of the individual environmental factors involved (mixing/aeration), due to the dependency of the oxygen transfer rate on agitation speed, and the inability to control pH and/or dissolved oxygen tension (DOT). This understanding is essential in order to reach a rational large-scale production (Seletzky et al. 2007). Recently, some approaches about the characteristic properties of shaken cultures trying to establish scaling criteria with an engineering basis have been reported (Hoopen et al. 1994; Silberbach et al. 2003; Seletzky et al. 2007; Klockner and Buchs 2012; Gamboa-Suasnavart et al. 2011). Therefore, the scaling-up from shake flasks to bioreactors is essential to obtain large quantities of final products. However, this process is troublesome and poorly understood, mainly due to the lack of knowledge concerning the influence of the operating conditions on mass transfer, hydrodynamics, and power input. On the other hand, it is difficult to define an engineering parameter of shake flasks to be scaled up to the bioreactor, since geometries are different, and commonly a biological parameter is used for this purpose, such as growth rate, productivity or morphology in filamentous cultures (Junker et al. 2004; Seletzky et al. 2007; Hoopen et al. 1994).

The relationship between agitation frequency, productivity, growth, and aggregation morphology has been widely studied in the filamentous bacteria *Streptomyces* sp., and mathematical models have been proposed to describe this relationship (Tough and Prosser 1996; Tough et al. 1995; Giudici et al. 2004; Roubos et al. 2001). Moreover, in submerged cultures of *S. lividans*, the aggregation morphology plays an important role in the production, secretion (Yun et al. 2001; Dobson et al. 2008; Anné et al. 2012), and post-translational modification of recombinant proteins (Vallin et al. 2006; Gamboa-Suasnavart et al. 2011). In the specific case of the recombinant production of the Ala–Pro–rich *O*-glycoprotein (also known as 45/47 kDa or APA antigen native of *Mycobacterium tuberculosis*) by *S. lividans*, the small and dispersed mycelial aggregates obtained in baffled and stainless steel coiled flasks improve the production and increase the degree of *O*-mannosylation of the recombinant protein, in comparison with the large aggregates obtained in conventional shake flasks (Gamboa-Suasnavart et al. 2011). This antigen is a candidate for generating a new vaccine against tuberculosis (Sable et al. 2011), due to its capacity to induce lymphoproliferative response associated with its *O*-mannosylation pattern (Horn et al. 1999; Dobos et al. 1996).

The purpose of this study was to establish the culture conditions in a laboratory bioreactor for obtaining a morphology of *S. lividans* similar to that produced in coiled and baffled shake flasks by using both, a biological parameter (morphology) and an engineering parameter (power input), and proposing scaling-up strategies for filamentous bacteria cultures based on engineering parameters. Moreover, this study would allow us to understand the relationship between morphology-production and post-translational modifications of recombinant proteins in *S. lividans*.

Materials and methods

Microorganism and culture conditions

Wild type *S. lividans* 66 strain 1326 (Kieser et al. 2000) was transformed with plasmid pIJ6021MT-45 carrying the *apa* gene under a thiostrepton-inducible promoter, and conferring resistance to kanamycin (Lara et al. 2004). Precultures were carried out as previously reported (Gamboa-Suasnavart et al. 2011). Coiled flasks (250-ml, filled with 50 ml of medium) consisted of conventional Erlenmeyer flasks with an inserted 30 cm stainless steel spring (1.3 cm diameter, 19 SW gauge). Baffled flasks consisted of conventional flasks with three indentations of 2.5 cm in the wall of the flasks. Culture medium was Luria–Bertani's with kanamycin (50 μg/ml), modified by addition of 34 % w/v sucrose (Lara et al. 2004). In this manuscript all coiled and baffled shake flask culture data was taken from previously reported data of our group and it was used as a reference for demonstrating the scale-up strategy (Gamboa-Suasnavart et al. 2011). The shake flasks cultures were carried out at 30 °C and 150 rev/min for 60 h (New Brunswick Scientific lab shaker, 2.5 cm shaking diameter), with addition of the inducer thiostrepton (10 μg/ml) at 16 h of culture.

Bioreactor culture conditions, control system and scaling up strategy

A 1.5-l bioreactor (Applikon Biotechnology®, USA) was used, containing 950 ml of the same shake flask culture medium. The bioreactor contained two 4.5 cm diameter Rushton turbines in a tank to turbine diameter ratio of 3:1. The bioreactor was inoculated with 50 ml from coiled shake flasks (12 h of culture), and the fermentation was maintained at 30 °C. Recombinant APA (rAPA) production was induced (thiostrepton 10 μg/ml) at the same biomass concentration achieved at 16 h in shake flask. DOT was maintained at least at 10 % of air saturation (to avoid oxygen limitation), by maintaining a constant total gas flow rate of 0.5 l/min, and by enriching the inflowing air with oxygen, according to a proportional-integral-derivative control algorithm in a ADI1030 controller (Applikon Biotechnology®, USA). The pH was controlled at 7.2.

Culture parameters (DOT, pH, temperature, and agitation) were displayed on line, and saved on hard disk for later analysis.

Based on the work of Tough and Prosser (1996), a correlation between Pg/V and bacterial morphology was proposed (Fig. 1a). Standard dimensions were assumed for the bioreactor Bioflo II (New Brunswick Scientific) as is shown in Table 1, and a air flow of 2.0 l/min (Tough and Prosser 1996; Tough et al. 1995). The power input was calculated from Eq. 1, and then divided by the operation volume to obtain the volumetric power input:

$$P = NpDi^5n^3\rho \quad (1)$$

In order to determine *Np*, Reynolds numbers were calculated from 100 to 1,100 rev/min (2), resulting higher than 1×10^4, indicating a turbulent flow (Rushton et al. 1950a, b). According to the relation proposed for Rushton turbines in turbulent flow, *Np* can be assumed as a constant value of 5.5 (Chapple et al. 2002):

$$\mathrm{Re} = \frac{\rho Di^2 N}{\mu} \quad (2)$$

In order to calculate the gassed power input, Eq. 3 was used as proposed by Hugmark (1980):

$$\frac{P_g}{P} = 0.1 * \left(\frac{Fg}{NV}\right) - 0.25 * \left(\frac{N^2 D_i^4}{gWV^{2/3}}\right) - 0.20 \quad (3)$$

Finally, it is important to consider that the previous equations are based on standard bioreactor geometries, including four baffles. To get a better approximation to the real Pg/V, a correction proposed by Dickey et al. (2004) was used to correct the Pg/V in terms of baffle number between this work and Tough and Prosser (1996). Results of these calculations are shown in Table 2. This correction was published online (www.chemicalprocessing.com/Media/MediaManager/Baffle_Effect.pdf). When two baffles are used, the calculated Pg/V must be multiplied by 0.85 (this work), and when no baffles are used the factor may decrease down to 0.2 (bioreactor used by Tough and Prosser 1996). Figure 1a shows the approach to *S. coelicolor* morphology as a function of corrected volumetric power input (Pg*/V). A corrected P_g/V and agitation rate is shown in Fig. 1b considering the bioreactor used in this work (1.0 l Applikon bioreactor).

Analytical determinations

Growth was followed by optical density at 600 nm, and biomass dry weight as follows: 10 ml of culture were filtered through a 0.45 µm pore size membrane (Millipore, USA), and washed once with one volume of distilled water. The obtained mycelia were dried for 24 h in an oven at 55 °C, then placed for 2 h in a desiccator, and weighed afterwards, as previously described (Gamboa-Suasnavart et al. 2011).

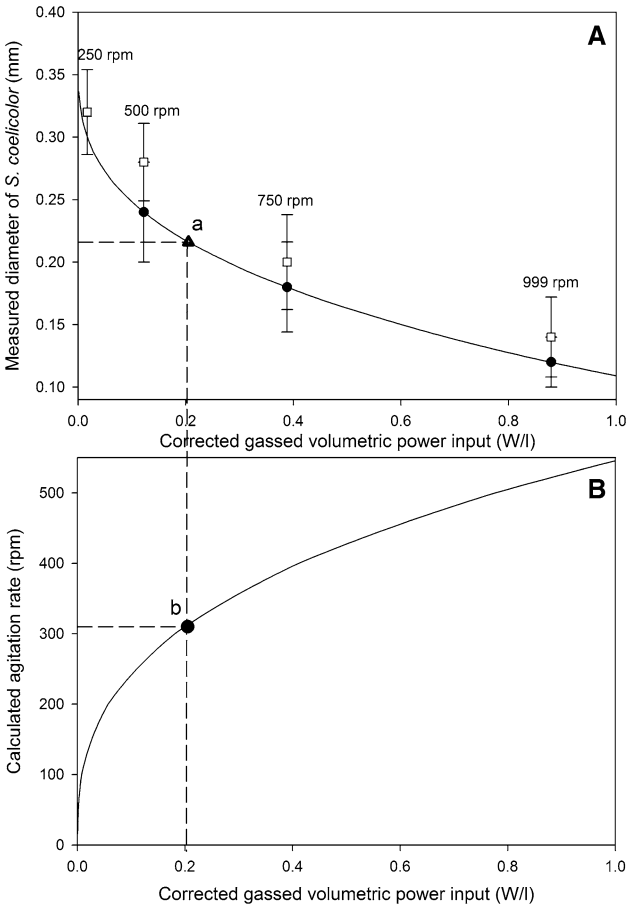

Fig. 1 **a** Relationship between corrected gassed volumetric power input (Pg*/V) and previously experimental (*closed circles*) and modeled data (*open squares*) of mycelia diameters of *S. coelicolor* (raw data was taken from Tough and Prosser 1996, of agitation and morphology radius, as is shown in Table 2). **b** Correlation between corrected *Pg*/V* and agitation speed in the bioreactor used in this work (the trend of *Pg*/V* data taken from Table 2 are shown, *dots* are not presented as several data of agitation speed for our system exceeded 1.0 *W/l*). The *dotted line* shows the searched aggregate diameter as the morphology on coiled shake flasks (**a**), and the equivalent *Pg*/V* and agitation in the bioreactor used in this work (**b**)

Image analysis

The characterization of mycelial aggregation was made on samples taken at 60 h from each culture, and fixed to avoid the loss of morphology (Gamboa-Suasnavart et al. 2011). These samples were analysed by microscopy, and the average diameter, area, and perimeter of each aggregate was determined. At least 100 clumps, aggregates or pellets were analysed for each sample, at least 3 samples were analysed per culture, and three cultures were carried out in the bioreactor. Coiled shake flask data were also reported

Table 1 Comparison of the bioreactors used for calculating P_g/V for *Streptomyces coelicolor* A3(2) growth (Tough and Prosser 1996) with the one used in this work

Parameter	Units	Tough and Prosser (1996), Bioflo II NBS (USA)	This work, Applikon (USA)
Number of baffles		0	2
V (Operation volume)	m^3	0.001	0.001
Di (Impeller Diameter)	m	0.045	0.045
n (Agitation speed)	rev/min	100–1,100	100–1,100
Np (Power number)	Dimensionless	5.5	5.5
Fg (Gas flow)	m^3/s	3.3×10^{-5}	8.3×10^{-6}
W (impeller width)	m	0.011	0.011

Table 2 Pellet radius reported and calculations derived from Tough and Prosser (1996) data, and calculations for the bioreactor used in this work

Agitation speed (rev/min)	Data derived from Tough and Prosser (1996)								Calculations in this work			
	Pellet radius (mm)*				Volumetric power input				Volumetric power input			
	Exp. Data		Modelled data		*P/V*[a] (W/l)	*Pg/P*[b] (−)	*Pg/V*[c] (W/l)	*Pg*/V*[d] (W/l)	*P/V*[a] (W/l)	*Pg/P*[b] (−)	*Pg/V*[c] (W/l)	*Pg*/V*[e] (W/l)
	Mean	SD	Mean	SD								
250	ND	ND	0.16	0.034	0.146	0.576	0.084	0.017	0.146	0.814	0.119	0.107
500	0.12	0.02	0.14	0.031	1.175	0.519	0.610	0.122	1.175	0.735	0.863	0.777
750	0.09	0.018	0.10	0.038	3.964	0.488	1.932	0.386	3.964	0.690	2.732	2.456
999	0.06	0.01	0.07	0.032	9.397	0.468	4.396	0.879	9.397	0.662	6.221	5.600

SD standard deviation

ND not determined

* Pellet radius reported by Tough and Prosser (1996) as a function of agitation speed

[a] Volumetric power input calculation using Eq. 1

[b] Ratio of gassed power input vs. power input calculation using Eq. 3

[c] Volumetric gassed power input. Calculated from P/V × Pg/P

[d] Corrected volumetric gassed power input. Pellet diameter (mm) reported by Tough and Prosser (1996) was graphed in Fig. 1a as a function of this value

[e] Corrected volumetric gassed power input. A trend of agitation speed was graphed as a function of this value in Fig. 1b for the bioreactor and aeration conditions used in this work

in Gamboa-Suasnavart et al. (2011), and are repeated here for comparative purposes. One-way ANOVA for independent samples and pair-wise comparisons using Tukey HSD (Test for Post-ANOVA) were carried out in order to assess the morphological statistical differences between cultures (Gamboa-Suasnavart et al. 2011).

Quantification, purification, and identification of rAPA

Total protein in the supernatant was determined using the Thermo Scientific Pierce BCA Protein Assay Kit (Thermo-Pierce, USA). Electrophoresis in 10 % SDS-PAGE, immunoblotting and purification of rAPA were carried out as previously described (Gamboa-Suasnavart et al. 2011). Briefly, culture filtrate was obtained by removing the mycelia at 8,000×*g* for 30 min at 4 °C, and filtration through 0.45 µm membrane filter (Millipore, USA). The supernatant was precipitated by ammonium sulfate (75 %), and proteins were recovered by centrifugation at 8,000×*g*, suspended in PBS, pH 7.4, and dialysed against distilled water overnight at 4 °C. Then, proteins were dialysed against acetate buffer pH 5 at 4 °C for 24 h, and centrifuged at 8,000×*g* for 10 min, the supernatant was dialysed against ConA-binding buffer for 24 h. The recombinant protein was purified by chromatography on a ConA-Sepharose column (Concanavalin A from *Canavalia ensiformis*, Sigma-Aldrich, USA), and eluted with 0.05 M of α methyl-mannopyranoside in ConA-binding buffer. A second purification protocol was also followed in those samples taken from the bioreactor: after the dialysis against

acetate buffer the proteins were centrifuged at 8,000×*g*, for 10 min. The supernatant was dialysed with 20 mM Tris–HCl, pH 8.3 4 °C. The rAPA protein was purified by anion-exchange chromatography (AKTA-Prime system, Pharmacia Biotech, USA) onto a HiTrap Q-Sepharose column equilibrated with 20 mM Tris–HCl, pH 8.3. Elution was then carried out with a linear 0–1 M NaCl gradient. Fractions were collected, analysed by SDS-PAGE, and quantified.

Protein digestion and MALDI-TOF analysis

Characterization of *O*-linked glycans at the C-terminal region of rAPA was made by MALDI-TOF analysis as previously reported (Gamboa-Suasnavart et al. 2011). rAPA was digested with LysC in order to obtain 8 peptides, and the C-terminal peptide (P8) was analysed. Mass was determined 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.

Reproducibility of growth cultures and MALDI-TOF analyses

Bioreactor and shake flasks cultures were made at least in triplicates. Figure 2 shows the mean value of the independent cultures, and the standard deviation among replicas. At least two MALDI-TOF analyses were done at the end of each independent culture.

Results

In a previous study three shake flask geometries were evaluated (conventional, baffled, and stainless steel coiled), in order to provide different shear and oxygenation conditions for *S. lividans* cultures expressing recombinant APA (Gamboa-Suasnavart et al. 2011). The largest and roundest pellets, with diameters up to 1.0 mm, were observed in conventional flasks; the smallest clumps, with diameters of 0.21 ± 0.09 mm, were obtained in both baffled and coiled shake flasks (Gamboa-Suasnavart et al. 2011). The production of rAPA is around 3 times higher in small mycelial aggregates than in larger pellets, and up to five mannose residues were found in the carboxy-terminal region of the protein, also named peptide P8 in coiled and baffled shake flasks, but in conventional shake flasks only two mannoses residues were found (Gamboa-Suasnavart et al. 2011). The aim of this study was to scale up this bioprocess to a 1.0 l bioreactor, taking into account rAPA productivity and quality (number of mannose residues in P8), and to achieve mycelial aggregate sizes similar to those obtained in baffled/coiled shake flasks. This was carried out by using the experimental data and the model proposed by Tough and Prosser (1996), which relates agitation rate to average diameter of *S. coelicolor* A3(2) (Table 2). These authors found *S. coelicolor* A3(2) aggregates with diameters of 0.16 ± 0.034, 0.14 ± 0.031, 0.10 ± 0.038, and 0.07 ± 0.032 mm for 250, 500, 750 and 999 rev/min, respectively in modeled data (as is shown in Table 2). Moreover, in experimental data, Tough and Prosser (1996) found aggregates with diameters of 0.12 ± 0.02, 0.09 ± 0.18, and 0.06 ± 0.01 mm for 500, 750 and 999 rev/min, respectively. Figure 1a shows the mean diameters and the agitation recalculated in terms of corrected volumetric gassed power input (Pg*/V) using Eqs. 1, 2, 3 and bioreactor geometry corrections (Table 2). The equation from this correlation resulted as an exponential decay (4):

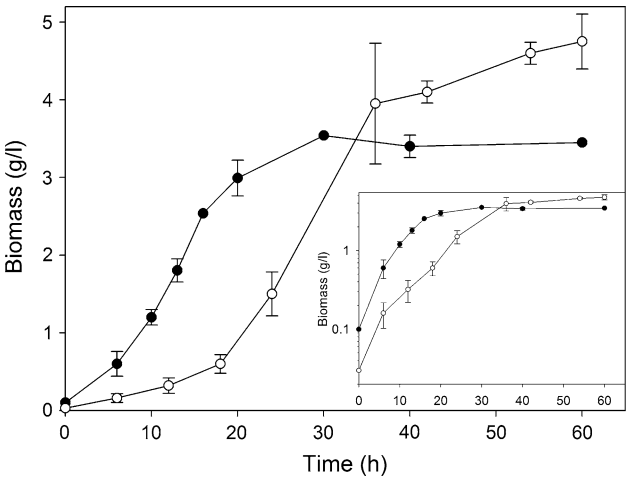

Fig. 2 Biomass growth of *S. lividans* producing rAPA from *M. tuberculosis*, in coiled shake flasks (*close symbols*) cultures carried out at 30 °C and 150 rev/min (previously reported by Gamboa-Suasnavart et al. 2011), and bioreactor (*open symbols*) cultures carried out at 300 rev/min, pH of 7.2 and DOT above 10 % of air saturation

$$Diameter = 0.112 + 0.193e^{-2.95(Pg*/V)} \quad (4)$$

An aggregate morphology of about 0.21 mm would be achieved by agitating *S. coelicolor* A3(2) with a Pg/V of 0.20 W/l (point "a" of Fig. 5a). To achieve the same Pg/V in this study (using an Applikon® 1.0 l bioreactor), 310 rev/min was needed (point "b" in Fig. 5b), in which gassed volumetric power input is correlated with agitation speed. To simplify the agitation control of all cultures, 300 rev/min was selected.

Figure 2 shows that the final biomass production in the bioreactor was higher (4.95 ± 0.35 g/l) than those obtained in baffled/coiled shake flasks (3.50 ± 0.20 g/l). However, the specific growth rate was not significantly different between bioreactors (0.09 ± 0.02 h^{-1}), and shake

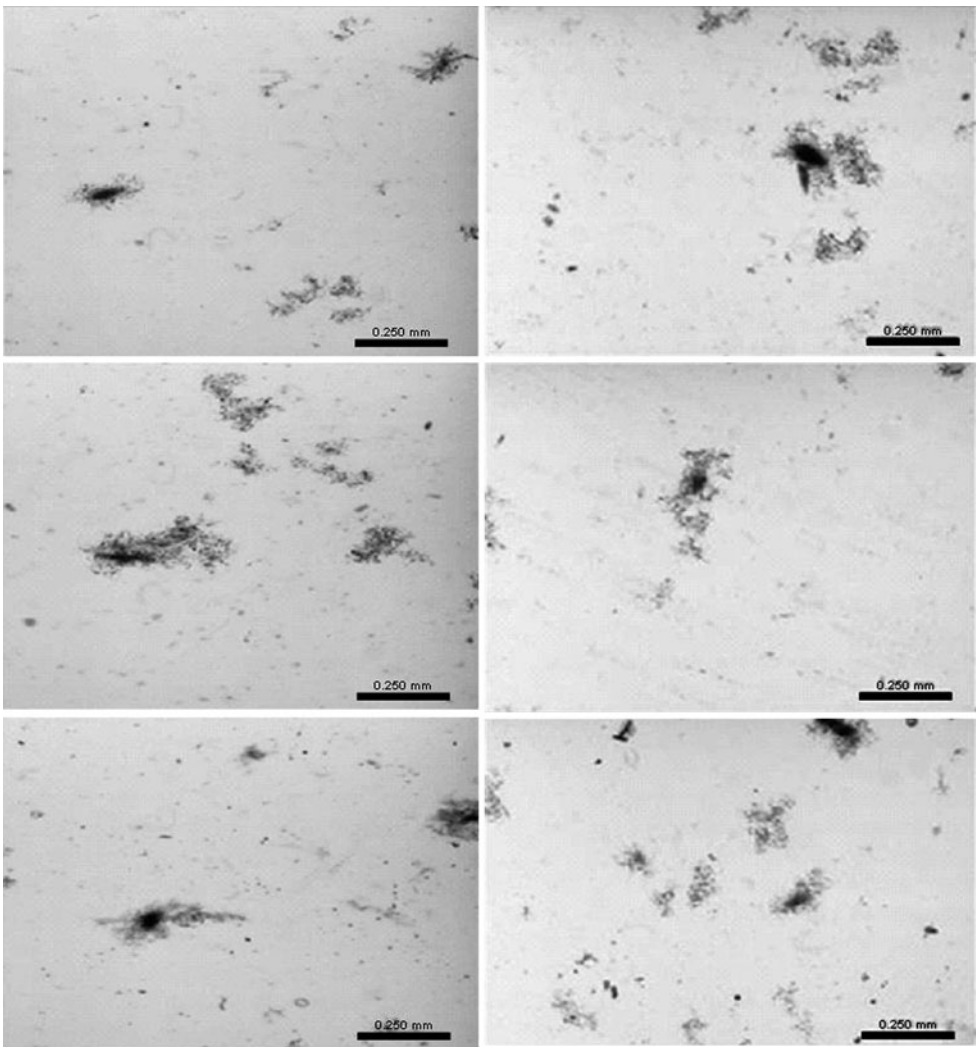

Fig. 3 Representative mycelia morphology of *S. lividans* cultured in: **a** Coiled and baffled shake flasks (previously reported by Gamboa-Suasnavart et al. 2011). **b** Bioreactor (*Bar* indicates 250 μm, 4× magnification)

Table 3 Morphological comparison between cultures carried out in coiled shake flasks and in bioreactor at 300 rev/min

	Area (mm^2)	Diameter (mm)	Perimeter (mm)
Coiled and baffled flask	0.029 ± 0.018	0.21 ± 0.09	0.89 ± 0.40
Bioreactor	0.019 ± 0.012	0.15 ± 0.01	0.58 ± 0.05

Data are presented as average ± standard deviation of image analysis of at least 100 clumps, mycelia or pellets for each sample

flasks (0.11 ± 0.01 h^{-1}). An increase in power dissipation rate promoting a higher biomass concentration was reported in *S. pristinaespiralis*, and *S. natalensis* in cultures carried out in shake flasks (Mehmood et al. 2011; el-Enshasy et al. 2000). It can be assumed that the dissipation energy in a 1.0-l bioreactor was slightly higher than in cultures carried out in baffled/coiled shake flasks. Furthermore, the doubtful "non-growth phase" in bioreactor cultures that lasted for almost 12 h are due to the size of the inoculum, since the bioreactor started with ten times less pre-germinated spores than coiled shake flasks (as seen in the inset of Fig. 2). Some reports demonstrate that high inoculum sizes promote dispersed forms in *Streptomyces*, while a small inoculum tends to pellet formation (Dobson et al. 2008; el-Enshasy et al. 2000), which is not the case in this work. No significant differences were found in morphology of aggregation between bioreactor cultures carried out at 300 rev/min (Pg*/V of 0.181 W/l) and coiled/baffled shake flasks (Fig. 3; Table 3). In comparison with the model used and predicting 0.21 mm, and considering an standard deviation of 0.06 mm of diameter of *S. coelicolor* A3(2) (equal to a standard deviation of 0.03 mm in aggregated radius, as presented in Table 2), the aggregation morphology of *S. lividans* in the bioreactor was not significant different with the diameter of aggregates of 0.15 ± 0.01 mm (Table 3). Moreover, these

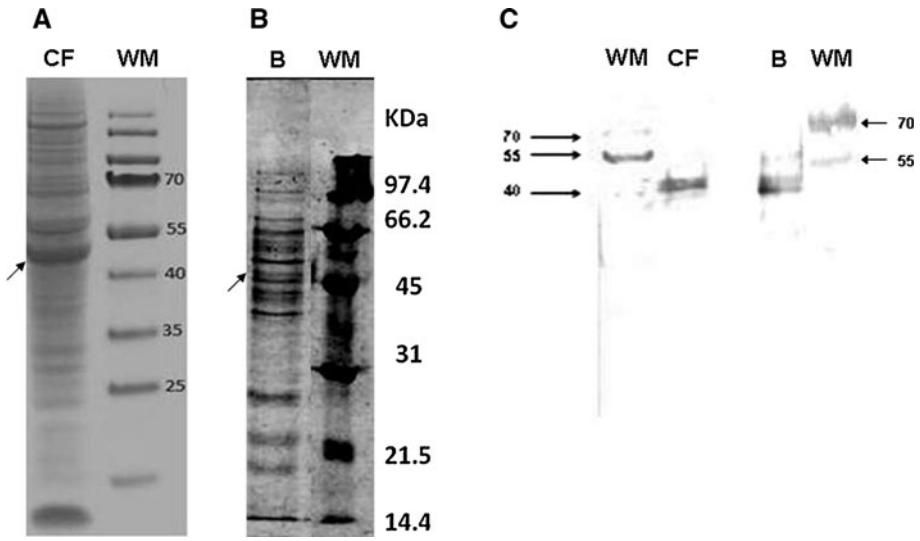

Fig. 4 **a** SDS-PAGE of total secreted proteins of coiled shake Flasks (CF) cultures carried out at 30 °C and 150 rev/min (previously reported by Gamboa-Suasnavart et al. 2011), **b** bioreactor (B) cultures carried out at 300 rev/min, pH of 7.2 and DOT above 10 % of air saturation. **c** Western blot of rAPA by *S. lividans* using the mAB 6A3, protein from coiled shake flask (CF) and bioreactor cultures (B). *WM* weight marker

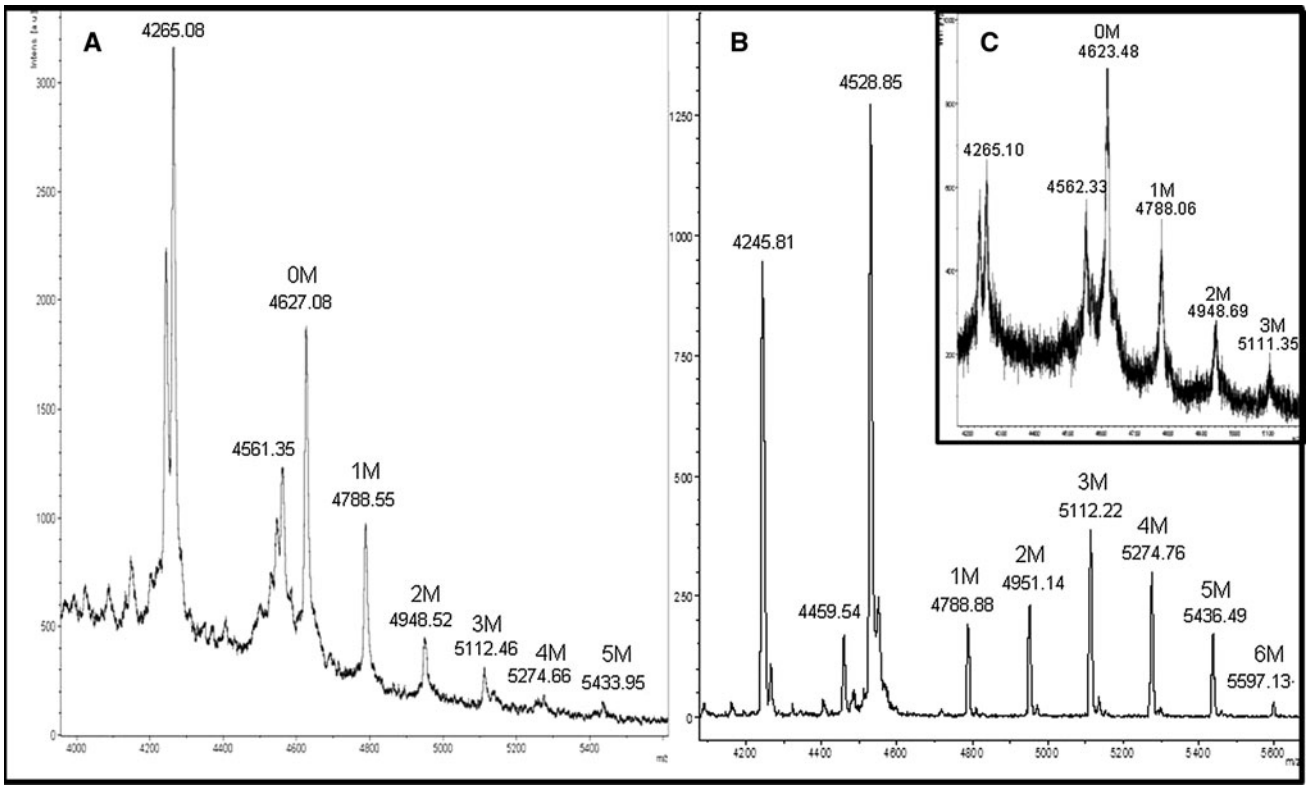

Fig. 5 MALDI-TOF analysis for the C-terminal peptide of the purified rAPA generated by LysC digestion, and obtained in coiled shake flasks (**a**) (previously reported by Gamboa-Suasnavart et al. 2011), and Bioreactor at 300 rev/min (**b**). Numbers above each units of atomic mass mean the number of mannoses linked to the peptide. In bioreactor cultures analysed by MALDI-TOF up to six mannoses were found, but the non-glycosylated peak was found. In the *inset* (**c**) the non-*O*-glycoform with a molecular mass of 4,623.5 Da is present in the purified protein by affinity cromatography using ConA

bioreactor morphology values fall within the size dispersion obtained in baffled/coiled shake flasks (0.21 ± 0.09 mm).

A densitometry comparison of volumetric total protein SDS-PAGE between coiled flasks and bioreactor supernatants shows a similar production of rAPA (Fig. 4a,b), and it was confirmed by mAB 6A3 in Western blots (Fig. 4c). Previously, Gamboa-Suasnavart et al. (2011) reported that a similar rAPA productivity was found in baffled and coiled shake flask. On the other hand, *O*-linked mannoses on P8 of rAPA produced in bioreactor cultures were characterized (Fig. 5) and compared with those reported in coiled/baffled shake flasks (Gamboa-Suasnavart et al. 2011). In native

Mycobacterium tuberculosis APA, C-terminal peptide presents heterogeneity from none to three mannose units attached to the Thr277 (Dobos et al. 1995, 1996). In the C-terminal peptide of the rAPA in *S. lividans*, none to four mannoses were found in 1.0 l conventional shake flasks cultures (Lara et al. 2004), and up to five mannoses in 250 ml baffled/coiled shake flasks (Gamboa-Suasnavart et al. 2011). For comparison purposes, Fig. 5a shows the MALDI-TOF of rAPA from coiled flasks previously reported by our group (Gamboa-Suasnavart et al. 2011). The rAPA from bioreactor cultures purified by anion-exchange chromatography was analysed by MALDI-TOF. Up to six mannose units were found (Fig. 5b) with a typical difference between each observed peak of 163 ± 2 Da, corresponding to a single mannose residue. However, the non-*O*-glycosylated C-terminal peptide was not found, corresponding with a theoretical molecular mass of 4,624.3 Da, and in shake flasks appears at 4,627.08 Da (Fig. 5a). In order to determine the presence of the non-*O*-glycosylated form, the 45/47 kDa band was excised from a culture extract SDS-PAGE gel and the Lys-C digestion was carried out. A typical MALDI-TOF analysis of non-purified APA is shown in the inset of Fig. 5b, showing the non-*O*-glycoform with a molecular mass of 4,623.5 Da, and also the forms with up to three mannose residues attached can be seen, but the noise is too high to observe the six glycoforms observed in purified rAPA by anion exchange. Results of this work combined with those previously reported by our group show that morphology might play an important role in the determination of *O*-glycosylation in recombinant proteins of *S. lividans*. Finally, this successful scale-up strategy can be useful to separately understand how hydrodynamic forces and oxygen/nutrient transfer inside *S. lividans* aggregates modify the ability to *O*-glycosylate recombinant proteins. The aim is to be able to design further production processes in order to control the amount of glycans added to bacterial recombinant glycoproteins, manipulating their antigenic properties.

Discussion

In this study, a mathematical model and experimental data of *S. coelicolor* cultures were used to predict the size of the aggregates in bioreactor cultures in a recombinant strain of *S. lividans*, based on the gassed volumetric power input. These data were used to obtain a similar morphology in the bioreactor as that reported previously in baffled/coiled shake flasks (Gamboa-Suasnavart et al. 2011), with the aim of maintaining biomass growth, recombinant protein production, and *O*-mannosylation degree. In this order, no significant differences were obtained in specific growth rate, morphology, and production between coiled/baffled shake flasks and the scale-up bioreactor. The observed narrower range of morphologies in bioreactor cultures, and also a smaller average diameter than in flasks (Table 2), even with no significant differences, can be an indication that the maximum local energy dissipation rate throughout the bulk in bioreactor is higher and more homogeneous than the distribution in baffled/coiled shake flasks. This is in agreement with Peter et al. (2006), who reported that at the same power consumption, the maximum energy dissipation rate in stirred tank bioreactors is ten times higher than those in shake flasks. Moreover, up to six mannoses attached to the C-terminal peptide were obtained in bioreactor cultures, an additional one to the number obtained in shake flasks. However, final biomass concentration was not similar, indicating that others factors like oxygen transfer rate, tip speed or energy dissipation/circulation function can be an influence on bacterial metabolism. Finally, it would be interesting to separately determine and quantify the effect of shear stress (at different agitation rates), dissipation/circulation function and DOT, in order to find the main operational (environmental) conditions that are affecting *O*-glycosylation and productivity in filamentous bacterial cultures, and to be able to propose an experimental methodology to explain the biological mechanisms that are involved in this phenomena.

Acknowledgments This work was financed by CONACYT-INNOVAPYME 181895, CONACYT 178528, 104951-Z and PAPPIT-UNAM IN-209113, IN-210013. RGS thanks CONACyT scholarship (316929). Authors thank Celia Flores, M. Sc., and Enrique Galindo, PhD. (Instituto de Biotecnología, UNAM), and Marisol Córdova, PhD. (Centro de Ciencias Aplicadas y Desarrollo Tecnológico, UNAM) for their technical assistance in image analysis. Authors thank Erendida Garcia, Chem. (Instituto de Química, UNAM) for MALDI-TOF analysis. We also thank Ana Delgado for reviewing the English version of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

References

Anné J, Maldonado B, Van Impe J, Van Mellaert L, Bernaerts K (2012) Recombinant protein production and streptomycetes. J Biotechnol 158(4):159–167

Buchs J, Maier U, Milbradt C, Zoels B (2000a) Power consumption in shaking flasks on rotary shaking machines: I. Power consumption measurement in unbaffled flasks at low liquid viscosity. Biotechnol Bioeng 68:589–593

Buchs J, Maier U, Milbradt C, Zoels B (2000b) Power consumption in shaking flasks on rotary shaking machines: II. Nondimensional description of specific power consumption and flow regimes in unbaffled flasks at elevated liquid viscosity. Biotechnol Bioeng 68:594–601

Chapple D, Kresta M, Wall A, Afacan A (2002) The effect of impeller and tank geometry on power number for a pitched blade turbine. TransIChemE 80(4):364–372

Dickey DS, Bittorf KJ, Ramsey CJ, Johnson KE (2004) Understand flow patterns in glass-lined reactors. Chem Eng Prog 100:21–25

Dobos K, Swiderek K, Khoo K, Brennan PJ, Belisle JT (1995) Evidence for glycosylation sites on the 45-Kilodalton glycoprotein of *Mycobacteria tuberculosis*. Infect Immun 63:2846–2853

Dobos K, Khoo K, Swiderek K, Brennan P, Belisle J (1996) Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. J Bacteriol 178: 2498–2506

Dobson LF, O'Cleirigh CC, O'Shea DG (2008) The influence of morphology on geldanamycin production in submerged fermentations of *Streptomyces hygroscopicus* var. *geldanus*. Appl Microbiol Biotechnol 79:859–866

el-Enshasy HA, Farid MA, el-Sayed S (2000) Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. J Basic Microbiol 40:333–342

Gamboa-Suasnavart RA, Valdez-Cruz NA, Cordova-Davalos LE, Martinez-Sotelo JA, Servín-González L, Espitia C, Trujillo-Roldán MA (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. Microb Cell Fact 10:110

Giudici R, Pamboukian CR, Facciotti MC (2004) Morphologically structured model for antitumoral retamycin production during batch and fed-batch cultivations of *Streptomyces olindensis*. Biotechnol Bioeng 86:414–424

Hoopen HJG, Gulik WM, Schlatmann JE, Moreno PRH, Vinke JL, Heijnen JJ, Verpoorte R (1994) Ajmalicine production by cell cultures of *Catharanthus roseus*: from shake flask to bioreactor. Plant Cell Tissue Organ Culture 38:85–91

Horn C, Namane A, Pescher P, Riviere M, Romain F, Puzo G, Barzu O, Marchal G (1999) Decreased capacity of recombinant 45/47-kDa molecules (Apa) of *Mycobacterium tuberculosis* to stimulate T lymphocyte responses related to changes in their mannosylation pattern. J Biol Chem 274:32023–32030

Hugmark G (1980) Power requirements and interfacial area in gas-liquid turbine agitated systems. Ind Eng Chem Process Des Dev 19:638–641

Junker BH, Hesse M, Burgess B, Masurekar P, Connors N, Seeley A (2004) Early phase process scale-up challenges for fungal and filamentous bacterial cultures. Appl Biochem Biotechnol 119: 241–277

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich

Klockner W, Buchs J (2012) Advances in shaking technologies. Trends Biotechnol 30:307–314

Lara M, Servin-Gonzalez L, Singh M, Moreno C, Cohen I, Nimtz M, Espitia C (2004) Expression, secretion, and glycosylation of the 45-and 47-kDa glycoprotein of *Mycobacterium tuberculosis* in *Streptomyces lividans*. Appl Environ Microbiol 70:679–685

Mehmood N, Olmos E, Goergen JL, Blanchard F, Ullisch D, Klockner W, Buchs J, Delaunay S (2011) Oxygen supply controls the onset of pristinamycins production by *Streptomyces pristinaespiralis* in shaking flasks. Biotechnol Bioeng 108:2151–2161

Peter CP, Suzuki Y, Buchs J (2006) Hydromechanical stress in shake flasks: correlation for the maximum local energy dissipation rate. Biotechnol Bioeng 93:1164–1176

Roubos JA, Krabben P, Luiten RG, Verbruggen HB, Heijnen JJ (2001) A quantitative approach to characterizing cell lysis caused by mechanical agitation of *Streptomyces clavuligerus*. Biotechnol Prog 17:336–347

Rushton JH, Costich EW, Everett HJ (1950a) Power characteristics of mixing impellers. Part I. Chem Eng Progr 46:395–404

Rushton JH, Costich EW, Everett HJ (1950b) Power characteristics of mixing impellers. Part II. Chem Eng Progr 46:467–479

Sable SB, Cheruvu M, Nandakumar S, Sharma S, Bandyopadhyay K, Kellar KL, Posey JE, Plikaytis BB, Amara RR, Shinnick TM (2011) Cellular immune responses to nine *Mycobacterium tuberculosis* vaccine candidates following intranasal vaccination. PLoS ONE 6:e22718

Seletzky JM, Noak U, Fricke J, Welk E, Eberhard W, Knocke C, Buchs J (2007) Scale-up from shake flasks to fermenters in batch and continuous mode with *Corynebacterium glutamicum* on lactic acid based on oxygen transfer and pH. Biotechnol Bioeng 98:800–811

Silberbach M, Maier B, Zimmermann M, Buchs J (2003) Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile. Appl Microbiol Biotechnol 62:92–98

Tough AJ, Prosser JI (1996) Experimental verification of a mathematical model for pelleted growth of *Streptomyces coelicolor* A3(2) in submerged batch culture. Microbiol 142(Pt 3):639–648

Tough AJ, Pulham J, Prosser JI (1995) A mathematical model for the growth of mycelial pellet populations. Biotechnol Bioeng 46:561–572

Vallin C, Ramos A, Pimienta E, Rodriguez C, Hernandez T, Hernandez I, Del SR, Rosabal G, Van ML, Anne J (2006) *Streptomyces* as host for recombinant production of *Mycobacterium tuberculosis* proteins. Tuberculosis (Edinb.) 86:198–202

Yun SI, Yahya AR, Malten M, Cossar D, Anderson WA, Scharer JM, Moo-Young M (2001) Peptidases affecting recombinant protein production by *Streptomyces lividans*. Can J Microbiol 47:1137–1140