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Chapter 6

Morphology-driven downscaling of *Streptomyces lividans* to micro cultivation

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ABSTRACT

Actinomycetes are prolific sources of secondary metabolites and industrially relevant enzymes. Growth of these mycelial microorganisms in small culture volumes is very challenging due to their complex morphology. Since morphology and production are typically linked, scaling down culture volumes requires better control over morphogenesis. In larger scale platforms, ranging from shake flasks to bioreactors, the hydrodynamics play an important role in shaping the morphology and determining product formation.

Here, we report on the effects of agitation on the mycelial morphology of *S. lividans* grown in microtiter plates (MTP). Our work shows that at the proper agitation rate cultures can be scaled down to volumes as small as 100 µl while maintaining the same morphology as seen in larger scale platforms. Using image analysis we compared the morphology of the cultures, which when the MTP was agitated at 1400 rpm approached those observed in shake flasks, while product formation was maintained.

Our study shows that the morphology of actinomycetes in microcultures can be controlled in similar manner as in larger scale cultures by carefully controlling the mixing rate. This could facilitate high-throughput screening and successful upscaling.

INTRODUCTION

The need to screen strains under many conditions increases the demand for optimized high throughput screening (HTS) methods. Downscaling of culture volumes, while maintain key factors like reproducibility and productivity comparable to shake flasks or small scale bioreactors, is necessary to make large screening efforts rapid and economically feasible (Long *et al.*, 2014). Development of an HTS platform for *Streptomyces*, which are multicellular filamentous organisms, is challenging and only a few tries in the literature can be found (Minas *et al.*, 2000, Siebenberg *et al.*, 2010, Sohoni *et al.*, 2012). In submerged cultures the filamentous mycelia manifest a complex morphology that has a profound impact on the regulation of product formation (van Dissel *et al.*, 2014). Successful downscaling of the culture volume therefore includes matching the morphology found in larger scale platforms (i.e. shake flasks or bioreactors).

Streptomycetes typically display a wide range of morphologies in submerged cultures (reviewed in (van Dissel *et al.*, 2014). Perhaps the most difficult morphology to scale down is that of aggregating, pellet forming Streptomycetes. This is exemplified by *Streptomyces coelicolor,* a model for *Streptomyces* growth and antibiotic production (Barka *et al.*, 2016), and by the related *Streptomyces lividans,* the ideal heterologous enzyme production host (Anné *et al.*, 2012). These streptomycetes are characterized by the formation of large multicellular aggregates or pellets. In a liquid culture, depending on the medium, the pellets exist as two distinct populations which differ in size (van Veluw *et al.*, 2012). The core of the pellets undergoes a developmental cycle, which is linked to the regulation of antibiotic production (Manteca *et al.*, 2008, Martin & Bushell, 1996). A mycelial pellet may be considered a self-immobilized biofilm (Petrus & Claessen, 2014), a term borrowed from granular sludge research, which deals with similar morphology dynamics (Liu & Tay, 2002).

The exact morphology, i.e. size, density and shape, is greatly dependent on the characteristics of the environment (Wucherpfennig *et al.*, 2010). The hydrodynamics, the characteristics of the agitated medium, seems to be of particular great importance as it influences many parameters associated with morphogenesis (Olmos *et al.*, 2013). Low agitation causes poor distribution of nutrients and reduced oxygen transfer rates, stunting growth and production, while strong agitation can cause cell death (Roubos *et al.*, 2001). The relationship between pellet morphology, hydrodynamics (and oxygen supply) and production has been well studied for bioreactors (Tamura *et al.*, 1997, Roubos *et al.*, 2001, Ohta *et al.*, 1995) and for shake flasks (Mehmood *et al.*, 2012, Dobson *et al.*, 2008), but has not been tried in an HTS setting which necessitates small culture volumes without affecting the growth properties. In this work we sought to match the morphology seen in larger scale platforms by controlling the hydrodynamics of the culture, down to 100 µl culture scale. The dimensions of microtiter plates (MTP), with small well diameters, requires extensive mixing forces to match the hydrodynamics. We applied an MTP vortex to reach high mixing rates and precise speed control, which allowed a successful scaling down, with native morphology and production found in larger scale platforms.

MATERIALS AND METHODS

Bacterial strains, plasmids

S. lividans 66 was used for morphological analysis and enzyme production and *S. coelicolor* M145 was used for antibiotic production. The plasmid pIJ703 (Katz *et al.*, 1983), which carries the *melC1* and *melC2* genes for heterologous tyrosinase production, was transformed to its host by protoplast transformation (Kieser *et al.*, 2000). Spores were harvested from soy flour mannitol agar plates and stored in 20% glycerol at -20^{0}C as described (Kieser *et al.*, 2000). The spore titer was determined by plating serial dilutions and counting CFUs.

Cultivation conditions

For cultivation in shake flasks, *S. lividans* was grown in 30 mL tryptic soy broth (Difco) with 10% sucrose (TSBS) in a 100 mL Erlenmeyer flasks equipped with a stainless steel spring. The flask was inoculated with 10^6 CFUs/ ml and cultivated at 30^{0}C in an orbital shaker with 1 inch orbit (New Brunswick) at 170 RPM. For the production of tyrosinase 25 µM $CuCl_2$ was added to the TSBS medium.

For antibiotic production *S. coelicolor* was cultivated in Yeast Extract - Malt Extract (YEME; (Kieser *et al.*, 2000)) but without sucrose (YEME0) .

100 µl media with 10^6 cfu/ml spores was added to wells of a V-bottom 96 well MTP (Greiner Bio-One, Germany). To minimize evaporation, the plate was covered with a custom molded silicone sheet made from MoldMax40 (Materion, USA), using the 96 well plate as a mold. An AeraSeal film (Excel Scientific, USA) was added to the top for sterility, while allowing gas exchange. The combined silicone sheet and AeraSeal film were fastened the MTP using masking tape. A Microplate Genie Digital (Scientific Industries, USA) was used for agitation. This microtiter plate vortex has an orbit of 1 mm with accurate speed control. The rotation speed was also checked using a Voltcraft DT-10L digital tachometer (Conrad, Germany). The entire setup was placed in a humidity-controlled incubator set to 70% RH and 30^{0}C. The evaporation rate was around 8 µL per well per day.

Image analysis

Image analysis was performed as described by whole slide imaging combined with automated image analysis using imageJ (Zacchetti *et al.*, 2016). In short, 100 µl sample was transferred to a glass microscope slide and covered by a 24x60 cover slip. The slide was mounted in an Axio Observer (Zeiss, Germany) equipped with an automated XY-stage, which allowed whole slide imaging using a 10x objective. The imageJ plugin for automated image analysis optimized for *Streptomyces* liquid morphology was used to obtain both the maximum Feret length in µm and a shape description with a value between 0 and 1 measured as the circularity, where 1 describes a perfect circle (Stojmenovic *et al.*, 2013) of each mycelial fragment or pellet found in the sample. Incorrectly analyzed pellets (e.g. out-of-focus mycelia) were removed manually. Further data processing and visualizing was done

in Microsoft Excel. The spread of a distribution was calculated as the standard deviation of the population, which indicates how spread out the measured particles are within a measurement. The deviation of the mean of each term was calculated from the differences between analyses of multiple samples.

Tyrosinase acitivity measurement

Tyrosinase acitivity was measured by the conversion over time l-3,4-dihydroxyphenylalanine spectrophotometrically at a wavelength of 475 nm, as described (Kieser et al., 2000).

Actinorhodin quantification

The production of actinorhodin by *S. coelicolor* was determined as follows. Culture supernatant (40 µl) was treated with 0.5 µl 5 M HCl to pH 2 to 3, extracted with a 0.5 volume of methanol-chloroform (1:1), and centrifuged at 5,000 rpm for 10 min. The concentration was calculated from the A_{542} (ε_{542}, 18,600).

RESULTS

The morphology of S. lividans in shake flasks

Submerged cultivations of streptomycetes are typically done in shake flasks, such as in physiological and metabolic studies. To reproducibly cultivate the filamentous mycelia, the shake flasks are equipped with a spring coil, which increases aeration and creates the shear forces in the vessel so as to obtain more favorable growth and promote fragmentation of the mycelial clumps. Fragmentation is the process where pellets or parts thereof are split off, which is needed for pellet multiplication in the culture. Baffled shake flasks or the addition of glass beads are also used for the same purpose.

We wondered if it would be possible to mimic these growth conditions and obtain similar growth characteristics in much smaller cultures volumes. As a reference, the morphological characteristics of shake flask cultures were investigated by image analysis, which allows a large number of pellet particles to be analyzed on multiple morphological relevant parameters. Around 500 aggregates were analyzed from a 24 h shake flask-grown culture, corresponding to the end of the exponential growth phase, which roughly corresponds temporally to the onset of antibiotic production (Nieselt *et al.*, 2010). Previous work comparing the maximum length of pellets revealed two different populations of *S. lividans* (van Veluw *et al.*, 2012). This separation is even more clear when also the circularity is taken into account, showing two distinct clusters of particles, that not only differ in size, but also in shape (Figure 1D, scatter plot). Pellets of population “a” (Figure 1D, falling within the yellow dotter oval) have similar lengths around 200 µm, but with a wide spread in circularity (Also see figure 1A as example). Most pellets are found in cluster “b” (Figure 1D, purple striped

oval), of which about half of the total pellet population were situated in the centroid around "C". The "average" pellet had a homogenous density, the majority showing a slightly oval shape (Figure 1, C). Increasing the pellet size was often accompanied by a loss of structural integrity (Figure 1B). Because of the effects of pellets on production and regulation it is important to capture all of these morphological characteristics in our effort to scale down.

Dependency of agitation rate on the morphology in micro-cultures.

The insight that pellet formation is mostly the result of the hydrodynamic forces and/or the supply of sufficient oxygen, often described by the power dissipation (P) and the k_La, prompted new experiments to match the environmental properties with those found in shake flasks. Both of these properties can be changed with (and are linked to) the agitation rate. A digital vortex, designed for microtiter plate (MTP) mixing, enabled setting the mixing

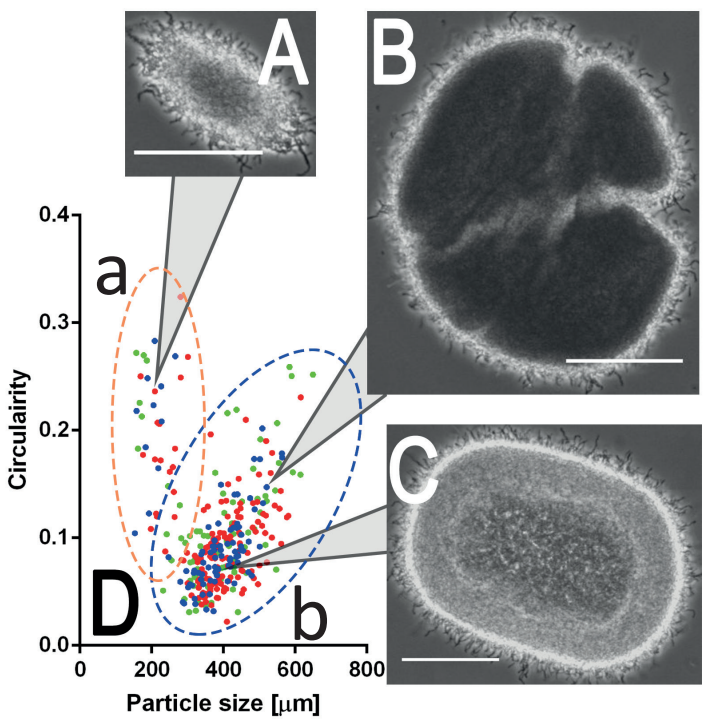

Figure 1. Morphological characterization of S. lividans in shake flask cultures. Spores of *S. lividans* 66 were inoculated at 10^6 CFU/ml into a shake flask (equipped with a coiled spring) containing 30 mL TSBS. The culture was grown for 24 h in an orbital shaker set to 30°C. Three 100 µL samples from two independent cultures were taken at 24 h and subjected to image analysis to obtain the maximum length and circularity of each distinguishable mycelial aggregate. Light micrographs A, B and C represent the three archetypes of the pellet morphologies seen in the culture, and corresponds to the indicated locations in the particle size (x-axes) and circularity (y-axes) scatterplot (D), for which the data was obtained by image analysis, the colors corresponds to data obtained from independent cultures. Bar, 100 µm.

rate, studying the effects on morphology and thus establishing whether a population could be obtained with morphological characteristics similar to those found in larger scale cultures.

In 100 µl MTP cultures and at low agitation rates, the mycelia failed to aggregate into the dense pellets normally observed in shake flasks, showing a more irregular shaped open morphology (Figure 2A, G). Preliminary experiments suggest that this may at least in part be caused by insufficient oxygen supply (see Discussion). Apart from the lower density, the average length of the mycelia is around 480 µm with a wide spread, indicating how narrow the measured particles are distributed, ranging from 80 µm to large aggregates of up to 1500 µm, likely resulting from fusion of multiple aggregates (Table 1).

At 1000 rpm the mycelia formed denser pellets, typical of *S. lividans* grown in shake flasks or in the fermenter (Figure 2H). However, the average pellet size of around 600 µm and the significantly smaller number of particles per culture volume suggest that the hydrodynamic forces in de micro-cultures were weaker than in shake flask-grown cultures (Figure 2B). At 1200 rpm the average pellet size approached that found in shake flasks, but the pellets had a more elongated, oval-shaped morphology, with an average roundness close to 0.2 (Figure 2C, I). At this agitation rate a few pellets of the vertical cluster appeared, indicating that the shear stress was sufficiently high to induce fragmentation (Figure 2C, inset). Increasing the mixing rate further to 1400 rpm lowered the circularity to the desired value of 0.1, with the average pellet length now closely resembling that found in shake-flask cultures (Figure 2D, J). Also the distribution of the pellet population closely resembled those formed in shake flasks, including the occurrence of the population of smaller oval-shaped pellets (Figure 2D, inset). When the agitation was further increased to 1600 rpm the cultures showed again an increase in length and a decrease in circularity (Figure 2I and J). Also the Feret diameter of the second population pellets decreases drastically, indicating that the mycelia might be exposed to a shear stress high enough to induce substantial cell damage (Figure 2I, picture). Increasing the agitation to 1800 rpm resulted in a similar trend as seen in 1600 rpm where

Table 1. Average length and circularity of the population of mycelial aggregates under different growth conditions for S. lividans.

Culture	Length		Circulairity	
	Average	Spread	Average	Spread
Shake flask*	372 ± 19	104 ± 24	0.11 ± 0.02	0.07 ± 0.03
800rpm	463 ± 58	310 ± 2	0.22 ± 0.00	0.07 ± 0.01
1000rpm	623 ± 88	189 ± 4	0.16 ± 0.00	0.06 ± 0.01
1200rpm	415 ± 36	100 ± 20	0.18 ± 0.03	0.05 ± 0.00
1400rpm	378 ± 51	104 ± 22	0.10 ± 0.00	0.06 ± 0.01
1600rpm	412 ± 62	170 ± 12	0.12 ± 0.02	0.08 ± 0.02
1800rpm	423 ± 3	135 ± 40	0.10 ± 0.02	0.07 ± 0.00

*shake flask was mixed at 170 rpm in a 1inch orbital shaker

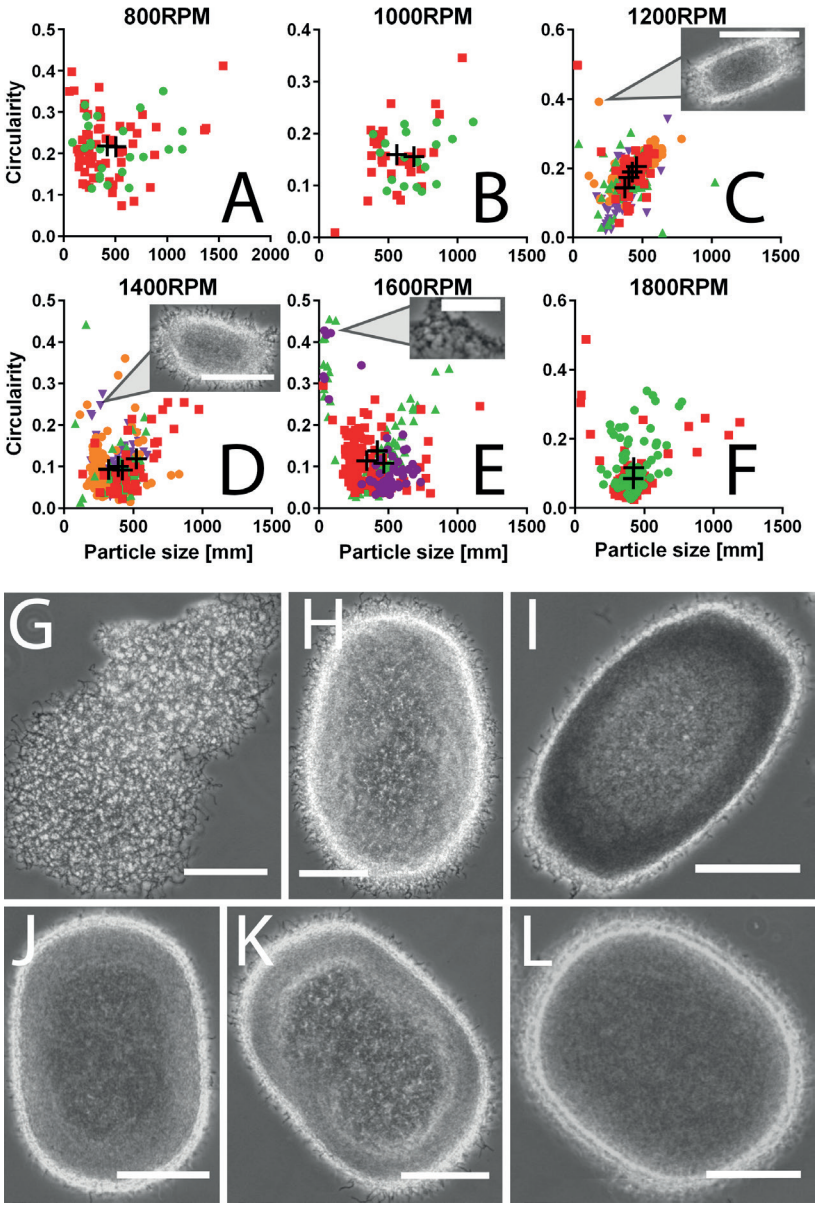

Figure 2. Growth of S. lividans in 100 µL MTP cultivation at different agitation rates. Spores of *S. lividans* 66 were inoculated at 10^6 CFU/ml into 100 µL in a 96 well MTP with V-shaped bottom. The MTPs were agitated using a digital MTP vortex, which was set in a humidified incubator with temperature set to 30°C. The agitation rate was changed between experiments ranging from 800 rpm (A,G), 1000 rpm (B,H), 1200 rpm (C,I), 1400 rpm (D,J), 1600 rpm (E,K) and 1800 rpm (F,L) and the effects on morphology of each aggregate after 24 h of cultivation was analyzed in respect to its circularity (y-axes) and maximum length in µm (x-axes) and are displayed in a scatter plot (A,B,C,D,E,F). Agitation rates were analyzed at least twice (replicates presented by different colors) and the centroids calculated (black crosses). C,D,E: image showing an aggregate of the smaller population. G,H,I,J,K,L: examples of a typical pellet found near the centroid. Scale bars: 100 µm or 50 µm (E).

larger pellets again appeared as part of the population. This may be explained by the culture fluid showing "out of phase" characteristics at high rotations speeds (Büchs *et al.*, 2000, Büchs *et al.*, 2001), which would result in a lower power consumption, and thus lower fragmentation rates and increased average pellet size.

Production of heterologous enzymes and antibiotics

Mycelia of 24 h old micro-cultures grown at an agitation speed of 1400 rpm generally had a morphology that was very similar to that observed for mycelia grown in shake-flasks. To analyze how similar the cultures were in terms of their producing capacity, we analyzed the production of tyrosinase, which is a good model system for extracellular enzyme production, and was heterologously expressed in *S. lividans* by the introduction of plasmid pIJ703 (van Wezel *et al.*, 2006). A similar amount of active enzyme was produced in shake flasks and in MTPs, although production started slightly earlier in MTPs (Figure 3). As the morphology appeared comparable, this is most likely the result of evaporation, which increases apparent biomass and substrate concentrations.

To study the effect on antibiotic production, we used *Streptomyces coelicolor* M145 as the model organism, as this strain produces pigmented antibiotics which are readily assessed spectrophotometrically. For this study, we compared the production of the blue-pigmented actinorhodin between shake flasks and micro-cultures (Figure 4). After 48h of growth both cultures had produced comparable amounts of actinorhodin, suggesting that also antibiotic production is very similar in the two cultivation systems.

DISCUSSION

High-throughput screening of actinomycetes for natural products or enzymes typically takes place in micro-scale liquid-grown cultures in an MTP-based setup. The alternative is solid-grown cultures, but it is very difficult to translate growth conditions from solid- to liquid-grown cultures. A drawback of screening of actinomycetes in submerged cultures is the formation of large mycelial networks, which show flocculation or attachment to abiotic surfaces and are associated with slow growth (van Dissel *et al.*,

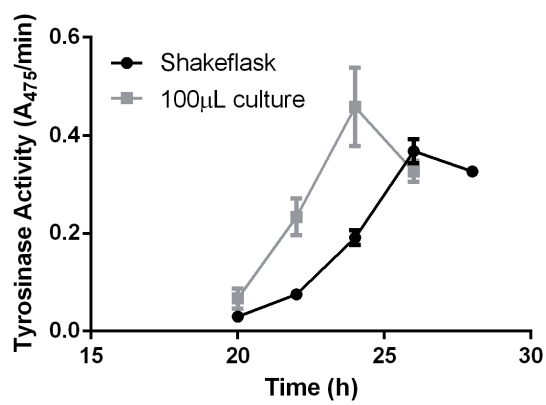

Figure 3. Tyrosinase production by S. lividans in shakeflasks and 100 μL cultures. Transformants of *S. lividans* 66 heterologously expressing the secreted enzyme tyrosinase from plasmid pIJ703 were grown in TSBS in either shake flasks or V-bottom MTPs. The graph represents the conversion rate of l-3,4-dihydroxyphenylalanine by the culture supernatant, which is indicative of tyrosinase activity. The shake flasks were run in duplicate, while the tyrosinase was measured in three different wells in the MTP.

2014). Additionally, cultures tend to be highly heterogeneous due to the large surface area of the mycelial clumps. Recently, we showed that aggregation of germlings increases culture heterogeneity (Zacchetti *et al.*, 2016). Because heterogeneity creates a distribution of morphologies, all contributing to production differently (van Veluw *et al.*, 2012, Martin & Bushell, 1996), a large population is often required to maintain reproducibility. As a lot of heterogeneity is the result of the environment, careful control is needed to mimic the morphology of a shake flask in small-scale cultivation platform.

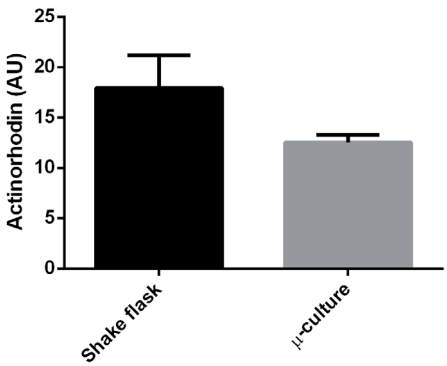

Figure 4. Actinorhodin production by S. coelicolor after 48 h of growth. S. coelicolor M145 was cultivated in minimal media for 48 h. The shake flasks were run in duplicate, while antibiotic production was measured in three different wells in the MTP. Actinorhodin was extracted by chloroform/methanol and measured spectrophotometrically at 542 nm. The average amount of actinorhodin concentration (in arbitrary units) and the standard deviation of three independent cultures are shown.

This study shows that the distribution of a heterogeneous mycelial population is highly dependent on the agitation rate in 96-well MTPs. Especially at insufficient mixing rates the mycelia failed to aggregate into a typical pellet structure. It is likely that this is at least in part the result of insufficient oxygen supply, which is important for antibiotic production (Chen & Wilde, 1991, Yegneswaran *et al.*, 1991). A k_La of at least 100 h^{-1} is needed for the production of pristinamycins by *Streptomyces pristinaespiralis* (Mehmood *et al.*, 2010). The relationship between oxygen supply and morphology is less well understood, but preliminary experiments where the oxygen supply was limited in a shake flask by reducing the gas exchange, resulted in pellets with a reduced density similar to what was found in poorly agitated MTPs (DvD and GPvW, unpublished results). Although the k_La was not measured in this study, initial calculations using equations for orbital mixing (Seletzky *et al.*, 2007) showed that the k_La could be as low as 40 h^{-1} when mixing at 800 rpm. This low value is suggestive of oxygen limitation as the cause of the morphology observed at low agitation rates and that in part the change in morphology by increased agitation is the result of an increased oxygen supply. While these observations are indicative of oxygen limitation as determining factor for mycelial morphology, oxygen transfer and hydrodynamic stress are coupled processes for orbital shaken cultivation methods (and to some extent also for bioreactors). At least for pristinamycin production hydrodynamic stress, described as the power input, was more descriptive for both pellet morphology and production levels (Mehmood *et al.*, 2012). How precisely agitation affects morphogenesis in MTP plates is as yet unclear and requires further study.

Detailed comparison of mycelial morphologies by image analysis allowed selection of

the appropriate culturing conditions to obtain a preferred average pellet size and structure. Comparison of maximal pellet length and circularity provided additional insights into the exact morphology of the pellets, which aided the down-scaling process. Besides providing the option of medium- to high throughput screening, the ability to grow *Streptomyces* with a native morphology on a small scale also allows studies that involve for example the addition of expensive or low abundance chemicals or enzymes.

CONCLUSION

The complex morphology displayed by filamentous actinomycetes in liquid-grown cultures greatly influences their productivity. Screening these bacteria for new therapeutic agents in an MTP-based setup without affecting normal growth and morphology would be a major advantage. This is in particular important in the light of the future upscaling, so as to maximize the chance that productivity is maintained. We have been able to translate growth and morphology from shake flasks to 100 µL microcultures by carefully tuning the rate of agitation. The resulting growth and average pellet size in standard HTS-compatible MTPs was reproducibly comparable to those in larger scale cultures, which is an important contribution to the state of the art.

LIST OF ABBREVIATIONS

HTS – High throughput screening
MTP – microtiter plate
rpm – Rounds per minute
k_La – specific oxygen transfer coefficient
P – Power dissipation

ACKNOWLEDGEMENTS

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