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Technical Report

Real-time monitoring of fungal growth and morphogenesis at single-cell resolution

Development times for efficient large-scale production, utilizing fungal species, are still very long. This is mainly due to the poor knowledge of many important variables related to fungal growth and morphogenesis. We specifically addressed this knowledge gap by combining a microfluidic cultivation device with time-lapse live cell imaging. This combination facilitates (i) studying population heterogeneity at single-cell resolution, (ii) monitoring of fungal morphogenesis in a high spatiotemporal manner under defined environmental conditions, and (iii) parallelization of experiments for statistical data analysis. Our analysis of *Penicillium chrysogenum*, the workhorse for antibiotic production worldwide, revealed significant heterogeneity in size, vitality and differentiation times between spore, mycelium and pellets when cultivated under industrially relevant conditions. For example, the swelling rate of single spores in complex medium ($\mu = 0.077 \pm 0.036 \text{ h}^{-1}$) and the formation rate of higher branched mycelia in defined glucose medium ($\mu = 0.046 \pm 0.031 \text{ h}^{-1}$) were estimated from broad time-dependent cell size distributions, which in turn were derived from computational image analysis of 257 and 49 time-lapse series, respectively. In order to speed up the development of new fungal production processes, a deeper understanding of these heterogeneities is required and the presented microfluidic single-cell approach provides a solid technical foundation for such quantitative studies.

Keywords: Filamentous fungi / Microfluidics / Morphogenesis / *Penicillium chrysogenum* / Single-cell analysis

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1 Introduction

The establishment of new fungal production processes including upscaling them from laboratory-scale (<100 L) to large-scale bioreactors (>100 m³) is a highly complex task [1]. Even after more than 70 years of production experience with the filamentous fungus *Penicillium chrysogenum*, the classical production organism for β -lactam antibiotics such as penicillin [2], process development and upscaling times are very long. The most underestimated aspect is knowledge of the many different process parameters involved in optimizing the cell physiology and mor-

phology in such a way that maximum production performance is guaranteed [3].

Filamentous fungi experience a complex morphogenesis during propagation and cultivation (Fig. 1A) and, thus, are extremely sensitive to modified process conditions (e.g., medium, pH, T, shear forces) [4]. In submerged cultures, the life cycle of *P. chrysogenum* starts with spore germination, which is further subdivided into spore swelling, germ tube emergence, and germ tube elongation [5]. The subsequent growth is characterized by elongation (due to vesicle transport) of the apical compartment, that is, the hyphal tip [6]. Branching occurs mainly at areas with high vesicle accumulation [7], resulting in new tips and an overall growing hyphal network. In addition to the specific cultivation conditions, there are strong indications that morphological changes during the production phase depend greatly on the history of the cells at all precultivation stages [8]. A better understanding of the underlying mechanisms inducing fungal morphogenesis is therefore required.

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Abbreviations: CI, circularity index; MSCC, microfluidic single-cell cultivation; PDMS, polydimethylsiloxane

Figure 1. Novel microfluidic approach for monitoring fungal growth and morphogenesis during typical steps in an industrial bioprocess setup. (A) The development of industrial fermentation processes for bulk production includes scaling up from shake flasks to laboratory- and large-scale bioreactors. In the case of filamentous fungi (exemplified for *Penicillium chrysogenum*), this sequential cultivation procedure is accompanied by great changes in fungal morphology and size. (B) Microfluidic setup and chip design. A cell suspension is pipetted onto a glass slide followed by the assembly of a PDMS pad on the top of the suspension to prevent cell washout during cultivation and to control the cell's environment through distinct inlet and outlet channels. The chip is then connected to the microfluidic periphery and mounted onto the microscope stage. (C) Cultivation principle. Fresh medium is continuously perfused through the system to establish constant cultivation conditions. With ongoing mycelium formation, the elasticity of the PDMS allows for an increase of chamber height and growth into the third dimension ($h_1 < h_2 < h_3$).

In recent years, simple microscopic imaging procedures have been established for the offline analysis of fungal morphology at distinct time points in cultivation (snapshot analysis). Typically, a sample of cells is placed on a microscopic slide, followed by microscopy and computational image analysis to characterize its fungal morphology [9, 10]. Recently, this image analysis was further automated to collect data in a high-throughput manner for statistical evaluation [11]. All these methods still depend on manual sampling and do not enable cultivation conditions to be controlled during microscopic observation.

Novel microfluidic chip systems enable the cultivation of single cells under defined and constant environmental conditions [12]. By combining these systems with automated time-lapse microscopy, image analysis and statistical data evaluation, it is possible to perform detailed studies on the growth, morphology, and metabolic activities of various eukaryotic and prokaryotic microorganisms. However, most of the currently available chip devices share the feature that cells are trapped within rigid structures, limiting their application to organisms generally featuring low size and shape variability [13], and accordingly, to cells at distinct morphological states [14].

By contrast, filamentous fungi change significantly in size and morphology during a typical life span, starting from spherical spores (typically smaller than $10 \mu\text{m}$) and developing into

simple branched hyphal structures ($< 100 \mu\text{m}$). Finally, these structures can form complex mycelia or pellets that reach sizes of up to several millimeters in diameter (Fig. 1A). As yet, no device is available that enables a systematic and coherent investigation of fungal morphogenesis along all morphological states under industrially relevant cultivation conditions and in a high-throughput manner.

2 Materials and methods

2.1 Organism

All experiments were conducted with the *P. chrysogenum* strain BCB1, which was kindly provided by Sandoz GmbH (Kundl, Austria) as spore suspension. The applied strain is an engineered strain based on the P2 strain of Panlabs [15].

2.2 Media and reference cultivation conditions

The reference cultivations were carried out as a three-step process. Preculture I (complex medium) was started by spore inoculation in 500 mL shaking flasks (100 mL culture volume)

at 25°C and 240 rpm. The biomass was used as the inoculum (13% v/v) for the subsequent preculture II (defined medium) in 500 mL shaking flasks (50 mL culture volume) at 25°C and 240 rpm. The main culture (defined medium) was inoculated by adding 15% v/v of preculture II and carried out as a batch process in a 1.5 L stirred bioreactor (1 L working volume). All media compositions and process parameters have been described in detail elsewhere [16]. For microfluidic single-cell cultivation (MSCC), biomass samples were taken from all process steps. Samples for the study of spore swelling, germination, and hyphal growth were obtained from preculture I and MSCC was performed with the same complex medium. Samples for the study of mycelium growth and pellet formation were obtained from preculture II and MSCC was performed with the same defined medium. Before application in the microfluidic device, the respective cultivation medium was filtered to prevent clogging.

2.3 Microfluidic chip assembly

The microfluidic system used in this study was designed for the real-time monitoring of fungal growth. The single-use device was made of PDMS (Sylgard 184 Silicone Elastomer, Dow Corning Corporation, Midland, TX, USA) and glass (D263, $t = 170\ \mu\text{m}$, Schott Glass, Malaysia). Before assembly, the inlet and outlet holes were punched. The glass slide and PDMS block were plasma oxidized for bonding [17]. Prior to the bonding, 2 μL of the cell suspension was taken from either a shake flask or bioreactor culture and pipetted onto the center of the glass slide. In the last step, excess medium was removed and the PDMS block was carefully placed onto the inoculated glass slide and finally sealed (Fig. 1B). The whole inoculation process took approximately 10 min before cultivation was started.

2.4 Microfluidic cultivation procedure and live-cell imaging

After assembly, the chip was mounted on an inverted microscope (Eclipse Ti, Nikon Instruments, Japan), equipped with a temperature incubator (PeCon GmbH, Germany). Microfluidic cultivations were performed at 25°C. Inlet and outlet were connected to a tubing and syringe pump system (NeMESYS, Cetoni GmbH, Germany) for continuous flow. For further information regarding setup and operation of the microfluidic system, the reader is referred to Grünberger et al. [17]. After flushing the system (60 min) at a flow rate of 200 nL min^{-1} , the desired positions for high-throughput investigations were selected (on average 50 positions, depending on the morphological state). During each experiment, the medium was perfused at a constant rate of 200 nL min^{-1} . Time-lapse series were taken at 100, 20, and 10 times magnification for the different morphological states. Phase contrast images and differential interference contrast images were recorded using an ANDOR LUCA R DL604 CCD camera with an exposure time of 200 ms and a time-lapse frequency of four pictures per hour.

2.5 Image analysis and determination of fungal growth parameters

Image analysis was performed automatically by specifically developed routines in MATLAB (Mathworks, R2014a), utilizing suitable functions of the Image Processing and Statistics Toolbox (Mathworks, R2014a). Before analysis, all time-lapse images were preprocessed by running the following steps: (i) conversion of raw images (“nd2” file format) into grayscale matrices; (ii) if required, image cropping, rotating, and intensity adjustment; (iii) object detection by segmentation applying Sobel approximation; (iv) image dilation to fill gaps in binary gradient mask; (v) filling of interior gaps in detected objects and removal of connected objects on image border; (vi) smoothing of desired cell objects and removal of smaller unwanted objects; (vii) marker-controlled watershed segmentation of cell objects. The resulting cell objects were analyzed with respect to different shape properties depending on cell morphology. Spore and hyphae sizes (corresponding to projected cell areas) were determined as a function of the total pixel counts (converted by $0.08\ \mu\text{m px}^{-1}$ to account for the optical magnification of 100). Mycelium sizes (corresponding to projected cell skeleton) were determined as a function of total pixel counts (converted by 0.40 and $0.80\ \mu\text{m px}^{-1}$ to account for the optical magnifications of 20 and 10, respectively). Spore swelling rates were estimated by fitting different growth models to the resulting time-series data (as depicted in Supporting information Table S1). Hyphal and mycelium growth rates were estimated by fitting exponential functions to the resulting time-series data.

3 Results and discussion

3.1 Monitoring fungal growth at single-cell resolution

We utilized a single use and highly flexible microfluidic system to study the growth of filamentous fungi throughout different morphological stages (Fig. 1B and C). The microfluidic chip ($20\ \text{mm} \times 15\ \text{mm} \times 10\ \text{mm}$) consists of a monolayer cultivation cavity (flexible dimensions), which is connected to one inlet and one outlet channel for continuous media supply. The cells are trapped between an elastic PDMS ceiling and a rigid $170\ \mu\text{m}$ thick glass bottom slide, ideal for live cell imaging and growth studies with full spatiotemporal resolution.

In comparison to unicellular organisms, fungi undergo a large size increase during their life span. For example, when *P. chrysogenum* spores were cultivated in chambers with fixed heights, the morphogenesis was significantly impaired (data not shown). To reduce such geometrical restrictions, our elastic PDMS chip material facilitates the continuous deformation of the cultivation chamber, depending on the fungus morphology and size (Fig. 1C). This approach enables parallel cultivation and monitoring of the morphogenesis of more than 100 individual fungal systems in one cavity, starting from one spore up to larger mycelia. To maintain constant cultivation conditions throughout the whole cultivation and all growth phases, fresh medium is infused continuously at $200\ \text{nL min}^{-1}$.

Figure 2. Heterogeneity of spore swelling. (A) Top row: Selected time-lapse images of two adjacent swelling *Penicillium chrysogenum* spores during the early-stage preculture of a typical production process (Fig. 1A) but in the microfluidic chip (Supporting information Movie S1). Middle row: Measurement of single spore projected areas following automated image analysis. After 11 h of cultivation, the red spore lyses that leads to contraction and a sudden decrease in spore size. Bottom row: Distributions of single spore volumes at corresponding time points. The two exemplary spores shown above are highlighted in red and blue. (B) Continuous monitoring of the growth of the two selected spores. The cell lysis event of the red spore leads to a step decrease in spore volume. (C) Distributions of swelling rates estimated from the time courses of 175, 60, and 22 spores monitored in three independent chip experiments and assuming exponentially increasing spore volumes.

3.2 Spore swelling

In a first experiment, spore swelling was studied by cultivating a total number of 257 *P. chrysogenum* spores in three biological replicates in our microfluidic chip under conditions equivalent to the first preculture of a typical production process (Fig. 1A). During the first 8 h of cultivation, the size of single spores (in terms of estimated spore volumes) roughly follows a normal distribution curve as depicted by one of the measured time-lapse series in Fig. 2A. This was caused by seeding the flow chamber with a heterogeneous population of spores that initiated the swelling process at different times during the preceding shake flask cultivation.

After 8 h, spores showed rather even size distributions due to an increased number of dying spores. As an example, the corresponding time-lapse images of one continuously growing (marked blue) and one dying spore (marked red) are shown (Fig. 2A). Cell death is accompanied by the release of cytoplasm into the culture media followed by cell contraction and a sudden decrease in spore size, which can be directly monitored online (Fig. 2B, Supporting information Movie S1). For this effect, although observed frequently, no correlation with a particular spore size or swelling rate was found (data not shown). Any significant trapping effect on spores in the microfluidic chamber can be excluded, because dead cells or cell fragments were also observed during spore cultivation in shake flasks followed by offline microscopic analysis of culture samples.

Swelling rates for each single spore were determined from continuous measurements of the increase in spore size over time (Fig. 2B). It is still an open question whether the swelling process is mainly controlled by the uptake of water or by the regeneration of the catabolic and anaplerotic pathways [14]. A constant diffusion of water over the spore surface as the rate-controlling step would result in a linearly increasing spore diameter. By contrast, if metabolic processes are involved to a major extent then, under balanced growth conditions, autocatalytic growth would result in an exponential increase in the spore size (in terms of the measured projected area or the estimated volume thereof). To test both hypotheses, we evaluated the measured time series data of projected spore areas following either a polynomial or an exponential model approach (Supporting information Table S1).

As a result, the spore diameter increased at an average rate of $k = 0.352 \pm 0.150 \mu\text{m h}^{-1}$, while the average swelling rate in the spore volume was estimated as $\mu = 0.077 \pm 0.036 \text{ h}^{-1}$. Both models fitted the data equally well (as represented by the comparable values of normalized mean squared errors) and therefore no direct conclusion about the spore swelling mechanism could be drawn. However, from the broad distribution of swelling rates in all three independent chip experiments (Fig. 2C, Supporting information Fig. S1), it becomes clear that swelling proceeds very differently among the single spores of a population. This fact strongly supports the hypothesis that metabolic processes are the major driving forces during spore swelling because a

constant water uptake should be reflected by a narrow distribution of swelling rates.

Since the volume is estimated by assuming the spores to be perfectly round spheres, we also determined the swelling rates directly from the measured projected areas (Supporting information Table S1). The resulting average rate was considerably lower and the underlying distributions partly overlapped (as represented by the SD).

To the best of our knowledge, these data represent the first estimates of the growth rate associated parameter of *P. chrysogenum* in the early stadium of its morphogenesis, including population heterogeneity. Determination of spore swelling rates during classical cultivation in a shake flask or bioreactor is not feasible due to the low sensitivity of all currently established quantification methods for fungal biomass in liquid media [18].

3.3 Spore germination and hyphal growth

In a second microfluidic experiment, we investigated the initiation of spore germination. One measurable quantity here is the circularity index (CI) [14], which is defined as $CI_i = 2\sqrt{\pi A_i}/p_i$. The terms A_i and p_i denote the area and the perimeter of a projected object (here single spore) measured at time point i . The CI is a measure of how well the projected spore area is approximated by a circle and it is, *by definition*, very sensitive to changes in the perimeter of the projected spore. Following automated image processing, the perimeter of dissected spore edges was strongly biased due to image fuzziness or incomplete separation of connected cells. The CI measures were thus not applicable for correctly determining the initiation of spore germination from automated image analysis (data not shown).

To enable a more robust recording of the germination process, we introduced the aspect ratio as another measure of spore circularity. The aspect ratio (AR) is here defined as: $AR_i = b_i/a_i$, with a_i and b_i denoting the major and minor axis of an ellipse, respectively, that is fitted to each spore object (Fig. 3A). Thus, a sudden decrease in the AR of individual spores below 1 during cultivation resulted from germ tube emergence and marked the initiation of tube elongation and hyphal growth. From these analyses, we frequently observed germination at one or two cellular positions, which is also consistent with previous studies [5].

Within the next morphogenesis stadium of *P. chrysogenum*, the growth of single hyphae was analyzed over time (Fig. 3B). A division of the projected hyphae area into active and nonactive (black) cell parts enabled correct rate estimations that only account for the viable biomass [19]. None of the existing methods permit this differentiation and their application would lead to a gross overestimation of active biomass in the early hyphal growth stadium and, accordingly, to an underestimation of the respective growth rates (e.g., 50 % in the given example). With our microfluidic chip system more elaborate studies on fungal growth kinetics, for example, the determination of branching frequencies [7, 14, 20], or even time-resolved investigations on intracellular processes can also be conducted. As one example, the septum formation in *P. chrysogenum* was analyzed in more detail and here an average septation time of $t_{\text{sep}} = 5.2 \pm 1.1$ h was estimated (Supporting information Fig. S2), which was not previously possible with standard offline microscopic analysis.

3.4 Mycelium growth and pellet formation

We next studied the growth of complex mycelia under the same microfluidic cultivation conditions. Due to the increasing size of the fungal cells, the image magnification was adjusted to monitor the growth of single mycelial structures (Fig. 3C, Supporting information Movie S4). The changes in size of these structures were observed over time by collecting the total number of pixel of projected cell skeletons (instead of projected areas). From a total of 49 single time series, the average growth rate of higher branched mycelia was estimated as $\mu = 0.046 \pm 0.031 \text{ h}^{-1}$, and this value fits well with our previous laboratory-scale bioreactor experiments ($\mu = 0.06 \pm 0.02 \text{ h}^{-1}$ [16]). It is noteworthy that the latter data represents the only information currently available in order to directly compare the results of our novel microfluidic approach with those from established cultivation procedures.

As already shown for the swelling process of single spores (Fig. 2B), there is also great heterogeneity in the growth of single mycelia (Fig. 3C). Under the given microfluidic conditions, all the cells of a mycelium receive a constant nutrient supply and potentially inhibiting by-products are continuously washed off [13]. Therefore, the observed heterogeneity is rather an effect of random intracellular metabolic processes, such as transport.

Clearly, we cannot rule out the fact that the restriction to only two dimensions for image analysis leads to an underestimation of the formed mycelium in the microfluidic chamber and thus in reality to higher growth rates (compare Supporting information Table S1). In addition, the already discussed influence of inactive biomass (Fig. 3B), which is not currently considered in any experimental setup, might also affect growth rate determination in this morphological state. Further clarification could be obtained by applying specific staining methods, such as membrane-selective fluorescent dyes, in combination with confocal microscopy [21]. Nevertheless, our chip system is primarily designed to enable the real-time monitoring of fungal growth and morphogenesis under various conditions (e.g., testing different media compositions), and all cellular parameters derived will be directly comparable among different chip experiments.

As expected, high-branched mycelium growth finally led to pellet formation and a corresponding switch to larger cell aggregates. Following automated image analysis, these aggregates resulted in larger objects of tightly connected pixels and could therefore be discriminated from the surrounding mycelial cells (Supporting information Fig. S3 and Movie S5).

The pellet morphology is typically a consequence of different environmental stress factors such as nutrient limitation when material transport from the outer to the inner parts of the fungal system is hampered [22]. The impact of pellet morphology on fungal growth and product formation is still under detailed investigation by many research groups [8], and the application of microfluidics is expected to lead to a substantial knowledge gain in this field.

4 Concluding remarks

In this study, we combined a microfluidic chip system with automated image analysis to provide data on fungal

Figure 3. Stages of spore germination, hyphal and mycelium growth along the sequential process phases of a fungal production process. (A) Top: Selected time-lapse images of spore germination during the late-stage preculture phase (Supporting information Movie S2). Bottom: Measurement of aspect ratio from single spores following automated image analysis and ellipse fitting. The formation of germ tubes is initiated after several hours of spore swelling and leads to a decrease in the aspect ratio as indicated by the dashed line. (B) Top: Selected time-lapse images of hyphal growth resulting from germ tube elongation and septum formation (Supporting information Movie S3). Bottom: Measurement of single hyphae sizes following automated image analysis. The resulting data enabled the unbiased estimation of hyphal growth rates by accounting for dead cell fractions (highlighted in black). (C) Top: Selected time-lapse images of mycelium growth during the main culture of an industrial bioprocess (Supporting information Movie S4). Bottom: Mycelium growth rates were estimated from the time courses of 49 mycelial structures monitored in one chip experiment following automated image analysis.

morphogenesis and growth kinetics at single-cell resolution. With easy and direct access to different process relevant parameters, the technology opens up unique possibilities for systematic screening studies and could replace labor-intensive vitality assays through the detection and monitoring of non-growing cells (as illustrated in Fig. 3B). We envision that this microfluidic single-cell approach will be integrated into routine experimental procedures for studying production-relevant parameters and their effect on morphogenesis and growth ki-

netics. For routine application, the parallelization of the chip into a multichamber system to isolate and analyze single spores and mycelia separated from each other, as recently shown for bacteria [23] and yeast [24], still remains to be optimized. Our results show that distinct heterogeneities do exist along the morphological states of *P. chrysogenum* that are relevant for the production of antibiotics. In order to increase productivity, a deeper understanding of these heterogeneities and of the underlying growth kinetics at the single-cell level

(covering spores and hyphal cells) is required [25] and the present work provides the first step toward studying these important growth characteristics quantitatively based on a solid methodological foundation.

Practical application

Filamentous fungi experience a complex morphogenesis during propagation and cultivation and, thus, are extremely sensitive to modified process conditions. In this study, we combined microfluidic single-cell cultivation with automated image analysis to provide quantitative data on fungal growth and morphogenesis. We emulated different environmental conditions that fungal cells are frequently exposed during scale-up from lab-scale to production-scale. Our results demonstrate that distinct heterogeneities do exist along the morphological states of the model organism *Penicillium chrysogenum* that might be of particular relevance for the production of antibiotics. The presented system is a sensitive and robust device enabling real-time analysis of cellular growth kinetics at single-cell resolution and routine application for complex filamentous fungi.

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The authors declare that they have no competing interests.

5 References

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