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# Controlling *Aspergillus niger* morphology in a low shear-force environment in a rocking-motion bioreactor

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## ABSTRACT

The filamentous fungus *Aspergillus niger* is an important production host in biotechnology. Shear force regimes are one of the key factors that affect macromorphology and product yield. While morphology changes under intensive agitation have been widely investigated, studies at a low shear force regime independently from oxygen limitation has remained a challenge. Therefore, in this study, a 2-dimensional rocking-motion bioreactor is used as an alternative platform for studying the macromorphology under a low shear force regime, but sufficient supply of dissolved oxygen. Talcum macroparticles were added at different concentrations to control the development of a certain macromorphology. Results showed that 0.25% and 1% ( $\text{ww}^{-1}$ ) of talcum led to a mixture of dispersed mycelia and loose clumps, similar to what is obtained in lab-scale stirred tank reactors. At lower talcum concentrations, distinct pellet formation was observed. Quantitative analysis of pellets showed that with 0.05% of talcum, 95% of the pellets had a diameter smaller than 850  $\mu\text{m}$  after 36 h. In case of 0.1% of talcum, 94% ( $\pm 5.0\%$ ) of the pellets had a diameter below 650  $\mu\text{m}$ . The presented approach makes it possible to achieve a certain morphology as, for example, observed in large scale cultivations to study the consequences for product synthesis.

## 1. Introduction

Filamentous fungi play a key role for the production of various enzymes and other bioactive compounds, including secondary metabolites. *Aspergillus niger* as one of the most important strains among filamentous fungi is extensively applied for citric acid production which yields over 1.6 million tons annually [1]. Moreover, *A. niger* is a reliable host for recombinant proteins with posttranslational modification [2]. *A. niger*, however, has some drawbacks for the application in industrial scale, as it develops a diverse macromorphology that depends on its growth environment. Morphology, in turn, directly affects mass transport into and out of the cellular structure, the rheology of the fluid phase, and – consequently – oxygen and substrate transfer, as well as production yield [3,4].

The development of a certain macromorphology during bioreactor cultivations is sensitive to multiple factors, including the dissolved oxygen availability [5], inoculum concentration [6,7], pH-value [7,8], osmolality [9,10], and – even more important – the hydrodynamic conditions, in particular the shear force regimes [11]. A distinguished

correlation between shear force regimes, morphology, and metabolic response linked to productivity has been observed in several filamentous organisms [12,13,14]. In order to assure sufficient mass transfer at high cell densities, a considerably high power input is required, which causes hyphal or pellet fragmentation, that can change viscosity and productivity [15]. The shear force environment on one side, and cellular stress responses on the other side can change the macromorphology and cause, for example, pellet formation of varying shape and compactness [16]. Apart from that, the shear forces vary in large scale stirred-tank bioreactors as zones appear with only considerably low power input and fluid flow, while high mechanical shear forces are present near the stirrer blades [17]. Thus, in contrast to lab-scale experiments, cells that are cultivated in a large scale environment experience an oscillating shear force regime, which can lead to the development of a different macromorphology than in lab-scale. Most of the lab-scale research, however, is focused on the effects of constantly high shear stress at intensive continuous stirring on the development of the macromorphology [13,18]. Since the stirring rate is a critical factor for achieving a certain gas-mass transfer, the investigation of the

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development of the macromorphology independent from oxygen limitation becomes impossible in stirred tank reactors. Other non-stirred bioreactor systems like bubble columns usually cannot achieve a sufficient gas-mass transfer coefficient.

One reactor concept that exhibits a comparably high gas-mass transfer is the 2-dimensional rocking-motion bioreactor (2D RMB) [19]. This bioreactor type has been used for the cultivation of shear-sensitive cells such as mammalian cells and heterotrophic algae [20]. Moreover, the system can guarantee sufficient oxygen transfer for a relevant cell concentration while a  $k_L a$ -value of up to  $600 \text{ h}^{-1}$  is achievable (in a 20 L bag with 12 L culture volume) [21]. The 2D RMB was used, for example, to investigate the effect of low shear stress on the development of the macromorphology of *A. niger* in fed-batch cultivations [22]. It was shown that under such cultivation conditions, the fungus grows in pellets instead of developing a dispersed mycelial form as it happens in stirred-tank bioreactor cultivations. Despite the mass transfer limitation inside larger pellets, pelleted morphology in *A. niger* is preferred for certain products such as citric acid [6], glucoamylase [23], endoglucanase [7], and secondary metabolites [3,4,10], respectively. Pellet formation is also aimed whenever it facilitates down-stream operation, e.g. during separation processes. Besides, pellet formation leads to a rather Newtonian behavior of the fluid phase, and an adequate mass transfer (in the culture broth) under the same power input in comparison with hyphal growth; thus, energy can be saved [24].

Furthermore, using *A. niger* as an example, it was also shown that the addition of microparticles, such as talcum, can affect the development of the macromorphology [22,25]. This effect is strain and microparticle-dependent [26–29]. Previous results showed that adding 1% ( $\text{wv}^{-1}$ ) of talcum microparticles to *A. niger* cultivations in a 2D RMB changed the morphology from heterogeneous to homogeneous pellets of smaller diameter [22]. Since, in a 2D RMB, no additional mechanical shear forces of a stirrer are created, it was possible to study the effect of talcum particle addition exclusively in a low-shear force environment, but under controllable cultivation conditions, in particular under a sufficient oxygen supply. The latter condition is not easily achievable in other low-shear cultivation environments, for example in shake flasks.

It was therefore investigated, if a quantitative relation exists between the talcum concentration and the development of the morphology (e.g. pellet fraction of the whole biomass, pellet diameter and pellet density), and whether it is generally possible to control morphology in a 2D RMB. In order to clarify this, different concentrations of talcum were added to cultivations that were conducted in a low-shear force environment while keeping otherwise ideal growth conditions.

If the pellet formation is controllable, a pre-defined macromorphology is achieved which can be investigated for its growth and product secretion performance. Finally, a favorable morphology can be identified, which shall be maintained in a production environment.

## 2. Material and methods

### 2.1. Strain

A recombinant strain of *Aspergillus niger* (ÖV4.10) was used in this study. Further information about the strain is provided by Richter *et al.* [30]. A conidia suspension of the organism was thoroughly mixed with an equal volume of 50% glycerol and kept as cryostock at  $-80 \text{ }^\circ\text{C}$ .

### 2.2. Media and inoculum preparation

Ingredients were obtained from CARL ROTH (Karlsruhe, Germany) and Sigma-Aldrich. Mineral salt medium (MM) was applied, which contained (per liter): glucose 7 g (Roquette, Lestrem, France),  $\text{KH}_2\text{PO}_4$  1.5 g, KCl 0.52 g,  $\text{NaNO}_3$  5.95 g,  $\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$  0.5 g, and 1 mL of modified Vishniac stock solution of trace elements [31]. This Vishniac stock solution contained (per liter): EDTA 10 g,  $\text{ZnSO}_4 \cdot 7 \text{ H}_2\text{O}$  4.4 g,

$\text{MnCl}_2 \cdot 4 \text{ H}_2\text{O}$  1.01 g,  $\text{CoCl}_2 \cdot 6 \text{ H}_2\text{O}$  0.32 g,  $\text{CuSO}_4 \cdot 5 \text{ H}_2\text{O}$  0.31 g,  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{ H}_2\text{O}$  0.22 g,  $\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$  1.47 g, and  $\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$  1.00 g. MM and Vishniac stock solution were sterilized separately by autoclaving at  $121 \text{ }^\circ\text{C}$  for 20 min. In order to activate the preserved spores from a cryostock, inoculation was conducted in a complex medium agar (MM + 0.5% yeast extract and 0.1% casamino acids, 1.5% agar, all  $\text{wv}^{-1}$ ). The Petri dishes were incubated at  $30 \text{ }^\circ\text{C}$  for 5 days. Spores were harvested after multiple washing with physiological saline solution (0.89% ( $\text{wv}^{-1}$ ) NaCl), followed by filtration through miracloth filters (pore size 25  $\mu\text{m}$ , Calbiochem, Frankfurt/Main, Germany) to remove any residual agar, and centrifugation. Freshly prepared spores were supplemented with 0.003% yeast extract (Biospringer®, Maisons-Alfort, France) to promote germination. This solution was used to inoculate the bioreactor ( $10^9$  spores per 1 L).

The feed medium consisted of 5% glucose, 0.5% yeast extract, 0.1% casamino acids (all  $\text{wv}^{-1}$ ) and 20 mM L-valine. All components were autoclaved separately. Talcum (talc – hydrated magnesium silicate,  $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$ ) microparticles with an average diameter of  $10 \mu\text{m}$  (40 g, 10 g, 4 g and 2 g for achieving final concentrations of 1%, 0.25%, 0.1% and 0.05%  $\text{wv}^{-1}$ , respectively) were added to 50 mM Na-acetate buffer; the pH-value of the mixture was adjusted to 6.5 with 1 M HCl solution.

### 2.3. Bioreactor cultivation

A 20 L 2-dimensional rocking-motion bioreactor CELL-tainer® CT20 (Celltainer Biotech, Winterswijk, The Netherlands) was applied in this study. It consisted of a rocker, a controller, and a single-use sterile bag which was equipped with pH and dissolved oxygen (DO) sensors. More information on the bioreactor set up was described previously [22,32]. 4 L of MM were transferred to the cultivation bag; then, before inoculation, the pH-value was adjusted to 3.0. The pH-value was kept constant throughout the whole cultivation by automated addition of acid (1 M HCl) and base (1 M NaOH). The rocker speed was controlled to keep the DO at least at 30%. The rocker speed was adjusted in a range between 5 and 15 rpm during the germination phase. Then, the rocker speed was increased to between 15 and 35 rpm to avoid oxygen limitation. The feeding and aeration rates were set at  $0.0046 \text{ L h}^{-1}$  and 1 vvm, respectively. All bioreactor cultivations were performed in duplicates.

### 2.4. Characterization of the morphology

Microscopy samples were taken regularly during the experiments. All samples were diluted to the same ratio using NaCl solution before Differential interference contrast (DIC) microscopy analysis. A Leica Microsystems DM 5000 microscope (Leica Biosystems, Nussloch, Germany) was used. At least, 150 of individual fungal agglomerates were diluted 1:10 with NaCl solution and poured into a Petri dish at each time point. The whole Petri dish was scanned slowly by microscopy to capture all the clumps/pellets. The pellets in images were further analyzed with Image J, version 1.53 n 7 to see whether controlling the pellet-size distribution becomes feasible [33]. The recently developed MPD-quantification plug-in was applied for more comprehensive analysis [34]. Since it is designed for the analysis of round, distinctively large pellets, some of the pre-defined variables were modified for a better adaptation to the actual macromorphology.

### 2.5. Analyses

In order to measure the cell dry weight (CDW), 15 mL of fungal biomass was filtered through pre-dried and weighted Whatman® filter paper (no. 41, WHA1441047, Whatman PLC, Keene, NH). The filter papers were dried in an oven at  $50 \text{ }^\circ\text{C}$  for 72 h and weighted for gravimetric CDW determination with three replicates for each sample. Since the trapped talcum in the hyphae interferes with the CDW measurement, the amount of talcum was separately measured by dissolving

2 mL of sample volume in an equal volume of 3 M hydrochloric acid for 2 h at 95 °C, removal of the liquid phase and washing as described for the CDW determination. This value, i.e. the mass of talcum that remained as solid fraction, was then subtracted from the previously determined CDW.

Additionally, cell-free supernatant samples of the filtration step were separated and centrifuged. Glucose concentration was measured in these supernatant samples using a Cedex Bio-HT® analyser (I&L-Biosystems, Koenigswinter, Germany). Short-chain carboxylic acids and alcohols were quantified with an Agilent 1200 high-performance liquid chromatography system, which was equipped with a refractive index detector and a HyperRez™ XP Carbohydrate H<sup>+</sup> column (300 × 7.7 mm, 8 μm) (Thermo Fisher Scientific, Schwerte, Germany) with 0.1 M H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.5 mL min<sup>-1</sup> at a temperature of 65 °C. Online off-gas measurement was performed with the Blue-In-One Ferm analyzer (BlueSens, Herten, Germany). Data was then used to calculate the respiratory quotient (RQ) according to Kurt et al [22].

### 3. Results and discussion

In order to study whether the development of the macromorphology can be changed and furthermore controlled by microparticle addition, *A. niger* was cultivated in the 2D RMB in fed-batch mode with different amounts of talcum. It was then investigated, if a quantitative relation exists between the talcum addition and the portion of pellets in comparison to dispersed biomass, pellet size or pellet density in a low-shear, non-oxygen limited growth environment as obtained in the 2D RMB.

#### 3.1. Growth characteristics

As it is shown in Fig. 1. A and 1. B, all the cultivations followed a similar trend in both, growth and substrate consumption. At 1% talcum concentration, however, the growth seemed to be faster in the early phase, although the glucose consumption rate for 1% talcum was in the same range as for the other cultivations. The cultures supplemented with 0.05% and 0.1% talcum had an average growth rate of 0.14 and 0.13 h<sup>-1</sup> between 12 and 20 h of cultivation, respectively. The CDW remained approximately constant after 40 h in the fed-batch phase of both cultivations. The substrate consumption rate in the batch phase was slightly higher at increased talcum concentration: 0.24 and 0.23 g L<sup>-1</sup> h<sup>-1</sup> for 0.25% and 1%, in comparison with 0.20 and 0.19 g L<sup>-1</sup> h<sup>-1</sup> for 0.05% and 0.1% talcum. The substrate-related biomass yield coefficient (by the end of the batch phase) was 0.47, 0.57 g cell/g glucose for the cultivations with pellet formation when applying 0.05% and 0.1% talcum, and 0.54, 0.67 g cell/g glucose in case of 0.25% and 1% talcum, respectively.

An increase in the final biomass concentration with talcum addition was already reported in literature. Gonciarz et al [35] observed an increase of the CDW concentration from 7 g L<sup>-1</sup> without talcum to 9 g L<sup>-1</sup> with talcum in *Aspergillus terreus* cultivations with highest values at talcum concentrations between 1.2% and 1.5%. A similar observation was made by Kaup et al [36]. The CDW concentration increased from 15 to 30 g L<sup>-1</sup> and 37 g L<sup>-1</sup> with talcum supplements of 1% and 1.5%, respectively. Dispersed growth, as obtained when higher talcum concentrations were applied, eventually caused higher enzyme secretion, metabolic productivity, and finally more biomass formation in comparison with the pelleted form. One obvious reason is a better nutrient and oxygen supply in free hypha. This observation seems to be valid at substrate excess, but not for the fed-batch phase, where growth, and thus differences in the process performance, are small.

The dissolved oxygen (DO) profiles of all cultivations are shown in Fig. 2. It is obvious that the change of the DO concentration correlates with the biomass formation. DO control was set to 30% of saturation, meaning that the rocking speed was increased whenever the DO level dropped below this setpoint. At cultivations with 1% of talcum, the DO decreased rapidly from 100% to the threshold value of 30% already within the first 10 h. After the manual increase of the rocking range (from 5- 15 to 15-35 rpm), the DO transiently increased and the setpoint of 30% was later maintained. A similar pattern was observed for all other cultivations. Volumetric oxygen uptake rates were determined to (average value in the batch phase from about 20 h): 0.15, 0.19, (-), and 0.24 mol L<sup>-1</sup> h<sup>-1</sup>, and in the fed-batch phase (around 36 h): 0.27, 0.29, 0.30, and 0.27 mol L<sup>-1</sup> h<sup>-1</sup> for 0.05%, 0.1%, 0.25%, and 1%, respectively (not sufficient values were gained for 0.25% talcum in the batch phase). By the end of the cultivation, the average volumetric oxygen uptake rate increased to 0.32, 0.32, and 0.4 for 0.05%, 0.1%, and 0.25%, but remained at 0.27 mol L<sup>-1</sup> h<sup>-1</sup> for 1% talcum.

The difference can also be caused by a changed gas holdup of the liquid phase or a changed gas-mass transfer due to an altered viscosity and surface tension among the cultures.

The importance of oxygen availability for the development of a certain morphology of filamentous organisms is known [37]. It was shown that under DO-controlled conditions (cascade control agitation), *Ganoderma lucidum* grew into dispersed mycelium, while uncontrolled DO led to the predominancy of clumps in STR cultivations [5]. In our work, it was crucial to avoid oxygen depletion to decouple the effect of oxygen limitation from shear stress on the development of the macromorphology. The DO concentration remained above the defined threshold value even at higher biomass concentrations while there was no need for the rocker to reach the maximum speed limit. Since the increase in biomass is associated with an enhanced oxygen demand

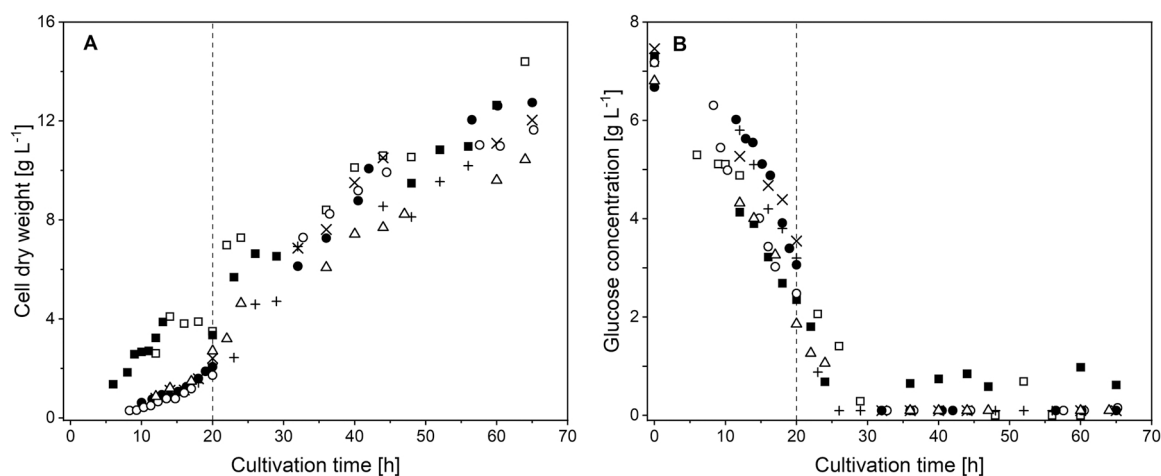
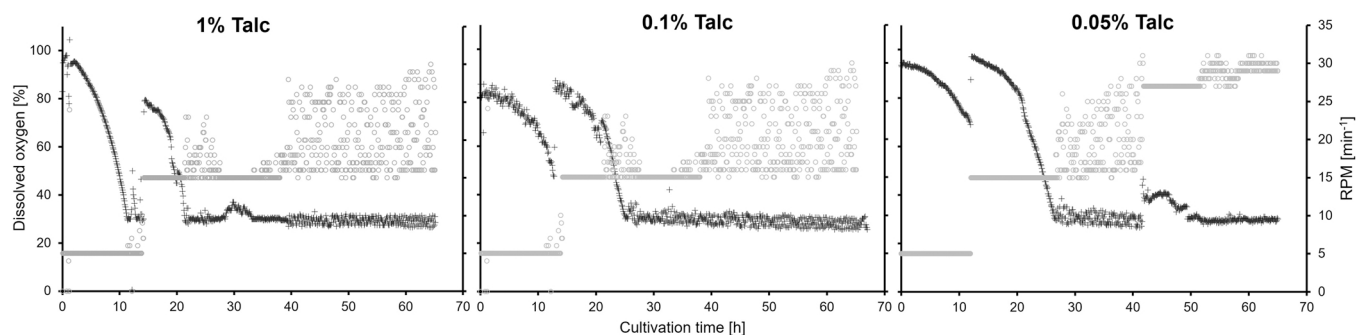


Fig. 1. Effect of talcum concentration on (A) cell dry weight and (B) glucose concentration, in *Aspergillus niger* cultivations in a 2-dimensional rocking-motion bioreactor with constant feeding. □ ■ 1% Talc, △ 0.25% Talc, × + 0.1% Talc, ● 0.05% Talc. Different symbols indicate duplicate cultivations.





**Fig. 2.** Dissolved oxygen (DO (%)) in *Aspergillus niger* cultivations in a 2-dimensional rocking-motion bioreactor with constant feeding. Black and gray shows DO% and rpm, respectively. In case of a talcum concentration of 0.05%, the rocker speed was maintained constant at 30 rpm to avoid pellet breakage in the late fed-batch phase due to sudden speed changes.

[18], it can be concluded that there was no oxygen limitation in the culture. Therefore, the appearance of a stationary phase when low talcum concentrations were applied, was not due to oxygen limitation, but might be associated with oxygen and substrate transfer resistances inside the pellets.

The growth activity of any cell with an aerobic respiratory system should be reflected by the RQ, which is the ratio of the rate of carbon dioxide production to the rate of oxygen consumed. The RQ in the fed-batch phase was about 1.0 in all cultivations independent of the talcum concentration (see [supplementary Fig. S1](#)), which is typical for aerobic growth if the carbohydrate source is mainly used for growth and energy supply without side product formation.

As shown in [Fig. 1. B](#), glucose depleted after 30 h in all cultivations. It was shown that the addition of talcum led to less accumulation of byproducts [22]. Lactate, succinate, acetate, and citrate were also detected in some of the samples of 0.05% talcum at low concentrations of 0.23, 0.16, 0.27 and 0.44  $\text{g L}^{-1}$ , respectively (data not shown).

### 3.2. Macromorphology in dependence of microparticle concentration

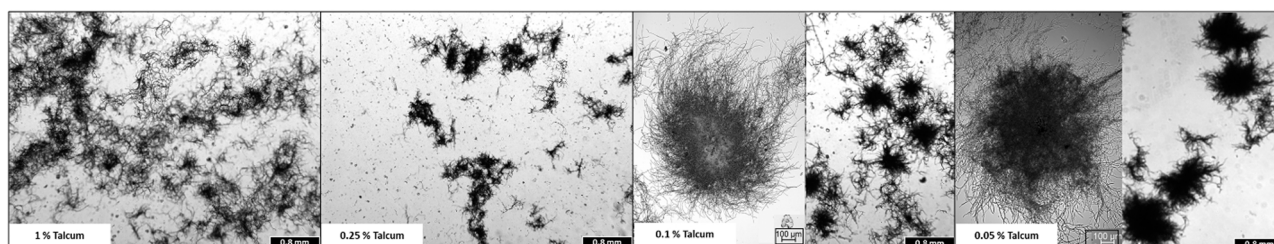
[Fig. 3](#) shows the development of the macromorphology at various talcum concentrations. At 0.25% talcum, an intermediate mixture of both, dispersed mycelia and clumps are seen, but the mycelial aggregation/clumps remained to be the dominant form. On the contrary, the macromorphology was shifted to a large fraction of pellets at lower talcum concentrations (0.1% and 0.05% ( $\text{wv}^{-1}$ )). If growth and macromorphological characteristics are compared, it becomes obvious that a higher growth rate throughout the cultivation is seen at cultures with a dominant and rather exclusive dispersed morphology, while those with a dominant pellet formation show lower growth rates and more side product accumulation. We believe that this observation is most likely due to a poor supply of substrate and oxygen in the pellet cores. This considerable difference in the batch phase might also be due to a higher accessibility of oxygen and nutrient supply for the individual spores in dispersed mycelia rather than inside an agglomerate or a clump.

Microparticle addition has been applied to control

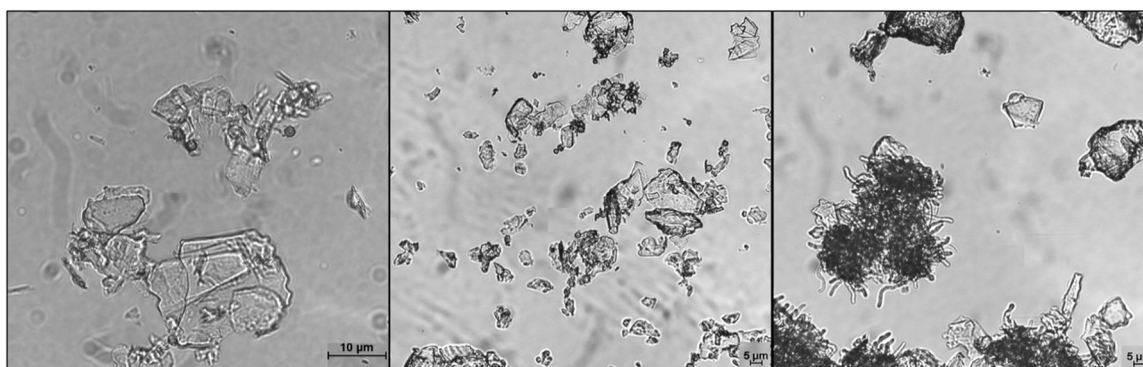
macromorphology before. The majority of previous studies about the effect of microparticles on the control of the pellet size of sporulating filamentous microorganisms described the agglomeration of coagulative spores as a main pre-requisite for pellet formation [22,38]. Kowalska *et al.* [29] compared 3 types of fungi, coagulative spores, coagulative hyphae and non-coagulative cultures supplemented with aluminum oxide. In their study, pellet size reduction was only observed in *A. terreus*, with coagulative spores. As *A. niger* is also categorized as a fungus with coagulative spores, it can be assumed that the effect of talcum on the spore germination might be an important reason for the entirely different evolution of macromorphology. The addition of microparticles had no significant effect on the pellet size or number in an *A. terreus* culture when it was added after the initial spore agglomeration phase [35]. How exactly talcum acts to prevent *A. niger* from growing into pellets cannot be fully answered yet. Authors of one of the most important studies in this field assumed a disturbance of spore aggregation by collision and a probable interference in the spores' initial interaction as primary reasons for pellet size reduction when microparticles were applied [25]. The microscopic images in our study showed that the hydrophobic spores are partly attached to the microparticles in the early cultivation phase ([Fig. 4](#)). Talcum as the agglomeration core hinders the growth and intense entanglement of later-developed hyphae, which possibly facilitate the detachment of outer parts from the aggregate due to mechanical shear or frictions caused by free talcum particles. This would also explain the strong dependence of the evolution of a dispersed morphology and the talcum concentration within a certain range. It can be concluded from our data that a ratio of  $4 \times 10^8$  (or less) spores per g talcum leads to a suppression of pellet formation, but at concentrations of  $2 \times 10^9$  (or more) spores per g talcum, pellets appear as the dominant macromorphological form.

### 3.3. Analysis of pellets

To answer the question whether there exists a quantitative relation between the amount of talcum and the pellet size distribution, images



**Fig. 3.** Selected DIC microscopy images of *Aspergillus niger* macromorphology with various talcum concentrations as obtained in a 2-dimensional rocking-motion bioreactor in the early fed-batch phase (36 h).



**Fig. 4.** Attachment of spores to talcum microparticles and the development of spore agglomeration in *Aspergillus niger* cultivations in a 2-dimensional rocking-motion bioreactor.

from three different time-points (end of batch phase, early and late fed-batch phase, Figs. 5 and 6, summary of data in Table 1) were further processed for the quantitative analysis of the mean pellet diameters. This was only possible for the cultivations with 0.05% and 0.1% talcum, as in the other cultivations with higher talcum concentrations, only dispersed mycelia or very small unstable clumps were the dominant morphological forms.

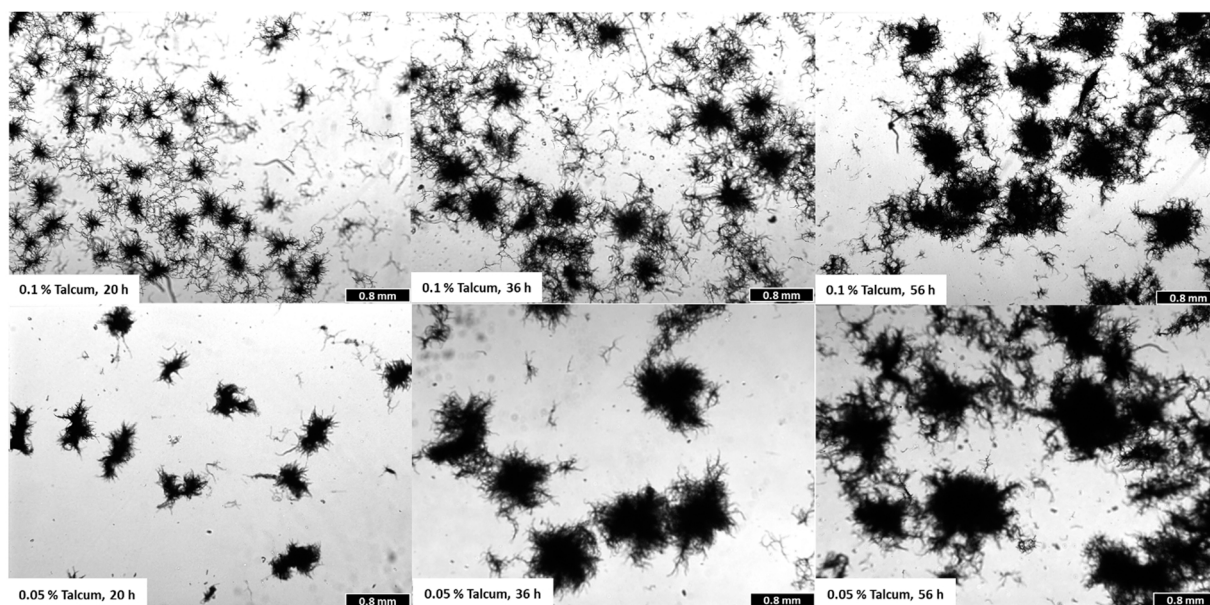
In the batch phase at 20 h of cultivation, a distinctive formation of uniform pellets with a diameter in the range of 150–550 µm was observed in both experiments (Fig. 6 A, D). 99% of the population expressed a size below 450 µm. The data formed a Gaussian distribution with 73% of the pellets' diameter being in between 250 µm and 350 µm with 0.05% talcum. With 0.1% of talcum, however, smaller pellets were formed, 70% of them had a diameter below 250 µm and the distribution was narrower. Thus, a concentration of 0.1% is leading to a more defined pellet size in comparison to all other concentrations that were applied in this study. The 95% confidence intervals and mean pellet diameter for all conditions (i.e., time and talc concentration) are shown in the supplementary material table S2. The results of the t-test can be found in table S3.

In the fed-batch phase with a constant feeding rate, the pellet diameters increased throughout both experiments as seen in the microscopic pictures (Fig. 5). At 36 h, when a talcum concentration of 0.05%

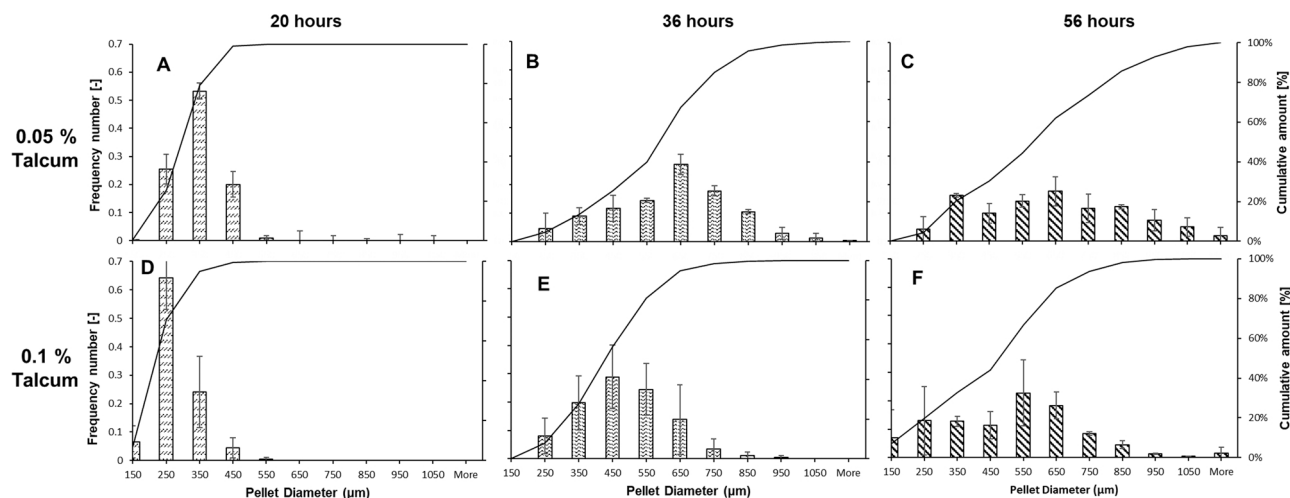
was applied, the pellet diameter range was in between 170 and 1070 µm (Fig. 6 B, E). It is noteworthy to mention that slightly more than 95% of the whole population had a diameter that was smaller than 850 µm. In case of 0.1% talcum, 94.9% of the pellet population exhibited a diameter that was smaller than 650 µm. This means that the pellets became smaller when a higher talcum concentration was applied. By 56 h the maximum size of pellets reached to 1.4 mm at the lowest talcum concentration (Fig. 6 C, F). Here, only 85.6% of the particles exhibited a diameter of 850 µm or less, a reduction by 10% in comparison to 36 h. The tendency of a narrower size distribution at a concentration of 1% talcum is maintained though.

The results show clearly that the pellet size can be controlled throughout the fed-batch phase by adjusting the talcum concentration. Significant differences in the mean pellet diameter were observable in comparison to the reference cultivation (no talcum addition), in which a wide variety of the pellet sizes occurred (data not shown). In another study, the addition of 1 g L<sup>-1</sup> of talcum into the pre-culture reduced the final diameter of *A. terreus* pellets from the maximum of 3500 µm down to 2000 µm in shake flasks (157 mL working volume). A further reduction to 900 µm was achieved at a concentration of 9 g L<sup>-1</sup> of talcum in the pre-culture [35].

Driouch et al. [25] cultivated *A. niger* in 250 mL baffled shake flasks (50 mL working volume) for 72 h at 120 rpm and 30 °C. The authors



**Fig. 5.** Development of pellets at 0.05% and 0.1% talcum in rocking-motion bioreactor cultivations with constant feeding after 20 (end of batch phase), 36 (early fed-batch) and 56 h (late fed-batch phase) in *Aspergillus niger* cultivations in a 2-dimensional rocking-motion bioreactor.



**Fig. 6.** Time course development of the mean pellet diameter at 0.05% and 0.1% talcum concentration in *Aspergillus niger* fed-batch cultivations in a 2-dimensional rocking-motion bioreactor. Samples were taken after 20 h (end of batch phase), 36 h (early fed-batch phase) and 56 h (late fed-batch phase). Data are shown as histogram plots (frequency number, bars) where the bars represent size intervals of 100 µm, and as cumulative size distributions (cumulative amount, lines). At least 150 pellets were measured in each sample.

**Table 1**

Comparison of growth kinetics and macromorphology for two different genetically engineered strains of *Aspergillus niger* at various talcum concentrations under the same cultivation conditions in a 2-dimensional rocking-motion bioreactor.

Strain	Talcum concentration (wv <sup>-1</sup> n %)	Pellet formation (%)	Specific growth rate $\mu$ (h <sup>-1</sup> )	$C_{biomass,max}$ (gL <sup>-1</sup> )	Image time-point (h)	Pellet width to length ratio (-) (if applicable)	Ref.
DS3.1S	0	90	0.24	31.2 <sup>a</sup>	60–120	0.80	[22]
DS3.1	1.00	15	0.12	35.9 <sup>a</sup>		0.75	
ÖV4.10	1.00	15	0.11	27.8 <sup>a</sup>		-	
ÖV4.10	1.00	14	0.2	13.51	36	-	this study
ÖV4.10	0.25	27	0.15	11.64	36	-	
ÖV4.10	0.10	67	0.13	12.04	20	0.61	
					36	0.78	
					56	0.67	
ÖV4.10	0.05	86	0.14	12.19	20	0.54	
					36	0.73	
					56	0.60	

<sup>a</sup>  $C_{biomass,max}$  was determined after 200 h, the talcum mass is not excluded from the data [22].

achieved to reduce the average pellet diameter from 1700 µm in control cultures to 800 and 700 µm with talcum concentrations of 0.04% and 0.06%, respectively. In the case of 1.0 g L<sup>-1</sup> talcum, the average pellet diameter was even smaller (500 µm). This is interestingly close to the obtained values of 650 µm with 0.05% talcum and 550 µm with 0.1% talcum in our study. The authors also observed that a concentration of 0.3% talcum led to a mixture of mycelia and small pellets while at concentrations higher than 0.5%, the macromorphology was fully dispersed [25]. The data suggest that the shear stress in the rocking-motion bioreactor is in the same order of magnitude as in baffled shake flasks, since a similar pellet formation pattern is obtained.

Although most of the cell growth at 0.05% talcum was accomplished during the fed-batch phase from 20 h on, the formation of uniform pellets before seems to be the basis for the achievement of a homogenous pellet size distribution thereafter. Pellets were more homogenous in size until 36 h, with a decreasing uniformity until the cultivation was terminated. Our hypothesis is that the reason might be a breakage of some bigger pellets when they reached a certain size and age. The smaller particles in the later phase of the cultivation were also mainly hyphal clumps rather than highly dense pellets. The change in nutrient availability and the cell age in general might have caused this. Substrate and oxygen deficiency is also common inside larger pellets [39,40], which could have led to pellet breakage [17,40] and therefore, formation of the bimodal distribution at the end of the fed-batch phase.

The previously obtained pellets during the early cultivation phase

were not circular and had rather elongated shape. This changed during the cultivations towards circular pellets at 36 h, while a rather heterogeneous mixture of both were finally observable. The width to length ratio was used to quantify this morphological feature (here, 1.0 describes a full circle). The determined values are summarized in Table 1. The roundness (measured by the MPD-quantification plug-in) was also assigned to provide a measure of how fluffy the pellets were at 0.05% and 0.1% talcum. The number decreases when angles are formed due to long hyphal growth around the main circle core. The average number was determined to be 0.66, 0.80, and 0.71 for 20, 36 and 56 h, at 0.05% talcum, respectively. At 0.1% talcum, however, the change was less; 0.71, 0.79, and 0.77 for the aforementioned time-points.

### 3.4. Impact of shear stress on macromorphology

The sensitivity of pellets to shear stress and accordingly a change in macromorphology has been observed in many studies. Fazenda *et al.* [5] observed an altered morphology of *Ganoderma lucidum* from pellets in shake flasks (batch phase) to dispersed and fragmented hyphae in the fed-batch phase of a stirred tank bioreactor cultivation where the hydro-mechanical stress is typically 10 times higher than in 2D RMB cultivations as applied in our study. Similar phenomena of *A.niger* pellet fragmentation was reported with both, Rushton turbine and elephant ear impeller at agitation speeds of 600–1000 rpm in a STR [41]. In another report, Maumela *et al.* [18] showed that the breakage of *A. niger*



pellets to fully dispersed mycelia is observable in a STR by enhancing the stirrer speed from 250 to 400 rpm. This was accompanied by an increase in the DO concentration from 8% to 30% of saturation. In a further study [13], the pellet diameter of *A. terreus* was reduced from 1,200  $\mu\text{m}$  in the seed culture to about 700  $\mu\text{m}$  when the agitation rate was 600 and 800 rpm in a 5-L STR. In this case, the highest product titers (in this case lovastatin) were obtained when fluffy pellets with a diameter of 2, 300  $\mu\text{m}$  were formed at a low agitation rate (300 rpm, impeller tip speed 1.02 m/s) and under aeration (1 vvm) with pure oxygen (in order to achieve an oxygen saturation level of about 80% v/v in the gas phase). Higher power input in an 80 m<sup>3</sup> large scale cultivation (energy dissipation/circulation of 29.3 kW m<sup>-3</sup> s<sup>-1</sup>) has also been associated with the cutting of the external layer of pellets of *A. oryzae* causing the formation of shorter hyphae and shaved-off clumps, where clumps only made up for 25% of the final biomass by the end of the batch phase [42].

The reproducibility had been reported to be the bottleneck in many experiments of fungal morphology research [25]. It was previously suggested that the addition of talcum creates more reproducible data in *A. niger* shake flask cultivations [25], which was confirmed in our previous work in a 2D RMB as well [22]. Additionally, since a high reproducibility is observed among the range of the applied talcum concentrations in our study, the bioreactor design and a low shear stress regime should also be considered as contributing factors. A poor reproducibility was observed in our lab-scale cultivations that were conducted in stirred tank reactors. There, a wide variety of morphology with different portions of small fluffy pellets was obtained (data not shown). This might be due to a non-uniform agglomeration of spores at inoculation. It is likely that the addition of talcum leads to a reduced spore agglomeration, and accordingly smaller pellets are formed. Whether the macromorphological form is maintained further or not highly depends on many factors including the shear stress. Further research is needed, however, to prove any hypotheses.

The goal of scaling down is the investigation of the cellular response to fluctuating cultivation conditions as they appear in large scale. For filamentous fungi, this is hardly possible without achieving the same or at least similar macromorphology in lab scale than what is observable in large scale. The aim of our study was to investigate whether it is possible to control the morphology with certain tools and under defined cultivation conditions in order to obtain any desired macromorphology and pellet distribution. If that is possible, any similar macromorphology to the large scale can be achieved in lab scale to study consequences of a different morphology on growth performance, substrate conversion, product secretion and any interaction between cells and environmental growth factors, e.g. media composition or in co-cultivations. The shear stress regime applied in the 2D RMB is, however, different from large scale stirred tank reactors, which are frequently applied in industry. Only a rough comparison of shear rates in STR and 2D RMB can be conducted as this depends on multiple factors like the viscosity, biomass, macromorphology, scale, and impeller design, among others. It was shown that the maximum shear stress that cells are exposed to in a lab-scale stirred tank reactor can reach up to and even beyond 10 Pa at the tips of the impellers as obtained with an Euler-Lagrange based computational fluid dynamics calculation [43]. Zhan *et al.* [44] reported the average shear stress in a 10 L (5 L working volume) 1D RMB for a Newtonian fluid (water) between 0.01 and 0.06 Pa (shear rate of 10–60 s<sup>-1</sup>). The values depend on the speed and angle of the rocker, which varied from 15 to 30 rpm and 4° to 7°, respectively. It is worth to mention that 1D RMBs cannot achieve the  $k_L a$  values of the 2D RMBs. Nevertheless, due to the complexity of calculating fluid dynamics in 2D RMB, no precise data has been obtained yet. In a recent study, fluid dynamics were simulated for an operation at lower angles in the same 2D RMB [32] as used in our study. The setup used there for the application of cell lines requires less gas-mass transfer, eddy formation at the angle of bags, as it occurred in this study with a higher rocking angle, hardly appeared and is not contributing to the shear stress. We believe, however, that the eddy formation, which leads to the necessarily

required gas-mass transfer in our study, is leading to the highest shear stress that occurs in the 2D RMB. Nevertheless, data from similar, not mechanically stirred systems can be taken into consideration for an estimation of shear stress as present in our cultivations when a very similar macromorphology is obtained. The shear stress in a 2D RMB seems to be in the same order of magnitude than the shear stress in shake flasks as seen at the similar macromorphology when no talcum was added. Giese *et al.* [45] showed that for 50 mL to 1000 mL unbaffled flasks, the shear rate is in the range of between 20 and 2000 s<sup>-1</sup> in dependence on the volumetric power input (shaking frequency), filling volume and fluid viscosity. This yields a quite broad range of shear stress if assuming a Newtonian rheological behavior like water. In another study, the shear stress was modeled with both baffled and unbaffled flasks (max diameter of 110 mm, 500 mL volume) at working volumes of 100, 150, 200, and 250 mL and agitation rates of 115 and 220 rpm with water as a model fluid. The average shear stress was calculated to between 0.1 and 1.8 Pa for unbaffled and 0.5 and 3.1 Pa for baffled flasks [46]. This is still substantially lower than the maximum shear stress in a STR, although the impact on cell damage / morphology is usually not well correlated with it under the typically applied turbulent fluid flow conditions, but both, the maximum shear stress and frequency of cells with which they are passing through this zone. The energy dissipation/circulation function (EDCF) considers the specific energy dissipation rate in the impeller swept volume and the frequency of exposure [47,48] and would be a better parameter for comparing conditions. This is, however, not easily applicable between a different reactor design and macromorphology.

#### 4. Conclusion

The possibility of a distinct control of fungal morphology independently from oxygen limitation was proven in a 2D RMB. It was demonstrated that not only obtaining a certain morphology becomes feasible at different talcum concentrations, but also the control of the pellet size is achievable under a comparably low shear stress. Pellet formation was prevented above a certain threshold concentration of talcum. Below it, the pellet size distribution was narrow in comparison to cultivations w/o talcum addition.

The DO profile in 2D RMB cultivations showed the possibility of decoupling the effect of shear stress and altered oxygen supply on the macromorphology as it is the case in stirred tank reactors. The presented concept allows to study effects of a certain morphology on the subsequent cultivation process, e.g. in scale-up. Vice-versa, for the purpose of scale-down, a similar morphology as in large scale cultivations can be achieved in the lab scale for studying consequences of the morphology on the process performance. Detailed mechanisms, however, remain to be examined.

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#### CRediT authorship contribution statement

**Tolue Kheirkhah:** Methodology, Investigation, Validation, Data curation, Visualization, Writing – original draft. **Peter Neubauer:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Stefan Junne:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare the following financial interests/personal



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## Data Availability

Data will be made available on request.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.bej.2023.108905](https://doi.org/10.1016/j.bej.2023.108905).

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