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Microbioreactor-assisted cultivation workflows for time-efficient phenotyping of protein producing *Aspergillus niger* in batch and fed-batch mode

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Abstract

In recent years, many fungal genomes have become publicly available. In combination with novel gene editing tools, this allows for accelerated strain construction, making filamentous fungi even more interesting for the production of valuable products. However, besides their extraordinary production and secretion capacities, fungi most often exhibit challenging morphologies, which need to be screened for the best operational window. Thereby, combining genetic diversity with various environmental parameters results in a large parameter space, creating a strong demand for time-efficient phenotyping technologies. Microbioreactor systems, which have been well established for bacterial organisms, enable an increased cultivation throughput via parallelization and miniaturization, as well as enhanced process insight via non-invasive online monitoring. Nevertheless, only few reports about microtiter plate cultivation for filamentous fungi in general and even less with online monitoring exist in literature. Moreover, screening under batch conditions in microscale, when a fed-batch process is performed in large-scale might even lead to the wrong identification of optimized parameters. Therefore, in this study a novel workflow for *Aspergillus niger* was developed, allowing for up to 48 parallel microbioreactor cultivations in batch as well as fed-batch mode. This workflow was validated against lab-scale bioreactor cultivations to proof scalability. With the optimized cultivation protocol, three different micro-scale fed-batch strategies were tested to identify the best protein production conditions for intracellular model product GFP. Subsequently, the best feeding strategy was again validated in a lab-scale bioreactor.

KEYWORDS
fed-batch, heterologous protein production, microbioreactor, microtiter plate cultivation, online monitoring

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Roman Jansen and Holger Morschett contributed equally to this study.

1 | INTRODUCTION

Starting with the commercial production of citric acid in 1919 where the first large-scale process utilizing filamentous fungi was established mostly without knowledge of basic molecular and bioprocess principles,[1,2] filamentous fungi have been established as one of the major workhorses in industrial biotechnology. Nowadays a billion dollar industry has been established, utilizing these cell factories for the manufacture of value-added products.[3] The natural portfolio of products is very diverse and consists of organic acids, secondary metabolites, antibiotics, proteins and enzymes.[3-6] A general advantage of filamentous fungi is their extraordinary secretion and production capacity. Especially *Trichoderma* and *Aspergillus* strains are known for their capability to secrete high amounts of enzymes, making them interesting platforms for industrial production. For example, up to 30 g L^{-1} of amylases and cellulases can be produced with *A. niger* and *T. reesei*, respectively.[7,8]

More than 250 species are known in the genus *Aspergillus*[9] with industrially relevant examples being *A. niger*, *A. oryzae*, *A. awamori*, *A. sojae*, and *A. terreus*. Generally, *Aspergillus* can withstand and endure harsh environmental conditions. With a wide range of tolerated temperatures (10–50°C), pH values (2–11) and water activities, these organisms are very robust.[10-12] As reviewed by Ward et al.,[13] members of the genus *Aspergillus* are capable of metabolizing various complex substrates, such as hemicellulose and cellulose, as well as pectin and xylan. In combination with their extraordinary production and secretion capacity, the utilization of renewable substrates allows for the efficient and potentially cheap manufacture of value-added products. Consequently, *Aspergillus* has become one of the key players for industrial biotechnology. Commercial applications are in different industries, such as food, pharma, feed, paper, pulp, and biofuels.[14]

Since 2010 more than ten genomes of various *Aspergillus* species have been made publicly available.[15] In combination with novel high-throughput gene editing technologies being adapted for filamentous microorganisms,[16] an increase in the product portfolio and subsequently the industrial manufacture of novel products is to be expected. This enables the utilization of *Aspergillus* as a multi-purpose cell factory in the future.

In contrast to bacterial organisms, filamentous fungi follow a distinctive life cycle. Beginning with spore germination under beneficial environmental conditions, spore swelling is followed by germ tube formation. These grow larger and form hyphae, compartmentalized by septa. At a certain size, hyphae start branching, leading to a complex interconnected hyphal network, the so-called mycelium.[14] Sporulation may again be initiated upon shifting to unfavorable environmental conditions. This change in morphology becomes especially apparent in submerged cultivation, creating challenging conditions for reproducible bioprocesses.

As thoroughly discussed by Veiter et al.,[17] there is often a complex interplay between process conditions, predominant morphology and productivity during biotechnological production with filamentous fungi. While pelletous growth has clear advantages with respect to culture broth viscosity and thereby also energy input needed for mixing and oxygen transfer, microfilamentous growth is beneficial for homogeneous nutrient supply. Although there is indication that pelletous morphology may be preferable for the production of organic acids while mycelial growth seems to support enzyme production,[18] there are no generally applicable rules established regarding preferable morphological state for manufacture of different products. Therefore, many screening experiments must be conducted making cultivation with an increased throughput highly desirable while integrated online monitoring can help to obtain better process insight and understanding.

Although microbioreactor (MBR) systems have proved to be valuable tools for such applications,[19] only few reports on their successful application for filamentously growing fungi can be found in literature, among these being for example Hortsch et al.,[20] Huth et al.,[21] or Jansen et al..[22] In the study presented here, MBR-assisted workflows for parallelized microscale cultivation were developed for *A. niger* producing green fluorescent protein (GFP) as a model for heterologous protein production. Special emphasis was put on achieving reproducible process patterns under batch, as well as strictly controlled fed-batch conditions. As literature reports about improved enzyme production in *A. niger* by tailoring processes toward mycelia rather than pellets,[18] maintaining microfilamentous morphology during microscale cultivation runs was targeted as the desired morphological state in this study.

2 | MATERIAL AND METHODS

2.1 | Chemicals, strain, and media

All chemicals were of analytical grade and obtained from either Sigma-Aldrich (Steinheim/Germany) or Roth (Karlsruhe/Germany). *Aspergillus niger anip7-gfp2* (formerly denoted as *A. niger* ARAn701) was kindly provided by Prof. Spieß and Mathias Papenfuß at the Technische Universität Braunschweig (Germany). The strain is deviated from the protease deficient *A. niger* AB1.13[23] and features the recombinant expression of GFP under control of the *glaA* promotor. Details on strain construction are provided by Driouch et al.[24]

Yeast extract peptone dextrose medium (YEPD) consisted of 10 g yeast extract, 20 g peptone and 20 g glucose per liter water and was set to pH 3.8 with sulfuric acid.

Potato dextrose agar medium (PDA) consisted of 39 g potato dextrose agar (Sigma-Aldrich, Steinheim/Germany) per liter water.

Sinha medium[25] consisted of 80 g glucose, 6 g NaNO$_3$, 1.52 g KH$_2$PO$_4$, 0.52 g KCl, 0.52 g MgSO$_4\cdot$7 H$_2$O, 50 mg ethylendiamintetraacetic acid (EDTA), 2.25 mg ZnSO$_4\cdot$7 H$_2$O, 11 mg H$_3$BO$_3$, 5 mg MnCl$_2 \cdot$ 2 H$_2$O, 5 mg FeSO$_4 \cdot$ 7 H$_2$O, 1.7 mg CoCl$_2 \cdot$ 6 H$_2$O, 1.6 mg Na$_2$MoO$_2 \cdot$ H$_2$O, 1 mg biotin, 1 mg pyridoxol, 1 mg thiamine, 1 mg riboflavin, 1 mg p-aminobenzoic acid and 1 mg nicotinic acid per liter water and was set to pH 3.8 with hydrochloric acid.

Linde medium[26] consisted of 140 g sucrose, 2.48 g NH$_4$NO$_3$, 2.45 g KH$_2$PO$_4$, 0.25 g MgSO$_4\cdot$7 H$_2$O, 0.23 mg CuSO$_4\cdot$5 H$_2$O, 1.09 mg ZnSO$_4\cdot$7 H$_2$O, 5.56 mg FeSO$_4\cdot$7 H$_2$O per liter and was set to pH 3.8 with hydrochloric acid.

Vogel medium[27] consisted of 10 g xylose (preculture only), 5 or 20 g glucose (mainculture only, depending on experiment), 6.6 g (NH$_4$)$_2$SO$_4$, 2.5 g KH$_2$PO$_4$, 0.2 g MgSO$_4\cdot$7 H$_2$O, 0.1 g CaCl$_2\cdot$2H$_2$O,

5 mg $C_6H_8O_7 \cdot H_2O$, 5 mg $ZnSO_4 \cdot 7 \, H_2O$, 1 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6 \, H_2O$, 0.16 mg $CuSO_4$, 0.5 mg $MnCl_2 \cdot 4 \, H_2O$, 0.05 mg H_3BO_3, 0.037 mg $MnSO_4 \cdot H_2O$, and 0.05 mg $Na_2MoO_4 \cdot 2H_2O$ per liter and was set to pH 3.8 with hydrochloric acid.

2.2 | Strain maintenance

For spore generation, 100 μl of an existing spore suspension was plated on PDA plates and incubated at 37°C for 5–7 days in an incubation chamber. After full sporulation, the spores were collected through addition of 1 ml cryoprotectant solution containing 20% (w v^{-1}) glycerol and 0.9% (w v^{-1}) NaCl and scrapping with a spatula. The suspension was filtered through a self-made cotton filter to remove loose mycelium. Subsequently, the spore concentration was determined utilizing a Neubauer chamber and adjusted to 10^8 spores ml^{-1} with cryoprotectant solution. Aliquoted stocks were stored at −80°C until further use.

2.3 | Microbioreactor cultivation

All microscale cultivations were conducted in either a BioLector I (batch and robotic dosing) or BioLector Pro (microfluidic feeding) MBR (m2p-labs, Baesweiler/Germany). Detailed description of the devices can be found elsewhere.[28-30] In short, utilization of the Bio-Lector platform enables cultivation in specialized microtiter plates under controlled environmental conditions, with a set shaking frequency, temperature, and humidity, while several process parameters are measured non-invasively by optical methods. Microtiter plates used for cultivation are sealed with specialized foils facilitating gas transfer while maintaining (cross-)contamination-free conditions.

Standard batch cultivations were run in 48-well FlowerPlates (MTP-FP84-BOH1) sealed with gas-permeable sealing foils (F-GPRS48-10) at 1000 μl liquid volume and 1300 rpm shaking speed. FlowerPlates are specialized microtiter plates for BioLector applications that provide high mass transfer similar to bioreactors as typically needed during aerobic cultivation.[31]

For robotic dosing experiments, the MBR was integrated into a customized Freedom Evo 200 liquid handling platform (Tecan, Männedorf/Switzerland). The robot was equipped with polytetrafluoroethylene-coated steel needles that were used to repeatedly dose 10 μl substrate pulses (80 g L^{-1} maltose) into the culture wells with continuous shaking of the cultivation MTP while initial culture volume was reduced to 800 μl.

Microfluidic feeding experiments were conducted in microfluidic FlowerPlates (MTP-MF32C-BOH1) sealed with special sealing foils (F-GPRSMF32-1) at 1400 rpm shaking speed, 35% headspace oxygen concentration and 800 μl initial culture volume. 400 g L^{-1} maltose was filtrated (0.2 μm cellulose acetate) to prevent potential blocking of microfluidic channels by any solid particles and served as feeding solution.

General conditions for all experiments were 37°C, ≥85% relative humidity and inoculation of cultures either to 10^5 spores ml^{-1} or with 10% (v v^{-1}) preculture suspension as stated in the individual experiments. Biomass and GFP were non-invasively monitored via scattered light at 620 nm wavelength, hereinafter referred to as "backscatter" and fluorescence (488/520 nm), respectively. In case of volume changes due to robotic or microfluidic feeding, measured values were normalized to the initial culture volume. Dissolved oxygen (DO) was measured via immobilized sensor spots on the bottom of the plate. It has to be noted that absolute values from intensity measurements (backscatter and GFP) often differ from device to device due to technical reasons and are therefore not comparable across different machines. To compensate for that issue, individual experimental runs have been executed with a reference batch process, which was used for data normalization and enabled cross comparison of cultivation data from different runs. Therefore, figures from Sections 3.3 to 3.5 show individual reference batches instead of referring to a single, general reference.

2.4 | Lab-scale cultivation

All stirred tank bioreactor cultivations in this study were carried out in 1.8 L stirred tank lab-scale bioreactors with two Rushton stirrers (DASGIP, Jülich/Germany). The reactors were equipped with probes for pH (405-DPAS-SC-K8S/225/120, Mettler-Toledo, Urdorf/Switzerland) and DO (Visiferm DO225, Hamilton/Switzerland). Upon reactor setup, distilled water and the carbon sources were sterilized directly in the glass vessel, whereas all remaining components were added aseptically afterward resulting in 700 ml initial culture volume. The medium was either inoculated to 10^5 spores ml^{-1} or with 10% (v v^{-1}) from a preculture. Reactors were always run at 37°C and 1200 rpm constant stirring. DO was regulated to ≥30% via adjusting the gassing with compressed air. In case of excessive foaming, 0.5 ml dosages of 10% (w v^{-1}) AF204 antifoam (Sigma, St. Louis) were added.

2.5 | Cell dry weight

1000 μl cell suspension was loaded onto a pre-dried (24 h at 80°C and subsequently cooled to room temperature in a desiccator) and pre-weighed centrifuge filter tube (SpinX, Costar, New York) with a 0.22 μm cellulose acetate membrane. The tubes were centrifuged for 3 min at 13,000 g. The cell-free supernatant was aliquoted and stored at −20°C for further analysis. The retentate was washed twice with 500 μl 0.9% (w v^{-1}) NaCl and the flow-through was discarded. The washed pellet was then dried at 80°C for 24 h, cooled to room temperature in a desiccator and weighted on a precision scale. Cell dry weight (CDW) was calculated from the mass difference.

2.6 | Offline pH

Offline pH was measured electrochemically in crude cell suspension utilizing an S20 SevenEasy pH meter with a 6.0234.100 micro

electrode (Metrohm, Filderstadt/Germany). All samples were measured at room temperature.

2.7 | Offline GFP

Offline GFP was measured in clear flat-bottom Greiner 96-well plates in an Infinite 200Pro photometer (Tecan, Männedorf/Switzerland). The photometer was set to an excitation wavelength of 488 nm and emission wavelength of 520 nm to detect GFP fluorescence in technical triplicates. A filling volume of 100 µl crude cell suspension was used for each measurement.

2.8 | Statistical analysis

The relative mean coefficient of variation of backscatter measurements was calculated as an indicator for reproducibility. The arithmetic mean $\bar{x}_i$ of eight biological replicates and the corresponding standard deviation s_i of each backscatter measurement was calculated for each measurement cycle i. The coefficient of variation $c_{v,i}$ was calculated according to Equation (1).

$$c_{v,i} = \frac{s_i}{\bar{x}_i} \tag{1}$$

The sum of $c_{v,i}$ for all data points with an increase in scattered light measurement above the limit of detection ($\alpha = 0.01$; $i = 1$) until the end of cultivation (n) was divided by the number of cycles (m) to calculate relative mean coefficient of variation (Equation (2)).

$$rmc_v = \frac{\sum_{i=1}^{i=n} c_{v,i}}{m} \tag{2}$$

3 | RESULTS AND DISCUSSION

3.1 | Establishment of a microbioreactor batch cultivation procedure for *A. niger*

Investigations on microtiter plate-based cultivation of *Aspergillus species* have been reported in literature and a wide variety of cultivation conditions (plate geometry, shaking speed, cultivation media, addition of morphology controlling agents, etc.) has been evaluated to obtain reliable workflows for reproducible cultivation in the desired morphological state. Very recently there has been a focus on the utilization of online monitoring of relevant parameters such as biomass to obtain deeper process insight when using such microscale systems.[21,22,26] As a starting point for establishing such cultivation procedure, a promising subset of conditions reported in Jansen et al.[22] with *A. giganteus* was tested for applicability with the *A. niger* strain addressed in this study. Individual cultures were always inoculated from defined spore material and results from selected conditions are shown in Figure 1.
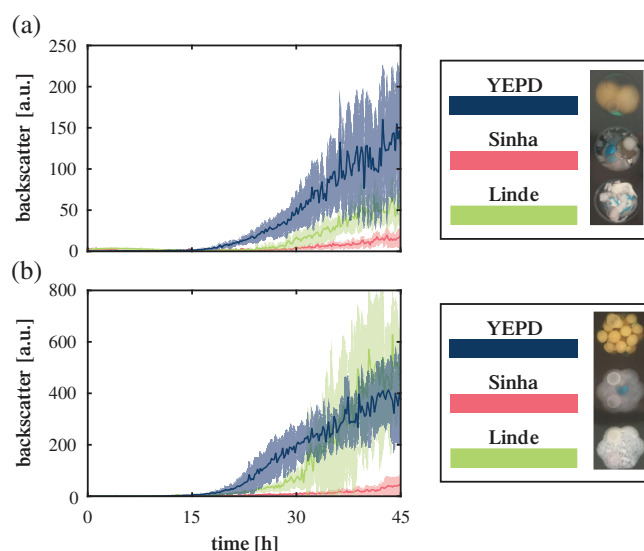

FIGURE 1 Microbioreactor cultivation of *A. niger* in selected media and different plate geometries. (a) Round Well Plate. (b) FlowerPlate. Cultivation at 37°C, 600 rpm, 1000 µl initial culture volume (YEPD, Sinha and Linde medium inoculated with 10^5 spores ml^{-1}) and ≥85% relative humidity. Mean and errors deviated from biological replicates ($n = 8$). Images from the bottom of the microtiter plate show corresponding macromorphology

While during 45 h of incubation only a marginal increase in backscatter intensity (as a measure for biomass) could be seen when applying Sinha medium, growth of *A. niger* could be observed in YEPD and Linde medium. However, reproducibility between biological replicates was exceptionally low irrespective of medium and MTP type used as indicated by relative standard deviations of up to 80%.

Microscopic imaging revealed that YEPD medium resulted in pelletous growth while Sinha and Linde medium resulted in the formation of mycelial clumps of varying size. As a general trend, smaller structures could be observed when using FlowerPlates instead of Round Well Plates. This was to be expected as such baffled shaken bioreactors feature a higher power input compared to non-baffled vessels[32] and thus introduce higher shear forces which are likely to break larger into smaller biomass structures.

Although the applied conditions enabled growth of *A. niger* during MTP cultivation in general, none of the tested parameter sets resulted in satisfying results. As shown for selected conditions (Figure 1), either irreproducible growth as shown by noisy backscatter measurements or pellet growth in the millimeter-range could be observed. This observation highlights a major problem in process development for filamentous fungi: Cultivation conditions can typically not be transferred from one strain directly to another but need individual testing and adaptation.

Based on indications from literature,[33] cultures were run in defined medium according to Vogel et al.[27] as an alternative setup and shaking speed was raised to 1300 rpm in FlowerPlates to force breakdown of pellet structures by increased power input and shear forces, respectively. Cultivation was again started by inoculation to 10^5 spores ml^{-1}.

Strikingly, under these conditions, highly reproducible growth behavior could be achieved with an rmc$_v$ of 8.3% (Figure 2a). While no

increase in backscatter values could be detected during the first 20 h of cultivation, total batch time until depletion of the carbon source was approx. 39 h. During the following stationary phase, no further changes in backscatter could be detected. The first process phase most probably represents the germination phase of the spores when transferred to fresh medium. As shown by microscopic imaging of culture broth at harvest after approx. 48 h of incubation, the applied settings were suitable to break down pellets and to obtain the desired microfilamentous morphology with free mycelium and only few loose aggregates.

The strain focused during this study features an expression cassette for intracellular GFP production as a model system for heterologous protein expression with *A. niger*. Despite biomass growth observed in the different media tested, no increase in GFP fluorescence could be seen along the complete runtime of the process (Figure 2b). This was to be expected since xylose used as sole carbon source acts as a strong repressor of the expression cassette and thereby, production of the GFP model protein does not take place under the conditions applied, yet.

Targeting a process that can be scaled-up beyond laboratory scale bioreactors, a protocol as described above might be problematic as long germination times, which contrast with high space–time-yields, have to be considered. In addition, the quantities of spores required for the inoculation of larger reactors cannot be provided at reasonable expense. Against this background an alternative seed train-based setup was tested in which the culture described above was harvested after 44 h incubation and the microfilamentous biomass was used as a preculture for 10% (v v^{-1}) inoculation of a subsequent main culture. In this way, the need for sporulation material can be drastically reduced for future applications at potentially larger scale. While keeping all other components of the Vogel medium identical, the carbon source was switched from xylose in preculture to maltose in the main culture to enable target protein expression.

Compared to the previous workflow, starting the main cultivation from a preculture instead of direct inoculation with spores proved beneficial as time until depletion of the primary carbon source was reduced to 15 h (−62%), mainly due to skipping of the spore germination phase (Figure 3a). In contrast to preculturing on xylose where backscatter remained constant after depletion of the carbon source (Figure 2a), in this case after depletion of maltose a further increase in backscatter by approx. 50% could be detected until stopping the cultivation. Although it was not tested if this observation was at least partially supported by a change in morphology, it might be speculated that further biomass growth took place upon metabolization of intracellular storage compounds or secondary carbon sources.
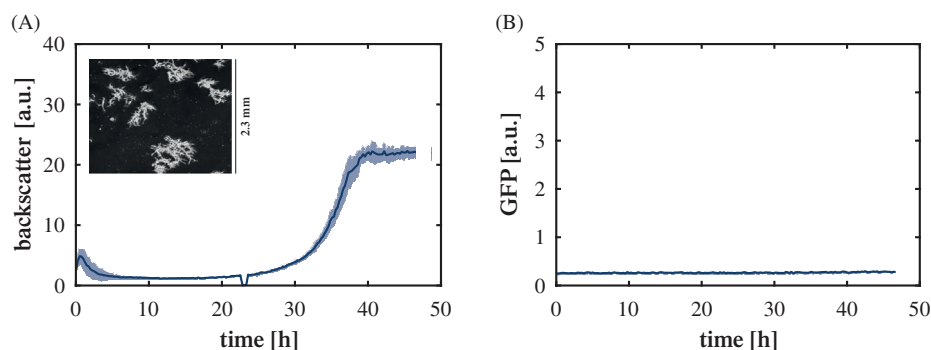

FIGURE 2   Microbioreactor cultivation of *A. niger* in defined Vogel medium. (a) Biomass by backscatter and microscopic image (40×) after depletion of carbon source. (b) GFP by fluorescence. Cultivation in a FlowerPlate at 37°C, 1300 rpm, 1000 μl initial culture volume (defined Vogel medium with 10 g L^{-1} xylose inoculated with 10^5 spores ml^{-1}) and ≥85% relative humidity. Mean and errors deviated from biological replicates (*n* = 16)
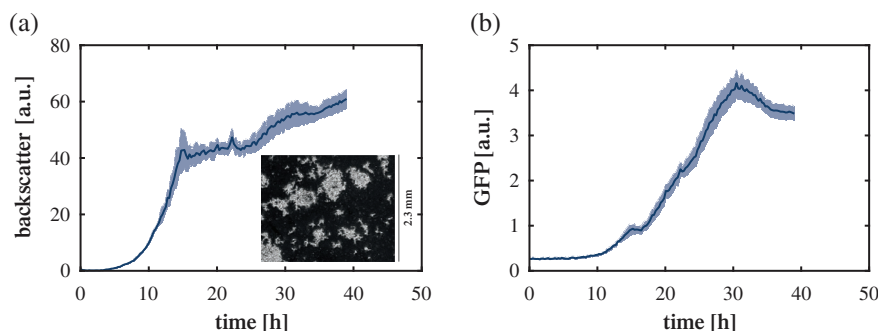

FIGURE 3   Microbioreactor cultivation of *A. niger* in Vogel medium after inoculation from a preculture. (a) Biomass by backscatter and microscopic image (40×) after depletion of carbon source. (b) GFP by fluorescence. Cultivation in a FlowerPlate at 37°C, 1300 rpm, 1000 μl initial culture volume (defined Vogel medium with 20 g L^{-1} maltose inoculated with 10% (v v^{-1}) preculture) and ≥85% relative humidity. Mean and errors deviated from biological replicates (*n* = 16)

While GFP expression was repressed during preculture on xylose (Figure 2b), induction took place upon the switch to maltose in main cultivation. Correspondingly, protein production could be tracked by online monitoring of GFP fluorescence during culture growth (Figure 3b). Despite it was noted that at first protein accumulation goes alongside with growth on maltose as monitored by backscatter measurement, a remarkable observation could be made: Incubation was continued for additional 24 h after depletion of the carbon source maltose and a monotonous increase of GFP fluorescence by approx. 195% was observed until reaching a maximum at 30.6 h process time. This timeframe goes far beyond reported maturation times for GFP[34] giving strong evidence for sustained expression of the target protein. Strikingly, around two thirds of total target protein production took place after metabolization of the initially provided maltose. Although this finding was not investigated in further details at this point, it seems very likely that heterologous protein expression during "stationary phase" might be driven by usage of secondary carbon sources or storage compounds as already hypothesized for biomass increase seen during this phase (Figure 3a).

3.2 | Biomass calibration for microbioreactor cultivation of A. niger

Despite A. niger exhibited a homogeneous microfilamentous morphology when the main culture was inoculated with 10% (v v^{-1}) mycelium, it needs to be checked if backscatter measurements can be used as a valid measure for biomass concentration. Therefore, a set of 21 biological replicates was cultivated under the exact conditions as applied above. At seven different time points distributed alongside the cultivation process, three wells were harvested per sampling event for offline CDW measurement and subsequent correlation to the respective backscatter readings.

For the sample time series acquired a linear correlation (Backscatter = 5.24·CDW − 3.37) between the both biomass measurement methods was revealed, while the lowest CDW taken into calibration was approx. 0.35 g L^{-1} (Figure 4). Consequently, backscatter may be
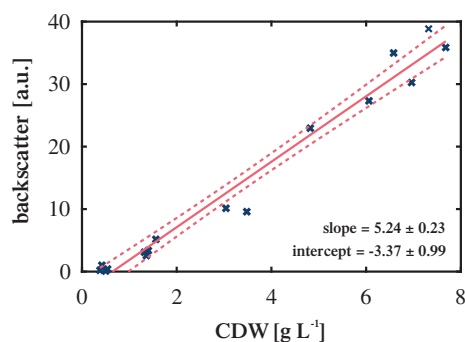

slope = 5.24 ± 0.23
intercept = -3.37 ± 0.99

FIGURE 4 Backscatter–CDW correlation of microfilamentous microscale batch cultivation. Cultivation in a FlowerPlate at 37°C, 1300 rpm, 1000 μl initial culture volume (defined Vogel medium with 20 g L^{-1} maltose inoculated with 10% (v v^{-1}) preculture) and ≥85% relative humidity. Mean and errors deviated from biological replicates (n = 3)

used as a valid online measure for A. niger biomass concentration. Here, it must be emphasized that calibration was not done by a dilution series of culture broth from one single point of the cultivation and thereby state of morphology (e.g. after harvest) but proved applicable along the complete culture process.

3.3 | Comparison of A. niger microbioreactor and lab-scale bioreactor cultivation

Having established a reliable setup for batch cultivation of A. niger in online-monitored microtiter plates (Section 3.1) enables for accelerated process development and phenotyping of such fungi. To use and apply the newly generated data sensibly, the transferability to conventional, stirred lab-scale bioreactors must be guaranteed.

Therefore, the cultivation conditions described at the end of Section 3.1 were applied both in a MBR as well as a lab-scale bioreactor. Bioreactor conditions were 700 ml liquid volume, 1200 rpm and 10 NL h^{-1} gassing with compressed air. To maximize comparability of the settings, pH in the reactors was not controlled just as for the microscale cultivations. Processes were run in parallel and sampled for offline measurement of CDW, pH, GFP fluorescence (Figure 5). Strikingly, a good overlay for all offline measurements between the different systems can be seen. CDW increased until 20 h cultivation time up to a value of ~12 g L^{-1}, while the pH decreased from approx. 3.3 to 1.8 upon maltose consumption. The GFP product titer increased during biomass accumulation in the first 20 h to a value of 21.84·10^2 a.u. for both systems, it further increased during the next 19 h up to 32.15·10^2 a.u. and 26.66·10^2 a.u. for the BioLector and bioreactor cultivation respectively, while the biomass concentration stagnated. The GFP signal increase after the main growth phase might be due to the consumption of overflow metabolites or storage compounds, as well as maturation effects of GFP. However, since GFP maturation time is expected within shorter times,[34] a potential delay of GFP chromophore development from previously built non-fluorescent material is regarded unlikely.

Even though cultivation scale differed by a factor of 700, a good overlay between lab-scale bioreactor and microscale cultivation can be seen for the process tested with respect to the offline measurements performed. Therefore, the developed microscale workflow may be regarded as a suitable tool for the prediction of lab-scale cultivation of the A. niger model strain used.

3.4 | Development of fed-batch processes for microscale cultivation of A. niger

The relevance of fed-batch cultivations already at a small scale has been discussed in literature such as Scheidle et al.[35] Of course, this also applies for filamentous fungi such as A. niger, since an increasing number of processes are performed in fed-batch mode, for example, to limit mass transfer and cooling capacity or for the sake
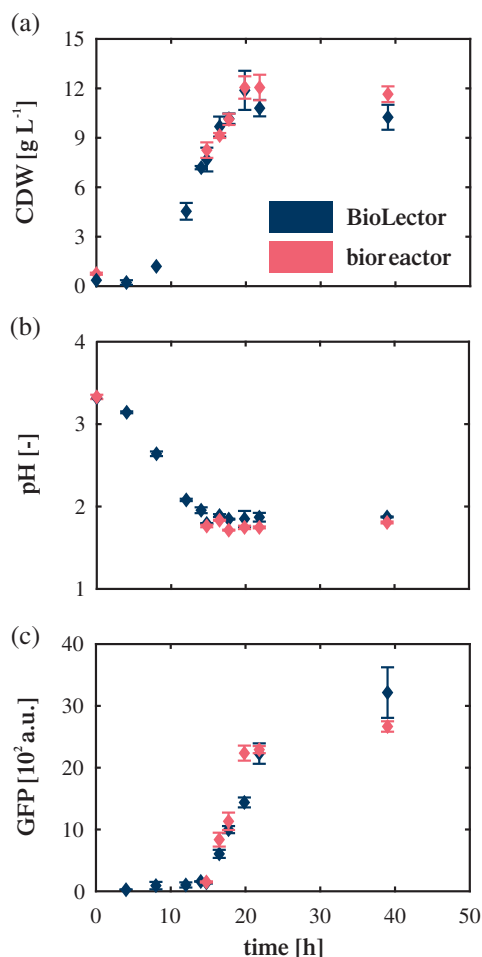
FIGURE 5　Comparison of *A. niger* microbioreactor and lab-scale bioreactor batch cultivation. (a) Biomass by CDW. (b) extracellular pH. (c) GFP by fluorescence. BioLector cultivation in a FlowerPlate at 1300 rpm, 1000 µl initial volume and ≥85% relative humidity. Bioreactor cultivation at 37°C, 1200 rpm, 700 ml initial volume and 10 NL min^{-1} gassing with compressed air (DO ≥30%). Common cultivation settings were 37°C and defined Vogel medium with 20 g L^{-1} maltose inoculated with 10% (v v^{-1}) preculture. Mean and errors deviated from biological replicates (*n* = 3)

of production related performance indicators.[36,37] Ideally, novel workflows allowing for microscale fed-batch cultivations described for bacteria should also be transferred and applied to filamentous MBR cultivations. Since *Aspergillus* as well as most other fungal species can metabolize various sugar polymers, the FeedER technology[38] or other enzymatic slow-release systems[39,40] are not applicable in the present case. Only recently, a further strategy enabling MBR fed-batch cultivation has been published: Through integration of polymer rings, diffusion-based fed-batch cultivation could be performed in MTPs without losing the possibility of conducting online measurements through the plate bottom[41] as is the case for standard MTP systems for diffusion-based slow-release.[42] However, for the cultivation of *A. niger* it seems advantageous to utilize FlowerPlates to increase shear forces toward microfilamentous morphology and to provide the mass transfer needed to avoid an oxygen limitation.

Therefore, two alternative strategies were tested to enable microscale fed-batch for *A. niger*: As the first one, robotic-assisted feeding was tested. Here, small pulses of a concentrated sugar stock are dosed into each cultivation well on either pre-defined time intervals or triggered by the respective DO signal.[43] The second strategy relies on microfluidic MTPs where cultivation wells can be flexibly supplied with the feedstock through microfluidic channels in the bottom of the plate.[30,44]

3.4.1 | Pulsed feeding by a liquid handling robotic platform

As a very simple approach, pulsed dosing with 15 predefined time points was evaluated. After an initial batch phase on 5 g L^{-1} maltose, a total of 15 g L^{-1} was added with 10 µl pulse size (0.8 mg maltose) at an hourly interval. The intermittent pulsed feeding strategy was compared to a batch cultivation where the same amount of total carbon source was provided right from the beginning (Figure 6).

In the batch process metabolization of provided carbon source was completed within 15 h as indicated by the rise of the DO signal. However, mass transfer provided by the system was not sufficient to keep the cultivation process fully aerobic as an oxygen limitation during the last 2 h of maltose consumption could be detected. In subsequent hours of cultivation, a further increase of backscatter values could be measured which is in good agreement with previous results. The course of the DO signal suggests metabolization of storage compounds or secondary carbon sources in the phase from approx. 25–35 h.

Target protein production as monitored by GFP fluorescence slightly increased up to a value of 1 a.u. being coupled to growth until the depletion of primary carbon source maltose as indicated by the spike of the DO signal after approx. 15 h. However, product formation increased strongly during the subsequent phase. GFP fluorescence reached its maximum of approx. 4 a.u. after 30 h of cultivation, at which time the DO signal also went back up toward 100%, indicating the end of metabolic substrate consumption. Please note, that it seems that approx. 80% of total GFP signal was observed after primary growth phase.

After 30 h, the GFP signal began to steadily decrease which might be explained by a change in intracellular pH: Under regular metabolic conditions, energy metabolites can be used to drive active ion channels for maintaining intracellular pH homeostasis within the neutral regime.[45] However, upon depletion of all carbon sources including storage compounds and/or secondary carbon sources, the resulting exhaustion of energy metabolite pools might result in the cessation of the activity of the ion pumps. As a result, the intracellular proton level begins to equalize to extracellular acidic levels resulting in a loss of GFP fluorescence.[46]

The pulsed feeding strategy was conducted as stated: Following an initial batch phase on 5 g L^{-1} maltose, pulsed feeding was started after 15 h. The backscatter increased linearly until the last pulse
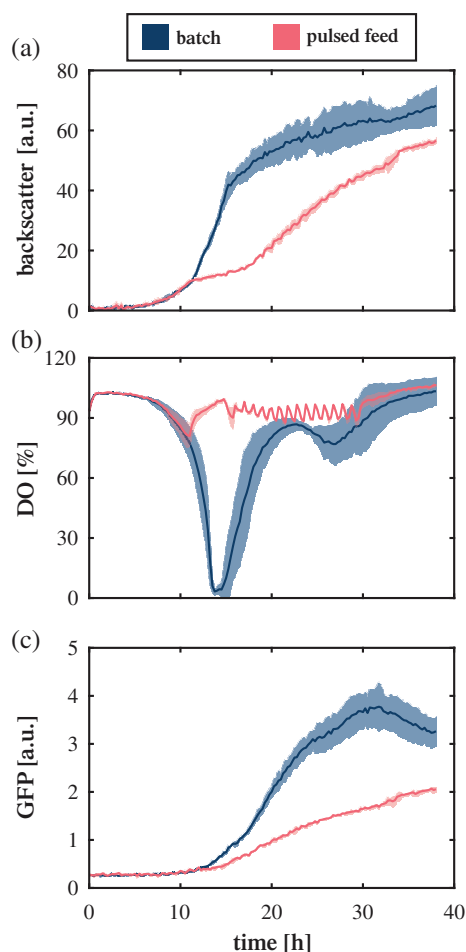
FIGURE 6 Robotic-assisted pulsed fed-batch cultivation. (a) Biomass by backscatter. (b) DO. (c) GFP by fluorescence. Cultivation in a FlowerPlate at 37°C, 1300 rpm, 800 μl initial culture volume (defined Vogel medium inoculated with 10% (v v^{-1}) preculture) and ≥85% relative humidity. Reference batch with 20 g L^{-1} maltose. Robotic feeding of 15 times 10 μl (0.8 mg maltose) after initial batch on 5 g L^{-1} maltose. Mean and errors deviated from biological replicates (n = 3)

was added after 30 h but reached approx. 15% less signal intensity than the batch reference. Throughout the whole cultivation, aerobic conditions could be effectively ensured. During the fed-batch phase, an oscillating DO signal could be observed between 100% and 90%. Strikingly, the maximum product titer achieved was only 2 a.u. and thereby reduced by 50% in contrast to the batch reference. Most likely, this was due to the feeding strategy, causing an oscillating effect between starvation and sugar excess. Such pulsing might not seem disadvantageous at first as the applied glucoamylase promotor system is strongly induced by maltose, but can on the other hand lead to intermediate accumulation of glucose from maltose hydrolysis. Contrary to maltose as an inducer, glucose acts as a strong repressor of the expression cassette as reported in Ganzlin and Rinas[47] where an oscillation between repression and derepression seems to be induced by the pulsed feeding. To

minimize this effect, the amount of maltose added per pulse needs to be reduced. Due to technical limitations of the robotic system, it is not possible to add smaller pulses than 10 μl without strongly reduced pipetting accuracy and the use of a less concentrated feedstock cannot be recommended as it results in significant dilution of the culture volume. Noteworthy, the technical implementation of this feeding profile for filamentous fungi seems to work quite well, even though the biological system in this case cannot benefit from it as it has been the case in studies on other biological systems reported in literature.[43]

3.4.2 | Microfluidic feeding

Microfluidic MTPs enable quasi-continuous feeding by nanoliter pulse addition through small channels in the bottom of the plate.[30,48] A cultivation with an initial batch on 5 g L^{-1} maltose was conducted and after 15 h different feeding modes were started. On the one hand a constant feed of 2 μl h^{-1} (0.8 mg h^{-1} maltose) was applied for 15 h. On the other hand, a regime with a total of 15 hourly pulses of 2 μl (0.8 mg maltose) was performed, which exactly resembles the previous pulsed feeding strategy. Thus, all processes featured the same amount of total maltose fed (15 g L^{-1}) as already realized during robotic feeding in Section 3.4.1. Again, a batch cultivation with the same amount of total carbon source was run in parallel to serve as a reference. Growth as well as DO levels and GFP product formation were monitored non-invasively as summarized in Figure 7. Additionally, photographic imaging was applied to verify that the fungi did not grow into the microfluidic channels of the MTP (Figure S1).

Complete consumption of the primary carbon source is likely to have occurred within 15 h for the reference batch, as indicated by the sharp rise of the DO signal (Figure 7d). At this point the backscatter is about 49 a.u., but slowly increases within the next 20 h, indicating a possible consumption of side products or storage compounds. The maximal GFP titer is reached after 25.5 h with a value of approx. 4.5 a.u. Afterward, the GFP fluorescence decreases strongly, as previously seen, most likely due to the loss of intracellular pH homeostasis. With respect to the measures described, the reference batch was very similar to previous results (Figure 6).

The fed-batch cultivations are prolonged due to carbon-limited feeding until 30 h but at harvest backscatter values comparable to the batch reference were achieved. Strikingly, the microfluidic pulsed feeding regime also resulted in GFP production patterns very similar to the batch reference which is in direct contrast to the robotic pulsed feeding where only reduced titers could be achieved (Figure 6). Despite pulses of 0.8 mg maltose were dosed in both cases, a subtle but probably important difference must be considered: Whereas during robotic dosing the full volume of the respective pulse is added within less than a second, microfluidic pulse addition takes approx. 1.5 min for a single substrate pulse. Thus, in the microfluidic application substrate oscillations are reduced

compared to the robotic workflow, so that performance loss as observed during robotic feeding could be circumvented. In case of microfluidic feeding at constant rate, the product formation was further increased up to 6.02 a.u. (+33%) confirming the hunch that strict carbon limitation and thereby avoidance of glucose repression or slowing down of growth in general is highly beneficial for target protein expression.

3.5 | Lab-scale validation of fed-batch processes

To validate the increased productivity achieved by constant rate feeding at microscale and to check for its transferability, lab-scale cultivations with identical feeding profiles were conducted. After an initial batch of 5 g L^{-1} that lasted approx. 8 h, a total of 15 g L^{-1} maltose was fed at constant rate over the duration of 15 h. Due to lack of

FIGURE 7 Microfluidic MBR fed-batch for *A. niger*. (a, d, g) Biomass by backscatter. (b, e, h) DO. (c, f, i) GFP by fluorescence. Cultivation in a microfluidic FlowerPlate at 37°C, 1400 rpm, 1000 μl initial culture volume (defined Vogel medium inoculated with 10% (v v^{-1}) preculture), 35% headspace oxygen and ≥ 85% relative humidity. Reference batch with 20 g L^{-1} maltose. Microfluidic feeding either for 15 h at 2 μl h^{-1} (0.8 mg h^{-1} maltose) constant rate or by hourly dosing of 15 times 10 μl (0.8 mg maltose) after initial batch on 5 g L^{-1} maltose. Mean and errors deviated from biological replicates (*n* = 4 in batch, *n* = 8 in fed-batch). * Masked oxygen limitation due to calibration offset
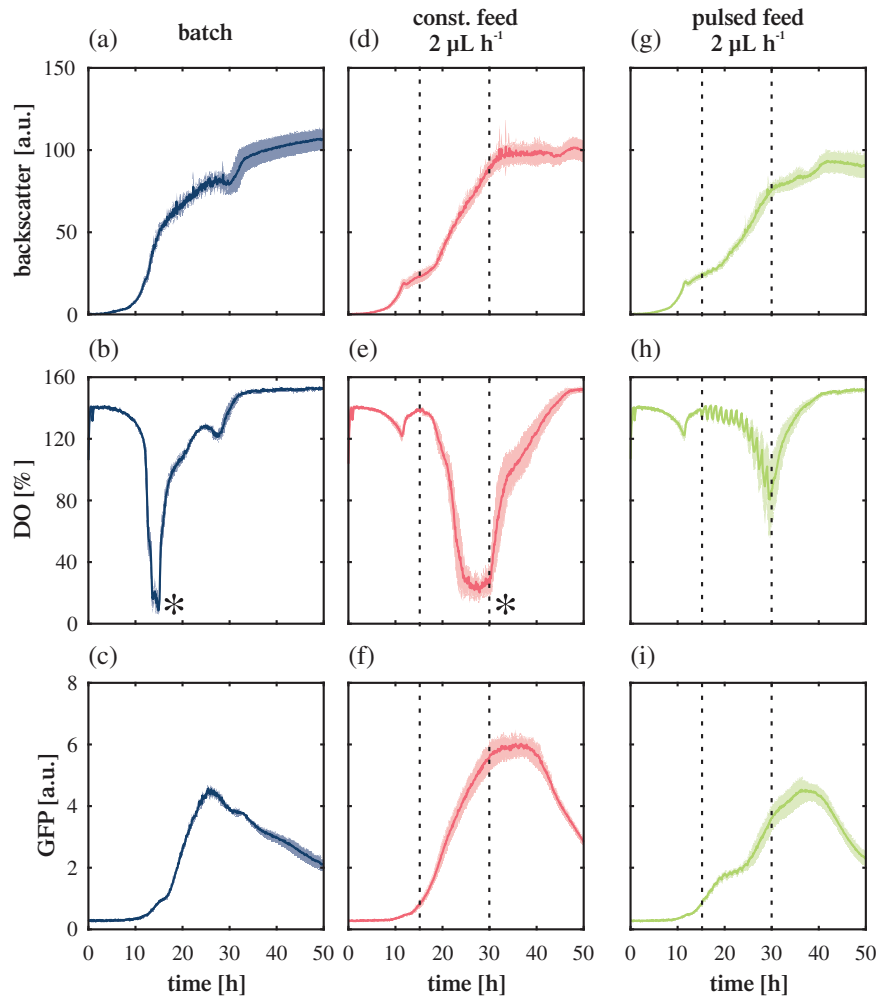

FIGURE 8 Lab-scale stirred tank bioreactor cultivation of *A. niger* in batch and fed-batch mode. (a) Biomass by CDW. (b) GFP by fluorescence. Cultivation at 37°C, 1200 rpm, 700 ml initial volume (defined Vogel medium inoculated with 10% (v v^{-1}) preculture) and 10 NL min^{-1} gassing with compressed air (DO ≥30%). Reference batch with 20 g L^{-1} maltose. Feeding for 15 h at 1.75 ml h^{-1} (700 mg h^{-1} maltose) constant rate after initial batch on 5 g L^{-1} maltose. Mean and errors deviated from technical replicates (*n* = 3)
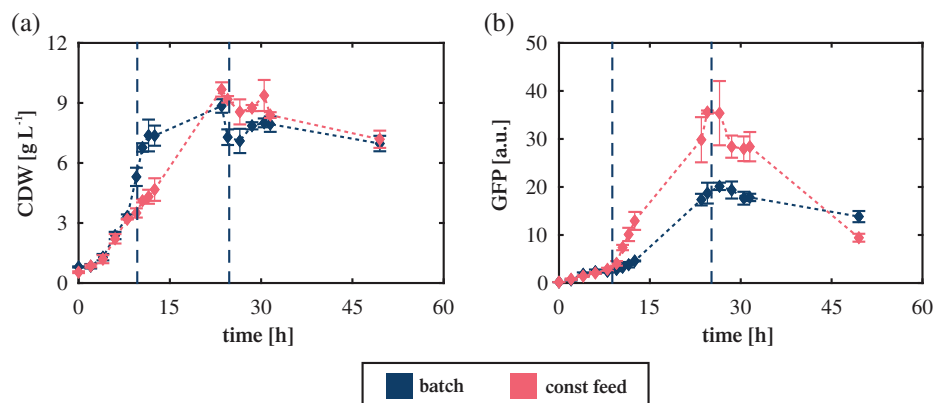
online monitoring, offline CDW and GFP fluorescence was determined (Figure 8).

Analogous to the MBR cultivation, the batch reached the stationary phase after approx. 14 h and thereby earlier than the fed-batch process. In both cases, very similar maximal CDWs in the range of 9 g L^{-1} were obtained. While the batch process achieved this concentration toward the end of the maltose consumption phase at 14 h (see DO data in Figure S2) the fed-batch cultivation reached its maximal CDW right at the end of the feeding phase after approx. 27 h. In both cases, a decrease of biomass concentration down to approx. 7 g L^{-1} during subsequent starvation until harvest could be observed.

Even though both cultivations resulted in comparable biomass concentration, the maximum product concentration was increased by 76% for the constant feed. Clearly, this improvement goes beyond the performance differences observed at microscale (33%). As the reason for this difference remained unclear from the data available, further investigation on the microscale protocol seems promising to decipher the relevant effect and maybe achieve identical results as in stirred tank bioreactors. Nevertheless, current results strongly suggest the valid application of feeding strategies for *A. niger* already at microscale as the general trend could be reproduced.

4 | CONCLUSIONS

Microbioreactor cultivation proved to be a valuable tool for micro-filamentous *A. niger* cultivation. Good transferability between batch microscale in shaken MTPs and lab-scale cultivations in stirred tank bioreactors enabled scalability of the results. Acquired offline parameters, such as CDW, pH and model product GFP resulted in a close overlay for both systems. Moreover, online measurements of scattered light for MBR cultivation were highly reproducible with 8.3% rmc$_c$ and showed a good correlation to offline CDW, which was not impacted by morphological changes. For reasons yet unknown approx. Two thirds of total GFP formation was observed after depletion of the initial carbon source. Based on the information available to now, it can only be speculated that this is attributable to metabolization of secondary carbon sources or storage compounds, which might affect protein expression or GFP maturation kinetics and should therefore be investigated in further detail.

In the next step, the implementation of different feeding strategies to enable microscale fed-batch cultivation was tested. Both robotic pulsed feeding as well as substrate addition via microfluidic channels resulted in reproducible fed-batch cultivation. Despite technical applicability, for the given biological system producing intracellular GFP, a clear benefit of microfluidic constant feeding with 2 μl h^{-1} could be seen, as protein production was increased by 33% in contrast to batch cultivation. While microfluidic pulsed feeding did not improve total product titer compared to the batch reference, robotic pulsed feeding even resulted in a reduced protein production (−50%). It therefore seems that stringent substrate limitation during microfluidic constant rate feeding is superior to pulsed profiles where product formation is repeatedly repressed by oscillations in substrate concentration.

Overall, microbioreactor cultivation, both in batch or fed-batch mode, enable accelerated phenotyping for filamentous fungi *A. niger* through miniaturization and parallelization. However, further effort seems necessary to improve comparability of fed-batch processes between both scales. As a next step, the combination of other workflows, such as automated morphology analysis, omics analysis and exometabolome fingerprinting with carbon-limited fed-batch cultivations would allow for deeper insights as well as better phenotyping at microscale.

AUTHOR CONTRIBUTIONS

Roman Jansen: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review & editing. **Holger Morschett:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; visualization; writing-original draft; writing-review & editing. **Dennis Hasenklever:** Investigation. **Matthias Moch:** Investigation. **Wolfgang Wiechert:** Conceptualization; funding acquisition; supervision; writing-review & editing. **Marco Oldiges:** Conceptualization; funding acquisition; project administration; supervision; writing-review & editing.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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