Ina Huth

Jens Schrader

Dirk Holtmann DECHEMA-Forschungsinstitut,
Frankfurt am Main, Germany

Technical Report

Microtiter plate-based cultivation to investigate the growth of filamentous fungi

Microscale bioprocessing techniques are rapidly emerging as a means to increase the speed of bioprocess design and to reduce material consumption. However, there is still a lack of suitable parallelized techniques to investigate the industrially important group of filamentous bacteria and fungi. Cultivation of filamentous organisms in shake flasks is still the favored technique for comparing and optimizing cultivation conditions of production strains at mL-scale. In this paper, the application of a microtiter plate-based cultivation system in combination with the filamentous fungus *Aspergillus niger* was investigated. A protocol for reproducible cultivation was developed and evaluated. Productivity of *A. niger* concerning the rose-like aroma compound 2-phenylethanol showed low standard deviations while regular and consistent morphologies appeared in the parallelized system. Furthermore, the effect of addition of microparticles on the morphology was investigated. The results can be used to accelerate the process development with *A. niger* and other filamentous organisms.

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1 Introduction

Filamentous fungi and bacteria are industrially important organisms to produce enzymes, antibiotics and pharmaceuticals as well as bulk and fine chemicals. In submerged culture processes, filamentous organisms typically exhibit a complex morphological life cycle that is related to production performance [1]. The morphology of these organisms can vary from dense spherical pellets to viscous mycelia. It has been shown that the formation of these morphological characteristics is strongly influenced by process parameters, including power input through stirring and aeration, mass transfer characteristics, pH value, osmolality and the presence of solid microparticles [1]. Investigations on morphological influences on the production of citric acid by *Aspergillus niger*, one of the most prominent fungal processes, resulted in contradictory conclusions about the benefits or disadvantages of pellet formation [2]. However, biomass as homogeneous as possible, present either in free hyphae or loose mycelia, is commonly regarded as desirable for fungal processes [3]. The

delivery of oxygen and nutrients to dispersed cells is unobstructed in contrast to the limited transfer to agglomerated cells which typically contain a core center of essentially dead biomass [4].

Cultivation of filamentous fungi in Erlenmeyer flasks is still the favored method for comparing different conditions at laboratory-scale although cultivation in microplates would be preferred to accelerate bioprocess development. Due to the broad set of parameters which influences the morphology of filamentous organisms, Erlenmeyer flask approaches are very time consuming. Furthermore, significant inter-flask variations, which are often observed in Erlenmeyer flask cultivations, make the comparison of product levels during fermentation difficult and less reliable [5]. Therefore, a method for systematic investigations of these factors in a parallelized microtiter plate (MTP)-based system is needed. Until now, only a few investigations concerning the cultivation of filamentous organisms in MTPs were performed. Langvad et al. [6] investigated the growth of different fungi in MTPs. The results showed a linear correlation between the absorbance at 630 nm and the cell dry weight. Experiments with different fungal strains resulted in a similar amount of about 4.2 mg/mL dry weight per absorbance unit. A special 16-well plate with an optimized design for the cultivation of filamentous fungi was used in an automated system to compare recombinant *Aspergillus vadensis* clones producing the enzyme feruloyl esterase B [7]. Alberto et al. showed that the highest enzymatic activity in the MTP-based cultivations was only approx. 10% of

Correspondence: Dr. Dirk Holtmann (holtmann@dechema.de), +49 69 7564–610, DECHEMA Forschungsinstitut, Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany

Abbreviations: 2-phenylethanol, 2-PE; MPEC, Microparticle-enhanced cultivation; MTP, Microtiter plate

the enzyme activity in shake flasks. Nevertheless, the qualitative results in both systems were comparable. A laser-based MTP nephelometer was used to measure fungal growth in microscale (300 μL /well) liquid cultivations [8]. The authors emphasized nephelometry as a direct method of measuring light scattered by particles in suspension in contrast to photometry as an indirect method. Since the scattered light intensity is directly proportional to the suspended particle concentration, nephelometry is a well-suited technique to record microbial growth and to be applied in studies on filamentous fungi. Linde et al. [5] compared in batch cultivations the reproducibility of the growth of the filamentous fungus *Aspergillus carbonarius* in 24-well plates as well as in shake flasks. Based on the comparison of quantifiable morphological parameters, the authors concluded that fermentation in 24-well plates is a more reliable screening method for metabolite production by filamentous fungi and possibly for screening metabolites in general. A method which combined 24-well plates with an agarized medium was used for the cultivation of *Trichoderma* strains. Supernatants obtained after a centrifugation step of the culture plates were evaluated for extracellular total cellulase activity [9]. A scale-down from shake flasks to microtiter plates was performed for the production of itaconic acid by *Aspergillus terreus* [10]. In both cultivation systems comparable product titers were achieved. Sohoni et al. [11] cultivated the filamentous bacterium *Streptomyces coelicolor* and compared its performance in a 24-square deepwell MTP to that in shake flasks and bench-scale reactors. The results, which were obtained in the MTP-based experiments, showed a substantial agreement in the specific growth rates as well as in volumetric antibiotic production rates compared to the results obtained in bioreactors. Furthermore, the incorporation of glass beads with a diameter of 3 mm in the MTP cultivation was investigated. The addition of beads resulted in an improved process performance in terms of oxygen transfer, homogeneity and product concentration. A study by Siebenberg et al. compared the production of novobiocin by a *Streptomyces* strain in a 24-square deepwell MTP and in baffled shake flasks [12]. For parallel cultivation batches in the deepwell plates, novobiocin accumulation showed standard deviations of 4–9%, compared to 39% in baffled Erlenmeyer flasks.

None of these investigations focused on morphological effects and on the investigation of the addition of microparticles to microplate cultures of filamentous fungi. A positive effect of microparticle addition was firstly described for the cultivation of the filamentous fungus *Caldariomyces fumago* in shake flasks [13]. The addition of talc microparticles prevented pellet formation on the one side and increased the maximum specific productivity of the enzyme chloroperoxidase by a factor of five on the other side. For this effect, the term “microparticle-enhanced cultivation” (MPEC) was introduced. Afterwards the principle was successfully transferred to the cultivation of *A. niger* [4, 14–16] and other organisms [17, 18]. The aim of this study was to develop a reliable method to investigate the growth behavior as well as the productivity of an *A. niger* strain in the presence of microparticles on microtiter scale. As model product, 2-phenylethanol (2-PE), a rose-like flavor compound produced from L-phenylalanine, was chosen.

2 Materials and methods

2.1 Chemicals

Chemicals (analytical grade) were purchased from Sigma-Aldrich (Munich, Germany) or Carl Roth (Karlsruhe, Germany). Talc particles were purchased from Sigma Aldrich (order number: 243604) and have a stated size of 10 μm . A detailed characterization of the particles is described by Etschmann et al. in 2014 [16].

2.2 Microorganisms and cultivation procedures

A. niger DSM 821 was obtained from DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cultivation on solid medium was performed at 30°C on oatmeal agar plates containing 30 g/L rolled oats and 15 g/L agar. The *Aspergillus* liquid medium consisted of 60 g/L glucose, 6 g/L L-phenylalanine, 5 g/L yeast extract, 1.84 g/L diammonium tartrate, 0.2 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.00132 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ [19]. MTP cultures (FlowerPlate® B, m2p-labs GmbH, Baesweiler, Germany) were incubated at 30°C and 1000 rpm in the BioLector (m2p-labs GmbH, Baesweiler, Germany). Apart from initial studies culture volumes in MTPs were 1.4 mL (overall nominal volume 3.2 mL per well). Inoculation was conducted by using suspended spores washed off from sporulating solid cultures by gently shaking an agar plate with 4 mL of a sterile solution of 9 g/L NaCl and 0.5 g/L Tween 40. The spore concentration was determined by using a counting chamber (Neubauer improved). Stocks of medium were inoculated with either 200.000 spores/mL, 20.000 spores/mL or 2.000 spores/mL (details in the descriptions of the experiments in the next paragraph) and divided to the wells of the MTP. MTPs were sealed using sterile gas-permeable sealing foils which allow sufficient aeration but reduce evaporation (gas-permeable sealing foil with evaporation reduction layer, m2p-labs GmbH, Baesweiler, Germany). The relative humidity (RH) was controlled at approximately 90 % to further minimize evaporation effects. In order to take daily samples from the cultivations, the MTP was removed from the BioLector and transferred to a laboratory clean bench once per day. The sealing foil was removed and samples of approx. 40 μL were taken from each well. After the sampling procedure (duration in total approx. 10 min) the MTP was sealed again and further incubated.

2.3 Analytics

2-PE was analyzed by a HPLC-10A (Shimadzu, Kyoto, Japan) with a SPD-M10Avp detector and equipped with a Luna 5 μm C18(2) 100 A column (125 $\times$ 4 mm) (Phenomenex, Aschaffenburg, Germany). Samples were centrifuged and the supernatant was diluted 1:15 with distilled water.

HPLC parameters: eluent 55% methanol, 44.9% distilled water, 0.1% orthophosphoric acid, flow rate 1 mL/min (isocratic elution), oven temperature 40°C, injection volume: 10 μL , detection wavelength 206 nm.

Figure 1. Effects of the amount of spores and of the addition of talc on the morphology of *A. niger* (left: cultivation conditions, right: resulting morphology in the MTP after 3 days, culture conditions: 30°C, 1.4 mL per well, 1000 rpm, humidity control ~90 %). In the table, the changes in the morphologies and the well-to-well reproducibility were summarized.

3 Results and discussion

The MTP-based system was investigated using *A. niger* DSM 821 to produce the rose-like flavor compound 2-phenylethanol (2-PE) from L-phenylalanine. The production in the MTP system using different filling volumes (1.0, 1.2, 1.4 and 1.6 mL) was tested in order to choose a process-suited culture volume for further investigations. The shaking speed was set to 1000 rpm. The highest product concentration as well as productivity was observed at the lowest volume of 1.0 mL. Most probably, this effect is caused by a reduced oxygen transfer rate at higher filling volumes [20]. Although the increase of the filling volume led to a decrease of both parameters, a culture volume of 1.4 mL was chosen for further investigations. It revealed that this volume is at least required to enable

continuous off-line product analyses of daily taken samples of approx. 40 µL without affecting culture conditions in an unacceptable extent. The shaking frequency of 1000 rpm appeared to be the upper limit compatible with the culture volume of 1.4 mL. The highest volume used in the experiment (1.6 mL) showed wetted sealing foil indicating a spillover of the liquid culture due to an imbalance between culture volumes and shaking frequencies.

In order to investigate the applicability and reproducibility of the MTP-based system, the effect of different spore concentrations used for inoculation and the adaptability of the MPEC-technique to the microscale should be examined. Since preliminary experiments (data not shown) using the spore concentration of 200 000 spores/mL established for shake flask studies showed imponderable large agglomerates, two different

Figure 2. Evaluation of the microparticle effect in MTP (culture conditions: 30°C, 1.4 mL per well, 1000 rpm, humidity control ~90%; picture shows MTP after 3 days of cultivation). In the table, the changes in the morphologies and the well-to-well reproducibility were summarized.

lower spore concentrations (2000 and 20 000 spores/mL) were compared in this experiment. In addition, the influence of the addition of 2% (w/v) talc particles at both spore concentrations was investigated. Although a pretest (data not shown) of the system has demonstrated that the well position within the FlowerPlate® has no influence on evaporation or further parameters of the cultivations, the six replicates of one condition were evenly distributed in a diagonal configuration. Figure 1 shows the applied conditions and a photograph of the resulting morphology after three days of cultivation. The morphological evaluation is summarized in the related. In order to characterize the well-to-well reproducibility the pellet size in the six wells with 20 000 spores/mL was statistically evaluated. An equal area of the photograph of each well (A1, B2, C3, D4, E1 and F2) was considered for the evaluation; in total 184 pellets were analyzed to get statistical reliable data. The mean value of the pellet diameter

amounted to 1.13 mm and the standard deviation to 0.18 mm (calculated by the $n-1$ method). The mean values in the individual wells varied between 1.07 and 1.28 mm. This evaluation shows the well-to-well reproducibility of the cultivation system in regard to the morphology of the filamentous fungus. Furthermore, it can be concluded that low spore concentration leads, on the one hand, to more finely distributed biomass but, on the other hand, to an unwanted mixture of mycelia and pellets. The addition of talc leads to a more homogenous growth per well and to an improved comparability in growth morphology between the individual wells. Due to finely dispersed biomass an estimation of the pellet size was not useful in these experiments.

In the next step, the amount of added talc particles at constant inoculum size was investigated. The effect of the addition of 0.5, 1 and 2% talc (w/v) to the cultivation of *A. niger* inoculated with

20 000 spores/mL is shown as photograph and recapitulated in tabular form in Fig. 2. Again, in the controls without the addition of talc, *A. niger* grew to small pellets. Similarly to the previous experiment, an equal area of each replicate was added for evaluation. According to that, 243 pellets in the control wells A5, B6, C7, D8, E5 and F6 were evaluated by calculation of the mean values as well as the standard deviation using the $n-1$ method. The mean value of pellet diameter was 1.01 mm overall and varied in the particular wells from 0.93 to 1.25 mm. The standard deviation amounted to 0.17 mm. These values are comparable to the aforementioned results under the same conditions. Thus, it can be concluded that the developed protocol using 20 000 spores/mL for inoculation at a constant culture volume of 1.4 mL in the FlowerPlate[®] are robust cultivation conditions and assure high reproducibility. Therefore, it can be concluded that the cultivation conditions are suitable for the investigation of the MPEC effect in the MTP-based system. The influence of initial spore concentration on agglomeration has often been the subject of studies by various research groups investigating different organisms, for example *P. chrysogenum* [21, 22], *Streptomyces tendae* [23] and *A. niger* [24–26]. In general, pellets are often formed at low spore concentrations, while at higher spore concentrations filamentous growth prevails. On the other hand, in *Aspergillus* cultivations usually the more spores are introduced to the submerged culture, the smaller pellets are formed. Most probably, the combination of different effects in the chosen MTP-based cultivation system (e.g. energy input, coagulation of the spores, volume-to surface ratios in miniaturized systems) leads to the effect that at low spore concentration a reproducible pellet formation can be measured. Figure 2 shows the effect of different talc concentrations on the morphology of *A. niger* after three days of cultivation. The addition of 0.5% talc (w/v) led to a more finely dispersed growth of *A. niger* compared to the cultivation without particles. Nevertheless, limitations in the well-to-well reproducibility became apparent. In five of the six wells of this talc concentration the cultivation method resulted in the formation of fine pellets (A6, B7, C8, D5, E6). The morphology in one well (F7) was in strong contrast to the other experiments under these conditions. In well F7, a huge biomass agglomerate was observed. The addition of 1% talc (w/v) resulted in homogeneous mycelia-like growth in all six wells (A7, B8, C5, D6, E7, F8). A further doubling of the amount of particles led again to a higher variation in morphology. Here a mixture of free mycelia and pellets of different sizes could be observed (A8, B5, C6, D7, E8, F5). The results of these experiments revealed that the addition of talc caused a more disperse growth of *A. niger* compared with non-supplemented cultures in all trials. Furthermore, the best homogenization effect and conformity was observed by the addition of 1% talc (w/v). In a previous study, we showed that the addition of microparticles to shake flask cultures of *A. niger* led to an increased productivity of up to 32% [16]. The highest 2-PE concentration of 1432 mg/L was measured after 144 h in cultures containing 2% talc (w/v). In order to evaluate the particle effect and the resulting homogenization effect on the productivity at microscale, the concentration of 2-PE during the cultivation in FlowerPlates[®] was measured (Fig. 3).

Analogously to the results obtained from shake flask cultivations, the addition of 2% talc (w/v) led to the highest level of 2-PE production, 754 mg/L 2-PE at 120 h. The overall

Figure 3. Evaluation of the microparticle effect in a MTP-based cultivation with different amounts of talc particles (culture conditions: 30°C, 1.4 mL per well, 1000 rpm, humidity control ~90 %, $n = 2$, independent biological replicates, error bars indicate absolute values of the duplicates).

productivity in the MTP was decreased compared with the previously described shake flask cultivations. The productivity in the MTP system was approx. 35% lower compared to the shake flask experiments. Furthermore, the improvement factor between the control and talc supplemented cultures varied between the cultivation systems. In the MTP-based cultivation, the addition of talc led to a 130% higher productivity. Nevertheless, the qualitative effects between the cultivation systems were similar.

4 Concluding remarks

The economic benefit, reliability and controllability of bioprocesses for filamentous organisms, in particular for filamentous fungi, are requirements for the large-scale production of a wide range of value-added products, including organic acids, enzymes, and antibiotics [27].

Beside the identification of highly productive strains, an initial screening should also consider morphological characteristics. Complex growth morphologies including cells adhering to the cultivation containment or the formation of large biomass agglomerates significantly affect the reproducibility of results and the subsequent scale-up procedure. Because of the high number of experiments which is therefore required, a parallelized screening technology assuring consistent results is needed. The MTP-based cultivation strategy developed in this work allows a fast screening of different strains and conditions of the industrially important genus *Aspergillus*. Considering the presented MTP-system and the results published by Sohoni et al. [11] concerning the cultivation of *Streptomyces coelicolor* in 24-well plates, it can be concluded that now various tools for an accelerated process design with different filamentous organisms are available. The cultivation system used in the current study is based on the development of the Büchs group [28, 29]. The investigations showed the applicability of the cultivation system to examine growth and productivity of filamentous microorganisms as well as the MPEC effect to improve the processes. The results of the

experiments described here can be used to accelerate the process development with these organisms. A further improvement of the cultivation system may be realized by combining it with automated image analysis. Furthermore, the application of cultivation plates with integrated pH and pO_2 measurements would enable a continuous monitoring of the cultivation conditions over time. Finally, further investigations on the interactions between different filamentous production strains, energy inputs, spore concentration, coagulation of spores, volume-to surface ratio as well as the transfer of the results obtained in MTPs into bioreactors should be investigated in detail.

Practical Application

The subject of this study is the miniaturized submerged cultivation of *Aspergillus niger* and the combination with the microparticle-enhanced cultivation (MPEC) method. Reliability and a great number of parallelized experiments are prerequisites for large screenings and facilitate the investigation of varying cultivation conditions or mutants. Due to their characteristics and especially to their complex morphology, filamentous organisms are difficult to handle and often exhibit large deviations between single runs. Therefore, the use of parallelized high throughput cultivation methods for these organisms is not prevailing. In this paper, the applicability of the microscale cultivation for the examination of the fungus *A. niger* is presented and gives an example for an effective microtiter plate-based cultivation of a filamentous organism. Additionally, the MPEC method, which was already known for influencing morphology and productivity as well as for improving reproducibility, is shown to be a supportive tool for the miniaturized cultivation.

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