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Establishing a Fed-Batch Process for Protease Expression with *Bacillus licheniformis* in Polymer-Based Controlled-Release Microtiter Plates

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Introducing fed-batch mode in early stages of development projects is crucial for establishing comparable conditions to industrial fed-batch fermentation processes. Therefore, cost efficient and easy to use small-scale fed-batch systems that can be integrated into existing laboratory equipment and workflows are required. Recently, a novel polymer-based controlled-release fed-batch microtiter plate is described. In this work, the polymer-based controlled-release fed-batch microtiter plate is used to investigate fed-batch cultivations of a protease producing *Bacillus licheniformis* culture. Therefore, the oxygen transfer rate (OTR) is online-monitored within each well of the polymer-based controlled-release fed-batch microtiter plate using a μRAMOS device. Cultivations in five individual polymer-based controlled-release fed-batch microtiter plates of two production lots show good reproducibility with a mean coefficient of variation of 9.2%. Decreasing initial biomass concentrations prolongs batch phase while simultaneously postponing the fed-batch phase. The initial liquid filling volume affects the volumetric release rate, which is directly translated in different OTR levels of the fed-batch phase. An increasing initial osmotic pressure within the mineral medium decreases both glucose release and protease yield. With the volumetric glucose release rate as scale-up criterion, microtiter plate- and shake flask-based fed-batch cultivations are highly comparable. On basis of the small-scale fed-batch cultivations, a mechanistic model is established and validated. Model-based simulations coincide well with the experimentally acquired data.

1. Introduction

Proteases are highly relevant in the technical enzyme market, especially in detergents.[1] *Bacillus* species belong to the main producers of proteases and are capable of secreting large amounts of extracellular enzymes.[2] However, mechanisms that control protease expression in *Bacillus* species are complex and knowledge is limited.[1,3,4] Transcriptome and proteome analyses showed that substrate starvation and limitation promote protease expression and secretion.[3,5,6] Substrate-limited fed-batch mode is characterized by continuously feeding one nutrient in limiting concentrations. This process mode is predominantly used for large-scale protease production.[1] The positive influence of substrate-limited fed-batch conditions compared to batch conditions in terms of protease yields was recently affirmed with a protease producing *Bacillus licheniformis* strain.[7]

In fed-batch mode, the feed rate has a direct impact on the metabolic activity of the cultivated organism.[8,9] By choosing an optimal feed rate, growth inhibition by elevated substrate concentration, overflow metabolism, and oxygen limitations can be prevented by simultaneously increasing biomass yield and product concentrations.[10–12] In addition, limiting substrate concentrations circumvent catabolite-repression, which can, e.g., increase product concentrations up to 420-fold when working with *Hansenula polymorpha*.[13] Due to these benefits, many fermentation processes are conducted in substrate-limited fed-batch mode using stirred tank reactors in combination with peristaltic pumps. Nonetheless, development projects usually start with small-scale shaken bioreactors such as microtiter plates (MTPs) or shake flasks.[14–16] Both systems are cost efficient, have a simple and functional design, and enable massive parallelization.[17,18] Characterization of the fluid dynamics and the maximum oxygen transfer capacities further improved the understanding of shaken bioreactors.[19–22] However, these systems were initially designed to be operated in batch mode, and thus, batch is the dominant operation mode for primary

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screening processes. Physiological conditions in batch mode are completely different from those in fed-batch mode. Scheidle et al. compared batch and fed-batch screenings of *Hansenula polymorpha* clones and showed that clones were ranked differently in the two process modes.[23] Therefore, optimal strains found in batch screenings may perform poorly under fed-batch mode in stirred tank reactors during scale-up. Thus, if the production process is operated in substrate-limited fed-batch mode, screening should be performed in substrate-limited fed-batch mode as well.[24]

Only in recent years, techniques were developed to realize continuous feeding in small-scale shaken bioreactors using microfluidic systems,[25,26] diffusion-based systems,[7,27–30] and enzymatic release systems.[31–34] The applicability of such systems, however, also depends on low investment and operating costs, compatibility with existing laboratory equipment, versatility, and reproducibility.[35] Enzymatic release systems fulfill most of these requirements, but show high variations in glucose release with moderately changing cultivation conditions.[36] Furthermore, many organisms, including *Bacillus* species,[37] secrete amylases and proteases, which may lead to uncontrollable glucose release kinetics.

Polymer-based glucose release disks (FeedBeads) for shake flask cultivations provide an alternative to the enzyme-based system and were already described by Jeude et al.[13] On basis of this principle, a commercially available polymer-based controlled-release fed-batch microtiter plate was developed (Feed Plate, Kuhner Shaker GmbH). Recently, Keil et al. provided a detailed characterization of glucose release kinetics with varying media conditions and temperatures.[38] When compared to the enzymatic release system, the polymer-based release system showed to be less sensitive against moderate changes of pH and temperature.[36,38]

In this study, glucose-limited fed-batch conditions were introduced at microtiter plate-scale with polymer-based controlled-release fed-batch microtiter plates. The reproducibility of glucose-limited fed-batch cultivations was examined using five individual polymer-based controlled-release fed-batch microtiter plates from two different production lots. The µRAMOS device enabled online monitoring of the oxygen transfer rate (OTR) within each individual well.[39] To investigate the effect of different initial cultivation conditions on a protease producing *Bacillus licheniformis* strain, the biomass concentration, the filling volume and the osmotic pressure were varied. Cultivations within the polymer-based controlled-release fed-batch microtiter plate were then scaled-up to the membrane-based fed-batch shake flask with the volumetric release rate as scale-up criterion. Finally, the acquired fed-batch online data were used to establish and validate a mechanistic model. The possibility of implementing defined glucose-limited fed-batch conditions in microtiter plates aims at showing the potential of the polymer-based controlled-release fed-batch microtiter plate for screening processes in early development stages.

2. Experimental Section

2.1. Strain and Media

The protease producing *Bacillus licheniformis* strain contains a plasmid for the expression of a subtilisin-like protease and a tetracycline resistance marker for selection and was kindly provided by BASF SE (Ludwigshafen am Rhein, Germany). Further information on the protease producing *Bacillus licheniformis* strain can be provided by BASF SE (Ludwigshafen am Rhein, Germany) upon request. The chemicals applied for media preparation were of analytical grade and purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma–Aldrich Chemie GmbH (Steinheim, Germany), Merck (Darmstadt, Germany), VWR (Darmstadt, Germany), and from AppliChem (Darmstadt, Germany). The composition of the complex Terrific Broth (TB) medium and the V3 mineral medium can be taken from Habicher et al.[7] The V3 mineral medium was previously described in publications from Meissner et al. and Wilming et al.[40,41]

2.2. Cultivation Conditions

Precultures were performed in 250 mL shake flasks and carried out on an orbital climo-shaker ISFX-1 from Adolf Kühner AG (Biersfelden, Switzerland) with a filling volume V_L = 10 mL, a shaking frequency n = 350 rotations per unit (rpm), a shaking diameter d_0 = 50 mm and a temperature T = 30 °C. For online monitoring, the in-house build RAMOS device was used. A commercial version is available from Adolf Kühner AG (Biersfelden, Switzerland) or HiTec Zang GmbH (Herzogenrath, Germany). Precultures were divided in two steps, whereby the first preculture was carried out in complex TB medium and the second in V3 mineral medium. A detailed description of the RAMOS-based two-step preculture procedure can be found in Habicher et al.[7]

Membrane-based fed-batch main cultures were performed in 250 mL shake flasks with a filling volume V_L = 16 mL. Shaking conditions and online monitoring was similar to the above described preculture procedure. For fed-batch main cultivations, no initial glucose was supplemented to the V3 mineral medium. A detailed description of the preparation of the membrane-based fed-batch shake flasks can be found in literature.[7,28]

Microtiter plate-based fed-batch main cultivations were conducted using 48-well round- and deep-well microtiter plates with a glucose-containing polymer on the bottom of each well (article number: SMFP08004, Kuhner Shaker GmbH, Herzogenrath, Germany; Figure S1, Supporting Information), a filling volume V_L = 0.5, 0.8, or 1.0 mL, a shaking frequency n = 1000 rpm, a shaking diameter d_0 = 3 mm, and a temperature T = 30 °C. For online monitoring of each individual well, the µRAMOS device was used.[39] The microtiter plate was covered with a sterile barrier (900 371-T, HJ-Bioanalytik GmbH, Erkelenz, Germany) to avoid contamination.

2.3. Determination of Glucose Release

Glucose release of polymer-based controlled-release fed-batch microtiter plates was measured with a filling volume V_L = 1.0 mL, a shaking frequency n = 1000 rpm, a shaking diameter d_0 = 3 mm, and a temperature T = 30 °C. The plate was covered with a Rotilabo sealing foil (article number: X172.1, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to eliminate evaporation. For each data point, three individual wells

(number of included wells (n) = 3) were harvested and sterile filtered (0.2 µm filter). The glucose concentration was analyzed by HPLC. The HPLC device (Ultimate 3000, Dionex, Sunnyvale, USA) was equipped with a protection cartridge (4 × 3 mm; article number: AJ0-4490, Securityguard Standard) and an ion-exclusion column (300 × 7.8 mm; article number: 00H-0138-K0, Rezex), both from Phenomenex (Aschaffenburg, Germany). Elution was carried out with 50 mM H_2SO_4 with a flow rate of 0.8 mL min^{-1} at a constant temperature of 70 °C. Glucose was detected by measuring the refractometric index with a Shodex RI-101 refractometer (Showa Denko Europe, Munich, Germany). Data analysis was done with the software Chromeleon 6.8 (Dionex, Sunnyvale, USA).

2.4. Offline Sample Analysis

Offline samples were analyzed regarding the optical density at 600 nm (OD_{600}), the pH-value, and the protease activity. A detailed description of the applied procedures can be found in Habicher et al.[7] The protease activity measurement is based on the method developed by DelMar et al. and on the experimental procedure described by Habicher et al.[7,42] The protease activity was measured from samples taken at the end of the cultivation. In order to normalize absolute protease activities, different methods can be chosen. In this study, absolute protease activities were normalized to the measured protease activity directly after glucose depletion at 11.5 h of the reference batch experiment (Figure S2, Supporting Information). The normalized protease activity is referred to as relative protease activity. For the comparison of fed-batch cultivations to batch, the final relative protease activity of the batch cultivation was taken as reference (Figure S2, Tables S2 and S3, Supporting Information).

It was assumed that cells and proteases were not able to penetrate into the polymer matrix. Hence, in addition to evaporation, swelling had a concentrating effect on cells and proteases. In Figure S1, Supporting Information, the experimental procedure to determine swelling and evaporation is presented. Two individual polymer-based fed-batch microtiter plates were used to determine evaporation (0.038 g per well) and swelling (0.089 g per well) under cultivation conditions after 70 h. The mass balancing procedure was performed with a precision balance (EWJ 3000–2, Kern und Sohn GmbH, Balingen, Germany). The measured mass difference was converted to volume with an assumed density of 1 g cm^{-3}. Evaporation was assumed to be constant for experiments with different filling volumes in microtiter plates. On basis of the determined values for evaporation and swelling, the measured protease activity and OD_{600} were corrected to the initial filling volume. In membrane-based fed-batch shake flasks, the volume loss was caused by evaporation and back diffusion of water into the reservoir [7]. As with polymer-based controlled-release fed-batch microtiter plates, protease activity and OD_{600} were corrected to the initial filling volume. The applied procedure can be taken from Habicher et al.[7]

Sterile filtered samples (0.2 µm filter) were used to measure the osmolality with the Osmomat 030 (Gonotec GmbH, Berlin, Germany). The device was calibrated with calibration standards of 0.85 or 2.00 osmol kg^{-1} (Gonotec GmbH, Berlin, Germany).

2.5. Fed-Batch Model

For the fed-batch model in microtiter plates, Monod kinetics were used. The equation for biomass concentration X is

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu \cdot X \qquad [\mathrm{g} \cdot \mathrm{L}^{-1} \cdot \mathrm{h}^{-1}] \qquad (1)$$

where μ is the specific growth rate. μ is calculated on basis of the Monod equation

$$\mu = \mu_{\max} \cdot \frac{S}{S + K_S} \qquad [\mathrm{h}^{-1}] \qquad (2)$$

where Ks is the Monod constant, $\mu_{\max}$ the maximum specific growth rate, and S the glucose concentration. In case of possible oxygen limited conditions, the Monod equation can be extended with an additional Monod term $O_2/(O_2 + K_{O2})$ to introduce the oxygen concentration O_2 as additional growth limiting substrate. The accumulated volumetric glucose release F_S is described with the empirical equation

$$F_S = \frac{(A \cdot \mathrm{e}^{B \cdot t} + C \cdot \mathrm{e}^{D \cdot t})}{V_\mathrm{L} \cdot 10^{-3}} \qquad [\mathrm{g} \cdot \mathrm{L}^{-1}] \qquad (3)$$

where A, B, C, and D are fitting parameters and V_L the liquid filling volume (Figure 5A). Glucose concentration is described with

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{1}{Y_{X/S}} \cdot \mu \cdot X - m_S \cdot X + F'_S \qquad [\mathrm{g} \cdot \mathrm{L}^{-1} \cdot \mathrm{h}^{-1}] \qquad (4)$$

where $Y_{X/S}$ is the biomass yield per consumed glucose and m_S the maintenance coefficient related to glucose. F'_S is the volumetric glucose release rate that is obtained by differentiating F_S with respect to time t. The oxygen concentration within the liquid is calculated with

$$\frac{\mathrm{d}O_2}{\mathrm{d}t} = -\frac{1}{Y_{X/O2}} \cdot \mu \cdot X - m_{O2} \cdot X + OTR \qquad [\mathrm{mol} \cdot \mathrm{L}^{-1} \cdot \mathrm{h}^{-1}] \qquad (5)$$

where $Y_{X/O2}$ is the biomass yield per consumed oxygen, m_{O2} the maintenance coefficient related to oxygen, and OTR the oxygen transfer rate that is calculated with

$$OTR = k_L a \cdot (O_{2,\max} - O_2) \qquad [\mathrm{mol} \cdot \mathrm{L}^{-1} \cdot \mathrm{h}^{-1}] \qquad (6)$$

where $k_L a$ is the volumetric mass-transfer coefficient and $O_{2,\max}$ the maximum dissolved oxygen concentration defined as

$$O_{2,\max} = L_{O2} \cdot p_\mathrm{amb} \cdot y_{O2} \qquad [\mathrm{mol} \cdot \mathrm{L}^{-1}] \qquad (7)$$

with L_{O2} as the oxygen solubility within the culture broth, p_amb the ambient pressure and y_{O2} the mole fraction of oxygen within air. $O_{2,\max}$ was assumed to be a constant parameter.

2.6. Model Simulation and Fitting

The system of ordinary differential equations (ODEs) for biomass X, glucose S, and oxygen O_2 was solved in MATLAB R2018a

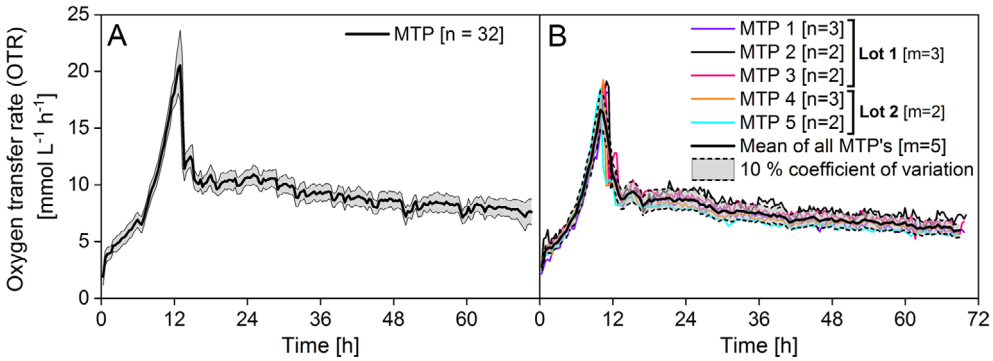

Figure 1. Reproducibility of individual fed-batch cultivations using polymer-based controlled-release fed-batch microtiter plates. A) Reproducibility of fed-batch cultivations using a single polymer-based controlled-release fed-batch microtiter plate (MTP). The mean oxygen transfer rate (OTR) of 32 parallel cultivations is depicted as black line (n = number of included wells). The shadow symbolizes the SD. B) Reproducibility of fed-batch cultivations using polymer-based controlled-release fed-batch microtiter plates from two different production lots (Lot numbers). The mean OTR of three plates from Lot 1 (MTP 1, MTP 2, and MTP 3; m = 3) and two plates from Lot 2 (MTP 4 and MTP 5; m = 2) are shown (m = number of included plates). Experiments on each plate were conducted in triplicates and inoculated starting with an individual glycerol stock followed by a two-step preculture procedure. Wells that could not be sealed tightly by the measurement device or had a noisy p_{O2} signal were excluded. The black line represents the mean OTR of all five MTPs from the two production lots. The shadow symbolizes a defined coefficient of variation (CV) of 10%. Cultivation conditions: polymer-based controlled-release fed-batch microtiter plate (48-well round- and deep-well), $OD_{600} = 0.5$, $V_L = 0.5$ mL, $n = 1000$ rpm, $d_0 = 3$ mm, $T = 30$ °C.

using the *ODE23tb* solver. The initial conditions for biomass concentration X_0 were deduced from the OD_{600} values on basis of an experimentally determined conversion factor with X [g L^{-1}] = 0.74 OD_{600}. The initial glucose concentration S_0 of 0.33 mg corresponded to the fitted volumetric glucose release F_S at time $t = 0$. The start value for the oxygen concentration $O_{2,0}$ was the maximum oxygen solubility $O_{2,max}$ (0.0002095 mol L^{-1}). Values and units of the constant parameters are listed in Table S1, Supporting Information.

The unknown parameters m_S and m_{O2} were fitted on basis of experiments with different initial biomass concentrations. The sum of least squares between the experimental and simulated data was globally minimized with the MATLAB minimization solver *fmincon* in combination with the global search function *gs*. Lower and upper bounds for m_S and m_{O2} were defined on basis of values from literature.[43–45] Model validation was done with experiments with different initial biomass concentrations and filling volumes.

3. Results and Discussion

3.1. Reproducibility of Microtiter Plate-Based Fed-Batch Cultivations

Screening processes in microtiter plates are usually performed without online monitoring. Therefore, clone rankings most often are based on final product concentrations or enzyme activities instead of yields, as, for example, $Y_{P/S}$. A solution for this problem is to define a constant volumetric glucose release rate and, if identical conditions are applied, to assume that the amount of released glucose after a certain time point remains constant in all wells. In doing so, it is of importance to investigate the plate-to-plate reproducibility of cultivations using the polymer-based release system.

Figure 1 shows the OTR curve of fed-batch cultivations with an initial OD_{600} of 0.5 and a filling volume of 0.5 mL. The OTR course is characterized by an initial batch phase, followed by an OTR drop, which marks the initiation of the glucose-limited fed-batch phase. In Figure 1A, the mean OTR of 32 parallel cultivations (n = 32) in a single polymer-based controlled-release fed-batch microtiter plate is depicted. The OTR-based well-to well mean coefficient of variation (mean CV) is 8.8%. In order to investigate the plate-to-plate reproducibility, five different MTPs from two production lots (Lot 1 and Lot 2) were used (Figure 1B). Due to the amount of investigated plates, the number of parallel cultivations on each plate was reduced. On basis of the mean OTR of all MTPs (Figure 1B, black line), the mean CV resulted in a value of 9.2%. Toeroek et al. also achieved a mean plate-to-plate CV of less than 10% using an enzymatic glucose release system with online biomass concentration monitoring.[36] However, different production lots were not considered in that study.

When determining the reproducibility on basis of cultivation experiments, it should be considered that the biological system and the online monitoring system add separate individual error. This error should not be attributed to the release system. Thus, glucose release experiments without cells resulted in a mean CV of only 4.5% using the polymer-based release system.[38]

3.2. Influence of the Initial Biomass Concentration on Microtiter Plate-Based Fed-Batch Cultivations

The effect of the initial biomass concentrations on the final protease activities in *B. licheniformis* fed-batch cultivations was investigated. In **Figure 2**, the OTR course of cultivations with an initial OD_{600} of 0.1, 0.5, 1.0, 1.5, and 2.5 is depicted. Independent of the initial biomass concentration, the OTR increases exponentially at the beginning of the cultivation (Figure 2A). In this phase, glucose accumulates and is available in excess (batch phase).[7] This is due to the generally low initial biomass concentration, which causes a low glucose consumption rate. By increasing the initial biomass concentration, the consumption rate increases as well,

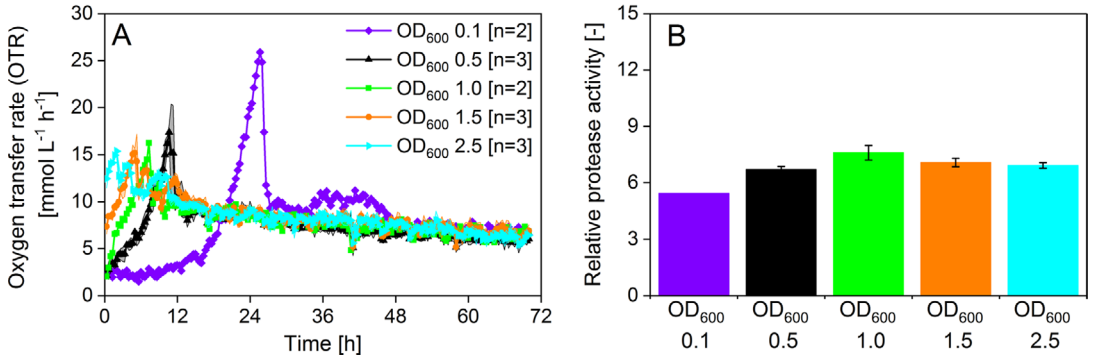

Figure 2. Influence of the initial biomass concentration (OD_{600}) on fed-batch cultivations of *Bacillus licheniformis* using the polymer-based controlled-release fed-batch microtiter plate. A) Mean oxygen transfer rate (OTR) over time. Each experiment was conducted in triplicates (despite OD_{600} 0.1, n = 2); however, wells that could not be sealed tightly by the measurement device or had a noisy p_{O2} signal were excluded. Shadows symbolize the SD of parallel cultivations (for n > 2). B) Mean relative protease activity at the end of the cultivation. Error bars symbolize the SD of the relative protease activity measured in parallel cultivations (n = 3). Protease activities were set in relation to the measured protease activity directly after glucose depletion at 11.5 h within the batch experiment, shown in Figure S2, Supporting Information. Cultivation conditions: polymer-based controlled-release fed-batch microtiter plate (48-well round- and deep-well), V_L = 0.5 mL, n = 1000 rpm, d_0 = 3 mm, T = 30 °C.

and thus, less glucose accumulates within the batch phase. Consequently, the length of the batch phase is shortened and the maximum OTR value is decreased (Figure 2A). Additional factors that influence glucose accumulation, which however were not relevant within this experiment, are the specific growth rate and potential lag phases.[46] Once the accumulated glucose is reduced to limiting levels, the OTR drops sharply, which marks the initiation of the glucose-limited fed-batch phase. Since the filling volume was kept constant within each well, the volumetric release rate was equal for all cultivations, which was reflected by a highly comparable OTR plateau of about 7.5 mmol L^{-1} h^{-1} within the fed-batch phase (Figure 2A). The slightly declining trend of the OTR plateau can be attributed to the glucose release that diminishes over time (Figure 5A). The secondary OTR peaks within the fed-batch plateau, for example, visible at 4 and 9 h within the experiment with an OD_{600} of 2.5, indicate the parallel consumption of the overflow metabolites acetate and 2,3-butanediol.[7] As overflow metabolites mainly derive from the preculture, the peaks become less pronounced with decreasing inoculation volume. Despite a low inoculation volume for the experiment with an initial OD_{600} of 0.1, a pronounced shoulder within the fed-batch OTR plateau is visible. Because of the low initial biomass, glucose accumulation was intensified and most probably caused additional formation of overflow metabolites that are consumed once entering glucose-limited conditions.

The experiments with an initial OD_{600} of 0.5, 1.0, 1.5, and 2.5 show no major difference of the final relative protease activity (Figure 2B). However, the experiment with the lowest initial biomass (OD_{600} of 0.1) has a decreased relative protease activity. One possible reason for this is the enhanced glucose accumulation within the batch phase, which possibly enhances overflow metabolite formation and culture acidification. Additionally, the prolongation of the batch phase cuts the time in which *B. licheniformis* is exposed to glucose-limited fed-batch conditions. It was shown in a previous study that under batch conditions protease biosynthesis is repressed, whereas under glucose-limited fed-batch conditions protease activities increase.[7] Thus, it is hypothesized that too low initial biomass concentration have a negative effect on the final relative protease activities.

3.3. Influence of the Initial Filling Volume on Microtiter Plate-Based Fed-Batch Cultivations

The glucose release rate of the polymer-based controlled-release fed-batch microtiter plate is defined by the composition of the silicone matrix and the amount of the embedded glucose crystals. The user cannot change these characteristics, although the filling volume can be varied in order to change the volumetric release rate. The experiment in **Figure 3**A–C shows the results of different filling volumes when using the polymer-based controlled-release fed-batch microtiter plate.

The initial increase of the OTR within the batch phase is highly comparable for the experiment with 0.5, 0.8, and 1.0 mL filling volume (Figure 3A). This is due to identical cultivation conditions with equal initial biomass concentrations. However, the maximum OTR, the length of the batch phase, and the level of the OTR plateau within the fed-batch phase directly correlate with the filling volume. While the batch phase is shortened with increasing filling volume, the maximum OTR as well as the level of the OTR plateau within the fed-batch phase are reduced. The filling volume that influences the volumetric glucose release rate (Equation (3)) solely causes these differences.

Final relative protease activities with different volumetric release rates are shown in Figure 3C (filled bars). The relative protease activities are inversely proportional to the filling volume. However, to investigate the physiological and regulatory effect of different volumetric release rates on protease biosynthesis with *B. licheniformis*, instead of the relative protease activity, protease yields have to be compared. In a previous publication, it was demonstrated that protease yields related to consumed oxygen ($Y_{P/O2}$) are a suitable alternative to the glucose based yields ($Y_{P/S}$).[7] Consequently, the overall oxygen transfer (OT), which is equivalent to the total consumed oxygen, was used to calculate $Y_{P/O2}$ (Figure 3B). The results highlight that the different volumetric release rates have no effect on $Y_{P/O2}$ and $Y_{P/S}$ (Figure 3C, dashed bars; Table S2, Supporting Information). This observation is in good agreement with the publication by Habicher et al. and underlines that the investigated volumetric release rates do not influence protease yield for this *B. licheniformis* strain.[7] In

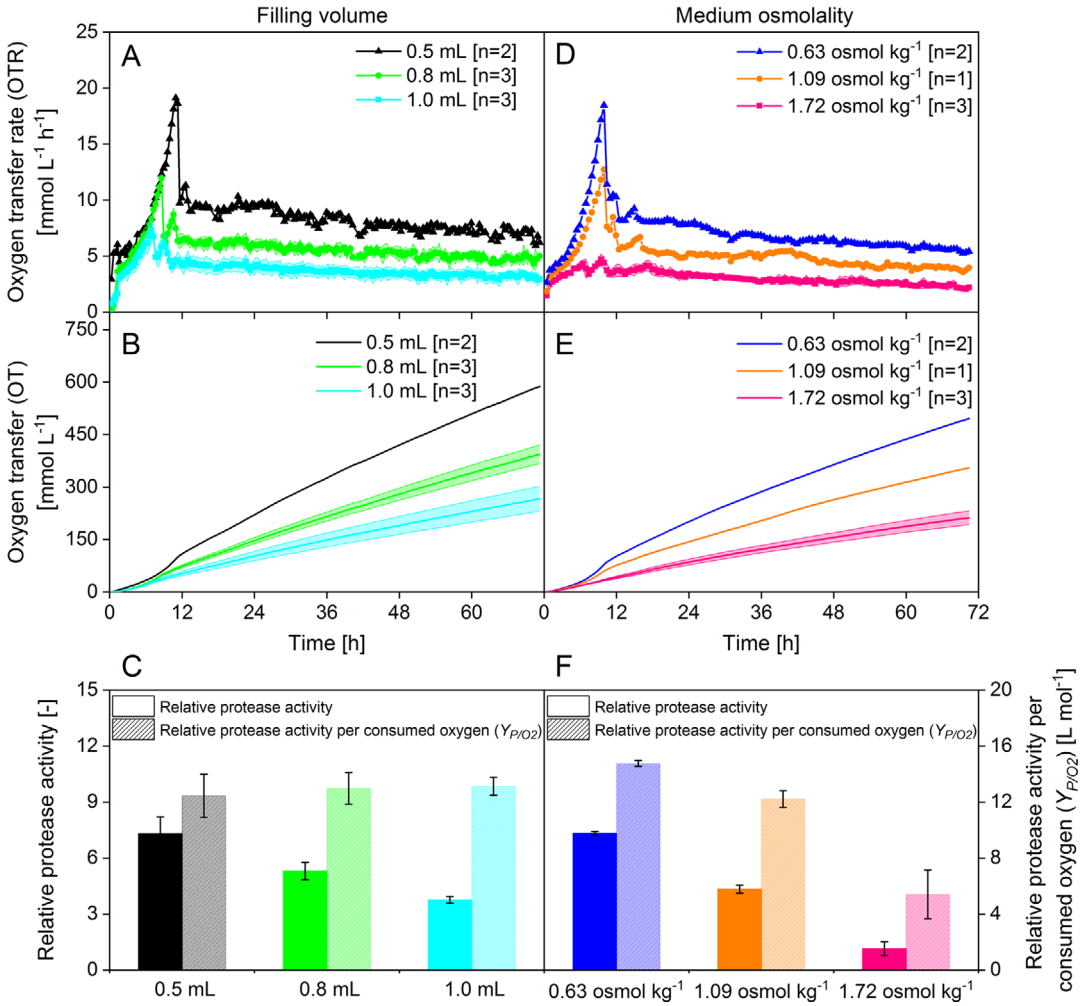

Figure 3. Influence of the initial filling volume and medium osmolality on fed-batch cultivations of *Bacillus licheniformis* using the polymer-based controlled-release fed-batch microtiter plate. A–C) Variation of the initial filling volume. D–F) Variation of the medium osmolality. A concentrated sodium chloride (NaCl) solution was utilized to increase the osmolality of the culture medium. The osmolality of the standard medium formulation (without the addition of NaCl) is 0.63 osmol kg^{-1}. A and D) Mean oxygen transfer rate (OTR) over time. Each experiment was conducted in triplicates, however, wells that could not be sealed tightly by the measurement device or had a noisy p_{O2} signal were excluded. Shadows symbolize the SD of parallel cultivations (for n > 2). B) Mean oxygen transfer (OT) over time. The OT is equivalent to the consumed oxygen. Shadows symbolize the SD of parallel cultivations (for n > 2). C) Mean relative protease activity (filled bars) and relative protease activity per consumed oxygen ($Y_{P/O2}$) (dashed bars) at the end of the cultivation. Error bars symbolize the SD of the relative protease activity measured in parallel cultivations (n = 3). Protease activities were set in relation to the measured protease activity directly after glucose depletion at 11.5 h within the batch experiment, shown in Figure S2, Supporting Information. Cultivation conditions: polymer-based controlled-release fed-batch microtiter plate (48-well round- and deep-well), OD_{600} = 0.5, V_L = 0.5 mL (if not stated otherwise), n = 1000 rpm, d_0 = 3 mm, T = 30 °C.

addition, the comparability between $Y_{P/O2}$ and $Y_{P/S}$ was confirmed. In contrast to the yields, however, increasing volumetric glucose release rates enhance the relative protease productivity (Table S3, Supporting Information).

In additional experiments with an initial OD_{600} of 1.5, the batch phase was artificially prolonged by supplementing 2.5 and 5 g L^{-1} glucose to the culture medium, while the filling volume of 0.5 mL and consequently the volumetric release rate was kept constant (Figure S3A, Supporting Information). The relative protease activity is slightly enhanced with glucose supplementation when compared to the experiment without initial glucose (0 g L^{-1}; Figure S3B, Supporting Information). Since the supplemented glucose is mainly used for growth, the fed-batch phase is initiated with different biomass concentrations, which could explain the higher final protease activity. Nevertheless, the protease activity per consumed oxygen ($Y_{P/O2}$) and per consumed glucose ($Y_{P/S}$) remains rather constant (Figure S3B, Table S2, Supporting Information).

3.4. Influence of the Medium Osmolality on Microtiter Plate-Based Fed-Batch Cultivations

To highlight the effect of increased osmolality, the V3 mineral medium was supplemented with a concentrated sodium chloride solution. In Figure 3D–F, the influence of media formulations

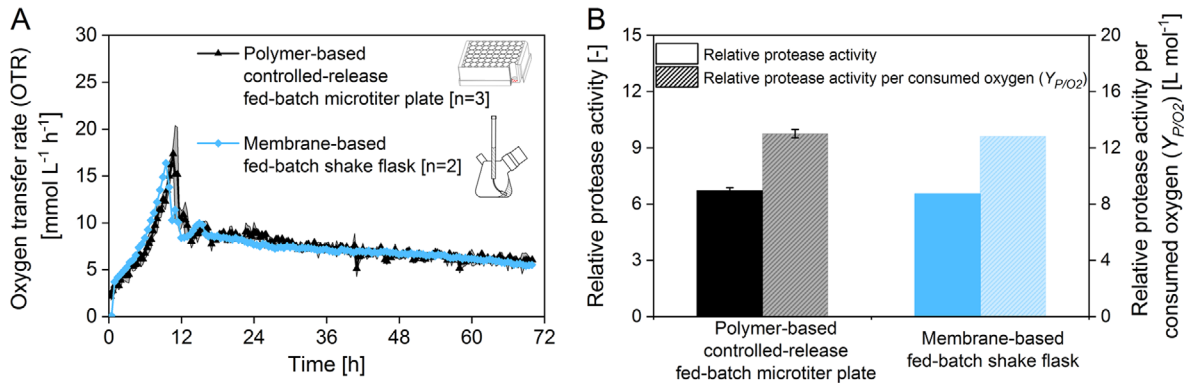

Figure 4. Scale-up of microtiter plate-based fed-batch cultivations to shake flasks with the volumetric glucose release rate as scale-up criterion. A) Mean oxygen transfer rate (OTR) of the polymer-based controlled-release fed-batch microtiter plate and of the membrane-based fed-batch shake flask over time. Shadows symbolize the SD of parallel cultivations (for n > 2). B) Mean relative protease activity (filled bars) and relative protease activity per consumed oxygen ($Y_{P/O2}$) (dashed bars) at the end of the cultivation. Error bars symbolize the SD of the relative protease activity measured in parallel cultivations (n = 3). Protease activities were set in relation to the measured protease activity directly after glucose depletion at 11.5 h within the batch experiment, shown in Figure S2, Supporting Information. µRAMOS cultivation conditions (microtiter plate): polymer-based controlled-release fed-batch microtiter plate (48-well round- and deep-well), OD_{600} = 0.5, V_L = 0.5 mL, n = 1000 rpm, d_0 = 3 mm, T = 30 °C. RAMOS cultivation conditions (shake flask): membrane-based fed-batch shake flask (250 mL Erlenmeyer flask), OD_{600} = 1.0, V_L = 16 mL, n = 350 rpm, d_0 = 50 mm, T = 30 °C. Setup membrane based fed-batch shake flask: V_L (reservoir) = 3 mL, S (reservoir) = 200 g L^{-1}, active diffusion diameter = 4.8 mm. Membrane: type, RCT-NatureFlex-NP; material, regenerated cellulose; thickness, 42 µm; molecular weight cut-off, 10–20 kDa.

with osmolalities of 0.63 (no sodium chloride added), 1.09, and 1.72 osmol kg^{-1} are shown. Within the batch phase, the increase of the OTR is reduced for experiments with increased osmolality (Figure 3D). However, the initial biomass concentration was kept constant, and thus, the osmotic pressure attenuates growth. Under osmotic stress, *B. licheniformis* activates mechanisms to accumulate compatible intracellular osmolytes.[47,48] Although this mechanism enables *B. licheniformis* strains to withstand high salt concentrations, growth yields diminish.[48] It was further found that the osmoadaptation goes along with an increased cellular demand for energy.[47] Thus, it is most likely that the higher energy demand slows down growth.

Within the fed-batch phase, the OTR level is inversely proportional to the medium osmolality (Figure 3D). As the filling volume was kept constant, the decreased OTR level within the fed-batch phase indicates that the osmotic pressure causes a reduction of glucose release.[38] In order to confirm this, final glucose concentrations were measured in wells with identical medium formulation but without biomass. The glucose concentration was 26, 17, and 8 g L^{-1} within wells with 0.63, 1.09, and 1.72 osmol kg^{-1}, respectively (Table S2, Supporting Information). The proportion between the final glucose concentrations correlate with the proportion between the final OTs (Figure 3E; Table S2, Supporting Information). Consequently, glucose release was affected by physically altered release characteristics within the polymer matrix. These results are confirmed by experiments with altered MOPS buffer concentrations (Figure S4A, Supporting Information). When comparing cultivations with similar osmolality using MOPS buffer and sodium chloride (1.08 ± 0.01 osmol kg^{-1}), the level of the OTR plateau within the fed-batch phase is highly comparable (Figure S4B, Supporting Information). Thus, the osmotic pressure affects glucose release.[38]

The volumetric release rate, which is highest for the lowest osmolality and vice versa, correlates with the final relative protease activity (Figure 3F, filled bars). The protease yield per consumed oxygen ($Y_{P/O2}$) and per consumed glucose ($Y_{P/S}$) does not give comparable results between the different volumetric release rates (Figure 3F, dashed bars; Table S2, Supporting Informaiton). Independent of the release characteristics of the polymer-based controlled-release fed-batch microtiter plate, the increased osmolality again has a physiological influence on *B. licheniformis*. Most probably, the osmotic pressure not only slows down growth, but also has a negative effect on protease biosynthesis.

3.5. Scale-up of Microtiter Plate-Based Fed-Batch Cultivations to Shake Flasks

To show scalability of microtiter plates to shake flasks, the polymer-based controlled-release fed-batch microtiter plate process was transferred to membrane-based fed-batch shake flasks. Consequently, the volumetric release rate within the membrane-based fed-batch shake flask was adjusted to meet the volumetric glucose release rate within the polymer-based controlled-release fed-batch microtiter plate. The results of both small-scale fed-batch cultivation systems are shown in **Figure 4**.

The OTR course of the membrane-based fed-batch shake flask and the polymer-based controlled-release fed-batch microtiter plate cultivation is highly comparable (Figure 4A). Especially the OTR level within the fed-batch phase of both cultivation systems underlines the fact that very similar volumetric glucose release rates are met. The fact that similar volumetric glucose release rates are achieved is also reflected by the similar amount of consumed glucose (Table S2, Supporting Information). The final relative protease activity, $Y_{P/O2}$, $Y_{P/S}$ and the protease productivity compare well in both systems (Figure 4B; Tables S2 and S3, Supporting Information). Additionally, mean pH and OD_{600} were related between the two systems at the end of cultivation. The pH was 7.07 and 7.02 whereas the OD_{600} 9.8 and 10.9 for the

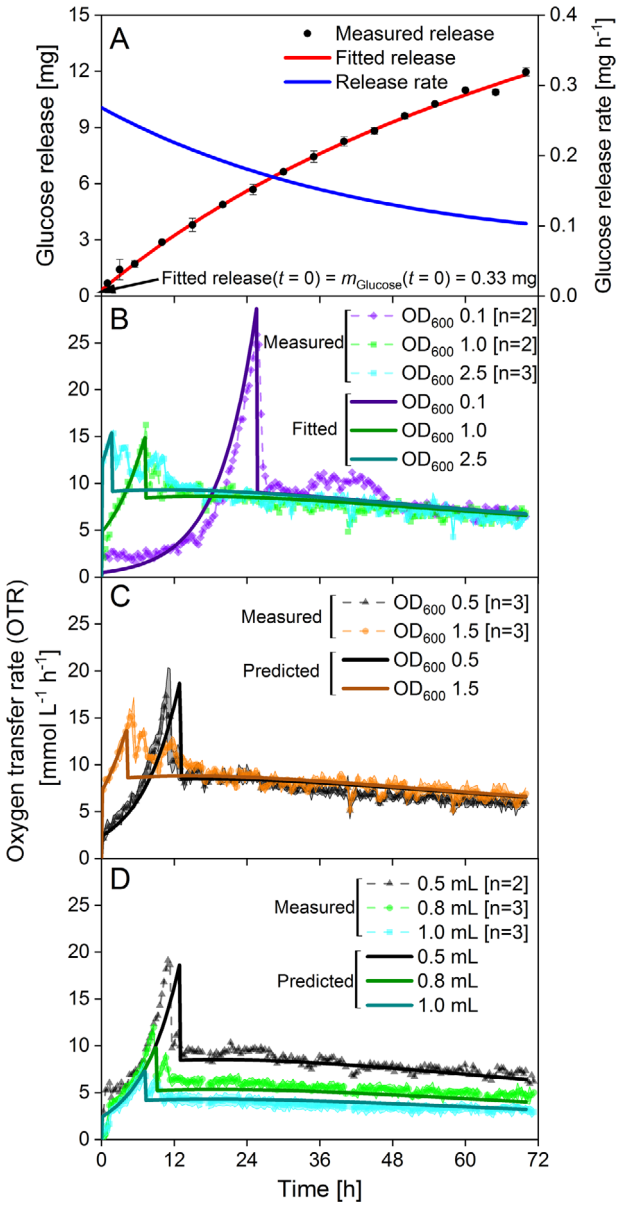

Figure 5. Glucose release of the polymer-based controlled-release fed-batch microtiter plate and model-based simulation of the OTR course of *Bacillus licheniformis* fed-batch cultivations with varied initial biomass concentration and filling volume. A) Glucose release was determined experimentally by measuring the glucose concentration in V3 mineral medium without cells over time. Each data point represents the mean of three individual wells (n = 3). The measured release was fitted and differentiated resulting in the glucose release rate. The intersection of the fitted glucose release with the *y*-axis ($t = 0$) corresponds to the amount of initially provided glucose. B) Maintenance coefficients m_S and m_{O2} were fitted to the experiments with an initial OD_{600} of 0.1, 1.0, and 2.5 by performing a global minimization (global search (*gs*) and *fmincon* MATLAB functions) of the sum of least squares based on the described fed-batch model (Equations (1)–(7)). C and D) The model with the fitted parameters m_S and m_{O2} was validated on basis of experiments with varying initial biomass concentration (Figure 2A) (C) and filling volume (Figure 3A) (D).

polymer-based controlled-release fed-batch microtiter plate and the membrane based fed-batch shake flask, respectively.

In a recent publication by Müller et al., the transferability of membrane-based fed-batch shake flask cultivations to stirred-tank reactors was validated.[49] Despite inevitable differences between the scales, such as pH control and initiation of the feed, comparable results were achieved when equal volumetric glucose release rates were applied. This demonstrates that glucose-limited fed-batch cultivations can be consistently scaled-up from microtiter plates to laboratory-scale stirred tank reactors. The microtiter plate-based fed-batch process, however, normally differs from a fed-batch process in laboratory-scale stirred tank reactors in terms of the feeding strategy and the total amount of fed glucose. Despite this fact, similar basic physiological conditions concerning catabolite repression, pH range, and oxygen availability are met between the scales. In contrast to batch mode, this basic comparability between the scales allows to investigate process parameters, such as osmolality, in early developmental stages using microtiter plates. Finally, if fed-batch cultivations with sophisticated feeding strategies conducted in laboratory-scale stirred tank reactors are transferred to production-scale stirred tank reactors, additional factors, such as mixing effects, have to be considered. These factors related to production-scale, however, are a subject of its own, and are not treated in this work.

3.6. Modeling Microtiter Plate-Based Fed-Batch Cultivations

Simulation of fed-batch cultivations with the polymer-based controlled-release fed-batch microtiter plates requires knowledge of the glucose release rate. Since the polymer-based release mechanisms are complex, glucose release was determined experimentally with V3 mineral medium without cells (**Figure 5**A). Glucose release slightly flattens over time, and thus, the release rate decreases from 0.27 to 0.1 mg h^{-1}. The volumetric release (F_S) and release rate (F'_S) are obtained by dividing the filling volume V_L (Equation (3)).

The unknown maintenance coefficients m_S and m_{O2} were fitted to the described model (Equations (1)–(7)) on basis of the experiments with an initial OD_{600} of 0.1, 1.0, and 2.5 (Figure 5B). The global minimum of the sum of least squares was found for $m_S = 0.0638$ g_S g_X^{-1} h^{-1} and for $m_{O2} = 0.0018$ mol_{O2} g_X^{-1} h^{-1}. When comparing the fitted values to another protease producing *B. licheniformis* strain from literature ($m_S = 0.0414$ g_S g_X^{-1} h^{-1} and $m_{O2} = 0.0011$ mol_{O2} g_X^{-1} h^{-1}),[50] the maintenance requirements are slightly increased. The higher maintenance requirements also explain the lower values for $Y_{X/S}$ and $Y_{X/O2}$ when compared to the same strain from literature. However, the fitted maintenance coefficients were within the physiological range of various *Bacillus* species.[44,45] Since the model equations did neither contain terms describing overflow metabolite production nor overflow metabolite consumption, secondary OTR peaks are observable for the measured but not for the simulated OTR course (Figure 5B).

To validate the established fed-batch model, additional conditions, such as different initial biomass concentrations and filling volumes, were simulated using the fitted maintenance coefficients. The model-output of experiments with varied initial biomass concentrations and filling volumes are shown in Fig-

ure 5C,D, respectively. In both cases, the simulated OTR course correctly reflects the initial batch phase, the initiation of the fed-batch phase, and the level of the fed-batch OTR plateau. It can be concluded that the determined maintenance coefficients in combination with the other model parameters are suitable to describe the OTR course of cultivations in polymer-based controlled-release fed-batch microtiter plates.

4. Conclusions

Polymer-based controlled-release fed-batch microtiter plates were successfully used for glucose-limited cultivations of a protease producing *B. licheniformis* strain. Reproducibility was shown by means of cultivation experiments using five individual polymer-based controlled-release fed-batch microtiter plates from two different production lots. µRAMOS-based online OTR monitoring provided the basis for visualizing the effect of changing initial cultivation conditions, such as biomass concentration, filling volume, and osmotic pressure. The initial biomass concentration has a direct influence on glucose accumulation, and thus, on the extend of the batch phase. The filling volume enables changing the volumetric release rate whereas the osmotic pressure physically influences the release rate. It was further shown that an increasing osmotic pressure negatively influences growth and protease yield. These results provide the basis for future designs of strain and media screening experiments with *B. licheniformis* using the polymer-based controlled-release fed-batch microtiter plate.

The high comparability between fed-batch cultivations with microtiter plates and shake flasks demonstrates the possibility of using different sizes of small-scale shaken bioreactors operated in fed-batch mode. Müller et al. showed the transferability of fed-batch cultivations with shake flasks to laboratory-scale stirred tank reactors.[49] Consequently, comparable results are achieved under glucose-limited fed-batch conditions in the scales relevant for the early stages of development projects (microtiter plates, shake flasks, and laboratory-scale stirred tank reactors). With the use of these bioreactor systems operated in fed-batch mode, a possible development scenario might be as follows: after a primary screening in polymer-based controlled-release fed-batch microtiter plates, the versatile membrane-based fed-batch shake flasks can be used to evaluate various substrate-limited fed-batch modes before continuing with process optimization in stirred tank reactors.

This work has shown that small-scale fed-batch shaken bioreactors that can be coupled with online monitoring devices, such as the RAMOS and µRAMOS devices, significantly increase information content. This enables creating and validating mathematical models in early development stages, which in turn improves process understanding. The established model can be used to determine the maximum oxygen transfer capacity (OTR_{max}) and the length of the batch phase as a function of the initial biomass concentration. On this basis, extended batch phases with possible oxygen limitations can be avoided. Hence, the model enables defining optimal cultivation conditions for screening processes with the polymer-based controlled-release fed-batch microtiter plate. A manuscript that aims at showing that with polymer-based controlled-release fed-batch microtiter plates the performance of *B. licheniformis* clones is ranked similarly to results from a laboratory-scale fed-batch stirred tank process is currently in preparation.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Bacillus licheniformis, fed-batch, microtiter plate, protease, shaken bioreactors

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