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Precultures Grown under Fed-Batch Conditions Increase the Reliability and Reproducibility of High-Throughput Screening Results

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One essential task in bioprocess development is strain selection. A common screening procedure consists of three steps: first, the picking of colonies; second, the execution of a batch preculture and main culture, e.g., in microtiter plates (MTPs); and third, the evaluation of product formation. Especially during the picking step, unintended variations occur due to undefined amounts and varying viability of transferred cells. The aim of this study is to demonstrate that the application of polymer-based controlled-release fed-batch MTPs during preculture eliminates these variations. The concept of equalizing growth through fed-batch conditions during preculture is theoretically discussed and then tested in a model system, namely, a cellulase-producing *Escherichia coli* clone bank containing 32 strains. Preculture is conducted once in the batch mode and once in the fed-batch mode. By applying the fed-batch mode, equalized growth is observed in the subsequent main culture. Furthermore, the standard deviation of cellulase activity is reduced compared to that observed in the conventional approach. Compared with the strains in the batch preculture process, the first-ranked strain in the fed-batch preculture process is the superior cellulase producer. These findings recommend the application of the fed-batch MTPs during preculture in high-throughput screening processes to achieve accurate and reliable results.

1. Introduction

For initial process development steps, such as clone selection, expression studies, or strain optimization, small-scale high-throughput culture procedures are the method of choice.[1,2] For such procedures, microtiter plates (MTPs) are most often applied due to their compact design and thus economical use of space and resources. Moreover, MTPs are easily integrated into fully automated process systems such as monitoring and liquid-handling devices.[3–7]

Most strain selection procedures start with colony picking from an agar plate, followed by at least one preculture step (**Figure 1**). The step of colony picking involves the risk of transferring different amounts of viable cells in diverging physiological and metabolic states from the agar plate into the preculture.[8] This always arises, regardless of which picking method (by hand or by a picking robot) or which picking instrument (inoculation loop, tooth stick, pipette tip, etc.) is applied. In alternative procedures, an additional cryoconservation step is interposed before performing the preculture.[9,10] Nevertheless, with most of the commonly applied methods, the transfer of inoculum from cryocultures to the preculture also carries the risk of generating unintended errors.[10]

A defined volume of preculture broth is usually transferred for main culture inoculation.[9] Regardless of the cause, if the biomass concentration or the metabolic state of the cells transferred is not consistent, the resulting growth of the main culture will be unreproducible. These diverging starting conditions carry over into the course of the main culture and, if the system needs to be induced, result in an undefined status of growth at the time of induction. However, the metabolic state of cultures at the time of induction is one of the key determining factors for product formation. Thus, diverging cell statuses will lead to unreliable results.[11,12] Based on such results, it is not possible to evaluate a clone bank with respect to the most productive strain.

To overcome this problem, several approaches have been reported: the most basic method is to cultivate all strains until

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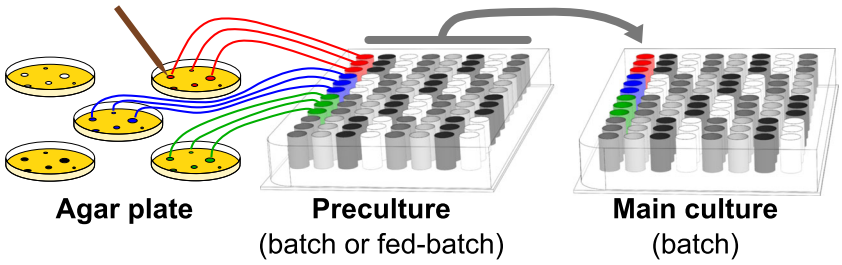

Figure 1. Usual inoculation procedure in high-throughput screening processes. In this work, the whole process is conducted in triplicate for each clone variant (symbolized for three clones by color). Each individual clone was streaked on an individual agar plate. Each agar plate contains various colonies of genetically identical clones.

substrate depletion (stationary phase) in preculture, with the assumption that all strains reach identical biomass concentrations. This approach bears the risk of acidification of the culture broth and therefore a considerable reduction in cell viability.[13] Due to the abundance of substrate present at the beginning of batch cultivations, the metabolic activity increases in line with the growth rate, but is not controlled by the operator. This may lead to oxygen limitation and cause undesired cell performance (e.g., anaerobic metabolism, reduction, or cease of growth).[14] Especially in small-scale reactors, oxygen limitation is rarely noticed. Furthermore, the stationary phase of batch cultivations is characterized by a transition of cells in terms of morphology and physiology.[15,16] Fast-growing strains remain in the stationary phase longer and are therefore more affected. All of these aspects may result in variations in lag times and growth rates in the main cultures.[17,18] Taking these factors into account, the method of growing strains until the stationary phase is not appropriate to achieve comparable growth in the main culture.

An alternative strategy is to monitor the biomass concentration online by means of scattered light or fluorescence intensity.[19,20] With well-known biomass concentrations, each culture can be inoculated with a customized preculture volume to achieve equal biomass starting conditions. This biomass-specific inoculation of the main culture, ideally with viable and exponentially growing cells, compensates for variations in growth characteristics during preculture.[4,21] Alternatively, an automated method to maintain cells at high growth rates until all cultures reach a predetermined biomass concentration is described.[22] This enables contemporaneous inoculation of the main culture with an equal number of cells in similar metabolic states. If these approaches are to be applied in high-throughput procedures, automated liquid-handling systems are unavoidable.[21,22] Unfortunately, these systems require considerable investment, and integration into an existing high-throughput process may be challenging.

Studier introduced an autoinduction medium to overcome all of these issues for a T7*lac* promoter system in *Escherichia coli* (*E. coli*).[13] However, this technique is limited to a specific organism and vector combination.

A sophisticated way to cope with the issue of unpredictable growth is to assure that the precultures feature similar biomass concentrations and states of growth at the point of transfer to the main culture. Jenzsch et al.[23,24] found that the application of the fed-batch operation mode in the early phase of stirred-tank fermentation greatly improves the batch-to-batch reproducibility. Subsequently, Huber et al.[8] presented the proof of principle of equalizing growth by applying the fed-batch operation mode in small-scale cultivation vessels. Šiurkus et al.[25] applied a similar method to maintain equalized growth during preculture.

The fed-batch operation mode is well-established in industrial bioprocesses. In comparison to batch operation, fed-batch operation prevents phenomena such as catabolite repression, overflow metabolism, and substrate inhibition. Additionally, due to the limited substrate supply under fed-batch conditions, metabolic activity is controlled and oxygen limitation is avoided.[26] Recent developments also enable fed-batch conditions in MTPs. Two typically applied systems are the enzymatic degradation of polysaccharides and polymer-based glucose release from a silicone elastomer matrix.[27–30] These techniques provide improved scalability of bioprocesses from MTPs to industrial-scale production.[28,31] Furthermore, the effect of different glucose release rates can be investigated at an early stage of process development.[29,32] However, all of these studies focused on the application of fed-batch conditions in the expression, or main culture. The effect of fed-batch operation during precultures on the subsequent main cultures and the resulting ranking of clones has not been thoroughly investigated.

In this study, polymer-based controlled-release fed-batch MTPs (FeedPlate; Kuhner Shaker GmbH, Herzogenrath, Germany) were applied to realize fed-batch conditions in precultures. The aim of this work was to investigate the influence of fed-batch precultures on the final clone ranking during a screening procedure in MTPs. An industrially relevant enzyme, cellulase celA2, was chosen as a model product to support the significance of the study for commercial process development. A simple Monod model is utilized to study the effect of unintended variations during colony picking on the main culture and to gain insight into whether a fed-batch precultivation will overcome this issue. The model is validated experimentally. Two screening processes are conducted in parallel, starting from colony picking, followed by one preculture, one main culture, and the product quantification step. The difference between the two screening processes is the operation mode of the preculture, which is either the batch or the fed-batch mode (compare Figure 1), while the main culture is always operated in the batch mode. Thirty-two cellulase-producing *E. coli* BL21 DE3 celA2 strains, generated by

site-saturation mutagenesis at three positions, serve as a model clone library. Scattered light, final cellulase activity, clone ranking, and the standard deviations among clones are evaluated to determine the influence of using the fed-batch preculture in comparison to batch preculture on the outcome of the screening.

2. Experimental Section

2.1. Clone Bank

All cultivations were conducted with an *E. coli* BL21 (DE3) clone bank for recombinant expression of the cellulase celA2. The clone bank was generated by site-saturation mutagenesis at three positions. The applied vector was a pET-28a(+)-plasmid. Cellulase production was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) using a T7*lac* promoter system. The total clone bank consists of 96 variants. For this study, 32 randomly chosen variants were employed. Wild type represents the clone expressing the original enzyme. Detailed information about the preparation of the clone bank is described elsewhere.[21,33]

2.2. Media

Agar plates were prepared with 5 g L^{-1} glucose, 12 g L^{-1} peptone (6681.4; Carl Roth GmbH, Karlsruhe, Germany), 24 g L^{-1} yeast extract (2904.4; Carl Roth GmbH), 12.54 g L^{-1} K_2HPO_4, 2.31 g L^{-1} KH_2PO_4, 1 mL kanamycin sulfate, and 15 g L^{-1} agar. Precultures and main cultures were cultivated in modified Wilms–3-(*N*-morpholino)propanesulfonic acid (MOPS) mineral medium.[34,35] The exact composition is described elsewhere.[21] For batch experiments (preculture and main culture), an initial glucose concentration of 20 g L^{-1} was applied. For fed-batch cultivations, an initial glucose concentration of 2 g L^{-1} was applied.

2.3. Microtiter Plates

96-well plates: For batch cultivations, black polystyrene MTPs with clear flat bottoms (96-round-well, black/clear; Falcon, NY, USA) were used. For fed-batch cultivations, fed-batch MTPs (product no. SMFP08002; 96-square well) from Kuhner Shaker GmbH were used.

48-well plates: For batch cultivations, round 48-deep well plates (m2p-labs GmbH, Baesweiler, Germany) were used. For fed-batch experiments, special custom-made round 48-well MTPs were kindly provided by Kuhner Shaker GmbH. The plate was manufactured following the production procedures for the 96-well plate already available.

As a permeable sterile barrier for all MTP cultivations in 96-well plates, "AeraSeal Film" (A9224; Sigma-Aldrich Chemie GmbH, Germany) was applied. For cultivation in 48-well plates, "Polyolefin sealing foil" (900371-T; HJ-Bioanalytik GmbH, Erkelenz, Germany) was applied.

2.4. Cultivation Procedure

Each clone variant (of 32 in total) was streaked on an agar plate and incubated for at least 24 h at 37 °C. For transfer from the agar plate to preculture, single colonies were picked. Picking was conducted by poking a sterile pipette tip one time into a single colony and subsequently mixing the media in the target well with the respective pipette tip. Cultivation conditions in preculture and main culture were 37 °C, with a shaking frequency of 1000 rpm and a shaking diameter of 3 mm. Humidified air (80%) was used inside the shaking hood to reduce evaporation. For cultivation in 96-well MTPs, the cultivation procedure was as follows: precultures were cultivated for approximately 18 h. For online monitoring of scattered light, batch precultures were carried out in a 150 µL culture volume in clear flat-bottom MTPs. For fed-batch precultures, 500 µL of culture volume was applied. The difference in culture volumes takes the increased cross-sectional area of the square well geometry into account as well as the liquid accumulating in the silicon matrix. The main cultures were performed at 150 µL of cultivation volume and cultivated for 23 h. The inoculation volume was 10 µL (6.67 vol%) of preculture broth. For induction, IPTG was added to a concentration of 0.1 mM in the broth after 4 h of cultivation. For cultivations in 48-round-well MTPs, a total filling volume of 800 µL per well and an inoculation volume of 53 µL were applied.

For online measurement of scattered light during cultivation, the device described by Samorski et al.[36] (BioLector; m2p-labs GmbH) was used. Scattered light linearly correlated with biomass.[20] The respiration activity monitoring system (RAMOS) was applied to determine metabolic activity. Therefore, oxygen transfer rates (OTRs) were calculated from the decrease in the oxygen partial pressure in the headspace of the reaction vessel. For determination of the OTR in 48-round-well plates, the µRAMOS technique was applied.[37,38] By integration of OTRs over time, the total oxygen transferred (OT) was calculated. OT is a good quantitative indicator for the total consumed substrate and therefore for the produced biomass (assuming a constant yield coefficient).[39] This approach is advantageous since the matrix at the bottom of the wells of the fed-batch MTP prevents the optical measurement of scattered light.

2.5. Offline Sample Analysis

Cell lysis was performed using the BugBuster 10× Protein Extraction Reagent kit (70921; Merck Millipore, Darmstadt, Germany). Additional benzonase (70746; Merck Millipore) and lysozyme (105281; Merck Millipore) were added to the reagent as recommended in the instructions, referred to as "protein extraction reagent." For preparation, 80 µL of the cultivation broth from each well was transferred into a 96-well V-shaped MTP (9292.1; Carl Roth GmbH) and centrifuged for 15 min at 964 × *g* (Rotina 35R; Hettich, Germany). The supernatant was removed with a pipette. The remaining pellets in the MTP were frozen at −20 °C overnight to support cell lysis due to ice crystal formation. Subsequently, the pellet was resuspended in 50 µL of the protein extraction reagent at room temperature for

20 min by shaking at 1000 rpm in a ThermoMixer (5382000015; Eppendorf, Germany). This cell lysate was used for the determination of cellulase activity.

For cellulase activity determination, the fluorescence-based 4-methylumbelliferyl-β-D-cellobioside (4-MUC) assay was applied, with some adaptations.[21,33] A 16.7 μM 4-MUC stock solution was prepared with 0.2 M potassium phosphate buffer (pH 6.5) and stored at 4 °C. Forty microliters of the cell lysate were transferred into a well of a 96-well MTP (polystyrene, flat bottom, black/clear; BD Falkon). Both the cell lysate in the MTP and the 4-MUC stock solution were preheated to 30 °C for 10 min. Afterward, 60 μL of the 4-MUC stock solution was added to each well and mixed thoroughly. Fluorescence was measured once per minute for 20 min with excitation/emission wavelengths of 365/455 nm in a Synergy 4 Microplate Reader (BioTek, Winooski, VT, USA). A calibration curve was prepared by correlating different 4-methylumbelliferone concentrations (0.03–0.2 mM) with measured fluorescence.

2.6. Benchmark Screening Procedure

To interpret and evaluate the mean standard deviation of the final cellulase activities (Figure 5), a benchmark was defined. Therefore, eight preculture wells were inoculated with a defined optical density. From each preculture well, one main culture well was inoculated. The standard deviation of the final cellulase activity for those eight wells was determined. This procedure was repeated six times, applying six different initial optical densities for preculture inoculation. The mean standard deviation of those six initial conditions was approximately 12%. This provides a benchmark for accuracy of the process depicted in Figure 1 without the error introduced by the picking process.

3. Theoretical Background

A simple model based on Huber et al.[8] is used with some modifications to mathematically investigate the concept of equalizing growth during precultures and thus minimizing variation in the main cultures. In this model, unintended variations occurring in colony picking are represented by varying inoculum concentrations of one clone in the preculture. The initial concentration of biomass in the main culture results from inoculation of 6.67 vol% preculture broth. The model is based on Monod kinetics and comprises the equations listed in Data S1 (Supporting Information).

4. Results and Discussion

4.1. Modeling: Preculture Cultivation

In the applied simple Monod model (Data S1, Supporting Information), different initial biomass concentrations (inoculum concentration X_0, ranging from 0.04 g L^{-1} to 0.4 g L^{-1}) represent variations in experimental colony picking. The increase in biomass is examined during the time course of precultivation in the batch operation mode (**Figure 2**A) and in the fed-batch operation mode (Figure 2B). The batch operation mode exhibits exponential growth for all initial biomass concentrations. The cultures with high inoculum concentrations (black and red curves) reach the stationary phase before the time point at which inoculum is transferred. At this time point, the culture with intermediate inoculum concentration is at the end of the exponential growth phase (blue curve), whereas the ones with the lowest inoculum concentrations (pink and green curves) are still exponentially growing and therefore have less biomass. The preculture in the fed-batch operation mode (Figure 2B) also exhibits exponential growth behavior for the initial time frame of cultivation ($X_0 = 0.4$ for 5 h; $X_0 = 0.04$ for 10 h). This is due to the accumulation of substrate during the initial cultivation phase since the biomass concentration is not yet sufficient to immediately consume the released substrate.[30] With increasing biomass, the accumulated substrate is consumed and the cultivations consecutively reach substrate limitation in the order of the initial inoculum concentration, beginning with $X_0 = 0.4$. From that point in time, the growth rate of the organism is defined by the feed rate and fed-batch cultivation is established. Once all cultures reach the fed-batch mode, each has consumed the same amount of substrate and therefore exhibits a similar biomass concentration. Furthermore, all of these cultures feature the same physiological and metabolic status and are in a condition of controlled growth. None of the cultures is faced with a deficiency in carbon source and thus none have entered the stationary phase. If the time of inoculum transfer were to be chosen in a way that all cultures have reached the fed-batch phase (in this example after 10 h cultivation time), the physiological state and biomass concentration in all cultures would be equivalent. In contrast, cultures grown in the batch mode manifest varying biomass concentrations as well as varying physiological states at the time of inoculum transfer. In Data S2 (Supporting Information), the modeled curves for the OTR and for the OT (batch: A + C; fed-batch: B + D) corresponding to the data in Figure 2A,B are depicted.

4.2. Modeling: Main Culture Cultivation

Figure 2C shows the growth characteristics of the main culture following batch preculture for 4 h until the time of induction. The three cultures with nearly maximal biomass concentration in the preculture ($X_0 = 0.4$–0.126 in black, red, and blue) only differ by the duration of the lag phase, following Equation 6 (Data S1, Supporting Information). However, the black curve exhibits a 65% higher biomass concentration compared to the blue one at the time of induction. For biomass concentrations ranging from $X_0 = 0.126$ to $X_0 = 0.04$ (blue, pink, and green curve), only the biomass concentration at the end of the preculture varied. Accordingly, the accumulated biomass concentration deviates at the time of induction. In conclusion, the highest modeled biomass ($X_0 = 0.4$, black curve, Figure 2C) is more than 400% higher compared to the lowest modeled biomass ($X_0 = 0.04$, green curve) at the time of induction. Furthermore, it is not possible to find a point in time at which the biomass of all cultures ranges in the target window of biomass suitable for induction. These major differences in biomass concentrations (and subsequently also in the

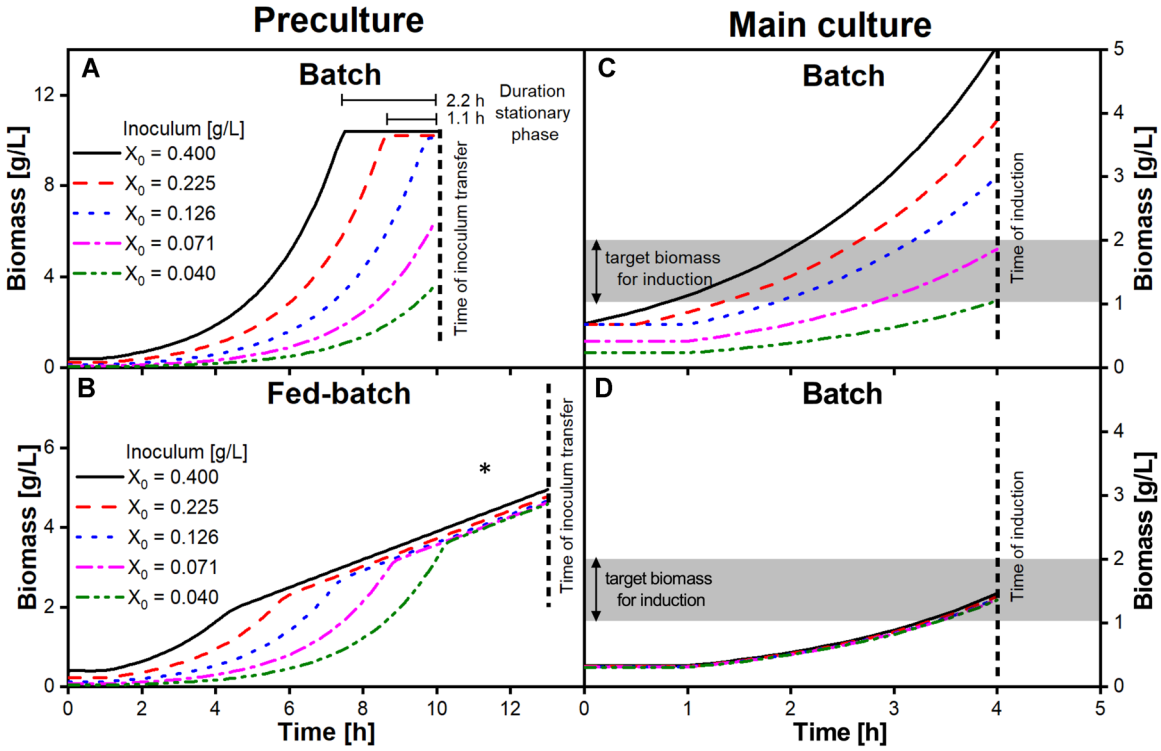

Figure 2. Model of an inducible microbial system obtained by applying simple Monod kinetics (varying initial biomass concentrations in the preculture $=X_0$ [g L^{-1}], $S_{0\_Batch} = 20$ g L^{-1}, $S_{0\_Fed\text{-}batch} = 2$ g L^{-1}, $\mu_{max} = 0.51$ h^{-1}, $K_S = 0.05$ g L^{-1}, $Y_{XS} = 0.5$ g_x g_S^{-1}, feed rate $= 1$ g L^{-1} h^{-1}).[8] A,B) Different initial biomass concentrations in a preculture caused by unintended variations in the process of colony picking from an agar plate. Preculture operated in (A) batch and (B) fed-batch modes (*flexible time period for inoculating the main culture). C,D) Main culture in the batch operation mode with initial biomass concentrations depending on the biomass concentration at the end of the preculture. The lag phase depends on the inoculation ratio and the status of growth in the preculture (Equation 5, Data S1, Supporting Information). Target biomass concentration for potential induction is indicated by the gray area.

remaining substrate concentrations) at the time of induction would lead to considerable variations in product formation. In this example, possible scenarios for one clone variant were investigated. If a whole clone bank is investigated, each clone would experience one out of a variety of scenarios. Under these conditions, a reliable clone ranking could not be obtained.

The main cultures following fed-batch precultures (Figure 2D) exhibit an aligned growth behavior for all cultivations, regardless of the initial inoculum concentration in preculture. This is due to the equalized biomass concentration and the identical growth status in preculture at the time of inoculum transfer. Furthermore, this aligned growth allows for induction of all cultures at one point in time and in the range of the target biomass. This model demonstrates the benefits of applying fed-batch in preculture for high-throughput screening.

4.3. Proof of Principle: Cultivation in Fed-Batch MTP

To experimentally demonstrate the benefits of equalizing growth with the help of the fed-batch MTP, experiments were conducted following the steps depicted in Figure 1. A single *E. coli* BL21 DE3 celA2 strain from one streaked agar plate was picked six times, inoculated into six preculture wells, and cultivated in the batch and fed-batch modes. The OTRs and the OT for the six batch cultivations are depicted in **Figure 3**A,C. The six cultivations of an identical strain display a different metabolic performance when cultured in the batch mode. This can be seen by focusing on two examples: for one cultivation (colored in black), the metabolic activity starts relatively quickly, whereas for another one (colored in green), the starting point is detected approximately 6 h later. The wide distribution of the OT curves over time in Figure 3C is consistent with the presumption of varying biomass concentrations in cultivations induced by colony picking. The OTRs during fed-batch precultures in Figure 3B likewise exhibit varying metabolic activities at the beginning of cultivation. Nevertheless, as soon as the initial batch phase is finished, the exponential increase in metabolic activity ends and the OTRs continue at a plateau level of approximately 10 mmol L^{-1} h^{-1}. This plateau indicates the fed-batch phase, resulting from the constant feed of glucose from the silicone reservoir. The OT also indicates a spread in biomass concentrations during the batch phase. As soon as all cultivations reach the fed-batch mode, the OT curves overlap, thus demonstrating the capability of fed-batch MTPs to equalize growth. The measured curves of the OTR and OT in Figure 3 are in good agreement with the modeled data in Data S2 (Supporting Information) and thus support the presumption of an equalizing effect during a fed-batch preculture.

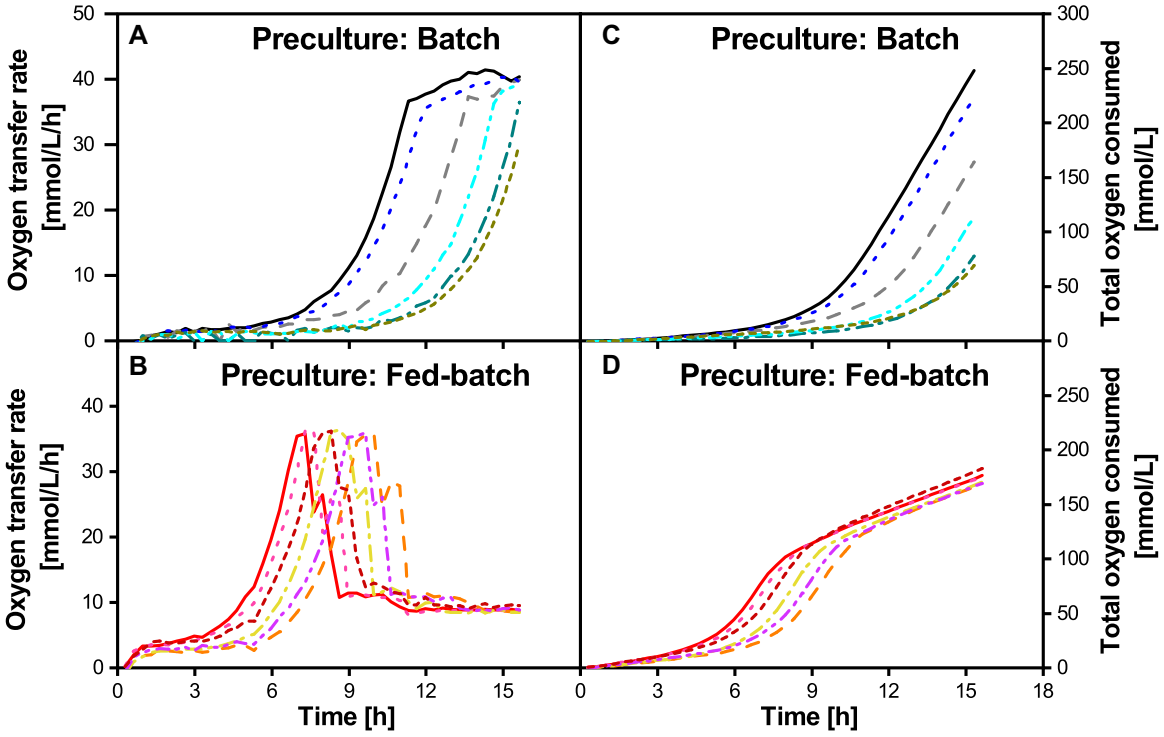

Figure 3. OTR and OT of one *E. coli* BL21 DE3 celA2 clone in Wilms–MOPS media. Six genetically identical colonies (indicated by different colors and line styles) were picked from an agar plate and each transferred into one well of a 48-round-well MTP. Differences in growth behavior are due to unintended variations during the colony-picking process. A,C) Batch operation mode and B,D) fed-batch operation mode. Culture conditions: $T = 37\,°C$; $pH_0 = 7.5$; shaking frequency: $n = 1000$ rpm; shaking diameter: $d = 3$ mm; culture volume: $V_L = 800$ µL per well; initial glucose concentration of batch: $S_{0\_Batch} = 20\,g\,L^{-1}$; initial glucose concentration of fed-batch: $S_{0\_Fed\text{-}batch} = 2\,g\,L^{-1}$.

The mean point in time for batch cultivations (Figure 3A) to reach an OTR of at least 10 mmol L^{-1} h^{-1} is 11.4 h, whereas in fed-batch cultivations the mean point in time is 6.2 h (Figure 3B). This observation indicates that the growth phase is initiated earlier in fed-batch cultures than in batch cultures. It is commonly known that lower osmolality is beneficial for the growth of *E. coli*.[40,41] Due to lower initial glucose concentrations in fed-batch media, the media have lower osmolality and are therefore more favorable for growth.

In batch cultures, strains (black, blue, gray, and light blue) with high metabolic activity exhibit a plateau of the OTR between 11 h and 15 h, which indicates an oxygen limitation. This is attributed to the abundantly available glucose in this operation mode. It is reported that oxygen-limited microorganisms have altered metabolisms (e.g., production of anaerobic by-products). This effect may lead to nonreproducible results.[14] In fed-batch cultivation, in contrast, the time spans of oxygen limitation and the formation of anaerobic by-products are reduced to a minimum since the fed-batch phase begins relatively quickly.

4.4. Application: Screening of a Clone Bank

An *E. coli* BL21 DE3 celA2 clone library was screened to identify the best cellulase-producing strains according to the procedure depicted in Figure 1, applying either the batch or fed-batch operation mode during preculture to enable comparison. The entire process from colony picking to the measurement of cellulase activity was performed in biological triplicate.

For two representative clones, out of 32 clones in total, the scattered light measured during batch preculture (**Figure 4**A) and during the subsequent main culture (Figure 4B) is shown for all three triplicates. The curves for all 32 strains are provided in Data S3 (Supporting Information). The curves obtained for preculture are similar to the results discussed earlier in this study. The triplicates of each clone (clone 19 indicated in blue and clone 26 in red) reveal three entirely diverse growth curves (Figure 4A). In Figure 4B, the impact on the course of the main culture becomes clear. For both clones, the continuous lines indicate the fastest-growing replicate of each clone in preculture. In the main culture, these replicates display the fastest growth as well. The same effect is observed for the replicates with medium growth (dashed curves) and those with the slowest growth (dotted curves). Furthermore, although the continuous and the dashed curve (fastest and medium growth) of clone 19 (blue curves) feature comparable biomass concentrations at the time of inoculum transfer from preculture to the main culture, growth progresses faster for the continuous curve in the main culture. This confirms the initial assumption of favored growth in the main culture with a prolonged residence time in the stationary phase during preculture prior to inoculum transfer. This behavior was considered in the theoretical model. The scattered light in the main culture does

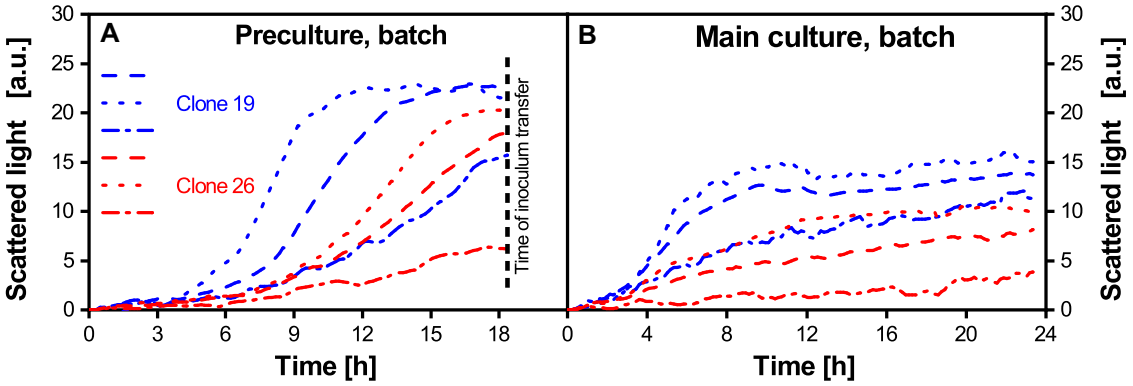

Figure 4. Scattered light for two (out of 32, complete data in Data S3, Supporting Information) representative cellulase-producing *E. coli* BL21 DE3 celA2 clones (blue and red) with site-saturation mutagenesis at three positions in Wilms–MOPS media. Per clone, three colonies (indicated by different line types) were picked from an agar plate. A) Each colony was transferred into one well of a 96-round-well MTP for preculture and cultivated in the batch mode for 18.3 h. B) The main culture was inoculated with 6.67 vol% of preculture broth and cultivated for 23 h. The main culture was induced with 0.1 mM IPTG after 4 h. Culture conditions: $T = 37\,°C$; $pH_0 = 7.5$; shaking frequency: $n = 1000$ rpm; shaking diameter: $d = 3$ mm; culture volume: $V_L = 150\,\mu L$ per well; initial glucose concentration of preculture and main culture: $S_{0\_Batch} = 20\,g\,L^{-1}$.

not reach the same level as in the preculture, since induced cells suffer from a metabolic burden.[39]

When conducting high-throughput screening experiments, the operator will most likely not work with triplicates, nor will they monitor the growth behavior online. Thus, it cannot be ruled out that the final values of biomass or product concentration/activity result from unintended variations during the screening process rather than high or low strain productivity. In this case, the selected strains in Figure 4 might have been evaluated to be suitable in the case of the continuous line. In the case of the dotted line, the investigated strain might be rejected due to a low biomass yield or poor productivity. This effect can lead to an unfortunate choice of strain, potentially missing promising candidates with high productivity.

To prevent this shortcoming, the fed-batch MTPs are applied. Therefore, the procedure for high-throughput screenings according to Figure 1 is performed, exchanging the conventional MTP with a fed-batch MTP for preculture. When these plates are used the capability of online monitoring of scattered light is lost due to the silicon matrix located at the bottom of each well. The scattered light of the subsequent main cultures and the corresponding final cellulase activities of the conventional and the modified fed-batch procedures are presented in **Figure 5**. The general trend of the scattered light in Figure 5A,B unambiguously reveals the equalizing effect of a fed-batch preculture. The scattered light curves of the main cultures that were subjected to preceding batch preculture are broadly distributed, indicating highly diverse growth behavior (Figure 5A). In fact, a remarkable number of strains exhibit no growth at all during the first 4 h of the main culture. This is due to poor growth in preculture, which leads to lesser amounts of biomass being transferred to the main culture. In particular, at the time of induction with IPTG after 4 h of cultivation, the measured biomass concentrations are highly diverse. This is relevant since biomass concentration and metabolic status of the culture at the time of induction have a huge influence on the performance of a strain.[12] In comparison, the main cultures produced with a preceding fed-batch preculture (Figure 5B) display equalized growth behavior for all 96 cultivations, especially during the first 4 h. As a result, at the time of induction, a comparable concentration of biomass was present in each well, with each strain being in a similar metabolic state. Consequently, there are no crucial anomalies in productivity due to inoculation at different phases of growth, which could impede screening. Furthermore, all strains are in the favorable, exponential growth phase at the time of induction.

In Figure 5C,D, the 32 *E. coli* BL21 DE3 celA2 clones are ranked according to their cellulase activity at the end of the main culture. The error bars for the cellulase activities indicate the deviation of the three biological replicates of each clone. The error bars for the screening performed with preceding batch preculture are larger than those with the fed-batch preculture. In the first case, the average standard deviation considering all clones is 64%; however, the mean standard deviation is reduced to 24% with the fed-batch preculture. Considering the benchmark standard deviation for the whole process of 12% (as described in Section 2), the conventional procedure results in more than five times higher standard deviation, whereas the new fed-batch preculture procedure reduces these variations. Both the biomass concentration at the time of induction as well as the uniform growth of the triplicates in the main culture account for the improved reproducibility in cellulase activity.

The best-ranked clone (clone no. 1) with preceding fed-batch preculture is ranked at position 16 when the conventional batch preculture was used. Thus, the results of the two approaches differ fundamentally. With the fed-batch preculture, the clone would be considered for further investigations, whereas with batch preculture, the clone would be discarded. A fraction of this clone library has already been part of a thorough investigation in a previous study.[21] Mühlmann et al.[21] eliminated the factor of nonequalized growth by automatically inducing cultures at a specific threshold of biomass concentration using a liquid-handling device. Considering only the clones of this current study, clone no.1 showed the overall highest cellulase activity in that study too. This strongly supports the

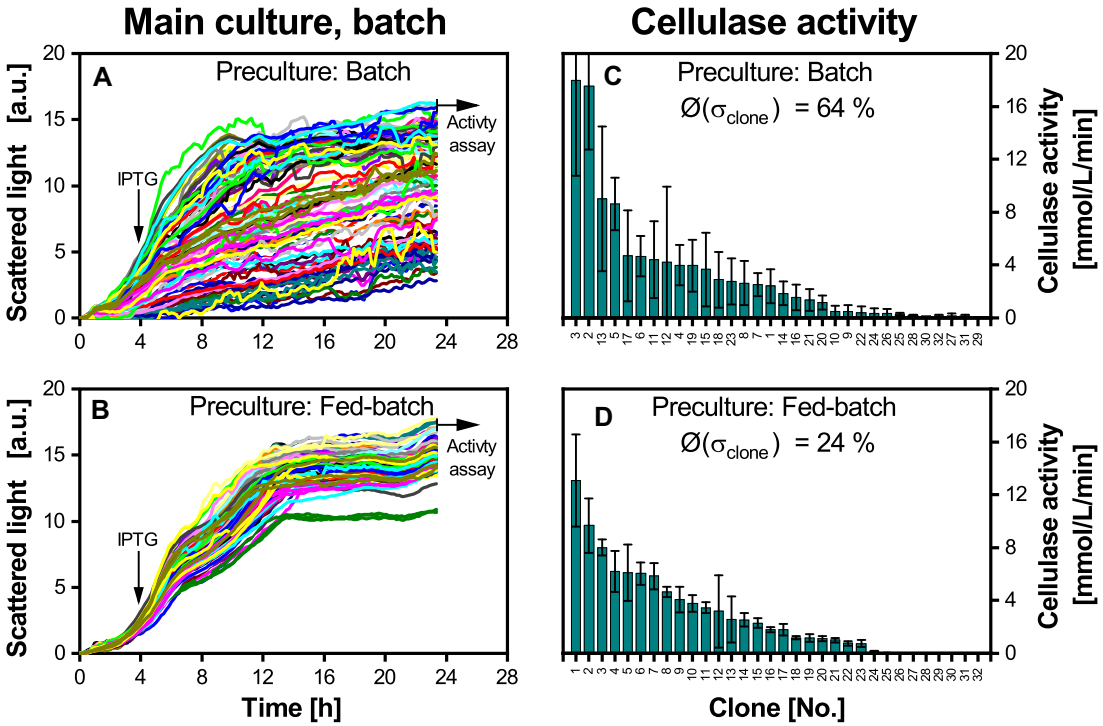

Figure 5. Scattered light and cellulase activity for the main cultures of 32 cellulase-producing *E. coli* BL21 DE3 celA2 clones in Wilms–MOPS media. Per clone, three colonies were picked from an agar plate for the preculture (three experimental replicates for each of the 32 clones) according to the scheme in Figure 1. The main culture was carried out in the batch operation mode, inoculated with 6.67 vol% of preculture broth, and induced with 0.1 mM IPTG after 4 h. Cell lysis and cellulase activity assay were performed after 23.5 h cultivation time. The error bars represent the standard deviation of the measured cellulase activity of the three biological triplicates. Since each replicate passes the whole screening process, the error bars of the cellulase activity of each clone correspond to that of the whole process. The average standard deviation ($Ø(\sigma_{clone})$) is the mean value of all standard deviations of all clones. Clones are ranked according to their cellulase activity in (D). The clone number always corresponds to the same clone in batch and fed-batch modes of the preculture. Culture conditions: $T = 37$ °C; $pH_0 = 7.5$; shaking frequency: $n = 1000$ rpm; shaking diameter: $d = 3$ mm; culture volume: $V_L = 150$ µL per well; initial glucose concentration of main culture: $S_0 = 20$ g L^{-1}. A) Scattered light of the main culture with preculture performed in the batch operation mode. B) Scattered light of the main culture with preculture performed in the fed-batch operation mode. C) Mean cellulase activity with preculture performed in the batch operation mode. D) Mean cellulase activity with preculture performed in the fed-batch operation mode. Clone 14 harbors the wild-type gene; clone 26 is an empty vector strain.

assumption that clone no. 1 from fed-batch preculture screening is the best cellulase producer of all investigated clones. In the batch preculture process, the factual best clone would not be detected. Actually, from those nine clones ranked first in the fed-batch preculture process, eight clones were in the top nine of the ranking of Mühlmann et al.[21] as well. Consequently, by applying the fed-batch preculture, the outcome of the overall screening is comparable to that of a fully automated and controlled screening procedure in a liquid-handling system.

5. Conclusion

The colony-picking step and the inoculation from cryocultures into precultures always involve a certain risk of creating unintended variations. The impact of these variations on a strain screening procedure decreases through the use of fed-batch conditions during preculture. The use of the fed-batch preculture allows for equalized growth in the main culture and therefore results in identical biomass concentrations at the time of induction. The final cellulase activity of each clone had a reduced standard deviation with fed-batch precultures compared to standard batch precultures. Applying the batch operation in preculture, there is a high probability for the best cellulase-producing clone not to be identified and thus not to be considered for further process development. The integration of a fed-batch preculture step into high-throughput screening procedures considerably increases the reliability of the final screening result and reduces the risk of missing the best-suited clones. The application of fed-batch conditions during preculture is highly recommended in order to achieve reproducible and reliable screening results.

Supporting Information

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

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Conflict of Interest

M. L. is an employee at Kuhner Shaker GmbH.

Keywords

bioprocess development, colony picking, fed-batch microtiter plates, high-throughput screening, precultures

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