J Ind Microbiol Biotechnol (2014) 41:513–525
DOI 10.1007/s10295-013-1396-x

BIOTECHNOLOGY METHODS

SIMB
Society for Industrial Microbiology and Biotechnology

Fed-batch operation in special microtiter plates: a new method for screening under production conditions

Anja Wilming · Cornelia Bähr · Claudia Kamerke · Jochen Büchs

Received: 7 October 2013 / Accepted: 22 December 2013 / Published online: 14 January 2014

Abstract Batch and fed-batch operation result in completely different physiological conditions for cultivated microorganisms or cells. To close the gap between screening, which is hitherto exclusively performed in batch mode, and fed-batch production processes, a special microtiter plate was developed that allows screening in fed-batch mode. The fed-batch microtiter plate (FB-MTP) enables 44 parallel fed-batch experiments at small scale. A small channel filled with a hydrogel connects a reservoir well with a culture well. The nutrient compound diffuses from the reservoir well through the hydrogel into the culture well. Hence, the feed rate can easily be adjusted to the needs of the cultured microorganisms by changing the geometry of the hydrogel channel and the driving concentration gradient. Any desired compound including liquid nutrients like glycerol can be fed to the culture. In combination with an optical measuring device (BioLector), online monitoring of these 44 fed-batch cultures is possible. Two *Escherichia coli* strains and a *Hansenula polymorpha* strain were successfully cultivated in the new FB-MTP. As a positive impact of the fed-batch mode on the used strains, a fourfold increase in product formation was observed for *E. coli*. For *H. polymorpha*, the use of fed-batch mode resulted in a strong increase in product formation, whereas no measurable product formation was observed in batch mode. In conclusion, the newly developed fed-batch microtiter plate is a versatile, easy-to-use, disposable system to perform fed-batch cultivations at small scale. Screening cultures in high-throughput under online monitoring are possible similar to cultivations under production conditions.

A. Wilming · C. Bähr · C. Kamerke · J. Büchs (✉)
AVT-Biochemical Engineering, RWTH Aachen University, Sammelbau Biologie, Worringerweg 1, 52074 Aachen, Germany
e-mail: jochen.buechs@avt.rwth-aachen.de

Keywords Fed-batch · Microtiter plate · Screening · *Escherichia coli* · *Hansenula polymorpha*

Introduction

Today, more and more compounds are produced by biotechnological fermentations [30]. Consequently, screening for e.g., pharmaceutically active molecules, new enzymes, and higher-yielding microbial strains producing desired chemicals becomes increasingly important. As these screening processes usually encompass thousands to millions of experiments, small-scale bioreactors such as shake flasks and microtiter plates gain in importance [6, 27]. Because of the high degree of parallelization, microtiter plates permit a vast amount of experiments in a rather short time since they can be handled automatically in high-throughput systems using modern robotics [11, 12, 16, 27].

Almost all screening experiments in shaken bioreactors are carried out in batch mode. High initial substrate and buffer concentrations in the medium are applied. Organisms such as *Escherichia coli* or *Hansenula polymorpha* may suffer from carbon catabolite repression, crabtree effect, and high osmotic pressures under these conditions [7, 34, 39, 40, 46]. During carbon catabolite repression, product formation is highly reduced and undesired overflow metabolites such as acetic acid or ethanol are formed. To circumvent these effects, most production processes are conducted in fed-batch mode [24, 28]. The substrates are fed at limiting concentrations and, therefore, reduced or no carbon catabolite repression and formation of overflow metabolites occur. The discrepancy between screening processes and production-scale processes is explained by the lack of established technology to perform fed-batch cultivations at small scale. Performance of a strain in batch mode does not need to be

the best under fed-batch conditions, which can lead to suboptimal strain selection with fatal economic consequences [6, 18, 33, 37]. Therefore, screening processes need to be carried out under fed-batch conditions [6, 18, 28, 33, 37]. Milli- or micro-scale process systems are needed, which enable fed-batch cultivations with continuous online monitoring throughout the process in order to harmonize process conditions in screening and production and to meet the demands of high-throughput experiments.

There are only a few systems on the market that allow screening processes in fed-batch mode and small scale below 100-ml working volume [44, 45]. Scaled-down stirred tank reactors have the advantage of elaborate options of process control but lack a high degree of parallelization. Consequently, high-throughput experiments are not possible, as for example in microtiter plates. Moreover, only pulsed feeding is possible causing temporarily exceeding substrate concencentrations immediately after the pulsing.

A different approach to realize fed-batch mode at small scale was chosen by Jeude et al. [18] and Panula-Perälä et al. [33]. They developed controlled release systems, which can be easily added to shake flasks and microtiter plates. Specifically, as a controlled-release system based on silicone elastomer discs, the FeedBeads were developed in 2006 as an effective, easy-to-handle system for fed-batch cultivations in shake flasks [18, 36]. The same principle is applied in the Feed-Deepwell-Microtiter plates (FeedPlates) [16, 37, 41]. In both cases, the substrate glucose or the pH-regulating agent sodium carbonate is embedded in the silicone matrix. Once the controlled-release system comes into contact with an aqueous medium, water diffuses into the matrix. Simultaneously, the embedded glucose or sodium carbonate is dissolved and diffuses out of the silicone material into the liquid. The release rate depends on material characteristics (e.g., hydrophobicity, degree of crosslinking, particle size of glucose or sodium carbonate crystals, and other material properties) as well as on the driving concentration gradient.

The EnBase system developed by Panula-Perälä et al. [33] is based on the enzymatic hydrolysis of starch. A gel layer including starch is covered with cultivation medium containing glucoamylase. Once starch diffuses into the liquid, the glucoamylase degrades the starch molecules and, consequently, glucose is released. The EnBase system has been further developed to the commercial EnBase Flo cultivation system [26]. Glucose is released by enzymatic hydrolysis from a complex medium. Successful scale-up to 100 l was shown for an *E. coli* process using this approach [26].

One disadvantage of the last two systems is the restriction to the substrate glucose. Considering the feeding rates, the EnBase system is more versatile than the FeedBeads as the feed rate can be more easily adjusted by changing the glucoamylase concentration. When using the FeedBeads, the user is restricted to the feed rate defined by the diffusional release characteristics of the used polymer matrix. By varying the filling volume, the attainable released glucose concentration can be adjusted to a small extent.

One option to realize active process control (controlled feeding of substrate and controlled release of pH adjusting agents) at micro scale offer microfluidics. Funke et al. [11] and Zhang et al. [48] showed successful miniaturized fed-batch cultivations in such microfluidic bioreactors. Both groups of authors proved that process monitoring and control are possible. Also for microfluidics, successful scale-up to 2 l was demonstrated [11]. However, all these microfluidic bioreactors are quite complicated and are still in the prototype stage and therefore up to now not available for routine laboratory use [11, 49].

For a deep understanding of the culture, online monitoring is of special interest. Monitoring in the BioLector device is realized by optical measurement of scattered light (biomass) through the transparent bottom of a microtiter plate [23]. In addition, measurement of DOT and pH is possible via optodes in special commercially available systems. Supplementing the BioLector technique with microfluidics results in a system that offers all the options of a lab-scale fermenter [12]. However, these promising systems are more costly and are not yet available. According to the manufacturer (http://www.m2p-labs.com/biolector-pro, 2013-12-17), market launch is planned for 2014. Currently, this device allows fluorescence measurement of fluorescent (fusion-) proteins (e.g., yellow fluorescent fusion-protein YFP, green fluorescent fusion-protein GFP) [16, 18, 22, 35]. The commonly used fusion-proteins YFP, GFP, and their derivatives are dependent on oxygen for their maturation process [9]. A highly reduced signal intensity was observed under oxygen-limited conditions in cultivations of recombinant *E. coli* strains while SDS page analysis revealed a steady increase of YFP concentration with time. With the onset of oxygen-unlimited conditions upon depletion of the carbon source (glucose), Drepper et al. [9] observed a sudden increase in the fluorescence signal intensity. This was explained by the maturation of the beforehand-accumulated, not-yet-maturated YFP as oxygen became readily available. As a consequence, the authors recommend the use of flavin mononucleotide-based fluorescent reporter proteins (FbFP), which do not depend on oxygen availability for maturation, unlike YFP [8, 9, 16]. Therefore, fluorescence signal intensity reflects the amount of formed protein—even under oxygen-limited conditions—making them ideally suitable to follow product formation online.

This study presents a first proof of concept for a disposable microtiter plate system, which allows 44 parallel fed-batch cultivations based on diffusion while being online monitored using the BioLector technique. In this fed-batch MTP (FB-MTP), a small channel filled with a

hydrogel connects a reservoir well filled with concentrated substrate solution and a cultivation well. The substrate diffuses continuously through the gel into the cultivation well. In this work, it is demonstrated that feed rates in the fed-batch microtiter plate can be adjusted in a broad range by simply adjusting the diffusion parameters like hydrogel characteristics, the substrate diffusion coefficient, the substrate concentration gradients, the diffusion path lengths, and the cross section area of the connecting channel [31, 47]. The potential of this novel FB-MTP system is shown by successful fed-batch cultivations with *E. coli* and *H. polymorpha* strains. Both microorganisms show catabolite repression when exposed to high carbon source concentrations [37, 42, 43]. Therefore, production processes are run in fed-batch mode in this new type of microtiter plate to enable the highest product formation [34, 39, 47].

Materials and methods

Strains

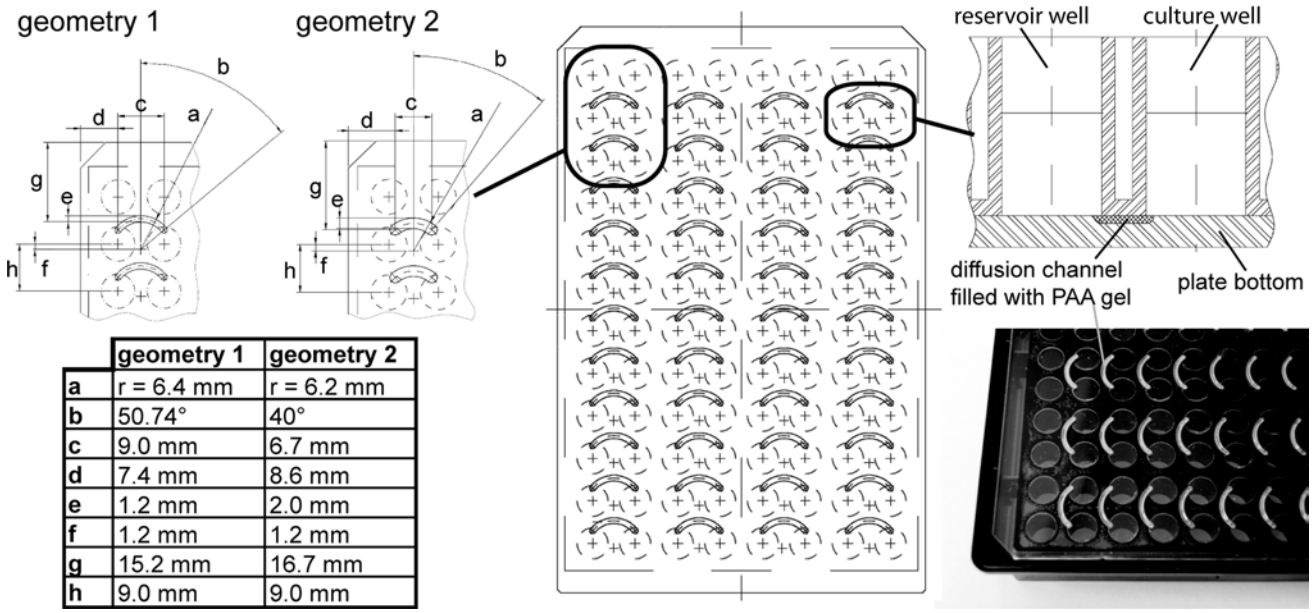
For fed-batch experiments, *E. coli* BL2 pRSET eYFP-IL6 [16, 35] and *E. coli* BL21 (DE3) pRhotHi-2-Ec FbFP [8, 19] were used to represent bacteria, as well as *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP) [1, 13] as a representative of the genus yeast. *E. coli* BL21 pRSET eYFP-IL6 is a genetically modified strain of *E. coli* BL21 (DE3) carrying one plasmid with the fusion protein consisting of interleukin-6 and the enhanced yellow fluorescing protein (eYFP). *Escherichia coli* BL21 (DE3) pRhotHi-2-Ec FbFP is also a genetically modified strain of *E. coli* BL21 (DE3). Due to the independency on oxygen availability of the flavin mononucleotide-based fluorescent reporter protein (FbFP), this strain is ideally suited for online monitoring of product formation in the BioLector. The used yeast *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP) is an uracil auxotroph strain transformed with the plasmid pFPMT21 including the gene for the green fluorescing protein GFP and additionally complementing the uracil auxotrophy. Both *E. coli* and *H. polymorpha* form overflow metabolites when cultivated with high glucose concentrations. In *E. coli*, formation of overflow metabolites is mediated by carbon catabolite repression [42]. In *H. polymorpha*, product formation is reduced as long as carbon catabolite repression prevails [37, 43]. These characteristics make them ideal model organisms to investigate the impact of fed-batch mode on fermentation performance [37].

Media

All chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), or Fluka (Buchs, Switzerland) and were of analytical grade.

Pre-cultures of both *E. coli* strains were cultivated on lysogeny broth [3, 4] in shake flasks (Erlenmeyer flask volume $V = 250$ ml, filling volume $V_L = 10$ ml, shaking frequency $n = 350$ rpm, shaking diameter $d_0 = 50$ mm, $T = 37$ °C, inoculation 1 % v/v glycerol stock) until mid-exponential growth phase was reached. Then, the pre-culture was centrifuged (10 min, 14,000 × *g*, 25 °C). In order to perform MTP cultivations, the resulting cell pellet was re-suspended in Wilms-MOPS medium as the main culture medium and diluted to the desired inoculation optical density (OD_{620}). The medium was prepared according to Scheidle et al. [38] with a base solution of $(NH_4)_2SO_4$ 5 g/l, NH_4Cl 0.5 g/l, K_2HPO_4 3.0 g/l, Na_2SO_4 2.0 g/l, 3-(*N*-morpholino)propanesulfonic acid (MOPS) 41.85 g/l dissolved in 900 ml of deionized water and adjusted to pH 7.5, volume adjusted to 947 ml and autoclaved. One milliliter of a trace elements solution [$ZnSO_4 \cdot 7\ H_2O$ 0.54 g/l, $CuSO_4 \cdot 5\ H_2O$ 0.48 g/l, $MnSO_4 \cdot H_2O$ 0.3 g/l, $CoCl_2 \cdot 6\ H_2O$ 0.54 g/l, $FeCl_3 \cdot 6\ H_2O$ 41.76 g/l, $CaCl_2 \cdot 2\ H_2O$ 1.98 g/l, $Na_2EDTA \cdot 2\ H_2O$ (Triplex III) 33.39 g/l] was aseptically added to the base solution. In addition, 10 ml of a 0.5 g/l $MgSO_4 \cdot 7\ H_2O$ solution, 1 ml of a 0.01 g/l thiamine-hydrochloride solution as well as 1 ml of a 0.1 g/l ampicillin solution were added to the base solution. Forty milliliters of a separately autoclaved glucose stock solution was added. The concentration of this stock solution is defined by the desired final glucose concentration in the medium. This cell suspension was then used to fill the culture wells of the FB-MTP. The reservoir wells were filled with Wilms-MOPS medium with the desired feed glucose concentration (50–700 g/l). No induction was performed in the cultivations with *E. coli*. Both *E. coli* BL21 (DE3) pRhotHi-2-Ec FbFP and *E. coli* BL21 pRSET eYFP-IL6 show considerable constitutive expression of either FbFP [9] or the eYFP-IL-6 protein [35], respectively. Cultivations were conducted with $V_L = 0.2$ ml, $n = 950$ rpm, $d_0 = 3$ mm, $T = 37$ °C, chosen according to Hermann et al. [15] and Huber et al. [16].

Pre-cultures of *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP) were cultivated on YPG medium [14] in shake flasks under the same conditions as *E. coli* until mid-exponential growth was reached. Syn6-MES medium was used for the main cultures conducted in MTPs and prepared according to Gellissen [14] and Kottmeier et al. [25]. The Syn6-MES-medium consisted of a base solution to which several mineral-, micro-element, trace-element, and vitamin solutions were added. The base solution consisted of KH_2PO_4 1 g/l, $(NH_4)_2SO_4$ 7.66 g/l, 2-(*N*-morpholino) ethanesulfonic acid (MES) 27.3 g/l, $MgSO_4 \cdot 7\ H_2O$ 3.0 g/l, KCl 3.3 g/l, NaCl 0.33 g/l. To one liter of base solution, 10 ml of a 150 g/l $CaCl_2 \cdot 2\ H_2O$ solution, 10 ml of a micro-element solution (Titriplex III (EDTA) 6.65 g/l,



	geometry 1	geometry 2
a	r = 6.4 mm	r = 6.2 mm
b	50.74°	40°
c	9.0 mm	6.7 mm
d	7.4 mm	8.6 mm
e	1.2 mm	2.0 mm
f	1.2 mm	1.2 mm
g	15.2 mm	16.7 mm
h	9.0 mm	9.0 mm

Fig. 1 Fed-batch microtiter plate for diffusion-driven fed-batch cultivations in microliter scale (*FB-MTP*): FB-MTP schematic (*mid*) with two different geometries of diffusion channel (*left*); cultivation system consisting of a reservoir well filled with highly concentrated feed solution that is connected to a cultivation well by a diffusion channel filled with polyacrylamide gel (*right*)

$(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$ 6.65 g/l, $CuSO_4 \cdot 5\ H_2O$ 0.55 g/l, $ZnSO_4 \cdot 7\ H_2O$ 2.0 g/l, $MnSO_4 \cdot H_2O$ 2.65 g/l), 10 ml of a vitamin solution (0.004 g D-biotin dissolved in 10 ml 2-propanole/deionized water (1:1) and mixed with 1.335 g of thiamine chloride dissolved in 90 ml of deionized water) and 10 ml of a trace element solution ($NiSO_4 \cdot 6\ H_2O$ 0.065 g/l, $CoCl_2 \cdot 6\ H_2O$ 0.065 g/l, H_3BO_3 0.065 g/l, KI 0.065 g/l, Na_2MoO_4 0.065 g/l) was added. $CaCl_2 \cdot 2\ H_2O$, micro-element, vitamin-, and trace-element solutions were sterile-filtered and aseptically added to the autoclaved base solution. A concentrated glucose solution is autoclaved separately and the needed volume is added to the base solution to reach the desired glucose concentration. The initial pH of the medium was adjusted at pH 6.4 using 5 M NaOH. The inoculation procedure and cultivation conditions were identical to that of *E. coli* cultures.

Design and construction of the fed-batch microtiter plate

The newly developed fed-batch microtiter plate (FB-MTP) consists of three elements: a black, 96-well microtiter plate (well diameter at the top 6.99 mm and at the bottom 6.58 mm) without bottom (microtiter plate body) (part no. 655000-06, Greiner Bio-One, Frickenhausen, Germany), a double-sided adhesive foil (Duplobond 3605.2 Plus, Lohmann, Neuwied, Germany) and a transparent 3-mm bottom plate made of polymethylmethacrylate (PMMA). Figure 1 shows a technical drawing of the bottom plate (Fig. 1 mid) as well as a magnified picture of the ready-to-use FB-MTP (bottom right). As illustrated in Fig. 1, small curved channels connect two wells, namely the reservoir well and the culture well. The channels are curved to generate a larger diffusion path length. To achieve a diffusion-driven release of the substrate into the culture well, the channels are filled with a polyacrylamide hydrogel (PAAm gel).

To fabricate the bottom plate, the channels were milled into the transparent PMMA plate with a CNC milling machine (Deckel Maho DMU 50T, Pfronten, Germany). Because the surface tension of the PMMA plate changed within milling, bending was prevented afterwards by moderate heating. Thereby, the bottom plate was slowly heated to 80 °C for 4 h and subsequently slowly cooled down for 12 h. Since the adhesive at the well bottom would interfere with the cultivation as well as the online monitoring, holes for the wells (diameter 6.6 mm) were cut out of the adhesive foil. To ensure a clean, precise, and reproducible cut, the holes were cut with a CO_2 laser at the Fraunhofer Institute for Laser Technology, RWTH Aachen University (Germany). For the assembly of the FB-MTP, the PMMA bottom plate, the adhesive foil, and the microtiter plate body were stacked and aligned carefully. The adhesive foil was placed on the microtiter plate body to match the cut out holes with the wells in the MTP body. On top of this, the bottom plate was placed carefully in a way that every channel connects two wells. By applying a pressure of 5 t with a hydraulic press, the three parts were bonded. During

this bonding process, it must be ensured by repeated visual inspection that air bubbles were removed between the microtiter plate body and the bottom plate to guarantee a high bonding strength.

In order to create generally different release kinetics, two different geometries differing in diffusion path length and diffusion cross-sectional area were realized, as depicted in Fig. 1 (top left). In geometry 1, the channels had a length of 11.33 mm, a width of 1.2 mm, and a depth of 1.2 mm, resulting in a cross-sectional area of 1.44 mm^2. In geometry 2, the channel length was 8.67 mm, and the depth and width were 2 mm, respectively. Here, the cross-sectional area accounted for 4 mm^2.

Preparation of hydrogel

The hydrogel, used as a diffusion barrier in the small channels connecting the reservoir and the culture wells, was a standard 5 wt % polyacrylamide gel commonly applied for SDS PAGE (PAAm gel). When preparing the hydrogel, all substances were cooled to 4 °C to keep the hydrogel liquid and pipettable. As ammonium peroxodisulfate and tetramethylethylenediamine (TEMED) act as polymerization starters, they were added last to the hydrogel solution. The prepared hydrogel was then pipetted into the channels of the mounted FB-MTP (18 µl for geometry 1; 25 µl for geometry 2). Because oxygen interferes with the radical polymerization reaction, the polymerization took place in a desiccator, which was extensively flushed with nitrogen gas. After an incubation of 90 min at room temperature, 200 µl of deionized water were added to each well to enable the hydrogel to reswell. This reswelling process is important to fully close up the channels and prevent convective flow of liquid between the channel walls and the gel body. The swelling process should proceed for at least 3 h, preferably 12 h. Afterwards, the deionized water was removed from the well by turning and shaking the MTP upside down.

Release kinetics and determination of filling volume

Since glucose is the most frequently used carbon source in microbial cultivations, it was used as substrate for the general characterization of the release kinetics in the geometry 1 and geometry 2 FB-MTPs. Under cultivation conditions (T = 37 °C, V_L = 200–300 µl, shaking diameter d_0 = 3 mm, n = 430–950 rpm), a reservoir well was filled with a glucose solution of the desired feed concentration (180–700 g/l) and the culture well with deionized water. The plate was sealed with a gas-impermeable membrane (Adhesive Plate Seals, Thermo Scientific, Epsom, UK)

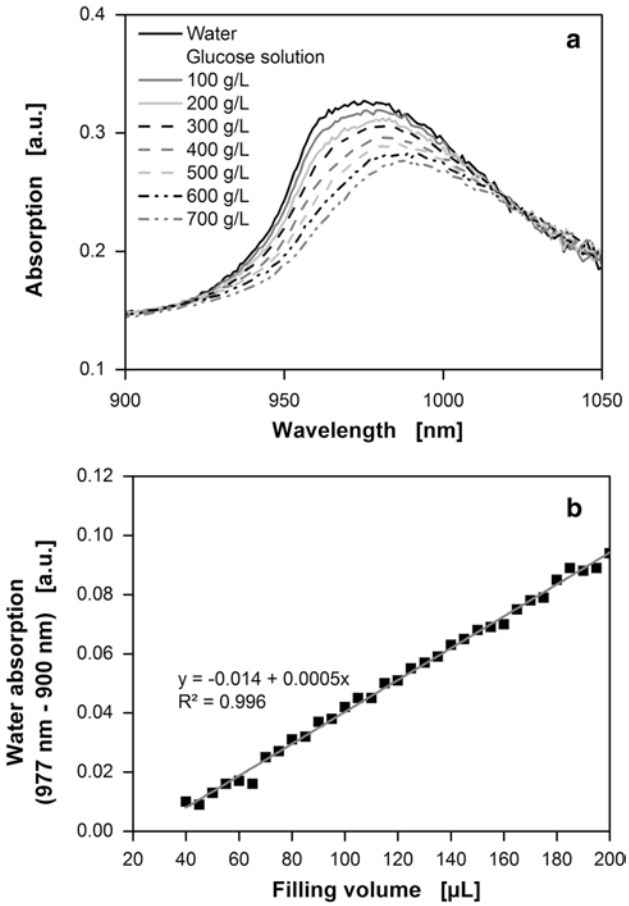


Fig. 2 Filling volume calculated by absorption; absorption spectra of 200 µl of water and glucose solutions (**a**); absorption as function of filling volume with absorption at 900 nm as a reference (**b**); water absorption determined with deionized water in Power Waveex spectrophotometer (BioTek), λ_{Abs} = 900 + 977 nm, filling volume 40 up to 200 µl, standard 96-well MTP

to prevent evaporation. For each measurement during the diffusion process, one fed-batch well pair was emptied to determine the glucose concentration in both wells. The glucose concentration was measured using an enzyme test kit (r-biopharm No. 10 176 251 035, Boehringer, Mannheim, Germany). In order to also determine the filling volume over the release time, a photometrical method was applied as described by McGown and Hafeman [29]. Figure 2a depicts the absorption spectra of pure deionized water as well as of concentrated glucose solutions (100–700 g/l) at a wavelength of 900–1,050 nm. The pure water showed an absorption maximum at 977 nm, which is supported by literature [29, 32]. Obviously, the absorption maximum shifts towards higher wavelengths with increasing glucose concentration at constant filling volume. To compensate irregularities caused by e.g., the liquid meniscus and other factors, a reference measurement at 900 nm was performed since water displays an absorption minimum at this

wavelength, as visible in Fig. 2a. All measurements were performed in duplicates and the median was calculated. Figure 2b proves a linear correlation of the water absorption on the filling volume. The photometrical determination of the filling volume was limited to the culture well. The glucose concentration in the culture well is so low that the absorption maximum of water is not affected. In general, the described method could also be applied to the reservoir well but calibration curves are needed for every glucose concentration used.

Online monitoring with the BioLector device

The BioLector technique allows online monitoring in shaken microtiter plates. Scattered light as signal for biomass and fluorescence measurements at different excitation and emission wavelengths to monitor the product formation were performed in this work (Table 1). For details on the BioLector technique, refer to Kensy et al. [22] and Samorski et al. [35].

Results and discussion

Glucose release kinetics

Prior to cultivation experiments, the glucose release at different feed concentrations in the reservoir well for the chosen polyacrylamide hydrogel was investigated. Figure 3 depicts the concentration-dependent glucose release from the reservoir well into the respective culture well of a geometry 1 plate. The experiment was performed as single assay, without technical replicates. The amount of glucose measured in the culture well is plotted over time.

At first glance, the glucose amount in the culture well rose nearly linearly for the tested glucose feed concentrations in the reservoir well over time after a lag phase of 2.5–5 h. While the glucose release increased with high glucose feed concentrations up to 500 g/l, no further increase in glucose release with a feed concentration of 700 g/l was observable.

Table 1 Settings of the excitation and emission wavelengths used for online monitoring in the BioLector

Signal	Excitation λ_{Ex} channel (nm)	Emission λ_{Em} channel (nm)
Scattered light	620	620
YFP	510	540
EcFbFp	460	490
GFP	485	540

During the observed lag phase, the glucose moved through the diffusion channel. After this diffusional lag phase, the glucose was available in the culture well. The time glucose needed to cross the diffusion channel depends on the diffusion coefficient of glucose in polyacrylamide gel, the driving concentration gradient, the gel matrix density, and the diffusion path length. Since all parameters were constant except for the driving concentration gradient, it was expected according to the Fick's law [10] that the duration of the lag phase depends on the initial feed concentrations. The results in Fig. 3 show that the lag phase was shortened by higher initial feed concentrations.

Considering Fick's law of diffusion [10], a proportional increase in glucose release was expected for increasing initial feed concentrations. Indeed, when increasing the initial feed concentration from 180 to 500 g/l, the release rate rose in an almost proportional manner. The scattering of the measuring values within an experiment was mainly caused by the sampling method. Thus, another well was emptied for each measuring value. For initial feed concentrations above 500 g/l, no further significant increase was observed. The non-proportional behavior of the release rate as function of the glucose concentration gradient will be explained later.

As mentioned before, the feedrates in the described FB-MTP can generally be adjusted in a broad range by simply adapting the initial feed concentrations. However, to meet the demand of very fast growing organisms, a second

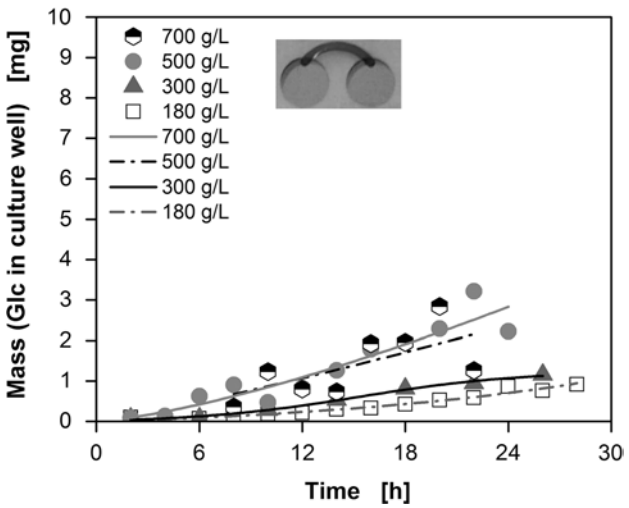


Fig. 3 Glucose release into culture well in fed-batch MTP geometry 1. Reservoir well: glucose feed solution 180–700 g/l, culture well: distilled water; both $V_L = 300$ µl, diffusion channel: width and depth 1.2 mm, length 9.0 mm, filled with 5 wt % PAAm-gel, $T = 37$ °C, $d_0 = 3$ mm, $n = 430$ rpm; every measurement point represents one well, which was completely emptied for the measurement. *Trendlines* were fitted using OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA)

geometry was developed in order to achieve a higher glucose release. Since the diffusive flux primarily depends on the concentration gradient as well as the diffusion path length and cross-sectional area, the channel length was reduced from 11.33 to 8.67 mm and, additionally, the cross-sectional area (channel depth × channel width) was increased from 1.44 to 4 mm^2 in FB-MTPs of geometry 2 (Fig. 1).

The total mass of glucose in the culture well (Fig. 4a) as well as the residual liquid volume in the culture well (Fig. 4b) in geometry 2 FB-MTP are presented over time in Fig. 4. The experiment was performed as a single assay. In Fig. 4a, after a lag phase of ~4 h, the amount of glucose in the culture well increased nearly linearly for the experiments with 50 and 100 g/l, whereas a sigmoidal progress could be observed for 300 and 500 g/l. The increase in feed concentration from 50 to 100 g/l resulted in a threefold increase in the final level (1 mg/0.15 ml = 6.7 g/l compared to 3 mg/0.15 ml = 20 g/l considering filling volumes from Fig. 4b) in mass of glucose diffused into the culture well during 50 h (Fig. 4a). For the 300-g/l experiment, a sevenfold increase compared to the 50-g/l experiment (46.7 g/l in 50 h) was cognizable. Surprisingly, a further increase to 500-g/l feed concentration did not lead to higher glucose release but to a slight decrease in glucose diffused into the culture well (40 g/l in 50 h). When comparing the glucose release for the two geometries, a 4.5-fold increase in glucose release for the 500-g/l feed concentration was achieved when changing from geometry 1 to geometry 2.

Figure 4b presents the changes of filling volume in the culture well monitored photometrically according to the method illustrated in Fig. 2b. For all conditions, the filling volume in the culture well decreased during the 50 h incubation, meaning a reduction by 20–25 %. This significant volume reduction cannot be explained by evaporation only, but rather by water diffusing through the hydrogel into the reservoir well driven by a gradient of osmotic pressure. Such a water counter diffusion had in general been reported for diffusive membrane processes [20, 21] as well as in particular for a dialysis fed-batch shake flask, where the substrate is released from a reservoir into culture medium through a membrane [2]. The investigation of water counter diffusion is crucial for estimating the glucose release rate since, by this effect, the glucose concentration is diluted in the reservoir well, thereby, reducing the driving concentration gradient for the glucose release.

The graph for the 500-g/l experiment differed significantly from those observed for the 50–300 g/l because it decreased faster after 18 h and shows a sigmoid slope. The reason for this observation cannot be explained at the time being. By this experiment, a water counter diffusion was also proven for the FB-MTP similar to the dialysis fed-batch shake flask [2]. Although the diffusion barriers are

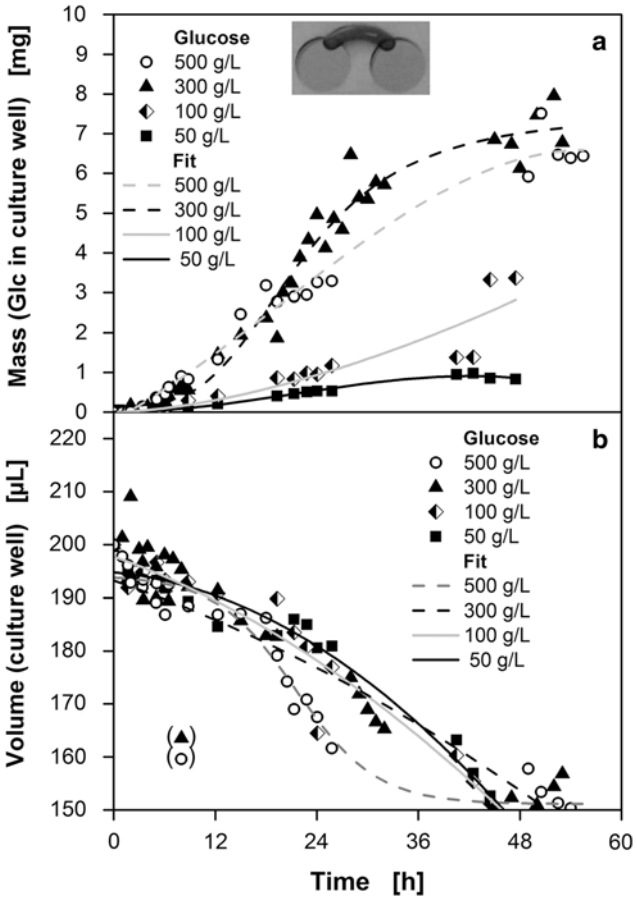


Fig. 4 Glucose release into culture well (**a**) and water counter diffusion from culture well into reservoir well (**b**) characterized in fed-batch MTP geometry 2. Reservoir well: glucose feed solution 50–500 g/l, culture well: distilled water; both V_L = 200 µl, diffusion channel: diameter 2 mm, filled with 5 wt % PAAm-gel, T = 37 °C, d_0 = 3 mm, n = 950 rpm; every measurement point represents one well, which was completely emptied for the measurement. *Trendlines* were fitted using OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA)

different (hydrogel and dialysis membrane), strong water counter diffusion was observed in both cases affecting the glucose release by dilution. This dilution may be the reason of the non-proportional increase of the glucose release with increasing initial feed concentrations.

The above characterization of the substrate release in the FB-MTP has demonstrated that it is possible to vary the feed-rate in a broad range by simply adjusting the initial feed concentration in the reservoir well and the channel geometry. In addition, this study presents a preliminary quantification of the substrate diffusion from the reservoir to the culture well and the water counter diffusion in the reverse direction. However, detailed investigations of these two mass fluxes are necessary for the mechanistic understanding and the mathematical description of the diffusion processes in the FB-MTP.

Cultivation experiments

Following the characterization of glucose release, results of fed-batch cultivations performed with the newly developed FB-MTP are presented as a proof of principle. Unless stated otherwise, experiments were performed in duplicates and the arithmetical mean was calculated.

Figure 5a depicts the scattered light signal for an *E. coli* BL21 pRSET eYFP-IL6 cultivation in FB-MTP geometry 1 representing the biomass formation while Fig. 5b plots the corresponding signal for product formation, the YFP signal, over time. In these fed-batch cultivations, the medium was initially supplemented with 5 g/l glucose. The experiment was performed in triplicate.

As can be seen in Fig. 5a, the scattered light signal showed a lag phase of ~6 h for the triplicates. Following the lag phase, an exponential increase of the scattered light signal was observed. At 12 h, a change in slope was visible

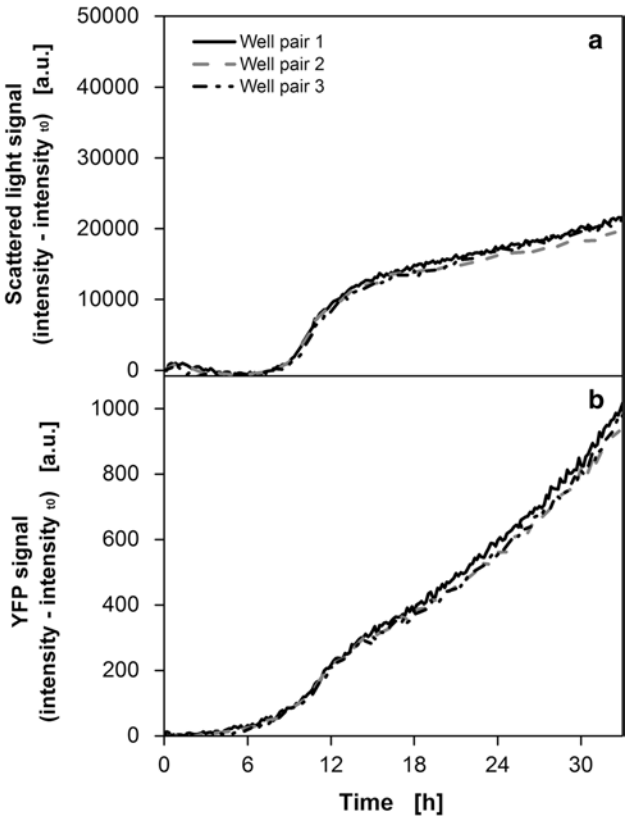


Fig. 5 Reproducibility of fed-batch cultivations of *E.coli* BL21 pRSET eYFP-IL6 in geometry 1. Three parallel cultures are shown; *scattered light* signals are depicted in (**a**) and product formation with YFP-signal in (**b**). Wilms-MOPS medium, Fed-batch: feed in the reservoir well $c_{Glucose} = 500$ g/l with initial $c_{Glucose} = 5$ g/l in the culture well. Cultivation parameter: $n = 950$ rpm; $d_0 = 3$ mm; $T = 37$ °C; $V_L = 200$ µl; scattered light signal absorption 620 nm; product IL6-YFP (IL6 fused to fluorescence marker protein) $\lambda_{EX} = 510$ nm, $\lambda_{EM} = 540$ nm; *E. coli* $OD_0 = 0.1$, 5 wt % PAAm-gel

transcending into a phase with linear slope. This phase lasted until the termination of the experiment.

The observed lag phase of ~6 h was longer than the diffusional lag phase (~2.5 h) according to Fig. 3. This lengthening of the lag phase can be explained by adaptation of the microorganisms to the new cultivation environment, here termed as biological lag phase. After the lag phase of ~6 h, the cultures grew exponentially until 12 h, metabolizing the initially added glucose (5 g/l) as well as the glucose released into the culture well until this time. During the biological lag phase, glucose accumulated since more glucose was released than consumed by the growing culture. When the accumulated glucose was depleted, only the steadily released glucose was available. The glucose depletion in the culture medium represents the end of the batch and therefore the beginning of the fed-batch phase at 12 h. Due to the glucose-limiting conditions in the fed-batch phase, the scattered light signal linearly increased as observed in Fig. 5a. Consequently, the glucose release defines the feed rate and thus the growth rate.

Figure 5b illustrates the course of the YFP signal and therefore the product formation. During the lag phase in the first 6 h, no YFP production could be detected. With onset of exponential growth at ~6 h, the YFP signal also increased exponentially equally to the scattered light until 12 h. When glucose limitation started and the culture had entered the fed-batch phase, the YFP signal changed to a linear slope with a final YFP signal of 1,000 a.u.

This experiment showed a successful fed-batch cultivation using the FB-MTP demonstrating the general proof of concept. Since the fed-batch experiments were conducted as technical replicates with identical initial glucose and feed concentrations, conclusions about reproducibility can be drawn. Deviations for scattered light and YFP signal were within the measuring error and therefore the reproducibility among the triplicates was excellent.

To illustrate the impact of fed-batch versus batch mode on *E. coli* (DE3) pRhotHi-2-EC FbFP, fed-batch experiments with 3 g/l initial glucose but varying concentrations in the reservoir wells (100–700 g/l) in a FB-MTP geometry 2 were compared to a batch cultivation with 10 g/l of glucose.

As shown in Fig. 6, the lag phase of the batch experiment lasted for ~8 h, followed by the exponential phase. This exponential phase transcended into the stationary phase at 14 h, which was characterized by the constant scattered light signal. All fed-batch experiments experienced a lag phase (~6 h) followed by an exponential growth phase with exponential increase in scattered light. The transition into the fed-batch phase was marked by the linear slope of the scattered light signal. Because only 3 g/l of initial glucose was used in the fed-batch experiments, the osmotic pressure was lower than in the batch experiment, in which

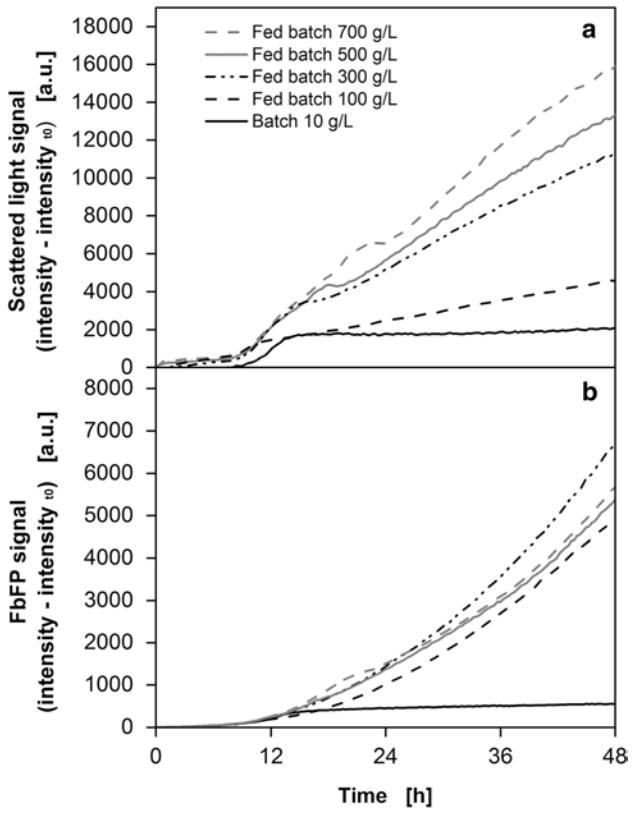


Fig. 6 Comparison of batch and fed-batch cultivation of *E. coli* (DE3) pRhotHi-2-Ec FbFP. Biomass formation as *scattered light* signal in (**a**) and product formation as FbFP signal in (**b**). Wilms-MOPS medium. Batch: $c_{Glucose}$ = 10 g/l, fed-batch: feed in the reservoir well $c_{Glucose}$ = 100–700 g/l with initial $c_{Glucose}$ = 3 g/l in the culture well. Cultivation parameter: n = 950 rpm; d_0 = 3 mm; T = 37 °C; V_L = 200 µl; scattered light signal absorption 620 nm; product FbFP (fluorescence marker protein) λ_{EX} = 460 nm, λ_{EM} = 490 nm; *E. coli* OD_0 = 0.4; 5 wt % PAAm-gel; geometry 2

10 g/l glucose was available. It is known that the lag phase of microorganisms is sensitive to elevated osmotic pressure [5]. The lower osmotic pressure led to a shorter lag phase in the fed-batch experiments compared to the batch experiment. A similar positive effect was described by Scheidle et al. [36]. Comparing the different fed-batch experiments, all showed the same lag phase but differed in the length of their respective exponential phases. Figure 6 clearly shows elongated exponential phases for higher feed concentrations and therefore a delayed transition into the fed-batch phase. This transition was visible in scattered light signal at 12 h for the 100-g/l feed experiment, at 14 h for the 300-g/l experiment, and at 18 and 22 h for the 500- and 700-g/l experiments, respectively. Because the lag phase (6 h) was longer than the diffusional lag phase of ~4 h (Fig. 4a), glucose accumulated during the lag phase in addition to the initially added glucose. Since the release rate increased with higher feed concentrations, more glucose accumulated during the lag phase for higher feed concentrations and therefore glucose became limiting at a later point. In the fed-batch phases, the slope displayed a dependency of the initial feed concentration in the reservoir wells. The higher the feed concentration, the higher the driving concentration gradient according to the Fick's law [10].

Figure 6b illustrates the respective formation of the fluorescence marker protein FbFP for the different fed-batch and batch experiments. In the batch experiment, product formation started with the beginning of the exponential growth phase. Upon the entry into the stationary phase, the product formation ceased and the FbFP signal remained constant at ~500 a.u. until the end of fermentation. Similar to the batch experiment, product formation started with the beginning of the exponential growth in all fed-batch experiments. With transition into the fed-batch phase, the FbFP signal further increased exponentially until termination of the experiment in contrast to the batch fermentation.

Comparing the batch with the fed-batch cultivations, a successive increase of final biomass from 2,000 a.u. in the batch experiment to maximally 16,000 a.u. for the 700-g/l fed-batch fermentation was observed, due to higher totally available glucose. The final FbFP signal also increased from 500 a.u. in the batch to maximally 7,000 a.u. for the 300-g/l fed-batch. Dividing the FbFP signal by the scattered light signal results in a FbFP/biomass yield. When comparing the batch experiment with the fed-batch experiments, the FbFP/biomass yield of 0.25 for the batch culture was significantly lower than those of the fed-batch fermentations (0.38–1.13). The enhanced yields of the fed-batch cultures can be explained by higher totally available glucose amounts and the change of operation mode. In the expression system used, the FbFP expression is under the control of the lac operon [9, 17, 19], which is regulated by carbon catabolite repression in *E. coli* [17, 42]. In batch mode, high initial glucose concentrations are responsible for repressing the FbFP expression. Only at the end of the exponential growth phase, was glucose below the repressing concentration. At this point, little glucose was left for product formation [2]. By contrast, limiting glucose concentrations occurred earlier in fed-batch fermentations. This benefit of fed-batch mode became less with increasing initial feed concentrations, explaining the decrease of FbFP/biomass yield within the fed-batch experiments. As seen in Fig. 6, this was most probably the case for initial feed concentrations equal to or higher than 300 g/l. Consequently, the 100-g/l feed concentration yielded the best specific product formation among the tested conditions for the used *E. coli* (DE3) pRhotHi-2-EC FbFP.

After successful application of the FB-MTP for fed-batch cultivation of a bacterial expression system, the FB-MTP was also used for the yeast *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP). This strain produces the green fluorescing protein GFP, its expression being repressed by

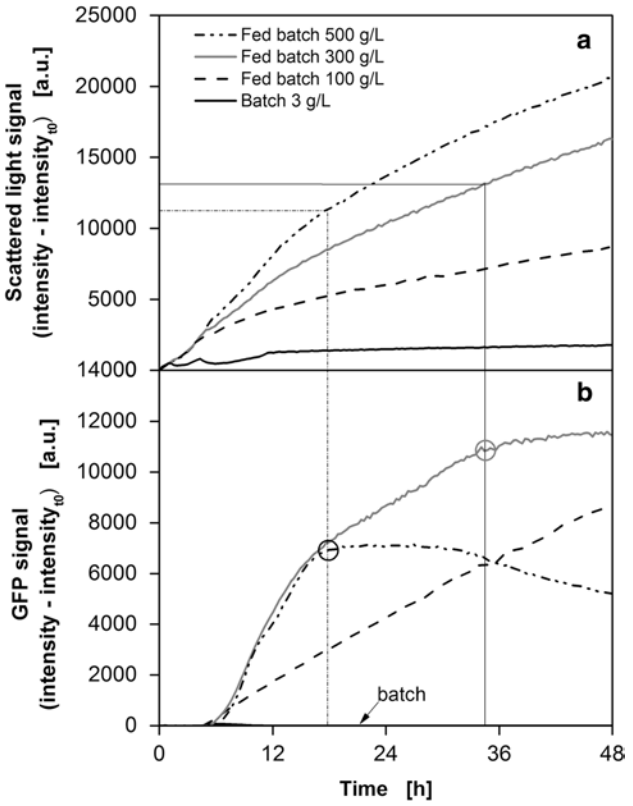


Fig. 7 Fed-batch cultivation of *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP). Biomass formation as *scattered light* signal in (**a**) and product formation as GFP signal in (**b**). Syn-6-MES medium. Batch: $c_{Glucose} = 3$ g/l, fed-batch: feed in the reservoir well $c_{Glucose} = 100–500$ g/l with initial $c_{Glucose} = 3$ g/l in the culture well. The *circles* indicate the time when the respective cultures enter a phase of more or less constant GFP signal (product concentration). Cultivation parameter: $n = 950$ rpm; $d_0 = 3$ mm; $T = 37$ °C; $V_L = 200$ µl; scattered light signal absorption 620 nm; GFP $\lambda_{EX} = 485$ nm, $\lambda_{EM} = 540$ nm; *H. polymorpha* $OD_0 = 0.8$; 5 wt % PAAm-gel; geometry 2

glucose [18, 39, 40]. As for the *E. coli* experiment, the initial glucose concentration in the culture well was kept constant at 3 g/l while varying the feed concentrations in the reservoir wells in a range of 100–500 g/l glucose for the fed-batch experiments. For the batch experiment, 3 g/l of glucose was also used. The scattered light signal and the GFP signal for product formation have been monitored online and plotted over time in Fig. 7 for the respective cultivations in the geometry 2 FB-MTP.

In Fig. 7a, the scattered light signal for batch and fed-batch cultures is displayed. The graph of the batch experiment showed a scattered light increase during the first 12 h. This growth phase can be divided into two phases, the first lasting for 4 h, and the second from 4 until 12 h. At 12 h, the transition into the stationary phase took place indicated by a constant signal. Obviously, no GFP was formed in the batch experiment throughout the whole fermentation (Fig. 7b) due to strong carbon catabolite repression of the FMD promoter [18, 39, 40].

The three fed-batch experiments showed an identical, exponential course until 4 h. As product formation starts at 6 h in all cases, it was reasonable to assume that the transition into the fed-batch phase, when glucose becomes limiting, takes place at this time. During the fed-batch phase, the slope for the 500-g/l fed-batch was steepest, followed by the 300- and 100-g/l fed-batch cultures, respectively.

A slightly different picture arises when looking at the GFP signal in Fig. 7b. The experiment with 100 g/l glucose in the reservoir showed a linear increase in GFP signal until the end of fermentation. The 300-g/l experiment showed a strong increase in GFP signal until ~18 h. At this time, a change in slope was visible and the GFP signal increased linearly from ~7,000 to 10,000 a.u. at ~32 h. After this point, the GFP signal remained constant until the end. A similar trend could be observed for the 500-g/l fed-batch experiment. In contrast to the 300-g/l fed-batch, no second phase was recognizable. Instead, a plateau commenced after the strong initial increase at ~16 h. This plateau lasted until ~33 h. Interestingly, the transition into the GFP plateaus for the 300- and 500-g/l fed-batch cultures occurred at the similar level of scattered light signal in a range of 11,000–12,800 a.u., indicating termination of product formation (indicated by circles in Fig. 7). Due to higher feed rate and therefore higher growth rate, the 500-g/l fed-batch culture reached the respective scattered light level sooner. A phase of decreasing GFP signal commenced the mentioned plateau at ~33 h.

While no GFP was produced in the batch culture, a maximum GFP signal of 9,000 a.u. was measured for the 100-g/l fed-batch, ~12,000 a.u. for the 300-g/l and ~7,500 a.u. for the 500-g/l fed-batch experiments. In relation to biomass formation, this equaled ~1.1 a.u. GFP/a.u. scattered light for the 100-g/l fed-batch fermentation, whereas ~0.7 a.u. GFP/a.u. scattered light were produced in the 300-g/l fed-batch experiment. As for *E. coli*, an initial feed concentration of 100 g/l turned out to be the best for high productivity for the used *H. polymorpha* RB 11 pC10-FMD (pFMD-GFP) strain.

Conclusions and outlook

In this work, a newly developed fed-batch microtiter plate (FB-MTP) was presented, which allows 44 parallel fed-batch cultivations in micro-scale by diffusion-driven substrate release from a reservoir well filled with highly concentrated feed solution to a culture well. This novel FB-MTP is characterized by its small size, high degree of parallelization, and its ease of use. Based on standard 96-well microtiter plates, it can easily be embedded in standard

laboratory processes. Only a microtiter plate shaker with temperature and humidity control is needed, which are nowadays available in almost every laboratory. In combination with the BioLector device, all wells can fully be monitored over time. Moreover, completely automated screening campaigns are possible when further combined with a robotic liquid handling system.

The results presented in this work demonstrate the proof of concept of the newly developed FB-MTP. The glucose release from the reservoir into the culture well as well as water counter diffusion were characterized providing a preliminary idea of the diffusion mechanisms in the FB-MTP. As a diffusion-based system, almost constant feed rates can be adjusted in a broad range by adapting diffusion parameters such as the geometry of the hydrogel channel, the density of the hydrogel, and the driving concentration gradient. Therefore it enables the easy evaluation of different feed-rates in a single experiment in order to identify the best conditions. Moreover, the FB-MTP exceeds other controlled-release systems since any desired soluble compound, even liquid substrates like e.g., glycerol, can be fed. Even feed supplementation into the reservoir wells of the FB-MTP allowing stepwise adjustment of the feeding rate during the cultivation is possible by manual or robotic injection. Thereby, it is now possible to perform a primary screening of different strains, media, and process conditions at an early stage of process development. The prior aim of the screening is the reproducible and reliable selection of clones and conditions allowing the highest productivity. The loss of highly producing strains during screening cannot be compensated in later development steps, while cell density can be optimized in the following process development. Therefore, the focus of the fed-batch experiments conducted in this study was a reliable selection rather than achieving high cell densities.

Fed-batch cultivations of *E. coli* and *H. polymorpha* using the novel FB-MTP proofed the positive effects of fed-batch mode to expression systems sensitive to carbon catabolite repression. With the novel FB-MTP, it is possible to feed glucose in limiting concentrations for continuous product formation and circumventing carbon catabolite repression. The higher productivity in fed-batch cultivations is the reason why production processes are usually run in fed-batch mode. In combination with the BioLector, the FB-MTP is a powerful tool to conduct screening processes under conditions similar to that of the perspective fed-batch production process providing more reliable results for the scale-up.

For future studies, the two mass fluxes glucose and water counter diffusion should be intensively investigated. More datasets are necessary for mathematical description of the diffusion mechanism in the FB-MTP. One of the most important topics of future experiments should be the applicability of the novel FB-MTP to other organisms. Also, the feeding of other compounds such as pH-regulating agents or liquid substrates should be demonstrated. To illustrate system flexibility, the combined feeding of two substrates should be investigated. In addition, the possibility to feed complex compounds such as yeast extract needs to be studied as many industrial production processes make use of cost-effective complex media. Finally, scalability has to be proven by cultivations in a fermenter with a medium reservoir and pumps using feeding rates realized in this study. Especially the discrepancy between theoretically reachable cell densities in the FB-MTP and the high cell densities achieved in production processes needs to be addressed as this is a prerequisite for direct scalability.

Acknowledgments The *E. coli* BL21 (DE3) pRhotHi-2—Ec FbFP was kindly provided by T. Drepper, Institute of Molecular Enzyme Technology, Heinrich-Heine-University Düsseldorf/Germany. The *H. polymorpha* RB 11 pC10-FMD (p_{FMD}-GFP) was kindly supplied by the company RheinBiotech. The authors thank the company Lohmann for the supply of adhesive foils and Mr. Gerhard Otto from the Fraunhofer ILT, RWTH Aachen University (Germany) for the modification of the adhesive foils. The authors also thank Mr. Alfons Will for preparing the technical drawings.

Conflict of interest All authors declare that they have no conflicts of interest.

References

1. Amuel C, Gellissen G, Hollenberg CP, Suckow M (2000) Analysis of heat shock promoters in *Hansenula polymorpha*: the *TPS1* promoter, a novel element for heterologous gene expression. Biotechnol Bioprocess Eng 5(4):247–252
2. Bähr C, Leuchtle B, Lehmann C, Becker J, Jeude M, Peinemann F, Arbter R, Büchs J (2012) Dialysis shake flask for effective screening in fed-batch mode. Biochem Eng J 69:182–195
3. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62(3):293–300
4. Bertani G (2004) Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J Bacteriol 186(3):595–600
5. Broughall JM, Anslow PA, Kilsby DC (1983) Hazard analysis applied to microbial-growth in food: development of mathematical-models describing the effect of water activity. J Appl Bacteriol 55(1):101–110
6. Büchs J (2001) Introduction to advantages and problems of shaken cultures. Biochem Eng J 7(2):91–98
7. De Deken RH (1966) Crabtree effect: a regulatory system in yeast. J Gen Microbiol 44(2):149–156
8. Drepper T, Eggert T, Circolone F, Heck A, Krauß U, Guterl J-K, Wendorff M, Losi A, Gärtner W, Jaeger K-E (2007) Reporter proteins for in vivo fluorescence without oxygen. Nat Biotechnol 25(4):443–445
9. Drepper T, Huber R, Heck A, Circolone F, Hillmer A-K, Büchs J, Jaeger K-E (2010) Flavin mononucleotide-based fluorescent reporter proteins outperform green fluorescent protein-like proteins as quantitative *in vivo* real-time reporters. Appl Environ Microbiol 76(17):5990–5994
10. Fick A (1855) Ueber diffusion. Ann Phys 170(1):59–86

11. Funke M, Buchenauer A, Mokwa W, Kluge S, Hein L, Mueller C, Kensy F, Büchs J (2010) Bioprocess control in microscale: scalable fermentations in disposable and user-friendly microfluidic systems. Microb Cell Fact 9:86
12. Funke M, Buchenauer A, Schnakenberg U, Mokwa W, Diederichs S, Mertens A, Müller C, Kensy F, Büchs J (2010) Microfluidic BioLector—microfluidic bioprocess control in microtiter plates. Biotechnol Bioeng 107(3):497–505
13. Gellissen G (2000) Heterologous protein production in methylotrophic yeasts. Appl Microbiol Biotechnol 54(6):741–750
14. Gellissen G (2004) Production of recombinant proteins: novel microbial and eukaryotic expression system. Wiley-VCH, Weinheim
15. Hermann R, Lehmann M, Büchs J (2003) Characterization of gas-liquid mass transfer phenomena in microtiter plates. Biotechnol Bioeng 81(2):178–186
16. Huber R, Ritter D, Hering T, Hillmer A-K, Kensy F, Müller C, Wang L, Büchs J (2009) Robo-Lector: a novel platform for automated high-throughput cultivations in microtiter plates with high information content. Microb Cell Fact 8:42
17. Jacob F, Monod J (1961) Genetic regulatory mechanisms in synthesis of proteins. J Mol Biol 3(3):318–356
18. Jeude M, Dittrich B, Niederschulte H, Anderlei T, Knocke C, Klee D, Büchs J (2006) Fed-batch mode in shake flasks by slow-release technique. Biotechnol Bioeng 95(3):433–445
19. Katzke N, Arvani S, Bergmann R, Circolone F, Markert A, Svensson V, Jaeger K-E, Heck A, Drepper T (2010) A novel T7 RNA polymerase dependent expression system for high-level protein production in the phototrophic bacterium *Rhodobacter capsulatus*. Prot Expr Purif 69(2):137–146
20. Kedem O, Katchalsky A (1958) Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. Biochim Biophys Acta 27(2):229–246
21. Kedem O, Katchalsky A (1963) Permeablility of composite membranes. 1. Electric current, volume flow of solute through membranes. Trans Faraday Soc 59(488):1918–1930
22. Kensy F, Zang E, Faulhammer C, Tan R-K, Büchs J (2009) Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. Microb Cell Fact 8:31
23. Kensy F, Zimmermann HF, Knabben I, Anderlei T, Trauthwein H, Dingerdissen U, Büchs J (2005) Oxygen transfer phenomena in 48-well microtiter plates: determination by optical monitoring of sulfite oxidation and verification by real-time measurement during microbial growth. Biotechnol Bioeng 89(6):698–708
24. Kim BS, Lee SC, Lee SY, Chang YK, Chang HN (2004) High cell density fed-batch cultivation of *E. coli* using exponential feeding combined with pH-stat. Bioproc Biosyst Eng 26(3):147–150
25. Kottmeier K, Weber J, Mueller C, Bley T, Büchs J (2009) Asymmetric division of *H. polymorpha* reflected by a drop of light scatter intensity measured in batch microtiter plate cultivations at phosphate limitation. Biotechnol Bioeng 104(3):554–561
26. Krause M, Ukkonen K, Haataja T, Ruottinen M, Glumoff T, Neubauer A, Neubauer P, Vasala A (2010) A novel fed-batch based cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. Microb Cell Fact 9:11
27. Kumar S, Wittmann C, Heinzle E (2004) Mini-bioreactors. Biotechnol Lett 26(1):1–10
28. Larsson G, Jørgensen SB, Pons MN, Sonnleitner B, Tijsterman A, Titchener-Hooker N (1997) Biochemical engineering science. J Biotechnol 59(1–2):3–9
29. McGown EL, Hafeman DG (1998) Multichannel pipettor performance verified by measuring pathlength of reagent dispensed into a microplate. Anal Biochem 258(1):155–157
30. Meyer H-P, Turner NJ (2009) Biotechnological manufacturing options for organic chemistry. Mini Rev Org Chem 6(4):300–306
31. Muhr AH, Blanshard JMV (1982) Diffusion in gels. Polym 23(7):1012–1026
32. Murray I, Williams PC (2001) Near-infrared technology in the agricultural and food industries. Chemical principles of near-IR technologies, 2nd edn. American Association of Cereal Chemists, St. Paul
33. Panula-Perälä J, Šiurkus J, Vasala A, Wilmanowski R, Casteleijn MG, Neubauer P (2008) Enzyme controlled glucose auto-delivery for high cell density cultivations in microplates and shake flasks. Microb Cell Fact 7:31
34. Riesenberg D (1991) High-cell-density cultivation of *E. coli*. Curr Opin Biotechnol 2(3):380–384
35. Samorski M, Müller-Newen G, Büchs J (2005) Quasi-continuous combined scattered light and fluorescence measurements: a novel measurement technique for shaken microtiter plates. Biotechnol Bioeng 92(1):61–68
36. Scheidle M, Dittrich B, Klinger J, Ikeda H, Klee D, Büchs J (2011) Controlling pH in shake flasks using polymer-based controlled-release discs with pre-determined release kinetics. BMC Biotechnol 11:25
37. Scheidle M, Jeude M, Dittrich B, Denter S, Kensy F, Suckow M, Klee D, Büchs J (2010) High-throughput screening of *H. polymorpha* clones in the batch compared with the controlled-release fed-batch mode on a small scale. FEMS Yeast Res 10(1):83–92
38. Scheidle M, Klinger J, Büchs J (2007) Combination of on-line pH and oxygen transfer rate measurement in shake flasks by fiber optical technique and respiration activity monitoring system (RAMOS). Sensors 7(12):3472–3480
39. Stöckmann C, Losen M, Dahlems U, Knocke C, Gellissen G, Büchs J (2003) Effect of oxygen supply on passaging, stabilising and screening of recombinant *H. polymorpha* production strains in test tube cultures. FEMS Yeast Res 4(2):195–205
40. Stöckmann C, Maier U, Anderlei T, Knocke C, Gellissen G, Büchs J (2003) The oxygen transfer rate as key parameter for the characterization of *H. polymorpha* screening cultures. J Ind Microbiol Biotechnol 30(10):613–622
41. Stöckmann C, Scheidle M, Dittrich B, Merckelbach A, Hehmann G, Melmer G, Klee D, Büchs J, Kang HA, Gellissen G (2009) Process development in *H. polymorpha* and *Arxula adeninivorans*, a re-assessment. Microb Cell Fact 8:22
42. Valgepea K, Adamberg K, Nahku R, Lahtvee P-J, Arike L, Vilu R (2010) Systems biology approach reveals that overflow metabolism of acetate in *E. coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. BMC Syst Biol 4:166
43. Van Wijk R (1968) Alpha-glucosidase synthesis respiratory enzymes and catabolite repression in yeast 1. Effects of glucose and maltose on inducible alpha-glucosidase synthesis in protoplasts of *Saccharomyces carlsbergensis*. Proc K Ned Akad Wet C 71(1):60–71
44. Weuster-Botz D (2005) Parallel reactor systems for bioprocess development. In: Technology Transfer in Biotechnology: From Lab to Industry to Production, vol 92. Advances in biochemical engineering-biotechnology. Springer, Berlin Heidelberg New York, pp 125–143
45. Weuster-Botz D, Altenbach-Rehm J, Arnold M (2001) Parallel substrate feeding and pH-control in shaking flasks. Biochem Eng J 7(2):163–170
46. Xu B, Jahic M, Blomsten G, Enfors SO (1999) Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *E. coli*. Appl Microbiol Biotechnol 51(5):564–571
47. Yankov D (2004) Diffusion of glucose and maltose in polyacrylamide gel. Enzyme Microb Technol 34(6):603–610

48. Zhang YK, Taiming L, Liu JJ (2003) Low temperature and glucose enhanced T7 RNA polymerase-based plasmid stability for increasing expression of glucagon-like peptide-2 in *E. coli*. Prot Expr Purif 29(1):132–139

49. Zhang Z, Perozziello G, Boccazzi P, Sinskey AJ, Geschke O, Jensen KF (2007) Micro-bioreactors for bioprocess development. JALA 12(3):143–151