Fed-Batch Mode in Shake Flasks by Slow-Release Technique

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Abstract: Most industrial production processes are performed in fed-batch operational mode. In contrast, the screenings for microbial production strains are run in batch mode which results in completely different physiological conditions than relevant for production conditions. This may lead to wrong selections of strains. Silicone elastomer discs containing glucose crystals were developed to realize fed-batch fermentation in shake flasks. No other device for feeding was required. Glucose was fed in this way to *Hansenula polymorpha* cultures controlled by diffusion. Two strains of *H. polymorpha* were investigated in shake flasks: the wild-type strain (DSM 70277) and a recombinant strain pC10-FMD (P_{FMD}-GFP). The oxygen transfer rate (OTR) and respiratory quotient (RQ) of the cultures were monitored online in shake flasks with a Respiration Activity Monitoring System (RAMOS). Formation of biomass and green fluorescent protein (GFP), pH-drift and the metabolite dynamics of glucose, ethanol and acetic acid were measured offline. With the slow-release technique overflow metabolism could be reduced leading to an increase of 85% in biomass yield. To date, 23.4 g/L cell dry weight of *H. polymorpha* could be achieved in shake flask. Biomass yields of 0.38–0.47 were obtained which are in the same magnitude of laboratory scale fermentors equipped with a substrate feed pump. GFP yield could be increased by a factor of 35 in Syn6-MES mineral medium. In fed-batch mode 88 mg/L GFP was synthesized with 35.9 g/L fed glucose. In contrast, only 2.5 mg/L with 40 g/L metabolized glucose was revealed in batch mode. In YNB mineral medium over 420-fold improvement in fed-batch mode was achieved with 421 mg/L GFP at 41.3 g/L fed glucose in comparison to less than 1 mg/L in batch mode with 40 g/L glucose.

Keywords: slow-release; fed-batch; shake flask; *Hansenula polymorpha*; overflow metabolism; catabolite repression; green fluorescent protein (GFP); high cell density cultivation (HCDC)

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INTRODUCTION

Biotechnological companies and academia nowadays run up to several hundred thousand individual experiments and more per year to find microbial production systems with new or optimal synthesis properties (Büchs, 2004; Suzuki et al., 2003). Shaken bioreactors are the most frequently used reaction vessels in biotechnology due to their simple bioreactor design and handling. Medium or fermentation parameter optimization and especially strain screenings are conducted simultaneously in large numbers.

Experiments in shake flasks are usually performed in batch operational mode where high initial substrate and buffer concentrations are applied (Kennedy et al., 1994; Kumar et al., 2004; Weuster-Botz et al., 2001). This is a great disadvantage if the microorganism undergoes metabolic phenomena such as specific inhibition by substrate excess, unspecific inhibition because of unfavourable water activity or osmotic pressure (Stöckmann et al., 2003b). Some microorganisms respond with overflow metabolism to high initial substrate concentrations and secrete large amounts of by-products like formic, acetic and lactic acid or ethanol under strictly aerobic conditions. Well-known representatives are *Escherichia coli* and the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (Suckow and Gellissen, 2002; Xu et al., 1999). The consequences of overflow metabolism may be a drift of pH or by-product inhibition on growth and on specific product formation (Klinke et al., 2004; Riesenberg and Guthke, 1999).

Catabolite repression of product formation occurring at elevated concentrations of a readily available carbon source is the most relevant case of negative metabolic phenomena during batch cultivation. Hardly any product will be synthesized on mineral media, if the operon responsible for product synthesis is intact and completely repressed by a specific substrate (Browning et al., 2005; Kramarenko et al., 2000; Stasyk et al., 2004; van Wijk, 1968). Therefore, in most screening procedures for catabolite repressed products

culture media with high amounts of complex components of undefined and changing composition are applied. Often compounds from complex media are slowly hydrolysed by swelling and dissolving processes making a key nutrient available with time. In fact, this is equivalent to a fed-batch mode for the strains (Losen et al., 2004; Rheinwald and Green, 1974). Commonly, the data obtained in this way is used for process optimization or scale-up without completely understanding the role of each media component or process parameter (Büchs, 2001; Clark et al., 1995; Humphrey, 1998).

Most large-scale industrial production processes are performed in fed-batch mode in order to avoid the above mentioned aspects and enhance biomass and/or product formation (Kim et al., 2004; Larsson et al., 1997; Riesenberg, 1991; Wittmann et al., 1995). Recently, a fed-batch system with pumps and 2/2-way valves for 16 shake flasks in parallel was established. Higher cell densities were achieved in comparison to batch cultures and physiological effects could be investigated in detail in small scale (Weuster-Botz et al., 2001).

An alternative way of feeding nutrients with controlled kinetics is represented by the slow-release technique. Entrapped nutrients are released from an artificial matrix or reservoir driven by diffusion. Polymeric delivery systems are used for drug release in medical technology and as fertilizers in agriculture. The intention is to deliver chemical compounds in the most effective concentration window (Baker, 1987; Carelli et al., 1989; Di Colo, 1992; McGinity et al., 1979). Several matrix delivery systems are chemically and mathematically described in literature (Higuchi, 1961, 1963; Hsieh et al., 1983; Langer, 1980; Langer et al., 1980; Langer and Folkman, 1976; Langer and Peppas, 1981; Narasimhan and Langer, 1997). Although there are plenty of reports on polymeric delivery systems, very little activity is observed in applying this concept in biochemical engineering (Baker, 1987; Kost and Langer, 2001; Langer, 1990). A single paper on the application of polymer based slow-release technique for microbial cultures was published by Lübbe et al., 1985, feeding NH_4^+ to *Streptomyces clavuligerus* from ethylene-vinyl acetate copolymer discs containing NH_4Cl (Lübbe et al., 1985). The intention was to improve cephalosporin production in comparison to batch cultures. However, the authors did not observe significant advantage of applying their slow-release polymer. This may be due to the fact that the released nitrogen source accumulated during the culture and nitrogen limited conditions were only obtained at the very end of the culture. This demonstrates that the design of the slow-release polymer is a crucial issue and must be carefully adjusted to the demand of the microorganisms. This is a very difficult task and requires a parallel online measurement technique (e.g. for OTR, CTR, pH) for efficient development. Such kind of technique was not yet available on small scale at the time when the investigation of Lübbe et al. was performed.

In this article, the effect of diffusion based slow-release silicone elastomers allowing a fed-batch mode is compared to regular batch fermentations with *H. polymorpha*. Polydimethylsiloxane (PDMS) was chosen as a matrix polymer because it does not contain any harmful components which could be extracted in aqueous solutions. The material shows a negligible shrinkage after vulcanization and has suitable mechanical properties (Schirrer et al., 1992). In medical sciences PDMS was used in a hybrid biochip for batch cultivation of bacterial cells and in applications with drug delivery systems for macromolecular drugs and proteins (Amsden, 2003; Carelli et al., 1989; Chang et al., 2003; Kajihara et al., 2000). *H. polymorpha* was chosen as a model organism because of its overflow metabolism at high initial glucose concentrations. The methylotrophic yeast is a well-known expression platform for peptides and proteins like hirudin and phytase (Faber et al., 1996; Gellissen, 2000; Hollenberg and Gellissen, 1997; Mayer et al., 1999; Weydemann et al., 1995). Several of its promoters were studied with β-galactosidase or GFP as reporter genes (Amuel et al., 2000).

MATERIALS AND METHODS

Organisms and Cultivation

H. polymorpha wild-type strain (DSM 70277) and RB11 derivate strain pC10-FMD (P_{FMD}-GFP) were maintained at $-80^{\circ}C$ in YNB medium. Stock cultures contained 200 g/L glycerol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). *H. polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) was kindly provided by Dr. C. Amuel (Heinrich-Heine University, Department of Microbiology, Düsseldorf, Germany) (Amuel et al., 2000; Gellissen, 2000).

Media and Solutions

Syn6-MES mineral medium basic solution consisted of 1.0 g/L KH_2PO_4, 7.66 g/L $(NH_4)_2SO_4$, 3.3 g/L KCl, 3.0 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L NaCl, 27.3 g/L 2-morpholinoethanesulfonic acid (MES). The basic solution was adjusted to pH = 6.4. To 1 L of basic solution was added: 6.67 mL calcium chloride solution (150 g/L $CaCl_2 \cdot 2H_2O$), 6.67 mL micro element solution (10.0 g/L $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.8 g/L $CuSO_4 \cdot 5H_2O$, 3.0 g/L $ZnSO_4 \cdot 7H_2O$, 4.0 g/L $MnSO_4 \cdot H_2O$, 10.0 g/L EDTA (Titriplex III)), 6.67 mL of vitamin solution (0.06 g/L D-biotin, 20.0 g/L thiamine hydrochloride), 3.33 ml of trace element solution (0.2 g/L $NiSO_4 \cdot 6\ H_2O$, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.2 g/L boric acid, 0.2 g/L KI and 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$). The medium resulted in a final volume of 1023.33 mL and no final pH adjustment was necessary (Gellissen, 2004). All reagents were of analytical grade and purchased from Carl Roth GmbH & Co., Karlsruhe, Germany.

YNB mineral medium consisted of 13.3 g/L $(NH_4)\ H_2PO_4$, 1.4 g/L yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ (Difco, Becton Dickinson & Co., Franklin Lakes, NJ). The medium had a pH of 4.3 (Stöckmann et al., 2003a).

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In batch cultures, glucose was added as specified in the figure legends. The fed-batch media were prepared without initial glucose content.

Cultivation, Cell Dry Weight Determination and Optical Density Measurement

Two hundred fifty millilitre-Erlenmeyer shake flask (Schott, Mainz, Germany) and modified 250-mL-Erlenmeyer shake flask of the Respiration Activity Monitoring System (RAMOS, HiTec Zang, Herzogenrath, Germany) were used with a filling volume of 12.5 mL (Anderlei and Büchs, 2001; Anderlei et al., 2004). The cultivations were all carried out at 37°C, 350 rpm and a shaking diameter of 50 mm on a Lab-Shaker LS-W (Kühner, Birsfelden, Switzerland). Cell dry weight (CDW) was determined by gravimetry. Optical density was measured at $\lambda = 600$ nm (OD_{600}) with an Uvikon 922 spectrophotometer (Kontron, Basel, Switzerland). In case of *H. polymorpha* 1.0 g of cell dry weight (CDW) per litre medium corresponded to an OD_{600} of 4.4. These samples were measured against fresh medium in a cuvette with a thickness of 1 cm at 25°C. The OD after inoculation was always adjusted to 0.5.

Online Measurement of OTR, CTR, RQ and μ_{max} With the RAMOS Device

The RAMOS device for the online measurement of the OTR, CTR and RQ in shake flasks was recently introduced by Anderlei et al. (Anderlei and Büchs, 2001; Anderlei et al., 2004). This device has already been successfully employed for several projects (Danielson et al., 2004; Hermann et al., 2001; Losen et al., 2004; Lotter and Büchs, 2004; Maier et al., 2004; Peter et al., 2004; Raval et al., 2003; Silberbach et al., 2003; Stöckmann et al., 2003a,b). The fermentations were conducted in specially modified 250-mL-Erlenmeyer flasks. The RAMOS device is designed and operated so that the hydrodynamic conditions in the liquid phase and the concentrations in the gas phase are identical to those in regular Erlenmeyer flasks cultures with cotton plugs (Anderlei et al., 2004).

Determination of Green Fluorescent Protein (GFP) Intensities

GFP intensities were determined fluorometrically in black 96-well microtiterplates (art.-no.: 655096, Greiner Bio-One GmbH, Frickenhausen, Germany) at a filling volume of 200 µL per well. The emission measurement was performed at $\lambda = 520$ nm after excitation at $\lambda = 485$ nm with a FLUOstar 0403 reader (BMG Labtech GmbH, Offenburg, Germany). All measurements were carried out in a linear interval in comparison to in-house purified GFP standards. The suspension and dilution medium was 100 mM phosphate buffer pH = 7.0. The fluorescence intensities were correlated against different protein contents of the GFP standard, determined with a standard Bradford test (Bradford, 1976). The GFP standard solution was kindly provided by Dr. J. Drossard (Institute of Molecular Biotechnology (Bio VII), RWTH Aachen University, Aachen, Germany).

Silicone Elastomer Discs

The applied polymer matrix consisted of PDMS and glucose. Sterile silicone elastomer discs with 20% (w/w) (art.-no.: 63269) were obtained from AC Biotec GmbH, Jülich, Germany (www.acbiotec.com).

Release Measurement of Glucose From Silicone Discs

The release of glucose under fermentation conditions was studied by adding one or more silicone elastomer discs (diameter $D = 22$ mm; height $H = 0.7$, 1.1, 1.8 or 2.9 mm) into 50 mL of culture medium. Contamination was prevented by adding 0.2 g/L NaN_3 (Merck KGaA, Darmstadt, Germany). The medium was not inoculated with any microorganism but all other conditions were kept as in cultivation. Samples of 50 µL were taken at minutes to hours intervals over a period of 1–3 days. The glucose released from the silicone elastomers was determined by a commercially available enzyme assay with hexokinase and glucose-6-phosphate dehydrogenase (Roche Diagnostics GmbH, Mannheim, Germany). Extinction of $NADH + H^+$ was measured in the linear interval at $\lambda = 365$ nm with a Powerwave X 340 reader (Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany).

High Performance Liquid Chromatography (HPLC) Measurements

Determination and quantification of glucose and other metabolites like ethanol and acetic acid were performed by HPLC, using an Organic Acid Resin HPLC pre-column (40×8 mm) and Organic Acid Resin HPLC separation column (250×8 mm) (CS-Chromatographie Service GmbH, Langerwehe, Germany). One millimolar sulphuric acid was used as eluant (flow rate: 0.8 mL/min). The temperature during chromatography was kept constant at 60°C. Substances were detected by UV absorbance at $\lambda = 210$ nm with an UV diode array detector UVD340S (Dionex Softron GmbH, Germering, Germany) and refractometric index with a Shodex RI-71 refractometer (Techlab GmbH, Erkerode, Germany). Analysis and peak calculation was performed with Chromeleon software version 6.4 (Dionex Softron GmbH).

Estimation of Glucose Release in Biological Experiments by Mass Balance Based on Cell Dry Weights, RAMOS and HPLC Data

The total amount of glucose released from the slow-release elastomer discs until a specific time of a culture experiment was calculated as a sum of four contributions as follows:

$$c_{glc}^{total}(t) = \sum \left(c_{glc}^{growth}(t),\, c_{glc}^{EtOH}(t),\, c_{glc}^{Ac}(t),\, c_{glc}^{residual}(t) \right) \tag{1}$$

The first term denotes the amount of glucose, which was utilized for growth. The second and third term represent the amount of glucose converted to the overflow metabolites ethanol and acetic acid. The fourth term is the measured residual glucose which had accumulated in the culture broth. For all times of the fermentation a material balance from the start of the fermentation was made. The first term of Equation 1 is based on pure growth of *H. polymorpha* using the following simplified reaction Equation 2 (Sonnleitner and Käppeli, 1986) and a statistical molecular weight of yeast cells of 23.85 g/mol ($M_{W,cells}$) with the composition $CH_{1.75}O_{0.5}N_{0.15}$ (Atkinson and Mavituna, 1983):

$$\phi \cdot C_6H_{12}O_6 + \alpha \cdot NH_3 + \beta \cdot O_2 \xrightarrow{\text{yeast}} \gamma \cdot CH_{1.75}O_{0.5}N_{0.15} + \delta \cdot CO_2 + \varepsilon \cdot H_2O \tag{2}$$

The Greek letters α, β, γ, δ, ε and ϕ represent stoichiometric coefficients. The absolute consumed molar amount of oxygen (β) until a specific time of fermentation can be calculated as integral of the OTR until this point. γ is known from biomass measurement. The oxygen consumption for acetate formation had to be subtracted from the integral as specified below. To calculate the amount of glucose for pure growth (ϕ), the stoichiometric factors for ammonia (α), carbon dioxide (δ) and water (ε) are required. These can be calculated performing oxygen, carbon, hydrogen, and nitrogen balances (Eqs. 3–6).

(1) Oxygen balance:

$$\phi = \frac{\gamma \cdot 0.5 + \delta \cdot 2 + \varepsilon - \beta \cdot 2}{6} \tag{3}$$

(2) Carbon balance:

$$\delta = \phi \cdot 6 - \gamma \tag{4}$$

(3) Hydrogen balance:

$$\varepsilon = \frac{\phi \cdot 12 + \alpha \cdot 3 - \gamma \cdot 1.75}{2} = \phi \cdot 6 + \alpha \cdot 1.5 - \gamma \cdot 0.875 \tag{5}$$

(4) Nitrogen balance:

$$\alpha = \gamma \cdot 0.15 \tag{6}$$

To receive ϕ for pure growth, Equations 4–6 were inserted in Equation 3, resulting in:

$$\phi = \gamma \cdot 0.179 + \beta \cdot 0.167 \tag{7}$$

By-product correction was made to calculate the amount of glucose which was used to produce ethanol and acetic acid (Albers et al., 1996) according to the following reaction Equations 8 and 9:

$$C_6H_{12}O_6 \xrightarrow{H.polymorpha} 2 \cdot C_2H_5OH + 2 \cdot CO_2 \tag{8}$$

$$C_6H_{12}O_6 + 2 \cdot O_2 \xrightarrow{H.polymorpha} 2 \cdot CH_3COOH + 2 \cdot CO_2 + 2 \cdot H_2O \tag{9}$$

Therefore, one mole of glucose has each to be taken into account for two moles of ethanol and acetic acid formed. One mole of oxygen is consumed per mole of acetate formed. This amount of oxygen has to be subtracted from the measured integral of the OTR, which is then used as an input in Equation 7.

RESULTS AND DISCUSSION

With slow-release technique a fed-batch mode in shaken bioreactors based on diffusion can be realized. It is established by adding polymer discs with carbon source to a regular batch culture without initial carbon source in the medium (Fig. 1). In this way, it is possible to get early information on process controlling parameters with an unrivalled easy handling of the release systems in shaken bioreactors.

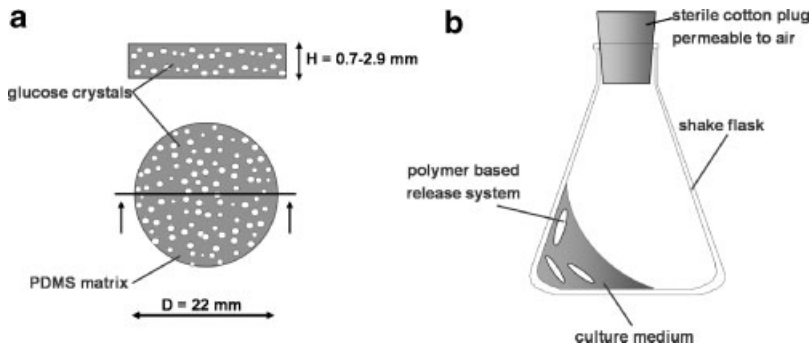

Figure 1. **a**: Cut and top-view of a silicone elastomer disc with enclosed glucose crystals. **b**: Regular shake flask filled with a culture suspension and three silicone elastomer discs as polymer-based release systems.

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Release of Glucose From Silicone Elastomer Discs

Glucose release from silicone elastomer discs was determined by adding a single disc to culture medium. Investigation of the release from discs with a height of 0.7, 1.1, 1.8 and 2.9 mm was done simultaneously in YNB medium. 2.9-mm discs were tested in a double set in YNB. In addition a single set of 1.8- and 2.9-mm discs were investigated in Syn6-MES medium. All measurements were performed in a threefold experiment. The course of relative glucose release from silicone elastomer discs is depicted in Figure 2a as average values. The absolute glucose release is shown in Figure 2b.

In all discs, a rapid release of glucose during the first 5 h occurred, which continuously decreased following a square root of time kinetic until glucose was almost depleted in the discs. Different release velocities were observed by variation of the disc height. The 0.7-mm disc released its whole glucose loading of 59 mg within 54 h (Fig. 2a). The kinetic followed a square root of time for at least 36 h. The 1.1-mm disc released 77 mg glucose (84%) within 54 h and 93% of glucose content within 75 h which results in an absolute amount of 83 mg. At 75 h, the 1.8-mm disc released 115 mg (76%) of its 151 mg glucose loading. The release of the 2.9-mm disc was 125 mg (51% of 243 mg total glucose) in 75 h. The repeatability in all experiments was very good, demonstrated by two data sets with triple determination each for 2.9-mm disc in YNB medium. The release of the 1.8- and 2.9-mm discs in Syn6-MES medium compared to the curve in YNB medium were almost identical (Fig. 2a).

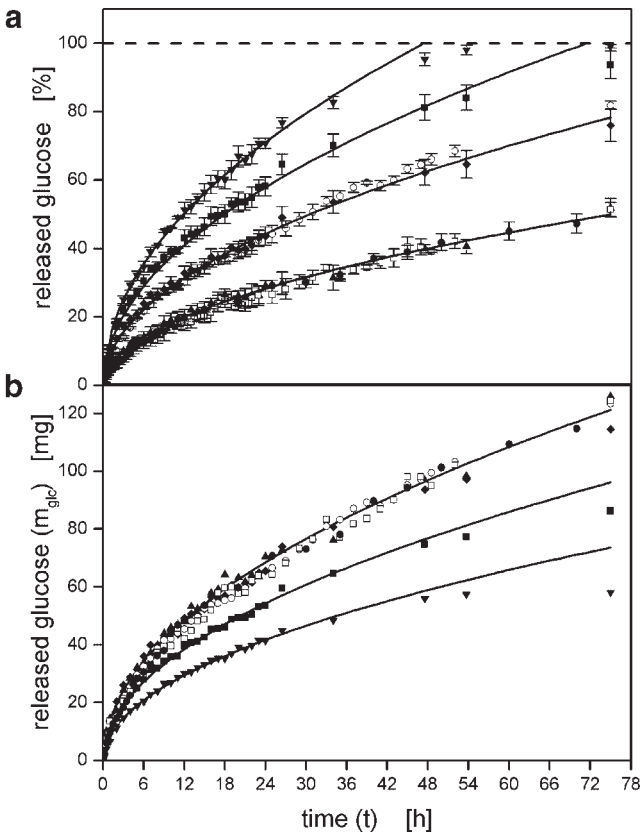

Figure 2. Glucose release kinetics. **a**: Comparison of relative glucose release from various silicone elastomer discs in YNB and Syn6-MES mineral medium. **b**: Absolute glucose release from various silicone elastomer discs in YNB and Syn6-MES mineral medium. Discs with a diameter of 22 mm, heights of 0.7 mm (▼), 1.1 mm (■), 1.8 mm (◆) and duplicate experiment of 2.9 mm in YNB (▲; ●). 1.8-mm discs (○) and 2.9-mm discs (□) were also tested in Syn6-MES. The line graphs represent square root of time fits, the respective mass flows are shown in Table I. All discs contained 20% glucose (w/w). Experiment conditions: culture medium with 0.2 g/L NaN_3; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 50$ mL.

Substance release following square root of time kinetics were, for example, demonstrated as a typical characteristic of matrix slow-release systems with homogenous dispersed drugs by Langer and Baker (Baker, 1987; Langer, 1980). The following equation from Langer can be used to approximate release from discs (Eq. 10).

$$M_{glc} = A \cdot (2 \cdot D \cdot C_s \cdot C_a \cdot t)^{0.5} \quad (10)$$

M_{glc}, mass of released glucose [mg]; A, surface of the disc [mm^2]; D, diffusion coefficient [mm^2/s]; C_a, amount of glucose per unit volume of matrix [mg/mm^3]; C_s, solubility of glucose per unit volume of matrix [mg/mm^3]; t, time [s].

This equation is only valid as long as the drug concentration remaining in the polymer is still above the drug solubility in the matrix. Thus, after a large percentage of the glucose has been released, this equation is not applicable any more. Assuming that all parameters of Equation 10 are constant for a disc besides time, the following equation (Eq. 11) was used to calculate a resulting mass released from a disc.

$$M_{glc} = z \cdot \dot{m}_{glc} \cdot t^{0.5} \quad (11)$$

z, number of discs [−]; $\dot{m}_{glc}$, mass stream of glucose [$mg/h^{0.5}$].

The glucose release velocity increased more or less linearly ($r = 0.95$) from 1.1 to 2.9 mm (Fig. 2b) according to the disc-to-disc ratios shown in Table I. A higher gap was found between 0.7-mm- and 1.1-mm discs which was probably due to other effects than surface dependence. Equation 11 in combination with the characteristic mass flow of glucose from Table I describes the release kinetic of glucose from the respective silicone discs following a square root of time dependency. Due to the high concentration gradient from the dissolving glucose crystals inside the disc matrix to the bulk phase of the surrounding medium, no dependency of the glucose release was found for 10–50 mL

Table I. Summary of the silicone elastomer discs characteristics (D = 22 mm; 20% (w/w) glucose).

Disc height (H) [mm]	Disc-to-disc surface ratio ($A_{disc}/A_{0.7\text{-mm disc}}$) [−]	Mass flow of glucose $\dot{m}_{glc}$ [$mg/h^{0.5}$]
0.7	1.00	8.3
1.1	1.03	13.1
1.8	1.09	13.7
2.9	1.19	14.0

The surface of the 0.7-mm discs is 809 mm^2.

liquid volume (data not shown). The released amount of glucose could be multiplied by simply using more than one silicone elastomer disc per shake flask. The average standard deviation was below +/−10%.

Influence of the Silicone Elastomer Matrix on Growth of *H. polymorpha*

The influence of the chosen silicone elastomer material on the growth of *H. polymorpha* (DSM 70277) was tested. Therefore, an experiment with the RAMOS device was performed in order to check differences in respiration of *H. polymorpha* as an indicator. Optical density, cell weight determination, pH and HPLC measurement were done as well. Four discs ($D = 22$ mm; $H = 1.8$ mm) without glucose content (placebo discs) were added to a batch experiment with 20 g/L dissolved glucose as a sole carbon source in Syn6-MES medium. The course of oxygen transfer rate (OTR) was monitored and compared to regular batch cultivations. In Figure 3 the OTR over fermentation time is shown. All curves had two maxima of about 0.05 mol/L/h. The first peak represented metabolized glucose. The second peak evolved because of overflow metabolism and represented ethanol which was metabolized after glucose was depleted. No difference was detected between flasks with and without placebo discs. The average integral of the OTR from each culture was 0.28 mol/L oxygen with an average standard deviation of 1.9%. This is additionally supported by identical final CDWs of 8.0 g/L (+/−5%), an OD_{600} of 35 (+/−3%) and a final pH 5.7 (+/−0.1). Therefore, the presence of silicone elastomer discs was proven to have no influence on the respiration, metabolism and growth of the microorganism. In the presence of silicone elastomer discs, glucose was metabolized and overflow metabolism took place in the same way as in regular batch cultures.

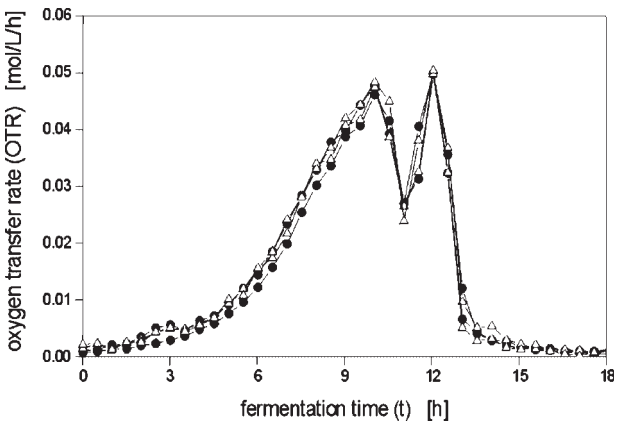

Figure 3. Influence of silicone elastomer discs on the cultivation of *H. polymorpha* wt (DSM 70277) in Syn6-MES medium. Course of the OTR in a regular batch (△) and in a batch in presence of four silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm) without glucose (placebo discs) (●). Duplicate experiments were performed for both conditions. Culture conditions: $pH_x = 6.4$; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL.

Growth Behaviour of *H. polymorpha* wt (DSM 70277) in Batch and Fed-Batch Cultures

H. polymorpha was cultured under batch and fed-batch conditions and its performance was compared. The batch experiment with 20 g/L initial glucose showed the expected fermentation pattern as already shown in Figure 3. The OTR rose up to a value of 0.051 mol/L/h (Fig. 4a). The subsequent decrease of the respiration activity at 8.6 h was due to exhaustion of glucose (Fig. 4d). The overflow metabolites ethanol and acetic acid were formed during this phase with 1.6 and 0.7 g/L, respectively. These compounds were obviously metabolized in parallel as carbon sources in the following time, resulting in a second OTR peak with a maximum value of 0.052 mol/L/h at 10.1 h (Fig. 4a). This change of metabolism was also clearly indicated by the respiratory quotient (RQ) (Fig. 4b). In the first phase of the fermentation the RQ rose to 1.6. Exceeding a RQ value of 1.2 is stoichiometrically only possible if reduced compounds like ethanol are formed, which is proven by the measurements. After the shift of metabolism from glucose consumption to consumption of the overflow metabolites ethanol and acetic acid, the RQ decreased to values significantly lower than 1.0. RQ values around 0.74 agreed nicely with the expectations based on fermentation stoichiometry. The biomass concentration (Fig. 4c) increased to a value of 7.8 g/L after 12 h and became constant after all carbon sources were exhausted. Respiration activity then reduced to very low values. The biomass yield ($Y_{X/S}$) was 0.39. The pH value sharply decreased during the growth of the microorganisms to 5.5. This is probably partially due to the fact that significant amounts of acetic acid were formed.

The experiment applying two slow-release silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm) showed an OTR pattern (Fig. 4a) typical for a fed-batch fermentation known from stirred tank fermentors equipped with a medium stock solution and a feed pump. In the initial phase of fermentation the glucose release from the silicone elastomer discs was larger than the consumption of the microorganisms. As a consequence, glucose was slightly accumulated to 3.1 g/L (Fig. 4d). Therefore, the OTR in this first phase of the fermentation increased exponentially as in the batch fermentation to 0.033 mol/L/h at 6.6 h (Fig. 4a). After growth of the microorganism proceeded to such an extent that glucose consumption exceeded release from the silicone elastomer discs and glucose concentration became limiting, the OTR sharply decreased at 7.1 h. From this point onwards the OTR reflected the supply of glucose from silicone elastomer discs. An elevated OTR level of about 0.01 mol/L/h was observed for the rest of fermentation, slightly decreasing over time. As a consequence of this modified form of glucose kinetics the formation of overflow metabolites was significantly reduced (Fig. 4d). Only very small amounts of ethanol and acetic acid could be detected with 0.1 and 0.2 g/L, respectively. This was also reflected in the RQ (Fig. 4b) which did not change from typical intermediate values to high and then to low values as in

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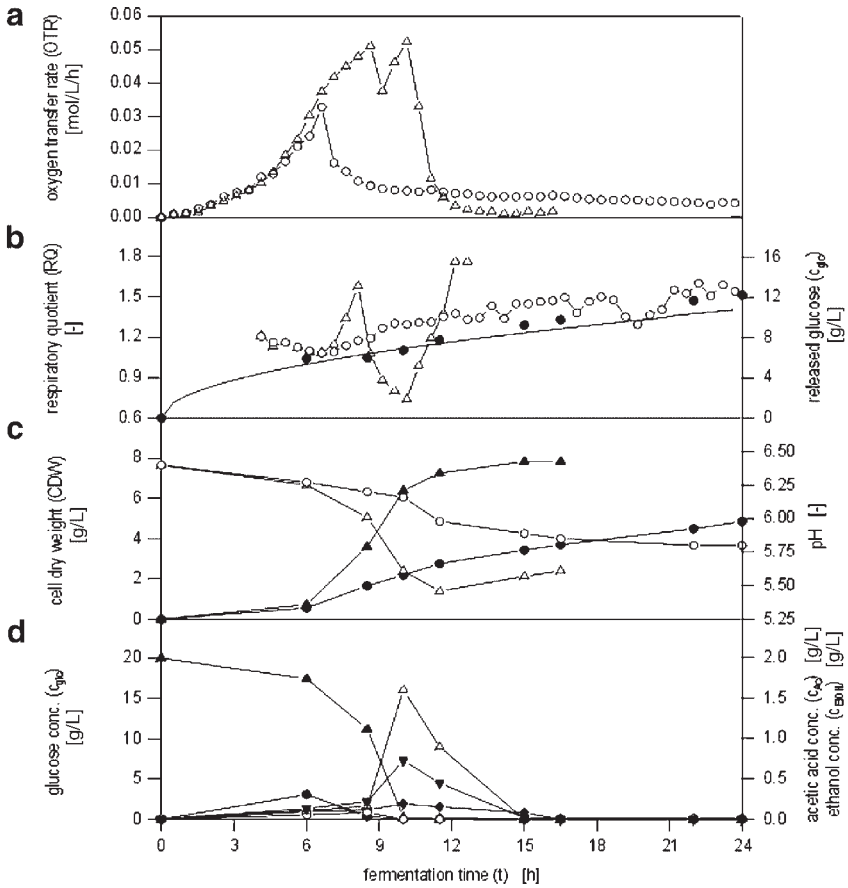

Figure 4. Comparison of batch and slow-release fed-batch mode in shake flask cultures of *H. polymorpha* wild-type strain (DSM 70277) in Syn6-MES medium. **a**: Oxygen transfer rate (OTR): batch (△), fed-batch (○); (**b**) respiratory quotient (RQ): batch (△), fed-batch (○); released glucose (●) calculated by Equation 1 with Equations 7–9 in comparison to the profile of two 1.8-mm disc calculated by Equation 11 (–); (**c**) cell dry weight (CDW): batch (▲), fed-batch (●) and pH: batch (△), fed-batch (○); (**d**) glucose: batch (▲); fed-batch (●), ethanol: batch (△), fed-batch (○); acetic acid: batch (▼), fed-batch (◆). Culture conditions: $pH_x = 6.4$; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL. Glucose was used as sole carbon source in batch with 20 g/L and fed-batch was performed with released glucose from two silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm; 20% (w/w) glucose).

the batch experiment. The RQ in case of the fed-batch experiment remained at a steady level slightly increasing with time. A dramatic change was also observed in the biomass formation and pH (Fig. 4c). Beginning with the time when glucose supply became limiting, the biomass only increased more or less linearly. Growth was steadily continuing until the termination of the experiment. In agreement with this, pH did not sharply decrease as in the case of the batch experiment but continuously decreased over time to 5.8.

The amount of released glucose (which is equivalent to the theoretical concentration in the medium if no microorganism were present and consumed glucose) can be estimated by using Equation 1 with Equations 7–9. The calculated value was plotted for each sample step in Figure 4b. For comparison the glucose concentration resulting from Equation 11 and Table I for two 1.8-mm discs was calculated. The resulting concentrations are depicted as a line in Figure 4b, fitting nicely to the calculated consumed glucose amounts. The total concentration of glucose consumption was calculated to be 12.3 g/L which resulted in a biomass yield ($Y_{X/S}$) of 0.40.

Figure 5 shows an experiment with 40 g/L glucose in a batch experiment and a fed-batch experiment using silicone elastomer discs. The OTR of the batch experiment with 40 g/L initial glucose showed a similar course as the batch with 20 g/L shown in Figure 4. The OTR rose to a maximum of 0.05 mol/L/h during the metabolism of glucose until 9.6 h (Fig. 5a). Subsequently, the OTR decreased because of glucose exhaustion. The RQ (Fig. 5b) rose to 3.1 until 9.6 h because of ethanol production (Fig. 5d). The ethanol amounts were significantly higher compared to the batch with 20 g/L (Fig. 4d). Due to a change of metabolism from glucose to ethanol consumption, RQ decreased from 9.6 to 15.6 h to a value of 0.55 while a second OTR peak was formed. This indicated the simultaneous consumption of the formed overflow metabolites ethanol and acetic acid which was proven by HPLC measurement (Fig. 5d). Temporarily 7.1 g/L ethanol and 1.3 g/L acetic acid was accumulated. The biomass increased exponentially and became constant at a maximum CDW of 11.4 g/L ($Y_{X/S} = 0.29$) (Fig. 5c). During the whole experiment pH sharply decreased due to exponential growth and acetic acid formation from 6.4 to 4.8. Afterwards it rose again to 5.2 because of acetic acid

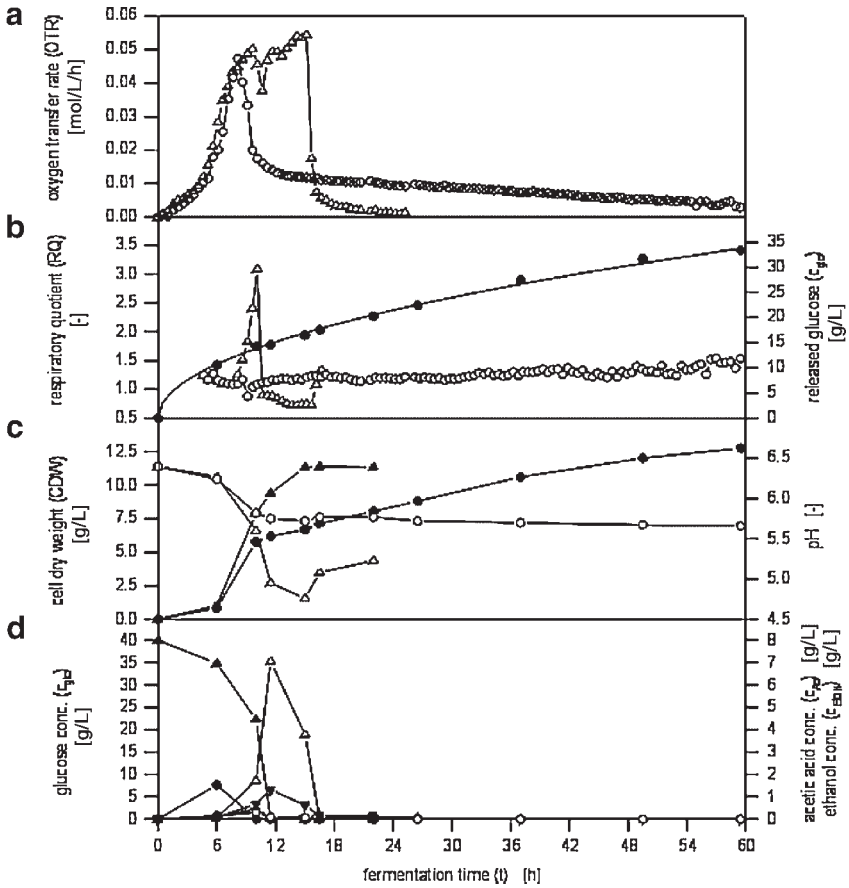

Figure 5. Comparison of batch and slow-release fed-batch mode in shake flask cultures of *H. polymorpha* wild-type strain (DSM 70277) in Syn6-MES medium. **a**: Oxygen transfer rate (OTR): batch (△), fed-batch (○); (**b**) respiratory quotient (RQ): batch (△), fed-batch (○); released glucose (●) calculated by Equation 1 with Equations 7–9 in comparison to the profile of four 1.8-mm disc calculated by Equation 11 (−); (**c**) cell dry weight (CDW): batch (▲), fed-batch (●) and pH: batch (△), fed-batch (○); (**d**) glucose: batch (▲); fed-batch (●), ethanol: batch (△), fed-batch (○); acetic acid: batch (▼), fed-batch (◆). Culture conditions: $pH_z = 6.4$; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL. Glucose was used as sole carbon source in batch with 40 g/L and fed-batch was performed with released glucose from four silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm; 20% (w/w) glucose).

consumption. When all carbon sources were depleted, the OTR decreased to zero within 10 h (Fig. 5a).

In case of the fed-batch experiment the glucose release of the four silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm) during the initial phase of the fermentation exceeded the consumption by *H. polymorpha*, leading to unlimited growth. The OTR increased to a maximum of 0.047 mol/L/h at 8.1 h while accumulated glucose was consumed. Subsequently the OTR decreased because of glucose limitation. An OTR level of 0.015 mol/L/h appeared according to the amount of glucose which was fed by the silicone elastomer discs. The OTR very slowly decreased during a time span of over 60 h (Fig. 5a). The RQ showed a more or less constant level slightly above 1.0, which indicates pure growth and no significant overflow metabolism. Only 0.4 g/L acetic acid and 0.3 g/L ethanol was accumulated in fed-batch mode (Fig. 5d). During glucose limitation phase biomass increased more or less linearly to 12.8 g/L until the termination of the experiment (Fig. 5c). The decrease of pH was much lower than in batch mode.

The amount of released glucose in theoretical concentration in the medium was determined using Equation 1 with Equations 7–9. The values were plotted for each sample step in Figure 5b in comparison to the glucose concentration calculated using Equation 11 and Table I for four silicone elastomer discs of 1.8-mm thickness. The results again are in very good agreement. The total glucose consumption was calculated to be 33.4 g/L, resulting in a biomass yield ($Y_{X/S}$) of 0.38, which was superior compared to 0.29 in batch mode.

Biomass Formation of *H. polymorpha* Wild-Type Strain (DSM 70277) in Batch and Fed-Batch Cultures

The cell yield was evaluated for several batch and fed-batch cultivations of *H. polymorpha* wild-type (DSM 70277) in shake flasks using Syn6-MES and YNB medium. Figure 6 shows the difference in biomass production of both operation modes and in both media after metabolizing different amounts of glucose. With a glucose concentration lower than 10 g/L in YNB medium, no difference of biomass yield could be found in either operation mode. Similar results were observed in Syn6-MES medium but with concentrations up to 20 g/L. It is obvious that Syn6-MES medium is superior to

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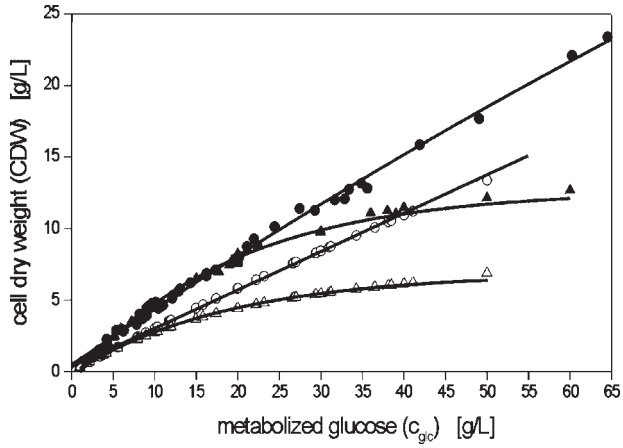

Figure 6. Cell dry weights of *H. polymorpha* in batch mode and slow-release fed-batch mode using silicone elastomer discs ($D = 22$ mm; $H = 1.8$). Glucose as sole carbon source. Syn6-MES medium: batch (▲), fed-batch (●); $pH_\alpha = 6.4$. YNB medium: batch (△), fed-batch (○); $pH_\alpha = 4.3$. Culture conditions: $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL.

the more commonly used YNB medium in terms of CDWs, due to its superior buffer capacity. At glucose concentrations higher than 10 g/L for YNB and 20 g/L for Syn6-MES medium significant differences in biomass yield were observed from batch to fed-batch cultures. The difference was due to the production and consumption of anaerobic by-products because of overflow metabolism. As the production of ethanol and acetic acid increased with the amount of directly available glucose in batch mode as shown in Figures 4 and 5, the relative biomass production decreased. Biomass production in fed-batch operation mode increased almost linear proportional to the amount of metabolized glucose. The highest cell density of *H. polymorpha* revealed in shake flasks was 23.4 g/L in Syn6-MES medium.

Green Fluorescent Protein (GFP) Formation of *H. polymorpha* pC10-FMD (P_{FMD}-GFP) in Syn6-MES Mineral Medium

The effect of operation mode on the product formation of the catabolite repressed expression system *H. polymorpha* pC10-FMD (P_{FMD}-GFP) was tested in Syn6-MES medium. The whole experiment was similar to the one with the wild-type strain depicted in Figure 5. Two peaks evolved in the OTR curve of the batch with 40 g/L glucose (Fig. 7a) as in the case of the wild-type strain (Fig. 5a). GFP was not found in presence of high glucose concentrations (Fig. 7b), which repressed the product formation. Only very low amounts of 2.5 mg/L GFP were produced after glucose exhaustion at 11 h (Fig. 7b). High acidic pH drift in batch mode (Fig. 7c) was due to the formation of overflow metabolites like acetic acid (Fig. 7d) identical to the experiment with the wild-type strain (Fig. 5c). 11.5 g/L CDW was obtained with 40 g/L metabolized glucose ($Y_{X/S} = 0.29$). During batch cultivation, 5 g/L ethanol and 0.7 g/L acetic acid were accumulated (Fig. 7d).

In comparison, the OTR of the fed-batch with five slow-release silicone elastomer discs formed only one OTR peak followed by a decreasing plateau at 17 h of 0.016 mol/L/h for 30 h to 0.005 mol/L/h (Fig. 7a). Only low amounts of 2.4 mg/L GFP were expressed in presence of glucose concentrations until 15 h of fermentation. In the following phase of glucose limitation after 15 h, strong GFP expression took place due to catabolite derepression (Fig. 7b). The GFP concentration increased linearly until 48 h. The maximum product yield in fed-batch with 87.9 mg/L was 35-fold higher than in batch. Both overflow metabolites were produced only in low concentrations in fed-batch operational mode with 0.6 g/L ethanol and 0.4 g/L acetic acid (Fig. 7d). This was similar to the wild-type strain experiment shown in Figure 5.

The amount of released glucose and its theoretical concentration in the medium were again estimated using Equation 1 with Equations 7–9. This was plotted for each sample step in Figure 7b in comparison to the glucose concentration calculated by Equation 11 and Table I for five silicone elastomer discs. Again a very good agreement was obtained. The total concentration of glucose consumption was calculated to be 35.9 g/L resulting in a biomass yield ($Y_{X/S}$) of 0.35, which was superior compared to 0.29 in batch mode.

Green Fluorescent Protein (GFP) Formation of *H. polymorpha* pC10-FMD (P_{FMD}-GFP) in YNB Mineral Medium

Batch and slow-release fed-batch cultivations in YNB medium was performed exactly in the same way as with Syn6-MES medium. A batch with 40 g/L glucose and fed-batch mode with five slow-release silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm) were performed for comparison. The course of OTR in batch mode, however, was at a lower level with a maximum OTR of 0.041 mol/L/h and with a different shape compared to fermentations in Syn6-MES medium (Fig. 8a). This shape is a general characteristic for product inhibition or suboptimal pH (Anderlei et al., 2004). Even less product was formed than in Syn6-MES experiments with GFP concentrations below 1 mg/L (Fig. 8b). The pH quickly decreased to a value of 2.6, which is below the optimal range known for *H. polymorpha* (Hansen and Hollenberg, 1996). A suboptimal pH after 12 h inhibited glucose consumption (Fig. 8d) and biomass formation (Fig. 8c) resulting in lower respiration. The biomass formation reached a maximum of 5.8 g/L which results in a biomass yield ($Y_{X/S}$) of 0.15 (Fig. 8c). Another consequence of pH-inhibition was slow by-product consumption of acetic acid and ethanol shown in Figure 8d. The same amount of acetic acid with 1.6 g/L was detected but less ethanol as in Syn6-MES cultures was formed with 1.5 g/L.

In the fed-batch experiment respiration continued at a relatively high level after 16 h and the accumulated glucose was quickly consumed (Fig. 8a). The GFP concentration in fed-batch rose after derepression at 16 h and increased nearly linearly until 421 mg/L (Fig. 8b). This product concentration

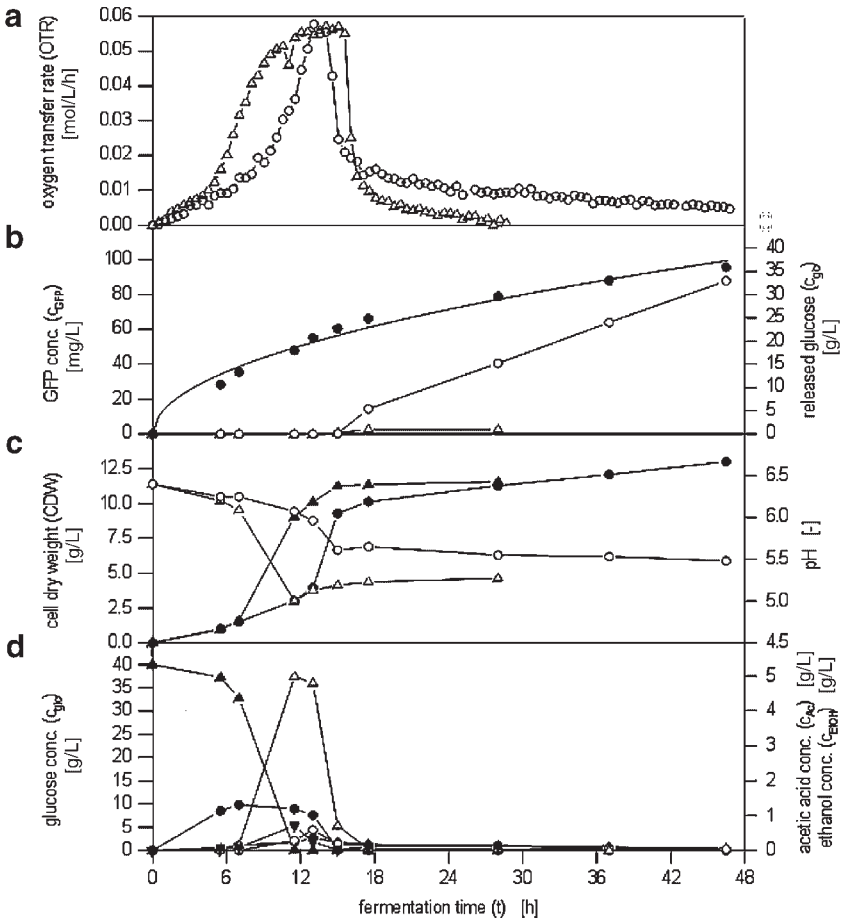

Figure 7. Comparison of batch and slow-release fed-batch mode in shake flask cultures of *H. polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) in Syn6-MES medium. **a**: Oxygen transfer rate (OTR): fed-batch (○), batch (△); **b**: green fluorescent protein formation (GFP): batch (△), fed-batch (○) and released glucose (●) calculated by Equation 1 with 7–9 in comparison to the profile of five 1.8-mm disc calculated by Equation 11 (–); (c) cell dry weight (CDW): batch (▲), fed-batch (●) and pH: batch (△), fed-batch (○); d: glucose: batch (▲), fed-batch (●); ethanol: batch (△), fed-batch (○); acetic acid: batch (▼), fed-batch (◆). Culture conditions: $pH_\alpha = 6.4$; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL. Glucose was used as sole carbon source in batch with 40 g/L and fed-batch was performed with released glucose from five silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm; 20% (w/w) glucose).

is in the order of magnitude of a conventional fed-batch fermentation in a fully equipped lab scale stirred tank fermentor (data not shown). The GFP yield in fed-batch was vastly superior to the batch with an improvement factor of minimum 420-fold. Compared to the fed-batch with Syn6-MES medium a 4.8-fold improvement was achieved. This was probably due to the fact that lower growth was achieved due to suboptimal pH. Therefore, more carbon source was directed into product synthesis, resulting in a higher expression rate. pH decreased linearly to a value of 2.1 according to the linear increase of biomass to 11.1 g/L (Fig. 8c). Low concentrations of overflow metabolites were detected with 0.4 g/L ethanol and to 0.2 g/L acetic acid (Fig. 8d).

The amount of released glucose and its theoretical concentration in the medium were estimated in the same way as in Figure 7. The revealed concentrations fit to the consumed glucose calculated by Equation 1 with Equations 7–9. The total concentration of glucose consumption was found to be 41.3 g/L resulting in a biomass yield ($Y_{X/S}$) of 0.27 which was superior compared to 0.15 in batch mode.

CONCLUSIONS

The advantage of slow-release fed-batch operation in shake flasks feeding glucose from silicone elastomer discs to *H. polymorpha* wild-type (DSM 70277) and RB11 derivate strain pC10-FMD (P_{FMD}-GFP) was demonstrated. Glucose release from the silicone elastomer discs by diffusion was absolutely repeatable (+/−10%). Minimization of overflow metabolism because of slow-release fed-batch technique led to higher biomass yields of up to 85% compared to batch cultures revealing a maximum CDW of 23.4 g/L after consumption of 64.5 g/L glucose.

GFP expression was significantly increased in comparison to the batch cultures with directly available glucose in high initial concentrations. Higher yield was obtained by adjusting low levels of glucose concentrations in both applied media. Catabolite repression was revoked which

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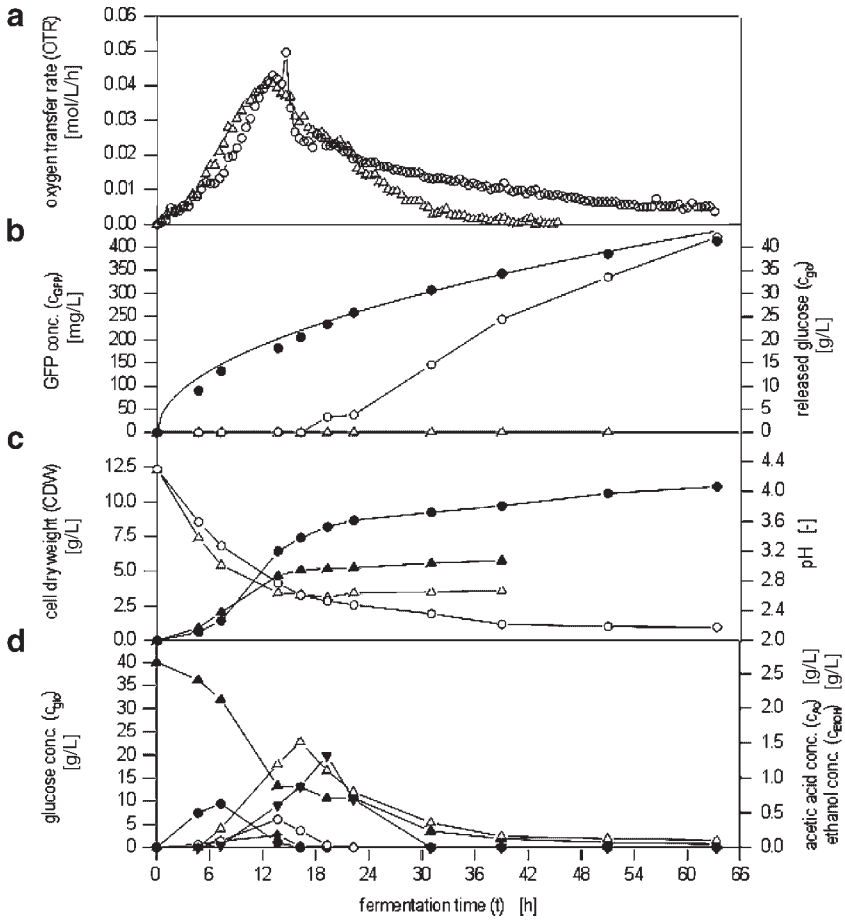

Figure 8. Comparison of batch and slow-release fed-batch mode in shake flask cultures of *H. polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) in YNB medium. **a**: Oxygen transfer rate (OTR): fed-batch (○), batch (△); (**b**) green fluorescent protein formation (GFP): batch (△), fed-batch (○) and released glucose (●) calculated by Equation 1 with Equations 7–9 in comparison to the profile of five 1.8-mm disc calculated by Equation 11 (−); (**c**) cell dry weight (CDW): batch (▲), fed-batch (●) and pH: batch (△), fed-batch (○); (**d**) glucose: batch (▲), fed-batch (●); ethanol: batch (△), fed-batch (○); acetic acid: batch (▼), fed-batch (◆). Culture conditions: $pH_x = 4.3$; $T = 37°C$; $d_0 = 50$ mm; $n = 350$ rpm; $V_L = 12.5$ mL. Glucose was used as sole carbon source in batch with 40 g/L and fed-batch was performed with five silicone elastomer discs ($D = 22$ mm; $H = 1.8$ mm; 20% (w/w) glucose).

in batch experiments is active until glucose is almost depleted. Finally, improvements in GFP expressions of 35-fold in Syn6-MES to over 420-fold in YNB medium were reached.

The application of easy to use diffusion based slow-release technique is a very promising option to improve bioprocesses. On the one hand, the application of release systems in primary screenings would most likely enable to find optimal functional strains for the expression of desired products. Well-functioning repression system of the microorganism usually leads to hardly detectable product formation in batch mode. This means that the optimum strain may not be found in commonly performed batch screening. On the other hand, the technique could accelerate process development by enabling a very high number of simultaneous, repeatable small-scale cultivations in fed-batch mode providing preliminary information for suitable feeding rates in scaled-up lab-scale fermentations. Currently microtitre plates with silicone elastomer depots immobilized at the bottom of the wells are under development. This will further enhance the applicability of fed-batch operation in screening procedures.

NOMENCLATURE

A_{disc}	surface of the disc [mm^2]
D	diffusion coefficient [mm^2/s]
C_a	amount of glucose per unit volume of matrix [mg/mm^3]
C_s	solubility of glucose per unit volume of matrix [mg/mm^3]
c_i	concentration of substance i [g/L]
CDW	cell dry weight [g/L]
CTR	carbon dioxide transfer rate [mol/L/h]
d_0	orbital shaking diameter [mm]
D	diameter of silicone elastomer disc [mm]
FMD	formate dehydrogenase
GFP	green fluorescent protein
H	height of silicone elastomer disc [mm]
HCDC	high cell density cultivation
$\dot{m}_{glc}$	mass flow of glucose [mg/h$^{0.5}$]
M_{glc}	mass of released glucose [mg]
MOX	methanol oxidase
n	shaking frequency [rpm]

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OD_{600}	optical density at $\lambda = 600$ nm [−]
OTR	oxygen transfer rate [mol/L/h]
PDMS	polydimethylsiloxane
RAMOS	Respiration Activity Monitoring System
RQ	respiratory quotient [−]
t	time [h]
T	temperature [°C]
V_L	filling volume [L]
YNB	yeast nitrogen base
$Y_{X/S}$	biomass yield coefficient for substrate [g/g]
z	number of applied silicone elastomer discs [−]

Greek symbols

α	stoichiometric coefficient for ammonia [−]
β	stoichiometric coefficient for oxygen [−]
γ	stoichiometric coefficient for cells [−]
δ	stoichiometric coefficient for carbon dioxide [−]
ε	stoichiometric coefficient for water [−]
ϕ	stoichiometric coefficient for glucose [−]
$\sum$	sum

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