Chemical Engineering Journal 160 (2010) 891–898

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Development of a single-use microbioreactor for cultivation of microorganisms

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ARTICLE INFO

Article history:
Received 11 August 2009
Received in revised form 18 January 2010
Accepted 21 February 2010

Keywords:
Microbioreactor
Single-use
Fermentation
Yeast
Continuous culture

ABSTRACT

Various microbioreactor prototypes have been presented in the literature. However, only a few have managed to become widely used in industry and even fewer (if any) have become an industry standard. We therefore present a versatile microbioreactor device for batch and continuous cultivation of suspended cells which combines the small working volumes known from microwell plates with the versatility of bench-scale reactors. Additionally, this device is designed for single-use which significantly reduces the workload before and after the actual cultivation.

The device presented here is cylindrical, consists entirely of polydimethylsiloxane (PDMS), and has outer dimensions of 14 mm diameter and 4.2 mm height. The reactor chamber is a cylinder with 8 mm diameter and 2 mm height resulting in a volume of 100 μL. Homogeneous mixing of the broth is achieved with a free-floating stirrer bar which also provides the updraft necessary to keep cells in suspension.

Temperature and pH can be tightly controlled to the desired set point; dissolved oxygen (DO) and cell density (via optical density, OD) are also measured on-line. Aeration is provided through a semi-permeable membrane which separates the gas from the liquid phase. pH is controlled by addition of CO_2 and NH_3 gas through the same membrane.

Last but not least, the reactor has been designed with simplicity in mind, such that the final design can be bought pre-sterilized and discarded with other lab-waste after use.

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1. Introduction

Efficient fermentations at industrial scale are usually preceded by a tremendous research effort aiming at optimizing the performance of the production strain and process. In such a screening procedure there is a demand for a high number of well-controlled lab-scale cultivations. In industrial-scale operations proper control of key variables can result in a higher output of the required product or the formation of fewer by-products. At laboratory scale, well-controlled cultivation conditions are essential to reach improved process understanding and to allow extrapolation of the results to larger scales. Besides the fermentation industry, research disciplines such as systems biology also depend on carefully controlled cultivation conditions in order to obtain relevant and useful experimental data.

Currently, many of these lab cultivations are done in bench-scale reactors or in shake flasks, both of which require comparatively large volumes. Additionally, both approaches have their distinct advantages and disadvantages with respect to their operation. Bench-scale reactors usually have volumes ranging from 1 L upwards. They have good control over the essential fermentation variables such as pH, dissolved oxygen (DO) and optical density (OD), but require a substantial amount of preparative work and also cleaning effort. Shake flasks on the other hand have smaller volumes (0.5 L upwards). They are easy to set up and clean, but do not offer anywhere near the same potential to control essential reactor variables compared to a bench-scale reactor. Microtiterplates can in this respect be seen as miniaturized shake flasks where the volumes are smaller (nL–mL) but the level of control is similar to shake flasks. Additionally, only bench-scale reactors offer the possibility of fed-batch or continuous (e.g. chemostat) cultivations.

A steady state condition reached with continuous cultivations at lab-scale is advantageous when obtaining physiological data or when designing an industrial operation. Steady-state allows for the quantitative treatment of bioreactions whereas the transients between two steady-states give information about the cellular metabolism. Such data again are useful when modeling non-ideal reactors, such as industrial fermentors are, as the effect of gradients of for example nutrients or oxygen on strain performance can be investigated [1]. This knowledge again can explain why some results obtained in the laboratory no longer are valid in production scale vessels.

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doi:10.1016/j.cej.2010.02.038

Continuous cultivations pose several requirements to the design of a reactor system: The volumetric inflow has to equal the volumetric outflow of the reactor—the reactor volume has to stay constant. This can be achieved rather easily for a lab-scale reactor, but for microbioreactors it means for example that no significant bulging of the membrane—as is typically used for bubble-free aeration—is allowed. Also, the concentration of cells has to be controlled to a certain level—most often this is done by setting the dilution rate D [h^{-1}] to a constant level whilst keeping the nutrient concentration in the inflow constant (chemostat operation). Moreover, bioreactions can only truly be regarded as continuous culture reactions if the culture medium composition of the outflow is the same as that of the reactor content i.e. if the assumption of an ideally mixed reactor holds. This in turn requires the mixing system to be efficient both in achieving a uniform distribution of for example nutrients and oxygen, but also in keeping the cells in suspension without the formation of dead zones. Finally, bioreactors for continuous culture should operate in parallel to rapidly provide statistically meaningful data and to enable direct comparison between different operating conditions.

Several research projects have addressed these issues [2–4], two of which are to be presented here. Akgün et al. [5] used specially prepared shake flasks with an outlet melted onto the side of the flask for the continuous cultivation of *Saccaromyces cerevisiae*. Centrifugal motion caused by an orbital shaker kept the liquid level inside the flask constant as the excess liquid flowed out through the overflow, but no means of control over the cultivation parameters was implemented. Another approach [6,7] used twenty-four 10 mL cylinders on a 24-well plate format. The individual cylinders are equipped with sensors for pH and DO. The whole system allows for the control of both pH and DO via sparging of the appropriate gases. Whilst this system allows a good degree of control over the fermentation, it does not support continuous cultivations.

Microbioreactors (commonly with volumes below 1 mL) are advantageous for running cultivations: they allow parallel cultivations under well-controlled conditions in disposable reactors [8] with a relatively low consumption of resources (nutrients, power, etc.). However, there definitely still is a need for more specifically designed and machined microbioreactors which enable high-performance cultivations [9]. Lee et al. [10] presented a 100 μL reactor fabricated out of polydimethylsiloxane (PDMS) which incorporated a novel mixing and aeration system as well as control over pH, DO and temperature. The supporting holder allows for the simultaneous operation of eight reactors. Batch cultures with *Escherichia coli* showed that the system is able to deliver reproducible results which are comparable to bench-scale reactors. Szita et al. [11] presented a microbioreactor consisting of various layers of poly(methyl methacrylate) (PMMA) and PDMS. The design mimicked larger bench-scale reactors with cylindrical shape, and also included an impeller for magnetically actuated stirring. They successfully demonstrated both the reproducibility and comparability with larger scale reactors in multiple batch cultivations of *E. coli*.

Finally, Zhang et al. [12] have described a polymer-based microbioreactor with integrated measurements of OD, DO and pH which was capable of achieving steady-state in continuous cultivations of *E. coli* at different dilution rates. They additionally also modified the surface of the reactor to reduce the amount of wall growth and included a heat barrier in the inflow channel to prevent the upstream migration of bacteria.

These examples illustrate that microbioreactor technology for microbial cultivations has reached a development stage where the operation of bench-scale reactors can be mimicked. The feasibility of pH [10,13,14] and DO [10] control has been demonstrated and sufficient mixing [15] has been proven for *E. coli*. However, no specifically designed microbioreactor as described above has yet been able to make the step from the laboratory environment to being a de-facto standard in industrial applications as the current microbioreactor systems available on the market build on microtiter platforms. Additionally, scaling-up at different k_La values and for different types of organisms still has to be demonstrated. Islam et al., for example, investigated the influence of three different scales of operation on the culture properties of *E. coli* and have suggested that k_La is a useful scale-up criterion for microbioreactors [16].

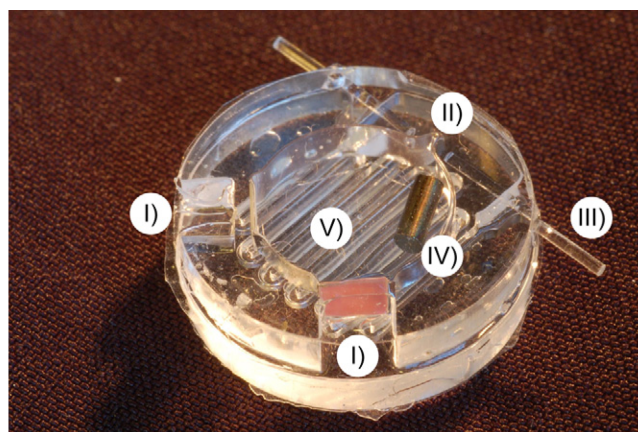

Fig. 1. Photo of the complete microbioreactor seen from the bottom: On the inside of the two windows the optical sensor spots (I, black for DO and white for pH) are attached. The magnetic stirrer bar (IV) lies freely inside the chamber. The two optical fiber stubs (III) show the location of the OD measurement (II). The two holes in the aeration layer are used to connect the aeration tubing to the meandering channel (V).

Incidentally, most of the work done to date deals with microbial cultures such as *E. coli*, only little is concerned with the cultivation of eukaryotic cells such as *S. cerevisiae*. This does not reflect the fact that many industrially relevant cultivations use *S. cerevisiae* as production organism. *S. cerevisiae* has been used in baking and alcoholic brewing for centuries, and lately also to a large extent in heterologous protein production. It is a favorable host in pharmaceutical production as it has obtained GRAS (Generally Regarded As Safe) status by the FDA. Furthermore, it is easy to modify genetically and easy to grow and handle in large-scale processes.

The work presented in this paper has focus on the mechanical aspects related to the development of a disposable microbioreactor with a relatively low complexity and cost, and which is sufficiently flexible to run both batch and continuous fermentations of microorganisms if desirable. Both design and fabrication as well as results are presented for the individual components of the microbioreactor. The final proof-of-concept is given with a batch cultivation of *S. cerevisiae*.

2. Microbioreactor design and fabrication

The general idea and layout of the microbioreactor concept is presented first to give the reader a general overview. Afterwards the individual components are presented individually in more detail.

2.1. Entire reactor

The main requirements for this microbioreactor design are the possibility to run cultivations of suspended cells in either batch or continuous mode. The reactor should be cheap, easy to use and disposable. Naturally, the cultivations have to be monitored and controlled online, so the appropriate apparatus has to be included.

The microbioreactor (Fig. 1) is 4.2 mm high and has an outer diameter of 14 mm. It comprises a reactor volume of 100 μL, pre-

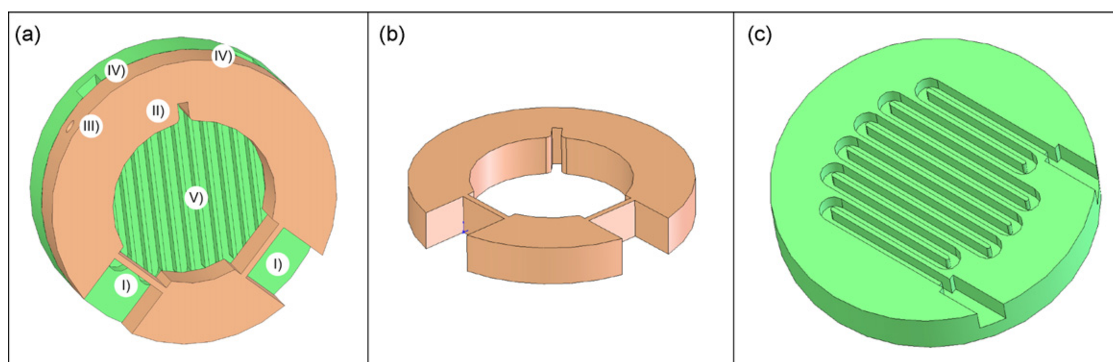

Fig. 2. Sketch of both main reactor layers as seen from below (without membrane layers): The reactor layer (orange) comes to lie on the holder, which leaves the aeration layer (green) on top. The two optical windows for the DO and pH measurements (I), the radial recess (II) for the OD measurement and the tunnels for the OD fibers (III) are located in the reactor layer, whereas the connections (IV) for the gas tubes and the meandering channel (V) are located in the aeration layer.

visions for the measurement of dissolved oxygen, pH and optical density as well as a magnetic stirrer bar for mixing. Additionally, an aeration layer is enclosed in the reactor for the bubble-less supply of oxygen. A specifically fabricated holder plate includes a temperature sensor and resistance wires for the adequate temperature control of the reactor contents.

The reactor consists entirely of PDMS (Sylgard 184, Dow Corning Corp., Midland, MI, USA. Mixing ratio of 10 parts silicone: 1 part curing agent) with the exception of the sensor spots and the magnetic stirrer bar. PDMS is commonly used in microbioreactor design for its favorable properties: It is optically transparent in the wavelengths required, is non-toxic, can easily be shaped with molding techniques and is permeable to gases such as oxygen, carbon dioxide or also water vapor.

Due to the fabrication technique used the reactor consists of 4 layers which are bonded together to form one single block of material in the final product.

The lower main layer (Fig. 2, brown part) is 2 mm high and contains the actual reactor volume. It is closed both on top and bottom by a membrane (80 μm thickness). It contains two windows of 1 mm thickness each, onto which optical sensor spots for the measurement of DO and pH are attached. Additionally, two tunnels for the insertion of optical fibers for the measurement of OD are present. These tunnels stop short of the reactor chamber forming a thin (0.5 mm) PDMS window between the broth and the fibers. This helps to ensure both sterility as there is no direct connection of the broth to the outside world and also the reproducibility of the measurements as the distance between fiber tips (and thus the path length of the light) is fixed.

On top of the upper membrane lies the aeration layer (Fig. 2, green part). It has a thickness of 2.2 mm and holds the meandering aeration channel (0.75 mm deep $\times$ 0.5 mm wide).

The whole setup was controlled via a LabView (National Instruments Corporation, TX, USA) interface using A/D cards (USB-6229 and PCI-4461, National Instruments Corporation, TX, USA) for in- and output of the signals. OD and DO were measured, whilst temperature and pH were both measured and controlled to the desired levels. All the measurement data are written into text files for further processing with other software, e.g. Matlab (The MathWorks, Inc., MA, USA).

Both the bottom and the upper main layer are fabricated by pouring liquid PDMS into a micromilled (CNC micromill, Minitech MiniMill 3/Pro, Minitech Machinery Coorporation, Norcross, GA, USA) PMMA mold and subsequent curing at 70 °C for one hour. Upon removal from the mold the two optical sensor spots were bonded onto their respective windows on the inside of the reactor using PDMS itself as the glue. In this manner no additional glue was used, hence eliminating the risk of chemical contamination of the culture. The PDMS membranes were then spin-coated onto the bottom of both the reactor layer and the aeration layers.

Finally, a magnetic stirrer bar was placed into the bottom (reactor) layer. The reactor layer and the aeration layer were then bonded together by applying liquid PDMS to the contact surface, clamping the two components together and curing for an hour at 70 °C. This resulted in a reactor which has a completely sealed culture chamber, a stirrer bar, two optical sensors and an aeration layer. Additionally, all materials chosen were suitable for γ-irradiation sterilization.

A reactor holder was fabricated to control the microbioreactor temperature. It includes resistance wires to heat the reactor and a temperature sensor. When the reactor is placed on the holder, the thin membrane on the bottom of the reactor offers virtually no thermal resistance and thus allows for the direct temperature measurement and heating of the reactor contents. With this setup no electrical connections to the single-use part have to be established which greatly decreases the risk of handling errors. An alternative offering slightly better temperature control would be to cast the wiring into the reactor wall and floor. Whilst this is entirely feasible with our reactor design and has also been tested, it comes with the cost of increased fabrication effort and the need of electrical contacting e.g. with battery contacts as proposed by Kortmann et al. [17]. In an effort to keep the single-use part of the reactor as simple as possible we have chosen not to follow this path.

2.2. Dissolved oxygen

Current lab-scale cultivation techniques have vessels which are only partly filled and have a headspace. They rely on sparging of air (bench-scale reactors) or surface-aeration (shake flasks or microtiterplates) for the aeration of the cultivation. On the contrary, most microbioreactors have well-defined volumes which are completely filled with liquid (no headspace). Also, due to the small size of microbioreactors sparging is not an option. Small air bubbles coalesce and form larger air bubbles which can displace culture liquid or block fluidic ports and disturb optical measurements.

For aeration, such microbioreactors therefore rely on diffusion of oxygen through a membrane [18] which leads to bubble-free aeration. Apart from preventing the formation of bubbles, it also greatly increases the quality of the online measurements. Moreover, the concept has been proven effective earlier [10,11,15]. Also, PDMS e.g. allows CO_2 to diffuse out of the reactor.

The aeration layer of our reactor (Fig. 2c) contains a meandering channel through which the aeration gas flows which maximizes the contact area between gas on the one side and liquid on the other side of the membrane. At the same time it supports the membrane in such a manner that it cannot bulge significantly in any direction.

This ensures a constant volume of the microbioreactor, even under overpressure, which is for example essential for chemostat operation. The aeration channels support the diffusion of CO_2 out of the reactor as all gas diffusing out of the reactor is transported away immediately, thus keeping the diffusion driving force high. Naturally, good mixing of the reactor contents is required to prevent concentration gradients in the liquid phase.

The concentration of dissolved oxygen was measured with a fluorescent optical sensor spot (SP-PSt3-NAU-D4-YOP, PreSens - Precision Sensing GmbH, Regensburg, Germany). Sine-modulated light (5 kHz) from a blue-green LED (505 nm, NSPE590S, Nichia Corporation, Tokushima, Japan) shone onto the sensor spot which was mounted on the inside of the reactor in direct contact with the culture broth. This sensor in turn emitted fluorescent light at 652 nm peak wavelength with the same sine frequency as the excitation light but with a certain phase lag. The emitted light was then collected with a silicon detector (Thorlabs PDA36A, Thorlabs Inc., NJ, USA) and the resulting voltage was read by LabView. A lock-in amplifier programmed in LabView then measured the phase shift between the outgoing and the incoming signal; this shift then translated directly to the dissolved oxygen concentration.

We have also conducted experiments where the optical DO sensor spot was mounted on the *outside* of the reactor and was only in contact with the culture broth via a thin membrane analogous to the aeration membrane. The transport characteristics through the membrane obviously reduce the reaction speed of the measurement—however, this is negligible compared to the speed with which the oxygen concentration in the culture broth changes. This has the advantage that it would allow for the production of an even cheaper reactor as one sensor spot can be reused. However it has the drawback that it also opens for new errors as the sensor spot itself bleaches out with use and ceases to function after some time. Thus, the quality of this external sensor spot would have to be checked continuously, and if necessary the sensor spot would have to be replaced before the cultivation. In order to achieve reliable operation we therefore have chosen to integrate the sensor spot into the reactor.

2.3. pH

The pH measurement relies to a large extent on the same principles as the DO measurement, with the obvious changes in sensor spot and light wavelengths. Light from a blue LED (465 nm, NSPB500S, Nichia Corporation, Tokushima, Japan) with a sine frequency of 44 kHz was shone onto an optical sensor spot (SP-pH-HP5-YOP-D4, PreSens - Precision Sensing GmbH, Regensburg, Germany) which in turn emitted fluorescent light at 520 nm and above. Further signal processing was then identical to the DO measurement.

The pH is then controlled with a simple on/off controller which adds ammonia (NH_3) or carbon dioxide (CO_2) to the aeration gas flow to increase or lower pH respectively [13]. As this method for pH control works by diffusion of gases through the membrane, no liquid is added to the reactor. Thus the total volume of the reactor is not changed which is highly desirable for reactors running batch or continuous cultivations.

2.4. Optical density

Optical density (OD) was determined by measuring the amount of light transmitted through the culture broth. This method is easy to establish, works on-line and has been well established [19]. Light at 600 nm wavelength was generated with a bright yellow LED (L600-10 V, Epitex, Kyoto, Japan). This excitation light was then led onto a 30–70 beamsplitter (NT45-317, Edmund Optics Ltd., York, UK) where 70% of the incoming light was guided to the microbioreactor for the measurement and 30% was directly fed onto a photodiode (IPL 10530, Integrated Photomatrix Ltd., UK) for reference purposes. The part of the light used for the measurement (70%) travelled in a fiber optics cable (500 μm core fiber, Edmund optics Ltd., York, UK) to the reactor, through the measurement window (0.5 mm thickness), then through the reactor with a path length of 0.5 mm and out through the second window into a second fiber optics cable and onto a photodiode (IPL 10530, Integrated Photomatrix Ltd., UK). In order to eliminate the effect of ambient light, the light intensity was sine modulated (47 Hz) and only the AC component of the measured signal was processed for OD analysis. By using the ratio of the reference and the measurement signal, possible variations in the LED signal strength can be eliminated.

2.5. Mixing

Thorough mixing of the reactor is essential both for the transport of oxygen and nutrients throughout the reactor and for keeping the cells in suspension. Only if the reactor contents can be considered homogeneously mixed can it also operate properly and generate useful results. In microbioreactors, liquid motion in the reactor becomes predominantly laminar, so mixing in this case has to rely on minimizing diffusion distances [20]. An additional problem is that some cell types sediment, for example yeast [21,22]. A homogeneous suspension of yeast cells will quickly sediment if there are any zones without uplift in the reactor.

In the microbioreactor that is presented here, mixing is achieved with a free-floating magnetic stirrer bar, similar as in shake flasks. In contrast to shake flasks it is essential that the stirrer bar does not rotate regularly in the middle of the reactor, since the reactor contents are then forced into a rotating motion which forces the heavier cells out towards the reactor walls where they then sediment into cell heaps on the reactor floor. As the stirrer is actuated slightly eccentrically it rotates both around its vertical axis, but also moves around on the floor of the reactor, ever so often to hit and then ricochet off the side wall.

Whilst testing different types of mixing in the microbioreactor this highly irregular and chaotic motion was observed to be very effective as it does not have any dead zones without mixing and can keep yeast (*S. cerevisiae*) cells in suspension over several days. Also, it does not require any specifically fabricated impellers nor any precisely machined rotation axis, thus simplifying fabrication and final cost.

2.6. Evaporation

Evaporation becomes an issue at this reactor scale as it can reach very significant rates [23,24]. Boccazzi et al. [25] measured an evaporation rate of 4.3 ± 0.4 μL/h through a 100 μm thick PDMS membrane with 10 mm diameter—a reactor with 100 μL volume would thus completely dry out within 24 h.

There are two obvious solutions to this problem: For batch reactors, it is highly effective to attach a water reservoir which is elevated above the level of the microbioreactor [15]. Any water which evaporates is then automatically replenished from the reservoir whilst keeping the concentrations (e.g. of cells) undisturbed.

Alternatively, evaporation of the reactor content can be minimized with pre-humidified aeration gas. This is particularly useful for continuous culture microbioreactors as it decouples the evaporation control from the flow control and thus permits to minimize the evaporation rate without affecting the dilution rate. In our design, the gas stream used for aeration is sparged through an external water volume before reaching the microbioreactor. This gives a high relative humidity of the gas and therefore minimizes the driving force for evaporation out of the reactor volume. This approach reduces the water losses through evaporation to tolerable

amounts; in our reactor, no change in reactor liquid volume could be observed over the course of several days for a microbioreactor operated in batch mode.

2.7. Fluidics

The necessary fluidic connections are currently made by first piercing the side walls of the reactor at the designated locations with a needle with 0.6 mm outer diameter (BD Microlance 3, Becton Dickinson, NJ, USA) in order to create a tunnel. The needle was then removed and a small copper tube (Spring spade terminal, beryllium copper, RS Components Ltd., Corby, UK, part no. 261-5238) is inserted in its place. Unlike the needle, the copper tube has the advantage of having a flat end which allows it to be mounted flush with the inner wall of the reactor and thus does not disturb the spinning bar. As the tube is tightly enclosed by the surrounding PDMS, the connection is leak-free at the pressures required for the cultivation of yeast. In order to make the connection more leak-proof, a self-sealing septum (Waters, MA, USA) can either be embedded in the PDMS or attached to the outside of the reactor.

When a needle is pulled out of the material, the material closes the hole so that no liquid can seep out. During the tests a reactor had been pierced several times before leakage started to occur. With this configuration, a continuous culture reactor can be constantly pierced by two tubes (one for the inflow of fresh culture medium and one for the outflow of the same amount of cultivation broth including cells). When the broth has to be inoculated or when direct access to the broth is needed for other reasons, a needle can be inserted into the reactor and removed again after use. This obviously makes handling very easy.

2.8. Temperature

Temperature was measured with a Pt 100 sensor (JUMO PCA 1.1505.1 M, JUMO GmbH & Co.KG, Germany) with outer dimensions of 5 mm × 1.5 mm × 1 mm, located in the reusable holder underneath the reactor. Due to the very thin reactor floor no significant time lag was to be expected for this measurement. The sensor was connected to LabView via a transmitter (JUMO dTRANS 04) which linearized and scaled the sensor signal. Also located in the holder was a meandering copper wire with 0.1 mm diameter. An electrical current flowing through the wire heated it up and with it the reactor contents. Temperature was then kept at the desired set point with an on/off controller switching the heating on and off. As microsystems typically have a very large surface to volume (*S*/*V*) ratio they tend to cool down very quickly, such that the temperature will drop below the set point within a matter of seconds. This calls for tight temperature control, but also allows for very quick temperature shifts in either direction.

3. Results

3.1. Dissolved oxygen

A good measure for the quality of the oxygen supply is the $k_L a$ which describes the transfer of oxygen (or another gas) into the reactor per unit volume. This is best measured with the dynamic gassing out method [19] where the culture liquid first is stripped of oxygen by e.g. flushing the aeration side of the membrane with nitrogen gas. Then, the aeration flow is changed to contain oxygen e.g. pure oxygen or ambient air and the rise of DO in the liquid are measured.

Out of this data the $k_L a$ is extracted as a first-order rate constant with

$$\frac{dC}{dt} = k_L a(C_s - C)$$

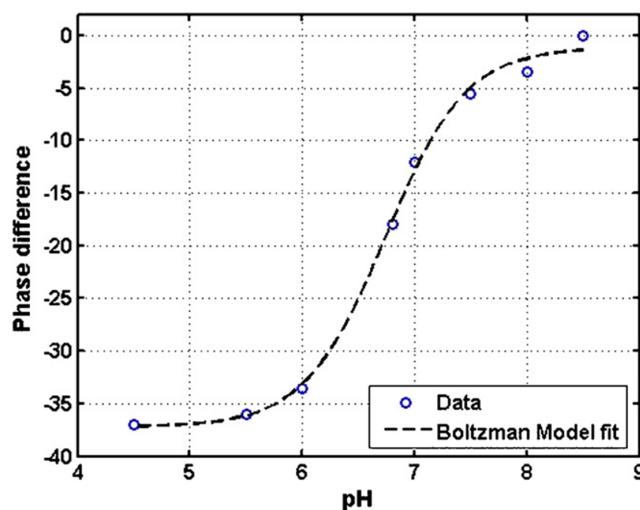

Fig. 3. Calibration of the pH sensor spot with the according Boltzman model. Characteristic are the decreasing sensitivities for high/low pH values.

where C_s is the saturation concentration (which depends on the aeration gas used) and C is the concentration of oxygen over time. With the current system we estimated (using the least-squares method in Matlab) the average (over nine different measurements) $k_L a \sim 63 \pm 7\,h^{-1}$ which is in line with other microbioreactor systems [19] and the values measured in shake flasks, whilst still being below the values for bench-scale reactors which can be as high as $200\,h^{-1}$ [16]. This result averages measurements in different reactors and at different operating conditions (mixing speed and aeration flow rate & pressure). Thus the relatively high standard deviation of 11% can be seen as a worst case variation which can be significantly reduced by standardizing mixing and aeration conditions.

When running cultivations of *S. cerevisiae* at maximum growth rates with ambient air, this level of $k_L a$ is not enough to keep the oxygen level sufficiently high. However, we have also observed that the amount of gas transferred into the reactor can significantly exceed the uptake rate of the liquid: When flushing the aeration channel at significantly higher gas flow rates so much air was seen to diffuse into the reactor that the liquid was displaced. This is in line with Zanzotto *et al.* who have shown that the transfer of oxygen into the system is primarily dependent on the oxygen uptake rate of the liquid phase and not on the diffusion through the membrane [19]. Also, this mechanism has been used actively for pumping [26,27] or bubble-removal [27] purposes. We thus expect that a careful stepwise increase of the aeration force will allow the $k_L a$ to be increased significantly before liquid displacement takes place.

3.2. pH

The ability to measure and subsequently also control culture pH is one important point distinguishing the microbioreactor from shake flask cultures. As soon as pH can be controlled cultures can be run without buffer which makes them directly comparable to e.g. bench-scale or pilot-scale systems. The chosen control scheme relies on the diffusion of CO_2 and NH_3 gases through the aeration membrane for a decrease or increase of pH, respectively.

The calibration data (Fig. 3) fit nicely to the Boltzman model and shows that pH can be measured with adequate precision in the range of pH 5.5–7.5 due to the characteristics of the sensor spots available.

Stepping-up/-down experiments (Fig. 4) show that pH can be controlled within ±0.1 of the desired set point which should be sufficient for many purposes. Also, the speed with which the pH can

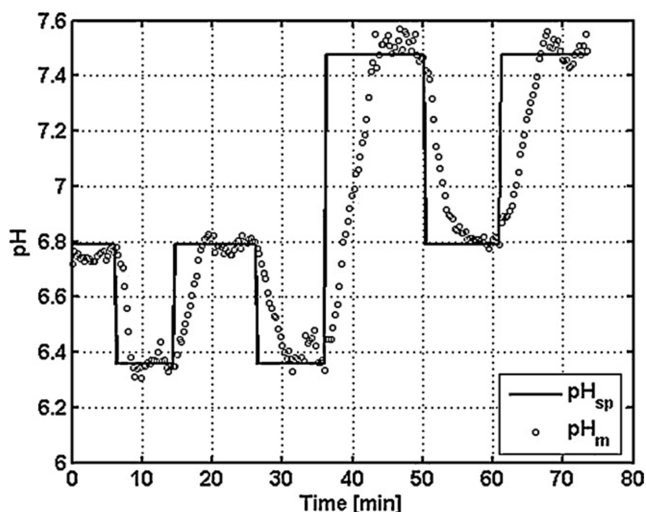

Fig. 4. Stepping up and down of the pH values demonstrating that pH can be manipulated in both directions.

be controlled is by far sufficiently quick for maintaining constant pH during a cultivation; it is also enough for dynamic stepping of the pH set point as a change by 0.5 pH can be achieved within 10 min.

3.3. Optical density

Two measurements are done to properly determine OD in the microbioreactor. Both the intensity of the reference light beam I_{ref} and that of the measurement light beam I_{meas} are measured with photodetectors and give voltage values. The effective intensity of the light passing through the broth is then

$$I = \frac{I_{ref}}{I_{meas}}$$

which decreases for increasing values of I_{meas} which corresponds to a thinner broth.

The voltage ratio then behaves proportionally to the concentration of cells in the broth (Fig. 5) within a certain density range. The linear range is determined by the intensity of the incoming light, the sensitivity of the photodetector and the light path length in the broth. With the setup described here the linear range lies between 0 and 20 g_{DW}/L.

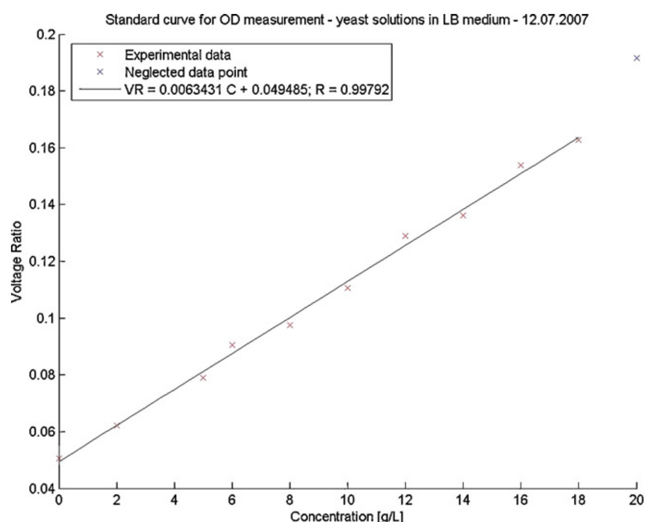

Fig. 5. Calibration curve for the OD measurement showing the linearity for concentration ranges up to 20 g_{DW}/L.

3.4. Mixing

We have currently used three indirect indications to quantify the quality of the mixing system: (a) the continuous measurement of OD, (b) mixing tests with dye and (c) visual inspection of the reactor for sedimented cells. For (a), baker's yeast was dissolved in pure water (without substrate) and the optical density of the broth measured over time. These measurements showed that the measured optical density stays constant over a period of several hours, which is long enough to prove that no sedimentation is taking place.

In addition, specific mixing tests (b) were performed with bromothymol blue solution (Bromothymol Blue solution, Sigma–Aldrich, St. Louis, MO, USA). With this method mixing can easily be observed as an addition of base induces a color change from yellow to blue; Addition of acid then reverses the color change. After the addition of base at 500 rpm, the solution again has a uniform color after approximately 1.2 s (Fig. 6). This forms a clear indication that the reactor contents are well-mixed.

Finally, the reactor was inspected for the presence of sedimented cells (c) after a cultivation with baker's yeast that lasted several days and was mixed with 600 rpm. After the cultivation the membrane on top of the reactor was cut away and the liquid was slowly removed. No sedimented cells could be observed in the reactor, proving that mixing in the reactor is sufficient.

3.5. Temperature

When controlling temperature, several aspects are important: On the one hand a certain set point has to be held with sufficient accuracy; Secondly the system has to be able to quickly change the temperature of the broth and thirdly the system has to be robust with respect to disturbances.

Temperature can be kept within ±0.2 °C [28] of the desired set point (Fig. 7). It can be increased more quickly than it can be decreased as heating is done actively whilst cooling is only due to the loss of heat to the surroundings. A temperature increase by 28 °C can be achieved within two min, whilst the corresponding cooling down to room temperature takes more than 30 min.

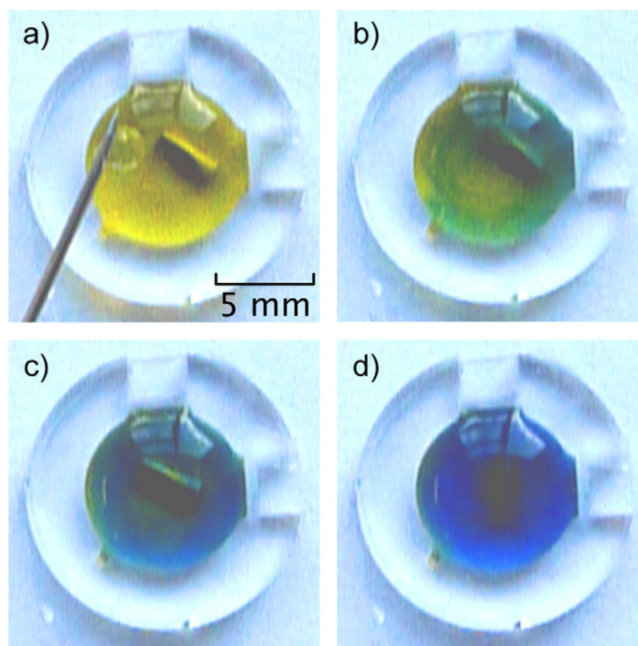

Fig. 6. Mixing in the microbioreactor at 500 rpm visualized with bromothymol blue solution. In (a) at time $t = 0$ a drop of base is added, in (b) and (c) pictures were taken 0.4 s and 0.8 s, respectively, after the addition of base, and mixing is proceeding. Finally, in (d) at $t = 1.2$ s the reactor contents are thoroughly mixed.

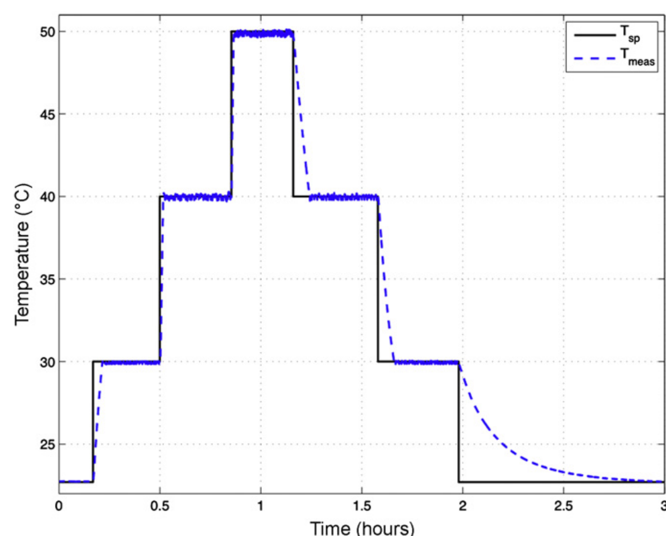

Fig. 7. Stepping temperature up and down to different set points. Observe that cooling down takes longer the closer to room temperature the set point comes.

An injection of cold water—to mimic the occurrence of a disturbance—which leads to a decrease in temperature of 0.5 °C was leveled out within 15 s. A temperature decrease of 2.5 °C was leveled out within 30 s.

3.6. Batch cultivation of S. cerevisiae

Batch cultivations of wild-type lab strain *S. cerevisiae* CEN.PK 113-7D [29] were conducted in the microbioreactor. For this purpose standard YPD medium (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract [30]) was prepared and adjusted to a pH of 5.5 with 2 M hydrochloric acid. After sterilization the medium was supplemented with 50 μg/L sterile-filtered streptomycin (Sigma, USA) to hinder bacterial contaminants to grow.

For inoculum preparation, a single colony of *S. cerevisiae* was picked from an agar plate and added to a 500 mL shake flask containing 100 mL of the prepared cultivation medium and incubated in a shaker at 30 °C and 700 rpm until the cells reached an OD_{600} = 10, typically after approximately 12 hours. A volume of preculture was withdrawn and diluted with fresh YPD medium to OD_{600} = 0.1 and used as inoculation broth for the microbioreactor. After the microbioreactor was completely filled with the inoculation broth it was directly placed onto the holder and connected to an external elevated water reservoir in order to compensate for possible evaporation during the fermentation. Temperature was controlled to 30 °C and the content was stirred at 600 rpm; Aeration was done with ambient air. As aeration takes place through the membrane, no particular precautions concerning the sterility of the gas have to be taken.

To further evaluate the performance of the microbioreactor, cultivations with the yeast *S. cerevisiae* were carried out. The resulting profiles of growth and DO over time were similar to those seen for bench-scale reactors. A representative time course is shown in Fig. 8. After a short lag phase the cells started to grow exponentially until approximately 13 h, when they enter stationary phase, most likely due to glucose depletion. A drastic decrease was seen for the DO level already around t = 10 h, were OD_{600} was approx. 10, which deviates from bench-scale cultivations where oxygen first is depleted at higher cell densities. Oxygen supply may be improved by exchanging the air with oxygen–air mixtures as discussed above in the oxygen transfer section. Growth rates within the expected range were obtained. Calculations based on three independent batch cultivations gave a maximum specific growth rate of 0.34 h^{-1} for growth on glucose with a standard deviation of 0.02, indicating

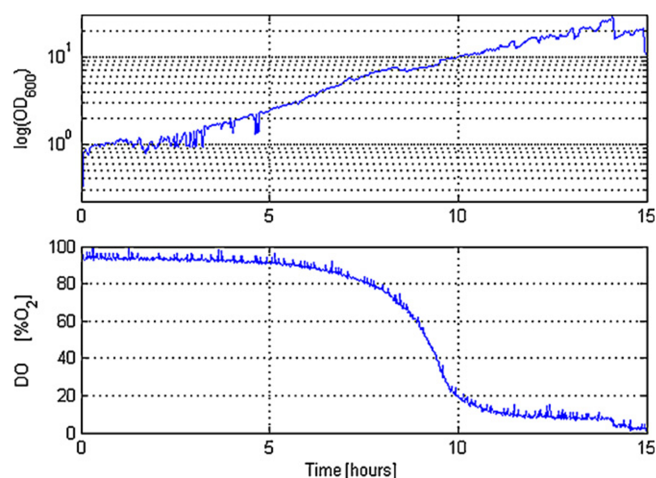

Fig. 8. A typical batch cultivation of yeast (*S. cerevisiae*) in the microbioreactor showing the functioning of the OD and DO measurements.

high reproducibility between batches. The measurement systems demonstrated reasonable readings; temperature control kept the broth within ±0.2 °C of the set point and the mixing system apparently kept the cells in suspension throughout the whole cultivation as no sedimented cells could be observed at the end of the cultivation. This shows that the basic traits of the reactor function as they are supposed to.

Whilst wall-growth of the organism may be an issue for certain organisms, no significant growth could be observed whilst running cultivations with yeast (*S. cerevisiae*) that lasted for several days; thus no specific precautions have been taken. However, techniques to modify the surface properties of PDMS exist [31], so it should be possible to also adapt this reactor design to more adherent organisms.

4. Discussion and outlook

As our aim was to develop a reactor with relatively low complexity which is suited for both batch and continuous operation of a fermentation with suspended microorganisms, the reactor has been designed such that complicated fabrication steps as e.g. clean-room work are omitted. Furthermore, material properties were exploited to enable fabrication of the whole reactor out of one material, and fluidic, optical and electrical connections were designed to be easy to handle providing consistent results. The reactor itself consists entirely of PDMS and only contains two optical sensor spots and one stirrer bar. In this form the reactor is gamma-sterilizable and can be delivered prepackaged similar to other single-use laboratory equipment such as syringes. The requirements for continuous culture and batch operation are being met with an appropriate mixing system which can also keep cells in suspension, an aeration membrane which cannot bulge and thus ensures a defined culture volume and the surrounding apparatus which enables the measurement and partly also the control of the most essential culture variables in addition to enabling different well controlled flows in and out of the reactor. As shown in the manuscript, the various components of the reactor work fine and allow the broth to be held within tight specification limits. From the experiments carried out thus far, it can be concluded that the reactor shows a performance which resembles that of previously presented similar, but considerably more complex reactors.

The proposed single-use microbioreactor should be a step towards increased miniaturization of well-controlled fermentation experiments, both in a research/development (screening) environment and in a production environment. The proposed design fits

fine also with recent requirements from industry, where there is for example an increasing interest for the production of pharmaceuticals in single-use equipment, mostly to reduce cleaning efforts and to reduce contamination from one production batch or one product to the next one. The proposed microbioreactors could fit well in such a production facility, for example to grow an inoculum under well-controlled conditions or to perform tests with microorganisms sampled from the full-scale fermentor. In addition, the proposed microbioreactors are ideally suited for process development studies, where the small size and the ability to control essential reactor variables form the key to cheap experimentation generating relevant experimental results that can be extrapolated to larger scales. In this context it is also important to emphasize that the volume of the proposed reactor design can be adjusted easily (e.g. increase to 5 mL), whilst technical solutions developed for mixing, aeration, sensing and control should not be modified.

There is of course still room for improvement of the proposed microbioreactor design. In the current version of the reactor, the fluidic tubes are manually inserted into the reactor. Obviously, this procedure is not error-free both during insertion and reactor operation. A future version of the holder would therefore also include a guiding and clamping tunnel for the fluidic connections which would define position, direction and insertion length of the tubes. This would then further facilitate the installation of the reactor.

Currently cultivations are run with a fixed concentration of oxygen in the aeration gas. In a next step closed-loop DO-concentration control will be implemented using similar control algorithms and devices as for pH control. This will for example also allow to investigate how a cultivation reacts to stress due to lack of oxygen, by controlling the DO-concentration at a low set point.

Also, the pH range which can be controlled now will not suit all kinds of organisms. Many will require a more acidic pH (e.g. *S. cerevisiae*) which requires a different kind of sensor spot. PreSens is working on a sensor spot which has exactly the same operating principles and conditions, but measures lower pH values. The availability of such a sensor spot measuring low pH values is a necessity for many bioprocesses.

Also, the comparison between this reactor design and scale with larger scale cultivations, especially bench-scale or even industrial cultivation remains to be done. This is a very important part of the further investigations as it is essential to know how to translate microbioreactor results into “real world” results. This comparison between reactor scales will be done by means of a wash-out curve where the maximal growth rate in the microbioreactor and a bench-scale reactor are compared. Similar maximum specific growth rates $\mu_{\max}$ in both systems would indicate that the overall growth conditions inside the microbioreactor compare well with larger scales. This assumption can finally be tested also in a ranking test where the productivity of different strains are compared to each other (“ranked”). If the microbioreactors deliver the same ranking as the bench-scale reactors then the validity of microbioreactor results for scale-up would be proven.

Acknowledgement

The PhD project of Daniel Schäpper is financed by the Novozymes Bioprocess Academy.

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