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Aerobic Batch Cultivation in Micro Bioreactor with Integrated Electrochemical Sensor Array

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Aerobic batch cultivations of Candida utilis *were carried out in two micro bioreactors with a working volume of 100 μL operated in parallel. The dimensions of the micro bioreactors were similar as the wells in a 96-well microtiter plate, to preserve compatibility with the current high-throughput cultivation systems. Each micro bioreactor was equipped with an electrochemical sensor array for the online measurement of temperature, pH, dissolved oxygen, and viable biomass concentration. Furthermore, the CO_2 production rate was obtained from the online measurement of cumulative CO_2 production during the cultivation. The online data obtained by the sensor array and the CO_2 production measurements appeared to be very reproducible for all batch cultivations performed and were highly comparable to measurement results obtained during a similar aerobic batch cultivation carried out in a conventional 4L bench-scale bioreactor. Although the sensor chip certainly needs further improvement on some points, this work clearly shows the applicability of electrochemical sensor arrays for the monitoring of parallel micro-scale fermentations, e.g. using the 96-well microtiterplate format.*
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Introduction

There is growing interest and increasingly widespread deployment of small scale bioreactor systems, both to speed up industrial bioprocess development as well as to accelerate our scientific insights in the functioning of micro-organisms at a molecular and genetic level. The current challenge in the further development of high throughput microreactor systems is the increase of the measurement and control possibilities.[1–5]

During this moment, there is a strong inverse relationship between the level of experimental throughput and the amount of information that can be obtained.[6] Cultivations performed in bench scale bioreactors yield reliable and information rich data, especially because these bioreactors are equipped with sophisticated measurement and control devices. However, bench scale bioreactors are expensive and time consuming to operate and this limits the number of experiments that can be carried out within a certain time frame.

The primary advantage of microtiter plates as a cell cultivation system is that they are designed for high-throughput and automated experimentation. Microtiter plates for cell cultivation typically contain 24 or 96 parallel wells with working volumes of 0.1 to 3 mL.[7,8] The major drawback of these systems is the limited possibility for measurement and control, compared to bench scale bioreactors. In microtiter plate cultivations, most often only the final product or the biomass concentration is measured and generally only the temperature is controlled during the cultivation.

The lack of sensing and control possibilities in microtiter plates is partially addressed by the integration of sensors in the wells. The 24- and 96-well plates with integrated optical sensors (optodes) for the measurement of pH or dissolved oxygen

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concentration, which are currently available, will increase the versatility of this tool for physiological studies.[9–12] However, optodes suffer from a small measurement range. Several industrially relevant species, e.g., many yeasts, are generally cultivated at a pH around 5, which is outside the working range of the optodes.

In the emerging field of miniature bioreactors that was reviewed by Kumar et al. and Lye et al.[3,13] the aim is to combine the small working volume and high-throughput possibilities of microtiter plates with the monitoring and control features of lab-scale bioreactors Arrays of such small scale bioreactors will enable us to gain better quality information both faster and cheaper.[6]

Several groups have reported successful batch fermentations on a millilitre scale with measurement of pH, dissolved oxygen, and biomass concentration[14–18] and even on a 5–250 microlitre scale.[19,20] In most of these designs, commercially available optical sensors with a limited measurement range are used for the online measurement of the pH and the dissolved oxygen concentration, thus limiting their applicability.

Clearly the monitoring performance in the current miniaturized bioreactors needs to be improved significantly before they can compete with bench-scale systems. As an example, the biomass concentration in miniaturized bioreactors is commonly determined by online turbidity measurements. This technique fails, however, at high biomass concentrations. Furthermore, off-gas measurement is state of the art in bench-scale systems and can provide essential, quantitative data on the performance of the cultivation, but was, until recently,[21] not available for sub-millilitre scale bioreactors.

It has been shown that electrochemical sensors are a viable alternative for optical sensing techniques. For the measurement of the pH, the Ion Sensitive Field Effect Transistor (ISFET) is superior to optodes, especially at pH values below 6. Biomass concentration can be measured using impedance spectroscopy. With this technique, only the viable cells are detected, while it has a much broader range than optical density measurement. Recently, an integrated sensor array has been developed, based on the 96-well microtiter plate format, containing sensors for pH, dissolved oxygen, temperature, and viable biomass concentration.[22,23]

Compatibility of a miniaturized bioreactor system to the current high-throughput cell cultivation platforms, i.e. the 24 and 96 (deep)well plates, is highly desirable because of the already available automation equipment. Taking this into account, we developed a parallelized micro bioreactor system with a working volume of 100 μL based on the 96-well microtiter plate format. In each of the micro bioreactors, a recently developed electrochemical sensor array[22,23] was integrated for the online measurement of temperature, pH, dissolved oxygen concentration, and viable biomass concentration. The cumulative CO_2 production during the cultivations was measured with a conductometric sensor.[24]

Replicate aerobic batch cultivations of the yeast *Candida utilis* were carried out in the 100 μL micro bioreactors. The results of the online measurements carried out during these cultivations were compared with results from a similar cultivation carried out in a 4L bench-scale bioreactor, obtained using conventional sensors and off-line measurements.

Materials and Methods

Micro bioreactor

Two wells were cut out of a 96-well plate and the bottoms were removed. Each of the two wells was mounted with

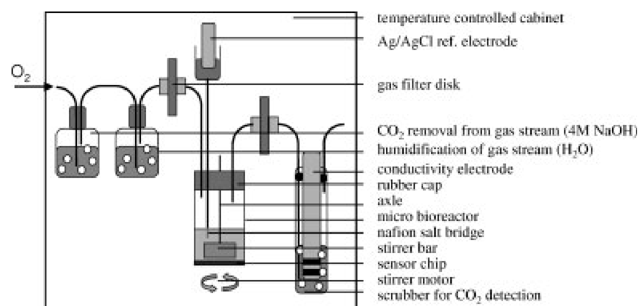

Figure 1. Schematic representation of the micro bioreactor setup, only one of the two parallel bioreactors is depicted.

Hysol® on a printed circuit board (PCB) that contained a sensor array. The wells were closed with rubber stoppers. The wells were equipped with small stainless steel stirrer bars (length: 4.75 mm, width: 1.91 mm, height: 1.54 mm, occupied volume 14 μL) (kindly provided by V&P Scientific Inc, San Diego, USA). A 0.6 mm hole was drilled through the stirrer bar for the stainless steel axle. The axle was fixed in the rubber stopper and fixed the stirrer bar in the middle of the micro bioreactor at 1 mm above the bottom. A magnetic stirring plate (KMO 2, IKA-Werk Janke & Kunkel KG, Germany) was used to drive the stirrer at a rotation speed of 200 rpm. The complete microreactor setup was placed in a temperature-controlled (temperature controller 6100+ West Instruments, Brighton, England) cabinet that was controlled at 30 $\pm$ 0.1°C via three 100 W light bulbs. A schematic representation of the micro bioreactor setup is presented in Figure 1.

Sensor array fabrication

The chips with integrated sensors for the measurement of pH, temperature, dissolved oxygen, and viable biomass concentration were fabricated on an oxidized silicon substrate. First, the Ta_2O_5 ISFETs were manufactured. Next, a lift-off process was used for the creation of the platinum structures for the other sensors. The impedance sensor used for the biomass measurements consisted of 200 $\times$ 200 μm electrodes with a spacing of 1200 μm. The ultra micro electrode array (UMEA) for measurement of the dissolved oxygen concentration consisted of a platinum macro electrode, covered by a photo structured layer of polyimide as to create arrays of recessed ultra micro electrodes with a radius of 2 μm. The temperature resistor was designed to have a nominal resistance of 500 Ohm. The sensor arrays were bond wired to a printed circuit board. Hysol® was used for packaging and for protecting the bond wires. Further details about the theoretical aspects, the design, the fabrication, packaging, and experimental characterization of the sensor arrays have been described previously.[22,23]

Readout of the sensor array

The readout of the individual sensors on the chip was accomplished through the use of a relay switch board controlled by a DAQ device (USB DAQ 6009, National Instruments, Austin, USA). The electromechanical relays sequentially coupled and uncoupled the sensors to the appropriate analyzer in order to prevent crosstalk. The outputs of the DAQ device were buffered using bipolar junction transistors to deliver enough switching power. A DC voltage

supply (E015-2, Delta Elektronika, Zierikzee, The Netherlands) delivered the switching power.

An impedance analyzer (HP4192A, Hewlett Packard, USA) was used to measure the response of the impedance sensor in the range of 10 kHz–10 MHz. The dissolved oxygen sensor was connected to a potentiostat (Parstat 263A, Princeton Applied Research, Oak Ridge, USA). The proper polarization voltage was determined from cyclic voltammetry and chosen high enough to ensure oxygen reduction, but not too high as to prevent interference from other redox reactions taking place. The ISFET was connected to an ISFET amplifier (home built). Both the output of the ISFET amplifier and the resistance of the temperature resistor were measured using a digital multimeter (34401A, Agilent, Santa Clara, USA).

A labVIEW™ (National Instruments, Austin, USA) software routine was used to control the relays, to communicate with the impedance analyzer and the digital mulitmeter and to log the signals. The LabVIEW™ routine was coupled to a Powersuite® (Princeton Applied Research, Oak Ridge, USA) software routine to control and log the dissolved oxygen measurements.

Salt bridge

The two salt bridges consisted of a stainless steel tube (inner diameter: 0.8 mm) that connected one reservoir in which the reference electrode (Ag/AgCl reference electrode, Radiometer Analytical, Copenhagen, Denmark) was placed, with the two micro bioreactors. At the side of the micro bioreactors 2 mm Nafion hollow fibre membranes (Perma Pure, Toms River, USA) (outer diameter: 0.03 inch) were slit over the metal tube and the fibre ends were closed with two component glue. The salt bridge was filled with a solution that had the same ion composition as the medium in the bioreactor; glucose was not added to the electrolyte solution in order to prevent growth in the salt bridge.

The voltage drop over the salt bridge, including the Nafion junction, was measured using a multimeter (digital multimeter 34401A, Agilent Technologies, Santa Clare, USA) and two reference electrodes at each side of the salt bridge (Ag/AgCl reference electrode, Radiometer Analytical, Copenhagen, Denmark). Stability and sensitivity in the range of pH 3- pH 7 was determined from ISFET calibrations performed with and without intervention of the salt bridge. The calibration was performed against a conventional glass pH electrode (PHM83, Radiometer Analytical, Copenhagen, Denmark), with medium. The pH of the solution was changed by the addition of either a 1 M NaOH or 1 M HCl solution to the medium.

Measurement of carbon dioxide production

The system for the measurement of the carbon dioxide production in the micro bioreactors has been previously described.[24] In short, for each micro bioreactor a mass flow controller (Brooks Instruments B.V. Veenendaal, The Netherlands; with a flow range of 0–3 mL min^{-1}) was used to blow a stream of pure O_2 gas (Linde gas, Schiedam, The Netherlands) over the headspaces at a flow rate of 600 μL $min^{-1.}$ The gas entered the headspace of the micro bioreactor via two syringe needles that were pierced through the rubber stoppers that closed the reactor. Before the gas entered the reactor, all traces of CO_2 were removed via absorption in a 4 M KOH solution. Subsequently the gas was humidified in a separate water bottle to minimize water evaporation from the micro reactor. Before entering the micro reactor the gas was sterilized via a disk filter with a pore size of 0.2 μm (Whatman, 's-Hertogenbosch, The Netherlands). The exhaust gas from the micro bioreactor was led through a stirred scrubber, containing 3 mL of a 80 mM NaOH solution. Herein, the CO_2 was stripped from the exhaust gas resulting in a conductivity change of the NaOH solution. This change was measured by a conductivity electrode (Consort SK20b; range 0.1 μS cm^{-1}–100 mS cm^{-1}), which was proven to be a quantitative measure for the amount of CO_2 that was produced in the micro bioreactor.[24] A custom made LabVIEW™ routine was written to log the conductivity signal via an RS232 interface. Gas impermeable Tygon tubing (Masterflex, Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used for all gas connections to prevent that CO_2 entered or left the system before the scrubber.

Micro-organism, cultivation medium, and preculture conditions

Candida utilis. CBS621 was obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. The yeast cells were transferred from a glycerol stock into a 100 mL shake flask filled with 20 mL mineral medium[25] and cultivated in an incubator (Certomat® BS-1 Sartorius BBI Systems GmbH, Melsungen, Germany) at 30 °C and 200 rpm. The medium was supplemented with 15 g L^{-1} glucose·1 H_2O and the ammonium was replaced by urea (2.3 g $L^{-1)}$ to reduce acidification of the medium during growth. The initial pH of the medium was 6.0. This medium was also used for the microreactor and bench-scale cultivations. Shake flask cultures at the end of their exponential growth phase served as precultures for the micro bioreactor cultivations.

Batch cultivation in the micro bioreactor

The two micro bioreactors were sterilized with 70% ethanol and thoroughly washed with sterile demineralized water. Batch cultivation was started by mixing 10 μL of the preculture with 90 μL of fresh medium and subsequently transferring this mixture into the micro bioreactors. Before these experiments, the sensitivity of the ISFET sensors for pH measurement was determined from two point calibrations using pH 4 and pH 7 buffer solutions (Radiometer Analytical, Copenhagen, Denmark). The sensitivity of the dissolved oxygen sensor was determined from a two point calibration in medium that was sparged with either pure oxygen or pure nitrogen gas. The sensitivity of the temperature sensor was also obtained from two point calibrations. The raw data from the biomass sensor was used without calibration.

Yeast cultivation in 4L bench-scale bioreactor

An aerobic batch cultivation carried out in a 4 L bench scale bioreactor served as reference system. First a preculture (0.4 L) was grown overnight in a 1 L shake flask at 30°C on mineral medium. The medium composition was the same as for the micro bioreactor experiment. The preculture served as inoculum for the batch cultivation in a 7 L bioreactor (Applikon, Schiedam, The Netherlands) with a working volume of 4 L. The bioreactor was equipped with sensors for

pH (Inpro 3030/120, Mettler-Toledo GmbH, Switzerland), temperature (PT-100), and dissolved oxygen tension (DOT, Mettler-Toledo GmbH Switzerland). All sensors were connected to a process control system (DCU3, Sartorius AG, Melsingen, Germany). The temperature of the bioreactor was maintained at 30°C by means of a thermo circulator (ADI 1018 Applikon, Schiedam, the Netherlands), which was controlled by the DCU3. The pH and the dissolved oxygen tension (DO) were measured but not controlled. Two Rushton turbine stirrers were used to mix the broth with a speed of 450 rpm. Air was sparged through the reactor with a flow rate of 3 L min^{-1} by means of a mass flow controller (Brooks Instruments B.V. Veenendaal, The Netherlands). The bioreactor was operated at an overpressure of 0.3 bar. The off-gas from the bioreactor was cooled in a condenser that was connected to a cryostat at 2 °C to minimize water evaporation. The CO_2 and O_2 concentrations in the off-gas were analyzed using a combined paramagnetic/infrared gas analyzer (NGA 2000 MLT 1, Fischer-Rosemount GmbH & Co Hasselroth, Germany). Commercial data acquisition software (MFCS, Sartorius AG, Melsingen, Germany) was used for online data logging. The biomass dry weight during the bioreactor cultivation was measured as described previously.[26]

Results and Discussion

Salt bridge characterization

For proper functioning of the pH and the dissolved oxygen sensors, an electrode of known potential which approaches ideal non-polarizability, generally referred to as a reference electrode[27] is needed. Although several microfabricated reference electrodes have been developed,[28,29] aqueous electrodes, like the aqueous silver/silver chloride reference electrode, remain the most suitable reference electrodes when long-term stability is required.

Because stable aqueous reference electrodes are too bulky for integration in the micro bioreactor a salt bridge was constructed for the electrical connection between the sensor and the reference electrode, which were specially separated. The junction, which closes the salt bridge, should be designed in such a way that the leakage across the junction is prevented as much as possible, and that the electrical resistance of the junction is minimized. To meet these constraints, an ion-selective Nafion hollow fibre membrane was chosen as a junction. No fluid leakage is to be expected and the electrical resistance of the junction is low because of its cation permeability.

Because the ion concentrations in the salt bridge and the micro bioreactor medium are assumed to be practically equal during the entire cultivation, the voltage drop over the salt bridge is expected to be very low and stable. The instability of the voltage drop over the salt bridge, including the Nafion junction, was measured to be less than 0.1 mV hr^{-1} within a 12 h period. Considering the fact that the ideal Nernstian sensitivity for an ISFET is −58.2 mV/pH unit at 20°C,[22] the stability of the voltage drop over the salt bridge was found to be acceptable for the intended application.

The stability of the salt bridge was furthermore tested by performing an extensive calibration in the pH range relevant to yeast fermentations, i.e., pH 3–pH 7. In the calibration procedure, the pH of the solution, starting at a pH of 6.3, was lowered first by adding 1 M HCl. Subsequently, when a pH of 2.5 was reached, the pH of the solution was increased by addition of 1 M NaOH until the pH reached 7.0. Instability of the salt bridge would result in nonlinear hysteresis behavior of the ISFET signal to pH changes. The results of the calibration procedure are depicted in Figure 2.

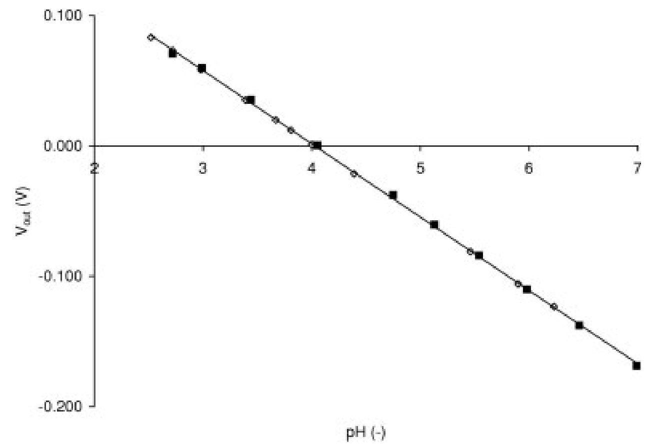

Figure 2. ISFET calibration.

First the pH of the solution was decreased by the addition of HCl (1 M) until a pH of 2.5 was reached (◇) and then increased by the addition of NaOH (1 M) until the solution had a pH of 7.0 (■).

As can be observed from these results, the output signal of the ISFET amplifier shows a linear relation with solution pH and negligible hysteresis over the whole measurement range. This indicates that the voltage drop across the salt bridge did not influence the pH measurement. Furthermore, it should be noted that the ionic strength of the solution was raised to ∼3 mM at the end of the calibration, because NaCl was formed by the subsequent addition of NaCl and NaOH. The measurements thus show that changes in ionic strength of the solution did not effect the pH measurement. The ISFET sensitivity equalled 56 mV pH^{-1} unit.

Online measurements during batch cultivations of C. utilis in the micro bioreactor

The array of two micro bioreactors was constructed and used for batch cultivation of *C. utilis* while monitoring the pH, dissolved oxygen concentration, temperature, biomass concentration, and carbon dioxide production of the two cultivations simultaneously. For highly parallelized systems, it is attractive from an economical viewpoint to use only one reference electrode for multiple micro-bioreactors. This idea was explored by constructing two separate salt bridges to each of the two micro bioreactors that originate from one vessel in which the reference electrode was placed.

A full measurement cycle, that included the readout of all the sensors on both sensor arrays, took almost 2 minutes. Because the duration of the measurement cycle was mainly determined by the time needed for data communication between the PC and the measurement equipment and not by the measurements themselves, this time interval could, in the future, be reduced significantly by adjusting the configuration.

In total 2, parallel batch cultivations (Batch 1A, 1B, 2A and 2B) were performed; however, because of software problems the dissolved oxygen concentration was only measured in Batch 1A and Batch 1B. Because of a malfunction in

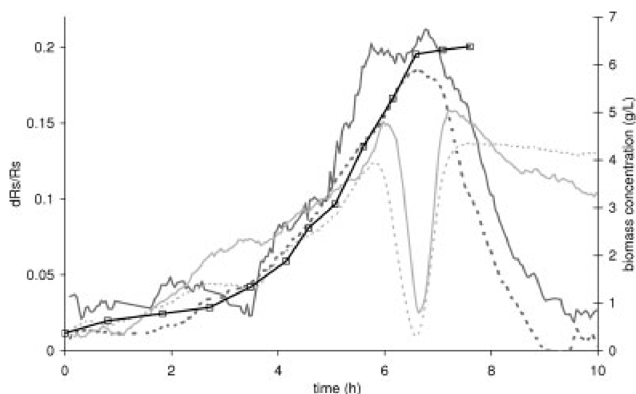

Figure 3. Readout of the biomass sensor during batch cultivation of *C. utilis* in the 100 μL bioreactors (1A = solid dark gray, 1B = dashed dark gray, 2A = solid light gray, 2B = dashed light gray) and off-line biomass dry weight measurement of the batch cultivation carried out in the 4 L bench-scale reactor (open squares connected with solid black line, right axis).

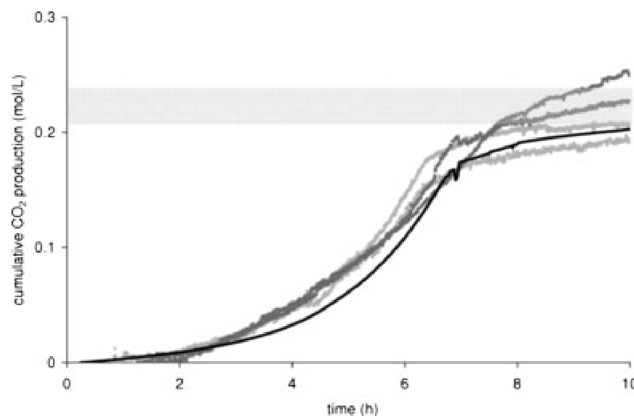

Figure 4. Online measurement of the cumulative CO_2 production, expressed per litre of culture volume, during batch cultivation of *C. utilis* in the 100 μL bioreactors (gray lines) and 4 L bench-scale reactor (black line).

The horizontal gray area indicates the expected cumulative CO_2 production at the end of the batch cultivation, based on the growth stoichiometry (see text).

the salt bridge, i.e. an air bubble obstructing the path in case of Batch 2A, no pH data are available for this cultivation. Apart from these issues all batch cultivations were carried out satisfactory.

Below the measurement data obtained from the batch cultivations carried out in the 100 μL bioreactors are compared with data obtained from a similar batch cultivation carried out in a bench scale bioreactor with a working volume of 4L and equipped with conventional sensors and off-gas analysis equipment. The bench scale fermentation was performed in duplicate and an additional two times with a different medium (doubled concentrations), of which the results have been published previously[23] and appeared very reproducible.

Biomass Concentration. The biomass concentration in the micro bioreactors was measured by impedance spectroscopy. For this measurement an alternating current (AC) electrical field was applied to the cell culture and the impedance was measured around the characteristic frequency. It has been shown previously that for this sensor, the total increase in impedance around the characteristic frequency was linearly dependent on the viable biomass concentration.[22]

In this experimental setup, the biomass concentration was determined from the difference in solution resistance, obtained from impedance measurements around 0.3 MHz and 2 MHz (dRs). This value is normalized by the resistance at 2 MHz (Rs), which is assumed to approach the resistance of the medium. The solution resistance was determined under the assumption that the conductivity between the platinum electrodes constituting the sensor could be modelled with a simple C(RC) circuit. To reduce random noise on the impedance curves, the resistance at each of the two frequencies was obtained by averaging 10 spectral points around that frequency.

From Figure 3, it can be inferred that the biomass concentration, expressed as dRs/Rs, as obtained during the four batch cultivations of *C. utilis* increased with time. The signals obtained appeared to be well comparable with the signal from the 4 L fermentation, although there was a lot of random noise on the signal from the micro bioreactor cultivations. Therefore, a moving average over 10 points was applied on the data. At the end of the batch, around 6–7.5 h after inoculation, the biomass signal collapsed and only in Batch 2A and Batch 2B, the signal recovered somewhat. The reason of this sudden collapse of the signal is not well understood. This phenomenon was not observed in performance tests of this sensor in a bench scale bioreactor.[23]

A possible explanation for the sudden collapse of the biomass signal at the end of the batch phase could be the decrease of viable cells due to mechanical shear, as was reported earlier for stirred micro bioreactors,[24] but this explanation does not comply with the recovery of the signal as was observed in Batch 2A and Batch 2B. A second explanation could be a shift in characteristic frequency caused by changes in cell size and shape at the end of the batch cultivation; however, this could not unambiguously be confirmed by more detailed inspection of the measured impedance spectra. Detailed analysis of the spectra showed that the resistance at 2 MHz (Rs) was constant, whereas the dRs increased during the batch cultivation as was expected. However, at the end of the batch culture both signals suddenly decreased. Fouling of the sensor, especially due to the high amount of cell debris and free-floating protein at the end of the batch is the most likely option for the observed phenomenon. Further investigation remains necessary to gain understanding of the observed behavior.

Carbon Dioxide Production Measurement. The CO_2 production was measured during the micro bioreactor cultivation by stripping the CO_2 from the off-gas in a scrubber and simultaneously measuring the resulting conductometric change in the alkali solution of the scrubber.

Figure 4 shows that the profile of the cumulative CO_2 production in the micro scale batch experiments was very reproducible and compared well with the 4L bench-scale cultivation. However, the growth rates calculated from the exponential increase in the CO_2 production curves were lower for the micro bioreactors then for the 4 L cultivation, i.e., $0.50 \pm 0.05\ h^{-1}$ and 0.6, respectively. Previously, it was hypothesized that this was caused by partial cell disruption caused by mechanical shear induced by the stirrer of the microbioreactor.[24]

The total amount of CO_2 that was produced at the end of the batch cultivation was very reproducible and compared well with the value obtained for the 4-L cultivation.

The total amount of CO_2 produced during the batch cultivations can be predicted by carbon-elemental balancing.[30]

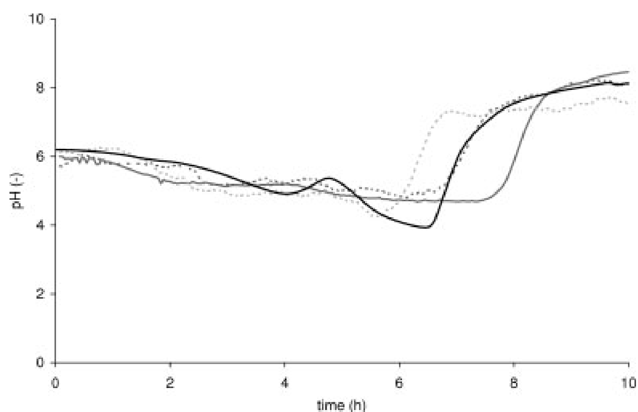

Figure 5. Measured pH during batch cultivation of *C. utilis* in the 100 μL bioreactors (1A = solid dark gray, 1B = dashed dark gray, 2A = solid light gray, 2B = dashed light gray) and 4 L bench-scale reactor (solid black line).

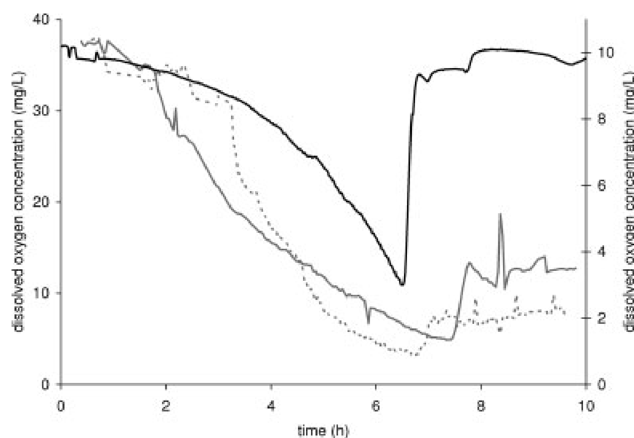

Figure 6. Measured dissolved oxygen concentration during batch cultivation of *C. utilis* in the 100 μL bioreactors (1A = solid dark gray, 1B = dashed dark gray, 2A = solid light gray, 2B = dashed light gray) and 4 L bench-scale reactor (black line, right axis).

Given an initial glucose concentration of 76 mM, an observed yield of biomass on substrate in the 4-L cultivation of 0.48 g biomass.g^{-1} glucose and a biomass carbon content of 1 mol per 25.6 g biomass,[31] it can be calculated from the stoichiometry of the conversion that the amount of CO_2 produced is equal to 0.20 mol L^{-1}. In this calculation, the formation of carbon containing by-products, in addition to biomass and CO_2, are not taken into account. However, due to the consumption of the nitrogen source urea an additional amount of CO_2 is produced. Assuming a nitrogen content of the biomass of 1 mol per 143 g biomass[31] it can be calculated that the additional amount of CO_2 produced due to the consumption of urea is equal to 0.023 mol L^{-1}, yielding a total amount of produced CO_2 of 0.22 mol L^{-1}. It can be seen from Figure 4 that the measured amount of CO_2 for both the bench- and microscale cultivations compared reasonably well (within 10%) with the calculated value of 0.22 mol L^{-1}. The gray area in Figure 4 indicates the error margin of this value, mainly caused by experimental variation in the amount of medium introduced in the microreactors. On average, the total amount of CO_2 produced in the batch cultivations was slightly lower than the calculated value. The most likely cause of this is the formation of small amounts of carbon containing by-products, like organic acids or glycerol, during the cultivations.

pH. The pH was measured in three of the four 100-μL cultivations and the data were compared to the pH data obtained from the 4 L batch cultivation (see Figure 5). It should be noted that for the microreactor cultivations ISFETs were used for pH measurement whereas in the 4 L scale a conventional glass electrode was used. It can be seen from Figure 5 that the pH in all batch cultures, including the 4 L bench scale cultivation, decreased gradually during the first 6 h, most likely because acids were produced. From the cumulative CO_2 measurements (see Figure 4), it can be inferred that after 6 h the cultures entered the stationary as a result of glucose depletion. Between 6 and 8 h after inoculation, i.e., during the transition between the exponential and the stationary phase the pH increased in all cultures, most probably due to consumption of the acids produced during the exponential growth phase. The exact time at which the pH was observed to increase was different for all cultures, which has most likely been caused by differences in biomass concentration in the inoculum or differences in the duration of the lag-phase. It must be noted that for Batch 1B and Batch 2B a moving average of 10 subsequent measurements was used to reduce the influence of random noise generated by the salt bridges, wiring, light interference or interfering electromagnetic fields in the environment.

Dissolved Oxygen Concentration. The measurement of the dissolved oxygen concentration in the micro bioreactors was based on amperometric detection of oxygen reduction. Although some oxygen is consumed in the measurement, the oxygen consumption of the sensor can be considered negligible compared to the oxygen consumption of the yeast culture in the micro bioreactor.[23] Figure 6 shows the dissolved oxygen concentration as a function of time in the two micro bioreactor experiments, i.e., Batch 1A and Batch 1B, and the bench-scale bioreactor. The oxygen concentration profile that was measured in the 4 L batch cultivation decreased exponentially in time due to the increased demand for oxygen of the exponentially growing yeast. The sharp increase in the dissolved oxygen concentration at 6.5 h indicated a sudden decrease of the oxygen consumption rate of the culture due to glucose depletion and after 8 h the dissolved oxygen concentration returned to its initial value.

Due to differences in kLa and aeration gas used (air for the bench-scale and pure oxygen for the microreactor) between the micro bioreactor and the bench scale reactor, the oxygen profiles for both reactor setups could only be compared qualitatively.

Although the measured dissolved oxygen concentration in the micro bioreactor decreased during the batch cultivation, the profile was not exponential whereas the biomass concentration and the CO_2 production measurements both indicate that the yeast indeed grew exponentially. Moreover, at the end of the batch phase, around 7.5 and 6.5 h for Batch 1A and Batch 1B, respectively, the dissolved oxygen concentration increased, but did not return to its initial value. These observations indicate that the effective electrode area must have decreased during the cultivation. This phenomenon can be caused by either physical degradation of the electrodes or by fouling of the electrode surface.

Similar behavior was not observed during performance tests of this sensor in a bench scale bioreactor.[23] Microscopic examination of the sensor arrays after conducting the batch cultivations in the microreactor did not reveal any signs of physical degradation of the electrodes. However, fouling of the electrode surface was clearly visible. After thorough cleaning of the sensor chip with acetone, the signal of the

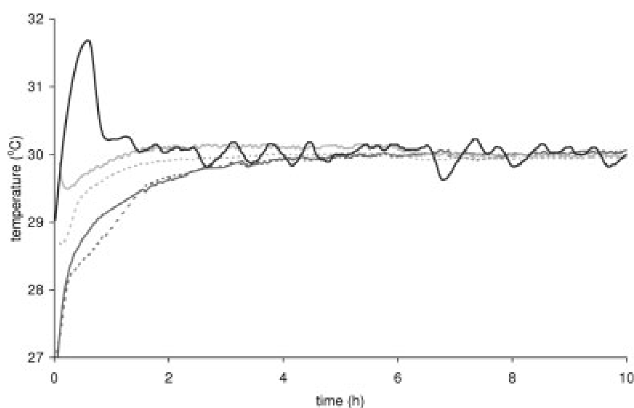

Figure 7. Temperature measurements during batch cultivation of *C. utilis* in the 100 μL bioreactors (1A = solid dark gray, 1B = dashed dark gray, 2A = solid light gray, 2B = dashed light gray) and 4 L bench-scale reactor (solid black line).

dissolved oxygen sensor returned to the calibrated value at 100% oxygen saturation. This indeed confirms that fouling has been responsible for the observed response of the sensor at the end of the batch cultivation.

What could be inferred from the oxygen concentration measurements in the micro bioreactor is that the oxygen concentration did not decrease to zero during the batch cultivation, indicating that oxygen limitation did not occur and thus that the oxygen transfer capacity of the micro bioreactor was sufficiently high for the applied cultivation conditions.

Temperature. Temperature is probably the easiest parameter to measure and control in micro bioreactors but is nevertheless of great importance. The complete micro bioreactor setup was placed in a temperature controlled cabinet and the temperature of the liquid phase of the reactors was measured by platinum thermistors on the sensor arrays. Figure 7 shows the results of the temperature measurement during the batch cultivations in the 100 μL microreactor as well as the 4 L bench-scale cultivation. It can be seen from this figure that the temperature signal from the integrated sensor array during the micro cultivation contained very little noise and that the result for the different cultivations were reproducible. The measured temperature was in all cases close to the setpoint of the thermostatically controlled cabinet, which was 30°C and was stable throughout the largest part of the cultivation process. The results of the temperature measurements also indicated that it took up to several hours before the setpoint temperature was reached. This effect was contributed to the relatively slow performance of the temperature controller of the cabinet in which the setup was placed. As can be seen in Figure 7, the temperature controller of the 4 L bench-scale reactor was much faster, but suffered from overshoot and oscillations around the setpoint. However, these changes in temperature were relatively small and did not affect the biological performance of the cultivation.

Conclusions

With this work, we have provided a proof of principle of the online monitoring of fermentation processes in two parallel microtiterplate wells with a working volume of 100 μL per well. The microtiterplate format was chosen to preserve compatibility with the current high-throughput cultivation systems. In each of the micro bioreactors an electrochemical sensor chip was integrated for the online measurement of the temperature, pH, dissolved oxygen concentration, and viable biomass concentrations. Furthermore, the cumulative CO_2 production during the batch cultivations was measured online.

Subsequently, the micro bioreactors were successfully applied to carry out batch cultivations of the yeast *C. utilis*. The online measurements obtained from the integrated sensor arrays in the microreactors were compared with similar measurements performed during a similar batch cultivation carried out in a conventional 4 L bench scale bioreactor. The measurements of cumulative CO_2 production, pH, and temperature in the micro bioreactor appeared very reproducible and compared very well to the results obtained in the bench scale bioreactor. The sensor for the measurement of the viable biomass concentration showed the exponential increase in biomass concentration during the batch cultivation. The reason for the sudden collapse of the signal of the biomass sensor at the end of the batch phase should be investigated further. The dissolved oxygen sensor initially performed well and showed oxygen sufficing conditions for the whole cultivation but the sensor suffered from fouling. Electrochemical cleaning steps might help in reducing fouling problems.

Although the sensor chip certainly needs further improvement on some points, this work clearly shows the applicability of electrochemical sensor arrays for the monitoring of parallel micro-scale fermentations, e.g., using the 96-well microtiterplate format. We believe that this work is an important contribution to the development of proper monitoring and control devices for high-throughput microreactor systems.

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