

The Hagen–Poiseuille pump for parallel fed-batch cultivations in microbioreactors

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ABSTRACT

We developed and applied a micropump for liquid flows of 0–150 nL/min. The operating principle of this pump is based on the Hagen–Poiseuille law. The main advantages of this pump are that an accurate continuous flow rate can be achieved, it is cheap to manufacture, easy to parallelize and easy to integrate in microtiter plates. Experiments at different flow rates showed that the flow rate remained stable in time and the measured flow rates corresponded very well to the Hagen–Poiseuille law. The applicability of the pump for reproducible feeding of microbioreactors was shown in a fed-batch system with a simple chemical reaction. Thereby the Hagen–Poiseuille pump was connected to a microbioreactor with a working volume of 100 μ L. The microreactor was filled with a H_2SO_4 solution which was fed with a Na_2CO_3 solution at a constant rate, while measuring the CO_2 release on-line. This resulted in a stable CO_2 production rate. Subsequently, the pump was successful applied to carry out anaerobic fed-batch fermentations of *Saccharomyces cerevisiae* in the microreactor system for periods up to 20 h.

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1. Introduction

For the majority of the industrial fermentation processes fed-batch cultivation is the preferred mode of operation. The term “fed-batch” refers to a technique where one or more substrates are supplied at a controlled rate to the bioreactor, enabling control of the growth rate under substrate limiting conditions. Fed-batch cultivation is the preferred mode of operation if substrate inhibition of product formation occurs or if product formation is maximal at a certain specific growth rate (Kim et al., 2004; Lee et al., 1999; Yamane and Shimizu, 1984).

Because the growth rate and oxygen demand of the culture can be controlled by the feed rate, fed-batch operation is often applied in high density industrial cultivations, to control the oxygen demand of the culture in order to prevent oxygen limiting conditions.

Furthermore, industrially relevant microorganisms, like *Escherichia coli* and *Saccharomyces cerevisiae*, secrete large amounts of by-products like acetate and ethanol under fully aerobic conditions when subjected to a high glycolytic flux i.e. at high substrate concentration. This phenomenon is known as “overflow metabolism”, “Crabtree effect” or “glucose effect” (Andersen and von Meyenburg, 1980; Postma et al., 1989). By-product formation not only decreases

the yield of the desired product, but can also inhibit growth. In these cases fed-batch operation will result in a significant decrease of by-product formation. In some cases hardly any desired product is formed at all at high substrate concentrations due to what is known as catabolite repression. Catabolite repression is the metabolic phenomena that the enzymes needed for product formation are repressed by a high concentration of a specific substrate, like glucose.

In the case that fed-batch cultivation is the preferred mode of operation for a certain process the selection of high producing microorganisms for this process should ideally be carried out under such conditions, i.e. fed-batch mode.

However, it is a common practice that high-throughput screening programs for industrially relevant microorganisms typically start with highly parallelized small-scale batch cultivations. Traditionally shake flasks are used during this phase of the selection process, however, the trend is towards smaller culture volumes like deep plate well or microtiter plates (Büchs, 2004). A major drawback of this first stage of the screening process is that, apart from the absence of control of important process parameters like pH, the screening is performed in batch cultures under conditions of substrate excess. Potentially high producing strains or mutants, which would perform well under substrate limited conditions but not under conditions of substrate excess, like strains possessing the metabolic phenomena that were discussed above, are then not identified during this stage. The reason that massive screening under fed-batch conditions has not been carried out so far, is that the current cultivation

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systems which would be suitable for fed-batch cultivation, i.e. lab-scale bioreactors, are laborious and thus costly to operate and require high investments in space and equipment. This limits the throughput of these systems and therefore only the best performers obtained in the first step can be tested under fed-batch conditions in lab-scale bioreactors.

However, some attempts have been made to develop fed-batch systems suitable for high-throughput screening. Early attempts to perform parallel fed-batch fermentations in shake flasks, date back to the 1950s (Dale et al., 1953). More recently, a shake flask system has been developed whereby a syringe pump coupled to a valve system providing intermittent feeding of glucose and pH control of 16 shake flasks of 100 mL (Weuster-Botz et al., 2001). To make further miniaturization of the system possible, a liquid handling system capable of intermittently feeding substrate to custom made miniature bioreactors of 10 mL was constructed (Puskeiler et al., 2005a,b).

Another way to accomplish controlled substrate supply is the “diffusion-feed” technique whereby the substrate is fed to a shake flask culture medium either from a submerged solid carrier (Jeude et al., 2006; Tyrrell et al., 1958) or from a compartment in the shake flasks filled with the substrate solution and separated from the culture medium by a membrane (Gerhardt and Gallup, 1963; O’sullivan and Pirt, 1973). However, an important disadvantage of the diffusion-feed systems is that a constant feed-rate is only guaranteed when the concentration difference across the membrane is maintained constant.

Recent attempts of downscaling bioreactors to microlitre size (< 500 μL) mainly focused on the integration of miniature sensors for pH, DO and biomass (Boccazzi et al., 2005; John et al., 2003a,b; Maharbiz et al., 2004; Zhang et al., 2006). To the author’s knowledge no fed-batch cultivations in microlitre cultivation systems, like microtiter plate wells, have been reported until now.

In order to perform a fed-batch cultivation in microtiter plate wells a substrate supply system is needed capable of handling flow rates up to a few hundred nL/min. Although syringe pumps would suffice for a proof of principle, they are too bulky and costly for a highly parallelised system; moreover, at the required low feed rates syringe pumps provide a pulsating instead of a constant feed flow. Another way of carrying out fed-batch cultivations in microtiter plates could be to supply the feed by means of a pipetting robot. The disadvantage is that a pipetting robot can only supply the feed in an intermittent fashion.

To circumvent the problems discussed above we developed a pressure based system for continuous feeding of microtiter plate wells. This system is based on the principle that by creating a pressure difference over a capillary tube with a defined diameter and length a certain flow rate through this capillary can be achieved (Fütterer et al., 2004; Savenije et al., 2003; Timmer et al., 2004). With this system flow rates from 0 up to a few hundred nL/min can be achieved by a capillary with an internal diameter of 20 μm , a length of 10–20 cm and a pressure difference of 0–1 bar. This supply mechanism allows continuous substrate feeding. Because of the simplicity of the supply system it is parallelisable and can be integrated in the bottom of a 96-well plate.

2. Theory

2.1. Hagen–Poiseuille pump

A pressure difference between the entrance and the exit of a capillary tube, forces the liquid in the tube to flow in the direction of the lowest pressure at a certain rate. The relation between the flow rate (φ (m^3/s)) through the capillary tube and the pressure difference (Δp (Pa)) over the capillary is described by the Hagen–Poiseuille

law (Eq. (1))

$$\Delta p = \frac{8\eta l \varphi}{\pi r^4} \quad (1)$$

where η (Pa s) is the viscosity; l (m) is the length and r (m) the radius of the capillary.

The length and the internal diameter of the capillary determine the range of liquid flow rates which can be obtained within a certain pressure range. Because pressure is relatively easy to apply and can be measured and controlled accurately, this variable was chosen to set the desired flow rate.

It should be realized that the Hagen–Poiseuille law is only valid for incompressible fluids at laminar regimes. The Reynolds number (Re (–)) characterizes the flow regime

$$Re = \frac{2\rho vr}{\eta} \quad (2)$$

where ρ is the density (kg m^{-3}) and v is the linear velocity (m/s). The flow regime in a tube is laminar at a Reynolds number smaller than 2000, at higher Reynolds numbers the flow regime transits towards turbulent conditions (Hellemans et al., 1980). The Reynolds number for aqueous solutions, as were used in our experiments, flowing at 150 nL/min through a capillary of 20 μm in diameter at 30 °C is never higher than 1. This is well within the laminar flow regime.

3. Materials and methods

3.1. Microbioreactors

Two wells from a 96-well microtiter plate served as microbioreactors. The entire 96-well plate was placed on a magnetic stirring motor (KMO 2, IKA-Werk Janke & Kunkel KG, Staufen, Germany). The microbioreactors were equipped with small stainless steel stirrer bars (dowels 1.93×2.46 mm; kindly provided by V&P Scientific Inc, San Diego, USA) operated at a rotation speed of 80 rpm.

Two mass flow controllers (Brooks Instruments B.V. Veenendaal, The Netherlands; with a flow range of 0–3 mL/min) were used to blow a stream of N_2 gas through the headspaces of the two wells at a flow rate of 600 $\mu\text{L}/\text{min}$. This was accomplished by two syringe needles that were pierced through the rubber stoppers that closed the wells and served as gas in- and outlets. Before the gas entered the wells 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 microreactors and sterilized via disk filters with a pore size of 0.2 μm (Whatman, ‘s-Hertogenbosch, The Netherlands).

For the fed-batch experiments the liquid was supplied to the two microbioreactors either by syringe or Hagen–Poiseuille pumps. The liquid was pumped into the wells via tubes that were pierced through the rubber stoppers (see Fig. 1).

The setup was placed in a temperature-controlled cabinet that was controlled (6100+ West Instruments, Brighton, England) at 30 ± 0.1 °C via three 100 W light bulbs. A custom made LabVIEW™ (National Instruments, Austin, USA) routine was used to measure and log the temperature via a DAQ card (Advantech-PCI-1710HGL, Milpital, USA).

3.2. Hagen–Poiseuille pump

Two fused silica capillaries (Bestler, Amstelveen, The Netherlands) (internal diameter: 20 μm , length: 0.2 m) were connected with Peek™ Nanotight® connectors (Upchurch Scientific, USA) to a liquid reservoir of 577 μL fabricated from PMMA. In between the reservoir and the two capillaries a pressure sensor (26PCCFA6D,

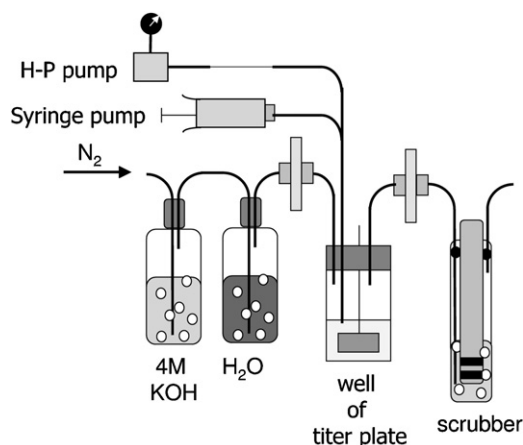


Fig. 1. Schematic representation of the measurement setup.

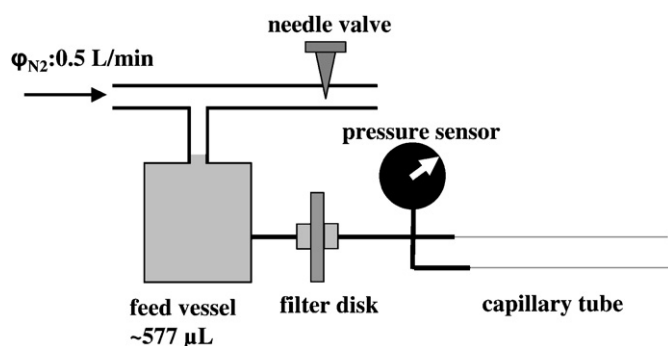


Fig. 2. Schematic representation of Hagen–Poiseuille pump.

measurement range ± 1 bar or 26PCDFA6D, measurement range ± 2 bar, Honeywell Sensing and Control, USA) and a disk filter (Millex[®] HV, 0.45 μm pore size, Millipore, Ireland) was placed to prevent particles entering the capillaries. A mass flow controller (Brooks Instruments B.V. Veenendaal, The Netherlands, with a flow range of 0–3 L/min) ensured a constant flow of pressurized air at a flow rate of 0.5 L/min over the liquid in the reservoir. The air-exit was squeezed with a needle valve to set the desired pressure (see Fig. 2). A custom made LabVIEW[™] (National Instruments, Austin, USA) routine was used to measure and log the pressure via a DAQ card (Advantech-PCI-1710HGL, Milpita, USA).

3.3. Flow rate measurement

The capillary of the Hagen–Poiseuille pump was connected to a transparent measurement tube ($254 \pm 12.5 \mu\text{m}$ ID, Teflon FEP, Upchurch Scientific, USA). The liquid flow rate (φ) was measured by measuring the movement of a Rhodamine B (1 μL , 1 mM) plug in the measurement tube alongside a ruler as a function of time. Before use, the exact internal diameter of the transparent measurement tube was obtained by connecting the tube to a syringe pump (KD Scientific 210, Antec Leyden B.V., Zoeterwoude, The Netherlands) with a 50 μL syringe (Hamilton, Reno, Nevada, USA) and measuring the displacement of the plug.

3.4. Carbon dioxide production measurement

In a previous paper (van Leeuwen et al., 2009) an on-line measurement technique, based on conductivity measurement, was reported for quantifying the CO_2 production rate in microbioreactors. This technique was used to measure the CO_2 production rate in both

the chemical and biological fed-batch experiments. The exhaust gas containing the CO_2 that was produced in each of the microbioreactors was led through a separate scrubber, i.e. a test tube containing 3 ml of 80 mM NaOH solution. A Teflon[®] coated stirring dowel (7 \times 2 mm) in the scrubber driven by a magnetic stirring plate ensured sufficient CO_2 transfer and liquid mixing. Herein the CO_2 was stripped from the exhaust gas. A conductivity electrode (Consort SK20b; range 0.1 $\mu\text{S}/\text{cm}$ –100 mS/cm) that was mounted in the test tube was used to measure the conductivity change of the NaOH solution. The measured conductivity change was used as a measure for the amount of CO_2 that was absorbed by the NaOH solution. The relation between conductivity and CO_2 accumulation was obtained for both probes by measuring the conductivity change when a 2% CO_2 calibration gas (Hoek Loos B.V., Schiedam, The Netherlands) was sparged through the scrubber at a flow rate of 600 $\mu\text{L}/\text{min}$. A custom made LabVIEW[™] routine was applied 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 in order to prevent CO_2 from entering or leaving the system before the scrubber.

3.5. Chemical fed-batch

To test the measurement setup the chemical conversion of carbonate to CO_2 was chosen as a model system. Either the Hagen–Poiseuille pumps or a syringe pump (KD Scientific 210, Antec Leyden B.V., Zoeterwoude, The Netherlands) equipped with two 50 μL syringes (Hamilton, Reno, Nevada, USA) was connected to two wells of a microtiter plate. The wells were filled with 100 μL 0.8 M H_2SO_4 . The pumps supplied a 0.52 M Na_2CO_3 solution to the wells at flow rates varying from 0 to 170 nL/min. Rubber stoppers were used to close the wells. The Na_2CO_3 solution was pumped via tubes through the rubber stoppers into the wells and the CO_2 production was measured.

3.6. Anaerobic fed-batch cultivation of *S. cerevisiae*

S. cerevisiae CEN.PK 113-7D was transferred from a glycerol stock into a 100 ml shake flask filled with 20 ml mineral medium (Verduyn et al., 1992) and cultivated in an orbital shaker (Certomat[®] BS-1 Sartorius BBI Systems GmbH, Melsungen, Germany) at 30 °C and 200 rpm under aerobic conditions. The medium was supplemented with 15 g/L glucose, 1H₂O and the ammonium in the medium was replaced by urea (2.3 g/L) to reduce acidification of the medium during growth. The initial pH of the medium was 6.0.

At the end of the exponential growth phase a 1 mL sample was taken, centrifuged (3 min, 13 000 rpm), decanted and resuspended in 0.5 mL of mineral medium. The medium for anaerobic growth was supplemented with anaerobic growth factors, Tween-80 and ergosterol, as described previously (Verduyn et al., 1990), the ammonium was replaced by urea (2.3 g/L) and no glucose was added.

The microtiter plate was sterilized with 70% ethanol and afterwards washed with sterile demi water. 100 μL of the resuspended cells was pipetted into each of the two wells. The wells were closed with the rubber stoppers containing the needles for gas supply and glucose feed. The glucose feed was supplied either by the Hagen–Poiseuille or the syringe pump at flow rates ranging from 35–175 nL/min.

3.7. 4L chemostat cultivation

A chemostat cultivation was used for the determination of the yields of all relevant products. *S. cerevisiae* was grown at 30 °C under anaerobic carbon-limited chemostat conditions in a 7L fermenter

(Applikon, The Netherlands) with a working volume of 4 L, on defined mineral medium (Verduyn et al., 1992) with 25 g/L glucose. The medium was supplemented with anaerobic growth factors Tween-80 and ergosterol as described previously (Verduyn et al., 1990). The dilution rate was set at 0.05/h. The pH was measured (Inpro 3030/120, Mettler-Toledo, Tiel, The Netherlands) and controlled at 5.0 with a 4 M KOH solution. The overpressure in the vessel was kept at 0.3 bar and the stirrer speed was 400 rpm. Anaerobic conditions were maintained by sparging the medium reservoir and the fermenter with pure nitrogen gas (0.5 L/min). Furthermore, Norprene tubing and butyl septa were used to minimize oxygen diffusion into the anaerobic cultures. The dissolved oxygen was measured with a DOT sensor (DOT, Mettler-Toledo, Tiel, The Netherlands). The off-gas of the fermenter was cooled in a condenser connected to a cryostat at 2 °C to reduce evaporation and dried with a Perma Pure dryer (Perma Pure Inc., Toms River, NJ, USA). The O₂ and CO₂ content in the off-gas were subsequently measured by a NGA 200 gas analyzer (Rosemount Analytics, Santa Clare, CA, USA). A process control system (DCU3, Sartorius AG, Melsungen, Germany) controlled the entire fermentation and MFCS software (Sartorius AG, Melsungen, Germany) logged the data to a computer.

Batch cultures were inoculated with 100 ml overnight shake-flask cultures, which in turn were inoculated from –80 °C glycerol stocks. The chemostat phase was initiated by starting the feeding 30–60 min after the end of the batch phase, as was observed by the CO₂ concentration in the off-gas. The cultures were considered to be at steady-state after 4–15 generations of glucose-limited growth.

3.8. Analytical methods

The biomass dry weight of the chemostat cultivation was measured as described previously (Visser et al., 2004).

4. Results and discussion

4.1. Flow rate measurements

For fed-batch cultivations to be carried out in standard 96 well microtiter plates the feed rate should not be much higher than 150 nL/min to prevent a too large increase of the culture volume in the wells. At a feed rate of 150 nL/min the volume increase is approximately 100 μL in 11 h which is a relatively short time period for a fed-batch cultivation. Therefore the range of flow rates used for calibration of the Hagen–Poiseuille pump was chosen between 0 and 150 nL/min. Because no standard method to measure flow rates in the range of 0–150 nL/min was available, we had to develop one. The method we used was based on the movement of a Rhodamine B plug in a transparent tube alongside a ruler. By connecting the capillary of the pump to the transparent measurement tube the liquid from the pump pushed the plug through the tube. By measuring the displacement of the plug in time, which took several hours for each measurement, the flow rate was measured.

However, a prerequisite for accurate flow rate measurements with this system is that the diameter of the measurement tube, and thereby the relation between the pumped volume and the displacement of the Rhodamine B plug, is accurately known. To obtain this relation a syringe pump was used. By measuring the displacement of the plug at a number of different known volumes the measurement tube was calibrated. Quadruplicate measurements resulted in a value of 59 ± 1 nL/mm displacement of the plug. This corresponded with an internal diameter of the applied measurement tube of 274 μm. The calibration together with the fact that a 1 mm movement of the Rhodamine B plug appeared to be easily measurable yields accuracy for this measurement technique of 1 nL.

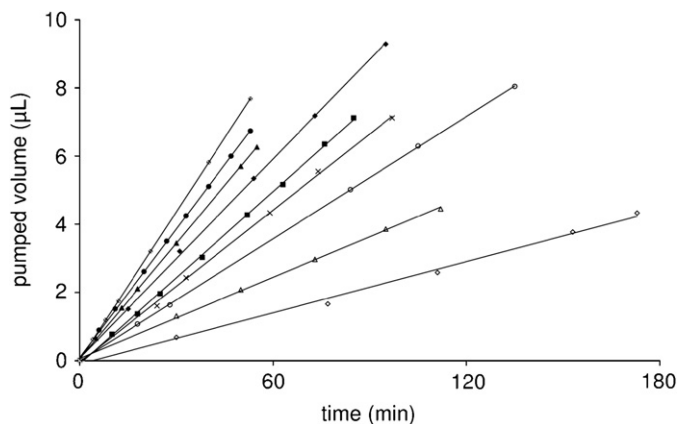


Fig. 3. Measurement of the pumped volume vs. time of the Hagen–Poiseuille pump at different pressures over the capillary: 0.27 bar (◇), 0.34 bar (Δ), 0.53 bar (○), 0.62 bar (×), 0.70 bar (■), 0.78 bar (◆), 0.99 bar (▲), 1.05 bar (●), 1.22 bar (X), all with a linear regression line.

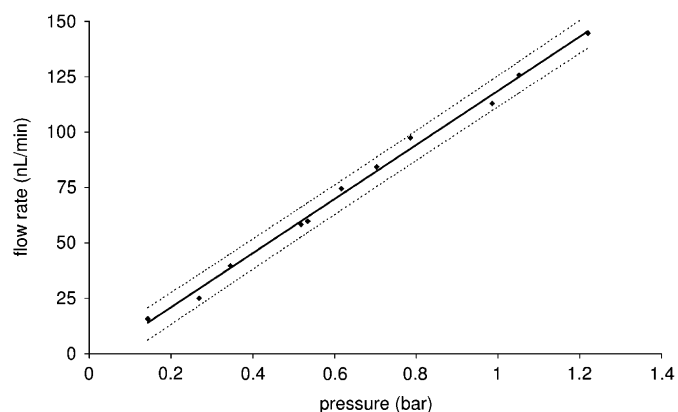


Fig. 4. Measured flow rate as a function of a pressure difference over a capillary (◆) and best fitting line based on the method of least squares (—) and the 95% confidence interval (---); the fit corresponds to the Hagen–Poiseuille law describing the flow rate as a function of pressure over a capillary with an internal diameter of 20.52 μm.

Subsequently this measurement device was used to characterize the Hagen–Poiseuille pump with respect to its accuracy and reproducibility. To do so the pumped volume was measured over time at different pressures. Fig. 3 shows the results of these measurements. It can be seen from this figure that the flow rates obtained by the Hagen–Poiseuille pump remain constant over time, indicated by the straight lines.

In Fig. 4 the experimentally obtained flow rates at varying pressures are compared to the Hagen–Poiseuille law. In the Hagen–Poiseuille law the temperature corrected viscosity of water was used (Vanýsek, 2006).

The internal diameter of the capillary was determined by fitting the Hagen–Poiseuille law to the data points (solid line in Fig. 4). The radius of the capillary (r) was determined to be $10.26 \pm 0.06 \mu\text{m}$ (average ± standard error). This experimentally determined radius appeared to be very well within specification of $10 \pm 1 \mu\text{m}$ as was provided by the supplier.

Adding more capillaries to the same pressure source yields parallel pumps. We attached 3 capillaries to the same pressure source by means of a cross junction. Subsequently the pumped flow rates were measured for at least 1.5 h at 0.52 bar pressure difference over the capillaries. This resulted in an average flow rate of 58 nL/min

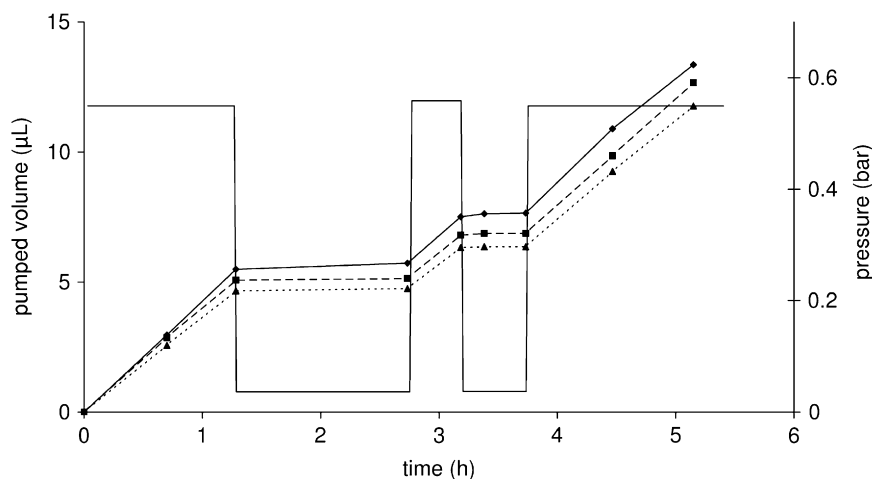


Fig. 5. Pressure controlled flow measurements of three parallelised pumps: pumped volume of pumps (■◆▲) and (–) pressure.

with a standard deviation of 2 nL/min for the 3 capillaries. This falls well within the 95% confidence interval for this pressure. From this it can be concluded that no pressure effects at the entrance of the capillary occurred due to the cross junction. Therefore further parallelisation up to 96 pumps, enough for the entire microtiter plate, seems to be very well possible.

By controlling the applied pressure difference over the capillary the flow rate of the Hagen–Poiseuille pump can be controlled. This makes it possible to use the pump as an actuator, for example for addition of acid or base for pH control. Fig. 5 shows the pumped volume of 3 pumps operated in parallel as a function of time when the pressure was switched between 0.55 and 0.05 bar. It can be seen from this figure that the flow rate responded very fast to the pressure changes.

The response time appeared to be so fast that we were not able to determine it. The total amount of liquid which was displaced by the three pumps was 13.3, 12.6 and 11.7 μL , respectively, after 5.2 h, which yields a standard deviation of 6.4% from the average. It must be noted that the deviation can have been caused both by differences in geometry of the capillary (length or internal diameter) and by errors in the displacement measurement itself.

4.2. Chemical fed-batch

In order to test the proper functioning of the CO_2 measuring setup in combination with the microtiter plate system, the reaction between H_2SO_4 and Na_2CO_3 , which results in the release of CO_2 , was used. This was done by continuous addition of a Na_2CO_3 solution (0.52 M), either via a syringe pump or via the Hagen–Poiseuille pump to a microtiter plate well that contained 100 μL of a 0.8 M H_2SO_4 solution. In the acidic environment the acid–base equilibrium of the carbonate is almost entirely at the side of CO_2 . This implies that as soon as the Na_2CO_3 solution is pumped into the microwell it is converted to CO_2 consuming two H^+ ions per added carbonate ion. The CO_2 that was produced in this way was transferred to the gas phase and carried by the N_2 towards the scrubber where it was trapped in the NaOH solution. In the scrubber the accumulated amount was quantified by means of the conductivity measurement.

The cumulative amount of CO_2 that was produced during the chemical fed-batch, was measured for at least 1.5 h after the start of the experiment. Different flow rates in the range of 20–175 nL/min. were applied. For the flow rate calculations of the Hagen–Poiseuille pump a viscosity of the Na_2CO_3 solution at 30 $^\circ\text{C}$ of 1.03 mPa s was used (Barthel et al., 1998).

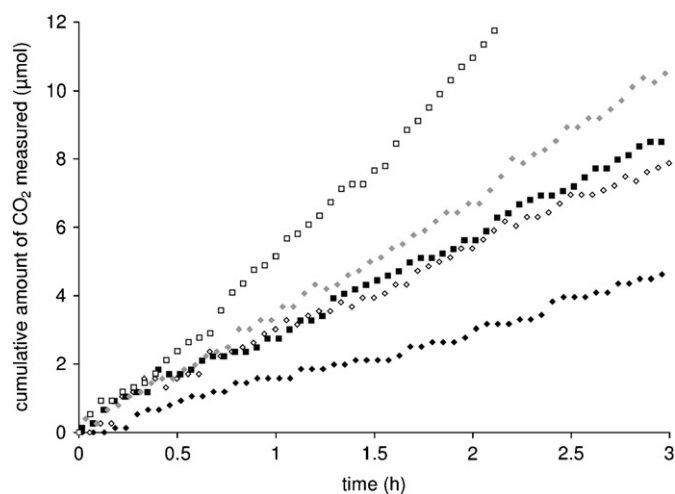


Fig. 6. Measurement of the cumulative amount of carbon dioxide that is produced from the addition of a sodium carbonate (0.52 M) solution in a sulfuric acid solution (0.8 M). The sodium carbonate was added either by the syringe pump at a flow rate of 83 (■) or 166 nL/min. (□) or by the Hagen–Poiseuille pump at flow rates of 53 (◆), 69 (◇) or 97 nL/min. (◇).

At a constant supply rate of the Na_2CO_3 solution the cumulative amount of CO_2 released should increase linearly in time.

Because the concentration in the feed was the same for all experiments, the CO_2 production rate should be linearly dependent on the flow rate of the pump. Fig. 6 shows a representative selection of the results from the chemical fed-batch experiments. In this figure the measured cumulative amount of CO_2 is plotted versus time for different feed rates. The graphs show a linear trend, as expected, although sometimes oscillations are observed in the measurement in time. These oscillations in the CO_2 measurement are most likely caused by temperature oscillations, because of inaccurate temperature control. It should be noted that the flow rate delivered by the Hagen–Poiseuille pump is a function of the viscosity of the pumped liquid, which is temperature dependent. Also the CO_2 accumulation measurement by means of conductivity appeared to be highly temperature dependent (van Leeuwen et al., 2009). From the carbon dioxide production measurements obtained from the experiments with the chemical fed-batch system at different feed rates the CO_2 production rate (R_{CO_2}) was determined as a function of the rate of Na_2CO_3 supply.

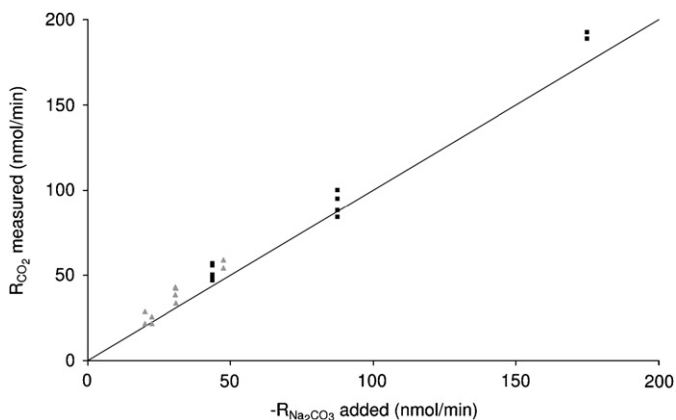


Fig. 7. The measured CO_2 production rate vs. the calculated rate of sodium carbonate supply. The sodium carbonate was added either by the syringe pump (■) or by the Hagen–Poiseuille pump (Δ).

The results are shown in Fig. 7. Note that the slope of the linear regression line through the measurement points should yield the stoichiometry for the chemical acid/base reaction. The slope of the regression line ($R_{\text{CO}_2}^{\text{measured}}/R_{\text{Na}_2\text{CO}_3}^{\text{added}}$) was calculated to be 1.09 ± 0.02 for the experiments carried out with the syringe pump, which compares very well with the theoretical stoichiometry of 1 mol of CO_2 per mole of Na_2CO_3 added.

However, it was found that if the Hagen–Poiseuille pump was used, a stoichiometry for the acid/base reaction of 1.23 ± 0.02 was obtained, which is significant higher than the theoretically expected value of 1. One of the reasons for this could be an incorrect internal diameter of the capillary. The Hagen–Poiseuille equation shows that, because the flow rate is proportional to the radius of the capillary to the fourth power, small variations in radius of the capillary strongly influence the flow rate. It is therefore necessary to determine the radius of the capillary accurately, as was done in the flow rate measurements described above. When the solution in the Hagen–Poiseuille pump is a base, (a 0.52 M Na_2CO_3 solution has a pH of approximately 12) and the capillary is made out of fused silica, then the base solution could etch the inside of the capillary, thereby increasing its radius. This could explain the too high value for the measured stoichiometry when the Hagen–Poiseuille pump was used. A detailed study of this phenomenon lies outside the scope of the paper, but this should be taken into account when applying this pump.

4.3. Anaerobic fed-batch cultivation of *S. cerevisiae*

In anaerobic cultivations CO_2 is a major degradation product and is directly related to cell growth and glucose consumption. From an anaerobic cultivation of *S. cerevisiae* CEN.PK 113-7D in a chemostat with a working volume of 4 L carried out at a dilution rate of 0.05/h the yield of CO_2 on glucose (Y_{S,CO_2}) was determined to be 1.5 mol CO_2 /mol glucose. This result compared reasonably well with a yield of CO_2 on glucose of 1.63 found earlier for a different *S. cerevisiae* strain (Verduyn et al., 1990).

Several fed-batch experiments were performed in the microtiter plate wells, whereby the cumulative carbon dioxide production was measured on-line. Both the syringe pump and the Hagen–Poiseuille pump were used to supply the glucose solution. The flow rate of the Hagen–Poiseuille pump was calculated from Eq. (1). Herein a viscosity of the glucose solution at 30 °C of 1.25 mPa s was used (Telis et al., 2007). In these experiments the initial biomass concentration and the initial liquid volume in the wells were approximately 5.7 g/l and 100 μL , respectively.

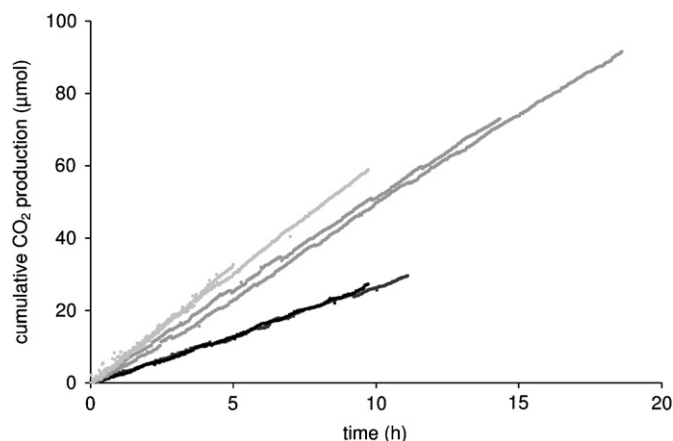


Fig. 8. The online measured cumulative CO_2 production as a function of time for anaerobic fed-batch cultivation of *Saccharomyces cerevisiae* at feed rates of 42 (■), 61 (●) and 88 nL/min (□) and a feed concentration of 0.92 M glucose solution. Each flow rate was done in duplicate.

Due to of the feeding of the glucose solution to the wells the liquid volume in the wells increased in time. With a glucose feed rate of 90 nL/min fed-batch cultivations could last up to 20 h before the wells were completely filled, i.e. at a volume of approximately 200 μL . Longer fed-batch cultivations, up to 83 h could be performed when the flow rate was reduced down to 20 nL/min (see Fig. 3).

It should be noted that when the glucose feed is supplied at a constant rate, all production and consumption rates are expected to be constant in time as long as the contribution of maintenance is negligible. It is known that the maintenance energy need of *S. cerevisiae* is small and even at relatively low growth rates the contribution of maintenance to the total substrate uptake rate cannot be observed (Verduyn et al., 1991, and references therein). Consequently, the contribution of maintenance was neglected in our experiments. If maintenance is negligible, fed-batch cultivation at a constant feed rate results in a constant CO_2 production rate, and furthermore in a linear increase of the biomass concentration in time and thus in an asymptotically decreasing biomass specific substrate uptake rate and specific growth rate.

Under anaerobic conditions the yield of biomass on substrate is relatively low, prolonged fed-batch cultivations could be performed without a large increase in the total amount of biomass and thus without a large decrease of the specific growth rate. Assuming an anaerobic biomass yield of 0.07 g biomass/g glucose (Tai et al., 2007) the initial growth rates in the fed-batch experiments were calculated to lay between 0.10 and 0.023/h for the range of glucose consumption rates ($-R_s$), i.e. 78–17 nmol/min, that were applied in the fed-batch experiments. These specific growth rates are well below the maximum specific growth rate of 0.3/h (Visser et al., 1990), thus ensuring glucose limited growth. After 20 h of fed-batch cultivation the growth rates were estimated to be 0.035 and 0.017/h for glucose consumption rates ($-R_s$) of 78 and 17 nmol/min, respectively.

As the cumulative CO_2 production is measured on-line in the fed-batch experiments, a constant CO_2 production rate is depicted as a linear increase in the cumulative CO_2 production in time.

Fig. 8 shows the on-line cumulative CO_2 measurements in time of the fed-batch cultivations performed with the Hagen–Poiseuille pump to feed the substrate to the microbioreactor. The $-R_s$ was set at 37, 54 and 78 nmol/min (corresponding to feed rates of 40, 58 and 84 nL/min with a glucose concentration in the feed of 0.93 M). All fed-batch cultivations were performed in duplicate. It can be seen from the curves shown in Fig. 8 that the increase in the cumulative amount of CO_2 proceeds linear in time, which indicates that R_{CO_2} ,

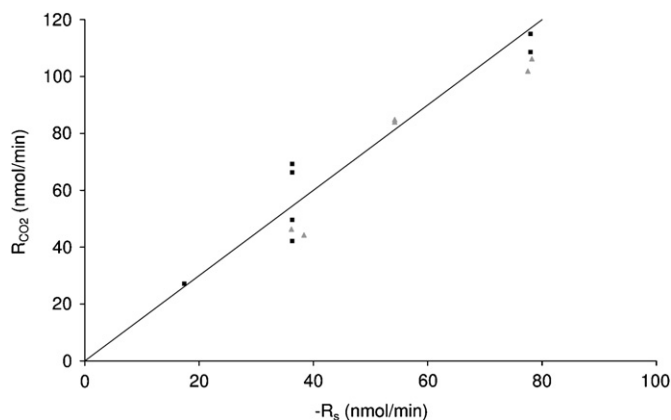


Fig. 9. CO_2 production rate (R_{CO_2}) as a function of the substrate consumption rate (R_s) in fed-batch cultivations of *Saccharomyces cerevisiae*, when using either a syringe pump (■), or the Hagen–Poiseuille pump (▲) for feed supply.

was constant as was expected. Even in fed-batch cultivations lasting 20 h, the R_{CO_2} remained constant. Increasing the glucose feed rate, and thus the glucose consumption rate $-R_s$, resulted in an increase in R_{CO_2} , as was expected. At the end of the experiment the culture was harvested and inspected under a microscope. No contaminations were found. The same was true for the fed-batch cultivations in which the syringe pump was used (results not shown).

From all fed-batch experiments carried out the R_{CO_2} was determined by linear regression and plotted against $-R_s$. The result is shown in Fig. 9. From these results the yield of CO_2 on glucose (Y_{s,CO_2}) was determined for each of the two pumps applied.

The Y_{s,CO_2} was determined to be $1.50 \pm 0.08 \text{ mol CO}_2/\text{mol glucose}$ and $1.40 \pm 0.05 \text{ mol CO}_2/\text{mol glucose}$ for experiments performed with the syringe pump and the Hagen–Poiseuille pump, respectively. From these experiments it can be inferred that the CO_2 yield on glucose determined from the fed-batch cultivations carried out on microlitre scale compared very well with the value of $1.5 \text{ mol CO}_2/\text{mol glucose}$ that was determined on 4 L scale for this strain.

5. Conclusions

We applied the Hagen–Poiseuille law to build an inexpensive, pulse free pump that can handle flow rates from 0–150 nL/min. The measured flow rates of this pump were found to correspond very well with the theoretical values of the Hagen–Poiseuille law. The pump was used to perform fed-batch experiments, both biological and chemical conversions, in microtiter plate wells of 100 μL whereby the CO_2 production rate was used for on-line monitoring of the fed-batch processes.

The chemical conversion Na_2CO_3 in an acidic environment into CO_2 was used as a model system in order to test the setup. Fed-batch experiments with the syringe pump for feeding the Na_2CO_3 yielded the theoretical stoichiometry for this reaction of 1, while with the Hagen–Poiseuille pump the measured stoichiometry was 1.2 which was probably caused by corrosion of the capillary by the alkaline solution.

Anaerobic fed-batch cultivations of *S. cerevisiae* with a constant glucose feed were performed in a volume of 100 μL and lasted up to 20 h. The cumulative amount of CO_2 produced in these cultivations showed a linear trend in time, as was expected. Both the syringe pump and the Hagen–Poiseuille pump were used for these fed-batch experiments. The yield of CO_2 on glucose (Y_{s,CO_2}) was determined to be $1.50 \pm 0.08 \text{ mol CO}_2/\text{mol glucose}$ and $1.40 \pm 0.05 \text{ mol CO}_2/\text{mol glucose}$ for the syringe pump and the Hagen–Poiseuille pump respectively. These yields correspond very well to the yield of $1.5 \text{ mol CO}_2/\text{mol glucose}$ that was determined in a 4 L chemostat cultivation.

The results of these experiments give proof of principle that fed-batch cultivation on microliter scale is possible. The pump we developed and applied for this purpose, the Hagen–Poiseuille pump, provides pulse free flows even at very low flow rates, the pump is easy to parallelize and is small enough to integrate in the bottom of a microtiter plate. We think that parallelization and integration of this system in the bottom of a microtiter plate can significantly improve high-throughput screening programs because it allows performing screening under industrially relevant, fed-batch, conditions.

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