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PAPER

A disposable picolitre bioreactor for cultivation and investigation of industrially relevant bacteria on the single cell level†

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In the continuously growing field of industrial biotechnology the scale-up from lab to industrial scale is still a major hurdle to develop competitive bioprocesses. During scale-up the productivity of single cells might be affected by bioreactor inhomogeneity and population heterogeneity. Currently, these complex interactions are difficult to investigate. In this report, design, fabrication and operation of a disposable picolitre cultivation system is described, in which environmental conditions can be well controlled on a short time scale and bacterial microcolony growth experiments can be observed by time-lapse microscopy. Three exemplary investigations will be discussed emphasizing the applicability and versatility of the device. Growth and analysis of industrially relevant bacteria with single cell resolution (in particular *Escherichia coli* and *Corynebacterium glutamicum*) starting from one single mother cell to densely packed cultures is demonstrated. Applying the picolitre bioreactor, 1.5-fold increased growth rates of *C. glutamicum* wild type cells were observed compared to typical 1 litre lab-scale batch cultivation. Moreover, the device was used to analyse and quantify the morphological changes of an industrially relevant L-lysine producer *C. glutamicum* after artificially inducing starvation conditions. Instead of a one week lab-scale experiment, only 1 h was sufficient to reveal the same information. Furthermore, time lapse microscopy during 24 h picolitre cultivation of an arginine producing strain containing a genetically encoded fluorescence sensor disclosed time dependent single cell productivity and growth, which was not possible with conventional methods.

Introduction

Industrial biotechnology is concerned with the production of chemicals, pharmaceuticals and proteins by using microorganisms growing on sustainable resources. Growth and production are the two key factors in biotechnological production processes which typically underlie continuous optimization. Assuming isogenic starting populations, optimal reactor control and mixing, uniform cell behaviour during growth might be expected. However, it has emerged in recent years that isogenic bacterial populations can be physiologically heterogeneous,[1–6] *e.g.*, comprising producing and non-producing cells. Hence there is strong demand to gain knowledge on population heterogeneity impacting industrial-scale cultivation.

Population heterogeneity may have two reasons: firstly, cellular heterogeneity, caused by either (a) emerged genetic differences,[7] (b) generally stochastic effects,[8] and (c) population based phenomena like quorum sensing,[9] and secondly, environmental heterogeneities at the microscale level, caused by insufficient process control and mixing. Particularly, cells are continuously exposed to fluctuating conditions as they travel through the various zones of bioreactors. Clearly, such heterogeneity directly affects cell metabolism.[10,11] Typically, cellular as well as environmental heterogeneities occur at the same time, leading to the problem that phenotypic screening and characterization is hard to accomplish. Until now, mainly due to missing experimental and analytical techniques, detailed knowledge on population heterogeneity and its impact on production processes is not available.[12,13]

To date, most of the heterogeneity studies are based on large cell populations, containing several billions of cells to collect statistically reliable data. To investigate the behaviour of individual cells of such populations, a well-established method is fluorescence activated cell sorting (FACS).[14] FACS is an ideal high throughput tool for screening and sorting of populations up to 80 000 cells per second.[15] However, FACS as well as other cytometric methods like Coulter counters are offline methods implying elaborate sampling routines prior to analytics. Furthermore, FACS is a snapshot analysis device and possibilities for investigating time dependent processes on the single cell level are limited. For example, FACS does not facilitate individual cell tracking, to determine proliferation, cell cycle events, growth rates and time dependent production.

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Due to its simplicity and applicability with conventional microscopes, cell culturing on small agar pads is frequently applied. It enables time-lapse investigations of microcolonies with single cell resolution, however, at very low throughput and minimal environmental control.[16] To overcome some of these limitations, automated microscopy and image recognition software have been applied to analyse multiple bacteria microcolonies on a single agar pad.[17] Nevertheless, lack of environmental and spatial cell growth control remain major drawbacks.

In contrast to FACS and agar pads, microfluidics and lab-on-a-chip devices do allow for long term cell analysis with the possibility to perform environmental and cell growth control.[18] Microfluidics offers homogeneous and well controllable microscale environments due to laminar flow and diffusion based mixing. Furthermore, due to massive parallelization of micrometre sized components, microfluidics has the potential for high-throughput bacterial cell analysis.

Two approaches have been described in the literature so far: Firstly, miniaturization of common bioreactor technology down to nanolitre volumes with integrated sensors for various process parameters. These microfluidic bioreactors allow environmental control but do not facilitate single cell resolution for longer time periods.[19] Secondly, microfluidic devices for biotechnological single cell studies including, *e.g.*, phenotypic population heterogeneity[20] and single cell growth.[20,21] Our intention is a combination of both approaches, to be specific, a microfluidic device allowing environmental reactor control at a defined culture volume and continuous single cell observation simultaneously.

To accomplish single cell analysis in microfluidic devices, cell trapping is essential. Different methods for single cell trapping have been applied so far, including contactless and contact based methods.[22,23] However, most methods were applied to eukaryotic cells.[24] Evidently, the typically 10–100 times smaller prokaryotic cells used in industrial biotechnology necessitate more precise microfabrication and well device control. Hence, only a few microfluidic methods for single bacteria analysis have been demonstrated till now. Keymer *et al.* and Mannik *et al.* developed microfluidic systems to investigate growth and motility of bacteria populations.[25,26] Wang *et al.* applied hundreds of 1 μm wide dead-end channels to determine the growth rate of individual *E. coli* cells over more than 200 generations, but no investigations on complete colonies were possible.[27] Kortmann *et al.* used elaborate dielectrophoretic (DEP) trapping to determine growth rates of single yeast cells and bacteria cells.[21] Walden *et al.* developed parallel trapping regions for bacteria cultivation of up to 300 cells in a monolayer.[28] The latter allows for population heterogeneity analysis of larger bacterial microcolonies. Unfortunately, the layout does not allow controlled spatial cell growth and could result in inaccurate growth rate quantification.

In this study a novel microfluidic bioreactor is presented allowing parallel analysis not only of individual bacteria but also of multiple microcolonies of up to 500 cells inside 1 pL sized bioreactors (PLBRs). The system was developed for single use with a focus on simplicity. In contrast to previously reported systems, single cells simply remain trapped inside the shallow bioreactor, not relying on sophisticated cell trapping mechanisms, and cells grow in a monolayer ideal for time-lapse microscopy. Analysis can be performed on many PLBRs in parallel by automated and image based microscopy. An innovative reactor design with overflow capability allows for continuous and non-restricted cell cultivation and observation, ideal for bioprocess investigations. Furthermore, it allows for steady medium infusion maintaining constant environmental conditions as well as defined medium switches within seconds to induce various cell reactions. Moreover, the high potential for parallelization makes the system an ideal tool to collect statistically trustworthy data.

This paper covers device principle, design, basic fabrication aspects and focuses on typical applications in biotechnological research, where other methods reach their technological limits. Three exemplary biotechnological investigations are presented indicating the versatility of the device. Firstly, as a proof of principle, the cultivation of two industrially relevant organisms, *E. coli* and *C. glutamicum*, was performed under constant conditions. By determining growth rates of three picolitre bioreactor cultivations, biocompatibility and reproducibility was investigated as a necessary step for further experiments. Secondly, defined short time medium changes were applied, to assess the biological response of the growing bacteria colony under starving conditions. Thirdly, a first example of combined growth and production studies with genetically encoded fluorescence reporters for *C. glutamicum* is shown. Utilizing one microfluidic system, we could investigate important bioprocess parameters, *e.g.*, cell growth, morphology, and productivity, at the same time.

Device principle and design

The presented microfluidic device is intended for the analysis of bacteria cells on the single cell level. We aim for the investigation of microcolonies of up to 500 cells. In contrast to previously reported systems, the device shown in this report has been designed for non-motile bacteria. For this purpose, novel picolitre sized bioreactors, in this paper referred to as PLBR, were designed and implemented into a microfluidic chip. The device can incorporate up to several hundred PLBRs connected to various inlet and outlet channels for supplying growth medium and waste removal. To minimize hydrodynamic resistance but enforce cells to grow in a monolayer, two different channel heights were included: The supply and waste channels have an approx. height of 10 μm, whereas the shallow cell cultivation region is approx. 1 μm high. This 1 μm culturing region restricts cell growth to a monolayer, ideal for image based live-cell and time-lapse microscopy. As illustrated in Fig. 1, each PLBR consists of a circular plateau with radially arranged channels. The front channel is elongated thereby forming the cell seeding inlet. The PLBR is located inside the centre of the supply channel, enabling a medium flow around the trap and reduced flow through the cultivation region (as evident from Fig. S1, ESI†).

As illustrated in Fig. 1 the experiment can be divided into three phases:

(a) During the "seeding phase", the device is infused with a cell suspension as shown in Fig. 1A. Cells are randomly seeded into the PLBRs with the fluid flow dragging cells through the seeding inlet into the circular reactors. Cells simply remain trapped in between the glass cover and reactor plateau, not relying on sophisticated trapping methods. Ideally each reactor is seeded

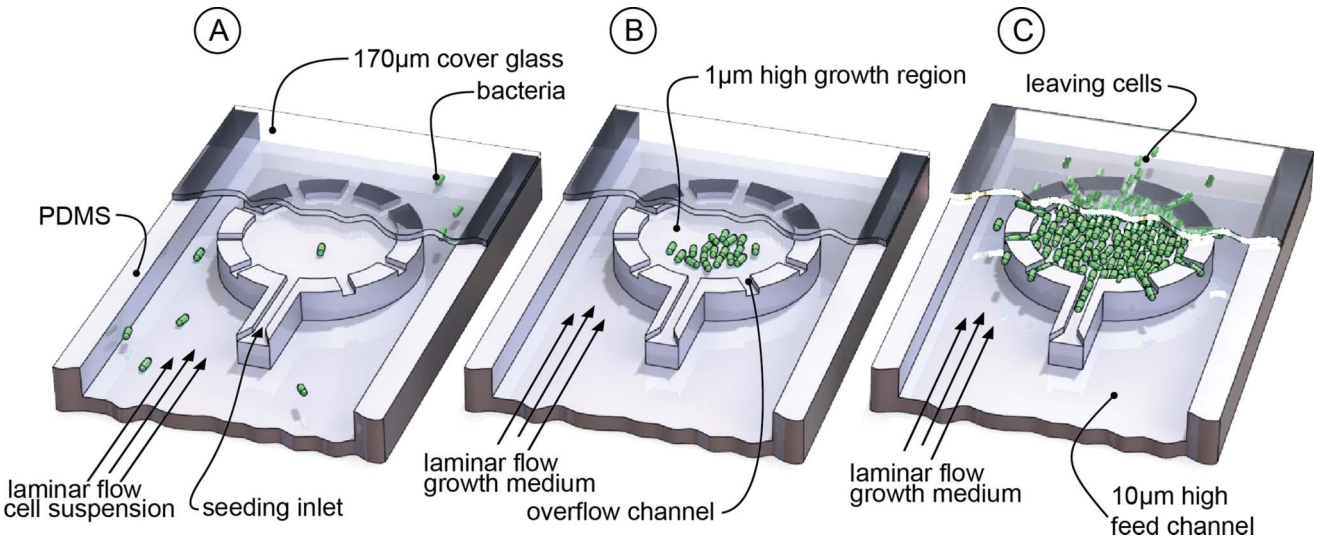

Fig. 1 Illustration of the picolitre bioreactor (PLBR) for cultivation of bacteria. The shallow circular PLBR has radially arranged channels and is placed inside a deeper supply channel. (A) During the "seeding phase" single cells are seeded into the PLBR. (B) Once a single cell is seeded, growth medium is infused initiating the "growth phase". (C) As soon as the reactor is fully packed, cells are pushed out of the overflow channels during the "overflow phase". Illustration is not to scale.

with one single mother cell, leading to isogenic microcolonies during cultivation.

(b) As soon as a single cell is seeded into the reactor, fresh growth medium is infused. This initiates the "growth phase", as illustrated in Fig. 1B. During this phase the growth of each microcolony can be followed over several generations by image based time-lapse microscopy.

(c) Depending on the size and shape of the cultivated organism, a maximum capacity of approx. 500 cells can be reached, until geometric constraints lead to the "overflow phase" of the PLBR. Excess cells are pushed out of the reactor chamber *via* the overflow channels and are continuously dragged away by the medium stream (Fig. 1C). This design allows for continuous cultivation and analysis.

Each device consists of a microfabricated polydimethylsiloxane (PDMS) chip bonded onto a 170 μm thick glass slide suitable for high resolution microscopy. Total processing time, including lithography for the mask as well as the chip fabrication, is usually accomplished within several days allowing for short innovation cycles. Since PDMS chips can be fabricated rapidly the system is designed for single use only and to be discarded after application. Hence elaborate cleaning and extensive autoclaving procedures can be omitted. A successfully fabricated device is shown in Fig. 2A. Each chip is 4 mm thick, 15 mm wide and 20 mm long. The total interior fluid volume is approximately 200 nL. As depicted, dispensing needles are connected to the device inlets and waste outlet. Six interconnected channel arrays (Fig. 2B) containing 5 PLBRs each (30 PLBRs in total) can be flushed with specific medium simultaneously. A gradient generator channel network was implemented, however, it was not utilized during this project and is intended for further studies. To verify structure resolution of the moulded PDMS chips, scanning electron microscopy (SEM) was performed prior to chip bonding with an SEM image of one PLBR shown in Fig. 2C.

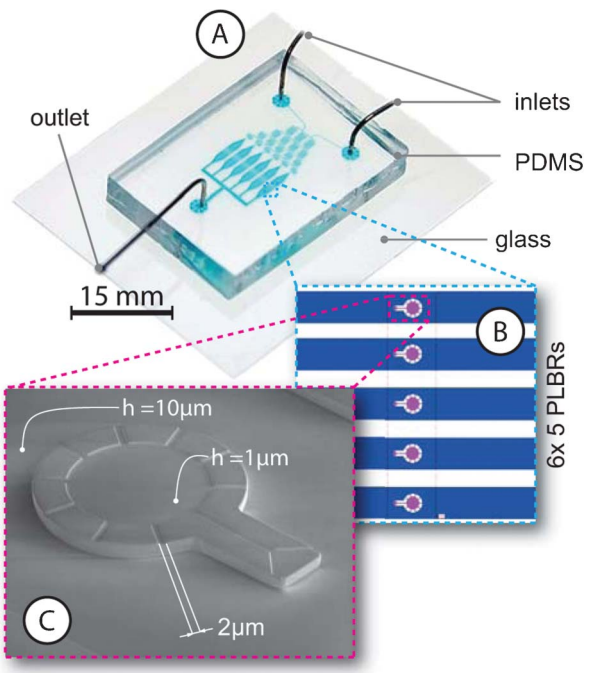

Fig. 2 Images showing the fabricated microfluidic chip device. (A) The PDMS microfluidic chip was bonded to a 170 μm glass slide and connected to silicone tubing. For purpose of illustration, the chip was filled with ink. Each chip is 4 mm × 15 mm × 20 mm (height × width × length) in size. The device consists of two inlets, one outlet, a microfluidic gradient generator for future studies and 6 linear arrays containing 5 PLBRs each (30 PLBRs in total). (B) CAD image of one PLBR array, containing 5 PLBRs in parallel. (C) SEM of a single PLBR with 1 pL cultivation volume. The height of the PLBR is approx. 1 μm and the supply channel height is approx. 10 μm. Seeding and overflow channels have a width of 2 μm.

Material and methods

Chip fabrication

To fabricate the mould for PDMS casting, a two layer SU8 process was carried out on a silicon wafer. For typical processing parameters the reader is referred to the literature.[29] In this report, only major processing steps and parameters are given.

Processing was performed under class 100 cleanroom conditions. A 4 inch silicon wafer was thoroughly cleaned in piranha solution, deionized water and eventually spin dried. Prior to resist spinning, a 30 min dehydration bake at 150 °C was performed. A 1200 nm thick layer of negative photoresist SU8 (SU8 2000.5/2010 mixture = 12 : 100, MicroChem Corp.) was spincoated onto the wafer. This layer was pre-baked for 1 min at 90 °C. To achieve the desired structure resolution, an electron beam written 5′′ lithography mask was fabricated and applied during wafer exposure. Exposure time was optimized with respect to SU8 thickness, structure resolution and mask aligner lamp intensity (SÜSS MicroTec AG, Garching, Germany), and was typically below 4 s carried out in vacuum contact mode. After a 1 min post exposure bake at 90 °C the wafer was developed in resist developer (mrdev 600, Micro Resist Technology GmbH, Germany) and eventually hard baked at 120 °C. A second SU8 layer (10 μm, SU8 2010) was spin coated onto the wafer. Lithography was carried out similar to the first layer and in accordance with the manufacturer's specifications. Processing resulted in a two layer relief structure ready for PDMS casting.

Moulding was carried out under conventional lab conditions. Polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer, Dow Corning Corporation, Midland, USA) was mixed with cross linker in a 10 : 1 ratio and degassed under slight vacuum. The liquid PDMS was cast over the mould and thermally cured for 3 h at 65 °C. The cured PDMS slab was cut into several chips. Uncured PDMS monomer residue was removed by washing the PDMS chips in pentane, acetone and eventually followed by a drying step overnight in accordance with Wang and co-workers.[27] Holes for the inlets and outlet were manually punched into the chip using sharp dispensing needles. The PDMS chip and a 170 μm thin glass plate (D263 T eco, 30 mm × 25 mm × 0.17 mm, Schott AG, Germany) were thoroughly rinsed with isopropanol ensuring sterile culturing conditions after assembly. After drying with nitrogen the PDMS chip and the glass plate were placed in oxygen plasma for 25 s at 50 watts (Femto Plasma Cleaner, Diener Electronics, Germany) and permanently bonded to the glass slide. Bonding was strengthened through two minute incubation at 65 °C. The inlet and outlet of the microfluidic chip were connected to silicone tubing (Tygon S-54-HL, ID = 0.25 mm, OD = 0.76 mm, VWR International) *via* dispensing needles (ID = 0.2 mm, OD = 0.42 mm). Chips were immediately used for microfluidic cultivation after fabrication.

Experimental setup, procedure and analysis

1 mL sterile glass syringes (ILS Innovative Labor Systeme GmbH, Germany) were used for medium supply. Medium flow control was realized with high precision syringe pumps (neMESYS, Cetoni, Germany). The chip was placed inside an in-house fabricated incubator for temperature and atmosphere control. The incubator was placed on a fully motorized inverted microscope (Nikon Eclipse Ti) suitable for time-lapse live cell imaging. In detail, the setup was equipped with a focus assistant (Nikon PFS) compensating for thermal drift during long term microscopy, Apo TIRF 100× Oil DIC N objective, NIKON DS-Vi1 colour camera, ANDOR LUCA R DL604 camera, xenon fluorescence light source for fluorescence excitation and high quality filters for the proper excitation of the chromophore EYFP and detection of its emission. Additionally, the objective was heated with an objective heater (ALA OBJ-Heater, Ala Scientific Instruments, USA).

The microfluidic device was flushed with fresh medium for 30 min prior to each cell seeding phase. A cell suspension with an optical density between 1 and 3, just transferred from a pre-culture at exponential growth phase, was infused to the system. Flow was stopped when satisfying amounts of PLBRs were seeded with a single cell. After seeding the flow was switched from bacteria suspension to growth medium, with a flow rate of approx. 10 nL min^{-1} per PLBR device (300 nL min^{-1} in total), to initiate the growth experiment. This flow rate was optimized to ensure stable trapping of the seeded mother cells inside the PLBRs. Medium switching was done manual as soon as the desired colony size was observed.

Time lapse images of individual PLBRs were typically acquired in 10 min time intervals. DIC microscopy images as well as fluorescence images were captured and analysed using the Nikon NIS Elements AR software package. Image analysis and data acquisition for the growth experiments was done as follows: the number of bacteria in each reactor chamber was counted and cell size measured manually at different time steps.

Additional methods and protocols for microfluidic experiments, bacteria sample preparation and bench-scale cultivation can be found in the ESI material and methods.†

Results and discussion

The *E. coli* strain BL21 is one of the most frequently used microbial hosts for recombinant protein production.[30] This strain is genetically modified to disable flagella functionality, and thus unable to move or migrate during cultivation. Likewise, *C. glutamicum* is one of the most important hosts for industrial amino acid production.[31–33] This bacterium is naturally non-motile. Therefore, both bacteria are suited for our PLBR single cell seeding principle, where cell migration is undesirable. Motile bacteria might actively leave the PLBR zone, and prevent proper cell counting studies.

Picolitre cultivation of *E. coli* BL21

A proof of principle experiment was performed to demonstrate the device functionality. During our microfluidic experiments *E. coli* BL21 was cultivated in complex LB-medium at 37 °C (± 0.2 °C) under aerobic conditions. We expect variations in oxygen to be minor in our device because of the high gas permeability of the PDMS, large surface area to volume ratio and the continuous influx of fresh growth medium. Recent studies suggest that even with bigger reactor chambers and more bacteria sufficient oxygen supply is guaranteed.[34,35] As expected we observed the three phases as explained in the device principle section.

(a) As depicted in Fig. 3A, the suspension of *E. coli* BL21 pre-grown in LB-medium was infused and within minutes the PLBRs were seeded with bacterial cells. Since filling was performed randomly, roughly 25% of the PLBRs were seeded with one single cell as desired. Since each device incorporates many PLBRs, enough PLBRs were available for microscopy and analysis. Chip designs with hundreds of PLBRs and improved seeding efficiency are currently under development. After switching to growth medium, a short adaption phase was observed and then the single mother cells started to grow. In Fig. 3D the first division event is resolved in more detail.

(b) During the growth phase (Fig. 3B) the microcolony could be analysed with respect to, *e.g.*, cell morphology, division rate, fluorescence related productivity, stress reactions and population heterogeneities on a single cell level. The constant environmental conditions and the possibility to induce instantaneous changes make this microfluidic system ideal for single cell analysis and bioprocess characterization. Performing image analysis, cell division was followed over several generations until the reactor was filled and eventually the overflow started, as shown in Fig. 3C.

(c) During the overflow phase cells were continuously pushed out of the reactor and eventually dragged away with the medium stream. If culture growth and cell removal are in balance, a nearly constant cell density can be maintained inside the PLBR, suitable for bioprocess studies (for a time lapse movie conforming to Fig. 3 watch Video S1, ESI†). However, depending on the organism's growth rate an ongoing increase in cell density inside the PLBR can be observed instead. Hence, in contrast to the relatively low cell density during the growth phase, the overflow phase can result in extremely high cell densities. At these high densities, individual cells are difficult to analyse. However, due to rapid chip production the overflow channels and reactor size can be easily tailored to specific requirements.

Growth quantification of *C. glutamicum*

For long-term growth rate experiments, defined constant and non-toxic environmental conditions have to be guaranteed. Therefore, it was essential to investigate the influence of our system on the physiological state of the cells, in this report indirectly measured by the growth rate. PLBR cultivation was performed with the wild type of *C. glutamicum* in minimal medium CGXII at 30 °C. For comparison the growth rates of three PLBR colonies on one chip were derived from time-lapse microscopy images by single cell counting. It can be seen in Fig. 4 that colonies grew exponentially with equivalent growth rates. It appeared that the growth curves are slightly shifted. This is potentially due to initially different cell-division cycle states of the captured "mother" cells and the adaption to the new environment. The maximum growth rate determined during our experiments was $\mu_{max} = 0.63 \pm 0.02\ h^{-1}$ ($n = 3$).

To the best of our knowledge this is the first study investigating growth of *C. glutamicum* on a single cell level in microfluidic devices. Literature values derived during typical shake flask cultivations are in the range of $\mu_{max} = 0.40\ h^{-1}$.[36] Applying a sophisticated continuously infused turbidostat bioreactor system only Baumchen *et al.* observed a nearly comparable growth rate of $\mu_{max} = 0.58\ h^{-1}$ using similar medium composition.[37]

The high PLBR growth rate of $\mu_{max} = 0.63 \pm 0.02\ h^{-1}$ supports our assumption that bacteria remain in good physiological state in

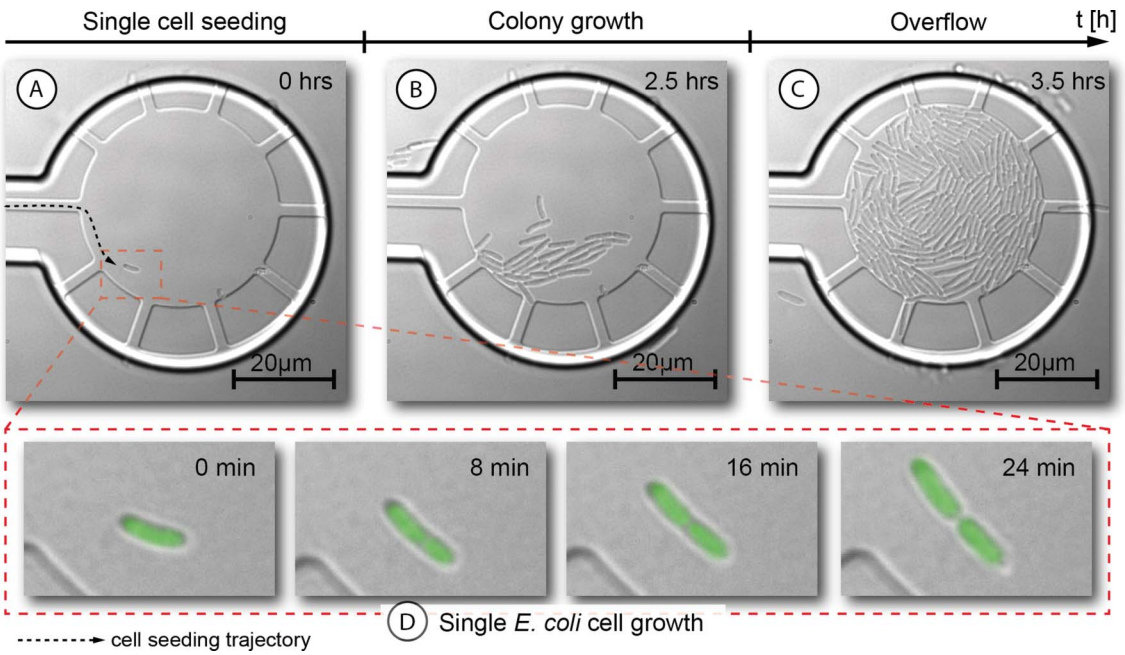

Fig. 3 Time lapse microscopy images showing the cultivation of *E. coli* BL21 inside a PLBR (see also Video S1, ESI†). (A) A single *E. coli* cell was seeded into the PLBR and complex LB growth medium was infused. (B) After 2.5 h of cultivation at $T = 37$ °C a microcolony of approx. 30 cells was formed. (C) After 3.5 h of cultivation the overflow phase was reached. Cells were pushed continuously out of the PLBR maintaining a constant density. (D) Time lapse image series showing the growth of the single mother cell after initiating the growth phase (for purpose of illustration the dividing *E. coli* cell was artificially coloured afterwards by image processing software). (A)–(C) The overflow channels have different lengths due to a slight misalignment during the two layer photolithography process. The functionality was not affected by this misalignment.

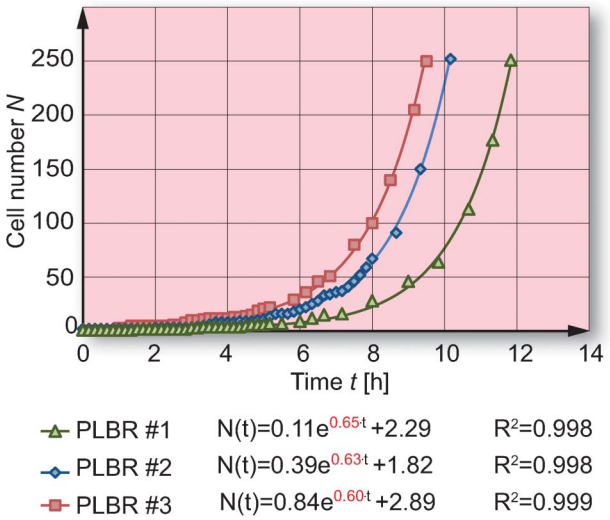

Fig. 4 Growth curves of *C. glutamicum* wild type microcolonies cultivated in three different PLBRs on one chip. Growth was followed by single cell counting of time-lapse microscopy images. The average maximal growth rate (μ_{max}) and corresponding doubling time (t_d) was determined by exponential data fitting as $\mu_{max} = 0.63 \pm 0.02\ h^{-1}$ ($n = 3$) and $t_d = 66$ min.

our microfluidic system. Actually, it also suggests that the microfluidic system offers better growth conditions than in typical lab-scale experiments. This is probably due to the continuous medium flow leading to more homogeneous conditions and the removal of secreted by-products. This aspect will be further investigated in more detailed future experiments.

Induced stationary phase during PLBR cultivation of *C. glutamicum* DM1800

In the following section, a comparison of a typical batch cultivation (1 litre) with our PLBR (10^{-12} litre) is shown. Results obtained during 1 litre cultivation will be discussed first, followed by our PLBR results.

A batch cultivation can be characterized by three phases, as illustrated in Fig. 5A. The process starts with the lag phase, in which freshly seeded cells adapt to the new environmental conditions. The lag phase is followed by the exponential growth phase where typically the maximum growth rate under the applied conditions is derived. Eventually, available nutrients are consumed and metabolic side products have increased, inducing the stationary phase. During this phase negligible growth is normally measured followed by cell degradation and cell death. Morphological variability of *C. glutamicum* has been known from prior microscopic observations, but no systematic process and time dependent investigations have been done so far.[33]

In a lab-scale batch cultivation (1 litre cultivation: for details see materials and methods in the ESI†) of the L-lysine producing strain *C. glutamicum* DM1800, cellular heterogeneity during the stationary and late stationary phase was observed (Fig. 5B). Two different sub-populations were seen as determined by the applied Coulter counter system, namely: cells larger than 1.3 μm and cells smaller than 1.3 μm in length. As depicted in Fig. 5B, over the entire cultivation time single cells larger than 1.3 μm and cells smaller than 1.3 μm in length can be observed. There is a pronounced change in the ratio of these two cell classes, with a clear predominance of small cells in the stationary phase. Similar results were obtained using *C. glutamicum* wild type (data not shown). The quantitative assessment of population heterogeneity required elaborate lab-scale cultivation, sample preparation and Coulter counter based cell counting. As can be seen in Fig. 5B, samples were taken, prepared and analysed during more than 160 h of cultivation. Despite the high relevance and interest in the observed population heterogeneity and its possible impact on industrial-scale cultivation, it becomes clear that processing time and effort is inappropriate for more detailed studies at lab-scale.

At this stage the developed microfluidic PLBR was applied to perform the same investigations during the late stationary phase. Obviously, our chip device that is continuously infused with fresh medium cannot be directly compared to a batch cultivation process. However, it can be used to artificially induce different environmental conditions within seconds simply by changing the medium and for instance mimic the stationary growth phase.

The chip was infused with the *C. glutamicum* DM1800 cells and PLBRs were seeded with single cells. The growth phase was initiated with fresh minimal medium CGXII at 30 °C. During the growth phase the measured maximal growth rate $\mu_{max\_PLBR} = 0.56 \pm 0.02\ h^{-1}$ was again significantly higher than the growth rate of $\mu_{max\_LS} = 0.37\ h^{-1}$, which was experimentally derived during the lab-scale batch cultivation described above. These findings are in accordance with previous PLBR cultivations and prove that better growth conditions can be maintained not only for the *C. glutamicum* wild type but also for the industrially utilized L-lysine producing strain DM1800. After reaching approx. 50 cells inside the PLBR, the medium was changed to a medium lacking glucose thereby artificially inducing the stationary phase. Time-lapse microscopy images revealed the same phenotypic differentiation as observed during the 1 litre lab-scale cultivation. Although no carbon source was available, the absolute cell number increased. Whereas one part of the culture almost stopped growing, the other part still continued to divide but into remarkable smaller cells, as exemplarily shown in Fig. 5C. A possible reason could be the formation of carbon storage pools like, *e.g.*, glycogen in some cells during the growth period under carbon excess. These storage pools are then used under carbon limiting conditions to continue growth for several generations. The amount of cells larger than 1.3 μm dropped notably after inducing the stationary phase, as shown in Fig. 5D. It can be seen that our PLBR system reduced the required experimental time drastically. Within 45 min of chip cultivation after inducing the stationary phase, the amount of cells smaller 1.3 μm equalled the quantity of cells larger than 1.3 μm. In contrast to that, 120 h of sheer experimental time were needed to obtain the same results at lab-scale batch cultivation. In particular the experimental time was reduced 160 fold. The slight increase of cells larger than 1.3 μm after approx. 8 h could be due to inaccurate cell measurement, as it became difficult to measure accurately 1.3 μm at densely packed colonies, as evident from Fig. 5C (12 h). Growth inside the PLBR completely stopped beyond 14 h of cultivation, which was comparable to the results obtained during the 1 litre cultivation.

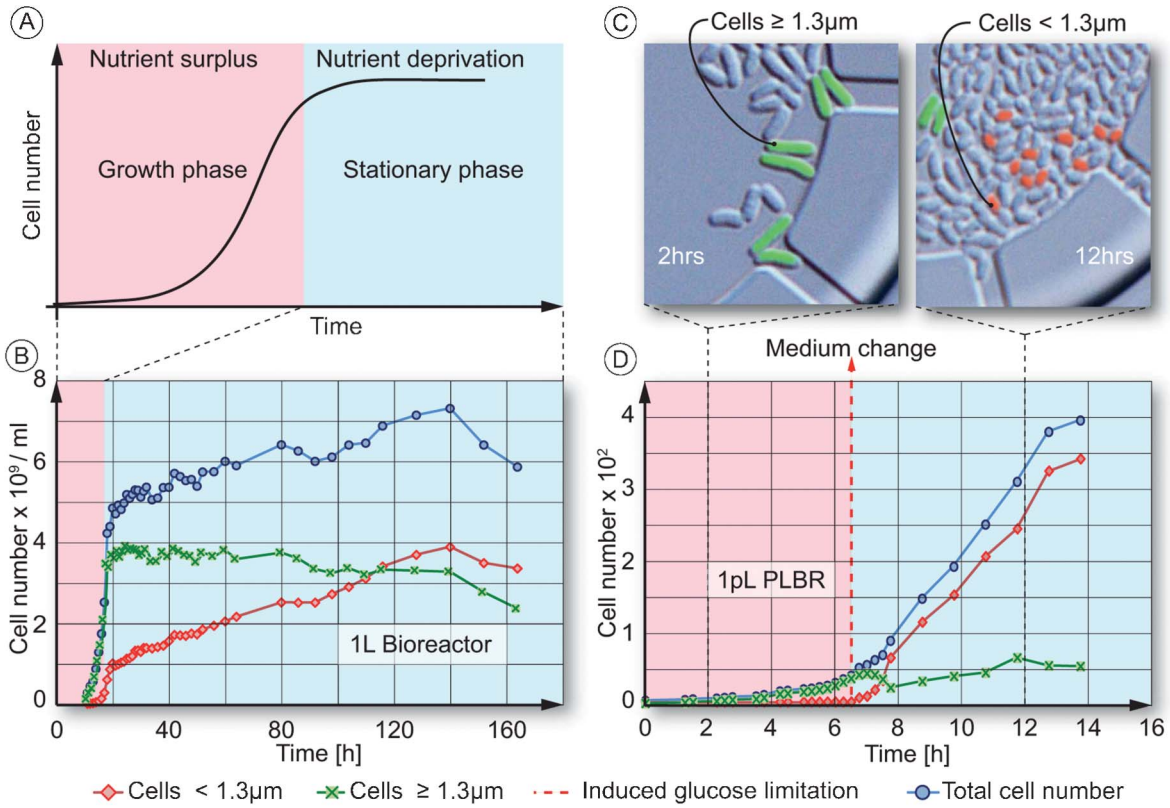

Fig. 5 Growth and morphology analysis of *C. glutamicum* DM1800 during 1 litre lab-scale batch cultivation and under PLBR cultivation after inducing an artificial stationary phase: (A) Typical growth curve during batch cultivation. (B) Experimentally derived growth curve of a 1 litre batch cultivation of *C. glutamicum* DM1800 following the exponential and late stationary growth phase. The number of small cells exceeded the number of larger cells approx. 100 h after reaching the stationary phase. (C) Time-lapse microscopy images showing the cell population at 2 h and after 12 h experimental time inside the PLBR. A few cells were artificially coloured exemplarily for purpose of illustration. (D) Experimentally derived growth curve of an isogenic microcolony inside the PLBR; The number of small cells exceeded the number of larger cells in approx. 45 min after artificially inducing the stationary phase.

Fluorescence based production studies

As described in the introduction, FACS is an ideal high-throughput system to sort and analyse microorganisms based on a fluorescence signal. However, FACS is limited to snap-shot analysis and time dependent analysis is impossible. In contrast, our microfluidic system allows fluorescence based productivity analysis on a single cell level for long time periods (here over 30 h) and tracking of individual cells is possible by image analysis.

As a proof of principle we cultivated the L-arginine producing *C. glutamicum* pSenLysTKP-argB(fbr) strain in our PLBR device. This wild type derivative contains a plasmid-encoded metabolite sensor, enabling yellow fluorescence protein (EYFP) expression in response to enhanced intracellular L-arginine concentration.[38] The plasmid contains in addition a feedback resistant acetylglutamate kinase, resulting in weak extracellular L-arginine accumulation as observed in shake flask cultivations. Using PLBR, constant environmental conditions were applied to analyse the characteristics of this strain with respect to the growth and EYFP signal. Fig. 6 shows a time-lapse image series of the isogenic microcolony inside the PLBR (see also Video S2, ESI†). Clearly an interesting change in cellular fluorescence and cell growth can be observed. It can be realized that the two cells in focus start to emit fluorescence after an adaptation phase apparently required to synthesize sufficient endogenous L-arginine to induce expression of the genetically encoded L-arginine sensor. EYFP emission continues up to 22 h, although some heterogeneity with respect to fluorescence intensity becomes apparent. With significant growth (μ_{max} = 0.46 h^{-1}) beginning after 22 h the number of fluorescent cells decreases, and at 30 h no single cell is fluorescent. A similar behaviour of fluorescence and growth was observed in additional PLBR cultivations, too, but not observed with a control carrying a vector devoid of argB(fbr). Currently, we cannot explain conclusively the time dependent fluorescence observed. One reason could be the rather weak production of the strain available. However, the experiment demonstrates the applicability of our system to investigate more complex biological phenomena such as time dependent production processes which was not possible before.

Conclusions

This report demonstrates an innovative microfluidic device for cultivation of bacteria on single cell level. A PDMS device was designed and fabricated for trapping of single bacteria cells in picolitre volume bioreactors (PLBR). The PLBR has a few important key characteristics: The system is a disposable low cost product, no intensive cleaning is required and the risk of

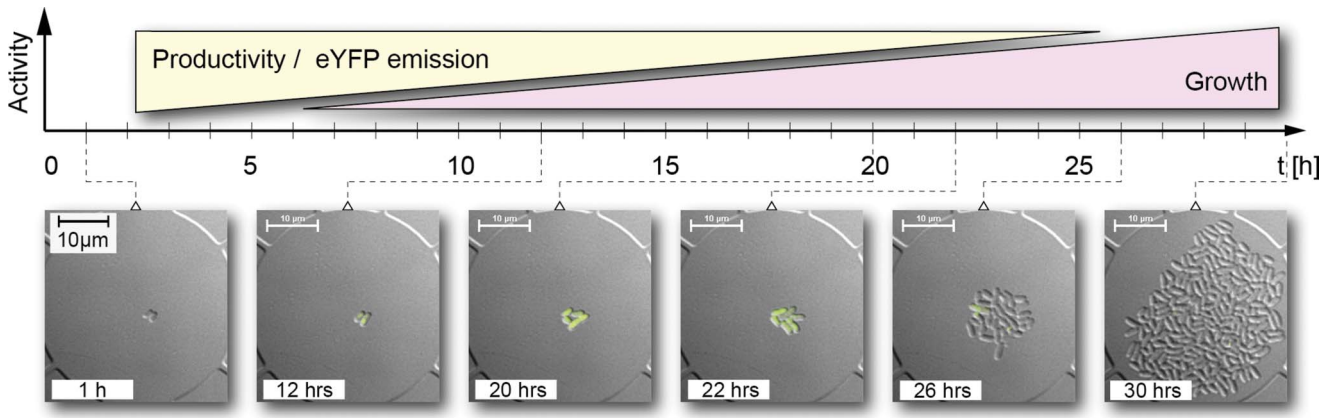

Fig. 6 Time-lapse image series showing growth and production of *C. glutamicum* pSenLysTKP-argB(fbr) during PLBR cultivation. The strain contains a metabolite sensor enabling EYFP expression in response to enhanced intracellular L-arginine concentration. The seeded mother cell starts to emit fluorescence after 12 h indicating production of L-arginine. While undergoing a change to maximum cell growth EYFP emission declined.

contamination is minimal. Due to an innovative design, bacteria are being trapped simply by the shallow bioreactor region, not relying on sophisticated technical cell trapping methods, and grow inside confined reactor regions allowing continuous analysis. Once the bioreactor is filled, radially arranged overflow channels provide well controlled cell removal not limiting experimental time. Moreover, fast medium changes facilitate to mimic every desired environmental situation of a lab-scale process. Due to picolitre volumes, low cell numbers and short response time, a fraction of experimental time is required for otherwise elaborate and time-consuming investigations. Furthermore, the high potential for parallelization makes the system an ideal tool to collect statistically trustworthy data.

The presented system is not limited to the analysis of cell growth under standard conditions, but could be applied to investigate many environmental changes, *e.g.*, medium composition, pH changes, temperature fluctuations and flow rates. Furthermore, if time-lapse images are recorded at appropriate time intervals, also division events of individual cells could be analysed in detail, rather than just counting cell populations. In-depth studies are planned to investigate population heterogeneity effects in more detail.

Additionally, the device carries great potential if combined with appropriate analytical techniques, for analysing substrate consumption and (by)-product formation of resulting micro-colonies. Hence future studies will also concentrate on quantifying the PLBR upstream regarding the cells exometabolome by untargeted (GC-TOF-MS) and targeted (LC-MS/MS) approaches as well as protein secretion by fluorescence labelling techniques.

As a final conclusion, our microfluidic PLBR is well suited for population heterogeneity studies on the single cell level and future bioprocess optimization strategies.

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