

Microbioreactors for nutrient-controlled microbial cultures: Bridging the gap between early bioprocess development and industrial scale use

Kartik Totlani¹, Rinke van Tatenhove-Pel¹, Michiel Kreutzer¹, Walter M. van Gulik¹, and Volkert van Steijn¹

¹Delft University of Technology Faculty of Applied Sciences

October 30, 2022

Abstract

It is common practice in the development of bioprocesses to genetically modify a microorganism and study a large number of resulting mutants in order to select the ones that perform best for use at the industrial scale. At industrial scale, strict nutrient-controlled growth conditions are imposed to control the metabolic activity and growth rate of the microorganism, thereby enhancing the expression of the product of interest. Although it is known that microorganisms that perform best under these strictly controlled conditions are not the same as the ones that perform best under uncontrolled batch conditions, screening, and selection is predominantly performed under batch conditions. Tools that afford high throughput on the one hand and dynamic control over cultivation conditions on the other hand are not yet available. Microbioreactors offer the potential to address this problem, resolving the gap between bioprocess development and industrial scale use. In this review, we highlight the current state-of-the-art of microbioreactors that offer the potential to screen microorganisms under dynamically controlled conditions. We classify them into: (i) microtiter plate-based platforms, (ii) microfluidic chamber-based platforms, and (iii) microfluidic droplet-based platforms. We conclude this review by discussing the opportunities of nutrient-fed microbioreactors in the field of biotechnology.

Microbioreactors for nutrient-controlled microbial cultures: Bridging the gap between early bioprocess development and industrial scale use

Kartik Totlani^a, Rinke J. van Tatenhove-Pel^b, Michiel T. Kreutzer^a, Walter M. van Gulik^b and Volkert van Steijn^{a*}

^a Department of Chemical Engineering, Faculty of Applied Sciences, Delft University of Technology, van der Maasweg 9, 2629 HZ, Delft, the Netherlands

^b Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, van der Maasweg 9, 2629 HZ, Delft, the Netherlands

Abstract

It is common practice in the development of bioprocesses to genetically modify a microorganism and study a large number of resulting mutants in order to select the ones that perform best for use at the industrial scale. At industrial scale, strict nutrient-controlled growth conditions are imposed to control the metabolic activity and growth rate of the microorganism, thereby enhancing the expression of the product of interest. Although it is known that microorganisms that perform best under these strictly controlled conditions are not the same as the ones that perform best under uncontrolled batch conditions, screening, and selection is predominantly performed under batch conditions. Tools that afford high throughput on the one hand and

dynamic control over cultivation conditions on the other hand are not yet available. Microbioreactors offer the potential to address this problem, resolving the gap between bioprocess development and industrial scale use. In this review, we highlight the current state-of-the-art of microbioreactors that offer the potential to screen microorganisms under dynamically controlled conditions. We classify them into: (i) microtiter plate-based platforms, (ii) microfluidic chamber-based platforms, and (iii) microfluidic droplet-based platforms. We conclude this review by discussing the opportunities of nutrient-fed microbioreactors in the field of biotechnology.

Keywords Fed-batch, Chemostat, Microbioreactors, Nutrient-limited growth, Industrially-relevant screening.

1. Introduction

Industrial biotechnology uses microorganisms to transform renewable resources like agricultural waste into products, resulting in more sustainable processes than the conventional chemical production from fossil feedstocks. Microorganisms naturally synthesize antibiotics, vitamins, proteins, and other valuable products, but typically in amounts insignificant for industrial scale production. Economic feasibility of bioprocesses hence hinges on the ability to enhance the performance of microorganisms. This requires modification of the microorganisms' metabolic pathways through genetic modification, either in a directed or a random way, and optimization of the cultivation conditions. Possible cultivation conditions are uncontrolled batch, controlled batch, fed-batch and chemostat (**Figure.1**). A general challenge in the development of bioprocesses is the identification of the optimum combination of modified microorganism and cultivation conditions. This requires studying the performance of a large number of modified microorganisms under dynamically controlled, nutrient-limited cultivation conditions, like fed-batch and chemostat cultures[1]. However, tools that afford high throughput as well as dynamic control over process conditions are only limitedly available.

The current bioprocess development strategy typically starts with studying a large number of modified microorganisms in the wells of microtiter plates (uncontrolled batch), with a small fraction of best performers progressing to the next phase[2]. Further selection is sequentially performed with tools that afford more control over cultivation conditions (controlled batch, fed-batch, chemostat), but have a lower throughput, as illustrated in **Figure.2**.

While microtiter plates enable high-throughput experimentation in an automated fashion[3], the low volume (typically 10 – 2,000 μL) in the wells of these plates impedes dynamic control over the cultivation conditions due to the difficulty in supplying minute amounts of nutrients to the individual wells during cultivation. Experiments in microtiter plates are hence commonly performed with all nutrients present from the start and without active control over parameters like pH and dissolved oxygen, resulting in growth of the microorganisms at the maximum possible growth rate[4]. However, these uncontrolled *batch* conditions are not comparable to the strict nutrient-limited conditions that are commonly imposed at industrial scale to control the metabolic activity and growth rate of the microorganisms[5], thereby enhancing the expression of the product of interest. Unfortunately, modified microorganisms that perform best under batch conditions are seldom the ones that perform best under nutrient-limited conditions. This has been demonstrated by Scheidle and co-workers, who compared the production of green fluorescence protein (GFP) by 224 different clones of *Hansenula polymorpha* under batch and fed-batch conditions[6]. Clearly, there is no correlation between best-producing clones under batch and fed-batch conditions, see **Figure.3(a)**. Additionally, the average yield achieved under fed-batch conditions is about 14-fold higher than that achieved under batch conditions. Similar observations, although less strikingly, were reported by Keil and co-workers[7], who compared the performance of 32 clones of cellulose producing *E.coli* under batch and fed-batch conditions, see **Figure.3(b)**. Both studies illustrate that identifying best performers under batch conditions with the purpose to use them under nutrient-limited conditions at industrial scale leads to ineffectiveness in bioprocess development.

Over the years, several technologies have been developed to address this shortcoming and transform the conventional bioprocess development strategy. The unsurpassed control over fluids in microfluidic channels offers the potential to overcome the impasse between throughput and level of control in microbioreactors[8]. These microbioreactors enable studying a large number of modified microorganisms in parallel under dy-

namically controlled conditions. An ideal microbioreactor should: (1) enable dynamic supply of nutrients and control over pH and dissolved oxygen, (2) enable integration with analytics to quantify biomass and products of interest and allow for selection and retrieval of best performers, (3) be scalable to enable high throughput experimentation, and (4) be robust and simple for easy adoption by the industry.

In this review, we present an overview of the emerging microbioreactor technologies, with the focus on nutrient feeding strategies. For more general reviews on microbioreactors or larger miniaturized bioreactors, we refer to Hemmerich *et al.* [8], Hegab *et al.* [9] and Schäpper *et al.* [10]. We start by illustrating feed strategies in microtiter plates. Next, we highlight microfluidic systems in which microorganisms are studied inside chambers. Finally, we elucidate the developments in the field of droplet-based microfluidics. After reviewing these three classes separately, we discuss the opportunities in the final section, highlighting where emerging miniaturized fermentation platforms can play a drastic role in the field of industrial biotechnology and beyond.

2. Microbioreactors for nutrient-controlled cultivation of microorganisms

2.1 Microtiter plate-based platforms

In recent years, significant effort has been made to modify microtiter plates to enable cultivation of microorganisms in fed-batch mode with passive and active control over nutrients. One strategy to continuously supply glucose to cells is by producing glucose inside the wells through enzymatic conversion of starch by glucoamylase, both added to the culture medium at the start of cultivation[15,16]. Different supply rates of glucose can be achieved by using different concentrations of the enzyme. A different strategy is to connect pairs of wells by a microchannel, with one well acting as a reservoir for nutrients, and the other as a cultivation chamber, see **Figure.4(a)** . The use of a polyacrylamide gel inside the connecting microchannel enables the slow and steady supply of nutrients to the culture well, with the feed rate depending on the diffusional properties of the gel[11]. Another strategy for nutrient release into the culture chamber is by loading wells with a silicon elastomer that contains crystals of glucose, see**Figure.4(b)** . In these so-called FeedPlates, release of glucose from the elastomer into the culture well is driven by a difference in osmotic pressure[12][17][18]. Since the silicon elastomer at the bottom of the wells hinders optical monitoring during cultivation, Habicher and co-workers[13] designed glucose-releasing rings to make the wells accessible for online monitoring, see **Figure.4(c)** . Apart from passive diffusion-based release strategies, active supply strategies have been developed such as the microfluidic BioLector system by m2p-labs[14][19][20]. **Figure.4(d)** illustrates two wells of a microtiter plate that are connected through a microchannel, with the flow from the nutrient well to the culture well actively controlled through pneumatic actuation. Controlled dosage is achieved by first filling the pump chamber with nutrient solution through opening and closing parts of the microchannel with an integrated micropump that inflates/deflates an elastic membrane, and subsequently emptying the pump chamber into the culture well. This technology allows fed-batch fermentations with a predetermined feeding profile[14]. Besides, it enables actively pH-controlled cultivations. A commercially available modified microtiter plate system (Micro-matrix) developed by Applikon Biotechnology (<http://www.applikon-bio.com>, Delft, the Netherlands) offers 24 parallel fed-batch fermentations with the possibility to feed nutrients whilst controlling parameters such as pH, temperature, and dissolved oxygen. While some of the modified microtiter plates discussed above integrate microfluidic channels, microbioreactors constructed as complete microfluidic devices present an interesting alternative and are discussed next.

2.2 Microfluidic chamber-based platforms

One of the first microfluidic bioreactors for carrying out nutrient-controlled cultivation was developed by Balagaddé and co-workers[21], who made a microfluidic chemostat. The design of one of the six microfluidic circuits integrated onto a single chip is shown in **Figure.5(a)** . Cells are continuously circulated in the growth chamber (loop) through the use of an integrated peristaltic pump constructed from pneumatically actuated membrane valves, also known as Quake valves. A solution of nutrients is periodically pumped into the growth chamber by opening and closing parts of the circuit using such valves, while effluent is removed from the growth chamber. This allows continuous cultivation of cells under nutrient-limited conditions, with

the cell growth rate directly controlled by the rate at which the chamber volume is refreshed by the nutrient solution (the dilution rate). An alternative strategy that does not require the integration of peristaltic pumps to supply nutrients and induce mixing was presented by Jensen[27]. They developed a microreactor with an external syringe pump for nutrient supply, while reactor effluent was collected in a pressurized water reservoir and mixing in the culture chamber was achieved by a ringed magnetic stir needle.

In addition to chemostats, fed-batch microreactors were developed. An example by Bower and co-workers[22] is shown in **Figure.5(b)**. The device comprises of three independent input channels which are connected to growth chambers via pressurized fluid reservoirs. The fed-batch process is achieved by partially filling up the chambers with cell solution, followed by the periodic supply of nutrients to the cells in the chambers through the actuation of the on-chip valves, until the maximum working volume is occupied, and the fed-batch process is complete.

A different strategy that requires less advanced integrated micro-pumps and valves is to culture cells inside perfusable chambers. An example of such a device, developed by Groisman and co-workers[23], is shown in **Figure.5(c)**. The chambers in which the cells are trapped are perfused by two surrounding nutrient supply channels. The shallow vias that connect the chambers to the supply channels ensure fluid to be exchanged, while cells remain trapped. The rate of diffusion of nutrients through these vias is much faster than the rate of nutrient consumption by the cells, such that the nutrient concentration in the chamber equals that in the supply channels, allowing direct control over nutrient-limited growth conditions. The use of an elastomeric material such as PDMS provides the means to load cells into the chambers, by injecting a cell solution into the device and subsequently pressurizing the device allowing cells to enter the chambers through the inflated vias. As cells remain trapped inside the chambers and do not leave as effluent, this type of perfusable device results in chemostat-like cultivation.

Continuous regulation of the number of cells inside the chambers during cultivation can be achieved by controlled inflation of the vias. This can be done by depressurizing a separate channel above the chambers, see **Figure.5(d)**. Automatic dilution of cells is then achieved using a feedback loop, with the pneumatic actuation controlled based on online measurements of the number of cells in the chambers[24][28]. While the primary feed strategy in the above two examples is based on diffusion, flow-through chambers have also been developed, notably by Grunberger and co-workers[25]. Cells are trapped inside shallow chambers located in a main channel, while nutrient solution is flown around and through the chamber, as illustrated in **Figure.5(e)**. Nutrient-limited growth conditions are primarily controlled through the concentration of nutrient solution, supplied using an external pump. The shallow nature of the chambers facilitates cells to remain trapped and to grow in a two-dimensional fashion, enabling accurate monitoring at single cell resolution. Besides in- and outflow, the perforations in the chambers also allow cells to leave the chambers once they are populated. Similar strategies even enable studies on co-cultures of cells in chemostat-like conditions, see **Figure.5(f)** [26]. For extensive reviews on applying chamber-based microfluidic devices for studying morphology, heterogeneity, growth, and communication of microorganisms in a high-throughput manner and at single-cell resolution, we refer to [29-31].

2.3 Microfluidic droplet-based platforms

The potential to use droplets as cultivation environments has been outlined decades ago[41]. The precise generation and control of droplets in microfluidic devices led to the development of droplet-based microreactors. Most efforts so far focus on batch processes, with all components encapsulated at the start. The typical workflow then comprises the generation of millions of droplets with cells and nutrients encapsulated, incubation of the droplets off-chip, and reinjection in a separate chip to analyse and sort the droplets[42-44]. While providing a high throughput, this type of workflow with off-chip incubation makes it cumbersome to periodically supply nutrients to all individual droplets and turn the system into a fed-batch or a chemostat[45]. An alternative workflow that may facilitate fed-batch or chemostat cultivation is based on on-chip (or in-tube) incubation. While operations on droplets can be precisely performed when operating microfluidic devices under steady state conditions, the inherent non-steady nature of such microreactors with different droplet operations to enable regular nutrient supply nevertheless makes their development an outstanding

challenge.

Jakiela and co-workers[32] presented the first example of a droplet-based chemostat by developing a device that comprises different circuits that can be isolated from each other with the use of off-chip solenoid valves. Cells were encapsulated inside droplets, which were transported back and forth in the main channel of the device. Each droplet regularly entered a circuit in which effluent was removed from the droplets through controlled break-up, as illustrated in the top of **Figure.6(a)** . The resulting droplets were supplied with nutrients in another circuit in which they were coalesced with nutrient droplets that were generated on demand, as illustrated in the bottom of **Figure.6(a)**. More recently, Jian and co-workers[33] developed a similar automated droplet-based chemostat, including a sorting step based on monitoring biomass growth via OD measurements (see **Figure.6(b)**).

Droplet-based fed-batch reactors can be made by spatially immobilizing the cell-containing droplets and supplying them with nutrients. One of the first examples of such a strategy was by presented by Leung and co-workers[34]. The device comprises 95 chambers, which can all be individually addressed through the pneumatic actuation of integrated Quake valves. After loading a cell-containing droplet inside each chamber of this multiplex device, nutrient droplets can be generated on demand and guided to the chambers as illustrated in **Figure.6(c)** , enabling studies under fed-batch conditions. A similar strategy based on immobilization of droplets in chambers and controlled supply of reagents through pneumatic actuation of integrated valves has been used by the group of Chang-Soo Lee[35,46,47] for the cultivation of cells and the development of bio-chemical assays, see **Figure.6(d)**).

The above examples show a trend towards enhanced control over cultivation environment by developing sophisticated devices with integrated/external valves, operated through multi-step actuation schemes. There is also another, almost opposite, trend visible in literature, in which control is achieved using (passive) geometrical features to keep device architecture and operation as simple as possible. An elegant example was demonstrated by Ismagilov and co-workers[36][48], who made a droplet-based fed-batch by compartmentalizing droplets inside chambers, which can be joined by manually sliding the top and bottom half of these so-called *Slip Chips* towards each other, as illustrated in **Figure.6(e)** . Baroud and co-workers[37] explored the use of cavities in the floor/ceiling of a microchannel in which droplets squeezed between the floor and ceiling can relax their shape, and thereby remain trapped. These cavities can be designed such that each allows immobilization of a cell-containing droplet, while leaving room for another droplet to be loaded for controlled supply, as illustrated in the left panel of **Figure.6(f)** . After loading those droplets (middle panel), supply is achieved by imposing coalescence (right panel). This is done by flowing a solvent through the channel in which the surfactant used for stabilization of the interfaces is less soluble. This strategy so far has been used for drug toxicity studies of cells with a single delivery of drugs[37]. Whether it can be used for repeated supply of nutrients to enable nutrient-controlled growth experiments under fed-batch conditions is yet to be explored. A recent example of a droplet-based fed-batch microreactor that does allow the repeated and controlled supply of nutrients to a cell-containing droplet immobilized in a chamber was demonstrated in the lab of the authors[39]. The design of the geometry in which the nutrient droplets are produced allows a robust periodic on-demand supply of droplets in a device free of valves, just through the use of a commercially available pressure pump[38], see **Figure.6(g)** . The authors demonstrated the cultivation of cells under fed-batch conditions, with the growth rate of the cells inside the immobilized cell-containing droplet controlled by the concentration of the nutrient-containing droplets.

Besides the droplet-based approaches in microfluidic channels, we conclude by highlighting a channel-free fed-batch approach in which cell-containing droplets are bio-printed in a yield-stress fluid. Nelson and co-workers[40] developed a bio-printing method to study the response to drugs injected into the droplets after 24h of incubation, see **Figure.6(h)** . Since the print-head enables injection of nutrients or extraction of effluent, this relative unexplored strategy is also potentially interesting for high-throughput studies of microorganisms under nutrient-controlled growth conditions.

3. Challenges of microreactors technologies

3.1 Microtiter plate-based platforms

While different modifications to existing microtiter plate-based platforms enable carrying out nutrient-controlled fed-batch cultivation experiments at microscale, there are several challenges that these platforms face before they are ready for adoption in biotechnological screening routines. Firstly, enzymatic glucose release-based systems are strongly influenced by operation parameters such as pH and temperature as the activity of the enzyme depends on them. Additionally, enzymatic release can only be used for feeding of glucose-controlled fed-batch cultivations. Diffusion-based feeding also depends on environmental factors such as media, pH, temperature, and geometrical factors as in the case of PAA filled microchannel [11]. Establishing specific nutrient feeding profiles is difficult in diffusion-based release strategies, let alone establishing active control with online feedback. Additionally, multiple glucose crystals can release due to the osmotic pressure difference leading to uneven glucose concentration due to this burst release. Finally, embedding microfluidic channels at the bottom of the standard microtiter plate as in the case of Microfluidic BioLector[14] poses difficulties in fabrication. The combination of Microtiter plates and microfluidic channels replacing the base of the plate with embedded micro-pumps and valves increases the complexity of the system. Additionally, systems like Microfluidic Biolector offer a maximum of 44 to 48 fed-batch experiments per plate, which could be treated as relatively low-throughput in screening routines.

3.2 Microfluidic chamber-based platforms

A challenge of microfluidic chamber-based platforms arises from the large surface to volume ration when miniaturizing, leading to biofilm formation at the solid walls. Other challenges arise from the chambers not being completely isolated, which may lead to cross contamination or to difficulties in screening based on secreted extracellular products. Besides, the large number of inlet ports required for operation in some of the platforms poses difficulty in scale out and accessibility of the technology to non-experts.

3.3 Microfluidic droplet-based platforms

A challenge of microfluidic droplet-based platforms is associated with the analytics and process control in microdroplet format. On-line measuring of process parameters such as pH, dissolved oxygen, nutrient or metabolite concentration, which forms the heart of any screening routine, can be difficult. One way to achieve this is by using different fluorescent-based readouts. Further on, implementing control over dissolved oxygen and assuring that the cultivation does not run under oxygen limitations can be a challenge. Fluorinated oils which often serve as the continuous phase can be used as oxygen source during cultivation[49]. Another challenge involves creating a simple and robust nutrient feeding strategy, making the technology accessible to non-experts[50]. As aforementioned, microfluidic droplet arrays are useful in carrying out non-steady assays in which nutrients can be added semi-continuously. However, these droplet arrays use multiple membrane based pneumatic valves which make the devices less robust, difficult to fabricate and possibly inhibit its easy adaptation by biotechnology and bioprocess engineering community. The barrier of adoption of droplet microfluidic methods by non-experts can be reduced by embedding the complicated chip operation workflow and associated experimental paraphernalia in “chip-in a box” type of systems[51]. Another challenge in implementation of droplet-based microfluidic platforms for long-term nutrient-limited fermentation is the possible leakage of molecules through the interface of the droplets[52][53]. Leakage of nutrients or secreted metabolites could not just lead to uneven growth rates but also selection of false positives during strain selection. Several studies have been performed to investigate mass transport through the oil-water interphase where droplets incubate inside PDMS devices. The nature of the molecules inside the droplets and the surfactants at the oil-water interphase are hypothesized as the most important parameter in understanding and controlling the leakage of molecules through microdroplets.

4. Opportunities for emerging microbioreactor technologies

Nutrient-limited microbioreactors can play a key role in the development of sustainable industrial biotechnology. Their small volume and therefore the possibility for high throughput-screening can accelerate the identification of robust and productive strains[54], while screening under nutrient-limited conditions ensures that industrially relevant strains are identified[6][7]. In addition, the screening-process itself is also more

sustainable and cheaper, as less reagents and consumables are needed[55].

The nutrient-feeding strategies described in this review also allow separation of growth- and production-phases by changing the medium, something that is not possible in batch-cultures. This is for example relevant for lipid-production in algae, where different media compositions for growth (stage 1) and lipid-production (stage 2) are used to improve lipid-production[56]. This separation of growth- and production is also relevant for the production of recombinant protein, as protein-production is typically induced after growth, by adding compounds like isopropyl β -d-1-thiogalactopyranoside (IPTG)[57]. To ensure selection of industrially relevant mutants, it is essential to incorporate these media-changes already at the start of the screening-procedure.

In the chemostat-like microbioreactors, the ability to remove part of the medium and the cells from the microbioreactor can also be used to miniaturize sequential batch evolution experiments. When the cells are kept in the exponential phase, such experiments select for mutants with a higher maximum growth rate in the imposed condition and are for example used to adapt cells to high concentrations of toxic compounds and low pH values[58]. When the cells are pulse-fed, sequential batch reactors can be used to select for mutants that produce large amounts of storage-compounds[59]. Miniaturizing such experiments can increase the number of parallel experiments that can be done, while decreasing the costs.

Overall, microbioreactors with nutrient-feeding strategies allow for high-throughput and low-cost cultivation in a broad range of cultivation set-ups (batch, fed-batch, chemostat, but also retentostat and sequential batch). Therefore, they have a high potential as new cultivation-tool for screening and selecting mutants in industrial biotechnology.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- [1] P. Neubauer, N. Cruz, F. Glauche, S. Junne, A. Knepper, and M. Raven, Consistent development of bioprocesses from microliter cultures to the industrial scale, *Engineering in Life Sciences*, vol. 13, no. 3, pp. 224-238, 2013.
- [2] S. Parekh, V. Vinci, and R. Strobel, Improvement of microbial strains and fermentation processes, *Applied microbiology and biotechnology*, vol. 54, no. 3, pp. 287-301, 2000.
- [3] W. Zeng, L. Guo, S. Xu, J. Chen, and J. Zhou, High-throughput screening technology in industrial biotechnology, *Trends in biotechnology*, 2020.
- [4] Q. Long, X. Liu, Y. Yang, L. Li, L. Harvey, B. McNeil, and Z. Bai, The development and application of high throughput cultivation technology in bioprocess development, *Journal of biotechnology*, vol. 192, pp. 323-338, 2014.
- [5] H. C. Lim and H. S. Shin, *Fed-batch cultures: principles and applications of semi-batch bioreactors*. Cambridge University Press, 2013.
- [6] M. Scheidle, M. Jeude, B. Dittrich, S. Denter, F. Kensy, M. Suckow, D. Klee, and J. Büchs, High-throughput screening of *Hansenula polymorpha* clones in the batch compared with the controlled-release fed-batch mode on a small scale, *FEMS Yeast Research*, vol. 10, no. 1, pp. 83-92, 2009.
- [7] T. Keil, M. Landenberger, B. Dittrich, S. Selzer, and J. Büchs, Precultures grown under fed-batch conditions increase the reliability and reproducibility of high throughput screening results, *Biotechnology journal*, vol. 14, no. 11, p. 1800727, 2019.
- [8] J. Hemmerich, S. Noack, W. Wiechert, and M. Oldiges, Microbioreactor systems for accelerated bioprocess development, *Biotechnology journal*, vol. 13, no. 4, p. 1700141, 2018.

- [9] H. M. Hegab, A. ElMekawy, and T. Stakenborg, Review of microfluidic microbioreactor technology for high throughput submerged microbiological cultivation, *Biomicrofluidics*, vol. 7, no. 2, p. 021502, 2013.
- [10] D. Schäpper, M. N. H. Z. Alam, N. Szita, A. E. Lantz, and K. V. Gernaey, Application of microbioreactors in fermentation process development: a review, *Analytical and bioanalytical chemistry*, vol. 395, no. 3, pp. 679-695, 2009.
- [11] A. Wilming, C. Bähr, C. Kamerke, and J. Büchs, Fed-batch operation in special microtiter plates: a new method for screening under production conditions, *Journal of Industrial Microbiology and Biotechnology*, vol. 41, no. 3, pp. 513-525, 2014.
- [12] T. Keil, B. Dittrich, C. Lattermann, T. Habicher, and J. Büchs, Polymer-based controlled-release fed-batch microtiter plate{diminishing the gap between early process development and production conditions, *Journal of biological engineering*, vol. 13, no. 1, pp. 1-15, 2019.
- [13] T. Habicher, V. Czotscher, T. Klein, A. Daub, T. Keil, and J. Büchs, Glucose containing polymer rings enable fed-batch operation in microtiter plates with parallel online measurement of scattered light, fluorescence, dissolved oxygen tension, and pH., *Biotechnology and bioengineering*, vol. 116, no. 9, pp. 2250-2262, 2019.
- [14] M. Funke, A. Buchenauer, U. Schnakenberg, W. Mokwa, S. Diederichs, A. Mertens, C. Müller, F. Kesy, and J. Büchs, Microfluidic BioLector-microfluidic bioprocess control in microtiter plates, *Biotechnology and bioengineering*, vol. 107, no. 3, pp. 497-505, 2010.
- [15] J. Panula-Perälä, J. Šiurkus, A. Vasala, R. Wilmanowski, M. G. Casteleijn, and P. Neubauer, Enzyme controlled glucose auto-delivery for high cell density cultivations in microplates and shake flasks, *Microbial Cell Factories*, vol. 7, no. 1, pp. 1-12, 2008.
- [16] C. Toeroek, M. Cserjan-Puschmann, K. Bayer, and G. Striedner, Fed-batch like cultivation in a micro-bioreactor: screening conditions relevant for *Escherichia coli*-based production processes, *Springer Plus*, vol. 4, no. 1, pp. 1-10, 2015.
- [17] T. Keil, B. Dittrich, C. Lattermann, and J. Büchs, Optimized polymer-based glucose release in microtiter plates for small-scale *e. coli* fed-batch cultivations, *Journal of biological engineering*, vol. 14, no. 1, pp. 1-12, 2020.
- [18] T. Habicher, E. K. Rauls, F. Egidi, T. Keil, T. Klein, A. Daub, and J. Büchs, Establishing a fed-batch process for protease expression with *bacillus licheniformis* in polymer-based controlled-release microtiter plates, *Biotechnology journal*, vol. 15, no. 2, p. 1900088, 2020.
- [19] A. Buchenauer, M. Hofmann, M. Funke, J. Büchs, W. Mokwa, and U. Schnakenberg, Micro-bioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices, *Biosensors and Bioelectronics*, vol. 24, no. 5, pp. 1411-1416, 2009.
- [20] M. Funke, A. Buchenauer, W. Mokwa, S. Kluge, L. Hein, C. Müller, F. Kesy, and J. Büchs, Bioprocess control in microscale: scalable fermentations in disposable and user-friendly microfluidic systems,” *Microbial cell factories*, vol. 9, no. 1, pp. 1-13, 2010.
- [21] F. K. Balagaddé, L. You, C. L. Hansen, F. H. Arnold, and S. R. Quake, Long-term monitoring of bacteria undergoing programmed population control in a microchemostat, *Science*, vol. 309, no. 5731, pp. 137-140, 2005.
- [22] Z. Zhang, P. Boccazzi, H.-G. Choi, G. Perozziello, A. J. Sinskey, and K. F. Jensen, Microchemostat-microbial continuous culture in a polymer-based, instrumented microbioreactor, *Lab on a Chip*, vol. 6, no. 7, pp. 906-913, 2006.
- [23] D. M. Bower, K. S. Lee, R. J. Ram, and K. L. Prather, Fed-batch microbioreactor platform for scale down and analysis of a plasmid DNA production process, *Biotechnology and bioengineering*, vol. 109, no. 8,

pp. 1976-1986, 2012.

- [24] A. Groisman, C. Lobo, H. Cho, J. K. Campbell, Y. S. Dufour, A. M. Stevens, and A. Levchenko, A microfluidic chemostat for experiments with bacterial and yeast cells, *Nature methods*, vol. 2, no. 9, pp. 685-689, 2005.
- [25] M. Kim, J. W. Lim, S. K. Lee, and T. Kim, Nanoscale hydrodynamic film for diffusive mass transport control in compartmentalized microfluidic chambers, *Analytical chemistry*, vol. 89, no. 19, pp. 10286-10295, 2017.
- [26] A. Grünberger, N. Paczia, C. Probst, G. Schendzielorz, L. Eggeling, S. Noack, W. Wiechert, and D. Kohlheyer, A disposable picolitre bioreactor for cultivation and investigation of industrially relevant bacteria on the single cell level, *Lab on a chip*, vol. 12, no. 11, pp. 2060-2068, 2012.
- [27] A. Burmeister, F. Hilgers, A. Langner, C. Westerwalbesloh, Y. Kerkho, N. Tenhaef, T. Drepper, D. Kohlheyer, E. von Lieres, S. Noack, A microfluidic co-cultivation platform to investigate microbial interactions at de ned microenvironments, *Lab on a Chip*, vol. 19, no. 1, pp. 98-110, 2019.
- [28] M. Kim, J. Bae, and T. Kim, Long-term and programmable bacterial subculture in completely automated microchemostats, *Analytical chemistry*, vol. 89, no. 18, pp. 9676-9684, 2017.
- [29] A. Grünberger, W. Wiechert, and D. Kohlheyer, Single-cell microfluidics: opportunity for bioprocess development, *Current opinion in biotechnology*, vol. 29, pp. 15-23, 2014.
- [30] A. Burmeister and A. Grünberger, Microfluidic cultivation and analysis tools for interaction studies of microbial co-cultures, *Current opinion in biotechnology*, vol. 62, pp. 106-115, 2020.
- [31] N. R. Wright, N. P. Rnnest, and N. Sonnenschein, Single-cell technologies to understand the mechanisms of cellular adaptation in chemostats, *Frontiers in Bioengineering and Biotechnology*, vol. 8, p. 1460, 2020.
- [32] S. Jakiela, T. S. Kaminski, O. Cybulski, D. B. Weibel, and P. Garstecki, Bacterial growth and adaptation in microdroplet chemostats, *Angewandte Chemie*, vol. 125, no. 34, pp. 9076-9079, 2013.
- [33] X. Jian, X. Guo, J. Wang, Z. L. Tan, X.-h. Xing, L. Wang, and C. Zhang, Microbial microdroplet culture system (mmc): An integrated platform for automated, high-throughput microbial cultivation and adaptive evolution, *Biotechnology and bioengineering*, vol. 117, no. 6, pp. 1724-1737, 2020.
- [34] K. Leung, H. Zahn, T. Leaver, K. M. Konwar, N. W. Hanson, A. P. Page, C.-C. Lo, P. S. Chain, S. J. Hallam, and C. L. Hansen, A programmable droplet based microfluidic device applied to multiparameter analysis of single microbes and microbial communities, *Proceedings of the National Academy of Sciences*, vol. 109, no. 20, pp. 7665-7670, 2012.
- [35] S. H. Jin, H.-H. Jeong, B. Lee, S. S. Lee, and C.-S. Lee, A programmable microfluidic static droplet array for droplet generation, transportation, fusion, storage, and retrieval, *Lab on a Chip*, vol. 15, no. 18, pp. 3677- 3686, 2015.
- [36] D. V. Zhukov, E. M. Khorosheva, T. Khazaei, W. Du, D. A. Selck, A. A. Shishkin, and R. F. Ismagilov, Microfluidic SlipChip device for multistep multiplexed biochemistry on a nanoliter scale, *Lab on a Chip*, vol. 19, no. 19, pp. 3200 - 3211, 2019.
- [37] R. F.-X. Tomasi, S. Sart, T. Champetier, and C. N. Baroud, Individual control and quantification of 3d spheroids in a high-density microfluidic droplet array, *Cell reports*, vol. 31, no. 8, p. 107670, 2020.
- [38] K. Totlani, J.-W. Hurkmans, W. M. Van Gulik, M. T. Kreutzer, and V. Van Steijn, Scalable microfluidic droplet on-demand generator for non-steady operation of droplet-based assays, *Lab on a Chip*, vol. 20, no. 8, pp. 1398-1409, 2020.
- [39] K. Totlani, Y.-C. Wang, M. Bisschops, T. de Riese, M. T. Kreutzer, W. M. van Gulik, and V. van Steijn, Fed-batch droplet nanobioreactor for controlled growth of *Cyberlindnera (Pichia) jadinii*: A proof-of-concept

demonstration,” *Advanced Materials Technologies*, vol. 6, no. 9, p. 2100083, 2021.

[40] A. Z. Nelson, B. Kundukad, W. K. Wong, S. A. Khan, and P. S. Doyle, Embedded droplet printing in yield-stress fluids, *Proceedings of the National Academy of Sciences*, vol. 117, no. 11, pp. 5671 - 5679, 2020.

[41] J. Lederberg, A simple method for isolating individual microbes, *Journal of bacteriology*, vol. 68, no. 2, p. 258, 1954.

[42] L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths, and J. A. Heyman, Single-cell analysis and sorting using droplet-based microfluidics, *Nature protocols*, vol. 8, no. 5, pp. 870- 891, 2013.

[43] M. Huang, Y. Bai, S. L. Sjöström, B. M. Hallström, Z. Liu, D. Petranovic, M. Uhlen, H. N. Joensson, H. Andersson-Svahn, and J. Nielsen, Microfluidic screening and whole-genome sequencing identifies mutations associated with improved protein secretion by yeast, *Proceedings of the National Academy of Sciences*, vol. 112, no. 34, pp. E4689-E4696, 2015.

[44] H. S. Kim, A. R. Guzman, H. R. Thapa, T. P. Devarenne, and A. Han, A droplet microfluidics platform for rapid microalgal growth and oil production analysis, *Biotechnology and bioengineering*, vol. 113, no. 8, pp. 1691-1701, 2016.

[45] B. L. Wang, A. Ghaderi, H. Zhou, J. Agresti, D. A. Weitz, G. R. Fink, and G. Stephanopoulos, Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption, *Nature biotechnology*, vol. 32, no. 5, p. 473, 2014.

[46] B. Lee, S. H. Jin, Y.-M. Noh, S.-G. Jeong, H.-H. Jeong, and C.-S. Lee, Scalable static droplet array for biochemical assays based on concentration gradients, *Sensors and Actuators B: Chemical*, vol. 273, pp. 1572-1578, 2018.

[47] H.-H. Jeong, B. Lee, S. H. Jin, and C.-S. Lee, Hydrodynamic control of droplet breakup, immobilization, and coalescence for a multiplex microfluidic static droplet array, *Chemical Engineering Journal*, vol. 360, pp. 562-568, 2019.

[48] W. Du, L. Li, K. P. Nichols, and R. F. Ismagilov, Slipchip, *Lab on a Chip*, vol. 9, no. 16, pp. 2286 - 2292, 2009.

[49] L. Mahler, M. Tovar, T. Weber, S. Brandes, M. M. Rudolph, J. Ehgartner, T. Mayr, M. T. Figge, M. Roth, and E. Zang, Enhanced and homogeneous oxygen availability during incubation of microfluidic droplets, *RSC advances*, vol. 5, no. 123, pp. 101871-101878, 2015.

[50] T. S. Kaminski, O. Scheler, and P. Garstecki, Droplet microfluidics for microbiology: techniques, applications and challenges, *Lab on a Chip*, vol. 16, no. 12, pp. 2168- 2187, 2016.

[51] V. Ortseifen, M. Viefhues, L. Wobbe, and A. Grünberger, Microfluidics for biotechnology: Bridging gaps to foster microfluidic applications, *Frontiers in Bioengineering and Biotechnology*, vol. 8, p. 1324, 2020.

[52] G. Etienne, A. Vian, M. Biocanin, B. Deplancke, and E. Amstad, Cross-talk between emulsion drops: how are hydrophilic reagents transported across oil phases? *Lab on a Chip*, vol. 18, no. 24, pp. 3903 - 3912, 2018.

[53] P. Gruner, B. Riechers, B. Semin, J. Lim, A. Johnston, K. Short, and J.-C. Baret, Controlling molecular transport in minimal emulsions, *Nature communications*, vol. 7, no. 1, pp. 1-9, 2016.

[54] Straathof, A.J., Wahl, S.A., Benjamin, K.R., Takors, R., Wierckx, N. and Noorman, H.J., Grand research challenges for sustainable industrial biotechnology, *Trends in biotechnology*, vol. 37, no. 10, pp. 1042-1050, 2019.

[55] Agresti, J.J., Antipov, E., Abate, A.R., Ahn, K., Rowat, A.C., Baret, J.C., Marquez, M., Klibanov, A.M., Griffiths, A.D. and Weitz, D.A, Ultrahigh-throughput screening in drop-based microfluidics for directed evolution, *Proceedings of the National Academy of Sciences*, vol.107, no. 9, pp. 94004-4009, 2010.

[56] Okoro, V., Azimov, U., Munoz, J., Hernandez, H.H. and Phan, A.N., Microalgae cultivation and harvesting: Growth performance and use of flocculants-A review, *Renewable and Sustainable Energy Reviews*, vol. 115, pp. 109364, 2019.

[57] Khani, M.H. and Bagheri, M., Skimmed milk as an alternative for IPTG in induction of recombinant protein expression, *Protein expression and purification*, vol. 170, pp. 105593, 2020.

[58] Mans, R., Daran, J.M.G. and Pronk, J.T., Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production, *Current opinion in biotechnology*, vol. 50, pp. 47-56, 2018.

[59] Stouten, G.R., Hogendoorn, C., Douwenga, S., Kiliyas, E.S., Muyzer, G. and Kleerebezem, R., Temperature as competitive strategy determining factor in pulse-fed aerobic bioreactors. *The ISME journal*, vol. 13, no. 12, pp. 3112-3125, 2019.

Figure legends:

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6







