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## Bridging the gap between bioprocess development and industrial use

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REVIEW

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

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## 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
chemostat, fed-batch, industrially-relevant screening, microbioreactors, nutrient-limited growth

## 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

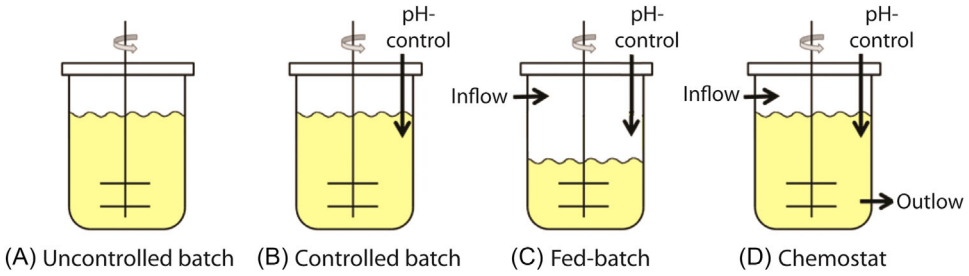


**FIGURE 1** Schematic overview of different cultivation methods. (A) In uncontrolled batch cultures, all nutrients are present from the start, and nothing is added or removed during the cultivation. (B) In controlled batch cultures, all nutrients are present from the start, and there is active control over parameters like pH and dissolved oxygen. (C) In fed-batch cultures, there is a nutrient-containing inflow into the reactor, which can be used to establish nutrient-limited growth. There is active control over parameters like pH and dissolved oxygen. (D) In chemostat cultures, a nutrient-containing inflow is used in combination with a nutrient- and cell-containing outflow to establish nutrient-limited growth. There is active control over parameters like pH and dissolved oxygen.

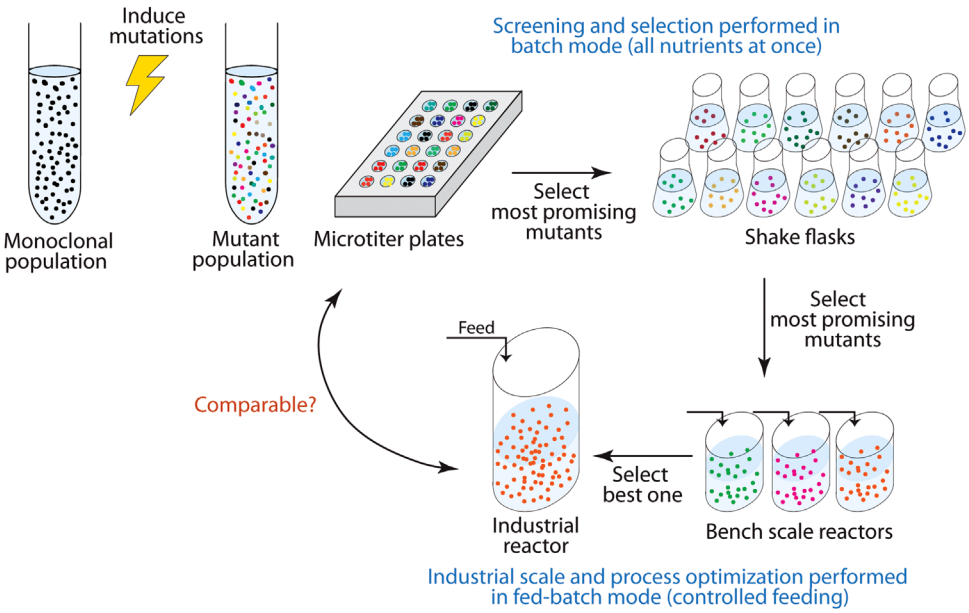


**FIGURE 2** Schematic workflow of conventional microbial strain screening applied in bioprocess industries. The bioprocess development strategy typically begins with creation of many mutants by directed or random modification. The mutants are screened for desired characteristics (e.g., productivity, yield, rate) in microtiter plates. These microtiter plates are operated as a batch: all nutrients are present at the start and there is no active control over cultivation conditions. The most promising mutants found under these batch conditions are studied in shake flasks with more control over process conditions. The most promising mutants are subsequently studied in bench scale reactors with active control over nutrient supply (fed-batch, chemostat) and cultivation conditions. The best performing mutants may eventually be used for industrial operation. The effectivity of the bioprocess development strategy benefits from the ability to perform screening and selection under nutrient-limited conditions in all steps. Microbioreactors have the potential to bridge the gap between screening and selection under uncontrolled batch conditions and industrial use under nutrient-limited conditions, by allowing nutrient-controlled fed-batch cultivation at high throughput (see Figure 1 from Teworte et al.[21]).

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 10–2000 $\mu$l) 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 3A. 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 *Escherichia coli* under batch and fed-batch conditions, see Figure 3B. 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 enable studying a large number of modified microorganisms in parallel under dynamically controlled conditions. An ideal platform 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 a strong focus on different strategies for nutrient feeding to establish a nutrient limited cultivation. More broader discussions such as those on applications, biosensors for analytics & process control, as well as investment potential of these platforms are out of scope for this review. For more extensive reviews with respect to different focus areas we refer to Long et al.[4] & Schäpper et al.[9] for applications; Hegab et al.[10] & Zeng et al.[3] for sensor integration, Hegab et al.[10] & Hemmerich et al.[8] for stirred microbioreactors and Long et al.[4] for larger miniaturized bioreactors. Here, we start by illustrating nutrient 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 their limitations as well as opportunities in the final section, highlighting where emerging miniaturized fermentation platforms can play a key role in the field of industrial biotechnology and beyond.

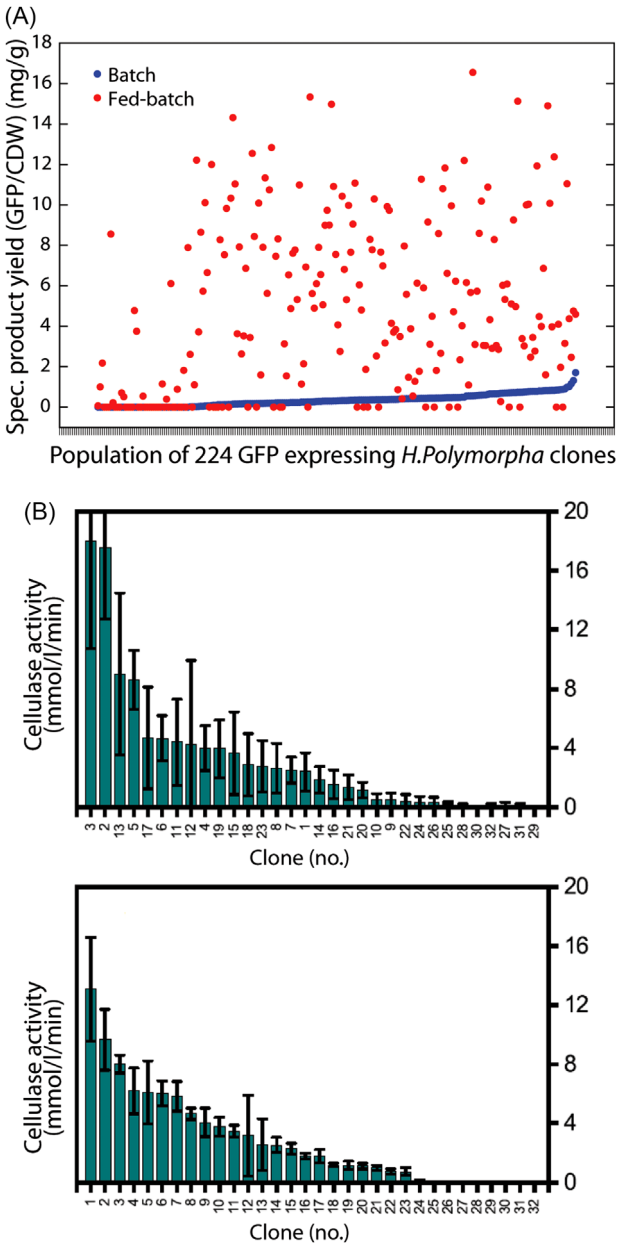


**FIGURE 3** Comparison of the performance of microorganisms under batch and fed-batch conditions, illustrating that clones that produce most under batch conditions are not the same as those that produce most under fed-batch conditions. (A) Green fluorescent protein (GFP) expression from 224 Hansenula polymorpha clones under batch (blue circles) and fed-batch (red circles) conditions. Adapted from Scheildle et al.[6] (B) Cellulose activity from 32 *E. coli* clones cultured under batch (top) and fed-batch conditions (bottom). Adapted from Keil et al.[7]

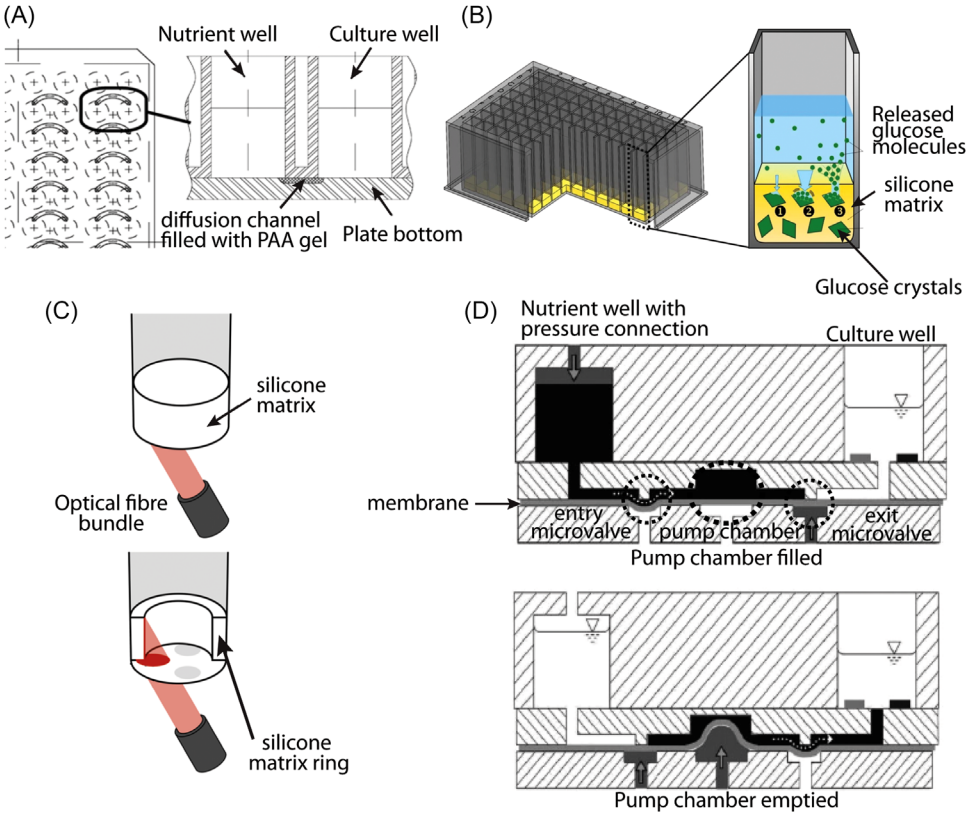


**FIGURE 4** Different types of modifications made to conventional microtiter plates to enable fed-batch cultivation. (A) Fed-batch microtiter plate showing a pair of wells connected by a polyacrylamide gel filled microchannel. One well acts as a cultivation chamber and the other as a reservoir for nutrients, with the nutrient supply controlled by the diffusional properties of the gel. Adapted from Wilming et al.[13] (B) Fed-batch microtiter plate with a glucose containing silicone matrix at bottom of each well. Adapted from Keil et al.[14] (C) Adaptation of the concept in (B) from a disc to a glucose-releasing ring, enabling optical accessibility during cultivation. Adapted from Habicher et al.[17] (D) Fed-batch microtiter plate with the bottom replaced by a PDMS microfluidic chip that connects pairs of wells (microfluidic Biolector). Precise delivery of nutrients from the nutrient well to the culture well is achieved through pneumatic activation of membrane-based valves, which allows the pump chamber to be filled first, before the nutrient solution is pumped into the culture well. Adapted from Funke et al.[18]

## 2 | MICROBIOREACTORS FOR NUTRIENT-CONTROLLED CULTIVATION OF MICROORGANISMS

### 2.1 | Microtiter plate-based platforms

In the 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.[11,12] 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 4A. 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.[13] Another strategy for nutrient release into the culture chamber is by loading wells with a silicon elastomer that contains crystals of glucose, see Figure 4B. In these so-called FeedPlates, release of glucose from the elastomer into the culture well is driven by a difference in osmotic pressure.[14–16] Since the silicon elastomer at the bottom of the wells hinders optical monitoring during cultivation, Habicher and co-workers[17] designed glucose-releasing rings to make the wells accessible for online monitoring, see Figure 4C. Apart from passive diffusion-based release strategies, active supply strategies have been developed such as the microfluidic BioLector system by m2p-labs.[18–20] Figure 4D 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.[18] Besides, it enables actively pH-controlled cultivations. A commercially available modified microtiter plate system (Micro-matrix) developed by Applikon Biotechnology

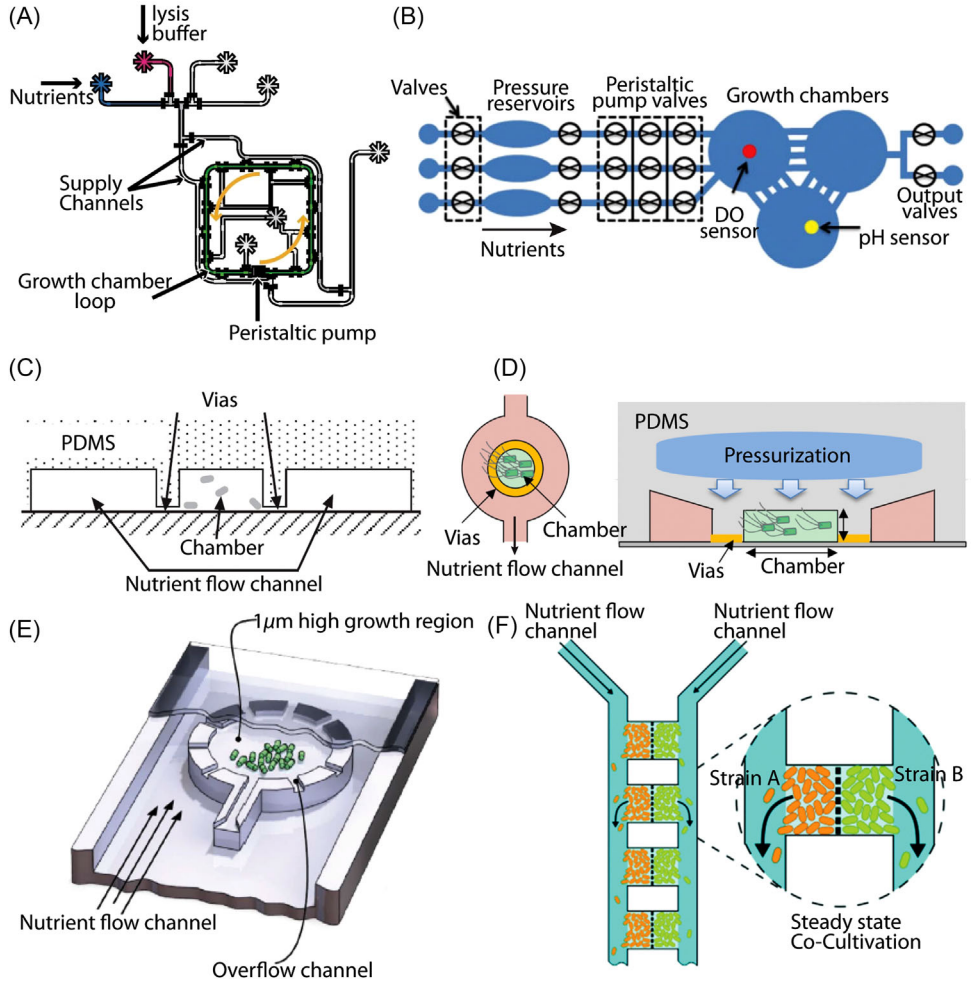


**FIGURE 5** Microfluidic chamber-based strategies for nutrient-controlled microbial cultures. (A) Microfluidic chemostat in which cells are cultured in a growth chamber loop, with the cell suspension circulating using an integrated peristaltic pump. Nutrient solution is periodically supplied to the growth chamber loop, while effluent is removed, through opening and closing of on-chip Quake valves (black lines). Occasionally, lysis buffer is pumped into segments of the growth chamber to remove cells that adhere to the walls. Adapted from Balagadde et al.[22] (B) Fed-batch microbioreactor with cells cultured in growth chambers and nutrients regularly supplied by pumping nutrient solution to the growth chambers through pneumatic actuation of the valves of the integrated peristaltic pumps. Dissolved oxygen and pH were measured online by the installed sensors. Adapted from Bower et al.[24] (C) Microfluidic chemostat-like cultivation in which cells are cultured in chambers, with nutrients supplied from the surrounding nutrient flow channels by diffusion through the shallow vias. The cells can be trapped in and released from the chambers by inflating the channels in the elastomeric device (PDMS) through an increase in pressure. Adapted from Groisman et al.[25] (D) Microfluidic chemostat with a similar working principle as in (C), but with the number of cells trapped inside the chambers automatically diluted by in/deflating the vias through de/repressurizing the channel above the growth chambers. Adapted from Kim et al.[27] (E) Microfluidic chemostat in which cells are cultured in a flow-through chamber, with the nutrient supply being controlled through the nutrient concentration. Once the chamber is populated, cells exit through the overflow channel, allowing non-restricted continuous growth. Adapted from Grunberger et al.[28] (F) Microfluidic chemostat based a on similar principle as in (E), allowing studies on co-cultures. Adapted from Burmeister et al.[29]

(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. A number of screening studies in such fed-batch microbioreactors have shown comparable results with that from a lab scale bioreactor.[12] Additionally, we refer our readers to a recent review by Teworte and co-workers[21] that dives deeper into automated liquid handling and control for microscale cultivation in miniaturized bioreactors. 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,[22] who made a microfluidic chemostat. The design of one of the six microfluidic circuits integrated onto a single chip is shown in Figure 5A.

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 and co-workers.[23] They developed a microbioreactor 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 microbioreactors were developed. An example by Bower and co-workers[24] is shown in Figure 5B. 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.

Even though it doesn't fall in one of the operating modes illustrated in Figure 1, 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,[25] is shown in Figure 5C. 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 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 5D. 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.[26,27] 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.[28] 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 5E. 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 5F.[29] 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 Grunberger et al., Burmeister et al. and Wright et al.[30–32]

## 2.3 | Microfluidic droplet-based platforms

The potential to use droplets as cultivation environments has been outlined decades ago.[33] The precise generation and control of droplets in microfluidic devices led to the development of droplet-based microbioreactors. 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.[34–36] 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.[37] 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, a critically important point in the development of microbioreactors with different droplet operations to enable regular nutrient supply is dealing with their inherent non-steady nature.

Jakiela and co-workers[38] 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 6A. 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 6A. More recently, Jian and co-workers[39] developed a similar automated droplet-based chemostat, including a sorting step based on monitoring biomass growth via OD measurements (see Figure 6B).

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.[40] 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

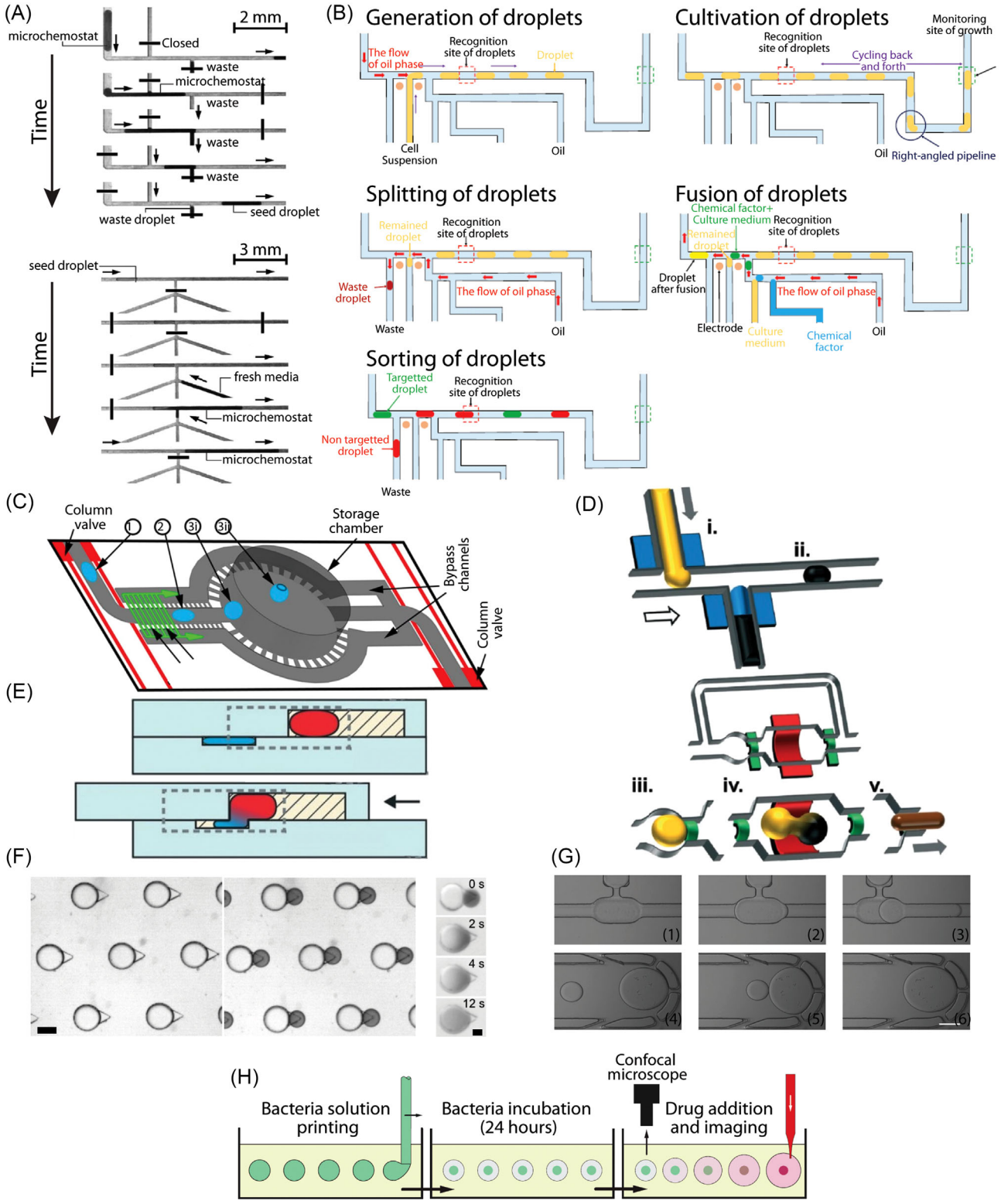


**FIGURE 6** Droplet-based microfluidic strategies for nutrient-controlled microbial cultures. (A) Oscillating trains of microdroplet chemostats with *E. coli* colonies growing inside them. After every incubation cycle, each microchemostat droplet is broken up into a seed and a waste droplet, after which a fresh nutrient droplet is coalesced with the seed droplet, mimicking a traditional chemostat. Adapted from Jakiela et al.[38] (B) Similar type of approach to study cells under chemostat conditions, with an additional sorting step. Adapted from Jian et al.[39] (C) Schematic of one of the chambers in a multiplex device, in which a cell-containing droplet is immobilized and supplied with nutrient-containing droplets to enable cell studies under fed-batch conditions. Adapted from Leung et al.[40] (D) Formation of cell-containing and nutrient-containing droplets using T-junctions controlled through integrated pneumatic valves (top). Chamber for the immobilization of droplets (middle), opened and closed through actuation of integrated pneumatic valves (middle). Strategy to immobilize a cell-containing droplet in a chamber and merge it with a

on demand and guided to the chambers as illustrated in Figure 6C, 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[41–43] for the cultivation of cells and the development of bio-chemical assays, see Figure 6D.

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,[44,45] who made a droplet-based fed-batch device by compartmentalizing droplets inside chambers, which can be joined by manually sliding the top and bottom half of these so-called *SlipChips* towards each other, as illustrated in Figure 6E. Baroud and co-workers[46] 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 6F. 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.[46] 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 microbioreactor 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.[47] 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,[48] see Figure 6G. 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[49] developed a bio-printing method to study the response to drugs injected into the droplets after 24 h of incubation, see Figure 6H. 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 MICROBIOREACTORS 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.[13] 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[18] poses complexity 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 24 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 ratio 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

nutrient-containing droplet (bottom). Adapted from Jin et al.[41] (E) Microfluidic SlipChip device illustrating loading of the two droplets in the wells (top). On moving the upper plate with respect to the lower plate the droplets are merged (bottom), enabling fed-batch cultivation. Adapted from Zhukov et al.[45] (F) Passive droplet trapping and coalescence within surface energy wells grooved on the floor of the microfluidic channel. Adapted from Tomasi et al.[46] (G) Fed-batch droplet-based microbioreactor comprising of droplet-on-demand junctions (left) for the controlled supply of nutrient droplets to a cell-containing droplet immobilized in a trap (right). Adapted from Totlani et al.[47,48] (H) Channel-free approach in which cell containing droplets are bio-printed in a yield stress fluid. This approach has the potential to repeatedly supply reagents to the cell-containing droplets and study cells under controlled conditions. Adapted from Nelson et al.[49]

**TABLE 1** Overview of advantages and limitations of the presented microbioreactor technologies

| Type of microbioreactor technology | Advantages | Limitations |
|---|---|---|
| Microtiter plate-based platforms | 1. Low adoption barrier | 1. Diffusion based feeding relies on environment |
| | 2. Easier analytical measurement & process control | 2. Challenging integration of microfluidic channels. |
| | 3. Possibility to integrate with robotic fluid handlers | |
| Microfluidic chamber-based platforms | 1. Devices are relatively simple to fabricate | 1. Biofilm formation on walls |
| | 2. Simple flowrate management | 2. Cross contamination between chambers |
| | 3. A large number of inlets can be used for multiple reagent injection | 3. Difficulty in scaling out due to large number of inlets |
| Microfluidic droplet-based platforms | 1. Better confinement & compartmentalization | 1. Difficulty in measuring and controlling pH, $DO_2$ |
| | 2. Non-steady assays can be performed | 2. Complicated fabrication involving membranes. |
| | 3. Higher throughput can be achieved with continuous flow | 3. Leakage through droplets during incubation |

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.[50] Another challenge involves creating a simple and robust nutrient feeding strategy, making the technology accessible to non-experts.[51] 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 the 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.[52] 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.[53,54] 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. Table 1 highlights major advantages and limitations faced by microtiter plate, microchamber and droplet based microbioreactor platforms.

## 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,[55] 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.[56]

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.[57] 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 $\beta$-d-1-thiogalactopyranoside (IPTG).[58] 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.[59] When the cells are pulse-fed, sequential batch reactors can be used to select for mutants that produce large amounts of storage-compounds.[60] Miniaturizing such experiments can increase the number of parallel experiments that can be done, while decreasing the costs.

One interesting perspective to consider is the role of automation and optimization algorithms during the screening and process development phase. Industrial bioprocess development can be accelerated by integrating data driven modeling and computer-controlled cultivation systems along with high-throughput microbioreactor devices. By designing better experiments, data that is generated during the screening phase can be used as model set for better models for later stages of process development. One of the earlier examples of computer-controlled cultivation experiments were those carried out in so called *A-stat*[61] and *D-stat* formats.[62] Here, chemostats were operated with a gradual change in dilution rate. The 2-mag system developed by the TU Berlin combines robotic liquid controllers for complex feeding profiles[63] with advanced model predictive control,[64] thereby trading off between throughput and process control. The *Robot Scientist* by the group of Ross King is yet another example of high throughput automation where experimental space can be covered much more efficiently.[65] Combining controlled high-throughput experimentation within microbioreactor platforms with complex feeding profiles and process control would be a promising approach to bridge the gap between screening conditions and those observed at the industrial scale. Therefore, they have a high potential as new cultivation-tool for screening and selecting mutants in industrial biotechnology.

## AUTHOR CONTRIBUTIONS

Kartik Totlani: Conceptualization; Data curation; Investigation; Methodology; Writing – original draft; Writing – review & editing. Rinke J. van Tatenhove-Pel: Writing – original draft; Writing – review & editing. Michiel T. Kreutzer: Supervison; Writing - original draft; Writing - review & editing. Walter M. van Gulik: Supervision; Writing – original draft; Writing – review & editing. Volkert van Steijn: Conceptualization; Data curation; Funding acquisition; Investigation; Project administration; Supervision; Writing – original draft; Writing – review & editing.

## CONFLICT OF INTERESTS

The authors declare no financial or commercial conflict of interest.

## DATA AVAILABILITY STATEMENT

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

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