Recent Advances in Fed-batch Microscale Bioreactor Design

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Abstract

Advanced fed-batch microbioreactors mitigate scale up risks and more closely mimic industrial cultivation practices. Recently, high throughput microscale feeding strategies have been developed which improve the accessibility of microscale fed-batch cultivation irrespective of experimental budget. This review explores such technologies and their role in accelerating bioprocess development. Diffusion- and enzyme-controlled feeding achieve a continuous supply of substrate while being simple and affordable. More complex feed profiles and greater process control require additional hardware. Automated liquid handling robots may be programmed to predefined feed profiles and have the sensitivity to respond to deviations in process parameters. Microfluidic technologies have been shown to facilitate both continuous and precise feeding. Holistic approaches, which integrate automated high-throughput fed-batch cultivation with strategic design of experiments and model-based optimisation, dramatically enhance process understanding whilst minimising experimental burden. The incorporation of real-time data for online optimisation of feed conditions can further refine screening. Although the technologies discussed in this review hold promise for efficient, low-risk bioprocess development, the expense and complexity of automated cultivation platforms limit their widespread application. Future attention should be directed toward the development of open-source software and reducing the exclusivity of hardware.

Keywords

fed-batch, microbioreactor, automation, microfluidics, synthetic biology, high-throughput, accelerated bioprocess development

Abbreviations

cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; DIY, do it yourself; DO(T), dissolved oxygen (tension); Fab, antigen binding fragment; GFP, green fluorescent protein; IMS, integrated microfluidic system; IPTG, isopropyl β-d-1-thiogalactopyranoside; LB, Luria-Bertani; mAb, monoclonal antibody; MBR, microbioreactor; MTP, microtitre plate; OD, optical density, OD_{600} optical density at wavelength 600 nm; OED, optimal experimental design; OTR, oxygen transfer rate; PDMS, polydimethylsiloxane; PI, proportional integral; PMMA, poly(methyl methacrylate); RAMOS, respiration activity monitoring system; RQ, respiratory quotient; SiLA, Standardisation in Lab Automation; µBR, microbioreactor

56 **1. Introduction**

57 The field of organic synthesis has enriched society since its inception in 1828
58 (Kauffman and Chooljian, 2001), facilitating efficient and economical production of a
59 myriad of useful natural products. With applications such as pharmaceuticals
60 (Nowrouzi et al., 2020), fertilisers, plastics, solvents, cosmetics and fuels (Walls and
61 Rios-Solis, 2020) etc., such products are extremely beneficial to everyday life
62 (Nicolaou, 2014; Wong et al., 2016). However, many rely on non-renewable
63 petrochemical feedstocks and the large number of steps required for more complex
64 natural products can hinder economic feasibility (Holton et al., 1994; Nicolaou et al.,
65 1994; Schneider et al., 2020). The construction of microbial cell factories for the
66 bioconversion of renewable feedstocks into such natural products has therefore gained
67 significant interest as an alternative production route.

68 Recently, substantial progress has been achieved in the field of synthetic and systems
69 biology, specifically applied to metabolic engineering (Malcı et al., 2020; Rios-Solis et
70 al., 2011). As a result, large libraries of strains expressing wide-ranging heterologous
71 biosynthetic pathways can now be built in unprecedentedly short timescales (Campbell
72 et al., 2017; Hemmerich et al., 2018). This major acceleration within the ‘design’ and
73 ‘build’ phases of the design-build-test-learn (DBTL) cycle has rendered the ‘test’ and
74 ‘learn’ phases increasingly rate-limiting in cell factory development (Campbell et al.,
75 2017; Hemmerich et al., 2018).

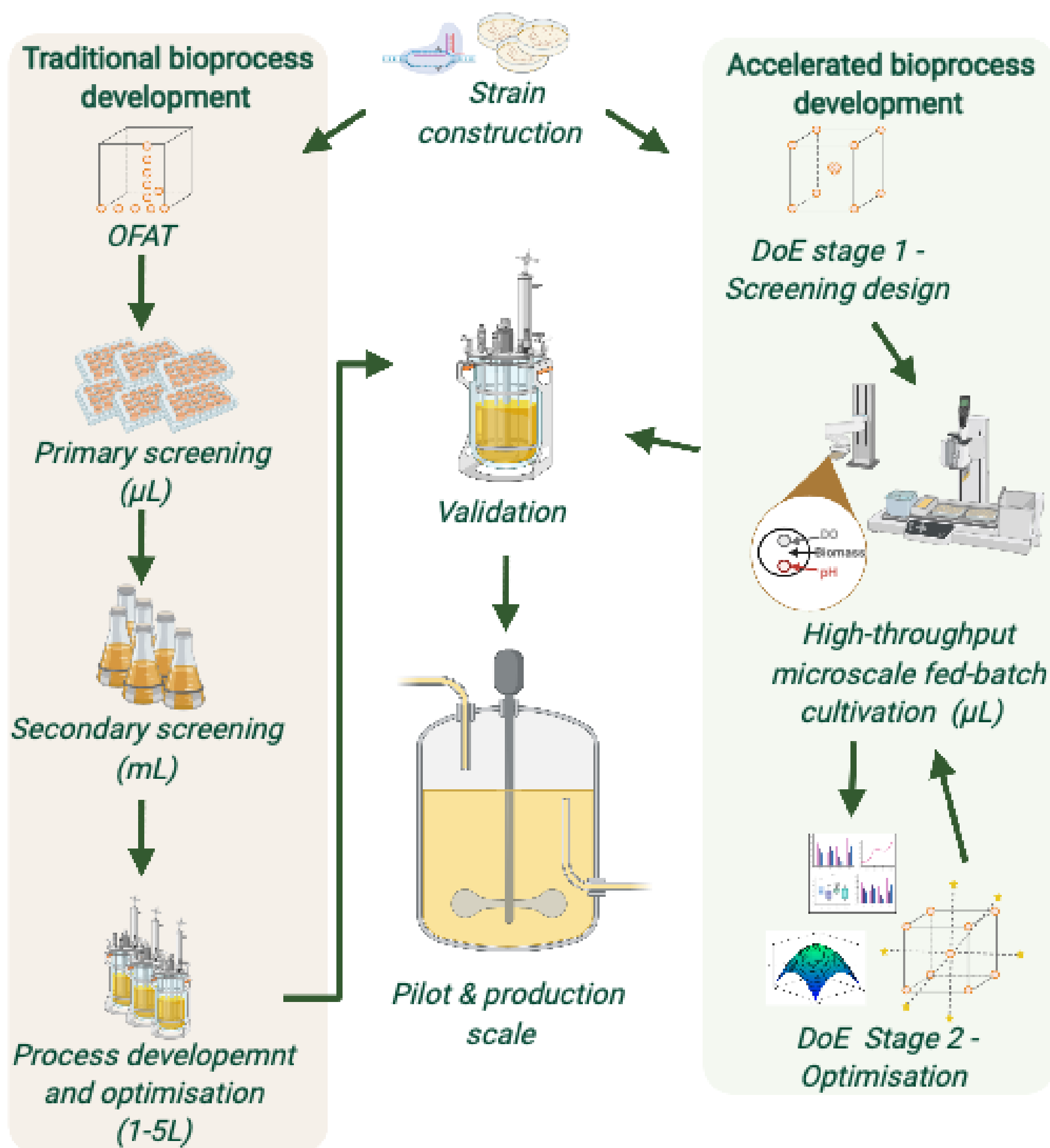
76 The initial phase of bioprocess development involves extensive screening of a wide
77 range of strains and processing parameters. The use of simple batch microtiter plates
78 (MTPs) or shake flask cultivations remains ubiquitous for this phase (Halim et al., 2014;
79 Rios-Solis et al., 2015a, 2011), largely due to their relatively low cost and high
80 throughput compared to laboratory scale stirred tank reactors. However, as a result of
81 small volumes and a lack of infrastructure for online monitoring and control (Rameez
82 et al., 2014), analyses are often restricted to endpoint assays, limiting process insight.

83 Advanced MBR systems are being increasingly employed in this context with the aim
84 of overcoming these key bottlenecks (Wilk et al., 2018). Microbioreactors typically have
85 a volume of 0.5–15 mL and are equipped with in-built sensors for the online monitoring
86 of critical process parameters such as pH, dissolved oxygen (DO), optical density (DO)
87 and fluorescence (Funke et al., 2010b, 2009; Hsu et al., 2012). With novel mixing
88 strategies, effective mimicry of larger laboratory bioreactors is possible, despite
89 dramatically reduced space and resource requirements (Heins and Weuster-Botz,
90 2018). Many units can be run in parallel, facilitating high-throughput screening
91 applications. By coupling MBR technologies with a strategic Design of Experiments
92 (DoE) approach, process insight can be further maximised, whilst minimising
93 experimental burden (Motta Dos Santos et al., 2016; Sandner et al., 2019). DoE
94 facilitates systematic estimation of factor interactions, which are omnipresent in
95 biological systems, and broader exploration of the design space. To ensure optimal
96 performance at industrial scale, DoE should be applied to optimise both genetic (e.g.
97 metabolic pathway optimisation) and environmental (e.g. media composition,
98 temperature, pH, DO) factors simultaneously in the early stages of bioprocess
99 development (Gilman et al., 2021; Kent and Dixon, 2019; Peng et al., 2017).

Although the online monitoring capabilities of MBRs have been improved dramatically, technical constraints and a lack of effective systems for substrate feeding and bioprocess control limit many systems to batch operation (Bareither and Pollard, 2011; Hemmerich et al., 2018; Krause et al., 2016). This is particularly problematic as fed-batch is the preferred mode of operation for industrial scale bioprocesses due to increased productivity, elimination of substrate inhibition (Du et al., 2014; Rios-Solis et al., 2015b), and the possibility to bypass the overflow metabolism observed in certain species (Ladner et al., 2017; Lattermann and Büchs, 2015). The major inconsistencies in cultivation strategy between micro and industrial scale cultivations can lead to the selection of suboptimal strains and process conditions in the earliest stages of bioprocess development (Funke et al., 2010b; Keil et al., 2019). The incorporation of process control strategies and fed-batch operation into high-throughput screening is therefore imperative to ensure closer mimicry of industrial scale cultivation conditions.

Several novel MBRs harbouring inbuilt feeding, control and sampling capabilities have been developed recently to overcome this critical bottleneck. Innovative internal and external feeding strategies and their potential to mimic different commonly used industrial feeding strategies such as pulsed, exponential, modified exponential, and linear feeding (Choi et al., 2014) have been investigated. Internal fed-batch strategies include diffusion and enzyme controlled feeds typically involving a biphasic culture medium separated by a semi-permeable membrane (Jeude et al., 2006; Krause et al., 2016) and the biocatalytic break down of a polysaccharide substrate (Krause et al., 2016; Panula-Perälä et al., 2008), respectively. External feeding has also been achieved by use of microfluidic (Bjork and Joensson, 2019; Funke et al., 2010b; Marques and Szita, 2017; Peterat et al., 2015) and automated liquid handling systems (LHSs) (Hemmerich et al., 2014; Huber et al., 2009b; Nickel et al., 2017). Such systems offer the inherent advantage of improved feed control, allowing industrially relevant pulsed, linear and exponential feeding strategies to be more effectively mimicked. The incorporation of model based optimisation algorithms to analyse process data in real time and re-determine the optimal cultivation strategy are also gaining significant interest to further expedite bioprocess development (Cruz Bournazou et al., 2017; Nickel et al., 2017).

A holistic method coupling novel fed batch MBRs with statistical DoE and model-based optimisation strategies is likely to be optimal for robust strain development and optimisation. Quality by design can be ensured by the strategic high throughput screening of a wide range of genetic and environmental factor combinations, whilst monitoring and controlling industrially relevant process parameters (N. Politis et al., 2017). This increased process insight has the potential to greatly expedite bioprocess development through a reduction in the number of screening stages required compared with the traditional approach. This concept is depicted in Figure 1 and progress towards it will be investigated in this review.



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141 **Fig. 1.** Bioprocess development summary. Traditional bioprocess development involves
142 primary strain and medium screening in batch microtiter plates (MTPs) using a one-factor-at-
143 a-time (OFAT) approach. This is followed by secondary screening in batch shake flask
144 cultures, where larger volumes increase manual sampling capabilities. Bench top bioreactors
145 with online monitoring and control capabilities are then used for more detailed process
146 development and optimisation studies prior to larger scale validation and optimisation. The
147 final optimal conditions are then scaled up to pilot and ultimately production scale. Accelerated
148 bioprocess development aims to reduce the number of steps by maximising process insight in
149 the early stages. Design of experiments (DoE) is used to strategically screen many factors in
150 the first stage, those with a significant effect on the response are then further optimised using
151 a response surface methodology. Advanced fed-batch MBR tools with online monitoring and
152 control capabilities are used to effectively mimic larger scale conditions, reducing the need for
153 shake flask and bench-top cultivations. The optimal conditions can be validated in laboratory
154 scale reactors before scaling up to pilot and production scale.

2. Internal feeding strategies

In internal fed-batch systems the substrate is released gradually within the culture vessel with no external feeding. The major advantage of these systems is their compatibility with existing infrastructure. As there is no need for advanced micropump, microfluidic or liquid handling robot technologies, cost and complexity can be reduced dramatically. Such systems typically exploit either diffusion or biocatalytic phenomena.

2.1 Diffusion controlled feeding

Diffusion controlled feeding involves the slow release of entrapped nutrients from a polymeric adsorbent or through an artificial membrane. The concept was introduced in a 1943 study by Hestrin *et al.*, involving the cultivation of *Bacillus subtilis* within a cellophane sac suspended in a concentrated medium (Hestrin et al., 1943). The nutrients within the medium diffused across the semi-permeable dialysis membrane where they could then be utilised by the cells. The dialysis cultivation method was further developed for a wide-range of species during the 1950s and 1960s as reviewed by Schulz and Gerhardt (Schultz and Gerhardt, 1969). More recently, this membrane feeding principle was coupled with a respiration activity monitoring system (RAMOS) to facilitate online monitoring of respiration activity and identification of metabolic phenomena such as oxygen limitation during fed-batch cultivation (Bähr et al., 2012). A later study by Philip *et al.* elucidated two parameters, initial substrate concentration in the reservoir and membrane geometry, as key factors affecting feed rate. This facilitated greater feed rate control and performance was found to be highly comparable to that of parallel laboratory-scale stirred tank bioreactor cultivations, despite a 100-fold scale-up in cultivation volume (Philip et al., 2017). A major limitation of the diffusion-controlled feeding approach using dialysis membranes, however, was its restriction to shake flask cultivations, which limited throughput.

Jeude *et al.* developed the FeedBead® technology (Figure 2), an alternative diffusion controlled approach involving the addition of silicone elastomer discs loaded with glucose crystals to the cultivation medium (Jeude et al., 2006). Although this technology was also initially developed for use in shake flasks, Scheidle *et al.* demonstrated the suitability of the FeedBead® technology for MTP applications (Scheidle et al., 2009). The researchers developed a modified RAMOS system, termed microRAMOS, which facilitated measurement of OTR in 96-deep-well plates via a specially designed lid. Interestingly, screening of a number of engineered *H. polymorpha* strains using the FeedBead®-microRAMOS platform resulted in the elucidation of entirely different optimal strains compared to parallel batch screening (Scheidle et al., 2009). This highlighted that batch screening is unlikely to be appropriate for the selection of optimised strains for fed-batch bioprocesses. In the device, OTR was measured as the sum parameter of all wells as the limited dimensions of MTP wells hindered installation of the necessary technical components to individual wells. As a result, detailed information on the state of each well could not be obtained (Scheidle et al., 2009). In response to these findings, Keil *et al.* developed an MTP FeedPlate® system incorporating an immobilised solid silicone matrix with embedded glucose crystals at the bottom of each well, as depicted in Figure 2. Fed-batch cultivation of *H. polymorpha* in these FeedPlates® resulted in a 245-fold improvement

200 in GFP production (Keil et al., 2019). The plates are commercially available in 24, 48
201 or 96 well format, allowing straightforward high-throughput cultivation in fed-batch
202 mode. However, external factors such as medium pH, temperature and osmolality had
203 a major influence on the rate of glucose release. As a result, precise control of the
204 substrate release rate was limited using this technology.

205 In 2016, an improved μ-RAMOS device was developed with the aim of overcoming the
206 bottlenecks of the original device (Flitsch et al., 2016). The updated system featured
207 gas inlet and outlet valves along with an optical sensor in every single well of a 48-well
208 MTP, facilitating simultaneous OTR monitoring for all 48 cultivations (Flitsch et al.,
209 2016). The technology was recently extended further for use with 96-deep-well MTPs,
210 allowing the researchers to achieve a 15-fold increase in experimental throughput
211 compared to the original shake-flask scale RAMOS system (Dinger et al., 2020).
212 Habicher *et al.* demonstrated the compatibility of the state-of-the-art μ-RAMOS and
213 FeedPlate® technologies for glucose-limited cultivations of a *Bacillus licheniformis*
214 strain engineered for protease production (Habicher et al., 2020). Online monitoring of
215 OTR improved the information content of the MTP cultivations considerably and
216 performance was found to be comparable at MTP and shake flask scale (Habicher et
217 al., 2020). Data generated using this platform could be used to generate mathematical
218 models during the earliest stages of development, dramatically improving process
219 understanding in line with quality by design principles (U. S. Food and Drug
220 Administration, 2009)

221 Wilming *et al.* developed an alternative diffusion based fed-batch system using 96-well
222 MTP (Wilming et al., 2014). Each culture well was connected to a reservoir well via a
223 diffusion channel filled with a polyacrylamide hydrogel, facilitating up to 44 parallel fed-
224 batch cultivations per plate. The reservoir wells were filled with a concentrated
225 substrate solution to achieve gradual diffusion driven feeding. The feed rate was
226 readily adjustable by varying the concentration in the reservoir and thereby the driving
227 concentration gradient (Wilming et al., 2014). However, the relationship between the
228 feed concentration and rate of glucose release was found to be non-linear. This non-
229 linearity, which complicated the fine tuning of the feed rate, was attributed to counter
230 diffusion of water. Despite this, the transparent base of the plates provided a major
231 advantage of compatibility with plate-reading technologies such as the BioLector
232 system (mp2-Labs, Germany) for measurement of biomass and fluorescence via
233 scattered light. Fed-batch cultivation of *E. coli* and *H. polymorpha* strains was
234 demonstrated using the system. Fed-batch cultivation of *E. coli* with optimal 300 g/L
235 glucose feed resulted in around fivefold and 14-fold increases in biomass and flavin
236 mononucleotide-based fluorescent reporter protein signal, respectively, compared to
237 batch control (Wilming et al., 2014).

238 **2.2. Enzyme controlled feeding**

239 Considering the control limitations of the FeedBead® technology (Jeude et al., 2006;
240 Keil et al., 2019), Panula-Perälä *et al.* aimed to develop a more tunable internal fed-
241 batch system by exploiting both diffusive and biocatalytic phenomena (Panula-Perälä
242 et al., 2008). The technology, patented as EnBase®, involved a solid phase containing
243 starch overlaid with the culture medium containing glucoamylase enzymes derived
244 from *Aspergillus niger*. The non-metabolisable starch gradually diffuses into the culture

245 medium, where it is broken down by the biocatalyst, releasing glucose (Panula-Perälä
246 et al., 2008). The release rate of glucose was controlled by varying the concentration
247 of glucoamylase.

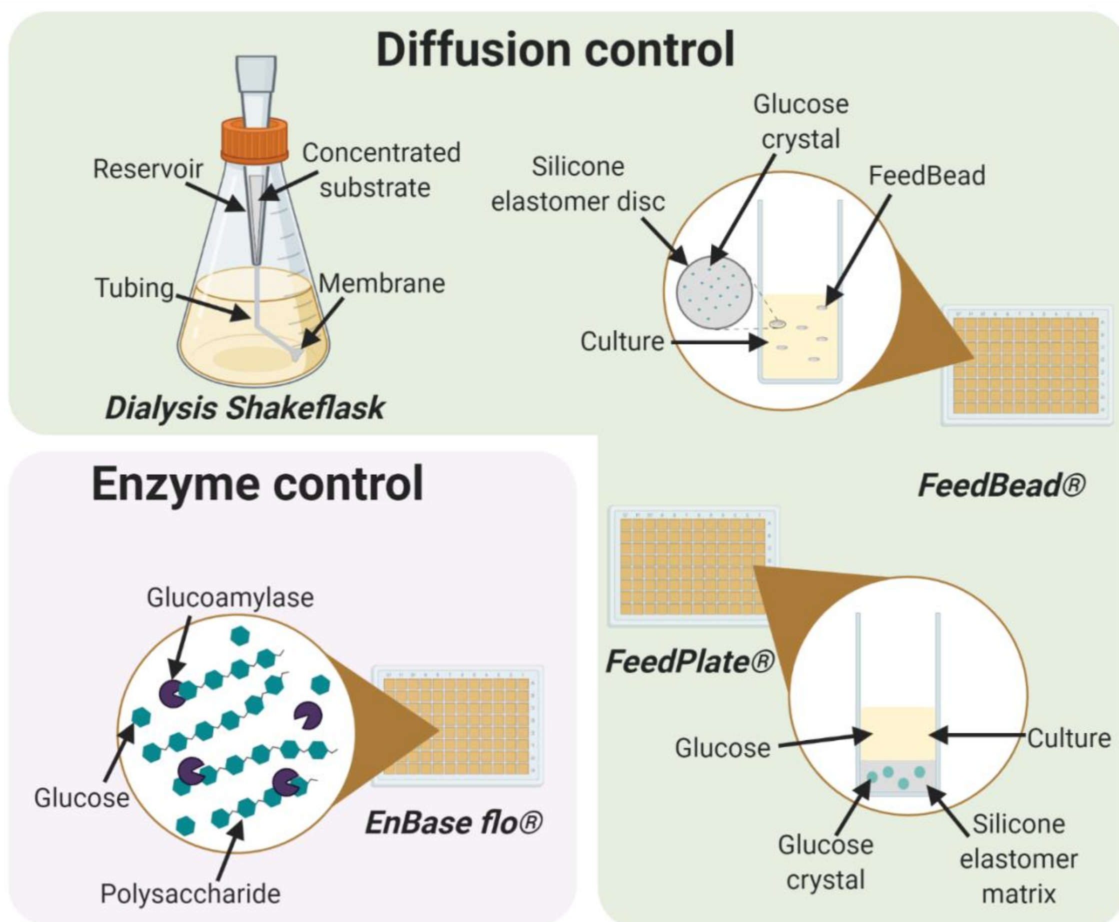
248 The poor solubility of starch in liquid culture necessitated a solid phase in the original
249 EnBase® process (Panula-Perälä et al., 2008). To eliminate the need for a biphasic
250 system, EnBase® Flo, featuring a fully soluble polymer substrate, was developed
251 (Krause et al., 2010). The glucose release method was coupled with a carefully
252 optimised combination of mineral salts and complex medium additives to generate high
253 cell densities and product titres (Krause et al., 2010). As proof of concept, several *E.*
254 *coli* strains were cultivated in the fed-batch EnBase® Flo medium in 24-deep-well MTP.

255 Glazyrina *et al.* investigated the scalability of the EnBase® Flo system by cultivating an
256 *E. coli* strain engineered for overproduction of the model enzyme, alcohol
257 dehydrogenase, at a range of scales from 3 mL to 60 L (Glazyrina et al., 2012).
258 Comparable growth rates and protein titres were achieved at all tested scales,
259 highlighting scalability. The EnBase® system also provided the additional benefit of
260 controlled glucose release during the initial cultivation phase in the larger scale
261 bioreactors, eliminating overflow metabolism entirely (Glazyrina et al., 2012).

262 The EnBase® technology was also made commercially available in a convenient tablet
263 form (EnPresso®, Figure 2). This EnPresso® system was coupled with a D-optimal DoE
264 approach for the optimisation of valinomycin production by engineered *E. coli* in 24-
265 well plates (Li et al., 2014). The DoE driven parallel fed-batch cultivation strategy led
266 to a 33-fold improvement in valinomycin titre compared to the original batch cultivation.

267 **2.3 Summary of internal feeding strategies**

268 Diffusion and enzyme-controlled feeding strategies provide a relatively simple and low
269 cost means of mimicking larger scale fed-batch processes. They provide a key
270 advantage of constant substrate feeding, but precise control of the feed rate is typically
271 not possible throughout the cultivation. As a result, more complex (e.g. exponential)
272 feed profiles cannot be implemented using internal feeding strategies (Panula-Perälä
273 et al., 2008). In addition, feeding is typically restricted to a single substrate, which may
274 lead to other nutrients within the culture medium becoming limiting. Enzyme based
275 feeding in particular relies on glucose as the carbon source which may not be optimal
276 for all processes. Furthermore, acid and base feeding is typically not possible in such
277 systems, limiting process control capabilities. However, these limitations may be
278 overcome by coupling internal substrate feeding with additional hardware, examples
279 of which are described in subsequent sections.



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281 **Fig. 2.** Summary of internal fed-batch technologies. Diffusion controlled systems include the
282 dialysis shake flask approach, where a concentrated substrate solution slowly diffuses from a
283 reservoir, through a membrane into the culture. FeedBead® and FeedPlate® systems
284 involve the use of a silicone elastomer matrix loaded with glucose crystals, which slowly
285 release glucose via diffusion. For enzyme control a polysaccharide substrate is broken down
286 by an enzyme.

287

288 3. External feeding strategies

289 In external fed-batch systems the substrate is fed from an external reservoir. The key
290 advantages of this strategy are increased flexibility and process control capabilities.
291 However, as additional infrastructure is required for feeding, external fed-batch
292 systems are inherently more complex and costly to operate.

293 3.1 Automated liquid handling systems

294 One approach to external substrate feeding involves the use of an automated liquid
295 handling robot such as the RoboLector® (mp2-Labs, Germany) or Ambr® (Sartorius
296 AG, Germany) systems. High-throughput sampling and addition of liquids to MTPs or
297 parallel MBRs becomes possible using a liquid handling workstation (Huber et al.,
298 2009a; Kensy et al., 2009). The RoboLector®, for example, includes the integrated
299 BioLector® (mp2-Labs, Germany) MBR screening platform. Microplates with inbuilt,
300 non-invasive optical sensors are used in the BioLector®, allowing online monitoring of
301 critical process parameters such as DO, pH and biomass online for 48 simultaneous

302 cultivations (Huber et al., 2009a). Therefore, the BioLector® can be used to mimic large
303 scale batch operations (L. Walls et al., 2021; L. E. Walls et al., 2021). The RoboLector
304 can then be readily programmed to feed acid or base in response to changes in pH
305 detected via these optical sensors. Fed-batch cultivation is also possible with the
306 system as demonstrated by Hemmerich *et al.* (Hemmerich et al., 2014). Pulsed feeding
307 of a mixed glycerol and methanol substrate at two different concentrations was
308 achieved using the liquid handling robot with the aim of optimising production of the
309 biosurfactant, *Rhizopus oryzae* lipase by an engineered *Komagataella pastoris* strain
310 (Hemmerich et al., 2014). Using the low and high substrate feeding strategies, biomass
311 accumulation was enhanced around three and fourfold, respectively, compared to
312 batch mode. Scale up of the low-rate fed-batch process from an 800 µL microscale
313 culture to 3 L bioreactor scale resulted in comparable lipolytic activity and activity yields
314 from methanol, two important response variables in the study. Automatic sampling was
315 programmed every 24 hours. Both feeding and sampling were achieved without
316 interruption to shaking, which minimised disruption to oxygen transfer and prevented
317 cell settling, allowing representative samples to be obtained (Hemmerich et al., 2014).

318 The key challenge associated with pulsed feeding strategies is the lack of a continuous
319 feed supply, which causes oscillations in cellular metabolism and limits comparability
320 to industrial scale fermentations, where exponential feeding strategies are more
321 commonly employed (Jansen et al., 2019). The enzyme controlled feeding strategies
322 described in Section 2.2 facilitated effective gradual release of the substrate (Glazyrina
323 et al., 2012; Panula-Perälä et al., 2008). However, whilst the feed rate could be
324 changed by varying the initial concentration of enzyme, scope for precise control during
325 the cultivation was limited. To address these bottlenecks, Jansen *et al.* developed an
326 automated feedback-regulated enzyme-based fed-batch system (FeedER). The
327 principle of FeedER is automated pH control and amyloglucosidase addition. As the
328 rate of glucose release depends on the concentration of the enzyme, a defined
329 exponential growth rate could be achieved through its controlled addition. This was
330 implemented through the RoboLector® platform with a Python-based process control
331 strategy. Biomass was measured using the inbuilt optical sensors of the BioLector®
332 FlowerPlate and the resulting values enabled online growth rate determination for 48
333 simultaneous cultivations (Jansen et al., 2019). When the growth rate dropped below
334 a predefined setpoint, amyloglucosidase enzyme was fed automatically using the liquid
335 handling system (LHS), facilitating the cleavage of dextrin present in the culture
336 medium to gradually release monomeric glucose. A major challenge associated with
337 enzyme-based methods, however, is the sensitivity of the enzyme to external
338 conditions such as culture pH. As acidification of the culture medium was found to have
339 a major effect on enzyme activity, tight control of pH was required to maintain effective
340 control of glucose release. In subsequent experiments, pH control was achieved by
341 automatic addition of ammonium hydroxide via the LHS when the measured pH
342 dropped below the setpoint. The system was demonstrated for the high-throughput
343 screening of fed-batch cultivations of three different model organisms (*E. coli*, *K.*
344 *pastoris* and *Corynebacterium glutamicum*) (Jansen et al., 2019).

345 Recently, the exploratory power of the RoboLector® platform as a screening tool for
346 microbial strain development was evaluated using enzymatic glucose release fed-
347 batch operation (Fink et al., 2021). A combination of *E. coli* strains producing various

348 antigen binding fragments (Fab) with various leader sequences were used for microbial
349 screening. The growth and expression characteristics as well as the strain rankings
350 were compared between a microbioreactor system (800 μL working volume), a
351 benchtop scale bioreactor (1.2 L working volume), and a stirred tank bioreactor (20 L
352 working volume). Deviations in clones and conditions observed at microscale were
353 found to be comparable to those at increased scale, highlighting good transferability.
354 Only small deviations in clone rankings for the *E. coli* – Fab – leader sequence
355 combinations were found and most of the strains showed the same growth and
356 expression characteristics across scales (Fink et al., 2021).

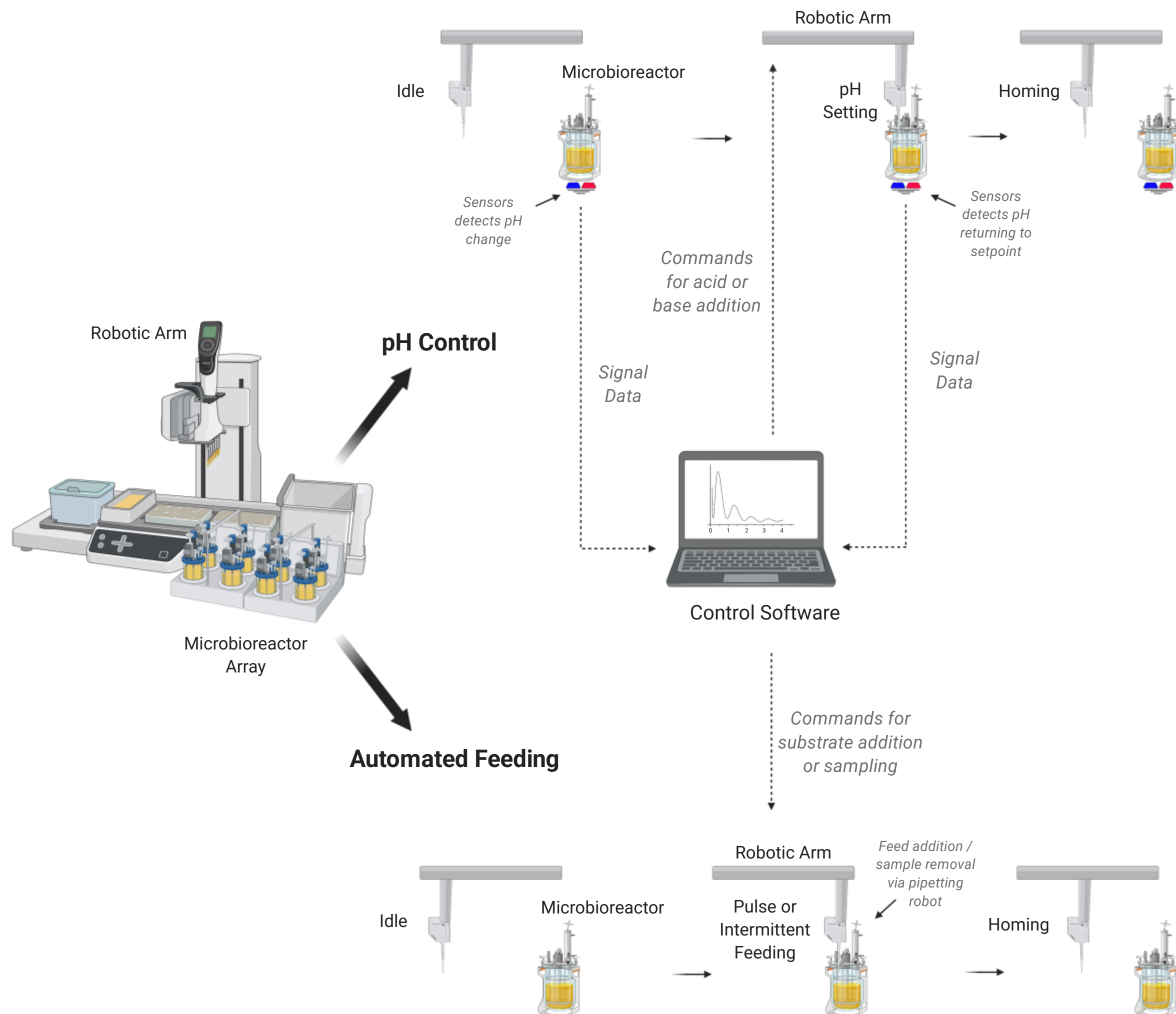
357 A major limitation of the RoboLector® system is the inability to precisely control DO due
358 to the MTP format. Oxygen transfer may be partially adjusted for the entire plate by
359 varying the shaking speed, however, control of individual wells is not possible. Also,
360 higher osmotic pressure or differences in medium composition were observed in
361 RoboLector® compared to larger scale cultivations (Fink et al., 2021). Ambr® systems,
362 which have largely become the industry standard MBRs for cell culture (Sandner et al.,
363 2019), are able to overcome this bottleneck. The Ambr® 15 is a high-throughput
364 platform which can run up to 48 cultivations in parallel. However, unlike MTP systems,
365 each of the 48 MBRs with a working volume of 10-15 mL is equipped with its own
366 sparger tube and impeller allowing control of DO in each individual MBR in response
367 to deviations detected via the online optical sensor. Controlled liquid addition and
368 sampling is possible using the liquid handling robot allowing control of pH and fed-
369 batch operation, as demonstrated in Figure 3. One limitation, however, was that the
370 analysis of all 48 vessels could take up to three hours, requiring a compromise between
371 experimental throughput and data volume (Sandner et al., 2019). Rameez *et al.*, 2014
372 demonstrated the ability of Ambr® 15 to mimic a previously developed larger scale
373 process. Recombinant antibody production in fed-batch Chinese hamster ovary (CHO)
374 cell cultures with bolus and intermittent feeding was studied. The resulting product
375 titres from the microscale cultures were within 10-15 % of those achieved in previous
376 3 L, 15 L, and 200 L fermentations (Rameez et al., 2014).

377 The Ambr® platform has undergone continuous improvement since its launch in 2010
378 (Bareither and Pollard, 2011) and an updated model of the platform developed
379 specifically for microbial systems was released in 2017 (Velez-Suberbie et al., 2018).
380 The addition of pumped liquid lines allowed the continuous addition of liquids to each
381 individual reactor. This overcame the limitations of intermittent feeding, facilitating the
382 implementation of continuous feed profiles and tighter pH control. Velez-Suberbie *et*
383 *al.* demonstrated continuous fed-batch operation with exponential feeding using the
384 system (Velez-Suberbie et al., 2018). The substrate and base solutions were fed *via*
385 the feed lines, whilst the liquid handler was employed for the addition of acid, antifoam
386 and the isopropyl β-d-1-thiogalactopyranoside (IPTG) inducer. Induction and feeding
387 were initiated in each vessel individually upon detection of a spike in pH, associated
388 with carbon source depletion, by the optical sensor. As each microreactor harboured
389 its own agitator, DO probe and sparger, the DO could be readily controlled to the
390 desired set point of 30 % (Velez-Suberbie et al., 2018). Growth and productivity were
391 found to be comparable to those of parallel 1 L bioreactor cultivations.

392 The bioREACTOR 48 platform (2mag, Germany) has been coupled with an LHS to
393 enable fed-batch and process control (Faust et al., 2014; Haby et al., 2019; Nickel et

394 al., 2017). The bioREACTOR 48 is a block of 48 miniaturised bioreactors (8-15 mL),
395 each with its own impeller, pH and DO sensor. Faust *et al.* coupled the platform with a
396 Freedom EVO (TECAN, Switzerland) LHS for the intermittent fed-batch cultivation of
397 *E. coli* (Faust et al., 2014). The authors also developed a modified enzymatic feeding
398 strategy to allow the use of an alternative substrate, sucrose. Sucrose was fed
399 intermittently to cultures containing beta-fructofuranosidase enzyme via the LHS,
400 allowing continuous release of metabolisable fructose and glucose. Comparison of the
401 intermittent glucose and enzymatic feeding strategies revealed very similar biomass
402 accumulation, however, GFP fluorescence was enhanced with continuous (enzymatic)
403 feeding. DO oscillations were substantially greater in the intermittently fed cultivations
404 (Faust et al., 2014).

405 High-throughput, parallelised MBR systems coupled with automated liquid handling-
406 based feeding capabilities are an ideal platform for the implementation of DoE, allowing
407 efficient exploration of the design space whilst reducing the experimental burden of
408 bioprocess development. Kensy *et al.* performed a four-factor full factorial DoE to
409 optimise fed-batch *K. pastoris* cultivations in a single microplate run using the
410 RoboLector® platform (Hemmerich and Kensy, 2013). A liquid handling station was
411 also used to implement a custom DoE for the optimisation of a fed-batch medium for
412 CHO cell culture (Rouiller et al., 2013). A total of 43 factors were screened in shaken
413 96-deep-well plates and multivariate data analysis was used to predict optimal media
414 formulation and identify key factors for further optimisation (Rouiller et al., 2013).



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416 **Fig. 3.** pH control and programmed feeding/sampling mechanism of an Ambr® 15
417 microbioreactor system. An automated handler (robotic arm) is connected to a control unit
418 and together they form a control mechanism. The pH of the cell culture is measured by a
419 sensor beneath the well. When the pH measurement is outside the dead band, the liquid
420 handler can add acid/base to the culture medium until pH returns to its setpoint. On the other
421 hand, feeding or sampling can be carried out at certain intervals by the automated handler.
422 The commands are sent from the control unit to the robotic arm to transfer the liquids from
423 the source to the destination. For pH control, commands are given as a response to the
424 signal data collected by the sensors while feed addition or sample collection is applied
425 periodically.

426 **3.1.1 Towards automated sample analysis for model-based control of automated**
427 **feeding**

428 Fed-batch processes are inherently subject to oscillations, pulses, and parametric
429 uncertainties. As a result, linear assumptions may lead to mismatch between
430 computational and experimental results or suboptimal feed regimes. Dynamic, non-
431 linear model-based experimental designs have the potential to provide more reliable
432 predictions for process development and optimisation (Abt et al., 2018). Traditionally,
433 sequential experimentation has proven to be an effective strategy for increasing the
434 robustness of non-linear dynamic models and minimising parametric uncertainty (Abt
435 et al., 2018). Although this necessitates cumbersome repeated experimentation, liquid

436 handling robots that allow autonomous operation of complex and parallel experiments
437 have enabled a dramatic increase in experimental throughput for controlled fed-batch
438 cultivations recently. However, the automation of offline analysis, data handling and
439 evaluation remains a significant bottleneck (Nickel et al., 2017; Sawatzki et al., 2018).
440 Frequent automated sampling of 48 simultaneous cultivations over a period of several
441 days generates hundreds of samples for offline analysis of critical parameters such as
442 biomass, substrate, and product content. In addition, the vast multivariate data sets
443 require statistical analysis and formulation for subsequent use in process control and
444 optimisation (Janzen et al., 2019; Nickel et al., 2017). Automation of these processes
445 is therefore imperative to maximising throughput.

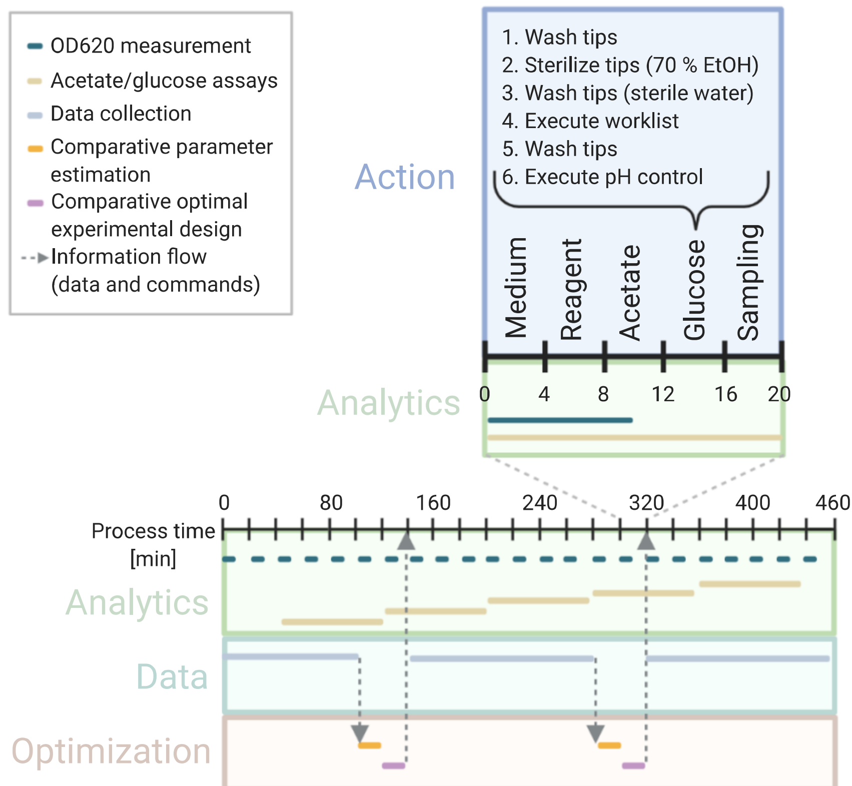
446 Researchers at Technische Universität Berlin developed a modular platform with the
447 aim of fully automating process control, substrate feeding, sampling, atline sample
448 analysis and data processing (Haby et al., 2019; Nickel et al., 2017; Sawatzki et al.,
449 2018). The bioREACTOR 48 system (2mag AG, Germany) was used for high-
450 throughput fed-batch cultivation in the platform. The system was made up of 48 parallel
451 reactors with a working volume of 8–15 mL each. Each reactor had optical pH and DO
452 sensors and its own impeller allowing control of DO. Feed addition, process control
453 and sampling were performed using a Freedom EVO LHS (TECAN, Switzerland).
454 Since pipetting operations occupied the entire capacity, an additional LHS (Hamilton
455 Microlab Star) was installed to automate the offline analysis of glucose and acetate
456 concentration (Nickel et al., 2017). Researchers achieved fed-batch cultivation using
457 the enzymatic EnBase® feeding strategy coupled with an optimal experimental design
458 approach (OED). Optimal experimental designs are a class of DoE applied to
459 determine the best set of experiments to maximise the identifiability of unknown model
460 parameters (Balsa-Canto et al., 2021). Nickel *et al.* employed an OED strategy to
461 minimise uncertainty of parameter estimates for a kinetic fed-batch *E. coli* model
462 (Nickel et al., 2017). Following depletion of glucose in an initial batch phase, additions
463 of acetate, glucose, Reagent A, and media were initiated via the automated cultivation
464 platform according to the OED. Process responses to such additions were monitored
465 and the resulting data was incorporated real time into sliding window optimal
466 experimental re-design (SWORD) algorithms (Cruz Bournazou et al., 2017) to
467 redetermine the optimal experimental design and appropriate subsequent input actions
468 (Nickel et al., 2017). SWORD aimed to minimise the uncertainty in parameter
469 estimation through the iterative re-design of experiments as more and more
470 information became available. Using this approach, a mean deviation of just 4.83 %
471 was observed for the prediction of 23 model parameters across eight parallel MBRs
472 (working volume 11/mL each) (Nickel et al., 2017). In another proof of concept study,
473 the method was found to reduce parameter variance by a factor of 50 (Cruz Bournazou
474 et al., 2017). A simplified description of the method is presented in Figure 4. With the
475 online generation of high-quality experimental data, model-based real time
476 experimental redesign and optimisation is becoming increasingly feasible.

477 Sawatzki *et al.* used the automated cultivation platform to screen the effect of
478 experimental conditions on recombinant endopolygalacturonase production by *S.*
479 *cerevisiae* AH22 (Sawatzki et al., 2018). A fractional factorial DoE with 16 different
480 factor combinations was implemented in triplicate using the built in bioREACTOR 48
481 system. A series of constant, linear, and exponential feeding strategies were included

482 in the experimental design. Following an initial batch phase, feeding was achieved *via*
483 small bolus substrate additions using the Freedom EVO® (TECAN, Switzerland) LHS.
484 Automated sampling was achieved using the LHS, with samples taken from each
485 reactor every two hours. The second LHS was used for atline analysis of OD and
486 glucose concentration and the remainder of the samples was stored at –20 °C for later
487 offline analysis of endopolygalacturonase and ethanol production. Multivariate
488 statistical methods were employed by the authors to analyse online and atline data as
489 it was generated for online decision making. Batch wise unfolded principal component
490 analysis was used to identify batches displaying abnormal behaviour and partial least
491 square regression models were built to predict enzyme activity (Sawatzki et al., 2018).
492 Haby *et al.* subsequently used the integrated automation platform to monitor the
493 production of recombinant mini-proinsulin by *E. coli* in 24 MBRs (Haby et al., 2019).
494 DO and pH values were measured online, OD was measured atline and glucose and
495 acetic acid concentrations were measured offline by the platform (Haby et al., 2019).
496 Model-based MATLAB algorithms were implemented to improve the efficiency of the
497 system's monitoring and optimise production patterns to identify the best-performing
498 phenotype (Haby et al., 2019). This platform demonstrated a major advancement
499 toward automated, computer aided bioprocess development.

500 Janzen *et al.* also attempted to construct a fully automated millilitre scale platform for
501 fed-batch cultivation, sample handling and storage (Janzen et al., 2019). Automated
502 cleaning and sterilisation procedures were integrated to maintain aseptic conditions
503 and eliminate reliance on antibiotics. The platform contained four blocks of 10 mL
504 bioreactors (bioreactor 48, 2mag AG, Germany) allowing up to 32 fed-batch
505 cultivations to be run simultaneously. Control of pH was achieved through automated
506 bolus additions of acid and base solutions. Cascade DO control was achieved by
507 altering stirrer speed and oxygen supplementation in each block of reactors.
508 Adjustments were made according to the reactor with the lowest DO level. Biomass
509 was measured atline using a microplate spectrophotometer and additional samples
510 were transferred to –20 °C storage for subsequent offline analysis of heterologous
511 product accumulation.

512



513

514 **Fig. 4.** An online optimal experimental design sequence performed on an array of eight
515 MBRs coupled to two robotic handling systems: one for substrate addition and another for
516 offline measurements, performed in 20-minute intervals. The amount of substrate added to
517 MBR in the array was determined by a control and modelling system, which analysed
518 experimental data in real time to decide on the next input action. Adapted from Nickel et al.
519 (Nickel et al., 2017)

520

521 3.2 Microfluidic and microvalve technologies for fed-batch microbioreactor 522 systems

523 A key challenge associated with automated LHSs is the intermittent nature of the
524 feeding. Feeding is achieved via discrete additions which cause major oscillations in
525 substrate availability and hence cellular metabolism (Heins and Weuster-Botz, 2018).
526 In industrial scale fed-batch processes, continuous feeding, in which the substrate is
527 fed continuously to maintain a more stable growth rate and avoid substrate
528 accumulation, is preferred. Recently, microfluidic technologies have been implemented
529 with the aim of developing more accurate scale-down models of industrial processes
530 (Blesken et al., 2016; Funke et al., 2010b; Marques and Szita, 2016). Microfluidic
531 bioreactor systems involve the controlled manipulation of small volumes of fluid.
532 Continuous delivery of solutions is possible *via* the liquid channels allowing industrially
533 relevant feed profiles and control strategies to be implemented in the absence of bulky
534 LHSs (Blesken et al., 2016).

535 Funke *et al.* developed a high-throughput microfluidic MBR device by modifying the
536 established BioLector® system described in Section 3.1 (Funke et al., 2010b). The
537 base of the FlowerPlate MTP of the BioLector® was replaced with polystyrene

538 microfluidic chips, each chip spanned a row of six wells, two reservoir and four
539 cultivation wells. The two reservoirs allowed the feeding of two different solutions to
540 each of the culture wells *via* fluid channels (Funke et al., 2010a). The microvalves and
541 pump chambers were grouped together and placed underneath the reservoir wells to
542 avoid interference with the optical sensor measurements (Figure 5A). Control of
543 medium pH *via* direct feedback has been demonstrated in the plates (Funke et al.,
544 2010a, 2010b). Deviations from the setpoint pH during *E. coli* cultivations triggered the
545 opening of the microvalves and hence supply of either acid or base from the
546 corresponding reservoir wells as appropriate (Funke et al., 2010a, 2010b). Opening
547 and closing of the valves was achieved by the application or release of pneumatic
548 pressure, respectively. In fed-batch *E. coli* cultivations, substrate concentration could
549 not be measured directly, requiring open loop control in which the substrate flow rate
550 followed defined feeding profile (Funke et al., 2010a, 2010b). This could not be
551 achieved using the initial design as flow rate was dependent on valve opening time,
552 channel length and pneumatic reservoir pressure. To overcome this, additional
553 microvalve and pump chambers were installed in the liquid channel between the
554 reservoir and culture wells to facilitate the pumping of a defined volume of liquid per
555 pump step (Funke et al., 2010a, 2010b) (Figure 5B). The required flow rate could
556 therefore be achieved by defining the necessary number of pump steps per unit time
557 (Funke et al., 2010b). Using this approach, *E. coli* was cultivated in fed-batch mode
558 with constant glucose feeding at a rate of 1 mg (2 μL) per hour for 22 hours (Funke et
559 al., 2010b). In a subsequent study, Funke *et al.* extended the application to exponential
560 feed profiles (Funke et al., 2010a). The resulting biomass, DO and pH curves were
561 comparable to those of a parallel cultivation in a 2 L stirred tank bioreactor. However,
562 the feed rate was deemed to be around 30 % lower than the setpoint due to calibration
563 challenges. Whilst feedback control is typically implemented through monitoring the
564 mass of the feed solution, this is not possible in an MTP, where the pump rate must be
565 calibrated prior to the experiment. As a result, improving the time stability of the pump
566 calibration was deemed necessary to improve comparability to larger scale.

567 In another study, *E. coli* was used as biocatalyst in fed-batch mode to produce
568 bioelectricity from glucose and urea in a microfluidic microbial fuel cell (MFC)
569 (Mardanpour and Yaghmaei, 2016). To construct the microfluidic MFC, a poly methyl
570 methacrylate plate with a single microchannel was used as the main body while nickel-
571 based anode and platinum loaded, carbon clothed cathode were used as the
572 electrodes at the top and under the main body as illustrated in Figure 5C. In this way,
573 biofilm growth was promoted by the hydrophilic nickel surface absorbing the anolyte
574 and facilitating the attachment of the cells. Using this inexpensive setup (<$1 per
575 device), the maximum power density of 5.2 μW cm^{-2} was achieved in the microfluidic
576 MFC with glucose-feeding thanks to extracellular electron transfer capability of *E. coli*,
577 whereas a maximum power density of 14 W m^{-3} was obtained with urea-feeding
578 (Mardanpour and Yaghmaei, 2016). Compared to similar studies reported previously
579 (Kerzenmacher et al., 2011; Oncescu and Erickson, 2011), these efficient energy
580 productions from the sources found in human excreta and urine show that microfluidic
581 fed-batch systems can be promising solutions for simultaneous waste-water treatment
582 with relatively low device costs.

To determine the most suitable microfluidic system to reproduce fluctuating conditions of large-scale bioreactors, Ho *et al.* compared three widely used microfluidic designs; mother machine, monolayer growth chambers, and negative dielectrophoresis (Ho et al., 2019). Mother machines consist of one (Wang et al., 2010) or two (Long et al., 2013) supply channels for fresh medium feeding and one growth channel that branches from the supply channel(s) and contains the cells monolayer growth chambers. The cells are held in a growth chamber fed with fresh medium by a single or multi supply channel allowing the cells to grow in a single layer (Grünberger et al., 2015, 2012). Negative dielectrophoresis systems are used to trap the individual cells in the centre of a supply channel (Fritzsch et al., 2017). In this way, a single cell can be isolated and cultivated without any mechanical force. These devices were first compared using computational fluid dynamics simulations with a range of frequencies as input signals and *C. glutamicum* was used to model the cells. It was followed by an experimental validation using a monolayer growth chamber (Ho et al., 2019). Among the systems, only the mother machine with two supply channels could reproduce low frequency signals down to 1 Hz to mimic oscillations in the large-scale conditions, whereas other designs lost 99% of the 1 Hz signals (Ho et al., 2019). This study indicates that device design of microfluidic systems plays critical role on quantitatively and sensitively reproducing inhomogeneities in a typical industrial scale bioreactor that might affect the process yield of fed-batch systems.

The microfluidic FlowerPlate technology was recently employed to optimise green fluorescent protein (GFP) production by *C. glutamicum* (Morschett et al., 2020). Morschett *et al.* developed a high-throughput parallelised workflow of pH-controlled, fed-batch cultivations with online monitoring of biomass, pH, DO and fluorescence in the microplates. The two reservoirs of each row were charged with a glucose-urea feed solution and 3 M phosphoric acid (single sided pH control), respectively. Fed-batch processes with different feeding strategies (pulsed, constant, exponential) were compared to a standard batch process. Gradual substrate feeding was found to improve GFP production substantially compared to batch cultivation with 2.27, 2.36 and 2.37-fold improvements observed for the pulsed, constant, and exponential fed-batch cultivations, respectively. Although yields of the pulsed and continuously (constant, exponential) fed cultivations were similar, strong oscillations in the DO concentration were observed in the pulsed process, indicating the cells were repeatedly exposed to substrate limitation. With constant feeding, however, no substrate limitation was observed, and the DO concentration of the cultures remained above 55 % throughout. As exponential feeding did not significantly improve GFP production compared to the simpler constant feeding approach, fed-batch with constant feeding was deemed the optimal strategy for the process investigated (Morschett et al., 2020).

The commercial micro-Matrix (Applikon Biotechnology, the Netherlands) platform is an alternative approach to near continuous feeding which facilitates independent liquid additions for each individual μBR via microvalves (Applikon Biotechnology, 2021). The state-of-the-art system, which is based upon a standard 24-deep well plate with a working volume of 2-7 mL, features integrated fluorophore sensors for pH and dissolved oxygen and independent gas and liquid additions for each individual well. As a result, pH, temperature and DO may be controlled separately in every well (Applikon

629 Biotechnology, 2021; Wiegmann et al., 2019). Microscale fed-batch cultivation of GS-
630 CHO cells according to six different feeding regimens was demonstrated with the
631 micro-Matrix system (Wiegmann et al., 2019). Relatively simple bolus and continuous
632 feeding strategies were compared to more complex approaches based on nutrient or
633 viable cell concentration. Interestingly, as for the microfluidic FlowerPlate system
634 (Morschett et al., 2020), the more simple feeding strategies were deemed optimal, as
635 increasing complexity did not correspond to any significant improvements in growth or
636 productivity (Wiegmann et al., 2019). The micro-Matrix has also been demonstrated
637 for microbial fed-batch cultivation using an *E. coli* strain engineered for production of
638 K4 capsular polysaccharide (D'ambrosio et al., 2021). Comparable product titres were
639 obtained for controlled DO-stat fed-batch cultivation using the micro-Matrix and 2 L
640 stirred tank bioreactors, highlighting the scalability of the system. Although gas
641 sparging enabled effective DO control in each individual well, evaporative losses were
642 exacerbated, resulting in a 31 % reduction in culture volume after just 48 hours
643 (D'ambrosio et al., 2021). For aerobic fed-batch cultivation, compensation for liquid
644 losses is therefore recommended (D'ambrosio et al., 2021; Wiegmann et al., 2019).

645 Although external hardware brings functionality to microfluidic devices, poor portability
646 of required operating systems can be a bottleneck. In addition, experiments are often
647 restricted to a single microplate per experiment due to costly and bulky hardware,
648 which limits throughput. The development of compact, integrated actuators with low
649 energy requirements is desirable to reduce reliance on bulky tubing and external
650 pressure sources. For decades, therefore, many researches have focused on
651 improving the design principles of microfluidic systems and developing integrated
652 microfluidic systems (IMS) using micromachines such as micro pumps, drivers, mixers,
653 and valves for multiplexed, high-throughput and automated biological applications
654 (Coluccio et al., 2019; Gencturk et al., 2017; Melin and Quake, 2007). An IMS with a
655 computer-controlled driver was developed for real-time tuning of bio-fluid mixing (Lam
656 and Li, 2012). This integrated device contained multiple independent modules, each
657 one integrated with two vortex micropumps, two Tesla valves and a micromixer to
658 produce an enough flow rate to mix the fluids containing cells or biochemicals. An
659 external digitally controllable driver was used to produce the electric signals to actuate
660 the micropumps and micromixers. This portable system without bulky parts set an
661 example of IMS to be used for precise and automated bio-fluid manipulations (Lam
662 and Li, 2012).

663 A microplate reader-compatible microfluidic system encompassing 30
664 microbioreactors was designed to allow high-throughput cell culture assays (Huang et
665 al., 2013). This integrated device consisted of four main components; a heater chip to
666 control culture temperature, a micro-scale sample loading part to add the cells,
667 pneumatically driven multiplex medium perfusion mechanism, and a medium collector
668 array that is microplate reader-compatible of subsequent analyses. Following the
669 device fabrication, the researchers used this versatile system to observe
670 chemosensitivity of human oral cancer cells (Huang et al., 2013). Tsai *et al.* developed
671 a digital hydraulic driver comprised of shape memory alloys and pneumatic cylinders
672 with a control capacity of 256 microvalves in parallel on a microfluidic chip for
673 integrated actuation (Tsai et al., 2017). In the follow-up study, the driver was integrated
674 into a 'functional lid' to realise an alternative mixing strategy in the microfluidic plates

675 (Tsai et al., 2018). The lid was placed on top of a 96 well plate to aspirate and release
676 liquid from the wells of the MTP, and gas exchange was ensured *via* its cavities (Figure
677 5D). Employing this portable IMS, CHO monoclonal antibody (mAb) cells were cultured
678 to compare the growth features with the static cultures. Researchers reported a better
679 culture growth with reciprocating mixing maintained by the driver integrated functional
680 lid (Tsai et al., 2018).

681 As many research groups have developed custom-made IMSs, they lack standard
682 design, therefore there are very limited research on evaluation the suitability of these
683 systems fed-batch cultivation. Standardised IMS construction systems or methods as
684 proposed by semiautomatic Microfluidic Device Assembly System, μDAS (Kipper et
685 al., 2017), can accelerate the expansion of the use of IMSs for more diverse
686 applications including fed-batch operations.

687 **3.3 Summary of external feeding strategies**

688 Recent advancements in novel MBR technologies with automated external feeding and
689 tight control of process parameters have enabled much closer mimicry of industrial
690 scale bioprocesses. Dramatic improvements in experimental throughput and precision
691 have been achieved through automation. Robotic LHSs have demonstrated potential
692 for effective high-throughput fed-batch cultivation at microscale. They can be coupled
693 with existing hardware and readily programmed to achieve wide ranging experimental
694 applications. Modification of the bioREACTOR cultivation platform by installing liquid
695 handling robots and analytical equipment enabled fully automated controlled fed-batch
696 cultivation with automatic sampling and atline sample analysis. The adaptability of the
697 RoboLector® platform was also demonstrated in a study by Mühlmann *et al.*
698 (Mühlmann and Büchs, 2018). With the aim of automatic feed media preparation and
699 cell cultivation, additional coolers, heater shakers, and vacuum stations were installed.
700 Pipetting operations may be pre-programmed to execute defined feed profiles and
701 repeated multiple times with high precision. This allows enhanced flexibility compared
702 to the diffusion or enzyme-based strategies outlined in Section 2.

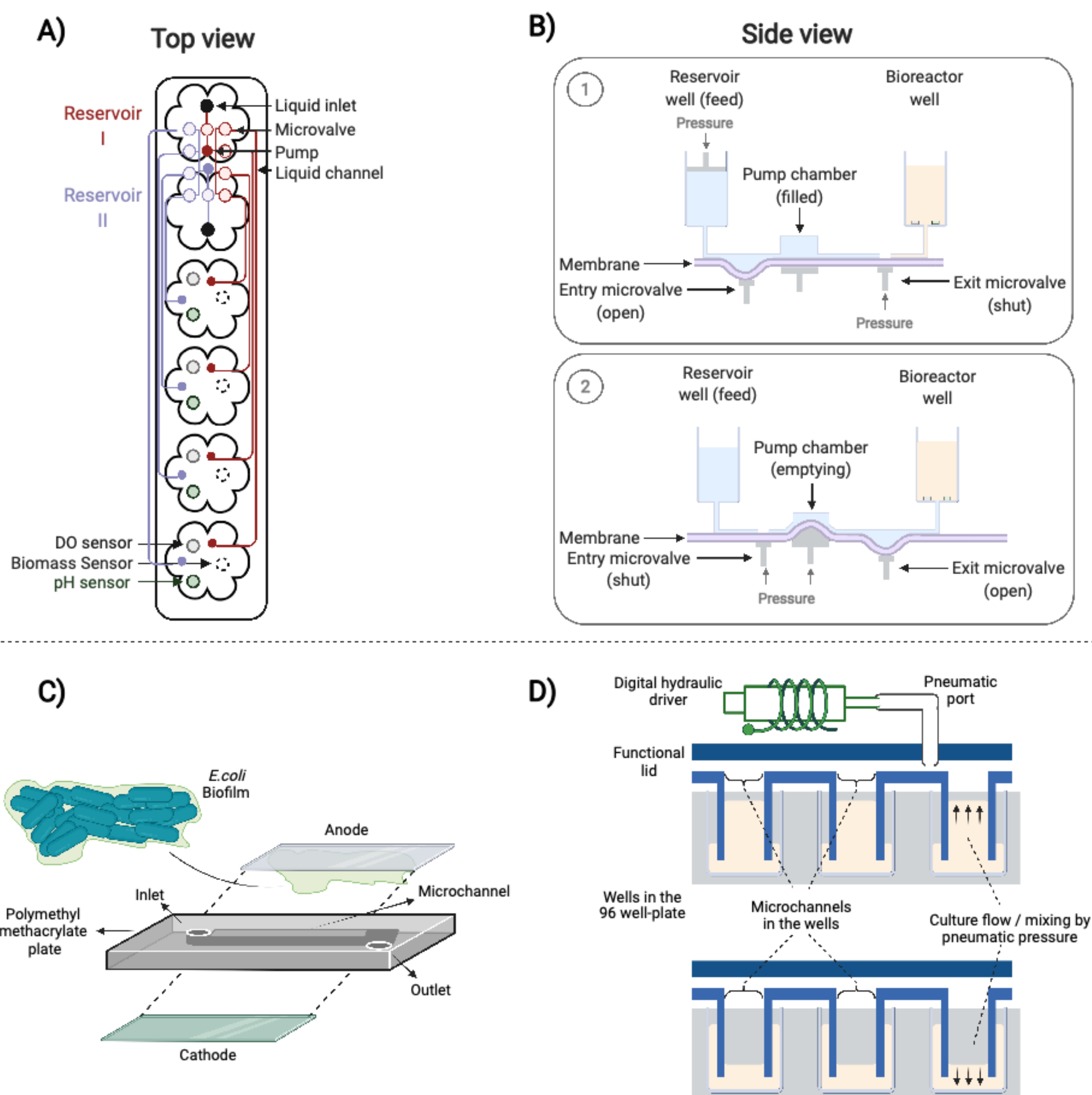
703 Another limitation of LHS-based feeding is its intermittent nature. Microfluidic devices
704 provide a continuous supply of feed to more closely represent industrial scale
705 conditions. Small volumes may be dispensed using microfluidic devices, rendering
706 them particularly attractive for the study of individual cells (Doong et al., 2018). This
707 can be beneficial for strain development as the study of isolated cells allows
708 intracellular effects to be distinguished from intercellular or population effects.

709 Automated parallelised MBR platforms with external feeding and non-invasive online
710 monitoring allow large, high quality data sets to be generated in a relatively short time
711 period (Bjork and Joensson, 2019). However, the investment is significantly greater
712 than more simple internal systems due to the high equipment costs and extensive
713 programming requirements. The expertise and sophisticated technological resources
714 may not always be available.

715 More affordable robotic platforms such as the Opentrons and OTTO have been
716 developed recently. These liquid handlers are distributed under MIT-licence allowing
717 the use of their software or hardware without any restrictions, therefore they provide
718 both free open-source software (FOSS) and free open-source hardware (FOSH) as a

719 part of open-source culture movement. In this way, these platforms can be
720 continuously improved by the community. Such platforms are highly flexible and could
721 be coupled to a microbioreactor system to enable fed-batch operation at a relatively
722 low cost (Bertaux et al., 2020; May, 2019). Recently an open-source Python platform,
723 named Pyhamilton, to program Hamilton robots such as Hamilton STAR, STARlet, and
724 Microlab VANTAGE was developed (Chory et al., 2021). With Pyhamilton, 480 *E. coli*
725 cultures were monitored in log-phase using 96-well plates. Automated cultivation
726 platforms could be improved dramatically if more open-source operating software
727 alternatives were developed for such platforms. Also, knowledge shared among the
728 users of automated cultivation platforms through online platforms or video-assisted
729 protocols (Velugula-Yellela et al., 2018) is expected to facilitate take-up of this type of
730 apparatus.

731



732

733 **Fig. 5.** A) View of a microfluidic well bioreactor system from above. The bioreactor well
734 contains sensors for pH, DO, and OD in the bottom of the well. Reservoir and substrate wells
735 are connected to the bioreactor well by membrane valves. B) Side view of a microfluidic well

736 bioreactor system showing a substrate (feed) being transferred from its reservoir to the
737 bioreactor well. 1) Pneumatic pressure is applied to the substrate liquid surface, causing
738 substrate to move through the entry microvalve underneath the well towards the pump
739 chamber. Pneumatic pressure is applied to the exit microvalve to keep it closed while the
740 pump chamber fills with substrate. 2) The entry microvalve is shut and the pump chamber
741 emptied by applying pneumatic pressure. The exit microvalve opens, allowing substrate to
742 flow into the bioreactor well. This microfluidic feeding mechanism is employed in
743 commercially available devices, such as the BioLector Pro® (m2p Labs GmbH). Adapted
744 from Funke et al., 2010b. C) Partially exploded view of the single channel microfluidic
745 microbial fuel cell (MFC) demonstrating the eight-cm main body and five-cm electrodes.
746 Hydrophobic nickel-based anode was used as a surface for biofilm growth while a carbon
747 clothed, and platinum loaded cathode was used to produce electric current. The cells were
748 fed with glucose or urea containing medium through the inlet. Adapted from Mardanpour and
749 Yaghmaei, 2016. D) Side view of the integrated microfluidic system containing a functional lid
750 with a digital hydraulic driver. The functional lid containing the microchannels is placed onto
751 the 96 well plate to release and aspirate the liquids as represented by the arrows. The gas
752 exchange is ensured by the pneumatic port connected with the digital hydraulic driver. In this
753 way, the flow of the liquid in the channel to create more homogenous culture is ensured.

754

755 **4. Conclusion**

756 Technologies for high-throughput fed-batch cultivation at microscale have advanced
757 substantially over the past decade. The key systems reviewed here are compiled in
758 Table 1. A wide range of feed mechanisms of differing complexity and hardware
759 requirements have been developed, rendering fed-batch cultivation increasingly
760 accessible. As fed-batch systems allow much closer mimicry of industrial scale
761 conditions than traditional batch cultivation systems, they allow risks associated with
762 bioprocess scale up to be minimised.

763 Despite being relatively low cost and easy to implement, diffusion and enzyme-
764 controlled feeding strategies have a major advantage of continuous substrate supply.
765 However, precise feed rate control is not possible throughout the cultivation and
766 feeding is typically limited to a single substrate. Implementation of more complex feed
767 profiles and control of process parameters such as pH is possible through the
768 introduction of external hardware. Automated liquid handling robots may be
769 programmed to perform liquid additions in response to deviations in process
770 parameters from specified set-points or according to predefined feed profiles. Recently
771 the affordability of automated liquid handling robots has improved dramatically,
772 however, the development of standardised operating procedures and intuitive software
773 for their straightforward operation is necessary for ensuring their widespread uptake.
774 Although their high precision and flexibility are advantageous, as feeding is *via*
775 intermittent bolus additions, industrially relevant continuous feed profiles cannot be
776 implemented. However, this could be readily addressed by coupling the LHS and
777 enzyme-controlled feeding strategies. Microfluidic technologies have also been
778 developed to facilitate continuous precise feeding of very small volumes.

779 By combining automated high-throughput fed-batch cultivation platforms with strategic
780 design of experiments and model-based optimisation strategies, process
781 understanding can be enhanced dramatically whilst minimising the experimental
782 burden. The incorporation of real time data to re-determine the optimal feed additions
783 and process control strategies shows great potential to enhance bioprocess

784 development. However, technology for online and atline analysis of critical process
785 parameters should be improved to fully realise the potential of model-based
786 optimisation. Parameters such as substrate utilisation and product formation, which
787 are critical to optimisation, are limited to offline assays in most cases. The development
788 of rapid, online alternatives to traditional techniques such as chromatography would
789 be particularly beneficial for re-design of experiments strategies.

790 Although the technologies discussed in this review show great potential for efficient
791 and low-risk bioprocess development, currently the high cost and complexity of
792 automated cultivation platforms limit their widespread application. Moreover,
793 standardisation of these technologies and methods is essential for their common use
794 and acceptance by the communities in academia and industry (Beal et al., 2020).
795 Future work should also focus on the development of FOSS and FOSH to improve
796 accessibility.

797

798 **Acknowledgements**

799 The authors acknowledge the funding from the British Council (Grant Number:
800 527429894), the Engineering and Physical Sciences Research Council (Grant number
801 EP/R513209/1), the YLSY Program of the Ministry of National Education of the
802 Republic of Turkey, and the Heinrich Böll Foundation.

803

804 **Conflict of interest**

805 The authors declare that they have no conflict of interest.

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1052 **Table 1.** Recently developed fed batch feeding methods and microbioreactors

Name	Manufacturer	Size	Controlled variables	Advantages	Disadvantages	References
Diffusion and enzyme-controlled feeding						
EnBase® FeedBeads® FeedPlate®	BioSilta Ltd Kuhner Shaker Kuhner Shaker	Microplate or shake flask	• Substrate concentration in gel • Initial concentration of enzymes in medium	• Easy to integrate into microplate or shake flask • High cell densities compared to standard methods • Control of growth rate • No additional equipment needed • Scalability demonstrated up to 150 L	• Limited substrate range for enzyme-based feeding • Limited process control due to working conditions of enzymes • Enzymes cannot be used for organisms producing amylases or proteases (Philip et al., 2017) • Limited shelf life of enzymes (Philip et al., 2017) • Addition of enzyme inhibitor or pH shift may be required to change active enzyme concentration	• (Glazyrina et al., 2012; Huber et al., 2009b; Krause et al., 2016; Li et al., 2014; Panula-Perälä et al., 2008; Philip et al., 2017)
High-throughput and real-time monitoring devices						
BioLector®	m2p Labs	0.8–2.4 mL	• Gases (O_2, CO_2, N_2) • Humidity • Agitation rate • Temperature	• Allows different feeding regimes to be studied • Online monitoring • Scalability to 1 L demonstrated	Further studies needed to demonstrate reproducibility and scalability	(Funke et al., 2010a, 2010b; Nickel et al., 2017)
Automated platforms						

Ambr15® RoboLector® Micro-Matrix bioreactor 48	Sartorius AG m2p Labs Applikon Biotechnology	10–15 mL 0.8–2.4 mL 2-7 mL 8-15 mL	• Impeller speed • Agitation rate • Proportional integral control of O_2, CO_2, N_2, air valves • Feed frequency and volume • Gas composition and flowrate (Applikon Biotechnology, 2021) • Built in impeller and pH and DO sensors in each reactor	• Scalability of 2, 3, 5, 10, 15 & 200 L demonstrated • Efficient screening of microbial strains to determine the promising candidates • Data transferability for larger scale cultivations • Automated preparation of feed media • Single-use, pre-calibrated • Parallel, high throughput: 24–48 vessels • Online measurement of OD and substrate concentration • Individual control of temperature, pH and DO for each individual well • Independent 200 nL liquid additions to each well via microvalves • Independent gas supplies to each well • Coupling with a LHS enabled	• Relatively high investment costs • Requires skilled users • Intermittent feed not representative of production scale conditions • Evaporative losses exacerbated by gas sparging • Only one MTP may be ran at a time using the system. • Almost impossible to avoid the deviations between microscale and industrial scale	(Alsayyari et al., 2018; D'ambrosio et al., 2021; Faust et al., 2014; Fink et al., 2021; Haby et al., 2018; Hemmerich et al., 2014; Rameez et al., 2014)

				precise fed-batch operation		
Microfluidic devices						
Microtiter plate (MTP), microvalves, micropumps, microfluidic chip (Funke et al., 2010b) 3D printed microchemostat (Kim et al., 2017) Microfluidic microbial fuel cell (MFC) (Mardanpour and Yaghmaei, 2016)	Custom Custom Custom	≤1 mL	• pH range (Funke et al., 2010b) • Feed rate (Funke et al., 2010b), • Mixing rate • Single cell microenvironment (Kim et al., 2017)	• User-friendly handling (Funke et al., 2010b) • Online monitoring • Microchemostat allows a single cell to be studied • Fine control of feed rate allows controlled growth rate • Biofilm growth to produce biofuel	• Need for auxiliary equipment • Setup challenges • Possibility of membrane fouling	(Funke et al., 2010b; Kim et al., 2017; Mardanpour and Yaghmaei, 2016;)

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