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Microdroplet screening and selection for improved microbial production of extracellular compounds

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Microorganisms produce extracellular compounds that affect the final product quality in fermentation processes. Selection of overproducing mutants requires coupling of the extracellular product to the producer genotype, which can be achieved by single-cell compartmentalization. Emulsions contain up to billions of microdroplets/mL which significantly increases the screening throughput compared to microtiter-plate-based selections. Factors affecting the success of screening in microdroplets include the nature of the producing organism (robust, no invasive growth), the product (not soluble in oil) and the product assay (preferably fluorescence based). Together these factors determine the required microdroplet production technique and sorting set-up. Because microdroplets allow relatively inexpensive ultrahigh-throughput screening, they are likely to become a standard tool in the strain selection toolbox of the fermentation industry.

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Introduction

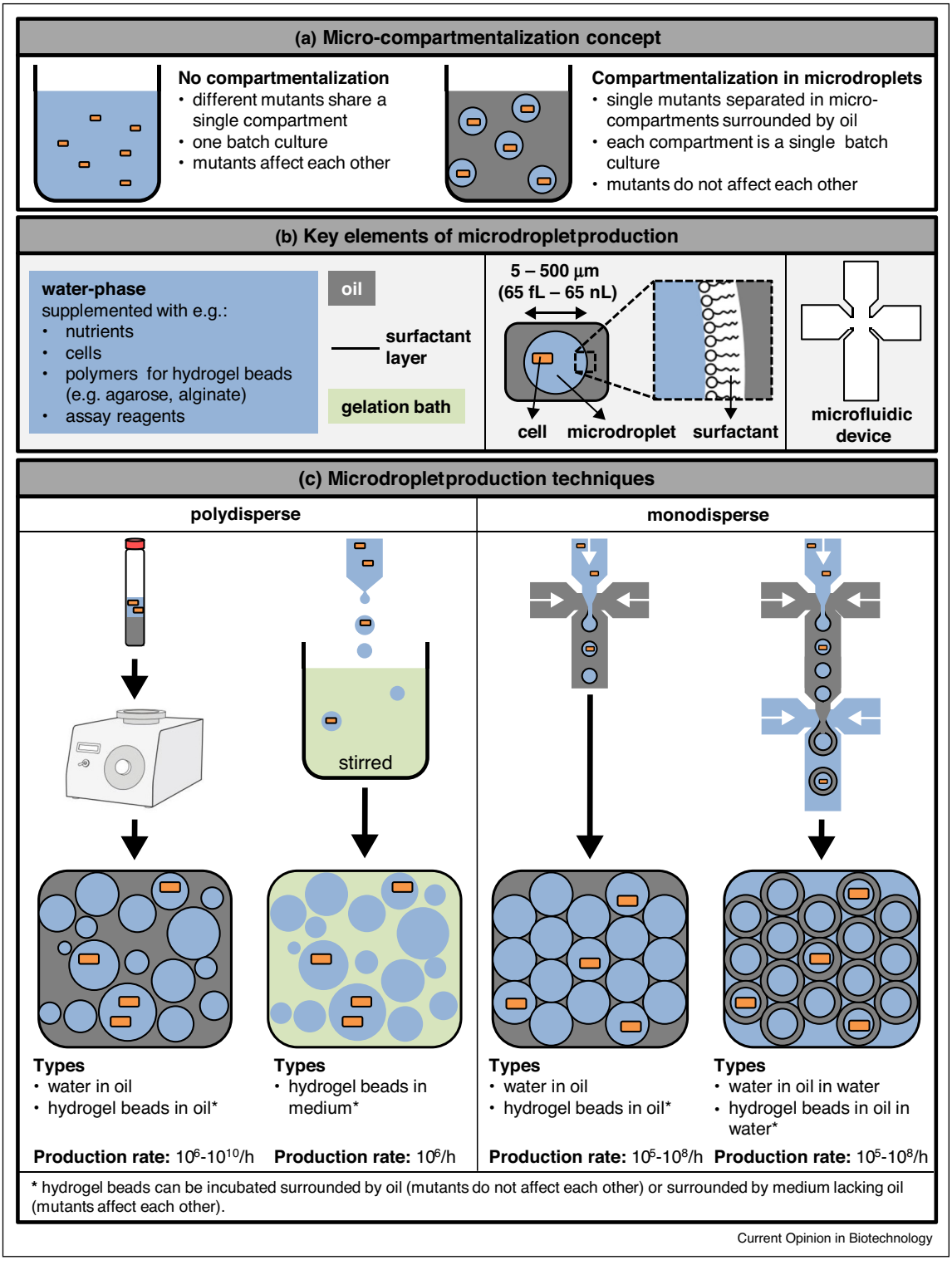
In many fermentation processes microorganisms produce extracellular compounds such as flavor volatiles, enzymes, polysaccharides and antimicrobials, which determine the quality of the final product. Mutant selections are regularly performed to alter the production profile of these compounds [1,2]. Selection systems in which mutants grow in a single compartment (e.g. shake flasks or batch reactors), often fail to enrich improved producers of extracellular compounds, because diffusion uncouples the product concentration from its producer cell (Figure 1a). To maintain such coupling, single cells can be cultured in separate compartments, for instance using microtiter-plates [3••]. The identification of natural mutants might require screening of over 10^4 cells (depending on bacterial species, strains and growth conditions), which is not always feasible with this set-up [4]. Alternatively, a comparison shows that single-cell compartmentalization in emulsions with up to 10^{10} microdroplets/mL allows cost-efficient screening of millions of cells [4]. Selection in microdroplets has been used for over ten years in academic laboratories [5–7] but it only slowly finds its way into the (food) fermentation industry. Here we discuss the potential of microdroplets as high-throughput screening platform for the identification of organisms with increased production of extracellular products. Other microdroplet-based assays such as cell-free systems are outside the scope of this review.

Production of microdroplets

Microdroplets usually have volumes of 65 fL to 65 nL (diameter of 5–500 μm) [5,8,9•,10] and they generally consist of a water-phase that contains the content of the microdroplet (e.g. nutrients, cells, assay reagents, hydrogel bead polymers), an oil-phase that can prevent cross-talk between compartments, and a surfactant that localizes at the water/oil interphase and stabilizes the microdroplet by reducing surface tension (Figure 1b). There are different types of microdroplets: water-in-oil (w/o) emulsions, water-in-oil-in-water (w/o/w) double emulsions and hydrogel beads, and all of them can be either monodisperse or polydisperse (Figure 1c).

Water-in-oil emulsions are generated by mixing an oil-phase containing oil-soluble surfactant and a water-phase. Mixing with a vortex shaker yields polydisperse microdroplets, while the use of microfluidic devices allows to produce monodisperse microdroplets (Figure 1c) [8]. Water-in-oil emulsions can be transformed into w/o/w double emulsions by re-emulsification of the primary w/o emulsion in a water-phase with water-soluble surfactant [6]. In both w/o and w/o/w double emulsions the microdroplets are surrounded by oil, which prevents cross-talk between compartments for compounds not soluble in the oil-phase. In this way every

Figure 1



Microdroplet types and their production techniques.

inoculated microdroplet acts as a single, monoclonal batch culture (Figure 1a).

Hydrogel beads are generated by adding polymers such as agarose or alginate to the water-phase of polydisperse or monodisperse w/o emulsions following gelation [11,12], or by dripping a solution containing polymers in a gelation bath [9•,13]. Next to incubation surrounded by oil, hydrogel beads can be incubated in medium. In that case hydrogel beads (or more complex beads featuring a surrounding shell architecture) resemble 'semi-open' vessels and can be engineered to specific molecular-weight cutoffs ranging from the low kD-range to pores almost of micrometer size [14].

The advantage of polydisperse microdroplets is that the required set-up is inexpensive, easy to use and the microdroplet production rate is high (10^6–10^{10}/h). Their disadvantage is that compartment volumes differ. Depending on whether cells do or do not grow in microdroplets, this leads to differences in either the absolute product amount or the product concentration in microdroplets respectively. When correction for microdroplet-volume is not possible, it is therefore hard to catch variants with only moderate levels of improvement. In those cases the use of monodisperse microdroplets is a powerful alternative, because they typically have less than 3% volume variation. However, compared to polydisperse microdroplets their production rate is lower (10^5–10^8/h) and a more advanced set-up is required [8].

Detailed protocols for the production of monodisperse and polydisperse microdroplets can be found in [8,15], and [5,16,17] contain information about advanced microfluidic emulsion processing.

Encapsulation of cells in microdroplets

To couple the product concentration to its producer genotype, encapsulation of a single cell-variant/genotype per microdroplet is required (Figure 1a). This encapsulation follows a Poisson distribution [8]. When the number of added cells is for example ten times lower than the number of generated microdroplets, 9% of the generated microdroplets contain a single cell, and less than 0.5% contain multiple cell-variants (Poisson distribution, $\lambda = 0.1$). When several rounds of enrichment are possible and variant libraries are huge, higher encapsulation ratios are often feasible. The λ parameter can also be adjusted to allow more than one cell-variant per microdroplet, for instance to co-localize producer and sensor cells [10,18,19••]. Special microfluidic devices can increase the percentage of microdroplets with single cells to 70–100% by aligning cells before microdroplet formation or post-encapsulation sorting [5,20], but good mixing of the inlet streams is required, especially for hydrogel beads [11]. A recent review that summarizes and discusses different encapsulation and cell-alignment techniques can be found in [20].

Strain selections using microdroplets

Organisms

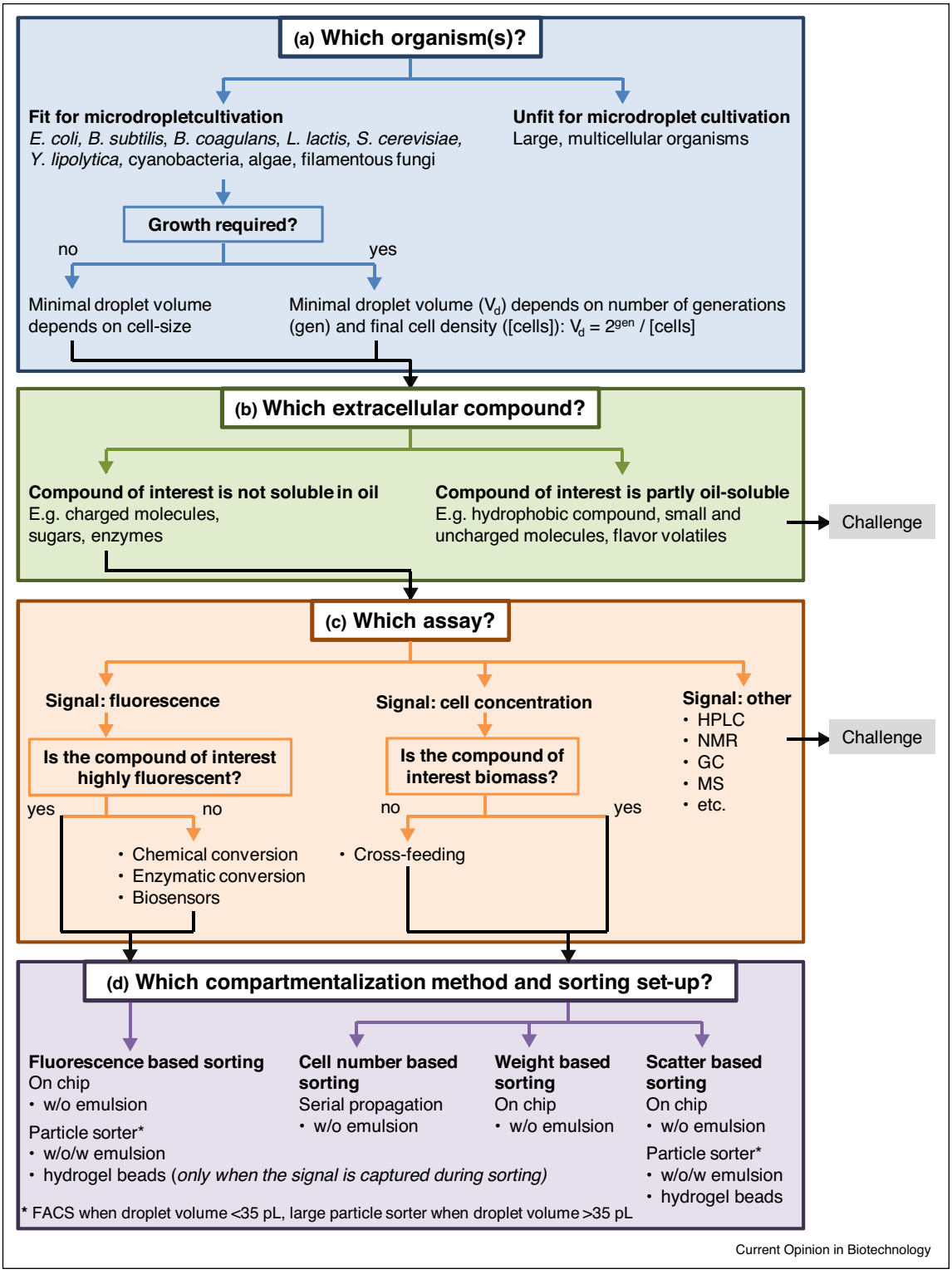
Factors affecting the success of screening in microdroplets include the producing organism, the product of interest, the product assay and the sorting set-up (Figure 2).

Micro-organisms can be cultured aerobically or anaerobically in microdroplets [32,33]. Successful strain selections in microdroplets include *Escherichia coli* [19••,23,28], *Saccharomyces cerevisiae* [12,23,26], *Bacillus subtilis* [13], *Bacillus coagulans* [29], *Lactococcus lactis* [1,9•,31], *Yarrowia lipolytica* [3••], cyanobacteria [21,24,25] and algae [21,27]. As microdroplets are typically inoculated with a single cell there is no competition between genotypes within a droplet. The platform is therefore also well-suited for slow growing micro-organisms. When cells clump, encapsulation of a single cell per microdroplet is not possible. If clumps consist of multiple genotypes selection is in theory possible, even though the selection efficiency will be reduced because the final product-concentration depends on a combination of genotypes. However, we are not aware of examples of successful selections in such a system. Mammalian cells and filamentous organisms can also be encapsulated and sorted, but they require microdroplets in the nL volume range which reduces the throughput [34,35]. For filamentous organisms apical growth of hyphae should be prevented, to avoid uncontrolled microdroplet coalescence [35]. When this is not possible, mutants can be selected using alternative approaches such as conventional microtiter-plate set-ups.

Detection of relevant phenotypes often requires growth in microdroplets. Best *et al.* for instance grew cyanobacteria and algae in microdroplets to select mutants that reached increased cell densities [21]. The minimal required microdroplet volume is determined by the desired number of generations per microdroplet and the final cell concentration in the medium (Figure 2a). Microalgae could for instance grow for 11 generations in 2 nL microdroplets, reaching a final cell concentration of 10^9/mL [27].

Hydrogel beads can also be taken up in growth medium before they are incubated surrounded by oil. This allows uncoupling of growth, product formation and product detection, making it a versatile selection system. Schmitt *et al.* for example grew single *L. lactis* cells in hydrogel beads taken up in growth medium. After micro-colony formation they induced the expression of lantibiotic production genes, re-emulsified the hydrogel beads in oil to prevent cross-talk between microdroplets and assessed the lantibiotic effectivity using co-localized sensor cells [9•].

Figure 2



Decision tree for strain selection in microdroplets.

Table 1

Overview recent literature on strain selections using microdroplets

Producer organism	Droplet characteristics			Product	Assay	Selection		Ref
	Type	Dispersity	Volume (pL)			Type	Throughput	
On-chip sorting								
L. lactis	w/o emulsion	Monodisperse	50	Riboflavin	Riboflavin auto-fluorescence	On-chip sorting	$2{\cdot}10^5$/h	[1]
Cyanobacteria	w/o emulsion	Monodisperse	65	Biomass (chlorophyll fluorescence)	Chlorophyll auto-fluorescence	On-chip sorting	$1{\cdot}10^6$/h	[21]
Algae	w/o emulsion	Monodisperse	34	Biomass (chlorophyll fluorescence)	Chlorophyll auto-fluorescence	On-chip sorting	$1{\cdot}10^6$/h	[21]
S. cerevisiae	w/o emulsion	Monodisperse	34	Tyrosine	Fluorophore binding aptamer	On-chip sorting	–	[22•]
S. cerevisiae	w/o emulsion	Monodisperse	221	Consumption of xylose	Enzyme assay *pyranose oxidase coupled to a H_2O_2-dependent fluorogenic conversion*	On-chip sorting	$1{\cdot}10^4$/h	[23]
E. coli	w/o emulsion	Monodisperse	221	Lactate	Enzyme assay *lactate oxidase coupled to a H_2O_2-dependent fluorogenic conversion*	On-chip sorting	$1{\cdot}10^4$/h	[23]
Cyanobacteria	w/o emulsion	Monodisperse	524	Ethanol	Enzyme assay *ethanol oxidase coupled to a H_2O_2-dependent fluorogenic conversion*	On-chip sorting	$4{\cdot}10^5$/h	[24]
Cyanobacteria	w/o emulsion	Monodisperse	10	Lactate	Enzyme assay *lactate dehydrogenase coupled to a NADH-dependent fluorogenic conversion*	On-chip sorting	$4{\cdot}10^6$/h	[25]
E. coli	w/o emulsion	Monodisperse	87/1,023	2-ketoisovalerate	Biosensor *E. coli, cross-feeding between two auxotrophic strains, sensor is fluorescent*	On-chip sorting	$1{\cdot}10^6$/h	[19••]
S. cerevisiae	w/o emulsion	Monodisperse	22	p-Coumaric acid	Biosensor *E. coli, transcription factor-based fluorescence*	On-chip sorting	$1{\cdot}10^6$/h	[26]
Algae	w/o emulsion	Monodisperse	2,145	Biomass (droplet weight)	Magnetic separation based on microdroplet weight	On-chip sorting	–	[27]
Particle sorters								
E. coli	agarose beads in oil	Monodisperse	65	pBAD promoters with desired characteristics	Promotor-dependent fluorescence	FACS	–	[28]
Y. lipolytica	w/o/w emulsion	Monodisperse	34	Riboflavin	Riboflavin auto-fluorescence	FACS	$4{\cdot}10^6$/h	[3••]
B. coagulans	w/o/w emulsion	Monodisperse	12	Lactate	Fluorescent pH indicator	FACS	$1{\cdot}10^6$/h	[29]
Siberian bear oral microbial community	w/o/w emulsion	Monodisperse	4	Antimicrobials against *S. aureus*	Biosensor *S. aureus, viability staining*	FACS	$1{\cdot}10^8$/h	[30]
S. cerevisiae	agarose beads in medium	Polydisperse	22	Antimicrobials against *S. aureus*	Biosensor *S. aureus, viability staining*	FACS	$1{\cdot}10^7$/h	[12]

Table 1 (*Continued*)

Producer organism	Droplet characteristics			Product	Assay	Selection		Ref
	Type	Dispersity	Volume (pL)			Type	Throughput	
E. coli	agarose beads in oil	Monodisperse	22	Antimicrobials against *S. aureus*	Biosensor *S. aureus, viability staining*	FACS	$1{\cdot}10^7$/h	[12]
B. subtilis	alginate beads	Polydisperse	65,000	Riboflavin	Biosensor *E. coli, aptamer based fluorescence*	Large particle sorter	–	[13]
L. lactis	alginate beads	Polydisperse	50,000	Effective lantibiotics	Biosensor *M. flavus, growth based fluorescence using DNA staining* or *L. lactis, growth based fluorescence using GFP expression*	Large particle sorter	$5{\cdot}10^5$/h	[9•]
Other								
L. lactis	w/o emulsion	Polydisperse	42	Biomass (cell number)	Serial propagation	Cell number	NA	[31]

Compounds

In microdroplets surrounded by oil, the oil-phase prevents diffusion of hydrophilic compounds (e.g. charged molecules, sugars, (poly-)peptides) between compartments, and therefore ensures coupling of the product concentration to its producer (Figure 1a). However, hydrophobic compounds leak into the oil-phase and therefore are not compartmentalized. One might circumvent this limitation by rapid enzymatic conversion of the hydrophobic product into a hydrophilic compound [24]. When assay reagents are oil-soluble, chemical modification can reduce their hydrophobicity to allow compartmentalization [36].

Product assays and microdroplet sorting

Assays for the produced extracellular compounds aim to couple the product-concentration to a measurable signal (Figure 2c). Developing these assays is often the most demanding task in a screening campaign. Assays that require direct analysis on, for example, an HPLC, MS, NMR or GC setup are less suited for the microdroplet format. Although compound libraries have been screened in microdroplets using MALDI-TOF MS [37,38], the method has a low-throughput. However, microdroplet-based screening campaigns are well suited for a wide range of spectroscopy methods (Table 1).

For w/o and w/o/w emulsions it is recommended to use assays with reagents that are not oil-soluble, and that can be added to the water-phase during microdroplet production. The addition of reagents after microdroplet generation and incubation is possible [23–25], but droplet fusion or liquid injection requires specialized equipment and know-how. Hydrogel beads are more flexible in this regard, because they can also be surrounded by a water-phase. This allows for instance viability staining of biosensors after incubation [12]. Once a measurable signal is obtained, microdroplets can be sorted either on-chip or with particle sorters (Figure 2d). For on-chip sorting generally w/o emulsions are used (Table 1). Particle sorters use water as carrier phase and therefore they can sort w/o/w double emulsions or hydrogel beads taken up in a water-phase (Table 1).

The required product assay, sorting set-up and compartmentalization method are highly intertwined (Figure 2c and d). Assay read-outs that are regularly used to select microdroplets are fluorescence emission and cell-concentrations.

Fluorescence

Direct selection for fluorescence is only possible when the product itself is highly fluorescent. Auto-fluorescence of riboflavin was for instance used to select *L. lactis* mutants producing four times more riboflavin [1]. When the compound of interest is not highly fluorescent, (bio)chemicals, enzymes or biosensors can couple the presence of the

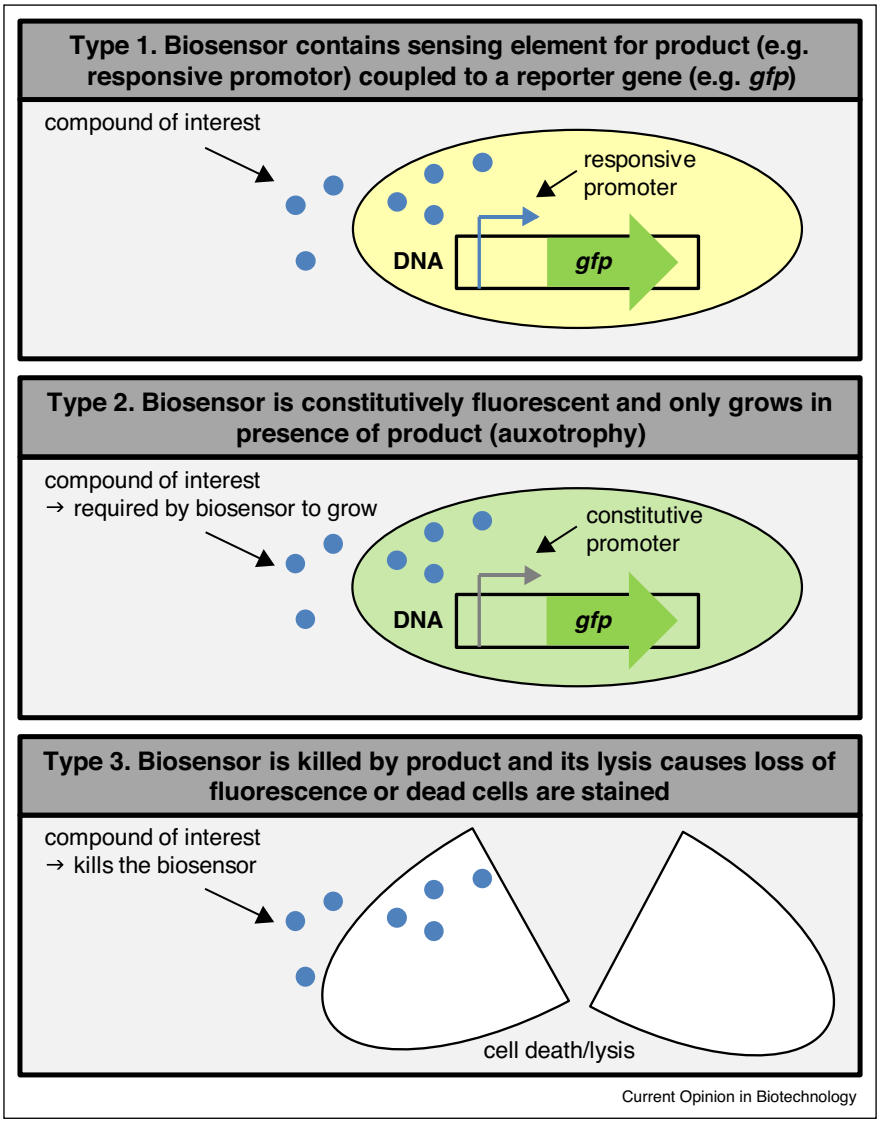
product to a fluorescence signal. Zhu *et al.* for instance used a chemical fluorescent pH indicator to select lactate producing *B. coagulans* strains [29]. Biochemicals which can be used are for instance fluorophore-binding aptamers, which yield a fluorescence signal when both a dye and the target molecule are bound [39]. Abatemarco *et al.* used these aptamers to select *S. cerevisiae* mutants that secreted 28-fold more tyrosine compared to their wildtype ancestor [22•].

Enzymes can also couple the presence of a compound to a fluorescence signal [23,24,36]. Hammar *et al.* connected lactate production of cyanobacteria to a fluorescence signal by coupling NADH production via lactate dehydrogenase combined to an NADH-dependent conversion of a fluorogenic substrate [25]. When the compound of interest is an enzyme itself, the addition of fluorogenic substrates can couple the enzyme activity to fluorescence [15,39–41].

Lastly whole-cell biosensors can also couple the presence of an extracellular product to a fluorescence signal. In general genetically accessible strains are used (Table 1), as biosensor development often requires genetic engineering. Biosensors are relatively cheap, since a high number of cells can be obtained in low-cost media, whereas other conversion systems (e.g. chemical or enzymatic) require tedious purification processes and analytical instruments such as chromatography and spectrophotometry [42,43].

We here classify biosensors in three types (Figure 3). Type 1 biosensors harbor reporter genes that respond to a

Figure 3



Biosensor types.

product based on promotor activation or repression [39]. The transcriptional regulator PadR was for instance used to couple the p-coumaric acid concentration to YFP production [26]. A more generic approach is the development of specific riboswitches, which was used by Meyer *et al.* to couple the presence of riboflavin to GFP production [13]. Type 2 biosensors are auxotrophic for the compound of interest and constitutively express a fluorescent protein [19••]. A computational analysis in *E. coli* predicted that auxotrophy-dependent biosensors can be generated for 53 metabolites [44], highlighting the broad applicability of this sensing mechanism. Growth of type 3 biosensors is inhibited when the producer strain releases effective antimicrobials. Fluorescent *Micrococcus flavus* cells were for instance used to select producers of effective lantibiotics [9•], and fluorescent *Staphylococcus aureus* cells were used to select antibiotic producers from the oral microbiota of the Siberian bear [30].

A recent review on different types of genetically encoded biosensors can be found in Ref. [39].

Once a fluorescence assay is established, microfluidic devices and particle sorters can be used for microdroplet selection [45•]. Abalde-Cela *et al.* sorted w/o emulsions based on their resorufin signal using a microfluidic device [24], and Zhu *et al.* used fluorescence activated cell sorting (FACS) to sort w/o/w double emulsions based on a fluorescent pH indicator to identify lactate overproducers [29]. While emulsions and double emulsions form 'closed' compartments with respect to compounds not soluble in the oil-phase, templated hydrogel beads or more complex beads featuring a surrounding shell architecture can also be incubated in a water-phase, resembling 'semi-open' vessels which can be engineered to specific molecular-weight cutoffs ranging from the low kD-range to pores almost of micrometer size [14]. Because of their big size, entrapment of biosensors is relatively easy. Duarte *et al.* for instance incubated GFP-expressing *E. coli* in hydrogel beads surrounded by oil, transferred the beads to a water-phase after incubation and sorted them based on fluorescence [28].

Cell-concentration

Compartmentalization of cells in microdroplets eliminates competition between mutants and therefore allows selection of mutants with a low growth rate, but a high biomass/cell yield [31]. An increase in cell concentration can also be coupled to the production of a specific product using cross-feeding. Saleski *et al.* for instance coupled 2-ketoisovalerate production by a lysine auxotrophic *E. coli* to the growth of a 2-ketoisovalerate auxotrophic sensor, which in turn secreted lysine to stimulate the growth of the 2-ketoisovalerate producer [19••]. In this way, cross-feeding creates a positive feedback loop, in which overproducers reach high cell concentrations.

Mutants with a high cell concentration can be enriched by serial propagation in microdroplets. Bachmann *et al.* for instance used this method to enrich *L. lactis* mutants that produced 71% more cells which coincided with a 26% higher biomass yield [31]. Next to serial propagation, microdroplets or hydrogel beads with increased cell concentrations can also be selected by sorting based on increased scattering of light [46], or weight-based magnetic sorting on a microfluidic device [27].

Conclusions

Screening in microdroplets allows selection for increased microbial production of extracellular compounds relevant to the (food) fermentation industry (Table 1). Multiple microdroplet production techniques are available (Figure 1c). They can be combined with different assays to generate fluorescence and cell concentration-based signals, which can be sorted in various ways (Figure 2). The optimal combination of production technique, assay and sorting system differs per screening question (Figure 2).

The applicability of microdroplets for screening is limited by the availability of suitable assays for the compound of interest. Current assays often require substantial tuning before they can be used in microdroplets, which is time-consuming [19••,36,39]. The most generic set-ups that are currently available use metabolite oxidases or dehydrogenases coupled to the generation of fluorescence [23–25], or viability staining of biosensors [9•,12,30]. Future research could focus on high-throughput screening for targeted aptamers [47] and (automated) biosensor development with a focus on enhanced sensitivity and specificity, increased dynamic ranges and improved transfer of sensing elements between organisms [39,48]. For filamentous fungi, future studies need to focus on increasing droplet stability, to prevent coalescence caused by apical growth of hyphae.

Screening in microdroplets is furthermore limited by the lack of a set-up to select for oil-soluble compounds. This is especially relevant for the food industry, because flavor volatiles are often oil-soluble. Possible solutions could focus on capturing and measuring oil-soluble compounds in microdroplets, for instance by measuring concentration gradients rather than absolute concentrations [49], or by capturing oil-soluble compounds in microdroplets by chemical or enzymatic conversion/modification [36].

Overall the recent technological progress on microdroplet production and sorting brings this technology within reach for strain selection in the (food) fermentation industry. While there are still a number of challenges, it has a high potential for (non GMO) strain improvement and due to its high throughput it should allow to select for phenotypes that are not accessible with conventional screening methods.

Conflict of interest statement

H.B. is employed by NIZO Food Research, a contract research organization. NIZO Food Research had no role in the decision to publish or the preparation of the manuscript.

R.J.v.T., J.H., B.T., O.P.K. and M.F. declare no competing interests.

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