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Published in:
FEMS Microbiology Letters

Link to article, DOI:
10.1093/femsle/fny258

Publication date:
2018

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Chen, J., Vestergaard, M., Shen, J., Solem, C., Dufva, M., & Jensen, P. R. (2018). Droplet-based microfluidics as a future tool for strain improvement in lactic acid bacteria. *FEMS Microbiology Letters*, *23*(1), [fny258]. https://doi.org/10.1093/femsle/fny258

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Droplet-based microfluidics as a future tool for strain improvement in lactic acid bacteria

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Key words: lactic acid bacteria, strain improvement, droplet-based microfluidics, high-throughput screening, starter culture, classical mutagenesis

Abstract

Strain development is frequently used to improve the performance and functionality of industrially important microbes. As traditional mutagenesis screen is especially utilized by the food industry to improve strains used in food fermentation, high-throughput and cost-effective screening tools are important in mutant selection. The emerging droplet-based microfluidics technology miniaturizes the volume for cell cultivation and phenotype interrogation down to the pico-liter scales, which facilitates screening of microbes for improved phenotypical properties tremendously. In this mini-review, we present recent application of the droplet-based microfluidics in microbial strain improvement with a focus on its potential use in the screening of lactic acid bacteria.

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Introduction

Lactic acid bacteria (LAB) is a group of Gram-positive bacteria that are ubiquitously used as lactic starter in dairy fermentation. The starter culture industry shares a billion-sized growing global market per annum (Vos 2011). As a food starter culture, LAB rapidly reduce pH in a given food matrix, which inhibits the growth of detrimental foodborne pathogens and spoilages. The metabolic activities of LAB, e.g., carbohydrate catabolism, proteolysis, and lipolysis dictate the formation of distinct flavor notes in different fermented foods. Other unique attributes of LAB have also extended the use to newer applications such as probiotics, bio-protection and plant material fermentation (Mozzi, Raya and Vignolo 2015).

With multiple advanced genome-engineering tools, precise genetic alterations on LAB chromosomes and plasmids can be achieved within a few days by experienced specialists (Joutsjoki *et al.* 2002; Levander and Svensson 2002; Papagianni 2012). Nevertheless, these GMO approaches have not been widely implemented in the starter culture industry due to the doubt of safe use from both the authority and the public (Derkx *et al.* 2014).

Traditional strategies are still dominating the strain improvement activity of industrial LAB, where mutagenesis screen, analog selection, and laboratory evolution are common non-GMO tools applied in the field (Derkx *et al.* 2014). Both analog selection and laboratory evolution have been proven as efficient tools for improving certain phenotypes. These methods rely on exposing cells to inhibitive analogs or stress conditions, which costs cellular fitness. Mutants with improved fitness compared to the general population can thereby be enriched on selection plates or through serial propagation with effectors. Successful implementation of either method relies on an increased cellular fitness that is genetically linked with a desired phenotype. Due to this constraint, the two methods are mainly applied in modifications of growth-relevant metabolic activities such as carbohydrate utilization, vitamin and cell wall synthesis and stress resistance (Burgess et al. 2004; Papagianni 2012; Chen et al. 2015a, 2015b;

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Kibenich, Kim Ib and Eric Johansen 2017). There are however many industrially relevant attributes of LAB that are not tightly associated with central metabolism, for which these two methods are not applicable (Johansen 2018).

In contrast, whole-cell mutagenesis by chemical or physical mutagens has a more general application in non-GMO strain development. The use of mutagens can increase mutation frequencies within two orders of magnitude compared to spontaneous mutations (Bates *et al.* 1989). Due to the random nature of mutagenesis, the use of this approach is only constrained by cellular genetic boundaries. The main drawback of random mutagenesis is though that phenotypes screening, without selective pressures, requires a large amount of screening activities to find desirable mutants. Fully automated liquid handling and colony picking have greatly accelerated the screening of mutagenesis libraries on miniaturized microliter plates (MTP). Nevertheless, for industrial-scale screening of LAB, automated screening on MTPs is still a slow and costly process (Johansen *et al.* 2015).

Droplet-based microfluidics is an emerging technology, where pL-sized droplets are used as containers for biological experiments. These droplets are water dispersed in fluorinated oil, of which the interface is stabilized by biocompatible surfactants (Figure 1A). This provides compartmentalization and each droplet can be considered as separate samples. Like in bioreactors or MTPs wells, microbes can proliferate and accumulate metabolites in droplets. Due to the small size of droplets, the screening can achieve a throughput above 1000 events/second. The emergence of the droplet-based microfluidics has been providing a more efficient screening avenue for the strain improvement with the classical mutagenesis technique (Figure 2). In this review, we will go through the recent progress of microbial strain optimization within the droplet-based microfluidics screening. Although the technique has not been widely applied for LAB, we think that the advances in other microbes are applicable in improvements of similar phenotypes for LAB, which could benefit the development of LAB-associated products in furture. We will furthermore address the problems that we consider critical for a wider application of the technique with regards to microbiology.

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Brief introduction of the droplets-based microfluidics for microbial library sorting

Monodisperse water-in-oil droplets (Figure 1A) can be generated by multiple methods, though a general approach is using an aqueous stream (containing cells and medium) and focusing it with fluorinated oil streams (containing surfactants). This is done at a T-junction on a chip termed a droplet generator, which is traditionally casted in PDMS (polydimethylsiloxane) (Figure 1B) (Mazutis *et al.* 2014).

The cell encapsulation in such droplet generating chips are influenced by the applied concentration of cells and the fact that the distribution follows the Poisson statistics (Collins *et al.* 2015). By adjusting cell density in the disperse phase, the ratio of singe-cell-loaded droplets to multi-cell-loaded droplets can be manipulated. For screening, it is ideal to have a low cell density in the disperse phase, which results in a high ratio of single-cell-loaded / multi-cell-loaded droplets. This comes at a cost, as the screening load is significantly increased due to the introduction of more empty droplets. Empirically, we use a $\lambda \approx 0.5$ (average of 0.5 cells in a droplet) for encapsulating LAB, which results in 30% singe-cell loaded droplets with an appropriate balance between the number of empty and multi-cell-loaded droplets (Chen *et al.* 2017).

Due to the use of biocompatible chemicals during droplet generation, microbial cells can normally proliferate in droplets (Figure 1 A). The emulsions of droplets are normally stored in closed Eppendorf tubes or syringes to avoid evaporation. Compared to a lab-scaled bioreactor, the cultivation of aerobes might be a problem due to limited gas exchange with the atmosphere over longer incubation periods in droplets (Kaminski, Scheler and Garstecki 2016). As LAB are facultative anaerobes, oxygen transmission is trivial for the cultivation in droplets.

The subsequent sorting of droplets is normally performed through the fluorescence-activated droplet sorting (FADS) (Figure 1C). The FADS utilizes a sorting chip that is embedded with two disproportionate-sized electrodes on sides of the sorting channel, which gives rise to an inhomogeneous electric field. Here an integrated

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system with a laser illuminates each droplet and records their fluorescent intensities on the chip. At values above a given threshold, the electrode activates and applies a dielectrophoretic force that pulls relevant single droplets into the collection channel, while the main population remains in the waste channel (Figure 1C) (Abd Rahman, Ibrahim and Yafouz 2017). Collected droplets can thereafter be recovered and validated at lab scale. Due to the small size of droplets, both droplet generation and sorting are operated at a kHz range. For microbes such as yeasts and bacteria, a 30-minute screening of 100,000 cell-loaded droplets that is derived from randomly mutagenized libraries normally leads to identification of target mutants with improved secretion of metabolites or proteins (Huang *et al.* 2015; Chen *et al.* 2017).

Droplet sorting in microbial screening for enhanced phenotypes

Confinement is the key feature in droplet-based screening, which provides opportunities to inspect microbial performance and analyze cell-to-cell interaction in a miniaturized compartment (Figure 2). For LAB used as starter cultures, many important contributions in food fermentation are associated with secreted metabolites such as lactic acid, aromatic compounds, vitamins, exopolysaccharides, and lantibiotic bacteriocins. Several studies have demonstrated the effectiveness of the droplet-based microfluidics in microbial library screening for improvement of metabolite and protein secretion (Wang *et al.* 2014; Huang *et al.* 2015). We will present them in this part.

Screening of enhanced metabolite secretion

LAB is well known for its capacity to raise micronutrient contents in fermented food, and a key example is the B-group vitamins. We previously explored the possibility to use droplet screening to find riboflavin over-secreting *L. lactis* mutants from random mutagenesis libraries. To minimize chances of accumulating harmful mutations, we applied mild chemical mutagenesis to *L. lactis*. Albeit the setup would result in a low frequent occurrence of variations, the high-throughput feature of droplet screening would allow easy identification of over-secreting mutants. After two iterative rounds of mutagenesis and droplet screening, we isolated a group of mutants, in which the best

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one secreted 4 times and 3.5 times more riboflavin compared to the starting strain in both the chemical-defined medium and bovine milk, respectively. We observed a high reproducibility for the strain improvement process. In each round, approximately four variations were introduced onto the chromosome of *L. lactis* by mutagenesis. To address the issue of screening low-error-rate library, we set strict sorting criteria, where top 0.05% cell-loaded droplets with the highest arbitrary units of fluorescence were sorted. A 30% true-positive rate of riboflavin over-secreting phenotype was found in the sorted droplets (Chen *et al.* 2017). Due to the non-GMO nature of the development process, these strains have potential use for production of riboflavin-fortified foods.

Instead of detecting secreted metabolites in droplets, the production can also be indirectly interrogated by detecting intracellular fluorescent readouts of metabolites with the assumption that accumulation extracellular of metabolites is proportionate to its intracellular concentration (Binder *et al.* 2012). Therefore, screening of bulk culture can be performed on FACS. In a parallel screening experiment for riboflavin over-secreting mutants, Wagner *et al.* found that mutants isolated with droplet screening yielded two times more extracellular riboflavin compared to mutants isolated through FACS without encapsulation (Wagner *et al.* 2018). The difference corroborates that the compartmentalization feature of droplets screening provides a better selection on secretion phenotypes compared to FACS (Figure 2).

Another important phenotype of LAB in industrial use is acidification of food by accumulation of lactic acid (Ardö and Nielsen 2014). LAB with a fast acidification phenotype is expected to reduce batch fermentation time or amounts of starter culture used for inoculation in milk fermentation (Chen *et al*. 2015). A coupled-enzymatic assay has been designed to quantify lactic acid secretion of microbes in droplets (Wang *et al.* 2014) (Figure 3). In this assay, lactic acid is first oxidized by lactate oxidase to generate H_2O_2. In the second reaction, the fluorescein resorufin (ex/em: 571/585 nm) is generated from oxidation of Amplex UltraRed (N-acetyl-3,7-dihydroxyphenoxazine) with H_2O_2 under the catalysis of horseradish peroxidase (Wang *et al.* 2014).

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Wang *et al*. validated the droplet screening of lactic acid production using an artificial *Escherichia coli* (*E. coli*) library, where high-lactate-producing *E. coli* strains were spiked into a bulk of low-lactate-producing strains at a ratio of 1:10,000 (Wang *et al.* 2014). With three iterative rounds of droplet screening, the high-producing strains were enriched 5,800 times. Even though the setup was not applied for a real mutagenesis library, the magnitude enrichment implies the effectiveness of droplet screening for enriching the lactic acid producing phenotype.

Screening of enhanced enzyme activity and protein secretion

One of the most pioneering applications using droplet screening in the biotechnology area is for enzyme engineering. The size of enzyme activity assay with pL droplets is at least six orders of magnitudes lower compared to the screening on MTPs, which tremendously saves the cost of expensive substrates, buffer reagents and plastic consumables (Agresti *et al.* 2010). The screening of enzyme activities in droplets is also facilitated by direct use of many fluorescence-based enzymatic assay system originally applied for MTPs screening (Wang *et al.* 2014; Huang *et al.* 2015).

The droplet screening has been extensively used for improving microbial glycoside hydrolases such as lactase and amylase. Lactase (beta-galactosidase) is an important glycoside hydrolase for hydrolysis of lactose. It is commercially produced from yeast or molds, but some attributes of LAB lactase have also attracted attention for the industrial application (Henriksen *et al.* 2016). For screening of the cytoplasmic enzymes such as lactase, it is critical to ensure the recovery of live bacteria after sorting. *Nir et al.* screened the microbial beta-galactosidase activity in hydrogel droplets (Nir *et al.* 1990). They entrapped single cells in agarose microbeads to form micro-colonies. The treatment of organic solvents allowed diffusion of the fluorogenic substrate fluorescein-di-beta-D-galactopyranoside (FDG) into cytoplasm for reaction. Live cells with high lactase activity could be recovered after screening due to the survival of microbes in the core of micro-colonies entrapped in the droplets when an appropriate permeabilization strength was used.

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Besides cytoplasmic enzymes, LAB also possesses several industrially important exoenzymes that are secreted or displayed on the cell wall surface, e.g., amylase and proteinase. Amylase secretion is mainly observed for the *Lactobacillus* species. The expression of amylases by *Lactobacillus* is important for starch ingestion in the human and animal gastrointestinal tracts, and the fermentation of starchy food (Lee, Gilliland and Carter 2001; Oguntoyinbo and Narbad 2015). Screening for more efficient amylase over-secreting *Lactobacillus* will enhance starch hydrolysis for assimilation. Amylase secretion is screened by examining formation of halos on chromogenic amylose agar plates, or with MTP-based colorimetric assays at low throughput (Castro et al. 1993). Fluorophore-labeled substrates for amylase activity assay are also commercially available (BODIPY-starch, ThermoFisher Scientific, Waltham, Massachusetts, U.S.) that can be adapted for high-throughput screening of microbial amylase secretion in droplets. Huang *et al.* screened a UV-mutagenized yeast library for better amylase secreting *Saccharomyces cerevisiae* in droplets using BODIPY-starch as the indicator (Huang *et al.* 2015). They screened 10^5 cells-loaded pL droplets in a few hours and collected 970 candidates, in which 122 mutants showed at least a 30% increase in amylase secretion compared to the starting strain in test-tube fermentation.

Cell-wall anchored proteinases are another important LAB exoenzyme. Milk protein is mainly composed of casein, which cannot be used by LAB as a nitrogen source without enzymatic degradation. Proteolytic degradation at the cell envelope by the proteinase lactocepin is therefore essential for the acidification in bovine milk, as the growth of LAB relies on peptide and amino acids released from casein digestion (Juillard *et al.* 1995). Proteolysis is furthermore important for the taste and flavor formation in cheese maturation. During cheese ripening, casein-derived free amino acids and aroma compounds generated from the amino acids catabolic activity of LAB dictates the organoleptic quality of cheeses. High proteolytic starter cultures are sometimes not appreciated for cheese ripening due to their concurrent generation of bitter peptides from the plasmin and chymosin derived peptides as these give an unfavorable bitter taste in cheeses (Ardö and Nielsen 2014). Genetic and biochemical

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characterization has clarified the fact that the caseinolytic specificity of the lactococcal lactocepin dictates the formation of bitter peptides during cheese aging (Ardö and Nielsen 2014). Protein engineering by amino acid substitution is one option to alter the substrate specificity and improves the proteolysis property of the proteinase (de Vos and Siezen 2005). For scouting proteinase variants, dye-labeled peptides enables the fluorescence-based assay of specificity in a high-throughput manner (Jones *et al.* 1997). Ng *et al.* investigated the substrate specificity of single-cell proteinase in droplets (Ng *et al.* 2016). The experiments comprised of multiplex substrates, which enabled a simultaneous screening of four cleavage sites of the cellular proteinase. They designed four oligopeptides substrates with different cleavage sites, where quenched fluorophores with distinct excitation/emission wavelengths were attached in a uncleaved form. The screening was conducted by inspection under fluorescence microscopy at low throughput. However, the concept can be adapted to the evolution of LAB proteinases with the droplet-based screening by using multiple read-outs systems. It could help the use of mutagenesis to eliminate unfavorable cleavage capabilities of the lactococcal proteinase but retain the useful specifications.

Screening of antimicrobial properties

As starter culture, LAB does not only contribute to the acidification and flavor formation of fermented food products but also provides significant antimicrobial properties against foodborne spoilages and pathogens during food preservation (Mozzi, Raya and Vignolo 2015). The fast growth of LAB in food matrix helps reach an environment with low nutrients, pH and redox potentials, which is not favorable for most other microbes. In addition, LAB can also secret a variety of metabolites functioning as antagonistic effectors (Klaenhammer 1988). The confinement feature of droplets provides possibilities to investigate and screen bacterial interaction and antimicrobial functions at a single-strain level, where the antimicrobial target is typically labeled with fluorescence markers, e.g., fluorescence proteins to indicate the growth in droplets (Terekhov *et al.* 2017). Terekhov *et al.* screened the human microbiota for microbial antagonists against *Staphylococcus aureus* (*S. aureus*) in

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droplets (Terekhov *et al.* 2017). The most enriched antagonists belonged to the *Streptococcus* and *Pseudomonas* species. The antagonistic effects of the isolates were confirmed by conventional inhibitory assays. Remarkably, the antagonistic role of *Pseudomonas aeruginosa* against *S. aureus* was not reported previously, which indicates that the high-throughput droplet screening can be an effective tool for identification of new protective microbes.

Challenges for general applications of the droplet-based microfluidics screening in lactic acid bacteria improvement.

The technological advancement of the droplet-based microfluidic screening has hitherto been validated for a limited number of applications in strain improvement, as the lack of commercial devices hinders the accessibility for non-specialists. The situation has recenlty been changed by the emergence of several dedicating start-up companies that supplies standard microfluidics chips, modular devices and other accessories (Holtze, Weisse and Vranceanu 2017). While these companies provide an essential service, several technological challenges remains unsolved before the technology should be considered mature. We will elaborate on these points in the following part.

Development of sensitive and biocompatible assays

A sensitive and non-invasive indication system is critical for a successful droplet sorting of live cells. In the screening of riboflavin over-secreting *L. lactis* mutants, we harnessed the auto-fluorescence feature of riboflavin for the indication of riboflavin production in droplets. Also, the culture medium used for screening was chemically defined, which also resulted in a low background fluorescence noise for droplet detection. Therefore, we achieved an efficient sorting and a high true positive rate of riboflavin overproduction (Chen *et al.* 2017). Auto-fluorescence is a scarce feature among primary microbial metabolites, and therefore assay development is prerequisite for application of the FADS. Wang *et al.* have demonstrated the feasibility of the oxidase-Amplex system for assaying metabolites in droplets with the FADS (Wang *et*

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al. 2014). Due to the issue of dye leaching, the detection requires injection of the assay mix into droplets after fermentation is finished, which requires critical design and manipulation of injection devices (Abalde-Cela *et al.* 2015). One alternative solution is to use riboswitch or synthetic RNA/DNA-based aptamers to construct metabolite-responsive fluorescent sensors for detection (Figure 3), as a wealth of metabolites-responsive riboswitch sequences are available (Mccown *et al.* 2017). Albeit native riboswitches are usually an off-switch, they can be inverted by engineering or in combinational use with the hammerhead ribozymes to become an on-switch sensor (Muranaka *et al.* 2009; Meyer *et al.* 2015). Even without prior knowledge about sequences of metabolite-binding nucleic acid, synthetic aptamer sequences can be discovered by the Systematic Evolution of Ligands by Exponential Enrichment procedure (Ellington and Szostak 1990), which facilitates the general application for detecting different compounds in droplets.

Riboswitch/aptamer sensors can be used for construction of whole-cell biosensors, in which a reporter gene (*gfp*) is typically transcribed by metabolites-responsive promoters in another microbe (Figure 4). In a parallel screening of riboflavin over-secreting microbes, Meyer *et al.* designed a whole-cell biosensor, in which a riboflavin responsive GFP expression cassette was generated by linking an flavin mononuleotide (FMN) riboswitch with a hammerhead ribozyme in between the promoter and the *gfp* gene (Meyer *et al.* 2015). The sensoring cell was engineered to use an orthogonal carbon source to circumvent the competition with the target microbe during the co-cultivation in droplets. By co-capsulation of the library and sensor cells, they successfully isolated several riboflavin overproducers from 30,000 candidates with the FADS. For the use of whole-cell sensors, transport activities should be considered for the analysis of low abundant metabolites in droplets (Figure 4A). If the cross-membrane diffusion of an analyte into sensor cells is not efficient, cell membrane could be a barrier to lift the detection threshold with whole-cell sensors compared to direct measurement of metabolites in droplets. Instead, the riboswitch/aptamer sequences are used for construction of *in vitro* biosensors. Abatemarco *et al.* designed

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in vitro responsive Spinach sensors, in which the ligand-binding aptamer sequences were grafted into the modular Spinach domain (Abatemarco *et al.* 2017). Upon binding with analytes, the Spinach sequence incorporates the dye molecule DFHBI and becomes fluorescent (Strack, Song and Jaffrey 2013). Due to the direct contact between sensors and analytes in droplets, these Spinach-aptamer sensors showed a very sensitive quantification of metabolites secreted by microbes in droplets (Abatemarco *et al.* 2017). With the sensors, the authors used the FADS to improve the tyrosine secretion in yeast by 28 folds (Abatemarco *et al.* 2017). Due to the *in vitro* use of these aptamer-based sensors, thorough tests of polynucleotide stability and optimal *in vitro* assay condition should be conducted before the application in droplets (Abatemarco *et al.* 2017).

Colorimetric assays are sometimes preferred over fluorometric assays if background fluorescences are high in culture medium. Especially for LAB, the fastidious nutritional demand requires supplement of complex substrates in the culture medium, which interferes with fluorescent signals. Absorbance-based colorimetric assays are generally incompatible with the droplet-based microfluidics due to the low sensitivity, which is caused by a very short path length of light through droplets. However, under some uncommon circumstances, it can also be applied for droplet sorting. Gielen *et al.* reported the successful use of a formazan color dye WST-1 for measurement of enzyme activities on a droplet screening system equipped with LED lights and photodetectors (Gielen *et al.* 2016). To improve the sensitivity of photodetection in droplets, they used large-sized droplets (180 pL), 100 times concentrated dye (2 mM), and a longer time for reaction (3 hours) to increase the assay sensitivity in droplets. With such compromises, they were still able to sort the droplets at a high frequency (300 Hz) with a low false positive rate.

Leaching of metabolites and fluorescent dyes

Unlike MTPs with rigid plastic barriers, molecules in droplets are segregated by surfactants at the water-oil interface. The small size of droplets dictates a high surface

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area to volume ratio. Solute leakages and droplets cross-communication are possible for relatively hydrophobic or small-uncharged molecules (Kaminski, Scheler and Garstecki 2016). We previously attempted to investigate the acidification of *L. lactis* in droplets with pH-responsive fluorescent dyes (data not shown). We could detect high fluorescent signals in acid droplets with lactic acid. Once they were mixed with pH-neutral droplets, the fluorescent signals decayed. At a low pH, lactic acid becomes protonated (uncharged) and tends to diffuse into neighboring neutral droplets with high pH. Therefore, the real screening of acidification in droplets did not succeed.

Solutes diffuse into neighboring droplets by droplets adhesion or through the fluorinated oil phase. The adhesion is caused by physical connection of droplets to form surfactant bilayers essentially due to a low solubility of some surfactants in fluorinated oil. If chemical potentials of adhesive droplets are mismatched, small molecules, e.g., water moves from the droplets with high potentials to the ones with low potentials until an equilibrium is reached (Thiam, Bremond and Bibette 2012). Diffusion through the oil phase is also caused by the mismatch of chemical potentials among droplets, but the mechanism is different. It is characterized by the partition of solutes into neighboring droplets through the continuous phase, in which a finite solubility of solutes takes places. Albeit the solubility of organic solutes in fluorinated oil is low, the presence of amphiphilic surfactants in oil increases their solubility (Gruner *et al.* 2016). This kind of leaching is commonly observed for secreted microbial metabolites in droplets, e.g., uncharged organic acids, volatile compounds, and alcohols during incubation. It is also a critical issue for assays, in which low-molecular-weight fluoresceins are used as indicators. In the oxidase-Amplex assay system, the generated fluorescent resorufin quickly diffuses into empty droplets through the continuous phase. This leaching illuminates neighbor droplets and interferes with screening. Therefore, the fermentation and the assay steps are sequenced, in which reaction mix is afterward injected into droplets for assay of metabolites (Wang *et al.* 2014). The screening is also finished within a few hours before a significant cross-contamination occurs. Some measures can help attenuate the diffusion rate such as using protective additives or

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reducing the amount of surfactants applied (Kaminski, Scheler and Garstecki 2016). Instead of using the conventional PEG-PFPE surfactant, the advancement of new surfactant development is also expected to solve the problem of droplet cross-talk in future applications (Pan *et al.* 2014).

Concluding Remarks

The predominant characteristics of FACS-based and MTPs-based screening are miniaturization and compartmentalization respectively (Figure 2). Combination of these two features in the droplet-based microfluidics technique enables the screening processes for improved microbial secretion of value-added metabolites and proteins both high-throughput and low-cost. The technique is especially of value for optimization of industrial production strains, where classical improvement approaches are still heavily implemented. For example, the use of classical mutagenesis/selection and other non-GMO tools is preferred for improvement of performance and functionality of food-associated LAB, even though the use of modern metabolic engineering or synthetic biology approaches for strain improvement is booming. Mild mutagenesis minimizes accumulation of irrelevant mutations, which is appreciated for strain development. Augmentation of screening load due to the low mutation rates, however, is a major obstacle for finding target phenotypes. With advancements of droplet-based microfluidics, isolation of desirable mutants from low-error-rate mutagenesis libraries becomes affordable. Development of feasible fluorescence-based assays for detection and new surfactants for stabilizing droplets and produced metabolites thereof are still the two most important tasks before we can fully implement the droplet-based microfluidics in the modern strain improvement process. Nevertheless, we can see initiatives have been being taken to mature the technique, and in some paradigms, it has been proved as an efficient primary enrichment tool that minimize the workload for the MTP screening, which reduces the cost of time and expenditure in a large screening activity (Wang *et al.* 2014; Huang *et al.* 2015; Chen *et al.* 2017).

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Acknowledgements

This work was supported by the grant NNF12OC0000818 from the Novo Nordisk Foundation.

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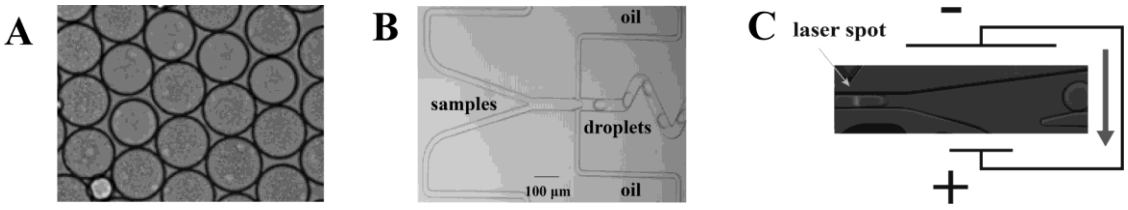
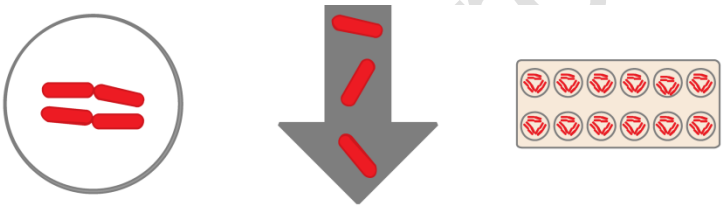


Figure 1. Illustrations of droplet manipulation. A, cell-containing monodispersed droplets. B, generation of droplets on polydimethylsiloxane chips. C, sorting of droplets on polydimethylsiloxane chips.



	Droplet	FACS	Robot & plate
Throughput	1K - 10K events / second *	10K events / second	10K events / day
Compatible fermentation type	Singe cell	Bulk	Single colony
Assay size	pL	cell size dependent	µL
Assay location	Intracellular Extracelluar	Intracellular	Intracellular Extracelluar
Assay type	Fluorimetry Possible colorimetry	Fluorimetry	Fluorimetry Colorimetry Luminometry
Operating cost	Low	Low	High
Commercial system availibility	✗	✓	✓

Figure 2. Comparison of microbial screening with the droplet-based microfluidics, FACS, and MTPs**.** The figure is inspired from the reference (Autour and Ryckelynck 2017). *, the throughput is dependent on the size of droplets.

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$$\text{lactate} + O_2 \xrightarrow{\text{lactate oxidase}} \text{pyruvate} + H_2O_2; H_2O_2 + \text{Amplex UltraRed} \xrightarrow{\text{horseradish peroxidase}} H_2O + \frac{1}{2}O_2 + \text{Resorufin}$$

$$\text{lactate} + O_2 \xrightarrow{\text{lactate oxidase}} \text{pyruvate} + H_2O_2$$

$$H_2O_2 + \text{Amplex UltraRed} \xrightarrow{\text{horseradish peroxidase}} H_2O + \frac{1}{2}O_2 + \text{Resorufin}$$

Figure 3. The coupled-enzymatic assay system to quantify lactic acid in droplets.

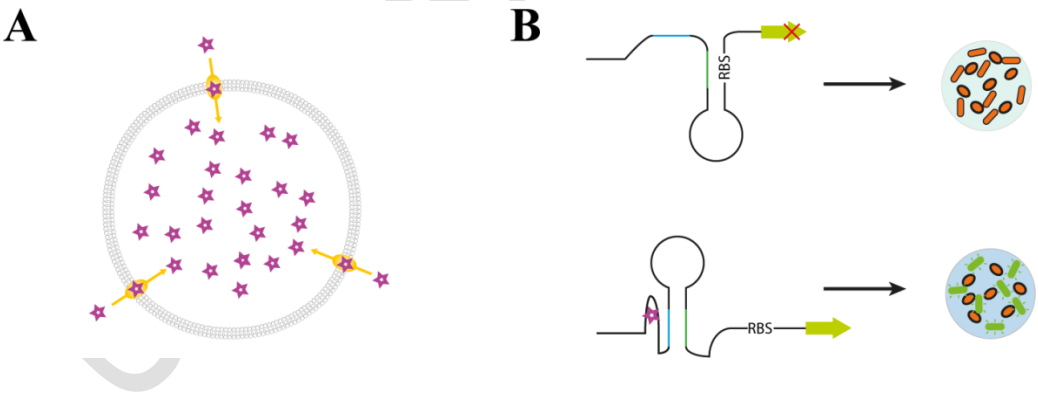

Figure 4. Illustration of analytes sensing using a riboswitch-based whole-cell biosensor. A, analytes transport by a host strain bearing biosensors. B, sensing analytes by the riboswitch-guide reporters. If no analytes are present, expression of reporters is not actuated due to a hairpin structure containing a sequestered ribosome-binding site. When analytes are available, the ribosome-binding site is released, and the expression of GFP occurs (Muranaka *et al.* 2009).

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