Technical University of Denmark

DTU

# High-throughput screening for industrial enzyme production hosts by droplet microfluidics

Sjostrom, Staffan L.; Bai, Yunpeng; Huang, Mingtao; Liu, Zihe; Nielsen, Jens; Jönsson, Håkan; Svahn, Helene Andersson

[Link back to DTU Orbit](Link back to DTU Orbit)

**DTU Library**
Technical Information Center of Denmark

# High-throughput screening for industrial enzyme production hosts by droplet microfluidics†

Staffan L. Sjostrom,[a] Yunpeng Bai,[a] Mingtao Huang,[b] Zihe Liu,[b] Jens Nielsen,[abc] Haakan N. Joensson[a] and Helene Andersson Svahn*[a]

A high-throughput method for single cell screening by microfluidic droplet sorting is applied to a whole-genome mutated yeast cell library yielding improved production hosts of secreted industrial enzymes. The sorting method is validated by enriching a yeast strain 14 times based on its $\alpha$-amylase production, close to the theoretical maximum enrichment. Furthermore, a $10^5$ member yeast cell library is screened yielding a clone with a more than 2-fold increase in $\alpha$-amylase production. The increase in enzyme production results from an improvement of the cellular functions of the production host in contrast to previous droplet-based directed evolution that has focused on improving enzyme protein structure. In the workflow presented, enzyme producing single cells are encapsulated in 20 pL droplets with a fluorogenic reporter substrate. The coupling of a desired phenotype (secreted enzyme concentration) with the genotype (contained in the cell) inside a droplet enables selection of single cells with improved enzyme production capacity by droplet sorting. The platform has a throughput over 300 times higher than that of the current industry standard, an automated microtiter plate screening system. At the same time, reagent consumption for a screening experiment is decreased a million fold, greatly reducing the costs of evolutionary engineering of production strains.

## Introduction

Enzymes are important for a wide range of industrial applications, for example, in the food, paper and biofuel industries.[1] Enzymes can offer several advantages in industrial processes compared to conventional chemical catalysts: they are derived from renewable resources, they are biodegradable, they typically offer stereoselectivity and substrate specificity, and they tend to operate under relatively mild conditions with respect to temperature and pH. However, in order for enzyme based catalysis to compete with traditional chemical catalysis in industrial processes, cost efficiency is a key issue.[2,3] The advent of modern biotechnology tools such as recombinant DNA technology has enabled new ways to improve enzyme function and production and has paved the way for improved enzymes and production strains.[4,5]

Enzymes for industrial scale use are typically produced in large-scale fermentations using microbial production hosts, also known as cell factories. Extensive engineering and optimization efforts are routinely employed to increase the productivity of the production hosts in order to make the production more commercially competitive.[4–6] However, cell metabolism is highly complex and the understanding of it is incomplete which limits the power of rational engineering approaches. As a complement, a directed evolution strategy may be employed where mutations are semi-randomly introduced to create a large library of microorganisms with diverse phenotypes.[1,3,7] Subsequently, the microorganism library is screened for variants with a desired phenotype, e.g. improved production of an enzyme. To efficiently screen a large library, a suitable high-throughput screening (HTS) system is needed.[2,3,6,8]

Several methods exist for the screening of microbe libraries. However, most methods are restricted to screening for specific phenotypes. For instance, variants can be evaluated by surface display technologies and fluorescently activated cell sorting (FACS) at throughputs of up to $10^8$ variants per day.[9,10] However, the range of reactions that can be screened with FACS is limited to cell survival and internalized or surface bound fluorescent probes and it cannot be used for secreted enzymes.[10–12] A standard FACS method is thus unfeasible for enzyme evolution in general, as the enzyme reaction product would diffuse away from the cell that produced the enzyme, decoupling the phenotype from the genotype. Today, the industry state of the

[a] Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, Royal Institute of Technology (KTH), Sweden. E-mail: helenea@biotech.kth.se

[b] Department of Chemical and Biological Engineering, Chalmers University of Technology, Sweden

[c] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Denmark

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3lc51202a

art technology for enzyme activity screening of microbe libraries are halo assays and microtiter plate based assays. These assay formats have limited throughput and high associated costs, reducing the total number of clones that can be sampled.[13–15]

Droplet microfluidics is a technology with strong potential for high-throughput screening of single cells.[16–19] Picoliter sized monodisperse aqueous droplets are generated in a continuous fluorinated oil phase and stabilized using surfactants to prevent coalescence.[20] These droplets can be manipulated at rates of thousands per second, using microfluidic devices manufactured by soft lithography.[21] Single cells can be encapsulated in such droplets, each droplet constituting the equivalent of a miniature test tube where each specific cell can be assayed. Microfluidic droplets can be manipulated in a multitude of ways including splitting,[22] fusion,[23] trapping,[24] injection of reagent[25] and sorting. Droplet sorting can be done passively on physical attributes such as size[26] but it is also possible to actively sort droplets by on demand activation of an electric field that exerts a dielectrophoretic force on a droplet.[27] This principle can be used to sort droplets based on their fluorescence using fluorescently activated droplet sorting (FADS).[28]

Droplet microfluidics has recently been demonstrated for directed evolution of enzymes.[28–31] The throughputs of these prototype systems are at least a 100 times higher than a microtiter plate robot and the reagent consumption is lowered by about a million fold, vastly reducing the total cost associated with enzyme evolution.[29] So far, efforts have been focused on model enzymes, differentiating between active and inactive enzyme variants,[28,31] screening for enzyme variants with increased turnover rate[29] and screening for enzyme variants with altered specificity.[30] The objectives of these studies were thus to improve the protein structure of an enzyme using evolutionary engineering.

In addition to improving the enzyme structure, directed evolution strategies can be employed to improve even more complex systems such as microbe production hosts. In this paper, we present a droplet microfluidic screening method for selection of improved production hosts of industrial enzymes. The method, as shown schematically in Fig. 1, involves encapsulation of single cells from a whole-genome mutated library together with a fluorogenic substrate in microfluidic droplets. The encapsulation inside the droplet confines the secreted enzyme and the fluorescent product to the droplet, linking the phenotype of each cell to its genotype. To demonstrate the system, we enrich a yeast strain based on its α-amylase production and we perform a library screening identifying several yeast clones with α-amylase production higher than the mother strain used to create the library.

## Materials and methods

### Materials

Glass slides were obtained from Thermo Fisher Scientific. HFE-7500 engineering fluid was obtained from 3M. EA
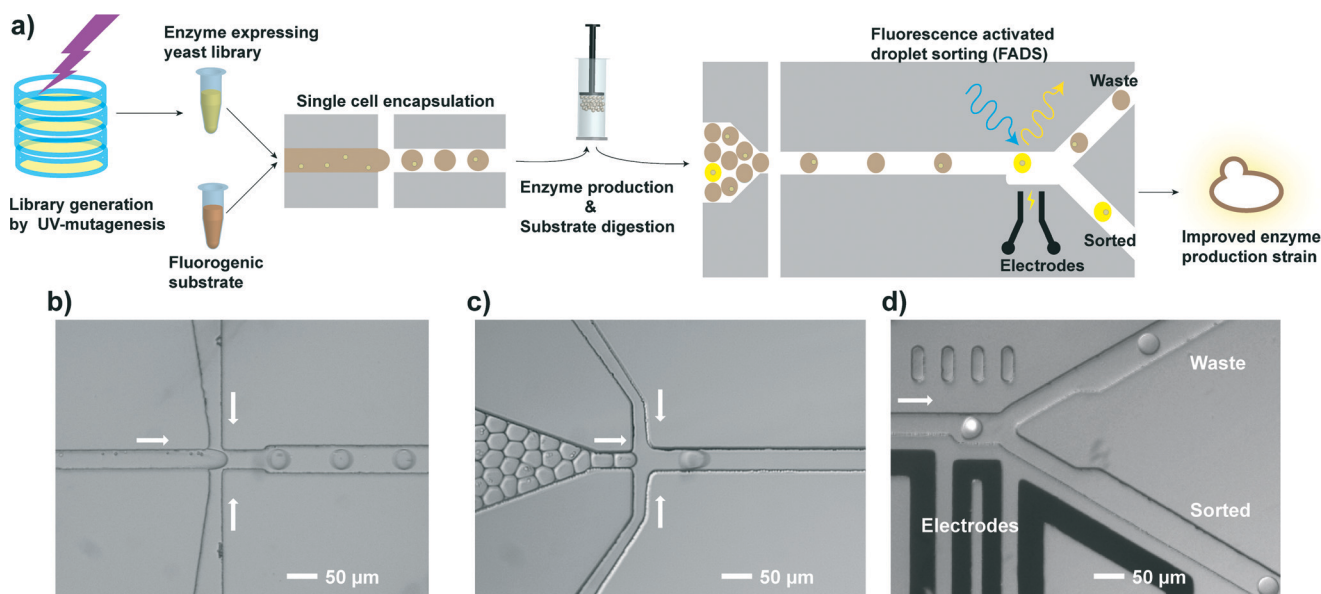


Fig. 1 a) Assay workflow. From left to right: a library of whole-genome mutated yeast cells was generated by irradiating yeast with UV-light on starch agar plates. Fresh media were added to the plates to solubilize the yeast. Once solubilized, the yeast was sonicated to disperse cell aggregates and diluted to an appropriate cell to droplet ratio. Subsequently, the cells were encapsulated in microfluidic droplets together with a fluorogenic enzyme substrate. Inside each occupied droplet, the encapsulated cell produced enzyme that digested the substrate and in turn increased the fluorescence of the droplet. After incubation, the emulsion was injected into the sorter circuit where the droplets were sorted on their fluorescent signal. The most fluorescent droplets, which contained the cells with the highest enzyme production, were recovered for further analysis. b) Micrograph showing droplet generation and cell encapsulation. c) Micrograph showing droplet reinjection into the sorter circuit. d) Micrograph of the sorting junction. Single droplet fluorescence was detected following excitation by the laser (the white dot). The default flow path of the droplet is to the top channel, "waste", as the waste outlet is at atmospheric pressure and a withdrawal of less than half the total in flow rate is applied to the "sorted" outlet. However, if the droplet fluorescence exceeds a predefined threshold an electric field is activated between the electrodes, pulling the droplet to the bottom channel. Arrows in micrographs indicate the direction of the flow.

surfactant and droplet destabilizer were obtained from RainDance Technologies. Polydimethylsiloxane base (PDMS) and curing agent were obtained from Dow Corning. Low melting solder was obtained from Indium Corp. Aquapel was obtained from PPG Industries. Fluorescein, α-amylase from *Aspergillus oryzae* and α-amylase from *Bacillus licheniformis* were obtained from Sigma-Aldrich. An EnzCheck Ultra Amylase kit (containing BODIPY–starch) and FUN1 cell stain were obtained from Invitrogen. An Ceralpha kit was obtained from Megazyme (Ireland).

### Manufacturing of chips

Microfluidic devices were manufactured in glass and PDMS using standard soft lithography methods[21] with injected electrodes. Briefly, a layer of SU8 was spin coated to appropriate thickness on a silicon wafer and cured with UV light through a channel-patterned photo mask to produce a master mold. Replica PDMS chips were fabricated by pouring 30 g of PDMS mixed with curing agent in a 1:9 ratio on top of the master mold, curing overnight at 65 °C. The cured PDMS slab was peeled off the master and holes were punched for channel inlets and outlets. The slab was cleaned and then exposed to oxygen plasma (FemtoCute, South Korea) to activate the surface and bonded to a glass slide, closing the microfluidic channels. Chips were surface treated by injecting Aquapel through the oil inlet, flushing out residual surface treatment with pressurized air. For the sorting chips, electrodes were fabricated by heating the chip and low melting solder to 95 °C on a hot plate. The liquid solder was injected into the purpose-designed electrode channels and interfaced for connection to an off-chip voltage source using an adapter, which was fixed in electrode channel inlets as the liquid solder solidifies.

### Experimental set-up

The microfluidic chip was placed on an adjustable *xy*-table on top of an inverted microscope. The syringes were connected to the microfluidic chip through polyether ether ketone (PEEK) tubing. All syringes were controlled using neMESYS syringe pumps (Cetoni GmbH) except the cell suspension syringe that was controlled using a Harvard Systems syringe pump. A 491 nm laser was focused through the objective lens (10×) onto a specific point on the chip that enabled single droplet fluorescence detection using a photomultiplier tube (Hamatsu), which captured emitted light through a band pass filter at 525 ± 20 nm. A high voltage amplifier unit (TREK Inc) is connected to on-chip electrodes and amplified a computer generated signal to create an electric field on chip.

### Operation of microfluidic circuits

The microfluidic system consists of a droplet generation circuit and a droplet sorter circuit (Fig. S1 and S2, ESI† for design schematics). The generation circuit generates 20 pL droplets at a rate of 2800 per second. The chip was operated with a total aqueous flow rate of 200 μL h⁻¹ and 1000 μL h⁻¹

HFE-7500 oil with 1% (w/w) EA surfactant for droplet stabilization. The emulsion was collected in a 1 mL plastic syringe (BD Plastipak) operated with withdrawal of 1000 μL h⁻¹.

The emulsion generated on the generation circuit was incubated in the syringe and subsequently injected onto the sorter circuit. The sorter circuit was operated with a flow rate of 30 μL h⁻¹ emulsion and 300 μL h⁻¹ HFE-7500 oil to space the droplets. Single droplet fluorescence was acquired for each droplet approaching the sorting junction. An electric field was activated if droplet fluorescence value exceeded a predefined threshold value by supplying a voltage to the on-chip electrodes. The threshold value was manually defined to sort a desired fraction of the droplets based on the fluorescence distribution of the droplet population. The electrodes were operated with 400 μs pulses of 800 $V_{p-p}$ square waves with a frequency of 30 kHz. Sorted droplets were collected in a syringe operated with a withdrawal rate of about 115 μL h⁻¹, which was fine tuned to collect only the sorted droplets.

### Droplet sorter validation

A binary mixture of high and low fluorescent droplets was created using the droplet generation circuit. Initially, approximately 15 μL of 20 μM fluorescein emulsion was collected in a syringe. Subsequently, approximately 735 μL of 4 μM fluorescein emulsion was collected in the same syringe. Note that it was difficult to capture exactly 15 μL of high fluorescent droplets resulting in some variation in the starting fraction of high fluorescent droplets between the experiments (ranging 1.9–4.4%). The droplets were subsequently sorted with the FADS circuit. After sorting, the sorted material was re-injected back to the sorter device and their fluorescence was acquired.

### Enzyme activity measurements in droplets

α-Amylase from *Bacillus licheniformis* was diluted in a reaction buffer from the EnzCheck Ultra Amylase kit. The enzyme solution was mixed in a 1:1 ratio with 200 μg mL⁻¹ BODIPY–starch substrate just prior to emulsification using an off-chip T-junction. Emulsion was collected for 15 minutes in a syringe and then reinjected at defined time points to measure the fluorescence of the droplets.

### Determining enrichment of yeast

Two defined yeast strains were used, MH34 and NC. They were grown in SD-2xSCAA medium over two nights at 30 °C and 150 rpm in an E-flask. The NC strain was stained with 20 μM Fun1 cell stain for 30 minutes at room temperature and subsequently mixed at a predefined ratio with non-stained MH34 at a ratio of 4:1. Subsequently, the α-amylase production screen method was followed.

After FADS, the cells were recovered from the emulsion and were analyzed using the Gallios Flow Cytometer (Beckman Coulter). Yeast cells were gated on forward and side scatter and were then classified either as Fun1 stained or non-stained based on their 610 nm fluorescence and side scatter.

## Yeast library preparation

A single yeast colony from fresh plate was inoculated in YPD medium, grown overnight using a rotary shaker at 200 rpm and 30 °C. The cells were centrifuged and washed with sterile water. The cell suspension was spread on starch agar plates (6.7 g $L^{-1}$ yeast nitrogen base without amino acids, 0.04 g $L^{-1}$ glucose, 10 g $L^{-1}$ starch, 20 g $L^{-1}$ agar) and exposed to 40 W UV light (UV cross-linker, Topac Inc., USA) at 254 nm for 2–8 s (4–16 mJ $cm^{-2}$) to introduce mutations randomly throughout the whole genome. Plates were incubated at 30 °C in the dark after UV treatment until colonies formed (~5 to 7 days).

## α-Amylase production screen

The yeast cells were sonicated for 3 × 10 seconds at 40 W using an ultrasonic probe (Vibra-Cell, Sonics & Materials, Inc.). The yeast was washed three times by centrifuging and exchanging media and then resuspended in fresh SD-2xSCAA[32] medium (pH 6.0) with 5% (w/w) BSA and incubated on ice for 15 minutes. The $OD_{600}$ of the yeast suspension was measured and the yeast was diluted to balance the cell to drop ratio. Afterwards, the yeast suspension was transferred to a syringe and injected to the droplet generation circuit. The flowing yeast cell suspension was mixed in a 1:1 ratio with 200 μg $mL^{-1}$ BODIPY–starch substrate just prior to emulsification using an off-chip T-junction. Emulsion was collected in a syringe for 30 minutes. Droplets were incubated at room temperature for 3 hours and subsequently sorted on fluorescence using the sorting circuit. After sorting was completed, the sorted material was recovered by removing excessive oil and adding 5 μL of droplet destabilizer and 200 μL of fresh media.

## Analysis of material from library screen

The sorted and the waste materials were recovered and seeded on starch agar plates. About 50 clones from each of the plates were picked at random, fermented in SD-2xSCAA medium and used for determination of the average α-amylase production and yield of the sorted fraction, the waste fraction as well as the library and the mother strain. The activity of secreted α-amylase was measured as described previously.[33] Briefly, Ceralpha kit was used with α-amylase from *Aspergillus oryzae* as a standard with an α-amylase protein conversion coefficient of 69.6 U $mg^{-1}$. The yield (α-amylase activity per gram of dry cell weight) was calculated with a conversion coefficient of 0.7 g per $OD_{600}$.

Furthermore, 60 individual clones were picked from the sorted material and analyzed for their respective α-amylase production and yield in tube fermentation.

Finally, the top clone from tube fermentation was analyzed for α-amylase production in batch fermentation and compared to the mother strain. Strains were inoculated to an initial $OD_{600} = 0.01$ and the batch fermentation was performed using a 1 L batch reactor (DasGip, Jülich, Germany) at 30 °C, 600 rpm agitation, 1 VVM (vessel volumes per minute) air flow and pH 6.0 in SD-2xSCAA medium.

# Results and discussion

## Characterization of the droplet sorter module

The workflow described herein relies on robust sorting of microfluidic droplets on their respective fluorescence. A FADS module was designed and tested for sorting of rare events. The function of the droplet sorter was investigated by sorting an emulsion consisting of a small fraction of highly fluorescent droplets (representing improved cell variants) from a background of low fluorescence droplets at a rate of 400 Hz. The sorted droplets were analyzed by re-injecting them and measuring their fluorescence (Fig. 2). Throughout three replicate experiments, it was found that a low fraction of high fluorescence droplets could be enriched to above 99% (Table 1). This corresponds to enrichment ratios[28] ranging 3437–8554 and false positive rates less than 0.0012 to 0.0029, validating the ability of the FADS chip to efficiently sort rare droplet events in a robust manner. A low false positive rate is arguably important for directed evolution experiments as the unwanted variants are expected to greatly outnumber the desired ones. If the false positive rate would be too high, the true positives would risk being overshadowed. Furthermore, it was observed in images that many false positives arose from a negative droplet mistakenly being sorted together with a true positive droplet, which implies that the false positive rate would be even lower should the fraction of true positives be decreased.

## Enzyme assay

As a pilot case, the method was used to evolve an improved yeast strain for production of α-amylase. To measure α-amylase activity in the microfluidic droplets, the commercially available enzyme substrate BODIPY–starch was used. The enzyme substrate consists of a starch molecule with
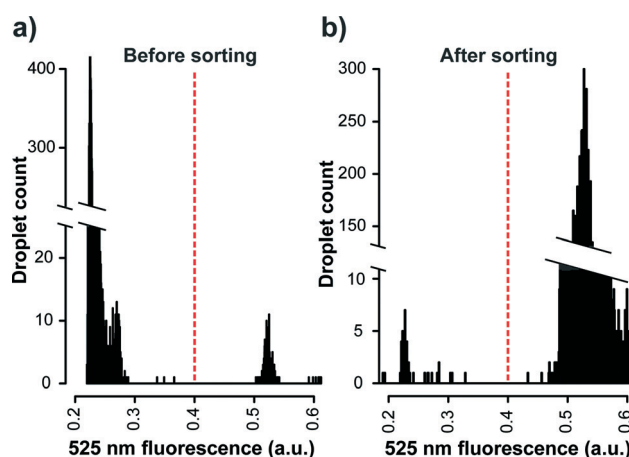


**Fig. 2** a) Fluorescence histogram of a binary mixture of 4 μM and 20 μM fluorescein droplets before the emulsion was sorted using FADS. b) Histogram of the remaining emulsion after sorting the binary mixture for droplets with a fluorescence of above 0.4 (sorting threshold indicated by the red dashed line). Both histograms show approximately 10 000 droplet events. Please note the broken Y-axis.

**Table 1** Results for sorting of three binary mixtures of high and low fluorescent droplets

| Fraction of high fluorescent droplets | | Enrichment ratio |
|---|---|---|
| Before sorting | After sorting | |
| 2.8% | 99.0% | 3437 |
| 1.9% | 99.4% | 8554 |
| 4.4% | 99.6% | 5410 |

covalently attached fluorophores that are quenched while attached to the long starch chain. As α-amylase hydrolyzes the starch substrate into smaller pieces, the fluorophores are gradually unquenched and the fluorescence of the substrate increases (Fig. 3a). The enzyme substrate poses unique challenges because of its non-ideal nature as well as the limited increase in fluorescence of about 3 times from an unreacted to fully hydrolyzed substrate. The resolution of the enzyme assay in droplets was tested by combining various concentrations of α-amylase together with the enzymatic substrate into droplets. The droplets were collected and incubated in a syringe and reinjected at defined points in time. It was found that the fluorescence of the droplets was correlated to the enzyme concentration and that a 2-fold
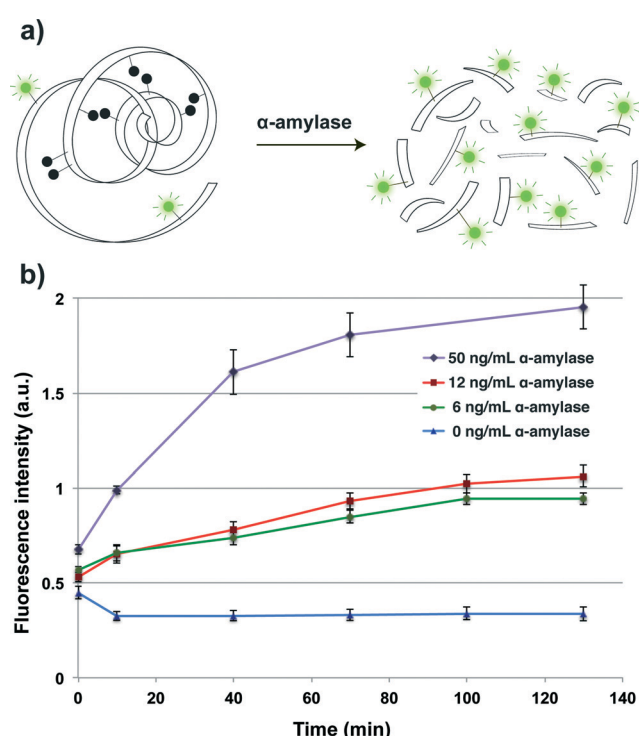


**Fig. 3** a) Schematic image of the BODIPY–starch. The substrate consists of a starch backbone with multiple quenched BODIPY fluorophores. As α-amylase hydrolyzes the starch backbone into smaller pieces, the fluorophores are unquenched conveying an increase in fluorescence. b) Droplets were generated containing α-amylase and BODIPY–starch substrate and droplet fluorescence was followed over time. Please note that lines between data points are only for guidance.

difference in enzyme concentration could be resolved after a 2 hour incubation time (Fig. 3b).

## Model selection for α-amylase expression

To validate the directed evolution workflow, an amylase producing yeast strain, MH34, was mixed at a 1 : 4 ratio with a non-amylase producing strain, NC, and the strains were separated according to their respective amylase production. The NC cells were stained with FUN1, a red fluorescent dye, prior to mixing with MH34 cells to be able to quantify the enrichment after the sorting.

Cell encapsulation into microfluidic droplets generally follows the Poisson distribution,[34] which implies that the cells need to be diluted to achieve predominately single cells in droplets. A cell to droplet ratio of 0.4 was used, which constitutes a tradeoff between higher throughput from having most droplets occupied by cells and the occurrence of adverse co-encapsulation events. Furthermore, since yeast grows by budding growth the offspring often remains attached to the parent cell.[35] Therefore, the cells were sonicated with an ultrasonic homogenizer to break apart cell aggregates prior to encapsulation.

The cell mixture was encapsulated in microfluidic droplets together with the α-amylase substrate and incubated off-chip for 3 hours to give the cells time to produce enzymes and to digest the substrate. Following incubation, the emulsion was reinjected to the FADS chip and sorted at a rate of 400 Hz, keeping the 5% most fluorescent droplets corresponding to 13% of the cell containing droplets.

The sorted cells were subsequently analyzed using a flow cytometer evaluating the fraction of red fluorescent FUN1 stained cells and non-stained cells, respectively. It was found that MH34 could be enriched 14 times over NC in one round of sorting (Fig. 4). This is in good agreement with the theoretically maximal enrichment,[28] $\eta_{max}$, of 13 (see ESI† for calculation). This result indicates that the enzyme screening and the sorting work very well, as $\eta_{max}$ is derived assuming that the only limitation to the enrichment is the Poisson governed encapsulation of cells. This is also in line with the sorter error rate being on the order of $10^{-4}$ indicating that it should not significantly affect the enrichment, which is more than ten times lower. A good agreement between $\eta_{max}$ and the actual enrichment was also reported by Fallah-Araghi et al. (2012)[31] and Baret et al. (2009).[28] In contrast, Kintses et al. (2012)[30] found an enrichment of ca. 1/20 of $\eta_{max}$, likely due to the fact that their sorting experiment involved a library rather than a binary mixture of two strains, the library being a more difficult model system.

## Yeast library screening for an improved amylase producer

The method was used to screen a yeast library with mutations randomly introduced throughout the genome, created by UV-irradiation mutagenesis, to select for cells with high α-amylase production. A total of about $3 \times 10^6$ droplets were sorted at a rate of 323 droplets per second over the course of
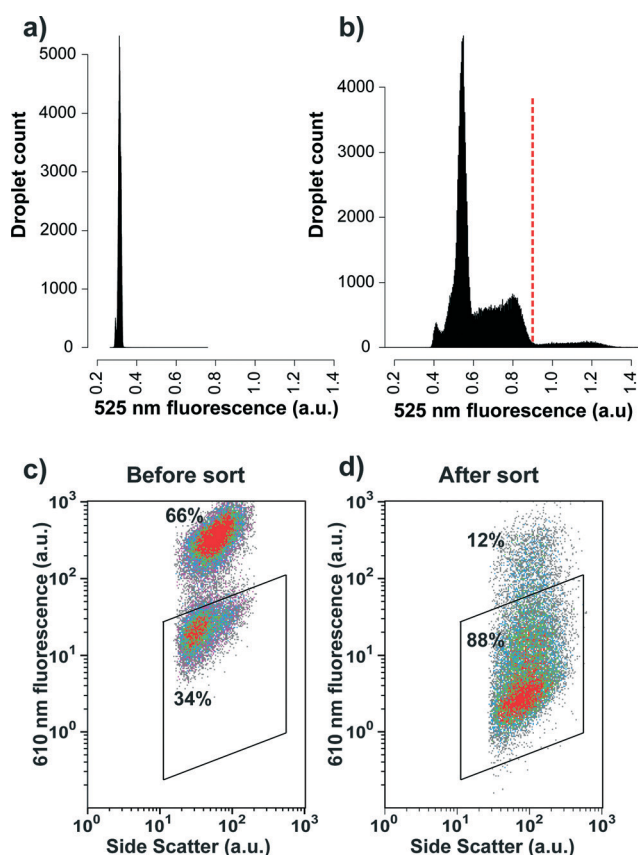
**Fig. 4** a) Histogram showing the fluorescence right after droplet generation of an emulsion produced from co-encapsulation of a mixture of NC and MH34 yeast cells with BODIPY–starch substrate. b) Histogram showing droplet fluorescence of the same emulsion as in a) after incubation for 3 hours and reinjection into the sorting circuit. The red dashed line indicates the sorting threshold of 0.9. c) Flow cytometer data from analysis sample mixture, differentiating between FUN1 stained red fluorescent NC yeast and non-fluorescent MH34 yeast. d) Flow cytometer data from the analysis of the sorted fraction. The gated areas encompass the non-stained cells. Color indicates density of events where red > green > blue > purple > grey.



**Fig. 5** a) Histogram showing the fluorescence distribution of the droplets from the sorting of a yeast library. Red line indicates the sorting threshold. b) Analysis of the α-amylase production and yield of polyclonal material from the yeast library, the sorted material and the waste material normalized to the mother strain used to construct the library. Production is measured in units of α-amylase activity per volume and the yield is α-amylase activity per gram dry weight of cells. c) Analysis of 60 clones picked from the sorted material normalized to the mother strain. d) The top clone indicated with a black arrow in c) was further analyzed in a large-scale batch reactor validating its improved α-amylase production.

2 h and 17 min and the cells in the 0.72% most fluorescent droplets were recovered for further analysis (Fig. 5a). Part of the polyclonal sorted material was cultivated and subsequently analyzed in bulk for α-amylase production and yield. We found that cells from the sorted fraction had on average a 63% higher enzyme production and a 35% higher yield compared to the original library as well as 8% higher enzyme production and 10% lower yield compared to the mother strain, which was used for the creation of the library (Fig. 5b). Production is measured in units of α-amylase activity per volume whereas yield is measured in units of α-amylase per gram dry weight of biomass. Since we select for α-amylase activity per volume rather than total yield on the glucose in the media, we expect to find variants that are primarily improved in production, which is indeed the case.

Sixty yeast clones were picked from the polyclonal sorted material and each was tested for α-amylase production and yield (Fig. 5c). Several variants with similar production and yield to the mother strain were found, which is expected.
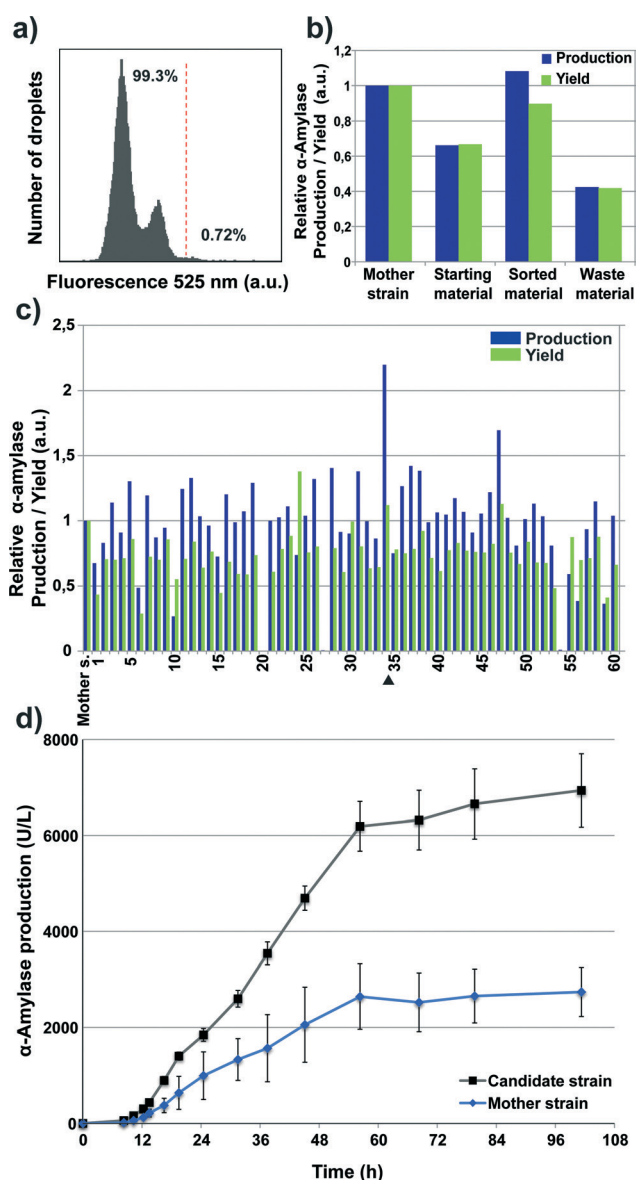
Several strains were also identified that were substantially improved. The top-performing clone was found to have more than 2 times the enzyme production compared with the mother strain. To verify that the phenotype of the top clone is stable and scalable, it was transferred to large-scale batch cultivation and compared to the mother strain. The experiment confirmed that the candidate strain had a more than 2-fold increase in α-amylase production (Fig. 5c), as well as a higher growth rate compared to the mother strain.

## Conclusion

In summary, we present a droplet microfluidics based method for single cell screening of microbe libraries for the selection of improved enzyme production hosts. The system provides an exceptionally high throughput compared to conventional microtiter plate based platforms, comparable to FACS based methods. However, in addition to a typical FACS method, the range of reactions that can be screened is vastly improved as FACS is limited to internalized or cell surface bound fluorescent probes. As a pilot case, we aimed to improve the production of secreted α-amylase, an industrially relevant enzyme used in biofuels production. A yeast strain with twice the α-amylase production of the mother strain was identified from a single round of selection from a whole-genome mutated library. The method can be readily applied for directed evolution by using the top strains as templates for generation of another library followed by another round of selection.

The method presented herein could have significant industrial value as the throughput of the droplet system is more than 300 times higher than an industry state of the art microtiter plate robot system while also reducing reagent consumption by a million fold. The system would be applicable to screen microbes for production of other enzymes or even other metabolites with minor modifications, provided that the metabolite can be linked to a fluorescent signal by coupling it to a fluorescent assay. In addition, the platform could find scientific value in understanding how the metabolism in production hosts can be augmented to increase production in general. Whole genome sequencing can reveal the mutations that bring about the improved phenotype. These mutations can potentially be transferred to other production hosts to similarly improve their phenotype using an inverse metabolic engineering approach.[36]

## Acknowledgements

## Notes and references

1 O. Kirk, T. V. Borchert and C. C. Fuglsang, *Curr. Opin. Biotechnol.*, 2002, **13**, 345–351.

2 J. A. Dietrich, A. E. McKee and J. D. Keasling, *Annu. Rev. Biochem.*, 2010, **79**, 563–590.

3 J. R. Cherry and A. L. Fidantsef, *Curr. Opin. Biotechnol.*, 2003, **14**, 438–443.

4 S. Ostergaard, L. Olsson and J. Nielsen, *Microbiol. Mol. Biol. Rev.*, 2000, **64**, 34–50.

5 J. Nielsen, *Appl. Microbiol. Biotechnol.*, 2001, **55**, 263–283.

6 E. Nevoigt, *Microbiol. Mol. Biol. Rev.*, 2008, **72**, 379–412.

7 F. H. Arnold and A. A. Volkov, *Curr. Opin. Chem. Biol.*, 1999, **3**, 54–59.

8 Y.-S. Jin, H. Alper, Y.-T. Yang and G. Stephanopoulos, *Appl. Environ. Microbiol.*, 2005, **71**, 8249–8256.

9 P. S. Daugherty, G. Chen, M. J. Olsen, B. L. Iverson and G. Georgiou, *Protein Eng.*, 1998, **11**, 825–832.

10 G. Yang and S. G. Withers, *ChemBioChem*, 2009, **10**, 2704–2715.

11 S. Bershtein and D. S. Tawfik, *Curr. Opin. Chem. Biol.*, 2008, **12**, 151–158.

12 E. Antipov, A. E. Cho, K. D. Wittrup and A. M. Klibanov, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 17694–17699.

13 B. Stevenson, S. C. Yip and D. Ollis, in *Enzyme Engineering*, ed. J. C. Samuelson, Humana Press, 2013, vol. 978, ch. 18, pp. 237–249.

14 R. Lauchli, K. S. Rabe, K. Z. Kalbarczyk, A. Tata, T. Heel, R. Z. Kitto and F. H. Arnold, *Angew. Chem., Int. Ed.*, 2013, **52**, 5571–5574.

15 B. S. Montenecourt and D. E. Eveleigh, *Appl. Environ. Microbiol.*, 1977, **33**, 178–183.

16 A. Huebner, S. Sharma, M. Srisa-Art, F. Hollfelder, J. B. Edel and A. J. Demello, *Lab Chip*, 2008, **8**, 1244–1254.

17 H. N. Joensson and H. Andersson-Svahn, *Lab Chip*, 2011, **11**, 4144–4147.

18 M. T. Guo, A. Rotem, J. A. Heyman and D. A. Weitz, *Lab Chip*, 2012, **12**, 2146–2155.

19 L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, *Nat. Protoc.*, 2013, **8**, 870–891.

20 J.-C. Baret, *Lab Chip*, 2012, **12**, 422–433.

21 J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller and G. M. Whitesides, *Electrophoresis*, 2000, **21**, 27–40.

22 D. Link, S. Anna, D. Weitz and H. Stone, *Phys. Rev. Lett.*, 2004, **92**, 054503.

23 M. Chabert, K. D. Dorfman and J.-L. Viovy, *Electrophoresis*, 2005, **26**, 3706–3715.

24 Y. Bai, X. He, D. Liu, S. N. Patil, D. Bratton, A. Huebner, F. Hollfelder, C. Abell and W. T. S. Huck, *Lab Chip*, 2010, **10**, 1281–1285.

25 S. L. Sjostrom, H. N. Joensson and H. A. Svahn, *Lab Chip*, 2013, **13**, 1754–1761.

26 H. N. Joensson, M. Uhlen and H. A. Svahn, *Lab Chip*, 2011, **11**, 1305–1310.

27 K. Ahn, C. Kerbage, T. P. Hunt, R. M. Westervelt, D. R. Link and D. A. Weitz, *Appl. Phys. Lett.*, 2006, **88**, 024104.

28 J. C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El-Harrak, L. Frenz, C. Rick, M. L. Samuels, J. B. Hutchison, J. J. Agresti, D. R. Link, D. A. Weitz and A. D. Griffiths, *Lab Chip*, 2009, **9**, 1850–1858.

29 J. J. Agresti, E. Antipov, A. R. Abate, K. Ahn, A. C. Rowat, J.-C. Baret, M. Marquez, A. M. Klibanov, A. D. Griffiths and D. A. Weitz, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 4004–4009.

30 B. Kintses, C. Hein, M. F. Mohamed, M. Fischlechner, F. Courtois, C. Laine and F. Hollfelder, *Chem. Biol.*, 2012, **19**, 1001–1009.

31 A. Fallah-Araghi, J.-C. Baret, M. Ryckelynck and A. D. Griffiths, *Lab Chip*, 2012, **12**, 882–891.

32  K. Tyo, Z. Liu, D. Petranovic and J. Nielsen, *BMC Biol.*, 2012, **10**, 16.

33  Z. Liu, K. E. J. Toy, J. L. Martínez, D. Petranovic and J. Nielsen, *Biotechnol. Bioeng.*, 2012, **109**, 1259–1268.

34  A. Huebner, L. F. Olguin, D. Bratton, G. Whyte, W. T. S. Huck, A. J. de Mello, J. B. Edel, C. Abell and F. Hollfelder, *Anal. Chem.*, 2008, **80**, 3890–3896.

35  A. Manukyan, L. Abraham, H. Dungrawala and B. Schneider, *Cell Cycle Synchronization*, Humana Press, 2011, vol. 761, ch. 12, pp. 173–200.

36  J. E. Bailey, A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner and P. S. Tsai, *Biotechnol. Bioeng.*, 2002, **79**, 568–579.