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Microfluidics for High-Throughput Screening and Directed Evolution in Agrochemical R&D

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Abstract: Directed evolution (DE) optimizes biomolecules through natural selection principles, revolutionizing the development of proteins, nucleic acids, and strains for various applications. However, conventional DE methods face limitations in screening throughput, which can prevent the identification of rare but optimal variants within a population. Droplet-based microfluidics enable the transfer of conventional screening methods into nanolitre-scale droplets, enabling high-throughput screening while preserving genotype-phenotype connections. This technology allows rapid screening of millions of variants, opening new possibilities for microbial strain engineering and metabolite production optimization. We discuss the integration of microfluidics into DE workflows and reflect on its potential applications in agrochemical research, including enzyme evolution, crop trait improvement, and natural product biosynthesis.

Keywords: Agrochemical R&D · Directed evolution · High-throughput screening · Microfluidics

1. Introduction

Directed evolution (DE) is a powerful technique that aims to optimise user-defined traits of biomolecules such as enzymes, proteins, and nucleic acids by mimicking natural selection in the laboratory. By iteratively generating genetic diversity and selecting for desirable traits, DE has enabled the identification of high-performance catalysts for industrial applications, and the engineering of proteins with enhanced stability, substrate specificity, or activity, as well as novel biosensors. For instance, DE has been successfully used to enhance enzyme kinetics for biofuel production, improve antibody affinity for therapeutic applications, and evolve nucleic acid aptamers for diagnostic use.[1–3]

Traditional DE methods use random mutagenesis and recombination to create diverse variant libraries, followed by high-throughput screening to identify improved variants (Fig. 1). While these methods can generate extremely diverse libraries in theory, practical screening limitations often restrict the accessible sequence space.[4] Conventional screening in microtiter plates, though advantageous for sample compartmentalization, is limited to about 10,000 samples a day, and to reach this number requires substantial investment in advanced fluid-handling systems. This creates a bottleneck when screening large libraries or searching for rare improvements, potentially extending the process to weeks or months.

Droplet-based microfluidic systems offer a transformative solution to directed evolution challenges by enabling ultra-high-throughput screening of large variant libraries (>10^6).[5] These systems can generate and process millions of reaction compartments within hours, vastly improving the scale and efficiency of variant evaluation. They provide precise control over conditions, miniaturize assay volumes, and allow efficient sorting based on various readouts. Crucially, they maintain a strong genotype-phenotype link through droplet encapsulation. This technology has already been applied to evolve more efficient enzymes, biosynthetic pathways, and RNA catalysts.[6,7]

2. The Microfluidic Workflow

Droplets can be simply generated within microfluidic systems by combining two immiscible phases: a continuous phase (also termed carrier phase) and a dispersed (or discrete) phase. Droplet generation is normally determined by the device geometry, the flow rates of each phase, and the physical properties (*e.g.* viscosity) of the involved fluids. The properties of the channel surface play a crucial role in droplet formation, with efficient and stable droplet generation occurring when the channel surface has a high affinity with the carrier phase.[5,8] This ensures that the carrier phase effectively wets the channel walls and thus prevents an unfavourable interaction with the discrete phase. Interfacial effects between the continuous phase and the channel surface as well as the interfacial tension between the two immiscible phases play an important role in droplet emulsification due to the high surface area-to-volume ratio at the microscale. Such droplets have volumes ranging from tens of femtolitres to tens of nanolitres and can be produced at rates of several kHz. Most importantly, both droplet size and droplet payloads can be precisely controlled through variation of the flow rates and composition of the input fluids. Water-in-oil emulsions are widely used in the field of droplet-based microfluidics, but several other emulsion configurations have been employed for specific applications, including oil-in-water emulsions and higher-order emulsions.[9] The most common design for droplet generation is a flow-focusing geometry, where the continuous phase meets the dispersed phase symmetrically *via* two side channels, and the dispersed phase is pinched (focused) on both sides until droplet break-up occurs.[10,11] (Fig. 2ai). This symmetrical design allows for exquisite control over droplet size and frequency, with more sensitivity to the flow rate of each phase. Importantly, the use of droplet generators in series or with tailored

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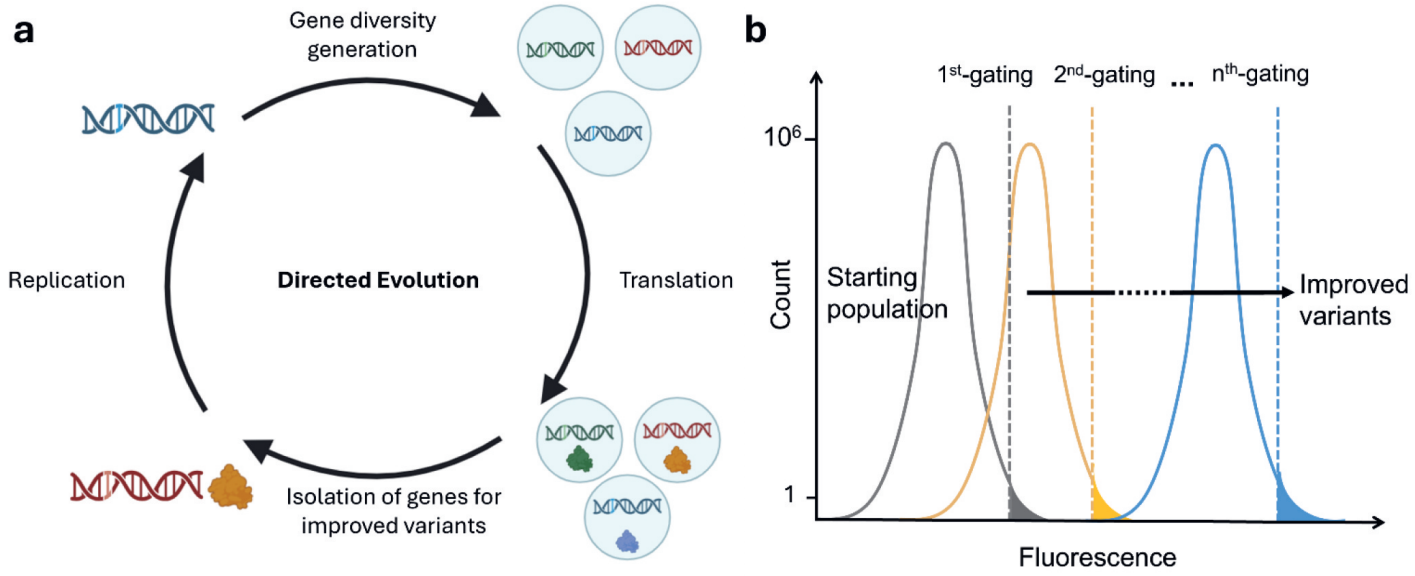

Fig. 1. Illustration of an iterative protein engineering process through directed evolution. (a) The process begins with the introduction of random genetic alterations into the gene encoding the target enzyme, generating a diverse set of mutants. The variants are subsequently expressed and assessed for desirable properties. The best-performing mutants are selected, their genetic changes are examined, and beneficial mutations are combined to create an optimized enzyme variant. The improved version serves as the basis for the next round of modifications. The cycle continues until the enzyme reaches the intended level of enhancement. (b) Schematic representation of population improvement by a stepwise selection process. The best-performing mutants are identified based on a fluorescent phenotype and beneficial mutations are isolated to create an optimized enzyme variant. The population generated at the conclusion of the directed evolution and selection process exhibits characteristics absent in the initial population.

geometries enable the generation of double (or higher) emulsions, which find many applications for biochemical analysis[12] and for the generation of functional microcapsules.[13] Furthermore, advances in microfluidic technologies have enabled the generation of monodisperse double emulsions with ultrathin shells and precise core sizes, (Fig. 2aii).[14] Hydrogel bead formation provides another robust strategy for encapsulating biomolecules or living cells within porous matrices. Hydrogels, such as agarose–alginate composites, offer tuneable mechanical properties that can be optimized for specific applications, including cell transportation or therapeutic delivery (Fig.2aiii). For example, hydrogels have been successfully used to encapsulate stem cells while maintaining their viability over extended periods. The degradation rate and swelling ratio of hydrogel beads can be fine-tuned by adjusting polymer concentrations and crosslinking agents, ensuring controlled release of encapsulated materials.[15,16] Additionally, hydrogel-encapsulated beads have demonstrated potential in proximity-driven encoded assays and biocompatible therapeutic systems.[17]

Following the generation step, droplets can be manipulated and processed in a robust manner, with a range of functional components allowing fusion, splitting, dilution, synchronization, sorting, payload mixing, incubation and storage.[18] To this end, a number of functional operations for droplet manipulation have been developed using bespoke channel architectures, and these different modules are often combined to fit a particular application/need. The most common operations are presented in Fig. 2b, c and include reagent dosing, mixing, and sorting.

Reagent dosing is most easily achieved through the precise control of the reagent flow rate ratios as laminar streams prior to droplet generation.[19] For some applications, however, the introduction of reagents at a later stage is needed. This can be achieved *via* droplet fusion using a specific channel geometry to induce droplet pair coalescence,[20] or in an active manner using an electric field to disrupt the interface between droplets.[21,22] Pico-injection also allows the controlled addition of picoliter volumes of reagents into droplets flowing at kHz rates[23] (Fig. 2bi). Here, the water-oil interface between the pico-injection channel and the passing droplet is disrupted using an electric field and a controllable amount of the new reagent stream enters each droplet during the transit time. Winding geometries are used to efficiently mix reagents within droplets *via* chaotic advection, achieving ultra-fast mixing on µs-ms timescales, with smaller droplets and higher flow rates leading to faster mixing[19] (Fig. 2bii). Droplets can be either incubated on-chip using various delaying geometries,[24] or immobilized within static traps[25] (Fig. 2bii).

It is especially important to note that droplets can be sorted according to their content (at kHz rates) based on a variety of readouts, allowing the selection of relevant droplet subsets from a much larger population (Fig. 2ci). Sorting based on fluorescence readouts is most widely employed due to its high sensitivity,[26-28] however a growing number of detection methods are being applied to droplet sorting, including absorbance[29] and mass spectroscopy.[30] Acoustophoresis and Fluorescence-Activated Cell Sorting (FACS) are advanced techniques employed in droplet microfluidics for manipulating and sorting biological samples. Acoustophoresis utilizes acoustic waves to gently manipulate droplets, making it particularly suitable for handling sensitive biological samples. Acoustic droplet sorting has been shown to sort droplets and particles at kHz droplet rates[31,32] (Fig. 2cii). More specifically, surface acoustic waves (SAW) generate acoustic streaming inside a channel changing the flow and thus deflecting droplets. Acoustic sorting is therefore independent of droplet content, properties or volume and uses low applied power to achieve sorting.[33,34] Using fluorescence-activated cell sorting (FACS) with double emulsions is a method that combines microfluidics and fluorescence-based sorting to enable high-throughput analysis and selection of encapsulated cells[35] (Fig. 2ciii). These microfluidic technologies can be integrated into a streamlined directed evolution workflow. Often for enzyme evolution, cells expressing a library of enzyme variants are encapsulated in droplets along with lysis reagents and fluorogenic substrates, incubated to allow the reaction to proceed, then sorted based on the intensity of fluorescence. The genetic material from selected droplets is subsequently recovered, sequenced, and utilized as the foundation for the next round of mutagenesis. Microfluidics technologies confer remarkable flexibility to this pipeline, enabling culturing of single cells in droplet-contained

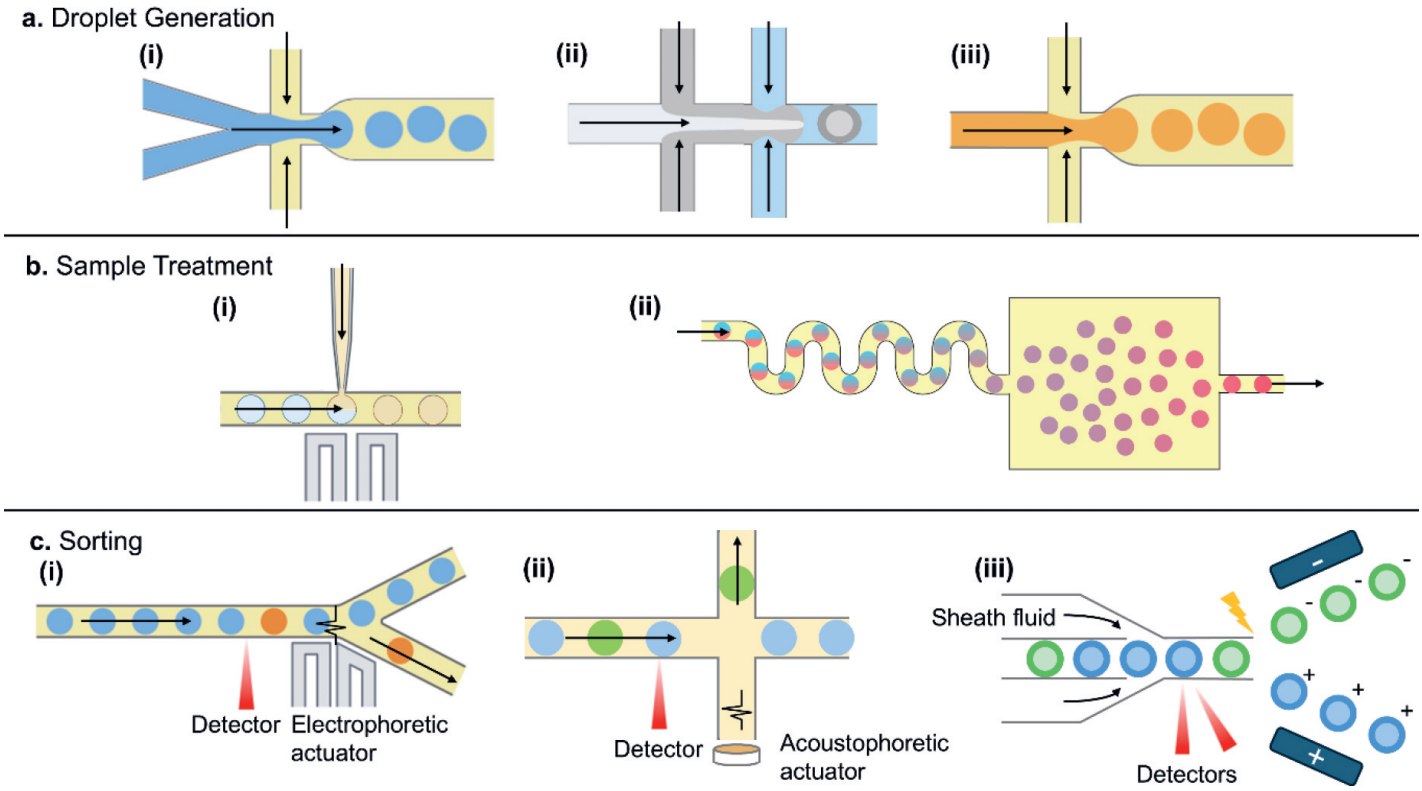

Fig. 2. Schematic representation of key operation units in droplet microfluidics for directed evolution screening. These modular devices can be integrated to create tailored microfluidic workflows for diverse DE experiments. (a) Droplet Generation. (i) Water-in-oil emulsions: Formation of aqueous droplets in a continuous oil phase, (ii) Double emulsions: Generation of water-in-oil-in-water droplets for increased stability, (iii) Hydrogel bead formation: Encapsulation of biomolecules in porous hydrogel matrices for enhanced functionality. (b) Sample Treatment. (i) Pico-injection: Precise addition of reagents into pre-formed droplets, allowing for multi-step reactions or timed reagent addition, (ii) Mixing channels and Incubation chambers allow optimal sample mixing after the droplet generation and subsequently a precise control of reaction conditions through integrated heating/cooling elements. Additionally, delay channels of varying lengths are used for precise incubation periods, enabling kinetic studies. (c) Sorting. (i) Dielectrophoresis: Fluorescence-Activated Droplet Sorting (FADS) that utilizes non-uniform electric fields to deflect droplets based on their dielectric properties and fluorescence signal, (ii) Acoustophoresis: FADS that employs acoustic waves to manipulate droplets, offering gentler sorting for sensitive biological samples, (iii) Fluorescence-Activated Cell Sorting (FACS): Sorting of double emulsions containing cells or cell-free expression systems, compatible with conventional flow cytometry equipment.

colonies, addition of reagents at precise timepoints, and compatibility with various microbial systems. By combining precise droplet manipulation, controlled reagent addition, and sensitive sorting techniques, microfluidic platforms significantly accelerate the directed evolution process compared to conventional methods.[36]

3. Emerging Trends with Potential Impact for the Agrochemical Industry

Droplet microfluidics enables directed evolution for the discovery and optimization of enzymes for trait development and biocatalysis, as well as strain engineering for natural product research. The technology's compatibility with various organisms, from lab strains to microbes that impact crops, makes it a versatile tool for advancing sustainable agricultural solutions and natural product development.

The production of agrochemicals using biocatalytic approaches, such as the use of enzyme or microbial fermentation for chemical transformation, allows crop protection chemicals to be produced at lower reaction temperatures and pressures, with fewer toxic solvents, and with regio- and stereoselectivity.[37] Microfluidics has facilitated efficient DE of enzymes for specific industrial applications.[38–40]

Moreover, droplet microfluidics has enabled the screening of microbial populations and metagenomic libraries containing more than 1,000,000 sequences.[41-43] This approach allows the discovery of rare enzymes, capable of catalysing reactions which were previously unattainable by biocatalysis, that can be immediately applied in biocatalytic processes or serve as promising starting points for directed evolution campaigns.

The potential of DE for enhancing insect-control traits and broadening the recognition spectrum of disease-control traits has been demonstrated using DE techniques that alter protein-protein or protein-DNA interactions.[44,45] However, these techniques are not readily adaptable for evolving enzymes, which is required for herbicide tolerance traits. Droplet microfluidics is amenable to evolving enzymes for herbicide tolerance traits through the DE of a target enzyme or sensitised microbial strain in the presence of an inhibitor herbicide active ingredient, or the engineering of an enzyme to metabolise a herbicide into non-phytotoxic products.

Trait proteins that are expressed in novel GM crops must undergo extensive safety and allergenicity testing before they can be commercialized. This requires the challenging task of producing tens of grams, and sometimes over 100 grams, of purified trait protein. Similarly, large amounts of engineered enzymes must be routinely produced for industrial biocatalytic applications. DE campaigns can be designed with microfluidic technology to maximise yield and solubility of enzymes for industrial production. For example, DE can be performed with encapsulated *E. coli* strains that contain fluorescent reporters that give a proportionate indication yield and folding of heterologously expressed proteins.[46,47]

Natural products are a rich source of active ingredients for crop protection which, for most, cannot be easily produced using traditional synthetic chemistry approaches. Harnessing these compounds for large-scale agricultural use has been hampered

by the challenge of producing them efficiently through microbial fermentation. Despite advances in metabolic engineering, achieving commercially viable titers of these complex molecules often remains elusive, necessitating innovative approaches to strain optimization and production enhancement. Droplet microfluidics emerges as a powerful tool for engineering microbial producers of natural products, enabling the rapid screening of vast genetic libraries to identify high-performing strains. By co-encapsulating individual producer cells with biosensor strains in picoliter-sized droplets, this technology creates miniature bioreactors for *in situ* detection of target molecules (Fig. 3). This technique has been used to increase the titer of industrially relevant chemicals produced by fermentation. For example, Bowman *et al.* screened libraries of three mutagenized microbe species to identify strains that produced increased amounts of natural products.[48] This includes the identification of a *Yarrowia lipolytica* isolate that produced 1.5 fold more of the polyketide triacetic acid lactone (TAL), which was screened using an *E. coli* biosensor that fluoresced in proportion to the amount of TAL present in the co-culture medium.

New-to-nature natural products can be discovered by using the method of co-encapsulating a library of mutagenized natural-product producing microbes with a biosensor strain. Schmitt *et al*. screened a library of antimicrobial lanthipeptides produced by *Lactococcus lactis* co-encapsulated with a fluorescent biosensor strain to identify novel lanthipeptide variants with improved activity against pathogens.[49] This method opens new possibilities for natural product discovery. However, it is important to note that agrochemical leads derived from natural products could be small molecules synthesized by enzymes in biosynthetic gene clusters, and not gene-encoded peptides. Therefore, to apply this approach in agrochemical discovery, appropriate mutagenesis strategies need to be developed and tailored to target these specific biosynthetic pathways.

Agricultural biotechnology applications focus on a diverse array of organisms, including plants, insects, microbial symbionts, and filamentous microbes, which present unique challenges for cultivation and analysis. Many economically important pathogens and natural product producers display filamentous growth, which can cause organisms to outgrow from droplets, leading to droplet coalescence.[50–53] Researchers have used varied methods to overcome this challenge, including adding gel-forming compounds to create a solid droplet substrate or forming droplets with a solid outer layer and a liquid,[53,54] or using larger nanolitre-scale droplets to increase the culture time before overgrowth occurs.[51] DE of plants in nanolitre droplets is conceptually feasible: protoplasts derived from plant cells can be encapsulated in droplets and sorted, and some directed evolution techniques can work directly in plant cells.[55-57] However, recovering and regenerating plants from protoplasts in droplets remains challenging. Alternatively, methods could be developed to perform DE on plant cell suspension cultures.

4. Discussion and Outlook

FADS is the main technological development that has enabled the high-throughput screening capability of droplet microfluidics, by providing the capability to screen millions of droplets for DE workflows. However, FADS is constrained by its reliance on fluorescent reporters, which can be challenging to develop for all desired enzymatic activities or strain phenotypes and may lead to the selection of variants optimized for reporter function rather than the intended phenotype. The innovation of Mass Activated Droplet Sorting (MADS), the integration of the direct detection of products and substrates by mass spectrometery, offers a more versatile and broadly applicable screening method for enzyme engineering and other applications in agrochemical development.[30] As this technology matures, it may broaden the application of droplet microfluidics within agrochemical research. Future ad-

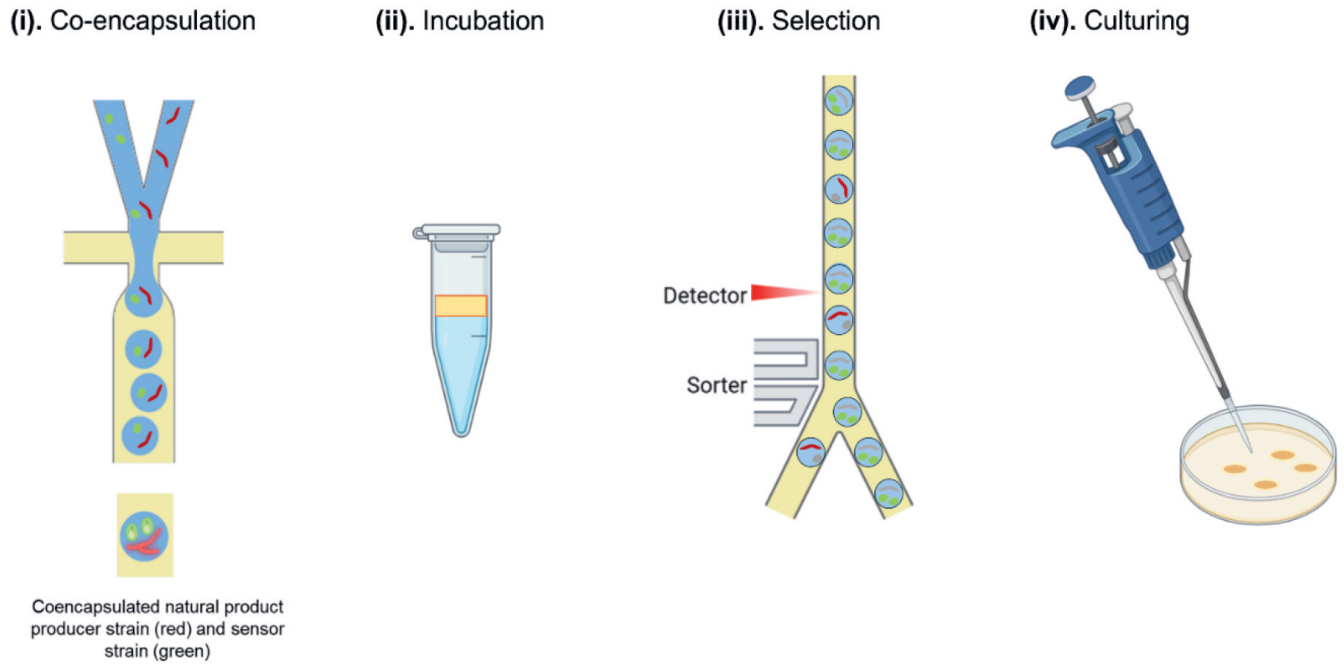

Fig. 3. Example of the microfluidic workflow for the directed evolution of natural product-producing microbial strains. (i) Co-encapsulation of the natural product producer strain (red) and the biosensor strain (green) within monodisperse water-in-oil emulsion droplets using a microfluidic device. (ii) Incubation of droplets under controlled conditions allows for metabolite production by the producer strain and its detection by the sensor strain. Throughout incubation, droplet integrity is critical to prevent leakage or crosstalk between droplets, which could lead to false positives or reduced selection accuracy, (iii) Selection is achieved by detecting the reporter signal (*e.g.* fluorescence) emitted by the sensor strain in response to the presence of the target compound. Droplets are sorted using fluorescence-activated droplet sorting (FADS), (iv) Selected droplets are broken, and producer strains are recovered and cultured for validation and further rounds of screening. The droplet format provides physical separation, chemical containment, and high-throughput capabilities essential for directed evolution of microbial strains.

vancements in microfluidic device design, detection modalities, and droplet stabilization techniques will be crucial in overcoming current challenges and expanding the applicability of both FADS and emerging technologies like MADS.

Strain engineering shows significant potential for advancing natural product discovery research. Most examples of strain engineering in this context rely on biosensors to screen for strains that produce increased titres of natural products. Realizing the full potential of strain engineering requires investment in biosensors that are sensitive, specific, and quick and easy to design and create. Titre engineering can lead to leakage of natural products from droplets when working with high-producing strains, potentially triggering false positive signals in neighbouring droplets. This risk could be mitigated by developing sensitive biosensors capable of identifying high producers early in an experiment. To facilitate the stepwise selection of high producers, it is crucial to design biosensors capable of detecting natural products across various concentration ranges. This approach allows for more precise and effective strain engineering in natural product discovery research. With appropriate biosensors, research can extend beyond titre increase. Screening for growth-enhancing natural product producers can be accomplished using syntrophic co-culture amplification, but this approach necessitates careful biosensor system design.[58] Microbial biosensors must be able to grow in the same medium as the target organism; developing a portfolio of biosensors in diverse microbes will enhance their utility. Molecular biosensors, such as RNA aptamers, offer a promising alternative to the challenges posed by co-culturing producers and living biosensors in droplets.[59] For screening extracellular production phenotypes, aptamers enable the detection of secreted metabolites and proteins, providing multiple advantages.

Beyond directed evolution, the high throughput screening of nanolitre volumes enabled by droplet microfluidics are enticing for the discovery of active ingredients for the agrochemical industry. However, significant challenges remain in applying this technology to small molecule screening for agrochemical discovery, including distinguishing individual compounds within droplets and controlling inter-droplet leakage. DNA-encoded libraries offer a potential solution for post-screen deconvolution, but overcoming the hurdle of segregating small molecules in droplets remains a formidable challenge.

5. Conclusions

Droplet microfluidics offers a game-changing paradigm for DE for agricultural biotechnology by improving screening throughput, enhancing precision, and reliably preserving genotype–phenotype linkage. Through strategies like biosensor-guided selection, co-encapsulation methods, and tailored microenvironments, microfluidic systems have overcome traditional DE limitations, enabling rapid identification and development of enhanced biological variants. As emerging detection modalities, such as MADS, expand the applicability of high-throughput screening beyond fluorescent assays, and as microfluidic platforms become increasingly accessible and modular, the integration of microfluidics into DE workflows are set to accelerate innovation across the agrochemical pipeline.

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Author Contributions

All authors wrote the main manuscript. V. V. and Z. D. prepared the figures. V. V., Z. D., G. S., and S. S. conducted the literature search. All authors reviewed the manuscript.

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