

Review Paper **Enzyme Functional Screening, Discovery and Engineering; Automation, Metagenomics and High-throughput Approaches**

Comment [AA1]: Valuable to provide further insight into the improvement of enzyme specificity, selectivity, and stability through the modification of enzyme acting or binding sites

ABSTRACT

Concerned with the construction and design of novel biocatalysts, the enzyme engineering served to overcome the limitations of native enzymes in order to create biocatalysts with tailored functions to facilitate industrial applications. The enzymes, being recognized by screening and discovery workflows and further tailored by engineering platforms, are of immense potential as improved biocatalysts. Functional metagenomics is a powerful tool to identify novel enzymes followed by the construction of metagenome-based enzyme libraries. And the subsequent screening of these enzyme libraries is in turn facilitated by ultra-high-throughput-based, for example FACS or microfluidics, enzyme engineering technologies. Relies on the compartmentalization of reaction components, in order to detect and measure assay signal within the reaction compartments, the enzyme engineering platforms are designed which include cell-as-compartment platforms, droplet-based platforms and micro-chamber-based platforms. The metagenomics approach and high-throughput screening by these three prime enzyme engineer platforms are the focus of this review.

Keywords: Biocatalysts, Compartmentalization, FASC, Microfluidics, Microchamber array

Abbreviations:

FACS (fluorescence activated cell sorting)

LbL (layer-by-layer)

IVC (in vitro compartmentalization)

GSBs (polymeric gel-shell beads)

BRAIN (Biotechnology Research and Information Network)

1. INTRODUCTION

With the greater specificity and higher reaction rates but the milder reaction conditions and additionally the capacity for regulation, enzymes are able to catalyze an immense range of reactions which makes them fascinating subjects for academic and analytical research as well as clinical and industrial applications for welfare of mankind. Through scale-up considerations and fermentation processes, many naturally occurring enzymes can be potentially accelerated to catalyze reactions for commercial applications including production of pharmaceuticals, biomaterials and fuels [1]. However, unlike the physiological conditions under which natural enzymes are evolved, the sequence and subsequent structural modifications are usually required for such commercial applications. An effort to optimize protein sequence in order to obtain desired phenotypic modification is protein engineering whose applicability range from biochemical study of enzymes [2], to the production of modified commercial catalyst [3]. The sequence-structure-function relationships, computational tools and mechanisms of natural biosynthetic processes are employed by protein engineers [4] and then the subsequent screening

followed by mutagenesis to screen strain with engineered properties of desired activity, specificity, stability.[5]. The screening usually is done by coupling variant enzymatic catalysis to a biochemical detection such as fluorescence or absorbance change of optical property indicating substrate consumption or product formation [6]. The increasingly growing applications of protein engineering include both research and development in the fields of pharmaceuticals and therapeutics [7], synthetic biology [8], biosensing [9], strain development and improvement [10] and production of chemical and fuels [11].

2. ENZYME ASSAYS AND FUNCTIONAL SCREENING: PAST, PRESENT AND FUTURE

A tool for enzymatic function analysis, the enzyme assay, examine the chemistry of a reaction step catalyzed by a particular enzyme followed by exhibiting its answer in terms of sample color change [12] or light signal [13], another way is biological screening event [14], or sometime both [15]. The designing of such assay test is molecular work based on free will of experimenter who, in order to arrive the final functional assay system (Fig. 1), can link different chemical insights and institutions, however must considering strict demands of efficacy. The idealized assays, although being simple, should not result in false positive or false negative and their success rate is based on type and nature of actual reaction being assayed, ease of implementation which in turn is based on cost and equipment and reagents availability. Besides the determination of enzymatic activity based on product formation or substrate consumption rate [6], alternative assay protocols are available for a number of enzymes and choice is primarily based on convenience, cost, availability of equipment and reagents and specially the sensitivity level requirement.

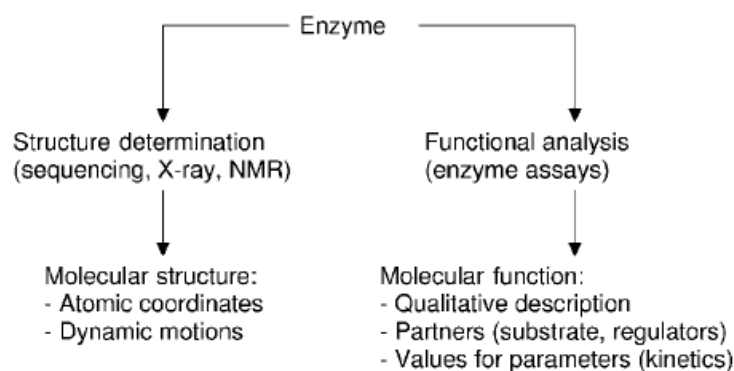


Figure 1; Enzyme Assay a tool for Functional Screening

With the basic purpose to understand enzymatic reaction mechanisms and chemical characteristics of substrates and their products, enzyme assay technology was designed based on classical bioorganic chemistry. Later translation of catalytic reaction into observable signal became possible following by substrate structure engineering and also by the use of chemical sensors, which ultimately leads to formulation of assay design for each known enzyme whose working principle implies the enzymatic turnover to be turned into a signal [16]. Although as earlier as the preliminary developments in Biochemistry, the study for metabolic reaction being catalyzed by enzymes became the central part of this subject, it was not until mid-nineteenth century that the progressive research led to the development of such methods which were capable of performing enzyme assay efficiently and precisely without the tedium of former repetitive assays. As the structural analysis tools are implied for visualizing enzyme structure the enzyme assay procedures tend to visualize enzyme function, however, unlike the structural determination methods which exploit the use of physical principles the assay procedures usually born out of chemical principles. Although employing the same basic principle the design of such system differs

substantially. One of the powerful weapon in enzyme assay armory was the Technicon system for automation in enzyme assay [17]. A great number of automated assays were performed by the said system at that time.

As concern with applicability of automation in enzyme assays, the generalized field of enzyme research dramatically reinforced with the foundation of fully-automated enzymatic assay systems with the capability to perform with in a day experiments that would formerly not only take weeks but also the tedium of repetitiveness. The present research with the interest of even more efficient but time saving enzyme assay tend to focus on high throughput screening [18] and the subsequent development of advanced combinatorial libraries [5] and robotics platforms [19, 20]. At present the enzyme discovery and their characterization is the useful purpose being served by the indispensable tools of enzymatic assay, and in turn invention of novel enzyme assays is the conjunction of research in wide field of enzyme discovery and engineering. The subsequent assays are applied to in various areas of investigation to distinguish active enzymes from microbial collection, to formulate enzyme mutant libraries and to identify commercial useful catalysts.

The actual potentialities of these systems are perhaps not yet fully realized. In research laboratories in general and clinical laboratories in particular, in order to deal with a great number of enzyme samples to perform standard assays routinely, computer operated automation and the ongoing developments in robotic platforms for fully automatized high-throughput screening [20], to process bulk data from multiple enzyme assays reveals a great operational development and overall efficiency of the laboratory.

3. ENZYME DISCOVERY: METAGENOME APPROACH TO SEARCH NOVEL ENZYMES

Before exploiting the platform of enzyme engineering, the search for existing novel enzyme of interest should be considered. Among the well-established approaches for enzyme searching one is metagenome approach [21]. In order to study community genetics of immense diverse microbial community, the DNA is extracted and analyzed following by generating small to large range inert DNA libraries using *E.coli* as transgenic host. The metagenome approach design can be summarized as selection of an environmental sample to extract complete genomic DNA followed by identification of enzyme encoded open reading frames along with their functional annotation for genetic information sequencing and exploration and then metagenome library construction by cloning and functional expression which are finally exploited by high throughput assays for screening novel enzyme activities (Fig. 2). This approach is interestingly valuable as without culturing the large communities of environmental bacteria, the communities' diversity and biochemical roles can be studied by either sequence-based analysis or by function-based screening for novel enzymes. This clearly demonstrate that the metagenome approach can be carried out in two ways; the sequence-based metagenomics and the functional metagenomics. (Fig. 3)

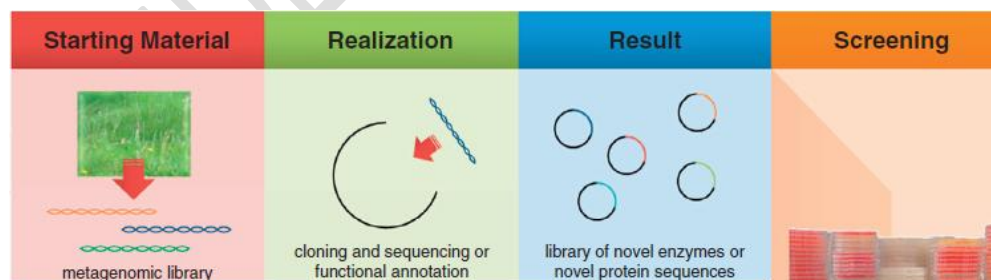


Figure 2; A workflow of Metagenomics approach [22]

3.1 Sequence-based Metagenomics

The next-generation sequencing platforms and advanced computational biology tools are served economical and accessible platforms to analyze library sequence data even in much larger sets followed

by searching public databases to find their sequence similarity to prior characterized genes and pathways. This result in searching and screening the genes of interest and among the rest many being marked as hypothetical or not known function whose range often vary between 40-50 % of genome searched [23].

As concerned with the applicability of this approach, the two prominent papers by venter et al. on marine metagenomes demonstrated the magnitude of microbial and protein diversity by revealing together the new species over 500, the protein encoding genes over 6 million and on other end the protein families with unknown function almost 2000 [24, 25].

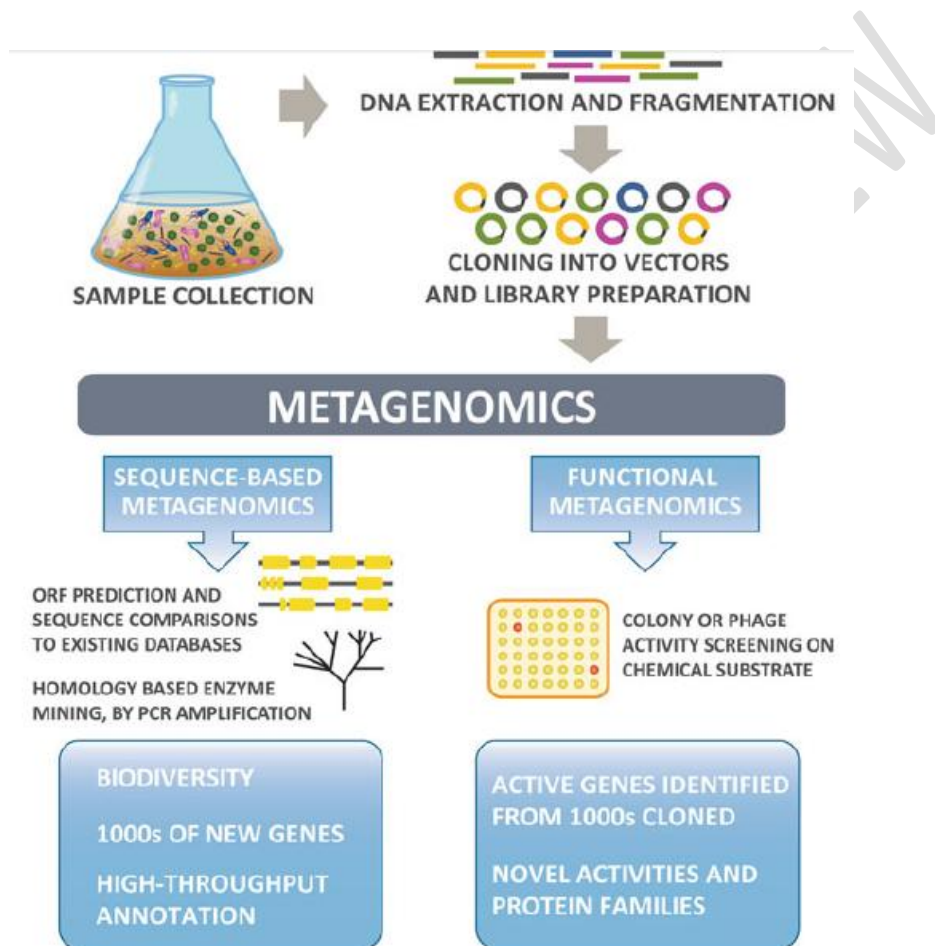


Figure 3; Comparison of two types of metagenomics [26]

3.2 Functional Metagenomics

The functional metagenomics, on the other way, comprised of a set of experimental approaches which are cultivation methods, meta-proteomics, meta-metabolomics, meta-transcriptomic and the screening of enzymes [27]. Directly assaying the metagenomics gene libraries following by the gene expression for the ability to hydrolyze or transform a given chemical substance are the basis of enzyme screening

approach. Usually, the native inducible promoters of *E. coli* are selected for expression of metagenomics enzymes and by using the chromogenic or insoluble substrates in agar the enzymatic activity is detected [28].

Alternatively in another approach the lambda phage-based expression vectors can be employed into which the environmental DNA fragments are cloned and then directly on these phage plaques the specific enzymatic activities are screened [29]. However, the metagenome libraries for enzymatic screening served for mining new enzyme activities by offering the potential to discover novel enzyme families whose sequence similarity is not for that of formerly characterized enzymes in such data banks like Uniprot or BRENDA. Additionally in order to adapt the unique environments for microbes, it offers a vast repository of novel enzymes with incredibly diversified characteristics.

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The expanded number of novel enzymes including many nitrilases, carboxyl esterases, cellulases and laccases have already achieved by enzyme screening approach of metagenome libraries. The screening of metagenomes of extreme environments have also contributed towards increasing repository of novel enzymes by revealing a vast biochemical diversity of "extremophiles" enzymes; the enzymes being able to tolerate extreme conditions such as extreme temperature, pH and high salt concentration, and the subsequent designing of molecular mechanisms for microbial adaptation to such extreme environmental conditions had already been reported by structural and biochemical characterization of these novel enzymes.

Industrial sector also exploits the metagenomics potential of enzyme discovery and the efforts of many companies including Henkel, DuPont, Degussa, Diversa and others have contributed towards savings and clear production in a multitude of industries by metagenomics screening of novel enzymes and then replacing conventional commercial processes with enzymatic processes. The company BRAIN, few years later, identified an immense molecular diversity for Subtilism Carlsberg: a well-known serine protease [30].

4. ENZYME ENGINEERING: THE THREE PRIME PLATFORMS FOR ENGINEERING BIOCATALYSTS

With the desired coverage, the screening of immense enzyme mutant libraries employs the use of protein engineering platform to achieve higher throughput and also in order to achieve the directed evolution, the maintenance of phenotype-genotype association [5]. The protein engineering platforms, except for those approaches that directly connect enzyme activity to infectivity or survivability [31], comprised of combinatorial operated three separate components for achieving the directed evolved workflow. The first to these is implementation of compartmentalization approach by which enzyme genotype (e.g., a bacterial cell transgene with plasmid encoding the variant) is segregated in spatial arrangement coupled with observable optical proxy for enzyme activity such as chemiluminescence or fluorescent product. Next, in order to detect and measure assay signal within the reaction compartments, an assay technology is used which result in high throughput assessment of enzyme function. Finally the screened enzyme mutants of interest are separated from the rest of members of same library by employing an isolation strategy. In short, the constraints by which the selection of screening strategy is dictated are; a production host being reliable and capable of efficient transformation, development of assay protocol, and the availability of equipment and analytical tools [32].

The development of compartmentalization techniques exploiting the use of cells as compartments or droplets to serve as cell like chambers has made the screening more advance with miniaturization the volume of reactions in microliters range, leads to higher throughput by FACS as well as flow cytometry. Currently, the introduction of microfluidics technology has enabled to reduce the reaction volume below to a few pico-liters [33]. In order to readily miniaturize and consequently increasing the throughput of a diverse range of enzyme assays, performed traditionally in micro-titer plate approach, the micro-chamber-based platforms are designed.

4.1 Cell-as-Compartment Platforms

The compartmentalization techniques have emerged as a concomitant of search for higher throughput. The natural reactions being compartmentalized within cells and organelles inspired the protein engineers which ultimately leads to the development of water-in-oil droplets to serve as cell like chambers which made the screening more advance with miniaturization the volume of reactions in microliters range. Consequently, the higher throughput can be achieved as immense number of variants can be screened in much shorter time [34].

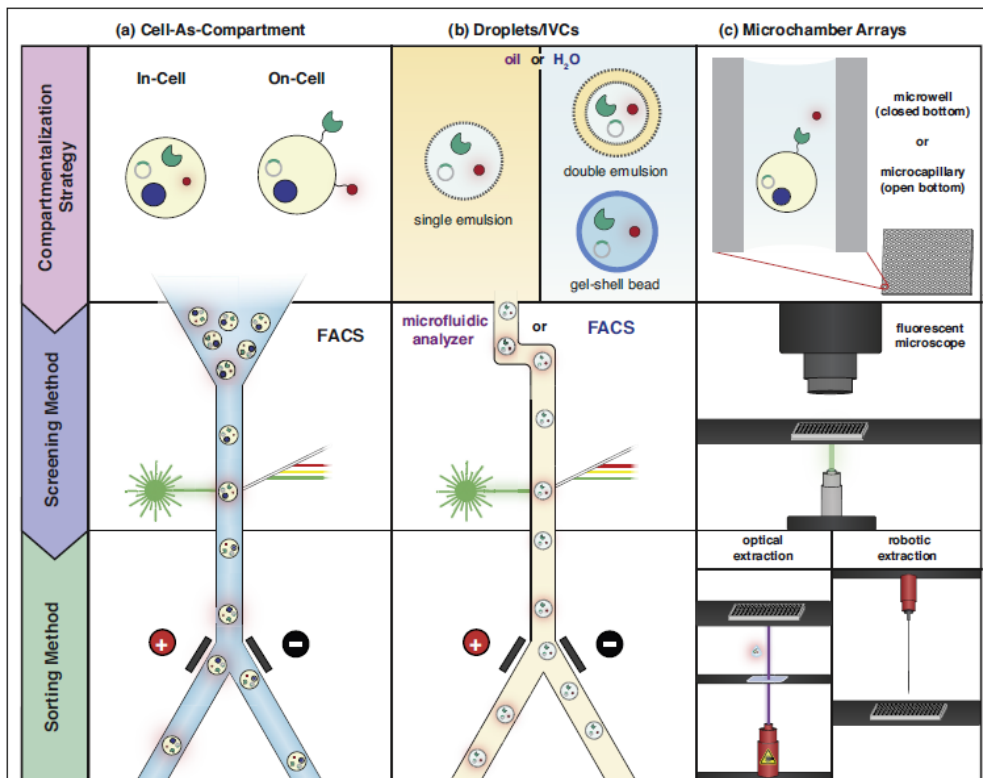


Figure 4; A comparisons of three prime platforms of enzyme engineering [5]

4.1.1 Nature's own Compartments

Any screening or selection method is actually a physically link between the genotype and its leading phenotype, and a prerequisite for it is typically a "compartment", may be a prokaryotic or eukaryotic cell. Thus, the change in sequence of nucleic acid can be detected and marked at the expression level of protein product. Many natural compartments are provided by the cell to couple the genotype to the phenotype. And by exploiting these nature's own compartments, several screening and sorting strategies have been established including cell-based screening, yeast surface display and mammalian surface display and in order to arrive at higher throughput of screening these have been coupled with FACS [35].

The cell itself can act as measurable and sortable compartment provided by the screening and sorting technology of fluorescence activated cell sorting (FACS) for directed evolution workflow by developing an assay strategy in which the assay signal is attached with or retained within the cell [5]

4.1.1.1 In-cell --- Intracellular Compartments

The intracellular compartments usually cytoplasm served as reaction vessel in many of straightforward compartmentalization strategies, for the enzymes that are intracellular in general and that uses biomolecules as substrate in particular. Followed by coupling the enzyme activity to the folding, expression, or trafficking of a fluorescently labelled protein, the intracellular enzyme assay have successfully been reported for DNA recombinase [36], inteins [37], protein chaperones[38] and proteases [39]. For the enzymes which require extra cellular substrate the in-cell enzyme engineering can also be employed provided the substrate introduced is cell permeable and enzymatic activity can be coupled with intracellular fluorescence as successfully applied for glycosyltransferases engineering in *E. coli* cytoplasm by introducing across the cell, the fluorescently labelled sugar substrates using dedicated transporters [40]. Also, the enzymatic activity can be detected even if the reaction substrate is not cell permeable, as done by coupling the activity to produce a detectable reporter protein, employing, for example, the system of three hybrid chemical complementation [41].

4.1.1.2 On-cell --- Surface Display As Compartment

Another generalized strategy for enzyme engineering is based on surface display that in order to provide access of wider range of substrates to enzymes employs the use of particle, cell, or virus display and it must ensure extracellular enzymatic activity to be turned into assay signal to maintain the usual genotype-phenotype association. The examples of applicability of this approach include the engineering of horseradish peroxidase [42] and the sortase A (SrtA); a bio-conjugation enzyme [43]. A related strategy but with the involvement of probes, referred as fluorescence resonance energy transfer (FRET), on any side of substrate can be employed for proteases and other bond breaking enzymes, the substrate being designed that after the enzyme processing the FRET activity is lost [44].

4.2 Droplet-Based Platforms

The in vivo compartments, although are efficient enzyme engineering units, a considerable limitation is demonstrated according to the range of enzymes that can be engineered because of the cell-restricted assay signal. These limitations are defeated, by developing engineering platforms where the diffusion of product and substrate is freely offered by exploiting specially designed man-made compartments and consequently the assay for variant activity (phenotype) of variant enzymes is spatially separated coupled with representing DNA or cell (genotype).

4.2.1 Man-Made Compartments

The requirement of transformation or transfections for employing the compartmentalization of the library in whole cells significantly limits the size of sample-able libraries. Also the scope of cell-as-compartments strategy is further limited by a number of requirements; the maintenance of cell viability, the buffer scope, the solvent and temperature compatibility [45]. Consequently, the in vitro compartmentalization (IVC) approaches have paved the way of cell-free directed evolution, especially in last two decades, the said field has quickly grown and expanded. In IVC, millions of micron-scale droplets can be generated with volume range from femtoliter to nanoliter by the lithographically-defined microfluidic devices at rate of thousands of droplets within a second. Then integrated microfluidic sorters are used in order to screen highly fluorescently marked droplets at kHz frequencies, by either dielectrophoresis or by some other sorting modalities. Alternatively, water-in-oil-in-water compartments also called double emulsions can be exploited being FACS-compatible as well as with flow cytometry. The polymers can also be used to create polymer-based compartments from scratch in a stepwise manner approach by polyelectrolyte multilayer assembly or self-encapsulation radical reaction.

4.2.1.1 Emulsion-Based Compartments

Followed by combining water with adjuvant surfactant containing mineral oil the initial in vitro compartments, water-in-oil droplets, were established. As mentioned above, millions of micron-scale droplets segregated such that each, representing a single library member, act as an independent micron reactor, and all comprising the whole library. The lithographically-defined microfluidic devices, with the control by fluid flow rates and channel dimensions, are used to generate droplets at rate of thousands of

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droplets within a second and the typical volume range from femtoliter to nanoliter [46]. In order to screen droplets, already highly fluorescently marked, the integrated microfluidic sorters are used at kHz frequencies, for that dielectrophoresis is usually employed [47], while other sorting modalities have also been developed [48]. A library of 10^8 picoliter sized droplets, encapsulating horseradish peroxidase enzymes displayed on yeas surface, has been screened employing this approach [49].

However shortly, the incompatibility of such droplets with FACS screening guides the development of more FACS-compatible water-in-oil-in-water compartments also called double emulsions [50], which served to extend the emulsion-based compartments applicability as being also compatible with flow cytometry. Double emulsions strategy was employed for paraoxonase enzyme engineering coupled with its increased activity, about 10^5 fold, for a G-type nerve agent being in situ generated [51]. Although, the barrier towards entry in droplet based screens is efficiently reduced by double emulsions, these are less sturdy than single emulsions, and in order to generate monodisperse droplets, require sophisticated parameters (surface chemistry, flow rates, viscosities etc.), and even then integrity may be compromised of the emulsions with FACS. In attempt to formulate more sturdy droplets, a refined polymeric gel-shell beads (GSBs) single emulsion approach, by constructing polyelectrolyte shell surrounding the agarose shell, has been developed. A library of 10^7 mutants of phosphotriesterases was screened per hour employing this strategy, and the resultant identified variant showed 20 fold faster and efficient kinetics [52].

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4.2.1.2 Polymer-Based Platforms

The polymers can also be utilized to create biocompatible and highly permeable artificial compartments, the so called polymer-based compartments. Establishing a stepwise manner approach they are made from scratch and either of the two approaches can be selected at a time the polyelectrolyte multilayer assembly [53] or another process the self-encapsulation radical reaction [54]. The polymer's contrasting electrostatic charges, such as of anionic alginate; cationic chitosan, are employed by the former technology, the polyelectrolyte multilayer assembly. Innermost layer, in fact the template of formed compartment, is removed on completion of the process of this layer-by-layer (LbL) assembly. So the capsule with the inner hollow structure enveloped by the rest of layers act as multilayer scaffold [53]. Yeast and bacterial cells encapsulation have already been successfully done by the following strategy [55]. The poly acrylate (ethylene glycol) can be utilized for free radical polymerization for the formation of capsules followed by the self-encapsulation approach. The Fenton reaction of hydrogen peroxide (H_2O_2) and ferrous ion (Fe^{2+}) generates a product, the free hydroxyl radicals ($\cdot OH$) which served to trigger the polymerization reaction.

4.3 Micro-chamber-Based Platforms:

The artificial compartmentalization approach is not restricted to the generation of droplets, another broad category of artificial compartments is micro-chamber, for enzyme engineering coupled with high throughput. Here each enzyme is maintained in physically separated vessel, this result in significantly miniaturizing the former micro-titer plate and the number of members of a library can be increased screening in a parallel manner by employing, for the diverse applications in the field of enzyme engineering, any of the two formats of micro-chambers can be exploited either micro-well arrays or micro-capillary arrays.

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4.3.1 Micro-well Arrays

The wells are fabricated onto a suitable medium; a glass or a polymer, the fabrication strategy such that the wells at micron-scale have bottom side closed while top side opened and furthermore loaded by discontinuous dewetting, this strategy usually achieved with micromaching or lithography [56]. Cells or functionalized microspheres both can be applied to spatially segregate single proteins in micro-well arrays, and the applicability of these arrays include protein screening [57], engineering [58], or analysis of single molecule [59]. An example of platform based on micro-well arrays, in order to release the fluorescein exploited, the hydrolysis of fluorescein and result in engineering of a lipase enzyme [58]. In

many instances in order to monitor enzyme activity employed with the production of fluorescent product, a 96-well-plate assays directly can be adapted in micro-well array technologies.

4.3.2 Micro-capillary Arrays

Micro-capillaries are also usually fabricated in a glass medium and micro-capillary arrays comprised of millions of spatially separated micro-capillaries can similarly be used to separate single particles or cells but unlike micro-wells, the micro-capillaries are bottomless high-aspect-ratio capillaries and also these can be loaded simply by capillary action. However, to ensure that on average a single particle or cell will occupy a single chamber, the concentration of these particles or cells is controlled in the library suspension, no matter which micro-chamber approach is being used. Using the Poisson statistics, this precise concentration is usually calculated [56, 60]. An example of platform based on micro-capillary arrays, engineered alkaline phosphatase by exploiting the hydrolysis of a substrate whose conversion into a product being fluorescently dephosphorylated was tracked for specificity and activity [60]. In order to engineer wide range of enzyme classes from proteases to amylases by miniaturizing a variety of assays, the GigaMax microcapillary array was used and within a day screening of about two millions variants at high throughput is reported [61].

5. FUTURE DIRECTIONS

The need for more assay types; The broader application of screening ~~following~~ followed by ultra-high-throughput is to incorporate continuous variation in assay designs leading to expanded portfolio of engineer-able classes of enzymes. Adding to it, the modifications in the hardware of instrumentation being applied for promised optical properties, for example absorbance, beyond total fluorescence would be worthwhile; already the contributing effort towards this goal have been made [62]. However, the need of the time is the greater collaborative efforts of scientists, as one should consider that the enzyme assays are interdisciplinary subjects of chemistry, biology, material science, microbiology and cell biology.

Small is better; Essentially the small amounts of reagents are needed as the volumes of microdroplets are usually in range of 10^{-12} - 10^{-15} L. Miniaturization in droplets compartments up to pico or femtoliter level, enable microfluidic to perform much larger numbers of experiments in relatively short time, giving the platform the extraordinary sensitivity and the improved precision for greater quantitative studies. Even the efficiency of this approach is more than colony screening and robotic liquid handling techniques, as concerned with performing the single reactions the microfluidic can carry out minimum 10^3 -fold more reactions than these techniques [63]. The ultra-high-throughput sequencing coupled with microfluidic to formulate more advanced sequence-function relationship technologies, in the future, may dramatically reinforced the current mutational studies by enabling more precise and deep mutational scanning for detailed survey and identifying each distant mutation dramatically effecting protein stability and fitness landscape [64]. Towards synthetic microdroplets formulation, a great contribution is the subsequent and ongoing developments for formation of droplet-on-demand variants which being fully unsupervised provide rapid accession to data obtained cumbersome in current approaches.

Biocatalysts discovery; The field of enzyme engineering is in turn based on the field of enzyme discovery which identify novel starting points for furthering engineering the desired characteristics for formulating the economical industrial biocatalysts. Functional metagenomics, as mentioned above, is a powerful weapon in enzyme discovery armory to identify novel enzymes without depending on existing homologs but by fragmentizing the genomic DNA randomly and the heterologous expression, which however is very slow approach and rarely hits (on estimation 1 in 10,000 variants) [65]. The use of highly sensitive high-throughput microdroplets-on-demand should enable this technology suited for screening the unprecedented sized metagenomics libraries by overcoming these odds.

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6. CONSIDERATIONS AND CONCLUSIONS

The traditional screening techniques in the field of enzyme engineering, although still represent a workflow, are being replaced by more advanced and promised directed enzyme evolution approaches at higher throughput. The automation and throughput screening of enzymes are being enhanced by technological advances leading towards deeper insights in sequence space of enzyme by establishing much faster, more efficient and reduced labor-intensive work-designs. The researchers can also exploit these combinatorial and diversity generation approaches coupled with high-throughput screening, for enhancing microbial performance under stress conditions or increasing microbial yield.

The platforms and workflows covered in the following review are of prime importance and consideration in the fields of enzyme screening, discovery and engineering which are not only exploited for commercial biocatalysts development, but also in metabolic engineering projects, facilitating synthetic biology and the combinatorial synthesis of biomaterials. However, one must consider that for an effective enzyme engineering screen the evaluation of high-throughput technologies revealed that the advantages and limitations are offered by each approach. These limitations are further considerable elements for enhanced performance of these technologies in future iterations.

Comment [AA7]: Long

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