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Directed evolution for enzyme development in biocatalysis

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Abstract

As an important sector of the chemical industry, biocatalysis requires the continuous development of enzymes with tailor-made activity, selectivity, stability, or tolerance to unnatural environments. This is now routinely achieved by directed evolution based on iterative cycles of genetic diversification and activity screening. Here, we highlight its recent developments. First, the design of "smarter" libraries by focused mutagenesis may be a crucial start-up for a fast and successful outcome. Then library assembly and expression are also key steps that benefits from modern molecular biology progresses. Finally, various strategies may be considered for library screening depending on the final objective: while low-throughput direct assays have been very successful in generating enzymes for important biocatalytic processes, even in bringing completely new chemistries to the enzyme world, ultrahigh-throughput screening methods are emerging as powerful approaches for engineering the next generation of industrial enzymes.

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Introduction

Directed evolution mimics natural evolution in the lab, in an accelerated fashion, and is used to modify proteins at will, to endow them with more desirable properties. In the past years, the technology has focused mainly on antibody and enzyme evolution, with the latter becoming a more and more substantial part of any industrial biocatalysis project. Directed evolution campaigns are based on iterative steps that are repeated until the desired variant is obtained (Figure 1). It starts in vitro by creating gene diversity by means of various molecular biology tools. The DNA library is then expressed in living organisms or cell-free systems and improved variants at the enzyme level are finally identified either by selection if the desired property is associated to a reproductive advantage of the clone, or most often in the field of biocatalysis, by a screening process that will assay every individual clone. Depending on how satisfactory the new properties are, the process can be iteratively repeated until the requirements are met [1].

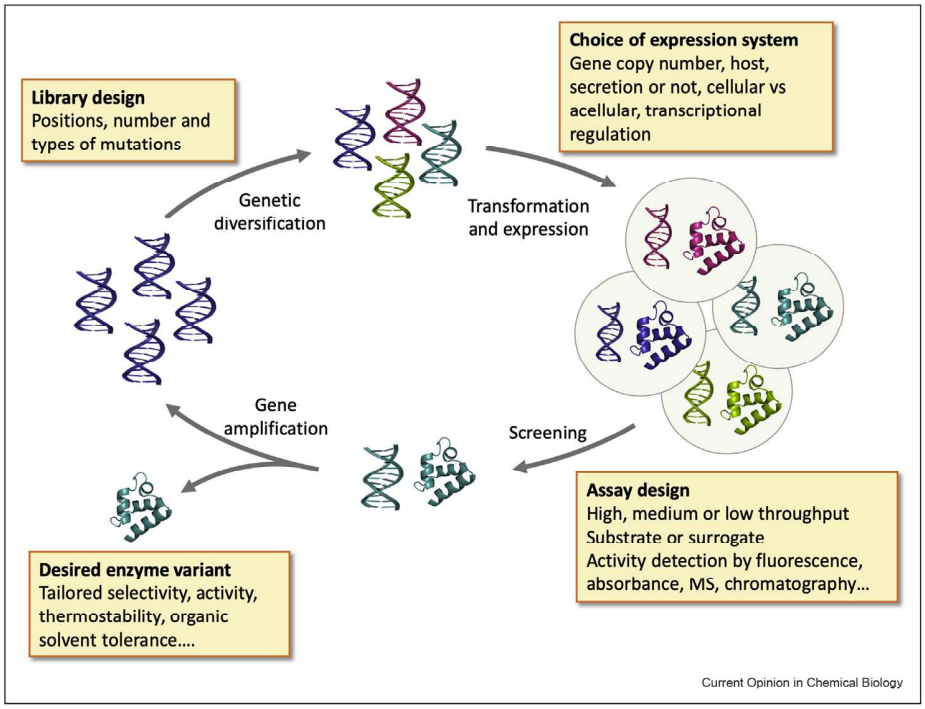
In this article, we will highlight recent advances on each of these three steps with a particular emphasis on the potential impacts for the future development and improvement of research in biocatalysis.

Library design, as smart as possible

To maximize the chances of a successful outcome in a directed evolution campaign, and since the risk of disrupting a protein fold upon mutation is high and will increase with the mutational load, there is a growing interest in controlling the number of mutations, and/or their positions in the structure, and/or eventually the nature of these mutations. Especially in cases where time-consuming screening is required, generating libraries of the highest quality is of prime importance [2].

While easy-to-handle and well-established methods for random mutagenesis on the full gene such as error prone PCR are still used and afford some control on the mutational load and the balance between the transitions and transversions, our increasing capacity to predict which residues may be mutated for evolving a specific property is facilitating the design of semi-rational libraries, also called smart libraries. Such focused mutagenesis methods are based on the assembly of genes that incorporate synthetic degenerate oligonucleotides targeting the diversification at specific positions (traditionally between two and six), for example active site residues if altering activity or substrate recognition of an enzyme is desired. The strategy is also useful for

Figure 1



Schematic view of the various steps of a directed evolution campaign. The starting gene encoding the protein that will be evolved (blue) is subjected to a gene diversification strategy; the quality and size of the resulting library will depend on the criteria chosen for its construction. The individual variants of the genetic library are expressed in an appropriate host or by cell-free in vitro transcription and translation (IVTT). Using an appropriate assay, a screening will then be implemented to identify one or more variants of interest, typically showing a desired activity, thermostability, or selectivity. These variants can be subjected to a new round of directed evolution, until the improvements are satisfactory enough to enable the biocatalytic process.

stabilizing an enzyme or increasing tolerance to organic solvents since there are many methods for predicting which residues should be mutated for stability improvement, the more successful being the consensus approach based on preferred residues in multiple alignments of homologues [3]. Another successful method is based on targeting residues with high B-factors and can be combined with the consensus approach [4]. In silico methods, which can assist in the identification of hotspot regions of the enzyme to mutate, are also quickly developing [5,6], and machine learning approaches may rapidly become unrivaled [7–10].

Controlling codon degeneracy may also reduce the subsequent screening effort. Saturation mutagenesis with NNK codon degeneracy encoding all 20 canonical amino acids has been widely used, but there is an increasing interest in using reduced amino acid alphabets such as NDT codon encoding 12 representative amino acids [11]. Specific codon degeneracy encoding amino acids with similar properties may also be an interesting approach. For example, NTK or MRK degenerate codons encode, respectively, 5 hydrophobic (L, F, I, V, M) or 6 polar neutral or basic (Q, N, S, H, R, K) amino acids. Alternatively, the recent development of massive parallel on-chip oligonucleotide synthesis affords the design of tailor-made library resulting from the assembly a large number of non-degenerate oligonucleotides, but at high cost.

Library assembly and expression

Focused mutagenesis generally requires the incorporation of one or more degenerate oligonucleotides into the gene library by assembling of pools of synthetic oligonucleotides with overlapping extremities or common PCR techniques [2]. Among recent developments, QuickLib is a fast and easy method based on PCR amplification of full linear plasmid followed by intramolecular Gibson-based recircularization, and allowing the direct incorporation of one or two degenerate oligonucleotides [12]. Golden Mutagenesis is another recently reported strategy taking advantage of asymmetric restriction enzymes for rapidly assembling multiple PCR fragments [13]. Library construction on the surface of microbeads by iterative ligation and split-and-mix cycles is also an interesting new strategy when combined with cell-free expression and water-in-oil droplet screening [14].

After assembly, the library must be integrated into an expression system (Figure 2). While high expression is usually desirable for maximizing screening sensitivity, the associated burden on the host may generate important fitness costs and genetic instability [15]. In these cases, a tightly repressed promoter, such as the arabinose inducible pBad may be advantageous. Eventually, cloning the library into a low-copy plasmid or the chromosome [16], or fusing the protein of interest to a ssrA degradation tag [17] are other ways to minimize toxicity problems.

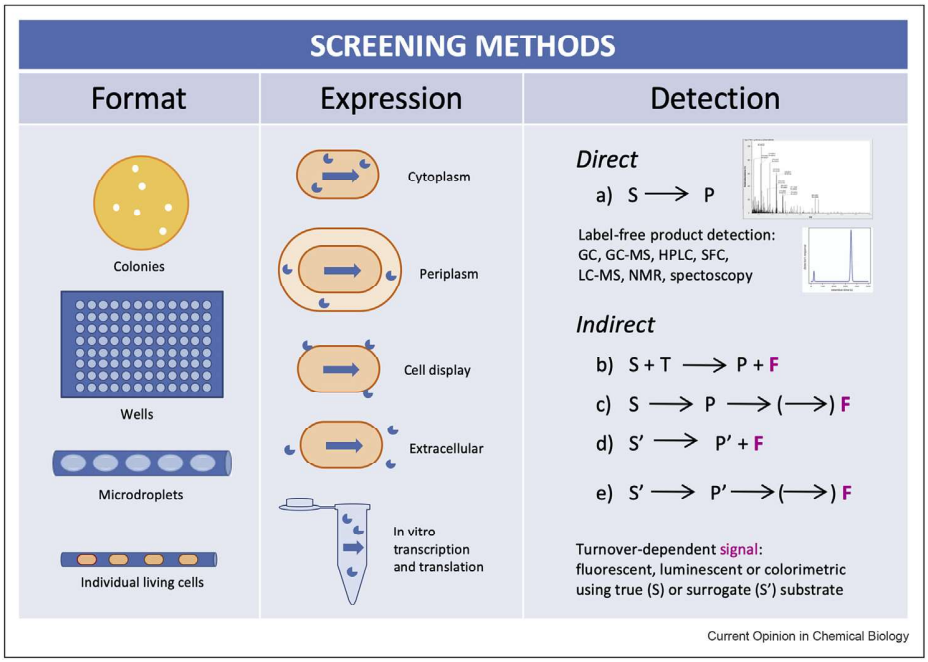
Enzyme variants are classically expressed in the cytoplasm, and subsequent activity screening is then performed with cell-penetrable substrate or after cell lysis. In some cases, secreting the variant in the periplasm of *Escherichia coli* may be advantageous since it will facilitate substrate access and eventually afford the formation of disulfide bonds. However, efficient periplasm targeting may require the use of co-translational signal sequence [18]. Extracellular secretion is also an attractive alternative although it is mainly developed in specific hosts like Gram-positive bacteria and *Pichia pastoris*. Displaying variants at the surface of cells may provide interesting benefits for FACS-screening of enzymes with high throughput [19–21]. Finally, cell-free expression is a potential option although it should be combined with miniaturized reaction volumes for minimizing costs.

Library screening, between limits and breakthroughs

Screening assays are of pivotal importance, not only because their robustness is one of the major predictors of success but also because the throughput rate determines the maximum library size that can be handled. An assay is typically designed to be as fast, as reliable, as sensitive, and as suited to the objective as possible, while keeping a good compromise between costs and quality of the assay. In this regard, the Z-factor is a useful dimensionless statistical parameter that combines data variability and signal dynamic range to provide a quality score for a certain assay [22].

While macroscopic screening formats like colonies on Petri dishes or bacterial culture in 96-well plates are relatively easy to handle, miniaturization in droplets or with single cells may greatly increase the assay throughput (Figure 2). On the other hand, the enzymatic assay can be conceptually designed with two

Figure 2



Schematic representation of screening assay types and formats for directed evolution. Direct assays utilize techniques that enable monitoring the target reaction directly (a): substrates or products may sometimes be detected by fluorescence or UV–vis spectroscopy, otherwise analytical chemistry technique can be employed; in this cases samples must be provided in the form of physically separated variants. Indirect methods rely on the development of a signal due to the reaction course but not necessarily generated by the target reaction itself. The signal may be caused by b) a co-substrate or co-product (e.g., NADH); c) a compound generated by one or more additional steps following product formation; d) the use of a so-called surrogate substrate, specifically chosen for the release, on reaction, of a detectable co-product molecule; and e) the end product of a cascade reaction initiated by the surrogate product.

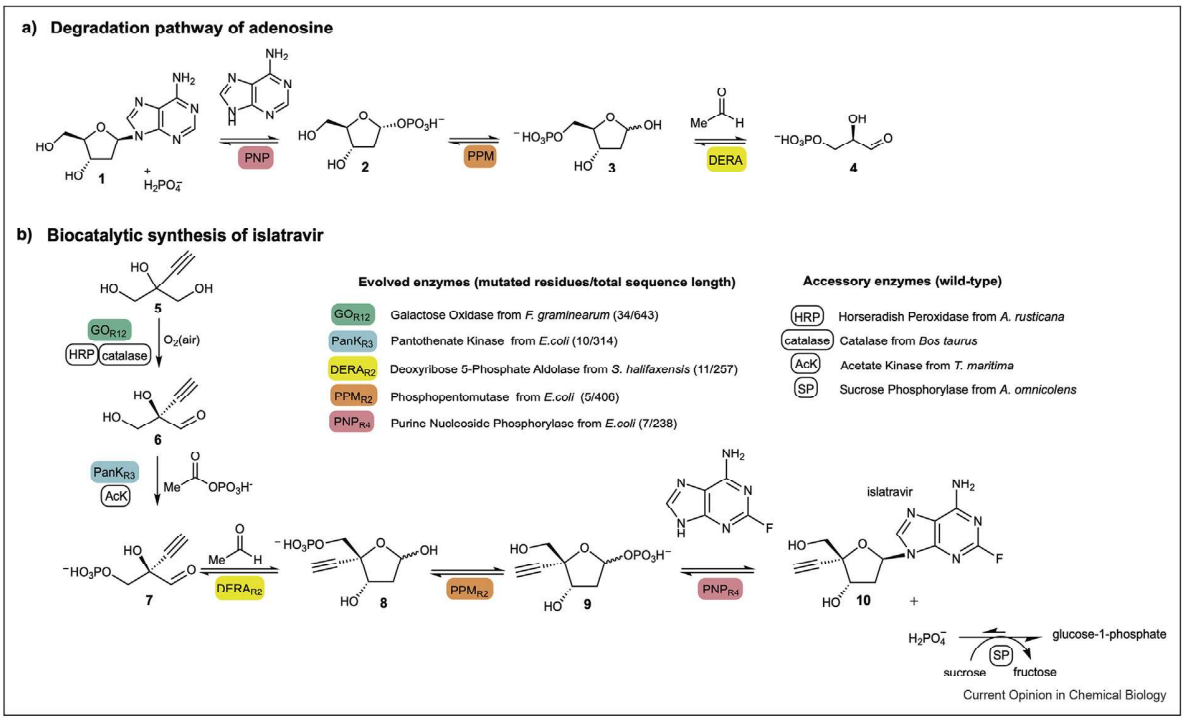
different strategies: direct assays will use "true" substrate and aim at directly monitoring the formation of the desired product, while indirect methods will rely on a signal developed as a consequence of the enzyme turnover (e.g., pH change, release of a fluorophore, etc.), and may require a cascade reaction or the use of a surrogate substrate. For enzymes catalyzing reversible reactions, screening assays may also be designed on monitoring the reverse reaction relative to the final objective.

Although direct screening based on common analytical techniques have a comparably low throughput, remarkable achievements have been reported. Noteworthy, Yang and coworkers have assembled a biocatalytic cascade enabling the synthesis of the anti-HIV islatravir with nine enzymes and starting from the simple building block 2-ethynylglycerol [23]. Five enzymes were evolved using a semi-rational approach combining in silico computational studies with library screening. Elegantly, a bacterial nucleoside salvage pathway was fully reverted into a biosynthetic route for islatravir (Figure 3) with a drastically reduced number of steps and excellent enantioselectivity. Arnold and co-workers also made extensive use of HPLC- and GC-based assays. The 2018 Chemistry Nobel Prize is very well known for the evolution of hemoprotein catalysts towards non-natural chemistries [24]. To name a few, impressive contributions to the field include the development of biocatalysts for the aminohydroxylation of olefins, for the amination of C(sp^3)-H bonds to yield chiral amines and for the production of heteroatom-substituted cyclopropanes [25–27].

While the higher reliability of these instrumental analyses compared with indirect assay methods is a major advantage, laborious sample workup, and an overall longer analysis time may speak against them. Therefore, considerable research efforts are devoted to streamline and simplify workflows. Leferink and co-authors, for example, have developed a completely automated pipeline for the GC–MS screening of monoterpene synthases mutants enabling to monitor up to 4 compounds per run and to screen up to 288 samples per day [28]. Recently, the coupling of microdroplets reactors to mass spectrometry afforded the direct screening of a 96-well plate in only 30s [29]. However, this notable microdroplet-MS screening remains of limited accessibility because of the high equipment cost and the need for a spatial separation of the variants in 96-well plates to allow recovery of the best variants.

Indirect assays cover a broader range of throughput values and do not always require a spatial separation

Figure 3



Striking example of a recent achievement in directed evolution. The native nucleoside salvage pathway (a), and its engineered version (b) for the biocatalytic synthesis of the islatravir, an investigational anti-HIV drug. The first two steps of the biocatalytic cascade (b) have been introduced to access compound 7 from 5, and are not part of the engineered nucleoside salvage pathway. The subscripts R for the evolved enzymes refer to the number of directed evolution rounds. Adapted from Ref. [23].

between variants. Still, it is not uncommon to use a mixed approach to integrate direct and indirect assays, especially when enantioselectivity is the target for evolution. An example is the evolution of the artificial retro-aldolase RA95.5−8 into stereocomplementary catalysts: high-throughput screening was initially performed by monitoring spectrophotometrically the substrate consumption at 350 nm and variants retaining at least 30% of the original activity were then subjected to chiral HPLC analysis [30].

Typical indirect assays are the chromogenic ones. Reactions that consume or release H_2O_2 are particularly interesting since H_2O_2 sensing using HRP is well established with several chromogenic substrates available. Recent applications concern the evolution of P450 fatty acid decarboxylases whose mutants could be screened via H_2O_2 sensing in a catalase-deficient host [31]. Similarly, the HRP-aided evolution of D-glycerate dehydratase has been reported. In this case, the product of reaction, pyruvate, was subjected to a pyruvate oxidase reaction releasing H_2O_2 that was detected via HRP-dependent conversion of a redox substrate (DA-64) into Bindschedler's green [32]. A pre-treatment of lysates was introduced to overcome the back reduction of Bindschedler's green by NADH.

Oxygen sensors also begin to be evaluated for the screening of oxygen-consuming enzymes (e.g., naphthalene dioxygenases) with promising first results [33].

For transaminases, a cascade assay based on the conversion of the amine donor 2-aminoethylaniline into indole and its subsequent colorimetric detection with the Ehrlich's reagent has been recently proposed [34]. The assay could be exploited for the screening of both amine donors and acceptors, and it was applied in liquid and solid format in a tryptophanase-deficient strain to avoid endogenous production of indole.

In the field of lipase engineering, the *Drosophila melanogaster* Lip3 has been recently evolved toward improved activity and solubility: a primary screening was performed on solid agar supplemented with 2% glyceryl trioctanoate and the colonies forming halos were used in a secondary screening in liquid format using p-nitrophenyl octanoate as chromogenic substrate [35].

Even a pH change specifically associated to the progress of a reaction can be exploited for the screening of a library. Using bromothymol blue, Gul and co-authors have developed an assay for the assessment of halohydrin dehalogenase mutants [36].

Among all, however, fluorescence-based assays are the most sensitive and may offer a wide range of screening options. An example of fluorescent, plate-based assay is the Suzuki−Miyaura cross-coupling developed for the screening of thermostable halogenase variants retaining their capacity to brominate tryptophan [37]. Phytase variants with improved pH tolerance were identified in continuous-mode screening using 4-methylumbelliferyl phosphate as fluorogenic substrate [38]. Aldehyde-forming enzymes, like the carboxylic acid reductase, can benefit from a novel cascade assay based on the chemoselective aldehyde-dependent cyclization of 2-amino-benzamidoxime derivatives to yield fluorescent dihydroquinazoline derivatives that can be easily detected down to the nanomolar range [39].

Screening assays were greatly improved, both in terms of throughput and costs, by combining fluorescence detection to miniaturized water-in-oil microdroplet reactors [40,41]. Stabilized by surfactants, such droplets can contain a single library variant on a nanoliter to picolitre scale, implying that the reagents consumed for the screening of thousands or even millions of clones, become almost negligible while the throughput can reach millions of droplets per hour. Compared with 96-well plates, however, hydrophilicity of enzyme substrate and reactants must be sufficiently high to avoid preferential solubilization in the oil phase [42].

An impressive achievement in this field is reported by Hilvert and co-workers about the fluorescence-activated droplet sorting supported evolution of cyclohexylamine oxidase (CHAO, a FAD-dependent enzyme) [43]. A library of 1.7×10^6 variants was expressed in *E. coli* and single cells were then co-compartmentalized with lysis reagents, substrate, and the reporter cascade, consisting of HRP and Amplex ultrared. Droplet sorting afforded the identification of a CHAO variant with a 960-fold improved catalytic activity in less than 2 weeks.

To avoid cell lysis and facilitate the recovery of clones of interest after droplet sorting, Bornberg-Bauer et al. combined the autodisplay of a sulfatase in *E. coli* with single cell encapsulation in microdroplets containing a fluorogenic substrate to improve the activity (30-fold increase) toward a disulphate [20].

If the desired activity of an enzyme is producing or can be coupled to the production of an amino acid, an interesting strategy to generate fluorescence is the activity-fed translation assay. In a proof-of-concept case, Soumillion and co-workers co-encapsulated a cell-free expression system with two plasmids, one encoding a penicillin acylase and the other encoding GFP. A small amount of methionine would initiate the expression of the acylase that will convert phenylacetyl methionine into methionine, thus activating the translation of both the enzyme and the GFP [44]. This amplification system allowed the detection of activity in droplets containing a single enzyme-encoding plasmid.

Bypassing microdroplet encapsulation while keeping high-throughput capacity may be possible when the fluorescent end-up product can be somehow associated to expression host. Vanella and co-authors used the activity of a yeast-displayed glucose oxidase to initiate polymerization of a fluorescent hydrogel around the yeast cell itself. Using macromonomers of alginate modified with aminofluorescein and tyramine, active variants producing hydrogen peroxide triggered an HRP-dependent radical reticulation of the alginate by tyramine crosslinking, resulting in the fluorescent encapsulation. Simple FACS-sorting afforded an overall screening throughput of 5×10^6 cells per hour [21].

Conclusion

With the growing quest for greener technologies using tailor-made biocatalysts, we expect that the coming years will witness an ever-growing development of directed evolution campaigns that will take advantage of smart library design combined with modern molecular biology to reduce the genetic diversity while increasing its quality and accelerating its assembly. The contribution of in silico methods is expected to become more and more relevant and is paving the way to automated workflows. On the other hand, problematic aspects of the most innovative screening technologies may be addressed enabling their regular use to evaluate in short-time large libraries. Even though areas like the pharma industry will most likely stick to classical analytical techniques, perhaps under novel high-throughput configurations, a leading role in the setup of novel assays will definitely come from fluorescence applications, most likely coupled to microfluidic technologies, as it is already happening.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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