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Reviews

Directed Evolution of Biocatalysts

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Abstract:

Biocatalysis is the activity to synthesize chemicals using biological entities, in particular enzymes. Natural enzymes often need to be optimized before being used in industrial processes, and directed evolution is an approach that is well suited for the creation of these improved industrial biocatalysts. Directed evolution involves creating a library of mutant genes and then sorting them based on the associated phenotypes. Main library creation and sorting protocols are compared here, with an emphasis on semirational approaches to library creation and fast screening or pool selection techniques. Representative reports of improved catalytic activity, enzyme stability, and specificity are discussed. Finally, an insight is given on recent technological developments to make enzymes more suitable for biocatalysis. These new technologies could favor the switch of numerous processes from chemistry to biocatalysis soon.

Introduction

The synthesis of new chemicals requires the design and implementation of complex production processes. Two options are generally possible when setting up a process for the synthesis of a new compound: chemistry or biocatalysis. Biocatalysis is defined as the use of biological entities, either purified enzymes or whole cells, to synthesize chemicals. Rewiring cells for the purpose of synthesizing chemicals is generally known as "metabolic engineering" and is not discussed here.[1,2] This review focuses on recombinant enzymes and on ways to make them fit the constraints of the production processes.

Many biocatalysis processes have a lower impact on environment than their chemical equivalents. The operation costs, purity of products, and synthesis of otherwise inaccessible chemicals can all be a motivation to opt for biocatalysis. The chirality of biomolecules also makes biocatalysis particularly suitable in the synthesis of chiral compounds.[3−5] But biocatalysis has some limitations, mainly in terms of the development time scale which is usually longer than that for chemical processes.

The first step in setting up a new biocatalysis process consists of searching among commercially available enzymes for those that might fulfill the technical requirements. If such enzymes are available, a large part of the work is already accomplished: the selected enzymes remain to be tested for the required activity and evaluated in the context of the process conditions.

In many cases, no suitable enzyme is commercially available to perform the target reaction. It is then necessary to identify an enzyme (from literature and database analysis, strain screening, or metagenomics), isolate the corresponding gene, express it in a production host, and eventually purify the protein. This upstream research, and in particular the expression and purification steps, can involve a costly and time-consuming process.

These natural enzymes (either commercially available or specifically isolated from nature) do not usually perform efficiently under harsh process conditions: the high concentration of substrates and products are often inhibitory to natural enzymes, and nonaqueous solvents, extreme pH, and high temperature are often required. One solution is to adapt enzyme formulation for example by adding surfactants in biphasic reactors or using immobilized enzymes.[6] Another option is to make use of enzymes isolated from extremophiles, microorganisms living at extreme temperature, pressure, pH, or osmolarity.[7] A third route, which we will discuss here, consists of generating enzyme variants which are more efficient in catalysing the reaction in the specific conditions of the industrial process. This procedure is referred to as "directed evolution".[8,9]

Directed evolution or "Darwinian evolution in the test tube" refers to a set of techniques that were first conceived two decades ago and are inspired by natural evolution.[10,11] Directed evolution is an ensemble of technologies aiming at optimizing existing biomolecules or creating new ones by first constructing a diversity of mutant genes and then sorting

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Table 1. **Main library creation technologies**

	error-prone PCR	saturation mutagenesis	massive mutagenesis	gene shuffling	synthetic shuffling
need for physical starting gene	one gene	one gene	one gene	several genes	no gene
large diversity/low cost per mutant ?	yes	no	yes	yes	yes
control over the diversity generated	very little (mutation rate)	complete	complete	little (starting sequences)	complete
need for double strand cloning	yes	yes/no (different technologies)	no	yes	yes

them based on their corresponding phenotype. Mutations associated with improvements are a rare event, and generating these positive mutants is not an easy task: a new domain has emerged at the interface of Science and Industry. The object of this review is to give an up-to-date summary of the different approaches that coexist to tailor enzymes having acquired new properties.

1. Begin by Generating Diversity

Various technologies exist to generate a diversity of enzyme mutants. Table 1 compares common library creation procedures that are discussed in detail in this section.

1.1. Rational Design Using Site-Directed Mutagenesis. Site-directed mutagenesis is the basic tool of protein engineering. It allows researchers to modify at will a handful of residues and has been a critical tool in understanding the role of individual residues of proteins. Various techniques have been described, yet most site-directed mutants are generated today using Stratagene's Quick Change protocol. Some success has been obtained by rationally designing several mutations that were confirmed as carrying the sought after improvement.[12,13]

Impressively, Dwyer and colleagues have reported that they succeeded, using such a rational design approach, in modifying a receptor protein so it now harbours an enzymatic activity.[14] This result is considered as opening the way for the generation of any enzyme activity *ex nihilo* and as the starting point of a new era for directed evolution.

However, such a success in designing an enzyme activity *de novo* has not been reproduced to date. In general, it remains difficult to predict the effect of a particular mutation on enzyme parameters, even when the 3D-structure is known. Therefore, success using a mutagenesis strategy based on rational design remains an exception.

To enhance the productivity of the mutagenesis process, and thus to increase the probability of finding positive mutations, many groups have used saturation mutagenesis (Figure 1). Saturation mutagenesis is basically a site-directed mutagenesis protocol adapted to the use of degenerate oligonucleotides (NNN or NNK mutagenic cassettes, with N = A, T, G, C and K = G, T for instance) to introduce a full diversity (the 20 amino acids) at a given position. Since its first description, several different technologies to generate saturation mutants have been described.[15−18] Saturation mutagenesis has proven useful to improve several enzymes. In a recent example, saturation mutagenesis was used to increase the thermostability of a bacterial phytase, for use as an animal food supplement.[19]

1.2. Random Mutagenesis Using Error-Prone PCR. To expedite the creation of sizable libraries, researchers usually turn to random mutagenesis. Although UV, chemical mutagens, or mutator bacterial strains can be used, the most common and convenient random mutagenesis technique is now based on error-prone PCR.[20,21] A starting gene is amplified over a million fold in an imperfect copying process that generates uncontrolled errors. The technique is a variation of standard PCR using unbalanced deoxyribonucleotides concentrations, high Mg^{2+} concentration, Mn^{2+}, low annealing temperatures, or a high number of cycles which are all error-triggering factors. When varying these factors, alone or in combination, the amplified DNA differs from the starting gene by a small number of point mutations.

Randomized libraries containing up to 10^{11} variants can be generated and used in selection processes not involving double-strand cloning steps, such as ribosome display. When the mutated genes need to be inserted in a plasmid vector for expression, which is the usual case, the diversity is usually limited to 10^7 individual clones.[22] The position and nature of the mutations are not controlled and are spread over the amplified sequence.

It should, however, be noted that the technique is actually far from random and suffers from a number of biases. Beyond differences in the occurrence of base substitutions (with transitions generally favoured over transversions), the most important bias stems from the very low probability of two or three substitutions occurring next to each other. This implies that starting from an AAA codon, codons such as

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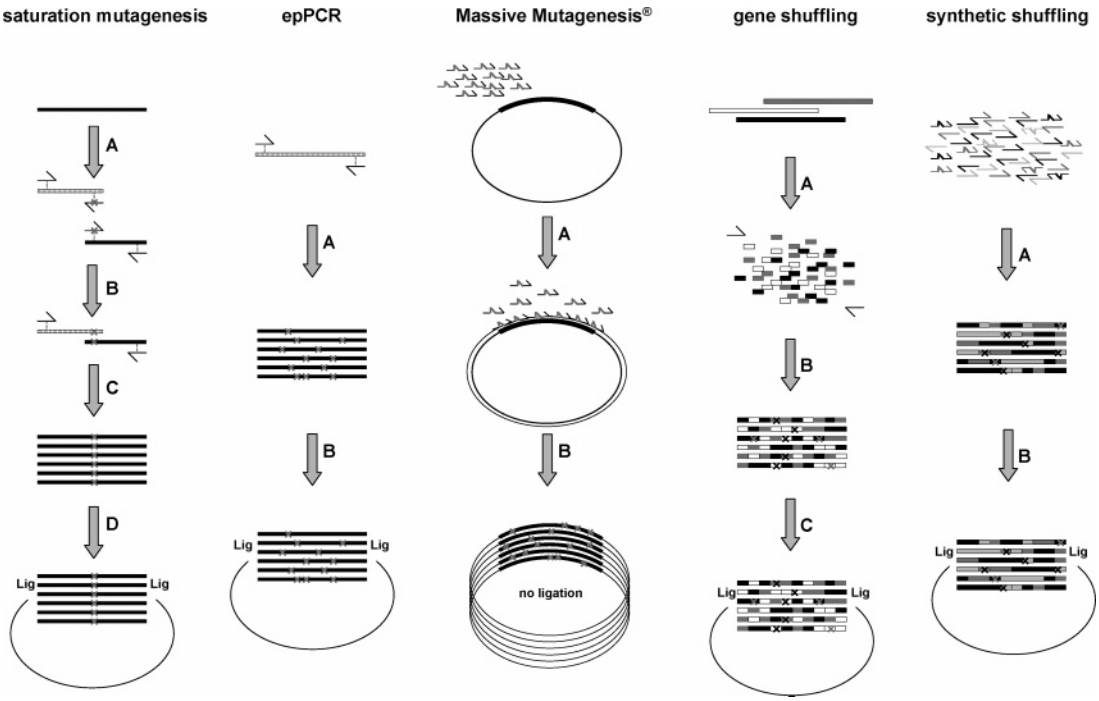

Figure 1. **Main techniques for library creation. Saturation mutagenesis: (A) degenerated codons introduced by PCR; (B) overlap PCR assembly; (C) set of degenerated gene fragments; (D) cloning into an expression vector. Error-prone PCR: (A) gene amplification under error-triggering conditions; (B) set of mutated gene fragments; (C) cloning into an expression vector. Massive Mutagenesis: (A) combinatorial incorporation of a set of mutagenic primers on a circular plasmid template; (B) specific degradation of the parental DNA strand (dotted circle). Gene shuffling: (A) DNAseI fragmentation of parental genes; (B) assembly of recombined genes using outer primers; (C) cloning into an expression vector. Synthetic shuffling: (A) synthetic assembly of oligonucleotides; (B) cloning into an expression vector.**

ATA, GAA, AAC will be obtained by random mutagenesis, whereas codons such as CCC or TAG will almost never be obtained. Instead of generating 63 new codons encoding the full range of 19 amino acids, random mutagenesis produces only 9 new codons, encoding 5 to 6 amino acids in average. Thus, about two-thirds of the possible diversity at each position will never be accessible using random mutagenesis. An extensive statistical analysis of 19 random mutagenesis methods demonstrates that all these methods fall far from exploring a large fraction of the sequence space and emphasizes the need for more efficient methods to generate diversity.[23] However, since the first description of error incorporation during PCR amplification, many successes have been reported using this easy to handle approach. Chen and Arnold were among the first to use error prone PCR to improve enzymes. As an early example, subtilisin E variants displaying a high activity in dimethylformamide were obtained using such methodology.[11] Since then, different parameters of various biocatalysts have been successfully modified using this technology: catalytic activity, activity in organic media, thermostability, specificity, enantioselectivity, etc.[24−29]

Random mutagenesis, the first technology to allow the efficient generation of large diversity, is therefore still in use in many laboratories. Some of its limitations, like the incompleteness of the diversity introduced, can be overcome in some cases by using a combination of technologies: Random mutagenesis is now commonly used in combination with saturation mutagenesis or gene shuffling.[30−32]

1.3. Gene Shuffling. Gene shuffling is a term which covers different diversity generation technologies, based on the recombination of homologous genes harvested from nature. The rationale is that the parental genes have been preselected by natural evolution as functional; hence their progeny has a good chance of containing improved genes due to additive or synergistic combinations.

Various *in vitro* gene shuffling techniques have been described. Most of these include a fragmentation step of the parental genes followed by the random reassembly of parental gene segments.[33] By contrast, the staggered extension process (StEP) is an amplification reaction with very short elongation cycles, resulting in frequent template shifting during elongation, which generates recombined amplicons.[34] All these approaches are based on sequence complementarity and require a high degree of homology between the parental

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sequences, thus limiting the scope of these techniques to well-known gene families. Other sophisticated gene shuffling techniques have recently been described and allow both the recombination of nonhomologous genes and the integration of structural features into the strategy.[35]

The limited access to natural diversity is an obvious limitation to gene shuffling procedures. Microorganisms found in nature and living under an immense variety of conditions can constitute a reservoir of new, potentially useful biocatalysts which can then be shuffled. However, only a minority (less than 1%) of these organisms can be cultivated in laboratory conditions. Technologies to increase this percentage of cultivable microorganisms by using microcapsules or microplates are presently being developed.[36,37]

An alternative method is metagenomics, which is a set of techniques that can tap into natural genetic diversity without the need of cultivating the microorganisms.[38] DNA is harvested and amplified from environmental vials using different methods and cloned in plasmid vectors. By using either a nucleotide sequence-based filter when cloning or an enzyme activity-based selection after cloning, metagenomics allows the direct harvest of genes encoding biocatalysts from a gram of soil, a litre of seawater, the intestine flora, or any other natural environment. Shotgun sequencing of a given environmental spot brings crucial information about the organisms this medium contains, and it can also reveal interesting candidate sequences.[39] Recently, a strategy called substrate-induced gene expression screening has been proposed to isolate catabolic operons or genes by fluorescence activated cell sorting.[40]

Since the first description of gene shuffling, many groups have demonstrated its power in enzyme evolution.[33] The major limitation of such template-based recombination processes remains the access to a collection of parental genes.

1.4. Generation of Diversity by *de Novo* Gene Synthesis. To bypass the requirement of access to natural DNA, techniques to create diversity without any starting gene have been proposed.[41] These techniques rely on the concatenation of a large number of variable oligonucleotides to synthesize *de novo* the gene library. This approach opens the way to the generation of custom-tailored diversity: one can introduce structural information or natural polymorphisms, identified from databases. Moreover, when a very large number of mutations per molecule is sought (over 10), this approach appears superior to any other, as the mutations are not introduced through a recursive process but in a single synthesis step.

But mutations due to errors in oligonucleotide synthesis constitute a major limitation in all these template-independent gene synthesis techniques. The error rate of oligonucleotide synthesis is often superior to 2 per kb, and errors are most frequently single base deletions or insertions, thus resulting in frameshifted sequences. Thus, with the exception of very small genes, the library will encode a large majority of nonfunctional, truncated proteins. Indeed, in one example, where diversity was introduced by gene synthesis of a 1.5 kb DNA fragment, only 20% of the resulting muteins had retained some activity, compared to 75% obtained by standard gene shuffling.[42]

1.5. Larger and Smarter Libraries: Massive Mutagenesis. Massive mutagenesis was first developed in the years 1999 and 2000 to give access to mutant libraries of unprecedented quality. Massive mutagenesis allows the generation of libraries composed of combinatorial site-directed mutants.[43] Figure 2 summarises the massive mutagenesis protocol, a template-based process starting with a plasmid containing the gene of interest and a mixture of a large number of mutagenic phosphorylated oligonucleotides.

Conceptually simple and experimentally robust, massive mutagenesis harbors a number of interesting properties. Any desired mutation can be accommodated, including all substitutions, deletions as well as insertions, just by adapting the oligonucleotide design. The frequency of single, double, or multiple mutants approximately follow a binomial distribution. The average number of mutations per clone can be finely tuned thus allowing an appropriate fitting to the plasticity of the protein of interest. Standard plasmids of up to 12kb, containing genes of up to 8kb, have been successfully mutated using massive mutagenesis. No labor-intensive and sequence-dependent cloning step is involved, which is a major simplification over all techniques that mutate a linear DNA. The size of the diversity is only limited by the efficiency of transformation, and high quality libraries of 10^7 to 10^9 can be routinely generated in a matter of days when working with high transformation yield plasmids. The process is template-based, accounting for the high quality of massive mutagenesis derived libraries, and less than 5% of the clones in the final library are inactive because of unexpected frameshifting mutations.

In large-scale mutagenesis projects, thousands of different oligonucleotides can be required. These oligonucleotides are usually synthesized individually and later mixed in equimolar amounts before including them in the amplification reaction. They are never needed individually though, and some parallelization is therefore suitable. We have recently shown that the use of chip-synthesized and eluted oligonucleotide mixtures could reduce the cost of large-scale mutagenesis projects while generating libraries with higher quality.[44]

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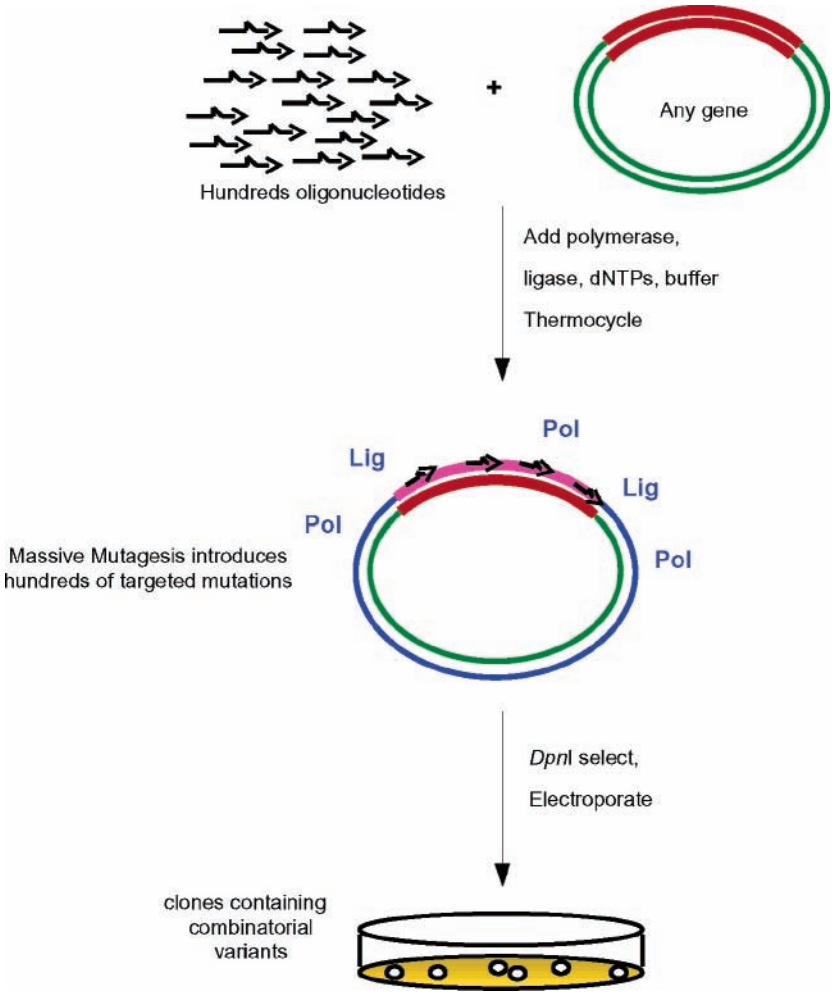

Figure 2. **Massive mutagenesis. Using a number of mutagenic oligonucleotides and a single-strand circular amplification reaction by a DNA polymerase and a DNA ligase, massive mutagenesis produces large libraries quickly, containing any custom-required combinatorial diversity.**

Contrary to random mutagenesis techniques, massive mutagenesis allows the targeting of precise mutations. Thus all relevant information concerning available structures, sequences, or models of the target enzyme can be incorporated while quickly producing very large libraries and therefore efficiently sampling vast regions of the sequence space. This technology allows, for example, combining precise substitutions, deletions, or insertions in catalytic site residues or in surface residues. Partial (e.g., with amino acids of the same class) or complete saturation can easily be introduced at any position.

Massive mutagenesis has limitations when wishing to introduce adjacent mutations (that can be mutually exclusive) or when targeting very small DNA regions: direct synthesis from pools of oligonucleotides is more adaptable in these cases.

2. Continue by Screening or Selecting To Isolate Positive Mutants

Once a library has been created, the next step is to isolate those positive mutants hiding in it. Because only a very small minority of the variants harbor the sought after improvements, a large number must be tested. The main techniques are shown in Figure 3 and are detailed in the following sections.

2.1. High-Throughput Screening. The simplest way to find improved enzymes is to assay variants one by one, by individual screening. In general, when using libraries of a high quality, the frequency of positive mutants exceeds $1/10^4$. Individual screening usually provides quantitative and precise data; the drawback is that it requires the design of an appropriate small-volume assay and involves some level of automation (colony picker, automatic liquid handlers).[45] 96-well plates are the basic screening format. 384 or 1536-well plates can sometimes be used instead; yet surface effects and evaporation must be taken into account when handling small volumes. In a move towards higher densities, microarray assays have recently been described.[46] Ideally, the assay must be quantitative, fast, cheap, sensitive, robust, and highly reproducible. Current high-throughput screening assays usually employ coloured or fluorescent substrates or products. This kind of assay does not provide complete information

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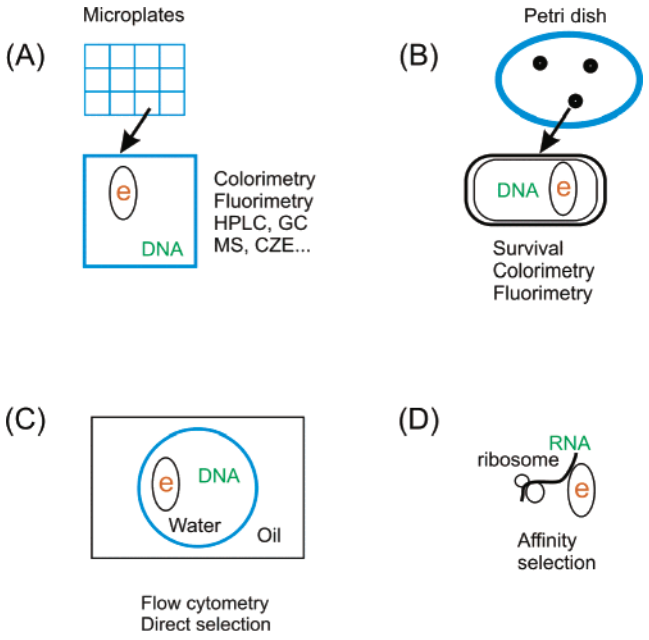

Figure 3. **Screening and selection methods to improve biocatalysts. (A) Individual screening assesses a large number of proteins one by one using microplates and automated equipment. (B) In vivo selection techniques: genotype and phenotype are linked in cells, and the catalytic activity of the enzyme of interest is detected from the survival, color, or fluorescence of the cells. (C) In vitro selection by compartmentalization. Microreactors such as oil droplets in inverse microemulsion replace cells and allow for an intermolecular, multiple turnover selection mode. (D) In vitro selection by ribosome display: a stable complex is formed among a nucleic acid (mRNA), a ribosome, and the encoded protein. Display techniques are inherently better suited to select for binding than catalytic activity.**

on the reaction, especially when surrogate substrates are used. In practice, a primary screen yields a limited number of candidates, including a high frequency of false positives, which are subsequently characterised in more detail.

Secondary screening is not a high-throughput activity: only a limited number of clones are tested. Once the false positives have been discarded, the enzymatic parameters of the selected candidates can be determined using the fluorescence or colorimetry assay already used for primary screening. Analytical techniques, such as liquid or gas chromatography, mass spectrometry, nuclear magnetic resonance, or capillary electrophoresis can also be used. The utilization of surface enhanced resonance Raman scattering (SERRS) has also been proposed.[47] These approaches are not specific to a particular system and are thus widely applicable. As they become cheaper and faster, these direct and general technologies should soon be used for the primary screen as well.

In the case of hydrolytic enzymes, which are a major class of biocatalysts with high commercial value, a large number of specific assays have been developed.[48] A range of colorimetric and fluorimetric assays use *p*-nitrophenyl, *p*-nitroanilide, or umbelliferyl derivatised substrates, such as 2-methyl decanoate *p*-nitrophenyl ester for lipases and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for proteases. Lipases and esterases can also be used as coupling enzymes: synthetic activity detection is then based on the *in situ* derivatization of acetaldehyde with a particular hydrazine yielding a fluorescent hydrazone.

Indirect assays can be used when specific substrates are not available for activity determination. For instance, acetic acid assays are based on the hydrolysis of an acetate which releases acetic acid. The hydrolysis is then quantified by measuring NADH increase spectrophotometrically at 340 nm after a cascade of reactions. "Adrenaline test" endpoint assays rely on the principle of back-titration. A periodate-resistant substrate is hydrolyzed by the enzyme yielding a periodate-sensitive product which will be oxidised. The activity level is proportional to the decrease in the deep red-colored adrenochrome formed by oxidation of adrenaline by residual periodate. This assay has been used recently to screen epoxyde hydrolases.[49]

To overcome the limitations associated with specific assays and individual high-throughput screening, the ideal solution would be to have *en masse* universal selection techniques allowing the direct isolation of positive mutants from pools.

2.2. Direct Selection from Mutant Libraries. Selection techniques were first developed to identify binders of a target ligand and are progressively being tailored to direct the evolution of enzymes. These techniques are based on the establishment of a link between each genotype (an easy-to-amplify nucleic acid sequence) and the phenotype it encodes (carried by the protein of interest).

2.2.1. Display Technologies. Protein display technologies have encountered outstanding success by allowing the quick identification of high affinity binders toward virtually any identified ligand. The link between the nucleic acid and the protein it encodes is at the molecular level. In ribosome display, a stable complex is formed between a messenger RNA and the enzyme it encodes during *in vitro* translation. mRNA-peptide fusion is conceptually very similar.[50] In other approaches the protein is expressed at the surface of an organism: in phage display, the protein is expressed in fusion with a coat protein of a filamentous phage, exposing the protein at the surface of the phage while the gene is trapped inside,[51,52] while, in cell-surface display, the enzyme is expressed on the outer surface of a bacterial or yeast cell.[53]

These display techniques are inherently adapted to a selection for binding, with many successes in the field of recombinant antibodies, in particular directed against tumoral markers. Several of these antibodies are already on the market, creating a new frontline in the battle against cancer. Several adaptations have been proposed to assay catalytic activities, for example by using transition state-analogues

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or suicide inhibitors. However no general approach has yet emerged.[54−56]

2.2.2. Direct Selection by Compartmentalization. Another way to achieve a link between the genotype and the phenotype is to use compartmentalization. Here, the mutant DNA and its associated proteins are individually gathered in a closed microcompartment. This approach has the additional advantage of linking, not only the enzyme and the gene but also the product of the enzymatic reaction. Compartmentalization is thus more adaptable to enzyme evolution than display technologies.

Natural cells are obvious compartments; when accessible, *in vivo* selection for survival can be a powerful and simple solution. Ideally, under appropriate culture conditions, a bacterium will grow if the plasmid it carries encodes an improved enzyme. A simple solution consists of generating a bacterial strain deficient in the enzyme of interest; expressing the enzyme on a plasmid will correct this deficiency. Under finely tuned selective pressure, only mutants with improved activity will allow the growth of the bacterial colony.[57] This approach is however limited by the need to build a specific auxotrophic strain prior to directing the evolution of the enzyme. As many genes are essential for bacterial metabolism, such strain construction is only possible in a minority of cases.

When such a life-or-death assay cannot be setup, another solution is to design an *in vivo* assay: the enzyme activity produces coloured or fluorescent cells, which can later be sorted by simple evaluation by eye of bacterial colonies. *In vivo* selections cannot be conceived for all enzymes or all properties though, in particular when the targeted substrate is not able to cross the bacterial membrane or when the product quickly diffuses outside of the cell.

To overcome this limitation, many efforts are currently invested in *in vitro* compartmentalization. *In vitro* compartmentalization uses water droplets of water-in-oil emulsions (or water droplets inside the oil globules of W/O/W double emulsions) as microreactors.[58] A library of genes and other necessary biochemical components is emulsified in such a way that the vast majority of droplets contain either one or zero gene. The genes are *in vitro* transcribed and translated inside the emulsion droplets. The enzymatic reaction that is assayed later occurs in the same droplet where the genotype and phenotype coexist. A 1 μL aliquot of emulsion can contain around 10^{10} droplets, and very large library sizes are thus easily accommodated. In particular, direct selection can be used in the case of enzymes that directly or indirectly act on DNA, such as polymerases.[59] In most other cases, the droplets must still be screened one by one. However, since fluorescence-activated cell sorters can be used, throughput can reach up to tens of million molecules per day, close to the efficiency of direct selection techniques.[60−62] Integrated microfluidic devices, allowing controlled droplet merging, splitting, testing, and sorting, hold much promise for the future: they could constitute a generic intermediate solution between screening and selection.[63]

As a possible alternative for selection of catalytic activity, the small-molecule yeast three-hybrid assay is the basis of a general approach termed "chemical complementation". This method potentially applies to bond formation or cleavage reactions. In this system, the binding of the substrate to a receptor is linked to the transcription of a reporter gene. This reporter gene is expressed through either the formation or the cleavage of a heterodimeric small molecule bridge between the DNA-binding domain/receptor fusion protein and an activation domain/receptor fusion protein. Although the principle underlying chemical complementation is quite general, one has to chemically synthesize a special bridging substrate or product for each new reaction that is selected.[64]

Directed evolution of enzymes is still at the beginning of the direct selection era, and robots for individual high-throughput screening are still in operation night and day. Generating robust and innovative direct selection systems will help reduce the development times of process enzymes, thus pushing the switch from chemistry to biocatalysis.

3. A Sample of Biocatalysts Improved by Directed Evolution

Various enzyme properties can be modified to adapt biocatalysts to industrial use. To illustrate this assertion, a number of examples are provided below in which enzyme catalytic activity, stability, or (stereo)specificity are targeted.

3.1. Increased Catalytic Activity. Increasing the activity of a biocatalyst is one of the main objectives of directed evolution in the domain of biocatalysis. An increased catalytic activity (k_{cat}) will allow the reaction to occur quicker and/or with a reduced quantity of enzyme; a reduced k_m means higher product purity: at the end of the reaction, substrate conversion continues to occur efficiently even if the substrate concentration is low.

For example, starting with a library of five randomised codons and using a powerful selection method, the k_{cat} of a phosphotriesterase has been multiplied by a factor of 63, making it the fastest hydrolase and one of the fastest ever enzymes.[65] *In vitro* compartmentalization has also been utilized to select ribozymes catalyzing multiple-turnover Diels−Alders cycloadditions from very large random RNA libraries.[66]

Semirational strategies have also been successful. Massive mutagenesis has been used to increase the specific activity of a hydrolase used in a chemical manufacturing process by

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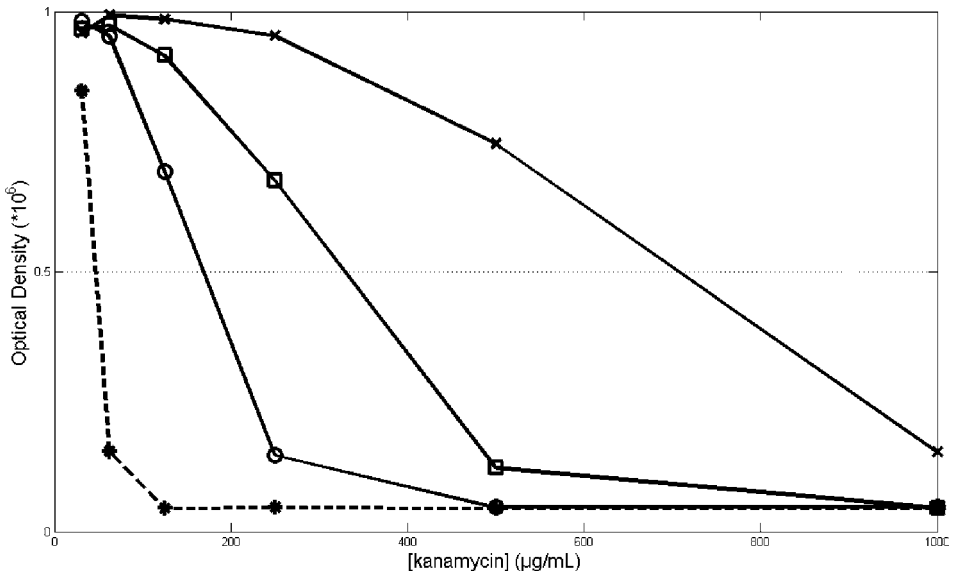

Figure 4. **Improved mutants of the APH gene. Optical densities of cultures with varying kanamycin concentrations are plotted for wild-type APH (*, dotted), the Q115W mutant (○), the S114H mutant (□), and the (Q115W, S114H) double mutant (×). The cultures were diluted 10^6 times before OD measurement. The Q115W and (Q115W, S114H) variants were *in vivo* selected as described in the text. As a control, the S114H variant was obtained separately by site-directed mutagenesis of wild-type APH.**

a factor of 60 (unpublished data). To illustrate the benefit of employing massive mutagenesis and an efficient selection system, we will now describe the improvement of the activity of aminoglycoside-3′-phospho-transferase-IIa (APH), which provides resistance to kanamycin. Kanamycin is an antibiotic of the aminoglycoside family, commonly used for research and medicine. All the residues of the APH gene were targeted, with the objective of generating each single mutant, most of the possible double mutants, and some mutants with a higher number of mutations. After three rounds of massive mutagenesis, 10 million clones were harvested, with an average of 2.5 mutations per clone, as determined from the sequencing of a sample of 48 individual clones. The library was transformed into *E. coli* strain BL21(DE3), and around one million independent clones were spread on LB agar plates containing different kanamycin concentrations (50, 100, and 300 μg/mL). At 300 μg/mL bacteria transformed by the plasmid containing the wild-type APH gene were unable to grow; however, approximately 100 colonies were obtained when the mutant library was transformed under the same conditions. To further confirm the ability of the selected mutants to grow at higher kanamycin concentrations, clones were picked and grown individually in liquid conditions at different kanamycin concentrations. EC50 was defined as the kanamycin concentration corresponding to an optical density equal to half that of wild-type APH in the absence of kanamycin. In particular, the Q155W mutant showed an EC50 3.5 times superior to that of wild-type APH (see Figure 4). It is important to note that this substitution (CAG to TGG) had almost no chance of being obtained using random mutagenesis by error-prone PCR, as two consecutive nucleotides are mutated. Neither could this mutation be obtained by gene shuffling: a database search shows that tryptophan is never found at position 155 among APH homologues. This substitution appears to be located on a flexible loop close to the active site (see Figure 5). This residue could then, arguably, have been targeted in a rational design project. This improved clone was subsequently used to create a secondary library from which a further improved mutant (Q155W, S114H) was isolated. This double mutant has an EC50 equal to 14.4 times that of wild-type APH. Again, the mutation (TCC to CAC) would not have occurred had error-prone PCR been used, and again, no homologue genes harbouring a histidine at position 114 were found, thus eliminating gene shuffling as an alternative route. Moreover, this particular mutation could not have been predicted, as position 114 is located very far from the active site of the enzyme (Figure 5). This double mutant associated with an enhanced resistance to kanamycin is a potential tool for molecular biology. First, a new generation of plasmid vectors carrying a higher resistance to kanamycin could be generated for research applications. Second, this improved mutant can be used as

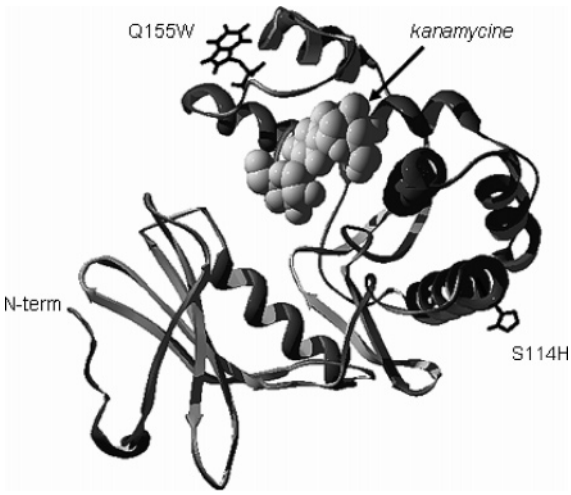

Figure 5. **Structural representation of the positive mutations obtained from the APH gene. The molecular surface of APH is calculated with a Swiss PDB Viewer. Mutations at positions 114 and 155 are indicated.**

a more sensitive sensor to select expressible variants of difficult-to-express protein, using a selection system based on protein fusion and comparable to the ones already described and based on the use of GFP.[67] This new, highly sensitive sensor has successfully been tested to increase the expression yield of proteins and will find an application in the domain of biocatalysis by allowing the bacterial expression of naturally difficult-to-express enzymes from other origins (fungi, yeast...). Such a bacterial expression system will facilitate the engineering of these enzymes as gene manipulation is less complicated in bacteria. In summary, these results leading to the improvement of the APH enzyme illustrate the relevance of engineering enzymes using a combination of large massive-mutagenesis-generated libraries and an *ad hoc* selection process.

Activity is probably the most targeted parameter in directed evolution. Modifying the specificity of an enzyme is a related activity and consists of directing the activity of an enzyme so it can use non-natural substrates or can produce new products.

3.2. Modified (Stereo)specificity. Enzymes in metabolic pathways often have a narrow specificity for their natural substrates and fail to catalyse reactions involving related and/or synthetic compounds. Broadening substrate tolerance is therefore an enzyme improvement commonly sought in biocatalysis.

Amine oxidases able to deracemize a broad range of racemic chiral amines mixtures were isolated by directed evolution, thereby opening a new route to produce chiral amines, compounds of great interest in organic chemistry.[68,69]

A known glutaryl-7-amino cephalosporanic acid acylase was evolved to accept the natural antibiotic cephalosporin C as a substrate, with potential implications in the production of semisynthetic cephalosporins.[70]

Cytochromes P450 consitute the largest family of enzymatic proteins in higher plants, with thousands of alkane substrates, but none of these enzymes accepts ethane or methane. A cytochrome P450 able to catalyse the direct oxidation of ethane to ethanol using dioxygen was evolved, bringing us closer to P450-catalysed methane oxidation, with major implications in the bioconversion of natural gas to fuels and chemicals.[71]

Added to their practical interest, these kinds of results can help refine existing enzyme classifications, suggest ways to alter specificities, and have interesting theoretical implications on the evolvability of promiscouous protein functions.[72,73]

Enantioselectivity is another form of modulation of enzyme function of prime importance for applied biocatalysis. Biocatalysis here appears as a particularly efficient competitor to chemistry, as biomolecules in general and enzymes in particular are mostly chiral.

The difference between an enzyme activity towards two enantiomeric forms can be exploited for the kinetic resolution of a racemic mixture into a mixture of two chemically and physically distinct and enantiomerically pure products. "Dynamic kinetic resolution" is the resolution of a racemic mixture in combination with *in situ* racemization of the unwanted stereoisomer or enantiomer.

The most seducing application of enzyme enantioselectivity is obviously the direct chiral synthesis by stereoselective conversion of prochiral substrates.

For all these applications, the natural enantioselectivity of enzymes is neither always sufficient nor always in the right orientation. These limitations may be overcome by directed evolution since enhancing and inversing the enantioselectivity of an enzyme are now accessible goals.

Benzoylformate decarboxylase, which catalyses the decarboxylation of benzoylformate and naturally produces benzaldehyde, provides a good example of what can be achieved using directed evolution. The wild-type enzyme harbours a secondary carbon−carbon bond forming activity which can be exploited to synthesize (*S*)-2-hydroxy-1-phenylpropanone from benzaldehyde and acetaldehyde. The enzyme from *Pseudomonas putida* has had this carboligase activity increased more than 5-fold after several rounds of directed evolution.[74] Another example is the isolation of a mutant of *Arthrobacter sp DSM 9971* D-hydantoinase, which was selected to efficiently synthesize 91 mM L-methionine from 100mM D,L-5-(2-methylthioethyl)hydantoin.[32] Much research is presently being conducted to direct the evolution of novel enantioselective enzymes.[75,76]

We anticipate that chiral synthesis will be a field of fast development for biocatalysis in the next decade.

3.3. Enhanced Stability. The low thermostability of natural enzymes is one of the main limitations to their industrial use. High temperature is often desirable in industrial processes because it accelerates the reactions, increases substrate solubility, and reduces the risk of contamination. Moreover, several results tend to show that thermal stability could be a good indicator of enzyme stability in general and hence of the enzyme half-life under various conditions.[77−80] Thus, thermostabilization of enzymes is a major goal of directed evolution.

The fusion temperature of an alpha-amylase isolated from *Bacillus licheniformis* and used in the starch industry has been increased by 13 °C by mutating five surface residues.[81] A triple-mutant esterase from *Rhizopus niveus* used in trans-

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esterification has been selected with an optimal temperature increased by 15 °C while maintaining 80% of wild-type activity.[82]

Thermophilic and hyperthermophilic organisms, mostly bacteria, offer a good source of thermostable enzymes which can be used as a good starting point for further optimisation by directed evolution.[83]

Temperature is not the only chaotropic factor: solvents, oxidants, detergents, etc. also destabilise enzymes. Directed evolution can also be used to stabilize enzymes in order to adapt them to process conditions involving these destabilising elements. For instance, an *Agrobacterium tumefaciens* enzyme used for D-amino acid production has been improved by increasing both its thermostability and its resistance to hydrogen peroxide.[84]

We have recently set up a novel and universal method to directly select thermostable mutants. This new system is based on the in-frame fusion of the protein of interest with a thermostable variant of the kanamycine nucleotydil transferase, used here as a thermostability reporter. This new system is based on the in-frame fusion of the protein of interest with a thermostable variant of kanamycin nucleotidyl transferase, which is used here as a thermostability reporter.[10,85] The selection system is based on the fact that the correct folding at high temperature (i.e thermostability), and hence the activity of this downstream reporter is directly dependent on the folding of the upstream protein of interest under the same conditions. A large library of mutants of the protein of interest is generated, and the subpopulation of mutants displaying an increased thermostability is selected in a single step by a simple transformation in *Thermus thermophilus*, a thermophilic organism. This system can potentially be used to improve the thermostability of any protein. As thermostability can be considered as an indicator of the protein stability in many conditions, this system will be useful in generating new enzymes with an extended half-life in biocatalysis processes.

Conclusion

In many domains a variety of new or improved proteins have been generated by directed evolution, and this approach currently revolutionizes the ways in which new antibodies, next-generation therapeutic proteins, drug targets, or enzymes for various industries are developed.

These successes have led the general opinion that the chemical industry should also benefit from directed evolution methods. These techniques seem indeed particularly suited to meet the growing need for better catalysts and thus fulfill regulatory and environmental requirements:[86] As natural enzymes cannot, in general, be directly used in industrial processes, the optimisation of their properties is very often necessary. The main parameters which need to be optimized are usually activity, stability, and specificity. Successful results have already been obtained in this field, and some examples have been given in this review.

Rationally designing several mutants, generating large random libraries using error-prone PCR, gene shuffling, creating wide or semirational libraries using massive mutagenesis technology are all approaches that have been used with various success in recent years. We think that methods which include some rational information data will be exploited more as our understanding of structure−function relationships of enzymes improves and as new bioinformatic tools are developed which allow the prescreening of mutations by browsing the sequence space.[87−89]

However, the constraints that weigh on the chemical industry are in some ways limiting the expansion of directed evolution in this field of biocatalysis. In particular, the process development time is critical, and biocatalysis is always benchmarked to the equivalent chemical processes. This is particularly true when working on products of pharmaceutical relevance, with schedules being restricted by intellectual property concerns.

A large part of the time necessary to set up a biocatalysis process is due to the directed evolution phase, which remains a tedious activity, requiring time and automated equipment. New technologies are still expected to accelerate developments and cut associated costs. In particular, technologies to directly select positive mutants from pools will allow time and cost reduction. Several methodologies of direct selection oriented toward activity or stability have been recently developed and have been described in this review.

As the technologies to direct the evolution of enzymes continues to mature, the time frames for setting up biocatalysis processes should progressively be reduced. This will progressively modify the balance in favour of biocatalysis. The next decade should see biocatalysis take an increasing place in the chemical industry.

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