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Research review paper

Beyond the outer limits of nature by directed evolution

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ABSTRACT

For more than thirty years, biotechnology has borne witness to the power of directed evolution in designing molecules of industrial relevance. While scientists all over the world discuss the future of molecular evolution, dozens of laboratory-designed products are being released with improved characteristics in terms of turnover rates, substrate scope, catalytic promiscuity or stability. In this review we aim to present the most recent advances in this fascinating research field that are allowing us to surpass the limits of nature and apply newly gained attributes to a range of applications, from gene therapy to novel green processes. The use of directed evolution in non-natural environments, the generation of catalytic promiscuity for non-natural reactions, the insertion of unnatural amino acids into proteins or the creation of unnatural DNA, is described comprehensively, together with the potential applications in bioremediation, biomedicine and in the generation of new bionanomaterials. These successful case studies show us that the limits of directed evolution will be defined by our own imagination, and in some cases, stretching beyond that.

Contents

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1. Introduction

Natural enzymes are highly efficient energetic models that can accelerate chemical reactions up to 10^{19} times, achieving kinetic perfection when the catalytic efficiency (k_{cat}/K_m) is close to the diffusion controlled limit (~10^8–10^9 s^{-1} M^{-1}) (Bar-Even et al., 2011). Unlike chemical catalysts that work under harsh conditions, enzymes show outstanding performance in very mild environments, close to the natural situations in which they typically act (*i.e.* atmospheric pressure, aqueous solution, and at room temperature). Indeed, they are considered as versatile biocatalysts with high regio-, stereo- and chemo-selectivity for tens of industrial, environmental and energy applications within the framework of the green chemistry paradigm (Alcalde et al., 2006). Despite these potential advantages, when we take enzymes out of their natural environment to use them in a defined biotechnology process, they just don't behave as we expect. Given that enzymes are fundamental to natural life, fulfilling key roles in strict metabolic pathways to guarantee cell survival and adaptation, they are simply not used to working in new artificial and often aggressive environments. Essentially, the millions of years of natural selection to which they have been put through have smoothly tuned their properties so that they perform a myriad of different reactions but generally, only for activities or in situations other than those of the synthetic applications we desire to manipulate.

Paradoxically, humankind has been using natural enzymes in a variety of processes (fermentations) for millennia, with the first recipe for beer attributed to the Sumerian civilization of the lower Mesopotamia around 2000 years B.C. However, the "art of biocatalysis" remained somewhat dormant until the first true-biocatalytic process using enzymes was reported at the beginning of the XX century (Bornscheuer et al., 2012). During this period we have witnessed two consecutive biotechnological revolutions, led first by the invention of the polymerase chain reaction (PCR) in the eighties and followed by the development of directed evolution in the nineties, opening the way for protein engineers to enter a wonderland full of surprises and challenges (Lutz, 2010; Dalby, 2011). Directed evolution allows scientists to guide selective pressure, allowing RNA, proteins, metabolic pathways, genetic circuits or even whole cells to be evolved in an iterative manner in order to sculpt ad-hoc properties for purposes other to those defined by nature. Although there is still much to learn about protein folding, expression and the mechanism of action of enzymes, from its origins directed evolution has been used to mostly improve inherent enzyme features, such as activity, selectivity, substrate scope or stability for the synthesis of pharmacological intermediates and fine chemicals (Reetz, 2010; Wang et al., 2012). More interestingly, enzymes are now also being designed *in vitro* to employ them outside of their biological context in order to produce biofuels, commodities and building blocks (Denard et al., 2015). From a global perspective, directed evolution has pioneered the development of synthetic biology, establishing strong links between metabolic engineering and systems biology (Alcalde, 2015). As more and more methods to generate mutant libraries appear (*e.g.* genetic drift, circular permutation, ancestral libraries, etc), as well as techniques for adaptive evolution based on mutagenic PCR and recombination, the design of high- and ultra-high throughput screening assays alleviate experimental loads (Benner et al., 2007; Gupta and Tawfik, 2008; Yu and Lutz, 2011). Recently, the coupling of directed evolution and computational/*in silico* algorithms has brought us closer to reaching some of our biotechnology objectives that just a few years ago appeared to be mere pipedreams (Damborsky and Brezovsky, 2014; Verma et al., 2012; Kiss et al., 2013; Kries et al., 2013).

Apart from the aforementioned studies, excellent directed evolution reviews have been recently published, including those covering a historical perspective (Cobb et al., 2013), molecular approaches for library creation and screening (Shivange et al., 2009; Packer and Liu, 2015), a conceptual view of directed evolution (Bloom and Arnold, 2009; Romero and Arnold, 2009; Tracewell and Arnold, 2009; Tee and Wong, 2013) or the use of these methods for metabolic engineering and synthetic biology studies (Abatemarco et al., 2013; Currin et al., 2014). In this current review, we provide an exhaustive update of the most successful directed evolution experiments aimed at adapting enzymes and other molecules to non-natural needs in cutting-edge areas of biotechnology, illustrating how the real impact of directed evolution can transcend the limits imposed by nature and go far beyond. In particular, we will summarize recent studies that combine directed evolution with *in silico* and rational engineering, in order to render a set of molecules to be used in different biotechnological fields, addressing the adaptation of enzymes to non-natural environments, catalytic promiscuity for non-natural chemistry, the use of unnatural amino acids and the design of artificial DNA. In addition, we will consider the pioneering applications of directed evolution such as xenobiotic detoxification, biomedicine and bionanomaterials (Fig. 1).

2. Non-natural environments

Directing enzyme evolution so that they can withstand organic solvents has been the subject of research for decades. Such behavior is necessary to tackle dozens of transformations in which substrate solubility is an issue, from bioremediation to organic synthesis. Indeed, the first directed enzyme evolution experiment reported aimed to enhance catalytic activity in organic solvents. In the beginning of 90's, subtilisin E was subjected to several rounds of error-prone PCR in order to improve its activity in the presence of high concentrations of dimethyl formamide (DMF). The activity of the mutant enzyme was 256-fold improved over the native counterpart in 60% (v/v) DMF (Chen and Arnold, 1993). Since this historic and pioneering study, many other evolution campaigns have been undertaken to improve catalysis in organic solvents. For example, p-nitrophenyl esterases were improved by mutagenic PCR and in vivo shuffling in order to induce the same activity in 30% (v/v) DMF as that of the native enzyme in aqueous solution (Moore and Arnold, 1996). Phospholipase A1 was also engineered by mutagenic PCR and DNA shuffling in 50% (v/v) dimethyl sulfoxide (DMSO) (Song and Rhee, 2001). There are also a few examples of the evolution of oxidoreductases, such as the $P450_{BM3}$ monooxygenase, an outstanding enzyme for organic synthesis (see below) that was evolved to improve its activity in tetrahydrofurane and DMSO. The combination of two co-solvents in the same screen gave rise to organic solvent promiscuity, allowing mutants to retain activity in other solvents with a different polarity and chemical nature (Wong et al., 2004). Following a similar strategy, a fungal laccase was engineered by iterative cycles of mutagenic PCR and DNA shuffling in yeast. After several generations in the presence of increasing ethanol and acetonitrile concentrations, the final variant harbored mutations at the surface of the protein that established new contacts, mainly through salt bridges or H-bonds. These adaptations reflected a structural reinforcement that permitted the laccase to retain its activity in the presence of organic solvents (Zumarraga et al., 2007).

More recently, ionic liquids (ILs), deep eutectic solvents (DESs) and concentrated seawater are being considered valuable alternatives to noxious organic solvents for a range of biocatalytic processes. ILs are ionic salts that are liquid at temperatures generally below 100 °C (Welton, 1999; Hernáiz et al., 2010) and they are largely recognized as green solvents because they have zero to low volatility (lower toxicity than conventional solvents) and they are poorly or non-inflammable. DESs are a class of IL analogues with similar physical properties as ILs but that are cheaper and easier to prepare (Smith et al., 2014). Concentrated seawater has also been proposed as a possible solvent for several reactions, avoiding the use of drinkable water (Grande and de Maria, 2012). Employing these special liquids in biotransformations can do away with the need for damaging organic solvents, although only once their deleterious effects on enzyme activity can be overcome.