Directed evolution of industrial enzymes

Directed evolution has emerged in just a few years as one of the most effective approaches to adapting bio-catalysts to the performance requirements of industrial and medical applications. Directed evolution mimics the processes of Darwinian evolution in a test tube, combining random mutagenesis and/or recombination with screening or selection for enzyme variants that have the desired properties[1]. A recent conference* that convened practitioners from industry and academia surveyed current efforts and highlighted the rapid progress the field has enjoyed. In addition to impressive demonstrations of enzyme engineering, there were a variety of challenging problems and approaches to laboratory evolution.

Why, what and how

The application of enzymes in industrial processes often calls upon properties not found in enzymes isolated from their natural environments. The enzyme technologist's wish list includes high overall stability under process conditions (high temperatures, organic solvents, detergents, pH and oxidants) as well as high catalytic activity to non–natural substrates (Torben Borchert, Novo Nordisk, Bagsvaerd, Denmark).

Screening natural diversity seldom yields a catalyst fulfilling all demands. Rational protein design is often not useful either, as the problems are usually complex and multifactorial, and there is seldom sufficient knowledge of the structure or function. Nature, however, adapts enzymes to new environments, and even to new tasks, without relying on molecular modeling, computational chemistry or X-ray crystallography. A survey of the types of problem solved during natural evolution highlighted the potential for evolutionary design processes (Frances Arnold, California Institute of Technology, Pasadena, CA, USA).

As might be expected, there are various ways to implement evolution in the laboratory. *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* have all been used successfully as host organisms for creating and expressing large (functional) enzyme mutant libraries. Even more choices are available for creating the DNA libraries (e.g. error-prone PCR, combinatorial oligonucleotide mutagenesis, DNA shuffling, StEP recombination, *in vivo* recombination), whether mutations should be targeted to specific regions or distributed throughout the gene, and for identifying improved variants (screens versus selections). These issues of experimental strategy surfaced throughout the conference and were the subject of lively debate by the practicing laboratories. There is certainly no agreement as to a single best approach and there may never be one, as they all address different needs and situations.

Directed evolution is particularly well suited to 'tuning' enzyme function; that is, improving an activity that already exists at some, albeit low, level. It was also shown to be useful for combining properties not necessarily found together in any naturally occurring enzyme. For example, the high thermostability of a thermophilic enzyme might be joined with the low-temperature activity of a mesophilic or even psychrophilic one, as was illustrated with a mesophilic esterase and a subtilisin that were stabilized by 17–18°C and whose catalytic activities were increased at all temperatures (Frances Arnold).

According to Doug Clark (University of California, Berkeley, CA, USA), enzymes from thermophiles and hyperthermophiles are stabilized by high pressures, more so than their mesophilic homologs. Interestingly, the thermostable subtilisin obtained by *in vitro* evolution displayed similar pressure-induced stabilization. Clark is hoping that natural and laboratory-evolved thermophilic enzymes will reveal their stabilization secrets under pressure.

Longer jumps in sequence space

Evolving entirely new catalytic functions will probably demand the simultaneous exchange of multiple amino acids, an event so rare that the new function is unlikely to appear in a completely random library of reasonable size. Finding out how to make these larger leaps in sequence space to create new functions or even to improve existing ones more efficiently remains a major challenge for directed evolution.

A very interesting approach to broadening access to sequence space (without creating all 'dead' enzymes) was discussed by Jeremy Minshull and Joe Affholter (Maxygen, Santa Clara, CA, USA): *in vitro* recombination of homologous genes (family shuffling). Family shuffling takes advantage of the conserved structures of homologous proteins ($>\sim70\%$ sequence identity) to generate functional chimeras with very different sequences.

Shuffling four homologous cephalosporinase genes and selection for antibiotic resistance yielded a 270–540-fold increased activity towards the target antibiotic (moxalactam)[2]. Minshull and Affholter went through an impressive list of more-recent family-shuffling experiments that have yielded improved industrial enzymes and therapeutic proteins. For example, shuffling and then screening ten human interferon-α genes generated one that inhibits mouse-cell proliferation hundreds of times more than the best parent.

Larger jumps in sequence space can also be obtained by random combinatorial mutagenesis directed to specific regions of the target gene. Combined with selection, this is the approach favored by Larry Loeb (University of Washington, Seattle, WA, USA), who presented his most recent results on the directed evolution of enzymes for cancer gene therapy. His group has evolved O^6-methyl transferases that are resistant to O^6-benzylguanine and thymidylate synthases resistant to 5-fluoroacil. It is hoped that the introduction of such enzymes into human bone-marrow cells will render the cells resistant to these agents during chemotherapy.

Screening technology

There was consensus among the participants that one critical phase of any directed-evolution experiment is deciding how to search for variants with the desired properties. For most practical problems, this search is both time-consuming and expensive. The First Law of Directed Evolution ('You get what you screen for') was repeated by several speakers, who emphasized the importance of establishing a screen or selection that reflects the desired result.

A striking example was presented by Volker Schellenberger (Genencor International, Palo Alto, CA, USA), whose first attempt to use a selection for secreted subtilisin variants yielded improved enzyme expression but no variant with the desired increased activity in laundry applications. He then set up the equivalent of washing

machines in microtiter plates and measured activity against surface-bound substrates. This screen yielded different molecular solutions than did a screen for proteolytic activity in solution.

Several speakers described multi-tiered screening systems in which a very-high-throughput, but relatively insensitive, screen (or selection) is followed by assays of increasing relevance to the desired product (Rowan Grayling, Proctor and Gamble, Cincinnati, OH, USA). To evolve an oil-desulfurization pathway, for example, a selection on minimal plates containing dibenzothiophene as the sole sulfur source was followed by a two-phase microtiter-plate assay and HPLC analysis of total sulfate produced in the aqueous phase (Jeremy Minshull).

Cell-surface display of proteins in combination with fluorescence-activated cell sorting (FACS) was described for the high-throughput screening of mutant libraries for directed evolution. Variants of the ompT protease on the surface of *E. coli* were selected using FACS for their ability to cleave a substrate labeled with a fluorophore and a quenching group. Cleavage yields a positively charged, fluorescent product that remains associated with the negatively charged cell surface (Mark Olsen, University of Texas, Austin, TX, USA).

Some new problems addressed by directed evolution

This year's conference delivered on its promise to discuss the engineering of industrial enzymes; enzymes that are already being commercialized by the leading manufacturers include improved α-amylases (Anthony Day, Genencor International), lipases (Torben Borchert) and subtilisins (Volker Schellenberger) for laundry applications. Many of the improvements were obtained using directed evolution. Evolved thermostable esterases and dehydrogenases (David Demirjian, Thermogen, Chicago, IL, USA) are also being commercialized.

Directed-evolution methods are now being applied to a number of interesting and very challenging problems, such as improving whole biosynthetic pathways, engineering enzymes involved in fatty-acid biosynthesis in plants and engineering a variety of enzymes for chemicals synthesis.

John Shanklin (Brookhaven National Laboratory, Upton, NY, USA) found that a bacterial unsaturated-fatty-acid auxotroph could be complemented by introducing certain soluble desaturase enzymes from plants, despite the fact that plants and bacteria have different strategies for introducing double bonds into fatty acids. He used this system to select for mutant plant 18:0-ACP desaturases with shorter chain-length specificity (14 and 16 carbon atoms) and that retain the Δ9 regiospecificity of the parent enzyme, effectively decoupling these two specificities. In other experiments involving the integral membrane desaturases and hydroxylases, he showed that the functional outcome of the reactions could be interchanged by exchanging key residues in the active site.

The chloroperoxidase of *Caldero-myces fumago* catalyses a variety of reactions, including halogenation, demethylation, sulfoxidation and epoxidation, in a highly stereoselective manner. The lack of functional expression in hosts other than *C. fumago* has not deterred Lowell Hager (University of Illinois, Urbana, IL, USA) from his goal of engineering this enzyme to resist inactivation by terminal alkenes and hydrogen peroxide. The development of a *C. fumago* integrative vector system has allowed his group to create random libraries, albeit of relatively small size, from which interesting chloroperoxidase variants have been isolated.

Frances Arnold reported the functional expression of horseradish peroxidase (HRP) in *E. coli* by directed evolution. This group uses the phenolic-coupling activity of HRP to monitor aromatic hydroxylation reactions for the large-scale screening of P450 monooxygenases by fluorescence imaging. Joe Affholter's account of the efforts at Dow Chemical to design a large-scale process for enzymatic conversion of chlorinated hydrocarbons to chlorohydrins underscored the demanding requirements for an effective catalyst for bulk-chemicals production, one of which is that the biocatalyst remain active for thousands of hours under process conditions. How to screen rapidly for such features remains an unsolved issue.

Novel allosteric nucleic-acid-based enzymes are evolving in the laboratory of Ron Breaker (Yale University, New Haven, CT, USA). His group is creating new molecular switches by fusing nucleic-acid-based binding domains (e.g. for ATP or FMN) to a ribozyme domain ('response module') that undergoes self-cleavage upon allosteric activation transmitted via a 'communication module' designed by directed evolution. Random sequences inserted between the binding and response modules were selected for their ability to function in allosteric regulation.

Although advertised as a directed-evolution conference, the presentations were peppered with examples of site-directed mutagenesis (perhaps to keep the competitive spirit high?). Our culture of scientific publication is one in which successes are reported; on the other hand, failures rarely are. Thus, nothing takes the place of a good presentation or discussion in which failures and successes are reported side-by-side. A number of the speakers readily admitted that their attempts at rational design had been unsuccessful, or at least extremely slow in yielding useful results. More informal discussions ('tears in beers') uncover the directed-evolution experiments that did not work. The failures appear to result from not heeding the First Law.

Challenges and prospects

Challenges for future directed evolution posed by the audience and speakers include developing workable strategies for the evolution of new catalytic functions, evolving complex biosynthetic or degradative pathways, evolving single enzymes and new enzyme pathways for large-scale chemical production, and evolving enzymes that are difficult to handle in terms of functional expression, stability and assay development. We still need to understand what makes a 'good' library for evolution (e.g. family shuffling versus optimization of single sequences), and we certainly need better high-throughput screening tools and new assay formats to address the many new problems to which these tools are now being applied.

Based on the reports we heard, however, *in vitro* evolution is a powerful and efficient tool for tuning enzyme performance in a wide range of applications. As a reliable catalyst-improvement technology, directed evolution will play a major role in removing bottlenecks to biocatalysis process development.

References
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