Review

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The continuously expanding applications of enzymes for the chemical, pharmaceutical and food industries create a growing demand for enzymes that exhibit higher operational stability, higher specificity and enantioselectivity, as well as for those that have new activities on natural and unnatural substrates. New and improved enzymes were traditionally isolated for these purposes by the screening of microorganisms from culture collections or from special or extreme environments. A promising alternative approach was the development of 'catalytic antibodies', especially for organic synthesis involving unnatural substrates.

The simultaneous development of new tools for the generation of very large mutant libraries and the accumulation of detailed data on structure-function correlation for many enzymes recently resulted in the isolation of new and improved enzymes using an 'in vitro enzyme evolution' approach (Fig. 1). The evolution process begins with the selection of a known enzyme and setting of an envisaged objective, ranging from preservation of existing activity and the addition of improved thermal stability, higher tolerance to the presence of organic solvents or modified optimal working temperature or pH, to higher specificity and enantioselectivity or altered specificity. A large mutant library, usually within the range of $10^4$–$10^7$, is then prepared by random mutagenesis of the entire gene, DNA shuffling or mutagenesis of a pre-selected subgenomic section of the gene. Cloning and expression results in potentially active enzyme molecules located either in cell cytoplasm, in the periplasmic fluid or on the phage or cell surface - these populations present a pool from which the best mutants have to be retrieved on the basis of detected enzymatic activity. Isolated mutants might subsequently serve as an improved starting point for additional rounds of mutagenesis to accumulate beneficial mutations for the best result.

Identification and isolation of the best mutant(s) is a crucial step in the process. In cases when the envisaged enzymatic activity or property is essential for viability and growth (e.g. overcoming increasing concentrations of antibiotics or providing an essential nutrient) selection might be applicable. Otherwise, screening based on the development of a detectable signal to identify the envisaged mutant on the basis of its targeted catalytic activity needs to be done. This process may be directed either towards the identification and isolation of very few highly active mutants from an essentially inactive background population, or towards the quantitative measurement of the activity exhibited by each cell or colony for the identification of most active mutants out of a moderately active background population.

The generation of large mutant libraries for in vitro enzyme evolution presents the challenge of effectively screening libraries of $10^4$–$10^7$ mutants on the basis of simultaneously assaying their biocatalytic activity. In this review, we highlight the main steps involved in this process, describe the alternative approaches to address this challenge, survey the state-of-the-art technology and assess achievements already made. It is anticipated that, as a result of the expected accomplishment of further improvements in high-throughput screening that will allow routine screening of whole libraries, the number of useful new and improved enzymes derived through in vitro enzyme evolution will expand rapidly in the near future.
**The screening process: the state-of-the-art**

The screening of a large cell population on the basis of biocatalytic activity primarily involves physical separation of the cells and their compartmentalization in a manner that allows the assay of either a single cell or a colony derived from a single cell. Given that the most common enzymatic assays are not sensitive enough for single-cell analysis, screening of populations comprised of colonies is predominantly used. The separation stage has a strong impact on the size of the effectively screened mutant population, often reducing its scope to a small fraction of the potential inherent in a whole mutant library. Main approaches to physical cell separation are dilution and spreading over agar in dishes, disks or plates, dilution into single cells in multiwell plates, flow cytometry, or a single cell on a single bead immobilization (Freeman, A. and Georgiou, G., US Patent no. 6,277,588, and Freeman, A. et al., unpublished results). With the exception of flow cytometry - allowing for single-cell analysis - incubation in growth medium for uniform colony development usually follows.

**Signal development**

The most crucial step in the screening process is signal development to identify the most active mutant(s), arising from exposure and incubation of the physically separated cells and/or colonies with the substrate of choice. The efficacy and accuracy of this step will be mainly affected by the type of signal developed, such as color formation arising from colored products such as p-nitrophenol, pH-change mediated by pH indicators, such as cresol red or bromocresol purple, fluorescence emission, or turbidity clearance because of protein digestion. Monitoring of the signal is expected to reflect specific activity, primarily relying on effective contact between the added substrate and the target enzyme. Diffusional limitations, potentially arising from low permeation of the substrate into the cell, might substantially reduce the signal, leading to the use of strategies such as cell permeation by treatment with organic solvent, or use of expression systems such as the Lpp-OmpA-protein fusion, displaying the enzyme of interest on the cell surface. Furthermore, when agar plating is used, product diffusion might become a major problem because it might reduce signal intensity as well as affecting the overlap of neighboring colonies. This problem led to the extensive use of soluble substrate and precipitating product combinations resulting in a colored or fluorescent insoluble product accumulated within the immediate vicinity of the active cell and/or colony.

**Signal detection**

Signal detection, primarily designed to identify the location of the most active cell and/or colony for isolation, might be easier when the envisaged mutant is rare, allowing qualitative or low accuracy quantitative monitoring. When many active colonies are closely packed on a solid phase (e.g. agar), or when there is a detectable background activity of the whole population, quantitative analysis leading to measured activity associated with each specific colony becomes vital. To meet this challenge, several digital imaging systems were developed for colored as well as for fluorescent products (see also http://www.maxygen.com). Imaging can be coupled with a microscope to allow analysis of a large number (e.g. several hundreds per cm²) of inspected colonies confined within a relatively small area. Quantitative measurement of the activity of populations compartmentalized by dilution into multiwell plates is readily performed by ELISA readers. It should be emphasized, however, that effective handling of a large number of mutants using dilution into multiwell plates requires the use of
of automated robotic systems\textsuperscript{23,25}. Optional routes for the design of a screening system are summarized in Figure 2. Screening based on the quantitative measurement of the activity exhibited by each cell or colony depends on the availability of sophisticated equipment, such as a flow cytometer or digital imaging integrated with robotic systems, as well as on trained personnel, thus mostly resulting in higher costs and screening of a low percentage of the screened population.

Alternative approaches

It should be mentioned that several molecular in vitro and in vivo approaches were also developed as an alternative to the ‘physical’ compartmentalization methods described above\textsuperscript{32}. These include compartmentalization and selection based on an in vitro evolution system confined within a water-in-oil emulsion\textsuperscript{33}, phage display coupled with substrate co-immobilization onto the phage, leading to modified identifiable phages carrying the envisaged activity and its product\textsuperscript{34,35}, and a three-hybrid system to detect biocatalysts in vivo\textsuperscript{36}.

A survey of the numbers of cells and/or colonies reported to be effectively screened using some of the routes described in Fig. 2 reveals that, in most cases, a small fraction of the generated libraries (estimated to contain $10^4$–$10^7$ mutants) was subjected to the screening stage for the envisaged activity or property (Table 1). The data in Table 1 reflect the impact of labor-intensive methodology on the size of the screened population. Incorporation of automated processing and digital imaging led to a substantial increase in the number of screened cells and/or colonies.

A new screening method recently developed in our lab, seems to provide a new option of screening for the ‘one in a million’ mutant in a single run (Fig. 3) (Freeman, A. and Georgiou, G., US Patent no. 6,277,588 and Freeman, A. et al. unpublished results). This method uses immobilization of a single cell on a single bead followed by bead immobilization on a physical support, providing a simple solution applicable in any conventionally equipped laboratory.

### Table 1. Examples of reported sizes of screened cell populations

<table>
<thead>
<tr>
<th>Number of screened cells or colonies</th>
<th>Compartamentalization</th>
<th>Activity detected</th>
<th>Signal</th>
<th>Automation +/- digital imaging</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>Multiwell plate</td>
<td>Lipase</td>
<td>Color</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>10 000</td>
<td>Agar</td>
<td>β Galactosidase</td>
<td>Color (ppt)</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>10 000</td>
<td>Multiwell plate</td>
<td>Hydantoinase</td>
<td>Color</td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td>30 000</td>
<td>Agar</td>
<td>β Galactosidase</td>
<td>Color (ppt)</td>
<td>+</td>
<td>16</td>
</tr>
<tr>
<td>35 000</td>
<td>Multiwell plate</td>
<td>Peroxidase</td>
<td>Color</td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td>200 000</td>
<td>Agar</td>
<td>Oxygenase</td>
<td>Fluorescence+</td>
<td>(ppt)</td>
<td>18</td>
</tr>
<tr>
<td>600 000</td>
<td>Flow cytometer</td>
<td>Protease</td>
<td>Fluorescence+</td>
<td>+</td>
<td>27</td>
</tr>
<tr>
<td>1 000 000</td>
<td>Immobilized cell on immobilized bead</td>
<td>β Lactamase</td>
<td>Color</td>
<td>+ UPR</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: UPR, unpublished results; ppt, precipitate.

This solution reduces tedious handling, allows the use of commonly employed, commercially available substrates as well as soluble products in a compact system readily coupled with digital imaging. A feasibility study has recently demonstrated handling and screening of one million cell populations.

The emerging capabilities of screening whole libraries on the basis of biocatalytic assay might simplify the common process of in vitro enzyme evolution by avoiding the need for multiple rounds of mutagenesis and screening limited to a small fraction (e.g. $10^3$ out of $10^5$–$10^7$ mutant population). It would be replaced with a single preparation of a hypermutated large population, as argued and demonstrated for the development of antibiotic resistance isolated via selection from hypermutated TEM-1 β-lactamase\textsuperscript{10,13,37}.

### Fig. 3. The ‘immobilized cell on immobilized bead’ approach to the screening of large cell populations for in vitro enzyme evolution.

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Concluding remarks

It appears that substantial progress has been made in the past few years in the development of screening tools, resulting in the successful identification and isolation of >40 improved and new enzymes (for details see http://www.che.caltech.edu/groups/fha). It should be emphasized, however, that most of these improved enzymes or enzymes that exhibit altered specificity remain very closely related in their 3D structure and activity category to their origin.

Remaining challenges for further improvement of screening methodologies to meet the challenge of whole library screening mainly include screening on novel detection methods, enabling analysis based on signal derived from natural or unnatural substrates that do not produce color or fluorescence, coupled with user-friendly, moderate cost combinations of automatic screening with microscopic digital imaging.

The ongoing efforts in the parallel development of new molecular methodologies, such as random mutagenesis confined to a predetermined section of the enzyme and DNA shuffling of a family of genes or subgenomic sequences, and the development of improved screening technologies are expected to merge into highly effective in vitro enzyme evolution, resulting in rapid growth in the number of useful new and improved enzymes.

References