

If you've got it, flaunt it – rapid screening for microbial biocatalysts

The main approaches to drug design are either 'rational', based on a knowledge of the 3D (though usually static) structure of the receptor of interest, or more usually by empirical test receptor binding, exploiting advanced robotic and data handling assay systems and often testing against several potential disease targets. The growth in both natural-product-based and purely synthetic combinatorial compound libraries is thus driving a High Throughput Screening (HTS) revolution in drug discovery that now sees large pharmaceutical companies able to test as many as 50 000 samples per day for biological activity, or 30 times as many as they could just three years ago.

Similarly, the development of improved biocatalysts using microbial strains and enzymes traditionally relies on screening large numbers of entities for optimum activity and then going into strain development programmes and modifying fermentation conditions to increase titre or activity. Thus, the emergence of exciting new technologies, driven by advances in HTS for drug discovery, has resulted in the recognition of a need for screening ever increased numbers of samples for biocatalytic activity, through automation, miniaturization, rapid single-step assays, and multi-assays, providing for greater product diversity and exploiting economies of scale to limit cost.

The challenge to industrial and academic researchers in developing technologies to meet these needs was the theme of a recent symposium* that brought together scientists from many of the established pharmaceutical companies, the often small but rapidly-evolving biotechnology screening and niche product development companies, and consultants and academic researchers working at the forefront of screening

*The meeting 'New Frontiers in Screening for Microbial Biocatalysts' was organized by W. J. J. van den Tweel, J. A. M. de Bont and C. P. van der Beek, under the auspices of the Applied Biocatalysis and Microbial Physiology Working Parties of The European Federation of Biotechnology, and was held in Ede, The Netherlands, 15–18 December 1996.

technology. Unusually, the majority of participants came from industry.

The advantages of biocatalysts, i.e. of using biotransformations compared with chemical synthesis, are well known, and include the shortening of synthetic pathways, the use of mild reaction conditions and inexpensive substrates, the potential to generate compounds of a given optical or regiospecificity in high yield and to use a wide substrate range (Klaus Kieslich; GBF, Braunschweig, Germany). Catalogues of known bioconversions are now available on a searchable CD-ROM. Although the use of biocatalysts has distinct attractions, random searching for novel microorganisms suitable for biotransformations is a time-consuming and laborious process. Thus a number of enrichment techniques have been developed to circumvent this, including those based on the isolation of microorganisms from environments correlated with the educts, such as alkane degraders from oilfields, and intuitive selection of strains from culture collections based on a priori knowledge (e.g. taxonomic relationships with strains previously investigated for similar phenomena). The effectiveness of such developed strains could be very great, with the *Rhodococcus rhodochrous* J-1 nitrile hydratase system yielding no less than 656 g l⁻¹ of acrylamide from acrylonitrile in a 10 000 t y⁻¹ process (Hideaki Yamada; Toyama Prefectural University, Japan).

Even where potential biotransformation systems have been identified, there is still much room for improvement. Screening ~300 commercial enzyme preparations would typically require 2–5 g of the substrate and where the products are analysed by HPLC or GC this takes, at least 8 min per sample, notwithstanding that one would probably need to try several temperatures, solvents and incubation times (David R. Dodds; Schering-Plough, Kenilworth, NJ, USA). Microbial screening requires even more substrate and is additionally disadvantaged by the normal requirement for an aqueous (or at least biocompatible) environment; as a result primary selection is typically

based on following up those strains exhibiting >5% of the desired bioconversion. Substrates derived from upstream processing that is optimized merely for their production may include contaminants inhibitory to the biocatalytic process under development, whilst the use of model chromogenic or fluorogenic substrates is often inconvenient and inappropriate since the biocatalyst must act on the actual substrate of interest. As large-scale processing is the most difficult area of biotechnology in which to motivate change, reaction conditions should accommodate the likely scale-up conditions as closely as possible, and for economic reasons the analytical methods should be direct, quantitative and minimize sample handling. Different parameters interact in complex ways, for example a solvent that is best at one temperature may be much worse at another temperature; only the most up-to-date, hierarchical experimental designs will pick this up.

Born to be wild – tapping into microbial diversity

The use of modern molecular methods such as PCR, nucleotide sequencing and rRNA analyses demonstrates unequivocally that only a minuscule number (probably less than 0.1%) of microbial species have as yet been cultured, which obviously leaves a substantial untapped resource for generating novel biocatalysts (Erko Stackebrandt; DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). The construction of phylogenetic trees provides a framework for establishing relatedness and the diversity of novel strains, to seek to maximize exploration of the biodiversity space. The need to learn how to culture uncultured microorganisms is now a high priority, and this may well be assisted by analysing their molecular taxonomy. However, there are as yet few rules governing the distribution of biocatalytic activities among the major taxa.

The search for novel biocatalysts might therefore be based on a focused screen using known enzyme producers or a more extensive random screen with the emphasis on making use of such biodiversity (Graham Byng; Panlabs Inc., Bothell, WA, USA). Increasing diversity typically involves the targeting of different habitats, selecting single representative strains chosen

by morphological comparison and employing different fermentation media and growth conditions. In an example study of microorganisms isolated from *Spartina alterniflora* cord grass in a salt marsh habitat, visual inspection and fatty acid methyl ester fingerprinting were used to distinguish 70 groupings in ~600 isolated strains. Dereplication at this point is considered essential to reduce the unnecessary workload that would ensue from having duplicate strains in downstream analysis.

Indeed, the importance of harnessing microbial diversity for accelerating drug discovery comes to the fore when coupled with the latest assay systems and sample presentation techniques. Modifying the growth conditions to mimic those in nature more closely would help persuade such organisms to make additional, and more interesting, secondary metabolites, and this proved to be true of biocatalysts too (Neil Porter; Biodiversity Ltd, Uxbridge, UK); increasing the efficiency of screening larger numbers of samples by using 384-well microtitre plates instead of the 96-well format, using homogeneous assays with just one process step in the assay and exploiting data-rich methods such as LC-MS were also important ways forward.

Sharing the burden – robotics and automation

The large step-changes in sample screening numbers require a concomitant increase in the generation of samples, which is unlikely to be met by traditional methods. Manually intensive methods of isolating and screening microbial strains for novel or desired catalytic activities or products have been the mainstay of the industry for many years, typically involving enrichment from a heterogeneous population (often derived from soil), and assaying for activity in culture tubes. However, after the initial enrichment and colony selection these methods tend to be repetitive and can be automated to reduce the resources in manpower and time needed to find a biocatalyst. Modern robotic systems can localize colonies (using image analysis), subculture and inoculate them into liquid culture, and can subsequently perform the liquid handling tasks required for assay of the broths for activity. Typically 10–100 000 strains may be assayed for a new biocatalytic activity, and this combination allowed the screening of 1000 strains per week

(Olgé Favre-Bulle; Rhône-Poulenc Industrialisation, St Fons, France). Even using object-oriented software, scheduling and exception handling are particularly important tasks for such robotic applications (Alain Donzel; Scitec, Lausanne, Switzerland).

Expression cloning

Although the classical approaches of microbial screening for novel enzymes may be sufficient to produce a biocatalyst with high activity, molecular biological techniques can be used to increase yields by expression cloning. Isolation of the enzyme by cloning serves a number of purposes, including separation from a complex mixture in the original host and making it possible to manipulate the gene for the enzyme of interest (and its regulation). Indeed, users are now beginning to demand mono-component enzyme preparations (Henrik Dalbøge; Novo Nordisk A/S, Bagsværd, Denmark). More than 200 different enzyme genes from fungi of interest have been cloned in *Saccharomyces cerevisiae* cDNA libraries by detecting expression with azurin-, PNP- and MUF-linked substrates. In this case enzyme expression could be increased by transferring the genes into a highly-transformable fungal host (*Aspergillus*) under the control of selected promoters. Similarly, screening for esterases using chromogenic or fluorogenic esters allowed the genes coding for ten esterases to be obtained from four different bacterial genera (Helmut Schwab; University of Technology, Graz, Austria). The cloning approach could be taken a step further by gathering published information about similar nucleotide sequences and using this to produce PCR primers that allow the isolation of similar enzymes (with perhaps slightly different properties) from non-culturable strains.

Molecular tagging

The ideal assay system for HTS would be a single manipulation (i.e. sample delivery) performed in a single well; it should be easily automated and preferably allow for the screening of a number of different activities simultaneously. One of the main challenges of isolating clones containing an enzyme activity of interest is developing simple assays sensitive enough to determine any level of activity that may represent an enzyme with optimal activity against

a related substrate. Novel colorimetric, luminescence and fluorescence methods have been developed as homogeneous assays applicable to HTS, with automated 'multiplex' (typically 10–20 compounds per well) processing, including variations on the theme of lanthanide measurement based on Dissociation Enhanced Lanthanide Fluoroimmunoassay (DELFLIA) and Homogenous Time-resolved Fluorescence (HTRF) (Stuart Webb; Wallac Oy, Turku, Finland). HTS multiplexing can be taken a step further by using a multiple label immunoassay, with as many as four different lanthanides to assay for different enzymes of interest. Separation-free/solution format HTRF screening can provide 2000 samples per hour throughput (Al Kolb; Packard Instrument Company, Meriden, CT, USA), although many advances in membrane filtration materials are making separation much less of an issue (Robin Hood; FSM Technologies, Clydebank, UK).

Many enzymes are difficult to screen because of their poor activity in aqueous environments. Thus a useful development was a series of new commercial substrates for lipase assays that are compatible with a range of reaction media. These consist of alkyl diacyl glycerols, containing a pyrene fluorophore and a trinitro phenylamino residue as quencher, that increase in fluorescence intensity upon hydrolysis. Assaying a range of these fluorescent derivatives allows lipases with different stereopreferences to be isolated (Albin Hermetter; University of Technology, Graz, Austria).

Getting real – microbial screening in practice

Lithium therapy, a major treatment for manic depression, is thought to act mainly through inhibiting *myo*-inositol monophosphatase (IMPase) [Axel J. Glanzhorn; Synthélabo Biomoléculaire (formerly Marion Merrell Research Institute), Strasbourg, France]. Natural products from 12 000 microbial fermentation broths and 30 000 synthetic compounds were screened with a robotic, automated, 96-well microtitre plate assay based on inhibition of IMPase activity using an ammonium molybdate/malachite green assay for measurement of inorganic phosphate. This resulted in the eventual isolation of one compound that acted as an irreversible inhibitor of IMPase.

Despite all of the systematic progress that will undoubtedly be made in developing processes by straightforward automated HTS, it is refreshing to note that some important breakthroughs should still come about through serendipity. Success in screening for microbial strains capable of generating artificial nucleoside analogues (some of which have been developed as anti-HIV agents) was initially achieved during an extensive screening programme by an accidental increase of the incubation temperature of *Enterobacter aerogenes* biosynthesizing adenine arabinoside. Investigation of the thermal characteristics of the process, which was prompted by this fortuitous discovery, revealed that the higher temperature (~50°C) was essential for reducing a deaminase activity in favour of the desired nucleoside hydrolysis (Takashi Utagawa; Ajinomoto Co. Inc., Japan). The use of higher temperatures during enzyme purification allowed use of the biocatalyst to be extended to the preparation of many other purine arabinosides. In general, a systematic approach based on enrichment or acclimation to obtain an activity, subsequent enzyme isolation and purification, followed by N-terminal sequencing and cloning, provided a rational approach that reproducibly delivered the goods (Yasuhisa Asano; Toyama Prefectural University, Japan).

Combining biodiversity and focused approaches

There are many advantages of utilizing microorganisms (e.g. thermophiles, halophiles) from so-called extreme habitats, where one may expect to discover unusual new enzymes with desirable properties (Jay M. Short; Recombinant Biocatalysis Inc., Sharon Hill, PA, USA). A novel approach to biocatalyst discovery and development, which is already being exploited commercially, involves preparing novel enzymes from these sources by elimination of the need to culture the microorganism. As discussed above, the vast majority of microorganisms have yet to be cultured or may be non-culturable under known conditions. Therefore, any method of using the biocatalytic potential of such non-cultured cells that eliminates the need for this step is of great value. This is achieved by isolating nucleic acids directly from environmental samples or primary enrich-

ment cultures and constructing gene libraries for HTS for particular enzyme activities. This 'Biopanning' of biodiverse environmental libraries provides cloned genetic material which can be further processed by directed evolution, for example by gene-shuffling, to combine the best properties of enzymes from a variety of sources.

Single cell selection methods

As a specific example of the development of biocatalysts for commercial processes, that for preparing the valuable intermediate (*R*)-2-(4-hydroxyphenoxy)propionic acid (HPOPS) is illustrative (Bernard Hauer; BASF AG, Ludwigshafen, Germany). Three strains were selected from a screening programme which analysed many thousands for the ability to hydroxylate the precursor POPS. The fungus *Beauveria bassiana* was chosen for strain improvement; by selection for POPS tolerance and UV mutagenesis the yield increased from 0.5 g l⁻¹ to 2 g l⁻¹ and by chemical mutagenesis to 7 g l⁻¹. Flow cytometric cell sorting was used to isolate elongated spores which correlated with a higher yield of HPOPS when cultivated and tested for hydroxylation of POPS in a microtitre plate assay. The current process operates at the 100 000 l scale.

Single cell selection is fundamental to the methods currently being used by the evolutionary biotechnology company Evotec (Björn F. Lindemann, Evotec Biosystems, Hamburg, Germany). Evolutionary strategies for efficient engineering of biocatalysts *in vitro* are being developed by generating genetic diversity through random mutagenesis; highly active variants are then located by bacterial display under physiological conditions, or by using the Fluorescent Enzyme Bead Assay System (FEBAS) where there is free-diffusion of the assay substrate but localization of the fluorescent product. In a miniaturized screening system consisting of 8000 compartments contained within a 4 inch wafer, volumes of less than 750 nl can be screened in drops of 0.5–3.5 nl volume dispensed at a rate of 3000 s⁻¹. Cells or beads can thus be assayed individually or in small groups using fluorescence correlation spectroscopy and confocal fluorescence scanning; cells presenting the product of interest can be identified by Confocal Nanospectroscopic Scanning (CNS) and a picker used to

select and remove them for cultivation. Clearly, one major advantage of this single cell/bead selection system is that it obviates the need to cultivate and assay all candidates using laborious large-scale screening methods.

The majority of the novel screening methods introduced at the symposium require some form of molecular tagging. However, analytical methods such as are used in earth remote sensing systems cannot enjoy such a luxury, and it transpires that infrared spectrometry in combination with the implementation of modern chemometric methods for multivariate analysis (DRASTIC – Diffuse Reflectance Absorbance Spectroscopy Taking In Chemometrics), which exploits natural spectral characteristics of molecules of interest for their identification, is sufficient to discriminate and quantify target determinands in complex fermentation broths *in situ* without separation or purification (Douglas B. Kell; University of Wales, Aberystwyth, UK). In a model fermentation system the carbocyclic nucleosides aristeromycin and neplanocin A, produced by *Streptomyces citricolor*, could be distinguished rapidly and quantitatively with these methods during a titre improvement programme.

One view had it that if today was the era of the multiwell plate, 'tomorrow' was that of molecular screening while the future would involve database mining and forced molecular evolution. Overall, the conclusion is that HTS methods will continue to be of great importance in the development of clean and efficient microbial biocatalysts, and that robotics, bioinformatics and data processing will be significant drivers in speeding the progress of biotransformations as agents of chemical change.

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