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Better Biocatalytic Processes Faster: New Tools for the Implementation of Biocatalysis in Organic Synthesis

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Abstract:

Recent advances in biology and process engineering have given rise to a number of new techniques ("tools") that will allow greater integration of enzymic and microbial catalysis in multistep industrial organic syntheses. These advances will enable a more systematic exploitation of the unique stereo- and regioselectivity of biological catalysts to carry out difficult, and often unique, chemical transformations such as asymmetric carbon−carbon bond formation and highly selective oxidations. In this mini-review four such tools are outlined, namely: (1) the use of directed-evolution methods, under real process conditions, to yield robust industrial biocatalysts, (2) the use of engineering and cost models for rapid process analysis and specification of development targets, (3) the use of microscale-processing techniques to accelerate data collection on competing biocatalyst and process options, and (4) the use of bioinformatics to aid biocatalyst identification and accelerate directed evolution. In each case we aim to highlight the key developments and define their role in delivering more efficient biocatalytic processes more rapidly. Where appropriate, areas requiring further research are also identified.

Introduction

In recent years biocatalytic processes, using whole cells or isolated enzymes as catalytic agents, have found increasingly widespread application.[1,2] This is particularly true in the pharmaceutical and agrochemical industries where the need for optically pure molecules is critical.[3] Biocatalysis has thus reached a particularly exciting time. Over 300 processes to date have been implemented in industry,[4] demand for complex chiral drugs is high,[5] and environmentally clean processes are increasingly required.[6,7]

Many of the most useful conversions concern the synthesis of products with multiple chiral centres where the use of biocatalysis circumvents the need for multiple chemical protection and deprotection steps. These are often difficult and reduce the atom efficiency of a process.[8] Until recently biocatalysis was applied primarily to hydrolytic and esterification reactions.[4] While many hydrolytic resolutions take advantage of the supreme stereoselectivity of enzyme action, they only yield 50% product formation at best. In situ racemisation/deracemisation to establish dynamic kinetic resolutions can improve the situation; nevertheless, asymmetric synthesis may prove preferable in many cases since it is a simple process to operate. Two new classes of reaction are now also being exploited. The first is asymmetric carbon−carbon bond formation. This key reaction in organic synthesis can be carried out enzymatically by transketolase[9−11] and aldolase[12−17] to create new chiral centres with superb optical purity. The second is selective oxidation. This exploits the regioselectivity of enzymes, usually operated in whole-cell format, to insert oxygen[18,19] or hydroxyl groups,[20] often

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asymmetrically, at specific positions on complex molecules again without the need for protecting groups. While many other complex and difficult conversions are being explored by academic chemists, biochemists, and microbiologists, these particular examples are now starting to be exploited commercially with the necessary bioprocess engineering in place.

Exploiting biocatalysis to effect such difficult chemistry has long been an ambition of biochemists and microbiologists. To implement such reactions in industrial chemistry, however, often requires a number of significant obstacles to be overcome. In particular low productivities and long development times are frequently not offset by the exquisite chemo-, stereo-, and regioselectivity to be gained by use of these catalysts. In this review we highlight four tools recently developed in the fields of biology and process engineering to help overcome these obstacles. These are: (1) directed enzyme evolution, (2) process and cost modelling, (3) microscale-processing techniques, and (4) bioinformatics. Given effective application, these tools will enable industrial process chemists to use biocatalysis alongside conventional chemistry for the most difficult of reactions.

Tools to Enhance Biocatalytic Process Development

(1) Directed Evolution for Process Compatibility. Advances in molecular biology, and more specifically directed-evolution techniques, have opened up the possibilities of engineering enzymes, enzyme pathways, and recently even whole-cell biocatalysts to have the desired biocatalytic properties for synthesis of novel pharmaceuticals and agrochemicals.[21−25] Directed evolution, in general, involves the construction of a library of genetic variants using polymerase chain reaction (PCR)-based methods[26] for either random mutation or the recombination of different genes or both. This is followed by the selection of the best variants according to some predefined criteria; in the case of biocatalysis this may be increased specific activity on a particular substrate or greater stability under extreme conditions. The best variants are then typically subjected to one or more rounds of the same mutation/recombination and selection procedure until no further improvement is obtained.

Naturally occurring enzymes have evolved to work in the cellular environment and are unlikely to operate efficiently under process conditions such as high substrate concentration, extremes of pH or temperature, or in the presence of organic solvent as is frequently required for efficient industrial synthesis. Directed evolution has already been used to address some of these problems. For example, subtilisin has been evolved to work in the presence of organic solvent (35% DMF), at extremes of pH (5.5 and 10), and also after heat treatment.[27] Despite this, most studies have been carried out under in vitro conditions that do not match the final operating environment found in industrial processes. The need for a convenient high-throughput assay encourages activity screening in growth media, either for a secreted protein or using a substrate that permeates the cellular membrane. This is unlikely to produce the best enzyme for industrial biocatalysis, where the enzyme may be isolated, immobilised, and repeatedly recycled. Although screening in media may appear to be similar to the use of an enzyme as a whole-cell catalyst, the conditions may still not reflect accurately those that occur when the process is scaled up to an industrial scale.[28]

Effective use of directed-evolution techniques to develop industrially relevant enzymes will depend on the possibilities for screening under true process conditions. It is here where the process-modelling approaches (as described in (2)) have the power to effectively direct the application of directed-evolution techniques and set the quantitative targets required to facilitate real process improvements. The potential use of microscale-process mimics (as described in (3)) and the application of laboratory automation to perform routine selection and screening procedures also have the potential to accelerate the process of directed evolution itself.

(2) Process Modelling. In the past decade at UCL a systematic approach to biocatalytic process design has been built.[29] This aids in the evaluation of process engineering techniques such as two-liquid-phase biocatalysis,[30] in situ product removal (ISPR),[31−32] and reactant feeding[10] that allow higher productivities to be achieved and in some cases have enabled commercialisation to go ahead. Examples of the growing number of techniques now available to enhance biocatalytic process productivity are given in Table 1. While some understanding of the implementation of each of these methods individually has been established, it is less clear how to evaluate their application alongside competing process options. To address this issue we undertook to examine the relationship between biocatalyst properties and the most appropriate process. On this premise we attempted to establish a systematic procedure that can guide the engineer from the basic biocatalyst data, via analysis of process constraints, through to a process concept.[29] This approach eliminates inappropriate cases early to focus on those most likely to be successful. However, such an approach does not take advantage of techniques such as

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Table 1. **Examples of the biological and engineering techniques now available to enhance the productivity of biocatalytic processes**

technique	example references[a]
biocatalyst modification	
mutagenesis/rational design	*Cedrone et al., 2000;*[70] Hornung et al, 1999[71]
directed evolution	Sieber et al., 2001;[59] *Bornscheuer and Pohl, 2001*[23]
metabolic engineering	*Chartrain et al., 2000;*[72] Wang et al., 2000[58]
immobilization	Carleysmith et al., 1980;[73] *Monsan and Combes*, 1988;[74] Vaghjiani et al., 2000[75]
nonconventional reaction media	
two-phase systems	Doig et al., 1998;[76] *Lye and Woodley, 2001;*[30] Schmid et al., 1998[77]
organic solvents	*Brink and Tramper, 1985;*[78] *Klibanov, 2001*[79]
ionic liquids	Erbeldinger et al., 2000;[80] *Roberts and Lye, 2002;*[81] *Sheldon, 2001*[82]
solvent-free systems	*Gill and Vulfson, 1994;*[83] Erbeldinger et al., 1999[84]
micellar systems	Eggers and Blanch, 1998;[85] Larsson et al., 1990;[86] *Shield et al., 1986*[87]
substrate feeding	Mitra et al., 1998[10]
catalyst recycle	Hoeks et al., 1992;[88] Mahmoudian et al., 1993[89]
deracemisation/resolution	Reetz and Schimossek, 1996;[90] Beard and Turner, 2002[91]
in situ product removal	*Freeman et al., 1993;*[31] Held et al., 1999;[92] *Lye and Woodley, 1999;*[32] Van Der Wielen et al., 1990;[93] Vincenzi et al., 1997[94]
process monitoring and control	Hack et al., 2000;[95] Schuster, K. C. 2002[96]

[a] References shown in italics represent useful reviews, all others are examples of particular applications.

directed evolution, and we now see it timely to integrate changes to the biocatalyst into this approach. To evaluate the benefit of any such changes quantitative methods are clearly required.

The use of modelling in chemical engineering[33,34] and increasingly many branches of bioprocessing is an established tool to quantitatively evaluate and optimise processes.[35−37] It has the advantage that, once the model is built, assessing process variants (via virtual experimentation) is both rapid and cheap. We are now applying such approaches to develop mathematical descriptions of bioconversion reaction kinetics. Frequently these follow few conventions, that is, Michaelis−Menten kinetics, since operation is well outside the concentrations for which the enzyme was naturally evolved. Consequently we have been developing models that are data-driven and empirical rather than fundamentally based.[38−40] The models describe the impact of catalyst, reactant, and product concentration(s) on reaction rate and biocatalyst stability. We have also developed a graphical representation of the outputs from such models, termed a "window of operation".[41] The window indicates the boundaries to reactor operation informed by physical and economic constraints on a case-by-case basis.[39,42] The impact of changing operation and downstream processing can also be evaluated in this way alongside changes to the biocatalyst. Informed by economic data this provides a powerful decision-making tool. More recently we have started to devise simple mass balance models to assess whole process limitations.

While modelling tools for particular application in the area of biocatalysis are in their infancy, their potential is clear. Provided some initial quantitative data is available, such process models will allow:

- options for different processes and process sensitivities to be evaluated
- with economic data, a first estimate at whether the process is viable (this may be particularly critical alongside competing chemical processes)
- bottleneck analysis to identify the primary constraints causing a process limitation (this will set the development programme)
- targets to be set for the selection of alternative catalysts or catalyst modification

Parallel developments in the area of Life Cycle Analysis[43] will increasingly be incorporated into these models to allow the environmental impact of a particular option to be assessed.

(3) Microscale-Processing Techniques. Parallel advances in molecular biology (such as directed evolution, described in (1)) together with the use of laboratory automation to rapidly screen biocatalyst libraries[44] are now placing severe pressures on the process development stages of the product life cycle. The use of automated, microlitre-scale experimentation, to obtain key process design data early, has the

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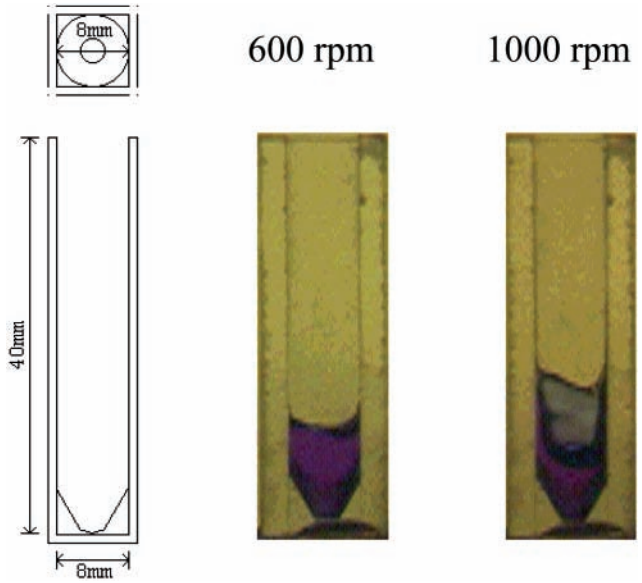

Figure 1. **Microscale reactor geometry showing a single well from a 96-deep-square-well microtitre plate (total well volume 2000 μL). Photographs show increasingly turbulent fluid motion with increasing agitation speed (orbital motion, 3 mm throw).**

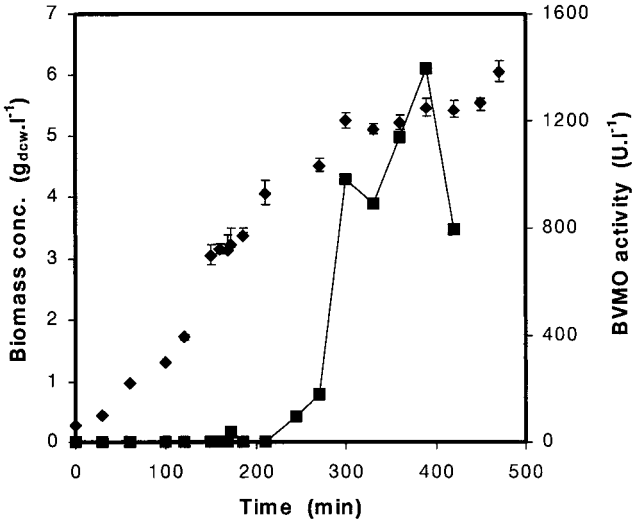

Figure 2. **Production of a recombinant *E. coli* biocatalyst and induction of BVMO activity at the 400-μL scale: (◆) biomass concentration, (■) BVMO activity. Cells produced by aerobic fermentation on a glycerol-based medium in a 96-deep-square-well-plate as shown in Figure 1. Cells induced by the addition of a 0.1% L-arabinose promotor after 160 min. BVMO activity measured using cyclohexanone as substrate.**

potential to overcome this bottleneck and radically improve the speed at which biocatalytic processes can be established. The advantage of such an approach is that it can utilise automation platforms virtually identical to those developed for combinatorial chemistry and high-throughput screening applications. Currently we are exploring the operation of whole biocatalytic process sequences, from biocatalyst production through to product recovery, in microwell formats.[45] The goal is the creation of a new process development tool that will:

- allow the "whole process" evaluation of larger numbers of biocatalysts in short periods of time
- reduce the quantity of often expensive synthetic substrates required for process development
- rapidly provide design data for use in process and economic models (as described in (2))
- speed the translation of new processes from laboratory to pilot-plant scale

In contrast to industry, which has invested heavily in laboratory automation for screening purposes, microscale-processing techniques will require an understanding of the fundamental engineering aspects of data collection in microwells such as mixing and gas−liquid mass transfer. This is crucial if the data collected at the microlitre scale is to be accurate, quantitative and, above all, capable of being related to larger scales of operation. Figure 1 shows the geometry of a single well, from a 96-deep-well microtitre plate, in which microscale-processing operations are typically carried out. The high-speed camera images show the increasingly turbulent fluid motion with increasing agitation speed. The creation of a deep vortex allows high oxygen mass transfer rates and short liquid mixing times comparable to those found in larger-scale reactors. It is the knowledge of these fundamental engineering parameters, and how they scale, that will speed the translation of laboratory processes to more focused pilot-plant trials.

Previously we have examined the production and use of a recombinant *Escherichia coli* biocatalyst, expressing cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*, at scales up to 450 L.[46] This biocatalyst has also been used to perform Baeyer−Villiger type oxidations of a range of bicyclic ketones at scales from 1 to 70 L.[47] Currently we are examining microscale versions of this process sequence. Figure 2 shows typical results for the production of the biocatalyst by aerobic fermentation and induction of the Baeyer−Villiger monooxygenase (BVMO) activity at 400-μL scale under previously optimised conditions. The cell growth rate, biomass yield, and specific BVMO activity calculated from the data in Figure 1 are very similar to those determined at the large scale.[46] The automation and linkage of both the fermentation and induction steps has subsequently allowed a wide range of media to be evaluated and the time of induction to be optimised. A number of other researchers have also begun to examine microscale fermentation processes for biocatalyst production with equally promising results. These have focused on the fermentation of *E. coli* [48] and Streptomycete strains[49] with

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some emphasis on the engineering of microwell systems.[20,50] Work on the storage and revival of microbial libraries in microwell formats has also been reported.[44,51] This is obviously important in relation to the exploitation of biocatalyst libraries prepared by directed-evolution methods.

Having produced the BVMO biocatalyst, we subsequently examined the ability to accurately collect biocatalyst kinetic data at the 250-μL scale.[52] The BVMO reaction is particularly interesting in this respect due to the stoichiometric requirement for molecular oxygen for product synthesis. Working with a typical substrate for this enzyme, bicyclo[3.2.0]hept-2-en-6-one, we were able to demonstrate the effects of microwell geometry and agitation rate on oxygen transfer. Results from experiments to quantify the effect of substrate concentration on specific biocatalyst activity were then validated against data collected at the 1-L scale. The microwell approach thus represents a 4000-fold reduction in scale and material requirements. In our case the experimental throughput following process automation (and hence parallel experimentation) is likely to be limited by the length of the GC assay used for analysis of the lactone products. However, for reactions such as hydrolysis in which there is a measurable pH change a number of groups have demonstrated the use of dissolved[53] or immobilised[48] pH indicator dyes for the in situ spectrophotometric determination of reaction kinetics. The instrumentation and analysis of microwell systems is clearly a key area for future research.

Finally, we have also considered microwell approaches to the recovery of bioconversion products. To date we have focused on the design and optimisation of equilibrium-based separation processes such as liquid−liquid extraction or solid-phase adsorption.[54] For example, using an XAD-4 resin, binding isotherms for the substrate and one of the regioisomeric products, (−)1(*R*),5(*S*)3-oxabicyclo[3.3.0]oct-6-en-2-one, of the BVMO bioconversion could be measured at the submillilitre scale. This enabled quantification of solute binding capacity and adsorption constants as a basis for the design of an ISPR process.[54] Microscale mimics of other commonly used downstream-processing operations will also be crucial if we are to achieve a "whole process" evaluation of recombinant biocatalysts in microwell formats.

(4) Role of Bioinformatics. The application of biocatalysis to organic synthesis begins with the need to identify a suitable enzyme, pathway, or microorganism that can perform the desired transformation(s). Several bioinformatics tools have emerged, both commercial and academic, that can meet this need. For example, enzyme transformation databases such as Synopsys (Synopsys Scientific Ltd., UK) can be searched by keyword and structure of substrates or products, using search tools such as REACCS, ORAC, and ISIS to identify biocatalysts that utilise these compounds. Similarly, metabolic databases such as KEGG, UM-BBD, EcoCyc, and MetaCyc[55−57] can be used to identify pathways in microorganisms that utilise or produce a target compound. The identified enzymes or pathways could then serve as the basis for the desired bioconversion, on which directed evolution can be used to improve its use under process conditions. Such enzymes or pathways can potentially be modified in the natural host by directed-evolution methods. Alternatively, a synthetic pathway can be constructed, inserted into, and modified in a different cell strain.[24,58]

Directed evolution strategies themselves are also set to advance rapidly as new genetic approaches are developed and integrated with bioinformatics and protein modelling tools. An increase in the availability of DNA sequences is expanding the number of matches to a given query sequence. These data immediately present further options for genes to include in any DNA shuffling strategy, where DNA recombination typically requires DNA sequences with at least 70% homology.[59] Knowledge of their sequence also readily permits the cloning of the identified genes for the DNA shuffling reactions via PCR.

Perhaps a more important role of bioinformatics in the longer term, will be an improved ability to predict function from sequence data as protein modelling methods evolve and as the elucidation of protein structures accelerates in the so-called protein-structure factories.[60] These developments will improve our ability to predict key residues towards which directed evolution could be biased. The available methods are already beginning to address these possibilities. For example, comparative protein modelling already permits the prediction of 3-D structures for proteins that have amino acid sequence homology to known protein structures.[61−63] This can be achieved to above 80% accuracy where sequence identity is above 30%.[64] De novo prediction of protein folds is also improving, with methods such as Rosetta producing

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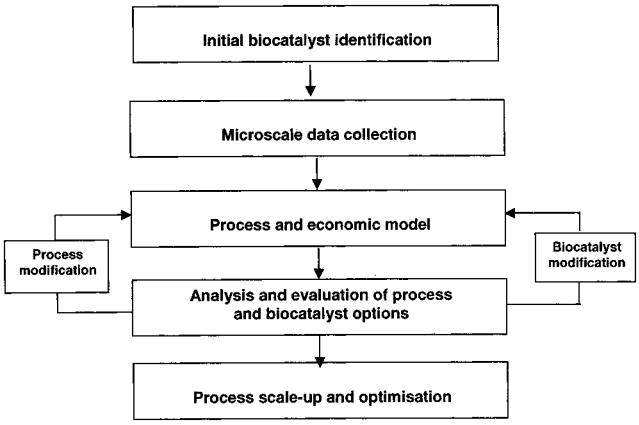

Figure 3. **Proposed process development paradigm demonstrating the use and interaction of microscale experimentation with process modelling and the role of directed evolution in biocatalyst modification. Bioinformatics tools can be used to both aid initial biocatalyst identification and accelerate directed-evolution approaches.**

fold predictions with enough accuracy to discern regions of sequence involved in the function of the protein.[65] More recently, computational modelling of enzyme active sites has predicted mutations that produce novel catalytic activity,[67] and a calculation of residue entropies has been proposed to correlate with residues that should be preferentially randomised during a directed-evolution experiment.[68] Together the above methods bring protein function prediction a step closer.

Towards a New Development Paradigm

The linkage and interaction of the four tools described in this review are schematically illustrated in Figure 3. Microscale-processing techniques will first allow the rapid collection of quantitative data on initially available biocatalysts and competing process options to overcome productivity constraints. This can be fed into process, economic, and environmental models for evaluation of the competing process alternatives. In most cases a modified process or catalyst will be necessary to meet the desired performance, and deciding which route to take through development will be critical. Ultimately, the tools described here will allow us to quantitatively define the required improvements to the biocatalyst or process or both and enable accurate predictions of their benefits to the selected route once implemented. This means that decisions will be based on a firmer foundation than at present. A second benefit of the process development cycle shown in Figure 3 is that it will allow, for the first time, design of the catalyst to match the process, rather than design of the process to be restricted by the constraints of the biocatalyst.[69]

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