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New Frontiers in Biocatalysis for Sustainable Synthesis

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Today enzymatic catalysis is widely used in the pharmaceutical industry and is expanding fast into the fine and specialty chemicals sector, driven by the need for evermore sustainable chemistry. This review highlights the increasing emphasis now placed on achieving better process performance metrics to reflect the demand for cost-effective and scalable processes, ready for direct implementation into industry. The review also highlights other developments at the frontiers of this field including flow chemistry and multi-step enzymatic reactions, which benefit sustainability.

Introduction

Biocatalysis uses enzymes to catalyze chemical reactions in a highly selective manner, usually under mild reaction conditions. The number of biocatalytic applications continues to expand and today the use of enzymatic synthesis is commonplace in the pharmaceutical industry [1] and developing rapidly in the fine and specialty chemicals sector. Aside from catalysis with high selectivity under benign conditions, there are several other reasons for this expansion. For example, most biocatalytic reactions occur in water, which makes for simplified liquid waste treatment. Likewise, enzymatic catalysts are renewable. Indeed, the renewable nature of enzyme catalysts should not be overlooked, since it brings significant benefits from and environmental perspective as well as stable catalyst costs. All these benefits were summarized recently in an important review highlighting the enormous potential of biocatalysis to satisfy the requirements of sustainable chemical synthesis [2](Table 1). However, despite this progress, some barriers to implementation still need to be overcome [3,4]. For example, in the pharmaceutical sector increasing development speed is paramount [5]. However, it is in the synthesis of lower value products that the major challenges for the future exist. An excellent recent review about the use of biocatalysis targeted at the production of bulk n-alkyl amines, highlighted the need for higher substrate loadings and higher productivities (space-time yields)[6]. Indeed, while many industrial chemical processes are extremely well optimized and thereby bring significant economic return, in many cases they are not sustainable due to poor selectivity, high energy

demand and the use of non-renewable reactants, reagents and catalysts. By contrast, enzymatic reactions discovered in the laboratory are frequently found to be good from the perspective of green chemistry, but are often limited in terms of economic potential (schematically illustrated in Figure 1). These demands therefore place significant emphasis on the need for translational research to convert laboratory reactions into industrial processes. Several recent trends reflect these needs (Table 2) and will be further discussed in this brief review.

Designing 'better' biocatalytic processes

Against this background, it is interesting that a recent trend in the field of biocatalysis has been the design and development of 'better' biocatalysts, biocatalytic reactions and biocatalytic processes. The target now is invariably to get enzyme reactions to operate closer to commercially-viable industrial conditions. Such conditions demand process performance metrics far from those seen in the laboratory. Nevertheless assessing the productivity (e.g. g product / L reactor. h), yield of product on biocatalyst (e.g. g product / g biocatalyst), yield of product on substrate (e.g. g product /g substrate), as well as product concentration (e.g. g product / L reactor) can be very valuable to benchmark processes and set targets for improvement [7,8]. Unlike chemical catalysis, where the process is in general designed to fit particular catalyst properties, in biocatalysis the option of engineering the enzyme (so called protein engineering) can enable enormous improvement in the biocatalyst properties [9]. Clearly, judicious selection of the starting point for protein engineering or evolution can also help the effectiveness of enzyme improvement [10]. Today, protein engineering technologies, using directed evolution linked with experimental screening, or using computational tools to guide protein modification, or even combinations of these approaches have gained widespread acceptance and considerable traction [11]. The latest developments in automated technology are now also contributing to the speed with which such methods can be applied [12]. In many ways such protein tuning technologies provide an extra degree of freedom in process design [13]. More recently such approaches have been used not only to assist in the adaption of a given enzyme to exciting new chemistry, but also assisting with modifying those enzyme properties that directly affect the process performance [14]. For example the synthesis of Vibergon using a ketoreductase that specifically targeted at high pH dynamic kinetic resolution (DKR) was recently reported [15].

A relatively new class of enzymes for synthesis of cyclic and acyclic amines are the NAD(P)H imine reductases (IREDs)[16,17]. A remarkable publication recently described the technical development required to scale-up such enzymatic systems for reductive amination [18]. Using a combination of protein engineering and process engineering the authors reported excellent volumetric productivities of 12.9 g/L.h for the reductive amination of model compounds cyclohexanone with cyclopropylamine to form a secondary amine. It is interesting that both protein engineering and process engineering were required to achieve such a result, and that the excellent volumetric productivities were achieved at such high turnover numbers (TONs), above 48 000. TON is defined as the number of moles of substrate (or reactant) converted by a mole of enzyme, before it is inactivated. In the example here it was calculated based on the molar

concentration of product formed divided by the molar concentration of enzyme used to form that product.

Another excellent example concerns the synthesis of aliphatic nitriles using an aldoxime dehydratase from *Bacillus* sp. (OxB-1) at substrate loadings of 1.4 kg/L [19]. This sets a new precedent for substrate concentration in biocatalysis and indeed the use of high substrate concentrations, and thereby resulting high product concentrations, is of vital importance not only to ensure cost-effective downstream processing, but also to minimize the water use in the process and hence reduce the *E* factor (kg waste/kg product)[20]. Likewise a new process for the synthesis of (S)-2-chloro-1-(3,4-difluorophenyl)ethanol using a ketoreductase has also been reported operating with substrate loadings of 0.5 kg/L [21], and an enzymatic process for the production of (R)-2-butyl-2-ethyloxirane reported at 0.3 kg/L [22].

Other process related improvements continue to focus on in situ product removal (ISPR) technologies with an excellent pilot scale demonstration reported using enzymatic reactive distillation [23], as well as more fundamental studies on enzymatic reactive crystallization [24-26], and an important review focused on in situ product crystallization for the improvement of biocatalytic processes [27].

Further work is still required to develop even better biocatalytic reactions and processes targeting not only high yields, but also to reduce the excess of co-substrates in two substrate reactions, as highlighted in a recent review defining future targets for accelerating the implementation of biocatalysis into industry [28].

Flow biocatalysis

A second major trend in biocatalysis in the last few years has been to embrace the development in synthetic organic chemistry towards flow synthesis [29]. Several excellent reviews have been published covering the fundamentals of this approach with a particular focus on biocatalysis [30-33]. It is clear that while enzymatic reactions are relatively slow, in many cases significant advantages can be gained from shifting to flow on account of a defined residence time, as well as reduced downtime for tank loading, emptying and cleaning. The former capitalizes upon the selectivity already at work when using enzymes for synthesis and the latter enables smaller reactor footprints, with the option of on-line instrumentation to control production in real time. Many of the flow approaches suggested to date rely on some type of enzyme entrapment, usually via immobilization on (or in) a support [34-36]. The drive towards a more generic method remains important [37], but dependent upon the required residence time, pressure drop considerations in flow can also be limiting. Hence in some cases alternative reactors will be required for continuous operation. A further driver for alternative reactors may come from the kinetic profile of the enzyme, where high Michaelis constants mean operation needs to take place in a plug-flow regime. Such an approach is possible in non-tubular systems using a series of stirred tank reactors. Several recent publications have highlighted the importance of choosing

the appropriate reactor configuration to best fit the kinetic profile of a given enzyme-catalyzed reaction [38,39]. Several reports have also indicated novel methods of control including using light-dependent activation.

In the last few years' interest in oxidative reactions using enzymes (driven by sustainability requirements) has expanded enormously [40]. Biocatalytic oxidation attracts particular interest as a sustainable route to alcohols and aldehydes [41]. An excellent recent example concerns the synthesis of (R)-1-(4'-hydroxyphenyl)ethanol using vanillyl alcohol oxidase [42]. Indeed oxidases are of particular interest since they do not require additional cofactors. The interest in oxidation has also led to several approaches attempting the difficult problem of oxygen supply to an aqueous biocatalytic system in flow. Such approaches have included the biocatalytic degradation of H_2O_2 using catalase to yield supersaturated concentrations of oxygen [43], tube-in-tube reactors operating up to 10 bar pressure [44], single tube reactors up to 34 bar pressure [45], and photo-biocatalytic methods to generate oxygen in situ in the liquid phase [46]. The best approach in a particular case is still unclear, but is of course dependent upon the economic requirements of a given reaction.

Multi-step biocatalysis

Many current biocatalytic methods use a single step in a synthesis that may contain many other catalytic (or even stoichiometric) reaction steps. From a green chemistry perspective it is of course important to try to make as many of the steps as possible biocatalytic, also to avoid the need for changing conditions (e.g. temperature, pH, pressure, solvent) in the middle of the synthetic scheme. Multi-step biocatalysis allows cascades of enzyme reactions to be integrated and huge progress has been made in this field in recent years [47,48]. An excellent example of a small cascade concerns the asymmetric synthesis of L- and D-homoalanine. Here for the first time, the synthesis of both enantiomers of an important unnatural amino acid was demonstrated using combinations of L-methionine ϒ-lyase, and D or L-amino acid aminotransferase [49]. More complex syntheses have also been proposed, and in some cases successfully demonstrated. In many of these cases, the use of modelling has been instrumental in designing a suitable experimental approach since the number of variables escalates very quickly [50]. A particularly interesting example concerns the seven enzyme system based around carboxylic acid reductases (CARs) [51]. In vitro such enzymes require the regeneration of cofactors, and in many cases this can become limiting. A recent study on an enzymatic cascade in vivo connecting the oxidation of secondary alcohols by an alcohol dehydrogenase (ADH), a Michael reduction by an enoate reductase (ERED), and a Baeyer-Villiger oxidation by a Baeyer-Villiger monooxygenase (BVMO), identified the limitations caused by cofactors, using kinetic modelling [52].

Tools for evaluation of green and sustainable biocatalysis

Whilst improvements are made to the economic return associated with implementing new biocatalytic processes, it is clear that systematic evaluation of reactions and processes is of vital

importance, if only to benchmark progress and set targets for those involved in research and development. A set of economic metrics has been developed in recent years with which to assess progress towards the ultimate goal of industrial implementation. Such metrics are of necessity somewhat simplified and therefore do not always give an accurate picture in every case, but they serve (especially at a very early stage in the laboratory) to guide further research and development. A parallel set of green chemistry metrics might be based on *E* factor [53] or the process mass intensity (PMI)). This is also simplified but can be combined with other metrics such as water intensity or solvent intensity. A suggested set of economic and green chemistry metrics (and their definitions) is given in Table 3. An increasing number of processes are being compared using still more detailed analyses such a life cycle analysis (LCA). Several comparisons between enzymatic and chemical conversions have now been reported, although a recurring challenge is the limited data on enzyme production, which can be of great importance in enzymatic reactions that have a low yield of product on biocatalyst. Meanwhile a prospective LCA was recently published showing the value of comparisons between alternative processes at an early stage, with a view to guiding further research and development [54].

Conclusions

Biocatalysis continues to develop as a major opportunity to improve the sustainability of methods for organic synthesis and production. In recent years, many new classes of enzymes have become available, and protein engineering techniques continue to open the possibilities for tuning enzyme properties dependent upon operation and scale-up needs. Increasing efforts on improving processes now mean that examples from the laboratory start to match the target metrics set by economic needs. Other developments are in the area of flow biocatalysis and also multi-step biocatalysis, both of which also deliver improved sustainability. Finally, developments in the benchmarking of processes using green chemistry and economic metrics also enable quantitative assessment of progress to be made.

Conflict of interest

Nothing declared.

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Table 1. Sustainable features of biocatalytic processes

Reactions	Few reaction steps (often circumventing protection and deprotection strategies)
	Good atom economy (effective chemistry based on Nature's efficiency)
	Mild operating conditions (safe and favors green chemistry metrics)
	Aqueous media (safe and improves waste disposal)
Catalyst	Renewable (expressed in bacterial and fungal hosts grown on sugar, air and water)
	Highly selective (the key feature of enzymes)
	Tunable (using directed evolution and protein engineering)

Table 2. Sustainability benefits of some recent developments in biocatalysis

Reaction intensification	Reduced downstream costs Reduced *E* factor
Flow biocatalysis	Reduction in reactor footprint Better control
Multi-step catalysis	Reduction in isolation steps and solvent changes

Table 3. Potential metrics to benchmark biocatalytic processes

Economic metrics	Productivity (g product / L reactor . h)
	Yield of product on biocatalyst (g product / g enzyme)
	Yield of product on substrate (g product / g substrate)
	Product concentration (g product / L reactor)
Green chemistry metrics	*E* factor (g waste / g product)
	C factor (g CO_2 equivalents / g product)
	Water intensity (g water used / g product)
	Solvent intensity (g solvent used / g product)

Figure 1. Generalized schematic representation of the need for improvements in conventional chemistry and biocatalysis for implementation of sustainable processes by achieving adequate economic and green chemistry (GC) metrics.