# Opportunities for the pharmaceutical industry: *key biotransformation technologies of the future*

Biotransformation is a broad and growing field of biotechnology and encompasses both enzymatic and microbial biocatalysis. Progress has been made in research on the key drivers of biotransformations, including the isolation, screening and characterisation of microbes and their enzymes, their utilisation in extreme environments, the manipulation alteration, augmentation of metabolic pathways and the use of combinatorial biosynthesis and biocatalytic methodologies for the development of new compounds. The convergence of biology and chemistry has enabled a plethora of industrial opportunities to be targeted, while discoveries in biodiversity and the impact of molecular biology and computational science are extending the range of natural and engineered biocatalysts that can be customised for clean industrial processes.

**By Praveen Kaul, Anirban Banerjee and Professor UC Banerjee**

During the past decade, there has been an increasing awareness of the enormous potential of enzymes for the transformation of man-made 'synthetic' materials with high chemo-, regio- and enantio- specificity. This trend is hardly surprising considering the increased integration of biological catalysis into a variety of industrial processes ranging from the manufacture of commodity chemicals to the synthesis of highly complex intermediates and drug substances. Largely the current expansion can be attributed to the recent progress in molecular biology, high-throughput screening techniques, advanced instrumentation and engineering. Increasing understanding of the mechanism of drug action on a molecular level has lead to the widespread awareness of the importance of chirality as the key to the efficacy of many drugs. In many cases where the switch from racemate drug substance to enantiomerically pure compound is feasible, there is an opportunity to extend the market exclusively of the drug. Increasing regulatory pressure to market homochiral drugs has led to the use of chemical and chemoenzymatic synthesis of chiral drugs[1]. Worldwide sales of chiral drugs in single-enantiomer dosage form is continuously growing at a brisk rate and is

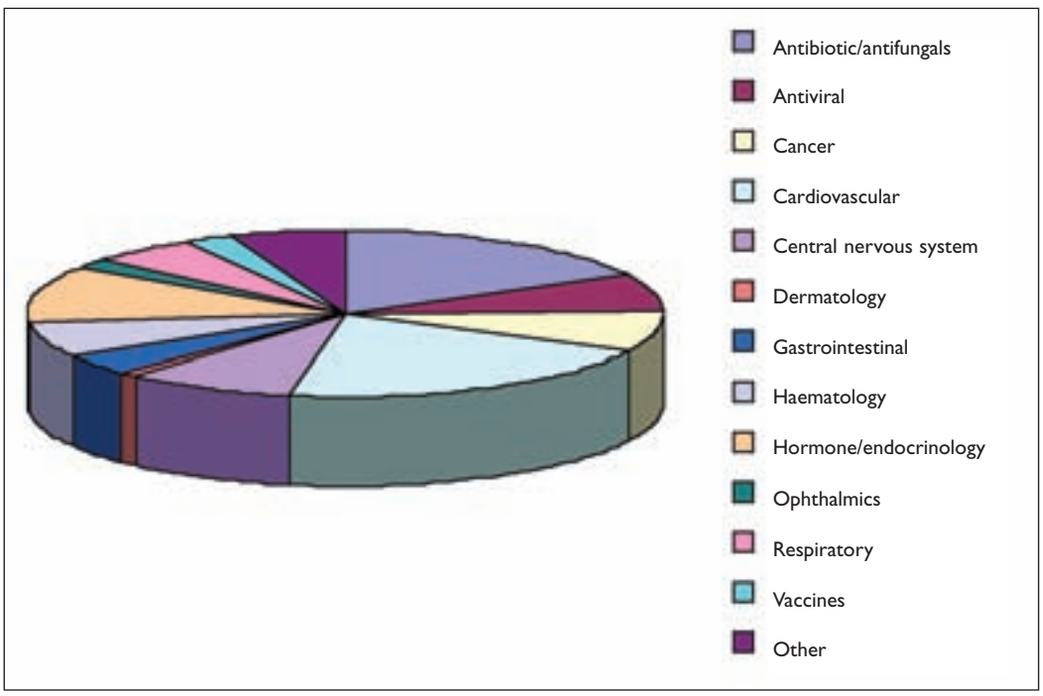


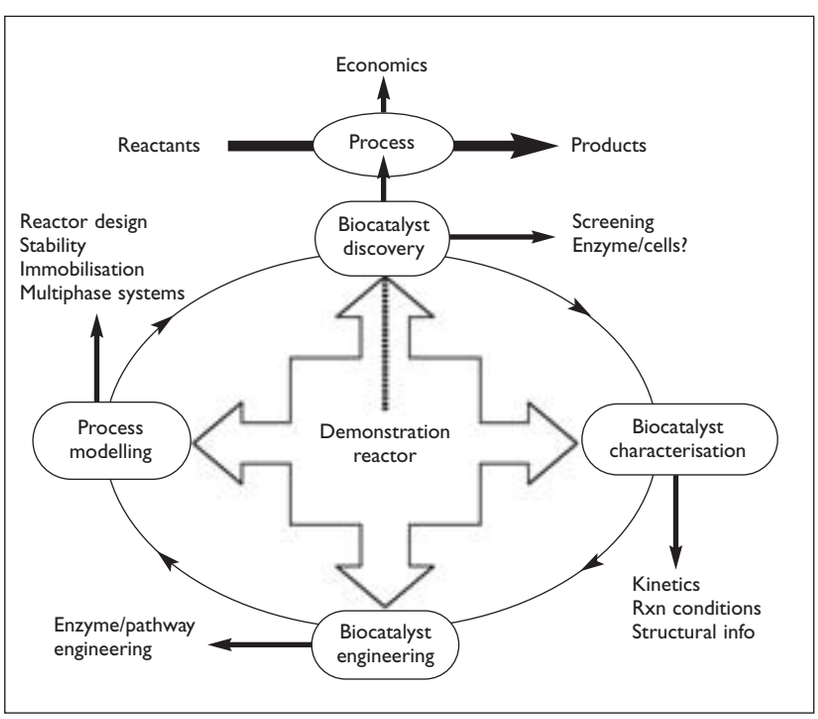

**Figure 1**
Projected sales of different classes of single enantiomer drugs for the year 2005

expected to touch annual sales of $171,865 (**Figure 1**)[2]. The success of biocatalysis depends ultimately on the economics of a specific process. It provides enormous opportunities and with the introduction of each new process, as experience and confidence accumulates, it becomes easier to develop and justify the next biocatalytic process.

## The biocatalysis cycle

Biocatalytic processes differ from conventional chemical processes, owing mainly to enzyme kinetics, protein stability under technical conditions and catalyst features. In the laboratory, new biocatalytic reactions often originate with new enzyme activities. For applications, a more rational approach is needed. The starting point will usually be a product, which can perhaps be produced by several possible biocatalytic reactions that convert a suitable substrate to the desired product. One or more biocatalysts must be identified or developed, a process must be set up and the resulting bioconversion will ultimately have to be economically feasible. The economic feasibility of a biocatalytic process depends on several factors (**Figure 2**). Depending on the type of the biocatalyst to be used, specific reactor and hardware configurations are needed[3]. In addition, biocatalytic processes are highly heterogeneous. In theory, this would necessitate specific designs of the catalyst-hardware interface[4]. But in practice, a limited number of hardware designs are found today in large industrial processes. In analogy to chemical processes, most biocatalysts are used in immobilised form as heterogeneous catalysts that can be recovered and reused. There are also processes, however, based on homogeneously suspended cells or enzymes, which are sufficiently inexpensive to permit single use, without recovery or reuse.

**Figure 2**
The biocatalysis cycle



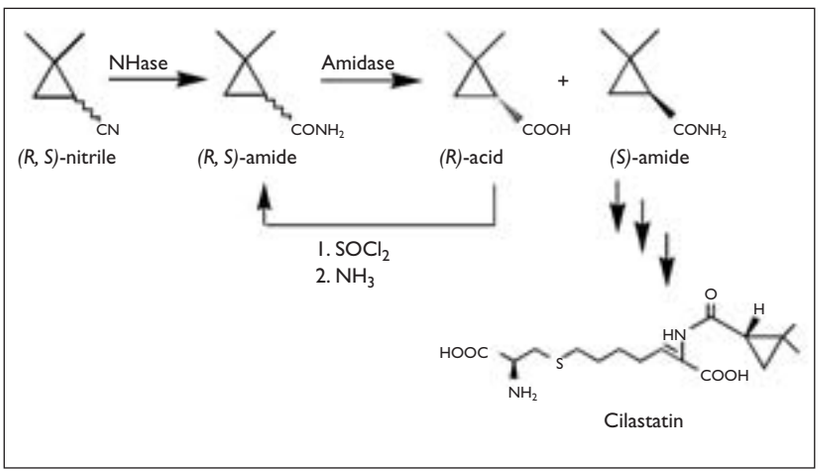



**Figure 3:** Route for production of cilastatin intermediate

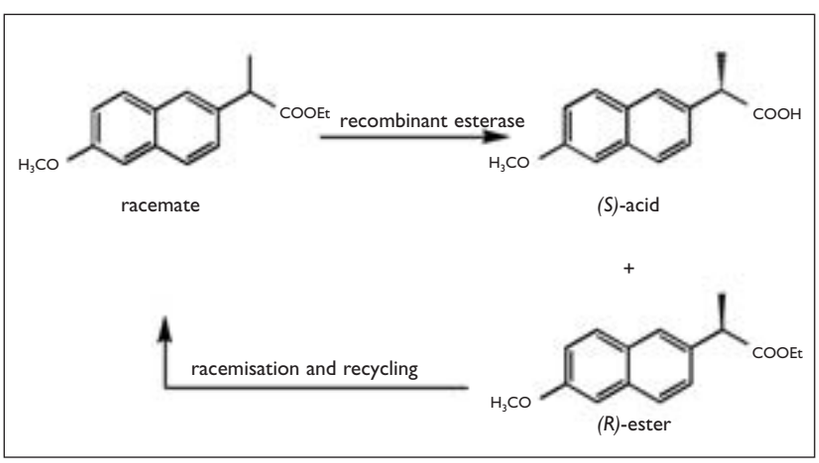



**Figure 4:** Resolution of naproxen by recombinant esterase of Chirotech

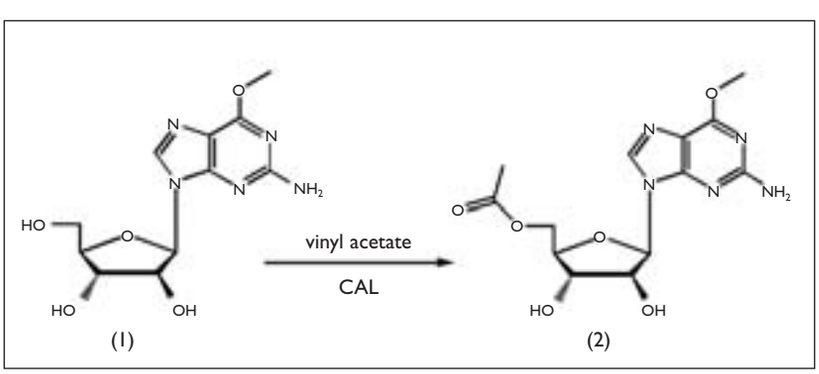



**Figure 5:** Lipase mediated production of anti-leukaemic agent

## Enzyme catalysed processes

A number of enzymes are known to produce a variety of chiral compounds through bitransformation. Nitrile hydrolases, oxidoreductases, lipases, hydantoinases, epoxidases and esterases are few among them. Hydrolases, in particular have shown to accept a wide range of substrates. An example is a process of Lonza for the synthesis of (S)-2,2-dimethylcyclopropanecarboxamide which is an intermediate for the production Cilastatin, a ß-lactamase inhibitor[5] (**Figure 3**). The product is produced at 15m$^3$ scale the yield is 35% with an ee value of >98%. The (R)-acid can be recycled chemically into the substrate for amidase, which leads to higher yields and minimises waste.

Chirotech has attempted to develop a biocatalytic route to the non-steroidal anti-inflammatory drug (S)-naproxen. They have screened and cloned an (S)-specific esterase, which allows the selective hydrolysis of (S)-ester. At the end of the reaction, the remaining (R)-ester and (S)-acid can be separated by centrifugation and the ester can be racemised. This process is currently in pilot scale at Shasun Chemicals (India) (**Figure 4**)[6].

A good example for combined *chemo-* and *regio-*selectivity of an enzymatic transformation is the acylation of purine (1) that is currently being developed by Glaxo Wellcome as an anti-leukaemic agent[7,8]. Using immobilised *Candida antarctica* lipase (CAL), type B and vinyl acetate (acyl donor), 99% conversion is achieved to the 5′-monoacetate (2), which renders the compound more soluble and thus increases bioavailability (**Figure 5**). This transformation is almost impossible to achieve by conventional chemical acetylation reagents because of their known preference towards N-acylation. Regioselectivity in this process is remarkably high, less than 0.1% of 3′-acetate and 0.3% of 3, 5′-acetate are formed[9]. The examples given here indicate the wide range of applications of biotransformation.

## Key to better biocatalysts

Until recently, scientists wishing to utilise biotransformations needed to rely upon the availability (via traditional screening efforts) of a particular organism or isolated enzyme to catalyse a desired reaction or synthesis of a particular product. Moreover, it was necessary to utilise reaction conditions that were predominantly aqueous in nature. Although such approaches remain critical today, new technologies have made an impact on our ability to utilise, tailor and develop new types of biotransformation reactions. These new technologies and the recent progresses made in these areas are briefly described below.

**1. High Throughput Screening (HTS)**

Biochemical engineers develop a process against limited capital and against very aggressive timelines. It starts with the assimilation of the biocatalytic machinery- enzymes, which are run through

a designed screen to obtain a potent and valid hit and has promising practical applications. The slowest step in finding a selective enzyme for a particular biocatalytic transformation is often the screening step and therefore the rate limiting step. Hence a rapid screening method is desired that can run in parallel with process optimisation experiments or a novel biocatalyst discovery programme. There are three important stages in a screening strategy: (i) designing the type of process and deciding the type of enzymatic activity desired; (ii) deciding which group of micro-organisms are to be isolated and screened; and (iii) designing an appropriate, convenient, sensitive and rapid assay that will allow as many micro-organisms as possible to be screened.

HTS has been found from the beginning to the development of robotic systems for sample handling. The most convenient reactions to test in HTS mode are those involving chromogenic or fluorogenic substrates[10-12]. Some enzyme reactions may be assayed by recording changes in physiological parameters as a function of reaction progress. The reaction of lipases and esterases on ester substrates liberates carboxylic acids, which results in a drop in the pH of the reaction medium. Provided the solution is not too strongly buffered, a pH indicator can be used to produce a reasonable signal upon reaction[13,14]. HTS for biocatalysis is often not only aimed at activity, but also for stereoselectivity, in particular enantioselectivity for the application of enzymes in fine chemical synthesis. We have recently developed an enantioselective method for screening nitrilase producing organisms using pH sensitive indicators[15].

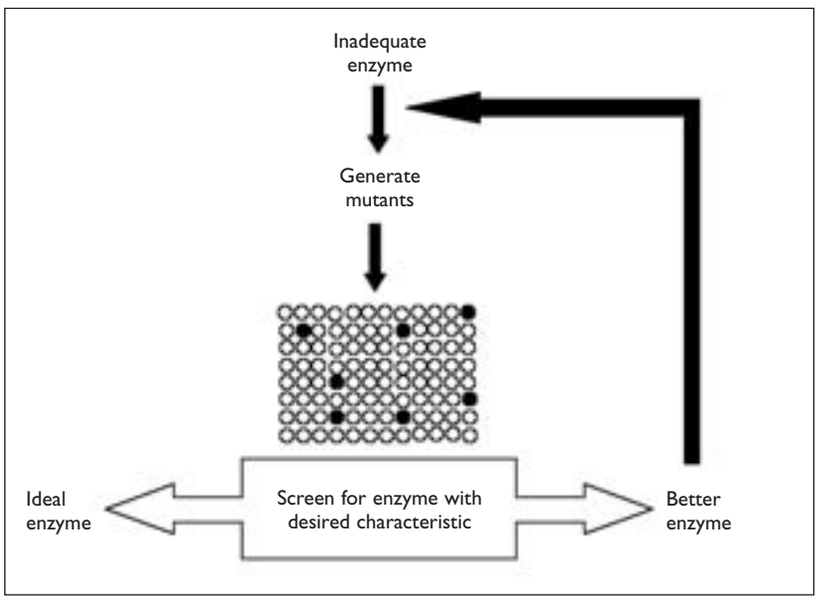



**Figure 6:** Scheme of events in a usual directed evolution approach

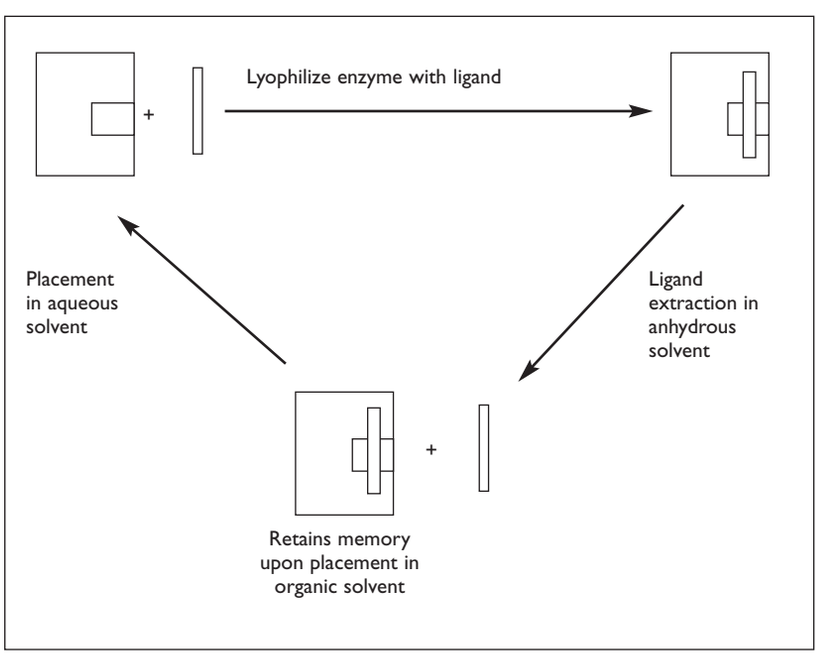



**Figure 7:** Schematic representation of the ligand-induced imprinting of the enzyme active site

**2. Directed evolution**

Directed enzyme evolution generally begins with the creation of a library of mutated genes. Gene products that show improvement with respect to the desired property or set of properties are identified by selection or screening, and the gene(s) encoding those enzymes are subjected to further cycles of mutation and screening in order to accumulate beneficial mutations. This evolution can involve few or many generations, depending on how far one wishes to progress and the effects of mutations observed in each generation (**Figure 6**).

The main requirements for successful directed evolution are: (i) functional expression of the enzyme in a suitable microbial host, (ii) the availability of an accurate screen and (iii) identifying a workable evolution strategy. The vast majority of possible evolutionary paths lead to poorer enzymes; the strategic challenge is to identify a path that results in the improvement of desired features. Recombination of mutations identified in parallel screens or selections can significantly accelerate laboratory evolution efforts. The DNA shuffling method[16] has been supplemented with two new methods for *in vitro* recombination, the staggered extension process (StEP)[17] and random-priming method[18]. Similar to rapid evolution of improved enzymes, improved metabolic pathways can be obtained by directed molecular

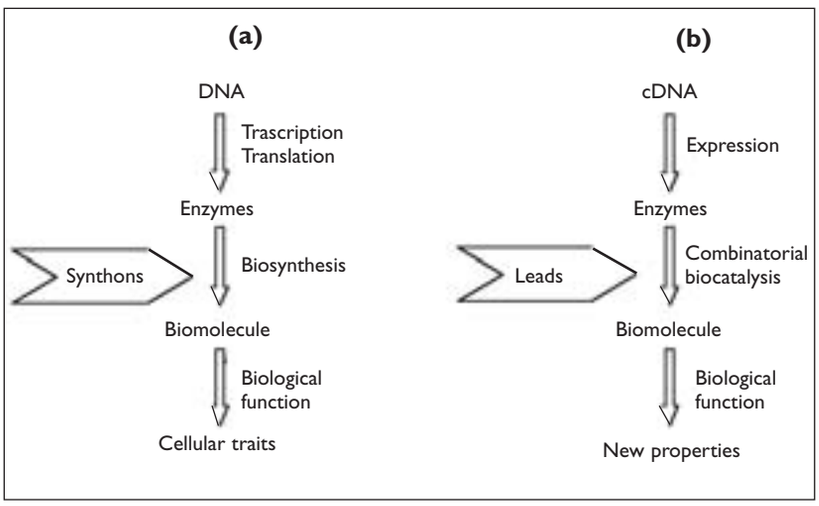



**Figure 8**
Pathways for combinatorial synthesis of new biomolecules from (a) synthons in nature and (b) from lead compounds *in vitro*

evolution. One striking example of this technique is the construction of an arsenate detoxification pathway[19]. Increased resistance of microbes to high levels of arsenate was achieved by gene shuffling and molecular evolution. An elegant strategy called compartmentalised self replication (CSR) has been demonstrated for directed evolution of enzymes involved in DNA replication[20]. This strategy links polymerase adaptive evolutionary improvement to amplification of its own gene. Genotype-phenotype linkage is affected by compartmentalisation in an emulsion. The progress this field has enjoyed reflects the rapid development of new experimental methods for creating genetic diversity and for searching through large populations for improved functions. Directed evolution is merging molecular biology, chemistry and engineering to forge many opportunities for future growth.

**3. Non-aqueous enzyme technology**

From a biotechnological perspective there are many potential advantages of employing enzymes in organic as opposed to aqueous media, including higher substrate solubility, reversal of hydrolytic reactions, modified enzyme specificity and improved enzyme thermostability. The absence of water is in itself conducive to new enzymatic reactions. However, adding alternative nucleophiles, such as alcohols, amines and thiols, leads to transesterification, aminolysis and thiotransesterification, respectively, reactions that are suppressed in aqueous solution[21]. By far the most significant advances have been made with isolated enzymes[22,23], where efforts are under way to understand the influence of organic solvents on enzyme structure, to activate and stabilise enzymes in dehydrated media for use in organic synthesis. The addition of small molecules to the freeze-drying process often results in improved catalytic activity and in some cases altered substrate specificities as compared to the lyophilised enzymes prepared in the absence of excipients. Substrate specificity can also be altered via lyophilisation in the presence of substrates as excipients, using a technique known as 'molecular imprinting'[24]. Molecular imprinting combined with non-aqueous conditions provides an avenue for altering enzyme substrate specificity, otherwise only attainable via protein engineering (**Figure 7**). The development of 'solvent free systems' has become of interest recently as a more environmentally benign technique for catalysing reactions that cannot be performed in aqueous solutions. For example, enzymatic glycosylation reactions usually give low levels of conversion, mostly because of competing hydrolytic reactions. But, when glycosylation was performed via condensation of sugar and alcohol in the absence of water by solid enzyme adsorbed on solid supports at elevated temperature, high conversion yields were achieved[25].

Finally, the use of enzyme catalysed acylation reactions in non-aqueous media as a replacement for existing chemical syntheses has become more attractive due to two important developments: the availability of activated acyl donors, and the more general application of chemoenzymatic synthesis strategies. Vinyl esters are now used routinely as acylating agents to yield highly selective transformations[26]. The ability of organic solvents, when used instead of water as reaction media, to affect and often enhance the catalytic properties of enzymes offers strategies for creating improved biocatalysts that sit alongside such techniques as site-directed mutagenesis, phage display, directed evolution and the production of catalytic antibodies. To take full advantage of the opportunities afforded by non-aqueous enzymology, several mechanistic issues need to be elucidated. A systematic inquiry should continue into the causes of diminished enzymatic activity in non-aqueous solvents and how to prevent it; in fact, there is no fundamental reason why enzymes could not be more active in such media than in water. Particular efforts are needed to develop a generally applicable, quantitative rationale for the solvent dependence of enzymatic selectivity[27,28] and to ascertain the whole scope and magnitude of this promising phenomenon. The structure-function relationship of the molecular memory of enzymes in anhydrous solvents warrants further investigation.

**Table 1:** Biocatalytic reactions available for combinatorial synthesis

| REACTION TYPE | SPECIFIC REACTION |
|---|---|
| Introduction of functional groups | C-C bond formation<br>Hydroxylation<br>Halogenation<br>Halohydin formation<br>Cycloadditions<br>Addition of amines |
| Modification of existing functionalities | Oxidation of alcohols to aldehydes/ketones<br>Reduction of aldehydes/ketones to alcohols<br>Oxidation of sulfides to sulfoxides<br>Oxidation of amino groups to nitro groups<br>Oxidation of thiols to thioaldehydes<br>Hydrolysis of nitriles to amides/acids<br>Replacement of amino group with hydroxyl group<br>Lactonisation<br>Isomerisation<br>Epimerisation<br>Dealkylation<br>Methyl transfer |
| Addition of functional groups | Esterification<br>Carbonate formation<br>Carbamate formation<br>Glycosylation<br>Amidation<br>Phosphorylation |

Specifically, it remains to be determined how the nature of the imprinting ligand is reflected in the modified enzymatic properties and how precise and fine-tuned the ligand-induced memory (due to cavities formed in the enzyme molecule) can be.

**4. Combinatorial biocatalysis**

Combinatorial biocatalysis harnesses the natural diversity of enzymatic reactions for the iterative synthesis of organic compound libraries[29]. Nature synthesises biomolecules of unparalleled structural complexity by encoding enzymes that catalyse a myriad of reactions. The products of these reactions induce a variety of important functions, which, in turn, play a major role in determining the important traits of the cell. Over evolutionary time, random mutations to DNA result in expression of modified enzymes, which may transform new precursors or transform existing precursors in different ways, to produce new biomolecules. If these biomolecules provide a survival advantage for the cell, the enzymatic pathways that produced them is preserved (**Figure 8**). Through this process, a vast majority of natural products have been produced, from which many important therapeutics and agrochemicals have been identified. Using just a few of these biotransformations, it is possible to generate a diverse library of unique chemical structures starting with a fairly simple lead compound (**Table 1**). For example, the use of lipases alone has lead to the generation of a library of derivatives of dibenzyl 1,2-phenylenedioxydiacetate[30]. Lipases and proteases have been used in concert in non-aqueous media to generate a library of acylated flavonoid derivatives[31]. The discovery of new natural resources and the expanding capabilities of combinatorial synthesis will ensure that the pipeline of new lead compounds continues to expand. Naturally, combinatorial biocatalysis and its role in drug discovery are expected to grow along with it.

## What next?

The broad field of biocatalysis is truly at the interface of chemistry and biology. Research in the field of biocatalysis is entering an exciting phase. Our ignorance of microbial diversity coupled with improvement in exploration and analytical technology suggest that many more developments will be forthcoming. Exteremophiles have endeared

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themselves to multibillion-dollar industries including agricultural, chemical synthesis and laundry detergents. The European Commission has supported research, training and the commercialisation of technology in this area since 1982[32]. From 1996-1999 it funded the 'extremophiles as cell factories' project (see www.tutech.de/ecf/ecf1_3.htm), which is now in a phase of industry-sponsored technology transfer to European companies. Additional opportunities are likely to result from further developments in catalytic antibodies and biomimetic catalysts. The increased awareness of chemists of the synthetic power of nature and the increased interest in synthetic chemistry from the biologists and biochemical engineers, will surely maintain the importance of biocatalysis in years to come. DDW

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