Industrial biocatalysis today and tomorrow

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The use of biocatalysis for industrial synthetic chemistry is on the verge of significant growth. Biocatalytic processes can now be carried out in organic solvents as well as aqueous environments, so that apolar organic compounds as well as water-soluble compounds can be modified selectively and efficiently with enzymes and biocatalytically active cells. As the use of biocatalysis for industrial chemical synthesis becomes easier, several chemical companies have begun to increase significantly the number and sophistication of the biocatalytic processes used in their synthesis operations.

Biochemists and microbiologists have long seen biocatalysis as an area with great promise for chemical synthesis, but industrial applications have been modest. In this review article we sketch the current state of industrial biocatalysis in several European industries and look ahead to new processes that are likely to develop, based on current academic and industrial research. Two parallel developments are apparent in industry: chemical industries are hiring increasing numbers of life scientists, and organic chemists are beginning to embrace biocatalysis as a tool in new and difficult syntheses. This will lead to more industrial applications of biocatalysts.

Work during the past decade has shown that there are surprisingly few barriers to the use of enzymes and whole cells as biocatalysts in organic synthesis[1,2]. Isolated enzymes are typically used for hydrolytic or isomerization reactions. Whole cells are often used for synthetic reactions that require cofactors which must be regenerated, because although cofactor regeneration *in vitro* is possible, it is generally easier and less expensive to regenerate cofactors in metabolically active cells. Both isolated enzymes and whole cells are used in industry today, and are an active area of research.

Enzymes are remarkable catalysts: capable of accepting a wide array of complex molecules as substrates, and exquisitely selective, catalysing reactions with unparalleled chiral (enantio-) and positional (regio-) selectivities. As a result, biocatalysts can be used in both simple and complex transformations without the need for the tedious blocking and deblocking steps that are common in enantio- and regioselective organic synthesis. Such high selectivity also affords efficient reactions with few by-products, thereby making enzymes an environmentally friendly alternative to conventional chemical catalysts.

These attributes have resulted in myriad applications, especially in the food and pharmaceutical industries where high reaction selectivity on complex substrates is critical. Examples include the production of high-fructose corn syrup, by the action of xylose isomerase[3] which catalyses the isomerization of D-glucose to D-fructose, and the preparation of semisynthetic penicillins catalysed by penicillin amidase[4]. Selective catalysis is now also becoming a requirement for the chemical industry, and recent advances in enzymatic catalysis have been extended to the synthesis of speciality chemicals and polymers[5,6] and of some bulk chemicals. For example, peroxidases are used industrially to catalyse the synthesis of phenolic resins for use as replacements of conventional phenol-formaldehydes[7], and nitrile hydratase is used to catalyse the hydration of acrylonitrile into acrylamide[8]. In both cases, nearly quantitative conversion of the reactants into products is obtained and under reaction conditions far milder than their chemical counterparts. Most commercial enzymatic processes today share several attributes, including high product concentrations and productivities, no undesirable by-products, and enzymes that do not require expensive cofactors.

Future biocatalytic processes generally will not be limited by the available technology or the nature of the substrates and the products. Instead, the feasibility of new biocatalytic processes will often be determined by the availability of the biocatalyst, the search for which is described in the accompanying papers in this issue by Walsh (pages 226–231), Arnold (pages 253–257) and Khosla and Harbury (pages 247–252). Consequently, a growing number of companies sees biocatalysis as an interesting option. As individual industries develop relevant experience, industrial biocatalysis will grow rapidly.

The biocatalysis cycle

Biocatalytic processes differ from conventional chemical processes, owing mainly to enzyme kinetics, protein stability under technical conditions and catalyst features that derive from their role in the cell's physiology, such as growth, induction of enzyme activity or the use of metabolic pathways for multistep reactions. 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 one of several possible biocatalytic reactions that convert suitable substrates to the desired product. Figure 1 illustrates the development of such biocatalytic processes. One or more biocatalysts must

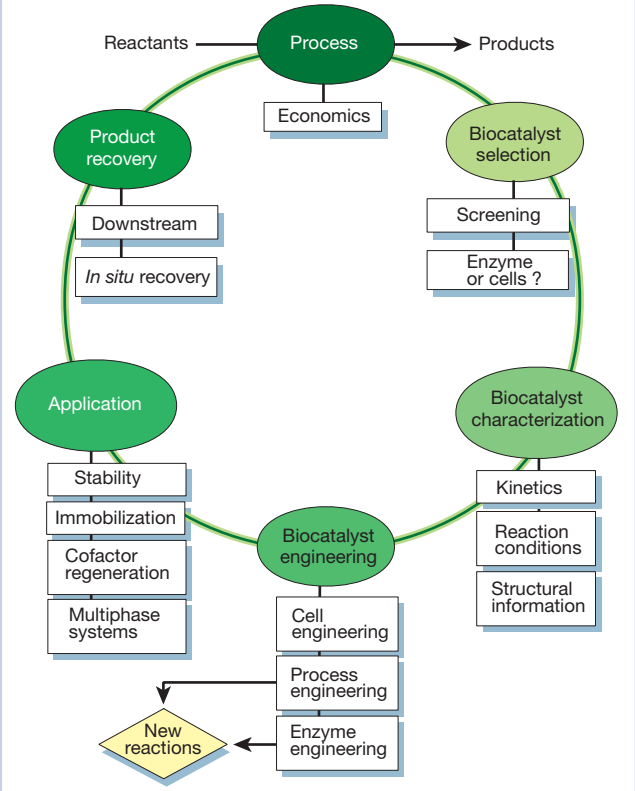

Figure 1 The biocatalysis cycle.

be identified or developed, a process must be set up, and the resulting bioconversion will ultimately have to be economically feasible. The development of such a process requires the input of many different specialists. Limiting aspects of the biocatalytic process are improved in an iterative manner, gradually leading to an efficient industrial process. In setting priorities for improvements at each process step, a detailed understanding of the costs and improvement potential of each of the partial steps in a process is vital.

The economic feasibility of a biocatalytic process depends on several factors (Fig. 1). Depending on the type of biocatalyst to be used, specific reactor and hardware configurations are needed (summarized in ref. 9). In addition, biocatalytic processes are typically highly heterogeneous. In theory, this would necessitate specific designs of the catalyst–hardware interface[10]. But in practice, a limited number of hardware designs is found today in large industrial processes, allowing the application of biocatalysts based on only a few concepts. In analogy to chemical processes, most biocatalysts are used in immobilized form as heterogeneous catalysts that can be recovered and reused. There are also processes, however, based on homogeneously suspended cells or enzymes, that are sufficiently inexpensive to permit single use, without recovery or reuse. In fact, several speciality chemical companies now use living cells as catalysts for reactions such as specific coenzyme-dependent oxidoreductions, as described below.

The biocatalyst

New processes can be based on the availability of an interesting new enzyme, or on the identification of desired products, after which a biocatalyst is then selected that permits conversion of available reactants. Such an enzyme might be available commercially, or it might have been described in the literature. Alternatively, it will be necessary to screen for organisms or enzymes that carry out the desired reaction, or completely new enzyme activities will be developed by protein design or directed evolution (see review in this issue by Arnold, pages 253–257).

For conversions that do not require regeneration of coenzymes, such as isomerization or hydrolysis reactions, both enzymes and whole cells can be selected. But when cofactors are required, whole cells are favoured because they enable cofactor regeneration. Reaction conditions for optimal enzyme function, high reactivities and long catalyst lifetimes are selected based on biocatalyst characteristics. The biocatalyst and the biocatalytic process are engineered for best performance — at the level of the enzyme (protein engineering for better activity, improved substrate range, enzyme stability), the host cell (solvent resistance, substrate import and product export, elimination of side-reactions), or the process. The biocatalyst (enzymes or cells) may be immobilized, and cofactors regenerated for coenzyme-dependent enzymes. The reaction medium, which may consist of an aqueous phase, an organic phase or a two-liquid-phase system, will be optimized to dissolve substrates and products while maintaining enzymatic activity.

Enzyme catalysis in organic solvents

The rapid growth of biocatalysis is a direct result of research and development in two key technologies: protein engineering, including molecular evolution[11,12], and enzyme engineering. Whereas the former provides enzymes with altered structure, function and selectivity, particularly in aqueous media, the latter, especially involving engineering of the enzyme microenvironment, provides striking improvements in nonaqueous environments. Indeed, it is now well known that enzymes do function in organic solvents, and many in neat (pure) solvents or in supercritical fluids in the absence of added water (see refs 13–15 and review in this issue by Klibanov, pages 241–246). Such an environment yields many potential advantages, including higher substrate solubility, reversal of hydrolytic reactions and modified enzyme specificity, which result in new enzyme activities that previously were only possible using genetic modifications or complex multistep pathways within whole cells. As a result, applications of enzymatic catalysis in organic solvents range from chiral resolution of pharmaceuticals, chemicals and their intermediates[16] to enantio- and regioselective polymerization[17].

Despite the advantages of nonaqueous conditions for biocatalytic transformations, enzymes nearly universally display low catalytic activities in these environments compared with native aqueous solutions. Nonetheless, recent developments have shown that biocatalysts can be engineered to function in neat organic solvents with activities and selectivities that are consistent with their aqueous-based counterparts. For example, subtilisin Carlsberg suspensions (subtilisin and other enzymes are insoluble in nearly all organic solvents) prepared simply by lyophilizing an aqueous preparation (see review by Klibanov, pages 241–246) in the presence of non-buffer salts[18] yield rate enhancements of more than 20,000-fold[19]. The mechanism of this activation has not been elucidated, although it is strongly dependent on the specific salt used. Specifically, kosmotropic (order-promoting) salts are expected to stabilize the folded form of enzymes during the lyophilization process as well as in the nonaqueous reaction medium, thereby leading to higher enzymatic activities in such solvents[20]. In addition to subtilisin, a number of other enzymes are activated by this technique, including those with very different catalytic mechanisms. Activation has also been achieved by the addition of crown ethers[21], transition-state analogues[22], and substrates and substrate mimics[23].

Activated biocatalyst preparations have found direct application in the pharmaceutical industry, where salt-activated biocatalysts have been used to synthesize a library of paclitaxel (taxol) derivatives[24]. The bacterial protease thermolysin was found to acylate selectively the 2′-hydroxyl of taxol in *t*-amyl alcohol (Fig. 2). Yields of the 2′-acyl derivatives approached 100% using KCl-activated thermolysin. For the specific acylation with divinyladipate, a taxol 2′-vinyladipate was generated, which served as the acyl donor for *Candida antarctica* lipase-catalysed hydrolysis of the terminal vinyl ester. The resulting taxol 2′-adipic acid derivative was nearly 1,700 times more soluble in water than the native taxol, a result of critical

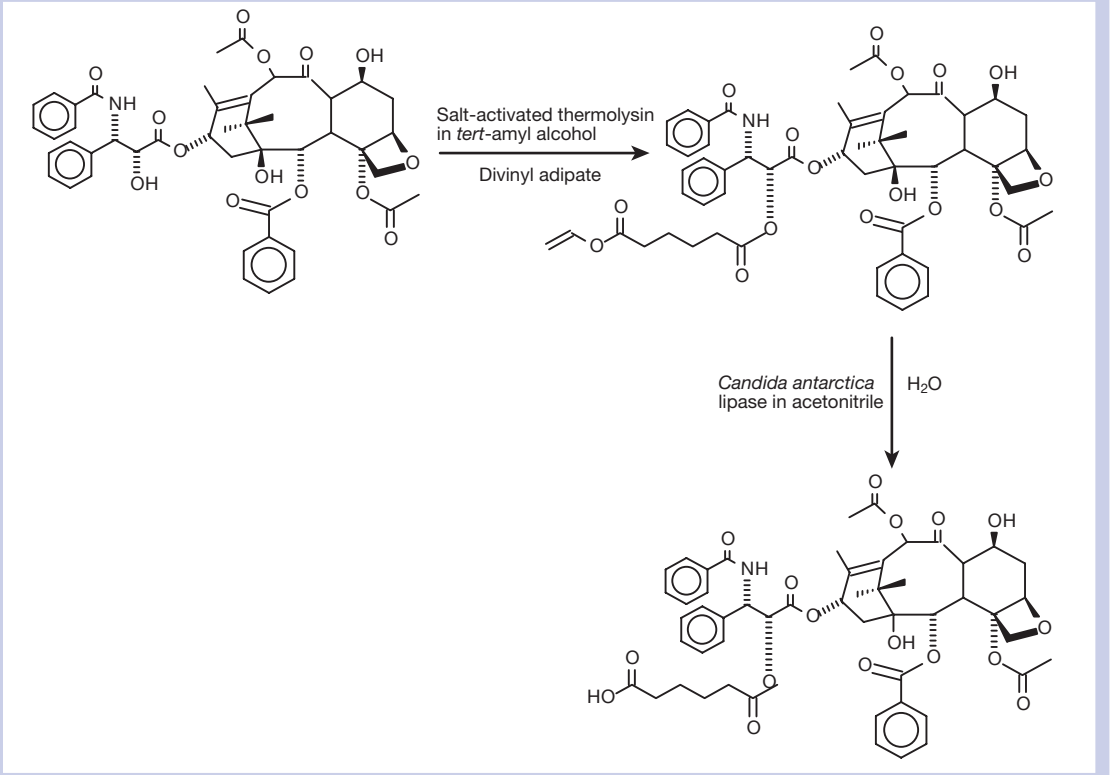

Figure 2 Synthesis of a library of paclitaxel (taxol) derivatives.

importance in the design of taxol prodrugs with increased bioavailability.

In the presence of low concentrations of a suitable surfactant, enzymes are able to dissolve in hydrophobic organic solvents, where they remain remarkably active and with secondary and tertiary structures nearly identical to that measured in water[25]. For example, in peptide synthesis, subtilisin Carlsberg and α-chymotrypsin were over 1,000-fold more reactive than their native suspended counterparts in suitable organic solvents. Solubilized enzymes have been used to generate 'biocatalytic plastics' (Fig. 3), wherein enzymes are incorporated into growing vinyl polymers to yield homogeneous immobilized preparations[26,27]. This technique enables the biocatalyst to be used in a form suitable for a specific function. Thus, suspension polymerization could be used to yield biocatalytic plastic beads of controlled sizes, for example as catalysts in packed-bed reactors. Thin-film formation could be pursued to give biocatalytic paints, coatings and films for applications ranging from antifouling coatings (for example to prevent surface protein and cell adhesion) to affinity materials for use in the synthetic, diagnostic and medical arenas.

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 polyester synthesis has been performed in neat solutions of diols and diesters[28]. Rapid polymer growth is obtained with nearly quantitative conversions.

Whole-cell catalysis with toxic solvents

A number of potentially interesting biocatalytic conversions being investigated today involve apolar substrates and products, such as aliphatic, aromatic and heterocyclic compounds. Such compounds are generally insoluble in water, and often they are toxic to whole cells[29–31]. Thus they cannot simply be added to an aqueous medium for whole-cell transformation. Several technical solutions to this problem have been developed.

One promising approach is to use two-liquid-phase media: an aqueous phase that contains the growing cells, and an apolar solvent that contains the substrate and newly formed product[32–34]. Biocatalysis in emulsions is a well established technology in research laboratories[35–37] and is expected to be equally applicable on larger scales[38–40]. Moreover, solvent-based processes enable the use of well established, industrial downstream-processing techniques. For two decades from the 1970s these systems were used with various catalytically active natural hosts, often pseudomonads, but lately recombinants harbouring genes for appropriate enzymes are gaining ground. Although not particularly solvent-resistant, *Escherichia coli* has been used effectively as a biocatalysis host. Examples include the oxidation of alkanes[34,35], aromatics such as toluene and styrene derivatives[41], and heterocycles[42].

A related engineering approach for processes based on multiphase reaction media is gas-phase biocatalysis[43]. Enzymes or intact cells form a solid phase and reactants are dissolved in the gas phase. This concept is suitable for reactants that can be brought into the gas phase at operating conditions, and has the advantage of simple downstream processing and efficient mass transfer, while the use of toxic or inhibiting solvents can be avoided.

A third approach to handling toxic compounds is to feed these into the bioreactor at limiting rates, so that such substrates are transformed without first accumulating, thus maintaining very low and non-toxic substrate concentrations. Products can be removed essentially instantaneously by continuous extraction techniques, either in suitable apolar solvents or by adsorption onto solid beds[44].

These techniques have now matured to a point where they can be used routinely to carry out whole-cell biotransformations on a wide range of substrate–product combinations. Perhaps surprisingly, *E. coli* may well be the preferred host. Such transformations are limited only by the availability of the required enzyme systems. If these can be found (from available strains, screening or selection) or generated (by protein engineering or directed evolution), a suitable whole-cell biocatalyst can be constructed[41,45]. By using highly water-insoluble solvents such as hexadecane, two-liquid-phase systems can be developed that contain 10–50% (by volume) apolar phase in an aqueous medium, and cause no damage to the *E. coli* host[39,46,47]. Recombinant strains can be grown to densities of 10–40 g l^{-1} (dry mass), either in batch, fed-batch or continuous-cultivation systems[30]. Overall enzyme activities for monooxygenases typically vary from 100 to 500

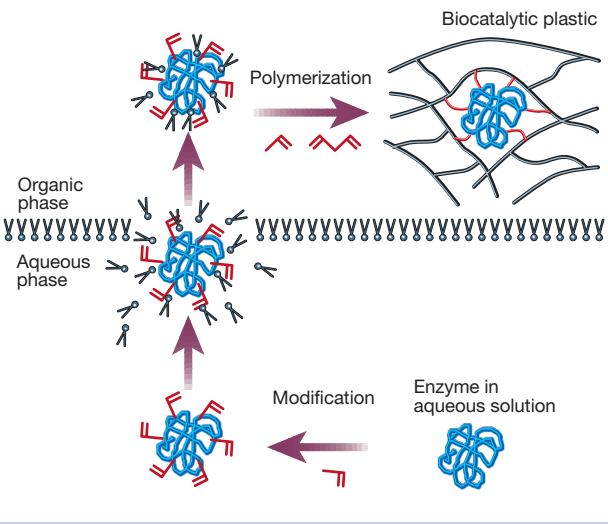

Figure 3 Production of biocatalytic plastics.

international units (micromoles converted per minute) per litre of aqueous medium, and these activities can be maintained for 10–20 hours[48,49]. These numbers lead to space-time yields of 5–25 tons of product per cubic metre aqueous medium per year, for typical small molecules with relative molecular masses of 100–200. Higher numbers might be reached, based on enzyme activities of 30–100 international units per gram cell (dry mass) and cell densities of 10–20 $g l^{-1}$ (ref. 48). Other parameters would, however, then become limiting, such as the practical volumetric energy and oxygen input and the heat removal capacity that can be achieved in large-scale industrial reactors.

Product extraction and purification

Most biocatalytic processes still operate in aqueous environments and often result in low product concentrations. This necessitates the development of special methods for product extraction. In contrast to industrial bulk chemical syntheses, bioprocesses are usually operated in batch mode; continuous processes are still the exception. Recovery of product from the reaction medium generally follows the biotransformation step as a separate batch operation, after separation of biomass from the reaction mixture either by centrifugation or filtration.

Even techniques as simple as precipitation of insoluble reaction products are used, for instance in the process for acrylamide production from acrylonitrile at product concentrations as high as 600 $g l^{-1}$ (ref. 50). Such *in situ* product-recovery processes, in which the product is separated from the reaction mixture during the biotransformation, have distinct advantages and are becoming popular (reviewed in ref. 51). These *in situ* product-recovery techniques are based on physical and chemical parameters of the reactants and range from crystallization through distillation to solid-phase or liquid-phase extraction. In general, *in situ* product recovery overcomes traditional limitations of biocatalytic processes such as substrate or product inhibition and product decay in aqueous reaction mixtures.

In the case of biocatalysis in mixed aqueous and apolar solvent phases, which is relevant to bioconversions of hydrocarbons and other apolar compounds[52], the phases are separated after the biocatalytic step, followed by subsequent product adsorption, liquid extraction or distillation, generally from the apolar phase[40,53,54]. These steps may in turn affect the selection of the reaction medium and reactor configuration, so that a biocatalytic process may be developed in several iterations through the biocatalysis cycle of Fig. 1. A number of recently developed processes are listed in Table 1.

Waste processing

The major waste products of aqueous bioprocesses are waste water, salts and biomass. Organic solvents represent minor waste products if used in emulsion processes or reactions in pure organic solvents: they can be recycled, apart from a minor fraction obtained as insoluble emulsion. The main waste products are treated in conventional industrial waste-water treatment systems. For processes based on recombinant, viable microorganisms, the treatment of biomass includes inactivation of the genetically engineered cells.

Biocatalytic processes at BASF

BASF (Germany) has several biotechnological processes in place for the large-scale manufacture of a number of important optically active building blocks and their derivatives. Critical for the development of these processes was a thorough understanding of the catalytic mechanism of the enzymes involved, such as lipases.

Lipase-based processes

Lipids are compounds that can be extracted in chloroform–methanol mixtures. They consist primarily of fatty-acid chains, which are linked by ester bonds to an alcohol or polyol backbone. Lipases belong to a large class of enzymes that hydrolyse the ester bond between the fatty-acyl side chains and the lipid backbone. Understanding the catalytic cycle of lipases has been of significant importance to their widespread use in different biotechnological applications. The lipase active site is composed of three different residues: serine, histidine and aspartate or glutamate.

The hydrolysis of an ester involves an acyl enzyme complex. The catalytic cycle starts by nucleophilic attack of the hydroxyl group of the serine side chain on the carbonyl carbon atom of the ester bond. The complex is resolved by the nucleophilic attack of water, the fatty acid is liberated and the enzyme is regenerated (Fig. 4).

As lipases are active in organic solvents, water can be replaced by other nucleophiles such as alcohols. The result of this reaction is a transesterification. For racemic alcohols only one enantiomer may be acylated, thereby leading to enantioselective transformations (see accompanying reviews in this issue by Walsh, pages 226–231, and Koeller and Wong, pages 232–240). Suitable acyl donors are vinyl esters, anhydrides or diketene. The reaction is irreversible and the separation of the remaining alcohol and the newly formed ester is simple. This principle is now used in many reactions to produce enantiomerically pure alcohols. BASF has recently extended its chiral synthesis capabilities to the enzymatic resolution of racemic alcohols. Some of the core technologies at BASF, such as condensation or hydrogenation of ketones, give access to the racemic starting compounds. A broad range of enantiomerically pure alcohols can now be offered (Fig. 5a).

Amines might also be used as nucleophiles[55]. Racemic amines are efficiently resolved using ethylmethoxyacetate as acylating agent (Fig. 5b). Using ethylmethoxyacetate the initial reaction rate is more than 100 times faster compared with butylacetate. The reason for this activating effect of the methoxy group is probably an enhanced carbonyl activity induced by electronegative α-substituents. Excellent yields and selectivity and minimal amounts of enzyme

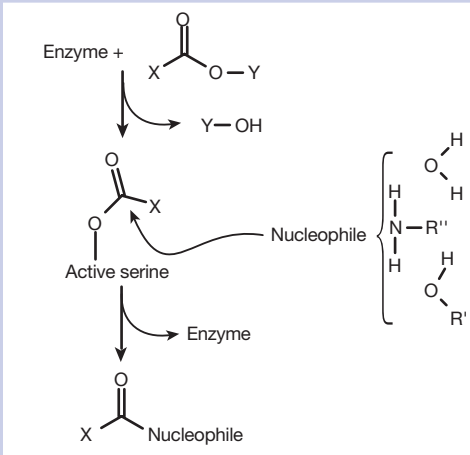

Figure 4 Reaction mechanism of lipase biocatalysis. The nucleophilic attack on carbonyl functionalities serves as target for reaction engineering approaches.

Table 1 Recently developed biocatalytic systems at several chemical companies

Figure	Product	Substrate	Reaction	Biocatalyst	Enzyme	Organism*	Scale (Tons yr^{-1})	Yield	Applicability	Source
Amides, alcohols, acids										
5a	Enantiopure alcohols	Racemic alcohols	Resolution	Enzymes	Lipases		Thousand	Excellent	Broad spectrum of alcohols	BASF
5b	*R*-Amide; *S*-amine	Racemic amines	Resolution	Enzymes	Lipases		Several hundred	Excellent	Broad spectrum of amines	BASF
5c	*R*-Mandelic acid	Racemic mandelonitrile	Hydrolysis	Enzymes	Nitrilases		Several	Excellent		BASF
Amino acids, penicillins										
6a	Non-proteinogenic L-amino acids	Racemic amino-acid amides	Kinetic resolution	Enzymes	Amidases	*P. putida*, *M. neoaurum*, *O. antropii*, rec. *E. coli*	Few to several hundred		Broad spectrum of L- and D-amino acids	DSM
6b	L-Aspartic acid	Fumaric acid	Addition of ammonia	Enzymes	Aspartic acid ammonia lyase	*E. coli*	Thousand		L-Aspartic acid	DSM
6b	Aspartame (L-α-aspartyl-L-phenylalanine methyl ester)	N-protected L-aspartic acid, D/L-phenylalanine methyl ester	Selective coupling	Enzymes	Thermolysine	*B. subtilis*	Thousand		Aspartame	DSM
6c	6-Aminopenicillanic acid (6-APA)	Penicillin G/V	Hydrolysis	Enzymes	Penicillin acylase	*E. coli*	Thousand		6-APA	DSM
6c	Semisynthetic penicillins	6-Aminopenicillanic acid	Selective coupling	Enzymes	Acylases	*E. coli*, *P. putida*	Few to several hundred		Semisynthetic penicillins and cephalosporins	DSM
***N*-Heterocyclic compounds**										
7a	6-Hydroxynicotinic acid	Niacin	Addition of water	Whole cells	Niacin hydroxylase	*A. xylosoxidans* LK1	Few	65 g l^{-1}	Insecticides	Lonza
7b	5-Hydroxypyrazine-carboxylic acid	2-Cyanopyrazine	Addition of water	Whole cells	Nitrilase/ hydroxylase	*Agrobacterium* DSM 6336	Development product	40 g l^{-1}	Antituberculosis drugs	Lonza
7c	6-Hydroxy-*S*-nicotine	(*S*)-Nicotine	Addition of water	Whole cells	Hydroxylase	*A. oxydans* NRRL-B-3603	Development product	30 g l^{-1}		Lonza
7c	4-[6-Hydroxypyridin-3-yl]-4-oxobutyrate	(*S*)-Nicotine	Complex reation	Whole cells	Several	*Pseudomonas* sp. DSM 8653	Development product	15 g l^{-1}	Analogues of Epibatidine	Lonza
Non-proteinogenic amino acids										
7d	*S*-Piperazine-2-carboxylic acid	(*R*,*S*)-Piperazine-2-carboxylic acid	Selective amidase	Whole cells	Stereospecific amidases	*K. terrigena* DSM 9174	Development product	9 g l^{-1}	Various bioactive compounds	Lonza
7e	5-Methylpyrazine-2-carboxylic acid	2,5-Dimethylpyrazine	Selective oxidation of a methyl substituent on an aromatic *N*-heterocycle (pyrazine)	Whole cells	Xylene oxidation pathway	*P. putida* ATCC 33015	Several	20 g l^{-1}	Synthon for antilipolytic drug	Lonza

*Organism: *A. oxydans*, *Arthrobacter oxydans*; *A. xylosoxidans*, *Achromobacter xylosoxidans*; *B. subtilis*, *Bacillus subtilis*; *E. coli*, *Escherichia coli*; *K. terrigena*, *Klebsiella terrigena*; *M. neoaurum*, *Mycobacter neoaurum*; *O. antropii*, *Ochrobactrum antropii*; *P. putida*, *Pseudomonas putida*.

characterize this new process, which has been used by BASF since 1993. The products, the *R*-amide and the *S*-amine, can be recovered and separated by distillation and have high chemical and optical purities.

The above process is applicable to a broad spectrum of amines, which are of considerable interest as chiral building blocks or as auxiliaries for the syntheses of bioactive ingredients. Several chiral amines (Fig. 5b) are now being produced in scales up to several hundred tons in a multiproduct facility. BASF is building a second-generation plant using this new technology, which is due to begin production in 2001 and will be operated according to cGMP (current Good Manufacturing Practice) standards. Another optically active amine that will be produced by this process is *S*-methoxyisopropylamine. It is a building block for the optically active corn herbicide Frontier x2. A dedicated plant for this amine will go on-stream in mid-2001 with an annual capacity of 2,000 tons.

For the success of lipases as industrial biocatalysts it has been important that they be available in commercial quantities, highly stable and active in organic solvents, and that they have a broad substrate range. Besides their usefulness in the production of alcohols and amines, lipases can be used for other reactions. For example, enolesters can be stereoselectively protonated and other reactions such as the cleavage of oximesters, C–C bond formation and synthetic Michael reactions (the nucleophilic addition of a carbanion to an α,β-unsaturated carbonyl compound) may be achievable. But significant further improvements are necessary to facilitate these reactions[56].

Nitrilase-based processes

An interesting class of building blocks are amino- and hydroxycarboxylic acids. Such compounds can be synthesized stereoselectively in high enantiomeric excess by nitrilases, a class of enzymes that catalyse the hydrolysis of a nitrile to the corresponding ammonium salt of a carboxylic acid (Fig. 5c). Again this class of enzymes fits nicely in existing product lines and synthesis capabilities at BASF. One such sector involves hydrogen cyanide chemistry, which provides access to the starting materials for subsequent enzymatic bioconversion to chiral α-hydroxy- or α-aminocarboxylic acids with nitrilases. For the α-hydroxynitriles (Fig. 5c, X = OH), both enantiomers can be used because cyanohydrins are known to racemize in aqueous solution through an equilibrium between aldehyde and hydrogen cyanide. By choosing the right pH conditions, quantitative enzymatic conversions can be achieved. Nitrilases therefore provide an extension of industrial hydrogen cyanide chemistry, giving access to a wide range of new intermediates. Microorganisms having nitrilase activity can be enriched from nature by using nitriles as the only nitrogen and/or carbon source in the growth medium. To find three new nitrilases, about 5,000 strains from enriched cultures and isolates were tested. One is currently used in a pilot plant to make *R*-mandelic acid on a multiton scale (Fig. 5c)[57].

Biocatalytic processes at DSM

The Dutch chemical company DSM has a long history in biocatalysis, starting with the pioneering work of Boesten *et al.*[58] in the mid 1970s

on the enzymatic resolution of D/L-phenylglycinamide into D-phenylglycinamide and L-phenylglycine using hog leucine aminopeptidase and an L-α-aminoacylamidase from *Pseudomonas putida*[59]. Currently, DSM uses biocatalysis, biotransformation and fermentation technologies in addition to chemical methods to produce advanced intermediates for the custom manufacturing arena[5,60,61]. Some examples of processes that make use of enzymes and that have been commercialized by DSM are listed below.

Production of non-proteinogenic L-amino acids

The demand for non-proteinogenic L-amino acids as building blocks for the synthesis of pharmaceutical compounds is considerable and these amino acids are not accessible by fermentation, in contrast to most of the 20 proteinogenic amino acids that are incorporated into polypeptides. The DSM process for the production of L-amino acids is based on the resolution of racemic amino-acid amides (**1** and ***rac*-1** in Fig. 6a), which are conveniently synthesized from aldehydes using the Strecker synthesis. Resolution is achieved with amino-acid amide hydrolases (amidases). Although the enzymes are strictly L-selective, both D- and L-amino acids (respectively **L-2** and **D-2** in Fig. 6a) can be accessed and the undesired enantiomer can be recycled.

The biocatalytic amidase toolbox of DSM consists of enzymes from *P. putida*, *Mycobacter neoaurum* or *Ochrobactrum antropii*, which vary in substrate range, specific activity and enantioselectivity[60]. As a result, a wide range of amino-acid amides can be resolved (***rac*-1** in Fig. 6a)[5,62–67]. The genes encoding the amidases have been isolated and over-production of the enzymes in *E. coli* has successfully been carried out, resulting in much improved biocatalysts[60].

Production of aspartame

The low-calorie sweetener aspartame (L-α-aspartyl-L-phenylalanine methyl ester; **5** in Fig. 6b) is produced on a kiloton scale by Holland Sweetener Company, a joint venture of Tosoh and DSM. The aspartame process uses a proteolytic enzyme, thermolysin, to catalyse the formation of the dipeptide from N-protected L-aspartic acid (Z-Asp; ***Z*-3** in Fig. 6b) and D/L-phenylalanine methyl ester (***rac*-4** in Fig. 6b)[5]. L-aspartic acid (**3**) for the production of Z-Asp is accessible by biocatalysis using aspartic acid ammonia lyase (aspartase), which catalyses the addition of ammonia to fumaric acid. Subsequently, the protective group is attached, resulting in Z-Asp[68].

The coupling of the two amino acids Z-Asp and D/L-phenylalanine methyl ester by thermolysin is not only the reversal of the hydrolytic reaction that is common to the enzyme, but also displays a remarkable selectivity. Out of the four possible conformations, taking both the α- and β-carboxyl group of Z-Asp into account, thermolysin selectively couples L-phenylalanine methyl ester to the α-carboxyl group of Z-Asp, thereby generating Z-aspartame (***Z*-5** in Fig. 6b). The Z-group is removed by catalytic hydrogenation and the remaining D-phenylalanine methyl ester can be racemized and recycled.

Green alternatives to produce semisynthetic penicillins and cephalosporins

DSM produces penicillin G/V by fermentation using *Penicillium chrysogenum* strains, which have been improved by classical strain improvement as well as genetic engineering. Most of the penicillin (for example, penicillin G; **6** in Fig. 6c) is converted to 6-aminopenicillanic acid (6-APA; **7** in Fig. 6c), which serves as a backbone for the synthesis of semisynthetic penicillins, providing a range of penicillin variants with differing antibiotic characteristics. The 6-APA core structure can be extended by chemical means or by the action of an enzyme to a core structure suitable for synthesis of semisynthetic cephalosporins, such as 7-aminodesacetoxycephalosporanic acid (7-ADCA; **8** in Fig. 6c). The coupling of side chains to the core structures generates derivatives of penicillins and cephalosporins that have traditionally been produced using complex solvent-based chemistry, typically conducted at temperatures as low as −80 °C to preserve the labile β-lactam ring.

Current processes use penicillin acylases to remove the side chain from penicillin G/V, providing a 'green' route to 6-APA[69]. For example, the β-lactam ring of penicillin G is converted chemically or enzymatically to phenylacetyl-7-ADCA, which is also accepted by the acylase, thus producing the nucleus for the expandase-catalysed synthesis of semisynthetic cephalosporins[70].

D-phenylglycine and D-*p*-hydroxyphenyl glycine, which are the side chains that are attached to the 6-APA or 7-ADCA back-bone in the semisynthetic antibiotics ampicillin, amoxycillin, cephalexine and cephadroxyl (respectively **9**, **10**, **11** and **12** in Fig. 6c), are also produced by DSM. The coupling of the side chain to the nucleus is largely performed chemically, but DSM has recently completed the development of a biocatalytic process using another penicillin acylase[4,5,71,72]. Under certain conditions of pH and by controlling precursor and product concentrations, the coupling of the two molecules can out-compete the hydrolysis reaction catalysed by the same enzyme.

Biocatalytic processes at Lonza

The Swiss custom-manufacturing company Lonza specializes in the production of many chemical compounds, including *N*-heterocycles. Lonza has developed a series of biocatalytic routes for the production of certain functionalized *N*-heterocycles where chemical synthesis is inefficient. The biotransformations described here can be divided into three types: the selective ring hydroxylation of aromatic *N*-heterocycles, the kinetic resolution of racemic *N*-heterocyclic carboxamides to the corresponding enantiomerically pure

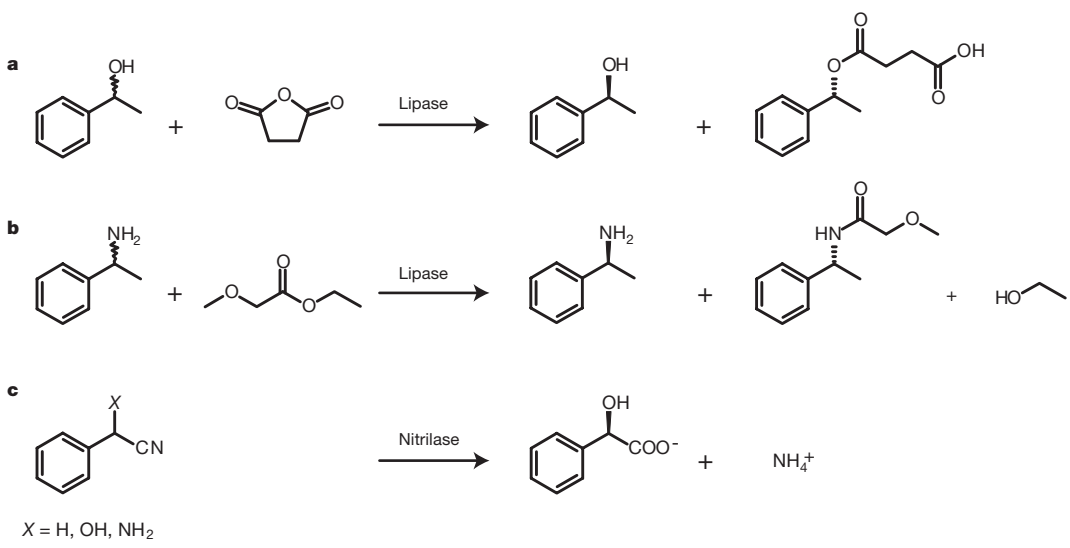

Figure 5 Recently developed biocatalytic systems at BASF. Lipases are successful biocatalysts used in the synthesis of **a**, enantiomerically pure alcohols, and **b**, chiral amines. **c**, Nitrilases are used in the synthesis of amino- and hydroxycarboxylic acids.

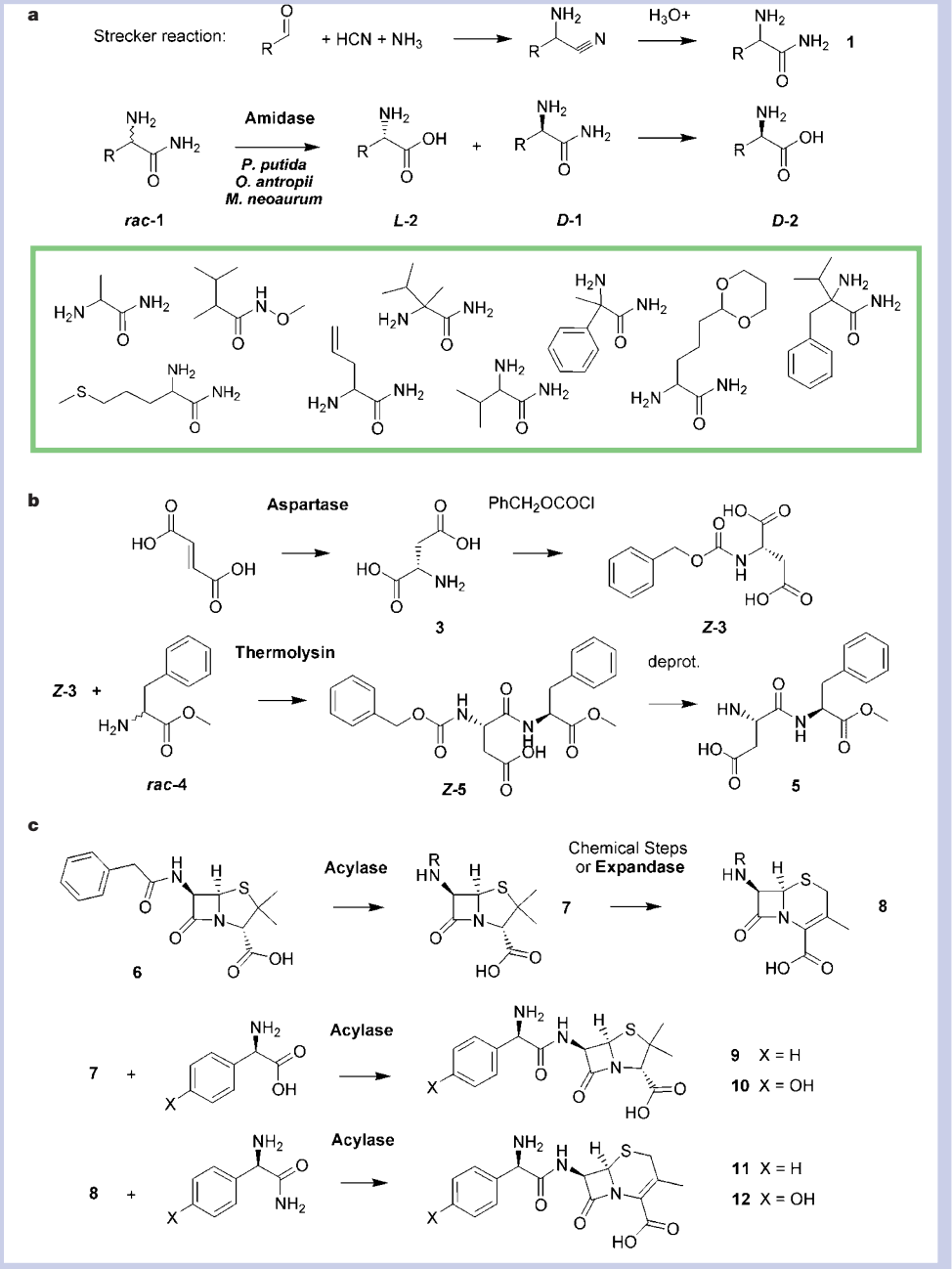

Figure 6 Biocatalytic processes at DSM. **a**, Amidase process for production of non-proteinogenic amino acids. **b**, Biocatalytic steps for the production of aspartame. **c**, Acylases for the production of semisynthetic penicillins and cephalosporins.

carboxylic acids with stereospecific amidases, and the selective oxidation of alkyl groups on aromatic *N*-heterocycles.

6-Hydroxynicotinic acid and 5-hydroxypyrazinecarboxylic acid

6-Hydroxynicotinic acid (**1** in Fig. 7a), a derivative of niacin (**2** in Fig. 7a), serves as a versatile building block predominantly in the synthesis of modern insecticides. The chemical synthesis of 6-substituted niacin results in the formation of by-products which make the cost prohibitively high. Lonza has detected microorganisms growing on niacin capable of accumulating up to 65 g l^{-1} 6-hydroxynicotinic acid at an overall yield of 90% (ref. 73). Remarkably, at niacin concentrations greater than 10 g l^{-1} the second enzyme of the pathway (6-hydroxynicotinate hydroxylase) is strongly inhibited, whereas the niacin hydroxylase remains unaffected (Fig. 7a).

A new two-step biotransformation in whole cells of *Agrobacterium* DSM 6336 was developed for the preparation of 5-hydroxypyrazinecarboxylic acid (**3** in Fig. 7b) from the starting material 2-cyanopyrazine (**4** in Fig. 7b)[74]. The 5-substituted pyrazine-2-carboxylic acid was then used for the preparation of novel antituberculosis drugs. In previous experiments, various 5-chloropyrazine-2-carboxylic acid esters, prepared from 5-hydroxypyrazinecarboxylic acid, were up to 1,000 times more active than previously described drugs against *Mycobacterium tuberculosis* and other *Mycobacterium* strains[75].

The biotransformation described involves the hydrolysis of the nitrile group to pyrazinecarboxylic acid (**5** in Fig. 7b) and the subsequent regioselective hydroxylation to 5-hydroxypyrazinecarboxylic acid by the same bacterial cells. The product concentration was as high as 40 g l^{-1}. To induce the required enzymes, the *Agrobacterium* sp. DSM 6336 was first grown on 3-cyanopyridine (**6** in Fig. 7b) as a carbon and energy source, followed by the bioconversion of 2-cyanopyrazine to 5-hydroxypyrazine-2-carboxylic acid (Fig. 7b).

Renewable functionalized pyridines derived from (*S*)-nicotine

(*S*)-Nicotine, which is present in concentrations of 2–8% in dried leaves of certain tobacco plants, was used as a starting material for the biocatalytic production of functionalized pyridines from renewable sources. These compounds are precursors in the synthesis of drugs such as analogues of epibatidine (**9** in Fig. 7c), an extremely effective analgesic molecule[76] that is produced by a tropical frog. The selective functionalization of nicotine at the pyridine or the pyrrolidine ring

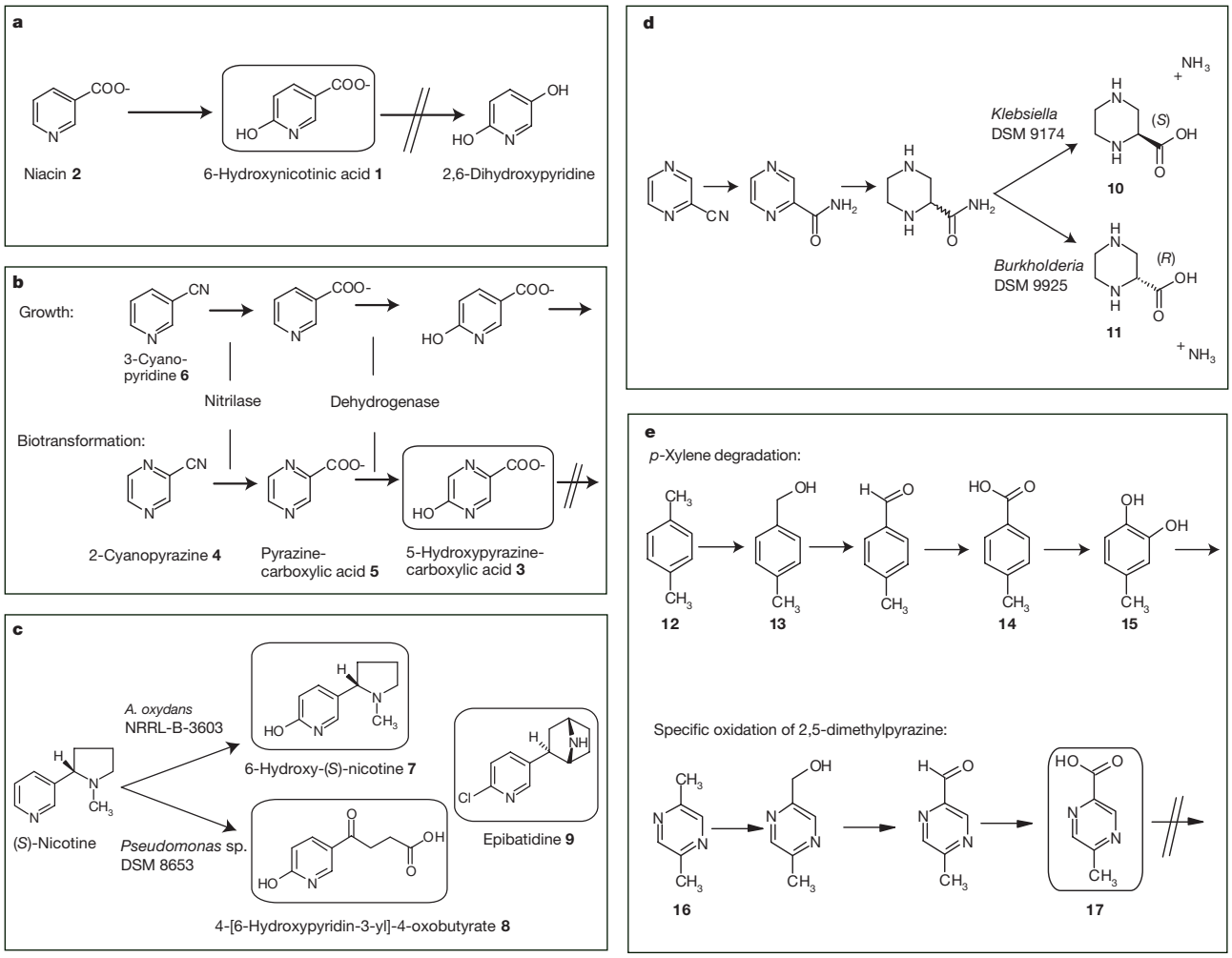

Figure 7 Biocatalytic processes at Lonza. **a**, Catabolic pathway for the production of 6-hydroxynicotinic acid with *Achromobacter xylosoxidans* LK1. **b**, Reaction sequence analogies of 3-cyanopyridine degradation and 2-cyanopyrazine biotransformation with *Agrobacterium* DSM 6336. **c**, Microbial metabolites of (*S*)-nicotine. **d**, Process for the production of enantiomerically pure piperazine-2-carboxylic acids. **e**, Enzymatic oxidation of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid with whole cells of *Pseudomonas putida* ATCC 33015 growing on *p*-xylene as sole carbon source.

by chemical means is difficult to control and this restricts the chemical preparation of semisynthetic pyridines using nicotine as starting material. Although it has long been known that microorganisms form a wide variety of metabolites of nicotine when grown on this heterocycle as the sole carbon and nitrogen source, these metabolites have not been used as starting materials for the chemical synthesis of new nicotine analogues. Using *Arthrobacter oxydans* NRRL-B-3603 and *Pseudomonas* sp. DSM 8653, it was possible to produce up to 30 g l^{-1} 6-hydroxy-(*S*)-nicotine (**7** in Fig. 7c) with an isolated yield of 51% and up to 15 g l^{-1} of 4-[6-hydroxypyridin-3-yl]4-oxobutyrate (**8** in Fig. 7c), respectively[77].

Preparation of piperazine-2-carboxylic acid

Non-proteinogenic amino acids such as piperazine-2-carboxylic acid are precursors of numerous bioactive compounds. The (*S*)-analogue (**10** in Fig. 7d) can be used for the synthesis of the HIV protease inhibitor Crixivan from Merck. The best current chemical route to the pure enantiomers of this amino acid is the classical resolution of the racemate by fractional crystallisation of diastereomeric salts. Soil samples containing the racemic carboxamide as the sole nitrogen source were used for enrichments to search for microorganisms with stereoselective amidases. As a result of this screening, *Klebsiella terrigena* DSM 9174 and *Burkholderia* sp. DSM 9925 were used for the preparation of (*S*)-piperazine-2-carboxylic acid and (*R*)-piperazine-2-carboxylic acid (**11** in Fig. 7d), respectively. For the production of enantiomerically pure acids the microorganisms were resuspended for various periods of time in buffer containing 10–20 g l^{-1} of the racemic carboxamide. The enantiomeric excess for both piperazine-2-carboxylic acids was greater than 99% (Fig. 7d)[78].

Enzymatic oxidation of methyl groups on aromatic heterocycles

Chemical oxidation reactions used for the industrial-scale preparation of heteroaromatic monocarboxylic acids from heteroaromatic compounds bearing one or more methyl groups are often nonspecific and lead to the formation of undesired by-products. To overcome this problem, Lonza has developed a biological oxidation method for this type of reaction.

Wild-type *Pseudomonas putida* ATCC 33015, capable of growing on *p*-xylene as the sole carbon and energy source, was used as the biocatalyst in these investigations. Both the biochemistry and genetics of the xylene degradative pathway have been studied extensively. *p*-Xylene (**12** in Fig. 7e) is oxidized by xylene monooxygenase to 4-methylbenzylalcohol and further oxidized by other enzymes to the aldehyde (**13**) and 4-methylbenzoic acid (**14**). The aromatic carboxylic acid is converted by toluene dioxygenase and a dehydrogenase into 4-methylcatechol (**15**) before cleavage of the aromatic ring by catechol dioxygenase. The cleavage product is then transformed into Krebs-cycle intermediates (Fig. 7e). Lonza has now demonstrated that

P. putida previously grown on *p*-xylene is capable of oxidizing many methylated five- and six-membered rings to the corresponding monocarboxylic acids, without decarboxylation or ring hydroxylation.

The performance of the enzyme was studied in greater detail on 2,5-dimethylpyrazine (**16** in Fig. 7e). The oxidation product, 5-methylpyrazine-2-carboxylic acid (**17**), is an intermediate for the production of an antilipolytic drug. High product concentrations and high yields were achieved with growing cells. For this reason a mixture of 75% (by volume) *p*-xylene and 25% 2,5-dimethylpyrazine was supplied as growth substrate in large-scale fermentations. After 54 hours, up to 20 g l^{-1} of 5-methyl-2-pyrazinecarboxylic acid was achieved (Fig. 7e)[42].

Conversions of steroids, fatty acids and oligosaccharides

A well established industrial application of biocatalysis is the biotransformation of steroids, the products of which are used in contraceptives and other steroid hormone derivatives. Basic building blocks for these important drugs can be derived from natural phytosterols from soya (typically a mixture of β-sitosterol (40%), campesterol (25%) and stigmasterol (25%)), from conifers (tall-oil: mainly β-sitosterol (70%), campesterol (10%) and β-sitostanol (15%)) and from rape seed (β-sitosterol (45%), campesterol (35%) and brassicasterol (12%)). Using mutants of *Mycobacterium* sp. devoid of steroid-ring degradation activities, Schering (Berlin and Bergkamen, Germany) uses these natural raw sources mainly for the production of androstendione and androsta-dien-dione on a scale of 200 m^3. The size of the world market exceeds 1,000 tons per year. These steroids are used as basic substrates for subsequent chemical and biotechnological syntheses of drugs. Biocatalytic processes with economic significance are hydroxylations (for example at the 11α or 11β positions with *Curvularia* sp.), dehydrogenations (Δ1-position; hydrocortisone to prednisolon) and reductions (17-keto-reduction). These processes are run at Schering at a scale below 100 tons per year[79].

Shimizu[80] has developed efficient fungal-based systems for the production of polyunsaturated fatty acids (PUFAs). These are precursors of a wide variety of metabolites such as prostaglandins, leukotrienes and hydroxy-fatty acids that regulate critical biological functions. PUFAs are required in every organ for the human body to function normally.

A promising strategy for the application of coenzyme-dependent enzymes in multistep whole-cell biocatalysis has been developed by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). This approach allows the production of oligosaccharides at high productivity, high product concentration and high yield, and is applied on a cubic-metre scale[81]. One product is globotriose, which is produced with a productivity of 5.22 g l^{-1} h^{-1} and a final product concentration of 188 g l^{-1}. The concept is based on the application of whole-cell coenzyme regeneration systems and recombinant enzymatic synthesis pathways in different non-viable but metabolically active microorganisms. Reaction intermediates as well as coenzymes diffuse freely among the permeabilized cells.

The next few decades

Cost of whole-cell biocatalytic processes

A detailed analysis of total production costs for the oxidation of *n*-alkanes to terminal *n*-alkanols in a two-phase, whole-cell bioconversion, followed by phase separation and a two-stage product distillation, showed that product costs would be of the order of US$8 per kg product for both fed-batch and continuous processes, based on a production scale of 10,000 tons per year (ref. 40). This required 800 or 2,000 m^3 of bioreactor volume (continuous and batch processes respectively), as well as a processing unit for phase separation, distillation of substrate and product from the carrier solvent, and final separation of product from substrate (also by distillation). The estimated cost distribution was as follows: equipment depreciation and maintenance, 18–22%; substrates and solvents, 20%; energy, 10%; waste handling, 5%; personnel, 2%. The single major expenditure comprised medium salts and the carbon source (glucose), which accounted for 40% of all costs owing to the limited biocatalyst activities and lifetimes.

A first estimate of whole-cell process feasibility can be based simply on the volumetric activity and catalyst stability that can be expected for a given (recombinant) whole-cell biocatalyst. The rule of thumb is that for products valued at less than US$20 per kg, the intended production should exceed 1,000 tons per year and volumetric activities should be above 100 international units per litre to warrant further work.

For the present time, biocatalytic processes will therefore undoubtedly be developed for products compatible with costs greater than US$20–30 per kg. But the above numbers demonstrate that there is significant scope for improvement by increasing biocatalyst activity and stability. This reduces medium costs, as well as the size of the fermentation equipment necessary for given production runs. Synthesis of products with values in the range of US$5–10 per kg may be feasible, and attempts to develop major products (50,000 tons per year or more) with values of US$2–5 per kg may be worth considering.

Although research on improved, multiphase bioconversion technology will be useful, it is the biocatalysts that present the most interesting target for improvement. Development of appropriate activities and selectivities enable biocatalytic processes in the first place, and improvements of activity and stability may make a process economically feasible. Thus it is not surprising that research efforts, both for whole-cell systems and isolated enzymes, have focused on improving enzyme properties such as substrate range and specificity, stability and, most interestingly, function in non-classical environments.

Biochips and combinatorial biocatalysis

Nonaqueous enzymology is maturing rapidly. The ability to place virtually any enzyme into an organic solvent and observe catalytic activity is now commonplace. The combination of catalyst engineering with directed evolution and gene-shuffling techniques will yield enzymes with tailored selectivities and synthetically relevant activities in essentially any suitable reaction medium. Increasing the stability of enzymes at solid interfaces and films and coatings[82] will result in more active and stable immobilized biocatalysts and biocatalytic materials for use in both aqueous and nonaqueous media. More active and stable enzymes at surfaces will enable protein-based chips[83] and nanomaterials to be developed for use in proteomics, functional genomics and microfluidic biocatalysis.

Finally, miniaturized biodevices may be combined with combinatorial biocatalysis[84] to generate libraries of biologically relevant compounds available for rapid screening. The high selectivity of enzymes for chemical-compound library synthesis enables a broad array of natural products and complex synthetics to be derived efficiently using biocatalysts. The mild reaction conditions and high selectivity of biocatalysts are critical for the generation of pharmacologically useful libraries from these lead compounds[85]. Combinatorial biocatalysis goes beyond drug discovery, and many of the same techniques used in pharmaceutical synthesis can be used to generate libraries of agrochemicals, biopolymers, nutraceuticals and separation media.

Continuous regeneration of coenzymes

From a process-engineering perspective, biocatalysts may be coenzyme dependent (such as oxidoreductases) or coenzyme independent (such as hydrolases). Coenzymes such as the pyridine dinucleotides (for example, NAD(P)H) or phosphorylated trinucleotides (such as ATP) are expensive and must be recycled. *In vivo* coenzyme recycling systems have been well established on the laboratory scale during the past two decades[86]. This approach is also used on the industrial scale where it is most easily achieved with intact and metabolically active cells as biocatalysts.

Highly efficient *in vitro* coenzyme recycling methods have been developed and have enabled the use of isolated oxidoreductases on

the laboratory scale (see refs 87 and 88 for reviews). An outstanding application on the industrial scale is the production of L-*t*-leucine in a membrane reactor by Degussa with enzymatic recycling of NADH using formate/formate dehydrogenase[89]. Recently, Clair and co-workers (ref. 90) reported a promising new approach for recycling of NADH in a crystalline preparation of horse-liver alcohol dehydrogenase (HLADH-CLEC). Crosslinking of enzyme crystals significantly stabilized the enzyme activity for two days in up to 90% polar organic solvents such as isopropanol. HLADH-CLECs were used in a small packed-bed reactor for the reduction of cinnamaldehyde to cinnamyl alcohol, with butanediol as the source of reduction equivalents and a calculated turnover number for NADH of 12,000.

Electrons can be supplied to oxidoreductases by electrochemical methods. For example, rhodium complexes have been used to channel electrons to NAD(P)H-dependent oxidoreductases, resulting in highly specific reduction of NAD^+ (refs 91, 92). Mediator proteins have also been used[79]. Attempts to avoid the use of labile and expensive pyridine nucleotides by using artificial redox mediators such as viologens or anthraquinone derivatives have been described for C–H bond-forming oxidoreductases derived from anaerobic microorganisms[93]. A new *in vitro* regeneration system for ATP has been described recently[94]. Here, the ATP-dependent formation of glucose-6-phosphate from glucose was achieved by coupling polyphosphate:AMP phosphotransferase and adenylate kinase. This allowed the *in situ* formation of ATP from AMP with phosphate groups derived from inexpensive polyphosphate.

Impact of biocatalysis in the chemical industry

The success of biocatalysis depends ultimately on the economics of specific processes. It provides enormous opportunities; and with the introduction of each new process, experience and confidence accumulate and it becomes easier to develop and justify the next biocatalytic process. This has been the experience at BASF, at DSM, at Lonza, and undoubtedly at other companies that have developed such processes.

Biocatalysis in organic solvents entails many of the same requirements and issues as standard organic chemical processes with respect to equipment, piping and tubing materials, chemicals handling, safety, storage, transportation, GMP and regulatory issues — all of which are already familiar to the chemical industry. The challenge now is to develop a range of useful biocatalysts that can be used in organic synthesis. As this review and the accompanying articles show, the basic tools are abundantly available. There now exist several specialized suppliers of new biocatalytic reagents, such as Novozymes, Genencor, and more recently Diversa and Maxygen. We anticipate that there will be accelerated growth of biocatalytic processes in companies that already have developed some expertise in this area, such as those represented by three of the authors. Other companies currently at an earlier stage in exploiting biocatalysis, such as Dow Chemical and Aventis, will surely expand their activities in this area in the coming decade. □

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