MICROBIAL TRANSFORMATIONS

1. Introduction

Biotransformations (also referred to as bioconversions) are reactions of organic compounds facilitated by either isolated enzyme or whole-cell biocatalysts. Biocatalysis is widely applied in industry for pharmaceutical, agrochemical, chemical, fragrance and flavor, nutritional, and bioremediation purposes. Since the last edition of this encyclopedia in 1998, significant new advances in methodology have occurred spurring a broader application of biotransformations. Many additional new enzymes from microorganisms and other sources have been characterized, and methods for their isolation, stabilization, and use have expanded. At the same time, advances in biocatalyst development via recombinant technologies have provided the basis for unprecedented biocatalyst engineering and use. Biocatalysis has been increasingly extended to reactions in nonaqueous systems, in which many organic reagents and precursors of interest are highly soluble and reactions that are difficult to achieve in aqueous solutions are possible. Additional advancements in the use and development of biocatalysis have drawn from the availability of more sensitive, rapid, and informative analytical techniques. Continued progress in biocatalysis will be achieved from the diverse areas of organic chemistry, analytical chemistry, biochemistry, molecular biology, microbiology, and engineering. Indeed, as this article will make clear, the most successful practitioners of biotransformations have an appreciation and understanding of the highly interdisciplinary nature of biotransformation development. Nevertheless, biotransformation techniques have evolved such that the synthetic chemist can readily incorporate these tools just as many other synthetic approaches.

Enzyme catalysts have several features that render them attractive as a class of reagents for organic synthesis. Enzymes are chiral catalysts. They often bind substrates very specifically and afford high regio-, stereo-, and enantioselectivities. The exquisite selectivity of enzyme-catalyzed reactions obviates the need to block undesirable reactions that commonly occur using traditional organic synthesis when multiple functional groups are present. Biocatalysis occurs under mild reaction conditions requiring no strong acids or bases, temperature extremes, rigorously controlled atmospheres, heavy metals, or other conditions commonly associated with chemical catalysts. Thus, labile and complex chemical structures can be modified more effectively. In addition, unique and multistep reaction pathways can be efficiently conducted within a single microorganism. Finally, the maturation of genomics, molecular biology, and *in vitro* evolution techniques have started to deliver the promise to provide highly efficient and tunable catalysts, tailored for specific synthetic goals. While routine harnessing of this potential is still years off, biocatalysis is today a viable alternative for conducting many synthetic reactions and processes.

While possessing unique reaction selectivity, many enzymes have evolved to acquire surprisingly broad tolerance toward the structure of organic molecules accepted as substrates in biotransformations. This tolerance is especially

relevant to catabolic enzymes involved in digestive, defensive, and similar degradation roles in living organisms, which by necessity must have evolved to handle highly diverse organic structures. Such enzymes, which include hydrolases and oxygenases, are most commonly used for practical organic biotransformations.

This distinction helps to counter a prejudice that enzymes are too specialized to be used for general organic synthesis: Although some enzymes react only with specific organic substrates, the substrate tolerance of other enzymes is remarkably broad. A well-known example is the reactivity of cytochromes P450 (1) with chemicals of broadly different structures; some human forms of this class of enzymes catalyze hydroxylations or dealkylations on up to 50% of pharmaceutical compounds tested with them (2). More general and convincing demonstrations are microorganisms capable of degradation and derivatization of synthetic organic compounds only recently introduced to the environment (3). This versatility may arise not only from the tolerance of individual enzymes, but especially from the huge number of enzyme structures that have evolved to catalyze various chemistries on organic compounds. Thus, almost any organic compound can serve as a reactant in enzyme-catalyzed transformations.

With this basic understanding of biotransformations, this article is devoted to practical applications of microbial biotransformations to organic synthesis of fine chemicals, food and consumer products, pharmaceuticals, pesticides, and other important commercial chemicals. It is intended as a practical and concise guide to an enormous literature and provides a basis for simple and productive experimentation by scientists of many disciplines who may benefit from biotransformation technologies, or desire to be more conversant with biotransformation specialists.

2. Applications of Microbial Transformations

2.1. Industrial Biotransformations. Microbial transformations have found use at all scales of industrial chemical processing (4–6). Microorganisms are of considerable economic importance in the manufacture of antibiotics, steroid hormones, alkaloids, vitamins, amino acids, organic acids, industrial solvents, nucleosides, nucleotides, fermented beverages, and fermented foods. The industrial success of biotransformation technologies ultimately depends on the economics of the given process. Historically, due to the ability to efficiently carry out sophisticated and highly selective transformations, biocatalysis has found many successful applications in the pharmaceutical and fine chemical industries, as documented in several excellent recent reviews (7–15). Modern advances in molecular biology, biocatalyst preparation–immobilization, and better compatibility with common organic solvent process streams and processing conditions have enabled new opportunities for developing highly efficient, economical bioprocesses that can successfully compete with conventional chemical production.

These advances have also built momentum for harnessing the fundamental advantages of biotransformations for bulk chemical applications (Fig. 1). Commodity chemicals produced by biocatalytic approaches are highlighted by the

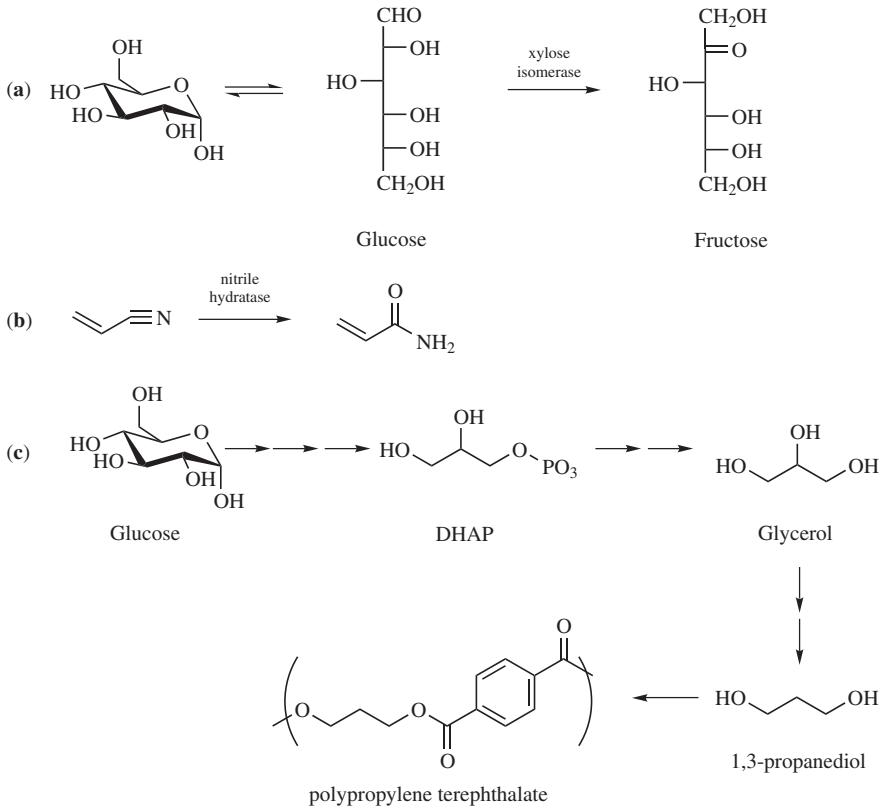


Fig. 1. Large-scale industrial bioconversions of (**a**) glucose isomerization to fructose catalyzed by microbial xylose isomerases, (**b**) synthesis of acrylamide from acrylonitrile, and (**c**) engineered metabolic pathway for the production of 1,3-propanediol from glucose for the production of polypropylene terephthalate.

production of acrylamide from acrylonitrile on the scale of 30,000 tonnes/year using nitrile hydratase from *Rhodococcus rhodochrous* (16). As further evidence of the adaptability of biotransformation processes to large scale, millions of tons of high fructose corn syrup are produced each year by the isomerization of glucose into fructose (17). Numerous additional large scale food and flavor applications employ microbial transformations, including L-ascorbic acid, citric acid, glutamic acid, various other amino acids, vinegar and malt (10). Additionally, the biosynthesis of 1,3-propanediol (and other monomer building blocks) from simple sugars provides an opportunity for a bioprocess to significantly impact the polymer industry: The demand for polymers based on 1,3-propanediol and additional specialty monomers synthesized using microbial catalysts is estimated to exceed 1 million tonnes/year in 10 years (18). Industrial-scale biotransformation processes such as these often offer a more economical, safe, and environmentally friendly alternative to chemical synthesis (19) due to the greatly reduced energy consumption, improved yields, and dramatic reduction of toxic waste, such as heavy metal catalysts.

2.2. Microbial Biotransformations for Drug Metabolite Production. Similar to other xenobiotics, drugs are recognized by the living organism as extraneous, potentially toxic agents, and therefore elicit a protective detoxification response. Upon administration to humans or animals, drugs typically undergo a complex series of metabolic transformations, primarily in the liver, evolved to promote elimination from the body. As a result of metabolic transformations, drugs can lose therapeutic activity, or result in metabolites with completely different pharmacological profiles, leading to unexpected and possibly toxic side effects (20). Since metabolites of several drugs have resulted in serious side effects, including deaths, detailed evaluation of key drug metabolites is an increasing important component of efficacy and safety studies on drugs prior to their approval for human use (21). Substantial quantities of metabolites of investigational drugs are therefore required for assay standards, structural characterization, and pharmacotoxicological studies prior to and during clinical trials.

Systematic studies of microbial transformations in mid-1970s revealed that microorganisms can closely mimic most of the metabolic transformations of drugs that occur in mammals (22). Fungi, which are eukaryotic organisms, are especially useful as model systems for mammalian metabolism studies, because they contain enzymes similar to hepatic enzymes responsible for metabolism in mammals (23,24). Species of *Cunninghamella*, and *Beauveria* have been especially successful as models for mammalian metabolism (23,25). Actinomycete and prokaryotic strains have also shown utility (26).

Recent developments in recombinant DNA technology have spurred the use of microbial transformations for the production of authentic drug metabolites (27,28). Many major enzymes of human metabolism, including cytochromes P450 and sulfotransferases, have been successfully cloned and expressed in heterologous microbial hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae* (29,30). These advances open new wide opportunities for large-scale production of authentic human metabolites using inexpensive and easy to maintain and grow recombinant microorganisms (28,31)

Microbial production of drug metabolites offer a number of practical advantages over alternative methods (26). For many metabolites, chemical synthesis can be quite difficult or even impossible, especially for drugs with complex molecular structures. Isolation of metabolites from body fluids (plasma and urine) or isolated liver tissue fractions of laboratory animals or human volunteers are complicated by the limited availability and high cost of tissues or subjects, low concentration of metabolites, and difficult purification. In contrast, microbial biocatalytic systems are easy to prepare at a low cost, and they can be readily screened in large numbers to identify those most suitable for the production of metabolites. Typical metabolic enzyme transformations that can be difficult to reproduce chemically, such as hydroxylations and stereospecific conversions, can be mimicked by microbial enzymes. In general, much higher concentrations of drugs can be used than in mammalian systems, and the stability of microbial biocatalysts tend to significantly exceed those of mammalian tissues or cells. As a result, and due to the fact that microbial fermentations can be easily scaled-up, significant amounts of metabolites can be synthesized, characterized, and isolated for use in pharmacological and toxicological studies.

Microbial systems have been extensively and successfully used for predicting mammalian metabolic pathways and preparative synthesis of mammalian metabolites. A representative example is the biotransformation of a tricyclic antidepressant, cyclobenzaprine, by *Cunninghamella elegans* (Fig. 2) (32). As a result of the microbial reaction, the drug was converted into six different metabolites, which were synthesized on a preparative scale, isolated, and their structures were determined by nuclear magnetic resonance (nmr). In a separate experiment, cyclobenzaprine was subjected to metabolic degradation by rat liver microsomes, which were found to produce a set of metabolites identical to those produced by *C. elegans*, albeit in different ratios compared to the microbial model system (32). Numerous similar examples have been summarized in recent reviews (26,33,34).

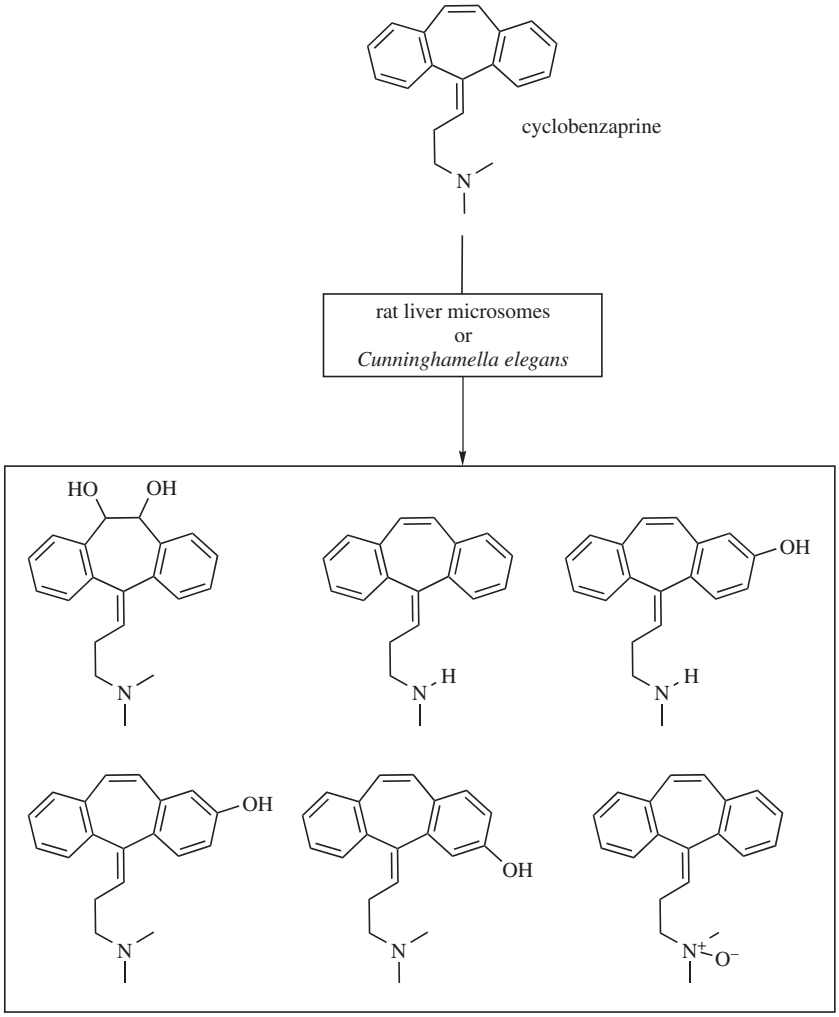


Fig. 2. Metabolism of cyclobenzaprine in rat liver microsomes and *C. elegans*.(32).

3. Chemistries for which Microbial Transformations are Commonly Utilized

Generally, microorganisms and their enzymes have been most frequently used to functionalize nonactivated carbon atoms, to introduce centers of chirality into optically inactive substrates, and to carry out optical resolutions of racemic mixtures (35,36). An outline of the types of chemistries catalyzed by microbial catalysts, which focused on the types of transformations that have most frequently benefited from biotransformation approaches, is offered below to help understand if biotransformations should be considered for a given synthetic problem.

3.1. Hydrolysis. Stereo- and regioselective hydrolysis are among the most widely applied microbiological reactions. Hydrolytic microbial enzymes are generally stable, even in organic process solvents, and require no cofactors for catalysis. Extensive application of hydrolysis for stereoselective resolutions of many esters, glycosides, epoxides, lactones, β-lactams, nitriles and amides have been covered in many reviews. As chirally pure drugs become more important to the pharmaceutical industry, stereoselective enzyme hydrolysis has become an important method to complement chiral separations and direct asymmetric synthesis (37). These techniques also extend broadly to applications in other fine chemicals, agrichemicals, flavors, and fragrances.

Numerous examples of specific hydrolytic reactions also exist. Selective hydrolysis has allowed removal of sugar moieties from pharmaceutically important cardiac glycosides and saponins (38). Selective and mild deacylation of steroids, opiates, and alkaloids have been reported using numerous organisms (39–45). As an important example, the hydrolysis of the amide bond of penicillins to give 6-aminopenicillanic acid (6-APA) is economically valuable since this acid is the principal intermediate in the chemical or enzymatic preparation and manufacture of semisynthetic penicillins. Although 6-APA was originally produced by direct fermentation in the absence of the side-chain precursors, the yields were low and the presence of penicillins that are also formed complicated its extraction and purification (46). The compound 6-APA is made on large scale by selective enzymatic hydrolysis of penicillin G (benzylpenicillin) or penicillin V (phenylmethoxypenicillin), which are produced in high yields by direct fermentation. 6-Aminopenicillanic acid has been prepared by conventional batch processing and by using immobilized cells, spores, and immobilized enzymes (47–49).

In a similar way, several cephalosporins have been hydrolyzed to 7-aminodeacetoxycephalosporanic acid (50), and nocardicin C to 6-aminonocardicinic acid (51). Penicillin G amidase from *E. coli* has been used in an efficient resolution of a racemic cis intermediate required for Loracarbef synthesis (52).

As mentioned above, microbial nitrile hydratase has been used in an industrial-scale production (3×10^4 tons/year) of acrylamide from acrylonitrile. Microorganisms, such as *R. rhodochrous* and *Pseudomonas chlororaphis* may catalyze complete hydrolysis of the nitrile to the corresponding acid by the action of nitrilases. They may also catalyze the stepwise hydrolysis of a nitrile to form amide and subsequent amide hydrolysis to the corresponding acid. Such enzymes are differentially induced sometimes within the same organism and features of the nitrilase gene of *R. rhodococcus* have been described (53). Significant recent

advances have been made in the further development of nitrile hydrolyzing enzymes (54).

3.2. Oxidations. Microbial oxidations have a long record of utility. Among the first demonstrations of microbial transformations was the bacterial oxidation of ethanol to acetic acid by Pasteur in the 1860s (55). Later in the 1930s, the oxidation of D-sorbitol, by *Acetobacter suboxydans* became an important step in the Reichstein-Grussner synthesis of L-ascorbic acid (56). In 1949, when cortisone and hydrocortisone were identified as potent antiinflammatory agents and no adequate synthesis existed to meet the sharply increased demand for these compounds, the problem of introducing functionality at C11 was solved via microbial hydroxylation progesterone to 11α-hydroxyprogesterone by *Rhizopus arrhizus* and also *Aspergillus niger* (57).

In the wake of these findings and the subsequent increased efficiency of this reaction by (*R. nigricans*) massive programs were launched to modify other sites of the steroid molecules by microorganisms in an effort to develop efficient syntheses of steroid hormones and to find new derivatives with more specific physiological activities than the parent compounds. As a result of this research, stereospecific microbial hydroxylations at practically all available carbon atoms of the steroid molecule were found (58,59). In addition, a stepwise β-oxidation carried out by bacteria allowed removal of sterol side chains (58).

Both mono- and polynuclear aromatic hydrocarbons can be oxidized by different microorganisms. Thus, *p*-cymene is converted to cumic acid and *p*-xylene to *p*-toluic acid (60) and a high yielding (98%) process has been developed in Japan for the production of salicylic acid from naphthalene (61). Microorganisms are also used to construct useful chiral synthons from various substituted benzenes. The technology of accumulating *cis*-dihydrodiols produced by the action of dioxygenases on benzenoid substrates (62). Mutant microbial strains accumulate the chiral diols that permit the highly enantiomerically controlled syntheses of agents, such as conduritols and pinitols (40–42,63).

Microbial Baeyer-Villiger reactions are well known in the steroid field, and applied for a variety of synthetic uses (64,65). As an early example of this reaction, the D rings of various steroids were converted into testololactone (66) by species of *Penicillium* and *Aspergillus*, and many other cyclic ketones are converted to their respective lactones by other organisms. Like many microbiological reactions, these conversions display the advantages of enantio- and regioselectivity.

Microbial oxidations continued to be important for a variety of oxidations to important acids, steroids, functionalized organic intermediates, and human and environmental metabolites of drugs and pesticides (67,68). Additional reviews of the microbial oxidation of aliphatic and aromatic hydrocarbons (69), terpenoids (70,71), lignin (72,73), flavors and fragrances (74), and other organic molecules (45,75–77) also have been published.

3.3. Reductions. Stereospecific microbial reductions have become a key approach for asymmetric synthesis of chiral alcohols in pharmaceutical, and other industries, and are among the most widely used microbial reactions in synthetic organic chemistry (39,44,45). Specific examples of practical applications to the reduction of cyclic and acyclic β-keto esters, aliphatic, and aromatic ketones as well as double bonds have been summarized (40–42). The efficiency of reduction of ketones to alcohols is often dependent on the size and nature of

substituents flanking the ketone functional group to be reduced. For example, the reduction of racemic decalone and hexahydroindanone derivatives and of related di- and tricyclic ketones by the fungus *Curvularia falcata* is highly stereospecific. The absolute configuration and stereoselectivity of the reduction is controlled by the steric size of groups flanking the ketone (78). Similar models often help to predict the stereochemistry of optically active alcohols obtainable from microbial oxidoreductases on ketone substrates.

As with other biocatalysts, new oxidoreductases with useful properties continue to be identified, such as the alcohol dehydrogenase from *Pseudomonas* sp. that transfers the *pro-R* hydride of reduced nicotinamide adenine dinucleotide (NADH) to the *si* face of carbonyl compounds to yield (*R*) alcohols (79). Microorganisms can also be used to implement selective reductions of β-diketones that are important in steroid syntheses (80). Reduction of 4-androstene-3,17-dione to 17β-hydroxy-4-androsten-3-one (testosterone) by yeasts is one of the earliest observed conversions of steroids (81). The microbial reduction of many simple and substituted aromatic carboxylic acids using whole cells of microorganisms, such as *Nocardia* sp. and *Aspergillus* have been described (82); the water solubilities of the substrates and the *in situ* regeneration of reducing cofactors by the living catalysts allows for high efficiency.

3.4. Additional Chemistries. Several additional biocatalytic chemistries have been of key importance to products. Asymmetric microbial acyloin condensation was discovered in 1921 (83) and utilized since 1934 in the synthesis of the natural (1*R*,2*S*)-ephedrine (84). In this thiamine pyrophosphate-mediated process, benzaldehyde is added to fermenting yeast and reacts with acetaldehyde, generated from glucose by the biocatalyst, to yield (*R*)-1-phenyl-1-hydroxy-2-propanone. The enzymatically induced chiral center helps in the asymmetric, reductive (chemical) condensation with methylamine to yield (1*R*,2*S*)-ephedrine. Substituted benzaldehyde derivatives react in the same manner (85). Similar asymmetric aldol condensations have been developed for the synthesis of unusual sugars for pharmaceutical and materials applications (40–42,86).

Asymmetric amination and hydrations by aspartase-producing bacteria have been used in the manufacture of L-aspartic acid (87), L-malic acid and L-Citrulline (88). Other L-amino acids have been obtained using related bacterial enzymes. Thus, by the addition of ammonium and pyruvate to the reaction mixtures, L-tyrosine has been produced from phenol, 3-(3,4-dihydroxyphenyl)alanine (L-DOPA) from catechol, L-tryptophan from indole, and 5-hydroxy-L-tryptophan from 5-hydroxyindole. Amination is also involved in the production of nucleotides, such as the flavor agent 5′-guanosine monophosphate (89). Likewise, hydration of DL-mixtures, L-isomers of other amino acids have been generated in 95–100% yields (60). Dehydration is also quite common for various applications. The selective biotransformation of hydroxy fatty acids to unsaturated fatty acids, elymoclavine to agroclavine, chanoclavine and other compounds; *cis*-terpin hydrate to α-terpineol; histidine to urocanic acid, or L-phenylalanine to phenylpyruvic, phenylacetic, cinnamic, benzoic and other acids are examples of this microbial chemistry in practice (38).

Transamination biocatalysts have been developed for either the synthetic production of chiral amines or for the resolution of racemic amines (90). The

reaction possibilities are illustrated for the stereospecific synthesis of (*S*)-α-phenylethylamine [enantiomeric excess (ee) of 99%] from phenylacetaldehyde by an (*S*)-aminotransferase or by the resolution of the racemic amine by an (*R*)-aminotransferase.

Isomerization using microbial enzymes has already been illustrated to be of considerable importance in the manufacture of high fructose syrup (91). Isomerizations as a common reaction throughout nature offers potential for development of additional processes. Dehydrogenation has assumed industrial importance in the synthesis of prednisone, prednisolone and their derivatives, all of which are more potent and have fewer side effects than the parent hormones (58). Finally, regioselective and mild carboxylations (92) and decarboxylations (93), N- and O- dealkylations (94,95), phosphorylations (96), and glycosylations (43) have also seen application. Overall, the diversity of selective, mild, and efficient reactions catalyzed by microorganisms and their enzymes, and the continuing discovery of new microbial enzymes—especially using modern DNA probe techniques—provides opportunities for new biotransformations to be developed.

4. Overview of Techniques

Success in the application of microorganisms and microbial enzymes as catalysts for organic reactions benefits from a working knowledge of simple microbiological laboratory techniques. Equipment for conducting sterile or aseptic techniques, and an understanding of suitable nutrient media required for growing catalytically active microbial cells are basic concepts. Different microorganisms and their contained enzymes have particular requirements for both optimal growth and catalytic activity. In addition to common environmental factors (temperature and pH), the ratios and amounts of carbon, nitrogen, phosphorus, trace minerals, special growth factors, and specific inducers of the desired enzyme activities are important for catalytically active cells (97).

Microbial enzymes always may be produced by the growing cell (constitutive) or only produced under certain conditions (nonconstitutive or inducible). Relevant to synthetic applications, it is possible (and necessary in the case of inducible enzyme pathways) to select growth conditions that favor the production of desired enzyme catalysts. Thus, environmental conditions can be controlled to favor specific single or multistep reactions, or eliminate potential side reactions. This is done by controlling the storage and preparation of a fresh microbial culture, the growth and transformation environment of the culture, the physical form of the organic substrate, and by establishing highly consistent and reproducible experimental protocols. Knowledge of the natural substrates of enzymes used to catalyze organic reactions, or even the identity of the enzyme itself, is not explicitly necessary.

It is also helpful, but not necessary to explicitly determine the location of the desired enzyme catalyst in the cell broth. Biotransformation enzymes may be present within (endo) or outside (exo) of the cells that produce them. Bacteria often contain water-soluble enzymes within the cytosol or excreted into the growth medium, and particulate enzymes bound to membrane structures. Yeasts

and fungi are more complicated; their enzymes are often compartmentalized within various organelles, including mitochondria, nuclei, vacuoles, as well as in cell wall membranes. *A priori*, there is usually no way to know the location of useful enzymes within the biotransforming cell. Therefore, experimental methods are designed to allow the transport of reactants to all possible catalytic centers, by favoring the highest possible solubility and dispersion in the reaction medium, and by enhancing permeability of the cells to the reactants.

With this basic understanding of important considerations for the use of microbial biotransformations, the rest of this section summarizes important concepts in the practice of microbial biotransformations, and in the design of successful biotransformation processes.

4.1. Selecting the Biocatalyst. Huge numbers of microbes coexist in almost all natural environments, particularly soils (estimated to have approximately billions of cells per gram of soil), waters, and sewage. The makeup of the microflora in these ecosystems is determined by the availability of oxygen and water, temperature, nutrients, and contaminants present. Widely different mixtures of bacteria, fungi, algae, and other microscopic life can be isolated from Nature by using different natural ecosystems as sources of inocula, and various isolation or selection techniques. This vast natural collection of microorganisms provides a virtually unlimited supply of diverse practical biocatalysts.

With access to a huge number of microbial strains, some intuition about narrowing the search for an acceptable biocatalyst can be valuable. Since microbial communities will evolve to best utilize the prevailing environmental conditions, considering the native ecosystem can help to narrow the search for candidate biocatalysts. Examples include the preferential isolation and use of thermophilic bacteria from hot springs for higher temperature bioprocesses (98) and selection of microorganisms for converting pesticides and organics by isolation from under leaky tanks storing the target compounds (99). The literature of the last 30 years also provides excellent leads to available organisms with specific, desirable enzymatic capabilities. Electronic databases of this literature, allowing relational and structure-based searching, provide an excellent tool for selecting good candidate biocatalysts (100). Other catalogues of chemical reactions catalyzed by microorganisms have been assembled with specific attention to groups of compounds, such as the alkaloids (101,102), the steroids (101,103), and nonsteroidal cyclic compounds including various drugs (101,104), and other xenobiotics.

While it may be possible to predict good candidate biocatalysts for a given transformation on a given substrate, the best catalyst most often must be identified from small-scale test reactions. This catalyst identification stage can generally be performed in two ways. The first strategy involves "screening" large numbers of individual reactions with pure cultures for a specified transformation. The second approach involves "selection" of a strain from a mixed culture, usually using its ability to grow on the test substrate as the selective pressure.

While the selection strategy from mixed cultures is commonly used for bioremediation studies, synthetic biotransformations greatly benefit from the use of pure cultures. Pure cultures are identifiable by their morphological, nutritional, and other characteristics that allow classification of organisms into taxonomic strata. Since pure cultures are definable reagents, they are easier to maintain,

possible to control, and their use helps ensure experimental reproducibility. Moreover, multistep reactions can be more easily studied and controlled with single biocatalyst strains compared with microbial mixtures. In essence, pure cultures are to biocatalysis what pure reagents are to chemistry. Microbial strains can be considered complex reagents and more straightforward results can be obtained from better characterized and purer reagents.

The complement of enzymes produced by microbial cells varies greatly at given times during the life cycle of the cell. The desired enzyme activity may be present continuously from the start of the growth cycle, or it may only appear or disappear in the late exponential, stationary, or cell death phases. The changes in enzyme activities during growth reflect the changes occurring within the cell and the culture medium as the organism grows and metabolizes nutrients. Thus, the optimum time for adding organic reactants or for harvesting cells must be established by experimentation. This is another motivation for using pure cultures for transformations.

A more recent, and growing option for selection of candidate biocatalysts is libraries of cloned enzymes in microbial hosts (54,105–108). Genes, coding for enzymes catalyzing a desired type of biotransformation chemistry, can be inserted into a selected microbial host. The host may allow more rapid screening, better tolerance of process conditions, and more options for optimization, compared to the original cell expressing the target enzyme. Individual recombinant strains can be further engineered to provide a more uniform microbial biocatalyst, and more defined enzyme inventory for screening for specific reaction chemistries. Advanced molecular biology techniques (109–111) can be applied to create a large focused subset of candidate enzymes for screening, optimize the best biocatalysts, and engineer for an industrial catalyst application. In this approach, the additional time to create the recombinant library and optimization approach can be considerable, but the screening and bioprocess performance can be significantly enhanced.

Rapid Screening of Microbial Catalysts. Even with a considerable narrowing of candidate microbial catalysts through rational selection, large combinations of microbial strains and conditions typically need to be empirically screened for biocatalyst selection and optimization. Therefore, efficient, flexible strain screening methods for evaluating a large numbers of microbial reactions are of key practical importance. Moreover, issues affecting the reproducibility and clarity of the large quantities of screening data for interpretation must be addressed prior to the screen. Fortunately, improvements in equipment, methods and analytical sensitivity make all of these goals more addressable.

Miniaturization and Automation of Microbial Culture Screening. Biotransformations, especially involving whole cells, can be labor intensive. The recent development of effective microscale equipment has recently made a huge impact on biotransformation screening and optimization efficiency. The challenges for rapid screening of large microbial (including clonal) libraries using numerous combinations of possible growth and reaction conditions are much easier to address using parallel arrays of microscale bioreactors. Limited quantities of organic compounds or precursors in the early development stage often further motivate miniaturized systems for biotransformation screening. Compatibility with modern laboratory automation, improved storage and

replication of stock cultures, decreased demands for incubation space, simplified sample processing by utilizing standardized parallel techniques and equipment are other advantages (112). Essentially, all of the protocols and guidelines mentioned in the following sections can now be scaled down for individual fermentation volumes of <1 mL. However, several issues require additional consideration when working with small volumes in nontraditional fermentor geometries.

Recent papers and reviews provide excellent coverage of the advances in the understanding of shaken microscale microbial cultures (113–116). The reliability and efficacy of these systems require attention to ensure reliable closure of individual wells to prevent cross-contamination and solvent evaporation, optimal well geometry, and the shake stroke to well diameter ratio (113,114). Consideration of culture morphology in small volume, high surface area fermentors, and biocatalyst form and preparation are also important. With less reaction sample available, automation can be useful to make microliter-scale liquid sample processing more precise as well as efficient. Subsequently, sensitive analytical techniques providing more information per analysis [eg, high performance liquid chromatography (hplc)/mass spectrometry (ms)] are increasingly favored to simplify data interpretation.

Miniaturized culturing systems for aerobic microbial growth based on the footprint of multiwell microtiter plates (Fig. 3) have been designed by many groups, and are commercially available (117,118). Such miniaturized fermentors–bioreactors have been successfully used in a variety of applications, including the production of secondary metabolites (119), whole-cell catalyzed bioconversions (120,121), and clonal libraries in *E. coli* (122,123) and yeasts (124,125). High throughput microbial culture techniques to screen for potential biocatalysts and optimize process variables will speed up the development of more robust industrial biotransformations (126,127).

A good example of the screening of resting cells in a microscale format is given by Semba and co-workers (128). The objective was to identify microbial catalysts with efficient *p*-hydroxylation activity on aromatic substrates. After an initial growth stage that isolated 23,400 strains from soil, colonies were regrown and induced on a solid media. Each grown strain was transferred to separate wells of a microplate containing 50 μL of buffer, phenol as a probe substrate for reaction, and glucose as a electron donor for the biocatalyst. A rapid dye indicator of the transformation of phenol to hydroquinone identified 1263 biocatalysts with different levels of the desired reactivity, for further ranking and development.

Biotransformation Analysis and Interpretation. As established earlier, screening of biocatalysts typically requires the execution and evaluation of large numbers of individual reactions. Once reliable and efficient biocatalyst reaction protocols have been established, the best improvement in the frequency of identifying new biotransformations comes from improving the throughput, sensitivity, and interpretation of reaction analysis.

A prerequisite for most high efficiency analytical methods is the development of rapid, parallel methods for the preparation of samples for analysis. The removal of catalyst and macromolecules (proteins, polysaccharides, polynucleotides, etc), and/or solvent exchange can be important steps prior to reliable use of many analytical techniques described below. Useful in this regard are

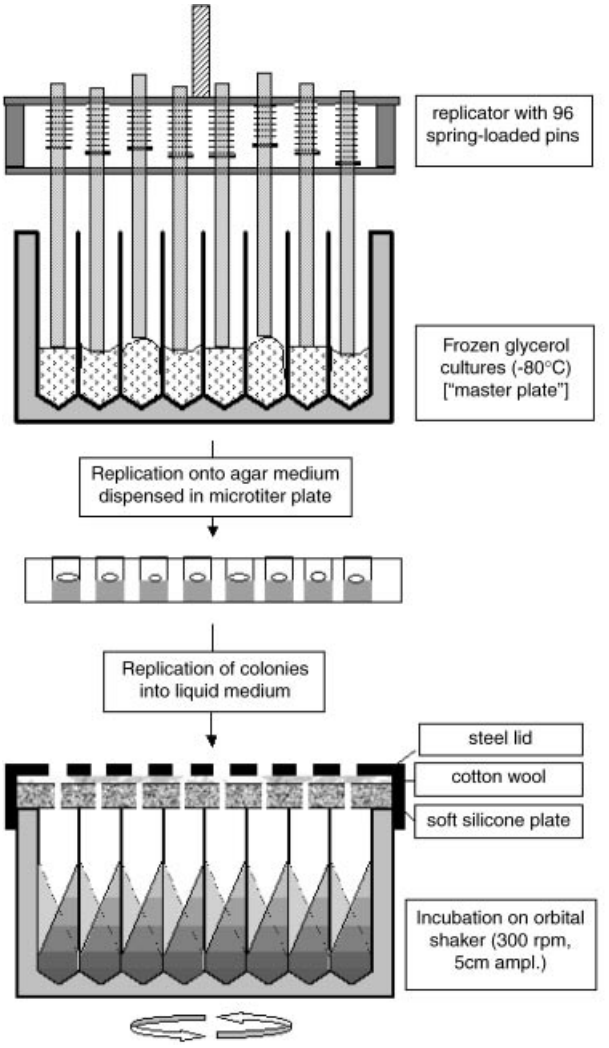


Fig. 3. System for the retrieval and growth of a microbial strain collection in a standard microtiter plate format. The spring-loaded replicator is used for the parallel sampling of stock cultures frozen in glycerol. The sampled cells are allowed to grow on an agar plate and transferred to a 2-mL square-deep well microtiter plate containing liquid medium, covered as shown and incubated on an orbital shaker.

the wide variety of filtration, ultrafiltration, liquid–liquid extraction, and solid-phase adsorption products that are now commercially available. Multicartridge manifold and 96-well plate-based techniques are particularly increasing in popularity due to the large number of samples that can be processed simultaneously with automated liquid handlers or manually.

Clearly, the best analytical method will be different for different lead molecules and different objectives. However, for almost all high throughput screening studies, thin-layer chromatography (tlc), gas chromatography (gc), and hplc have been the proven workhorses. Improvements in laboratory-scale ms equipment,

however, have made ms an important addition to the biotransformation practitioner's repertoire, either in direct flow injection mode, or in tandem with other analytical techniques [eg, liquid chromatography (lc–ms)].

Traditionally, TLC has been the primary method for analysis of biotransformations. The tlc is well-suited primarily because it is an inexpensive method for fast parallel analysis, and, thus, can be used to analyze a large number of reaction samples simultaneously. This technique can work very well as an initial screen if a sensitive, and preferably specific, indicator reagent is available for visualizing reaction components. However, its application to highly polar compounds can be complicated if they cannot be simply extracted from an aqueous reaction into a volatile organic solvent. Moreover, improvements in equipment and methodology for other, higher resolution and more informative techniques, such as hplc and ms, have increasingly made them attractive for the rapid characterization of complex reaction mixtures.

Gas chromatography is another commonly applied tool for analysis of biotransformation mixtures. In combination with a mass spectrometer, gc can provide rapid and sensitive quantitative analysis and structural information for reaction products present in the reaction mixture. Like tlc, gc usually requires extraction of the reaction mixture into a volatile organic solvent for application to the column. Moreover, application of gc is limited to compounds that are volatile or can be derivatized to a volatile substance. Many nonvolatile, functionalized organic compounds, or thermally labile compounds, are poorly analyzed by gc; for analysis of unanticipated products of biotransformation screens, these limitations can be undesirable. Nonetheless, for many volatile test substrates, gc is the method of choice.

In contrast to gc, hplc analyses are not limited by the molecular weight, volatility, thermal stability, or organic extractability of test compounds and derivatives, and therefore are applicable to a very broad range of substrate molecules and their derivatives. The general versatility of hplc methods makes them very attractive for analysis of biotransformation screens and reactions. Moreover, a wide variety of high resolution separation columns with different stationary-phase chemistries, and analyte detection methods are commercially available. However, typical analysis times of 15–45 min per sample for a serial analytical technique have limited its use for biotransformation screening from large biocatalyst collections.

Recently, very rapid, high throughput hplc methods have been described for the analysis of large libraries produced by combinatorial chemistry or natural product discovery (129,130). These approaches apply "universal" solvent gradients to separate a broad diversity of compound classes and use automated instruments for processing multiple samples. Such approaches have also been adapted for general application to biotransformation screening (131-133). Several hundred to thousands of injections a day, yielding resolution adequate for biocatalyst screening, can be performed using 1–10-min run times. Proper sample preparation, however, is more critical; sharp solvent gradients at high column pressures on high efficiency, small particle size packed columns result in a higher susceptibility to plugging with microbial debris or precipitated proteins.

One of the most powerful methods for rapid identification and structural characterization of biotransformation products is ms. The advantages of ms

analysis include broad applicability, high sensitivity, large information content, relative ease of interpretation, and very small volumes of sample required. With appropriate sample preparation to minimize interferences from the biotransformation medium, ms can deliver specific molecular weight information even from minimally processed samples from a biotransformation mixture either via direct injection, or postseparation. Careful attention must be paid to impurities that may interfere by suppressing ionization of the desired analytes. Integrated gc–ms or hplc–ms analysis allows resolution of complex samples with simultaneous mass spectral characterization of individual components eluting from the chromatographic column. Especially coupled with high throughput separation methods described above, hplc–ms yields a broadly applicable, rapid analysis (~5 min per sample), giving an unparalleled degree of information. However, although equipment costs are decreasing rapidly, ms remains a very expensive and technically demanding tool, especially in comparison with techniques, such as tlc.

Additional recent advances in hplc with direct nmr detection of the eluent (134–136) can provide immediate structural characterization of biotransformation products, but this method is currently cost-effective for only a limited number of postscreening biotransformation analyses.

Thus, careful consideration of analytical strategies for biotransformation analysis is very important, especially in the common case when a large number of biocatalysts and several test compounds result in a considerable number of analyses to be performed. Analysis and interpretation can easily be the most time- and labor-consuming step of the process—and an important one, since undetected or unidentified products are lost, along with the work to produce them. For convenient initial screening for major transformations or degradation, tlc is a proven, cost-effective, efficient parallel technique. Both gc and hplc can be more informative, but are more expensive to run and will typically require more time per sample. The techniques of gc–ms or hplc–ms are both very expensive, but will likely give the most information per unit time, and greatest level and clarity of information. Selection of the best methods will ultimately depend on the type of test compounds and expected products, number of samples, time and resources available, stage of the biotransformation development, degree of information needed, and cost of missed information.

4.2. Using the Biocatalyst. *Addition of Organic Compounds to Reaction Mixtures.* Since microbial growth and biological reactions typically take place in aqueous environments, there is a natural tendency to restrict biotransformation reactions to aqueous media, and therefore to water-soluble organic substrates. In fact, biotransformations occur equally well with both lipophilic and hydrophilic substrates as long as an adequate concentration of reactants can be delivered to the biocatalyst. More directly, the key to success with biotransformations of lipophilic compounds is the enhancement of compound availability to the catalytic site of the appropriate enzyme.

It is generally assumed that access to the active site of microbial enzymes is possible only for compounds dissolved or dispersed in the reaction medium. But, for whole cell reactions, once contact with the cell occurs, substrates can penetrate the cell wall and membrane by passive or active transport. Cell surfaces and membranes, as well as enzymes themselves, have hydrophobic domains

that facilitate transport, binding, and reaction with lipophilic compounds. In addition, microorganisms produce a variety of endogenous emulsifiers that promote these reactions.

Several methods have been developed to improve the solubility and dispersion of reactants in water. The delivery and dispersion of lipophilic substrates is accomplished using chemical agents or physical methods that have a minimal impact on the bulk aqueous reaction medium. The most common method for adding water-insoluble substrates to a bulk aqueous reaction medium is in water-miscible organic "carrier solvents". Preferably, these solvents should have low toxicity to the biocatalyst and excellent solvation capacity. Common carriers include many of same solvents used for organic compound transfer and storage, such as dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ethanol, methanol, and acetone. This technique works with most water-miscible solvents. The use of these carrier agents is usually well tolerated by most microbial strains and isolated enzymes. However, there are some exceptions. For example, microsomal cytochrome P450 enzymes tolerate only very low levels ($<1\%$) of organic carrier solvents (2). Thus, additional strategies for compound delivery must sometimes also be considered.

Alternatively, dispersants, eg, surfactants, cyclodextrins, polymers, or solid resins, are frequently used. For example, the *Nocardia coralline* catalyzed oxidation of sterols dispersed with several different surfactants were compared with the performance of substrates sonicated to reduce their particle size (137). Cationic, nonionic, and anionic surfactants were used at 0.01% concentration. Some cationic and anionic detergents significantly inhibited cell growth. Most nonionic surfactants did not inhibit growth and provided good emulsification. Emal 10C, Emulbon T-83, Sorbon T-40, and Tween 80 surfactants significantly stimulated the oxidation of soy sterols.

Large molecule solubilizing vehicles can be used to solubilize substrates and improve cell permeability. Cyclodextrins enhance solubilities of water-insoluble substrates by forming soluble complexes (138) and poly(vinylpyrrolidones) (PVPs) also disperse many types of aromatic compounds in aqueous media by formation of coprecipitates (139). For example, for the hydroxylation of ellipticine by *Aspergillus alliaceus*, the addition of 60-g/L PVP allowed approximately two-fold increase in product yield and 20-fold increase in fermentor productivity (139).

Inert supports may also be used to adsorb and deliver a variety of compounds within the lattices of inert materials, eg, zeolites, molecular sieves, diatomaceous earth, and polymers, eg, divinylbenzene–polystyrene (101,140). Lipophilic compounds are adsorbed to the supports from solvent solutions. After evaporation of the solvent, bound compounds are added directly to incubation mixtures. The resulting ultrafine particle sizes and large surface areas promote a high degree of dispersion of lipophilic substrates. Potential toxicity of reactants or products is frequently reduced due to controlled exposure of the cells, yet adsorbed substrates are often efficiently biotransformed by cell enzymes. Stability, recovery, and purification of the product can also be greatly enhanced by sorbing the product to a solid support *in situ*.

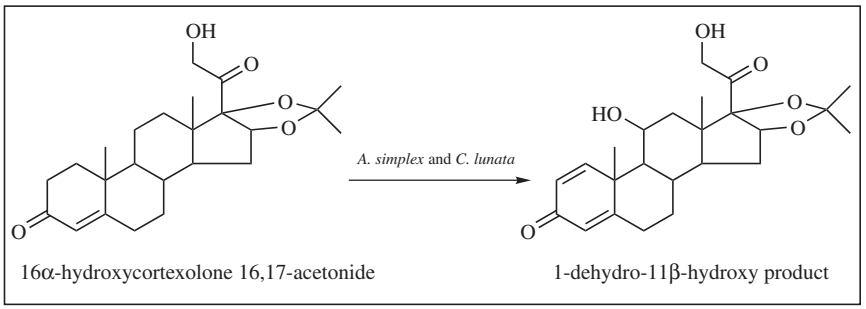
Physical milling or sonication has also been used effectively to disperse hydrophobic feedstocks. For example, finely milled progesterone wetted with

suitable amounts of 0.01% aqueous Tween 80 surfactant allowed 20–50 g/L of steroid substrate to be dispersed in the aqueous fermentation medium, and allowed nearly complete conversion to hydroxylated product (141).

A good empirical comparison of appropriate protocols for lipophilic substrate delivery to biocatalysts is offered by Lee and co-workers (142). They examined in detail the aggregation and solubilization phenomena of steroid substrates. A mixed culture of *Arthrobacter simplex* and *Curvularia lunata* catalyzed the simultaneous l-dehydrogenation and 11β-hydroxylation of 16α-hydroxycortexolone-16,17-acetonide.

Substrate was prepared: (*1*) in 0.1% (w/v) aqueous Tween 80 surfactant; (*2*) as a suspension in cold solvent; or (*3*) as solutions in hot and cold solvents. The substrates were added to the cultures immediately after preparation and again 25 h later. Best yields (60–90%) were obtained with hot solvents and cold DMF (Fig. 4). Yields were related to the particle size of the substrate. Hot solvents gave dispersions with 0.5–2-μm particles; those from cold solvents ranged from 10 to 100 μm. Apparently, ultrafine, amorphous particles are more accessible to enzyme active sites than crystalline forms. This may be due to improved rates of compound dissolution and improved cell permeability.

Timing of Substrate Additions. When growing cells are used for biotransformations, the time of addition of the organic substrate profoundly influences the yield of product. Toxic substances, such as many antibiotics and antitumor compounds, often inhibit growth and enzyme production if they are added early in the growth cycle. In many cases, however, it is advantageous to add at least small amounts of substrate at the beginning of the growth phase to promote



16α-hydroxycortexolone 16,17-acetonide 1-dehydro-11β-hydroxy product

Substrate delivery system	Conversion to product, %
Hot borate +ethanol+water solution	90
Hot ethanol+acetone solution	67
Hot ethanol solution	65
Cold dimethylformamide solution	60
Cold ethanol+acetone suspension	30
Cold ethanol suspension	20
Cold aqueous Tween 80 suspension	5

Fig. 4. Effect of substrate delivery system on the microbial conversion of 16 α-hydroxycortexolone-16,17-acetonide by a mixed culture of *A. simplex* and *C. lunata.* (142).

enzyme induction. The addition of substrate during the late logarithmic growth phase minimizes toxicity effects while promoting enzyme induction. At this point in their growth cycle, cells are still capable of enzyme synthesis, while the proliferation of the biomass is less likely to be inhibited by toxic substrates. The same reasoning applies to substrates added in toxic solvents.

The timing of substrate addition must take into account the physiological state of the microorganism. The position of the cell in its growth cycle determines its enzyme capabilities. In general, enzyme levels will be determined by the competing rates of enzyme expression and degradation–inactivation. Enzymes of interest may be expressed only at specific times during the growth cycle, eg, late log, or stationary growth phases. Looking for reaction when the enzyme is not present would be futile. Enzyme concentration may be subject to the presence of inducers of expression, fluctuations in the pH or temperature of the medium, the amount and kinds of carbon and nitrogen nutrients in the medium, and the degree of oxygenation of the medium. The optimal time for substrate addition is difficult to predict and is best determined experimentally.

Toxic substrates or substrates in toxic solvents may be added incrementally by "dosing" (143). Dosing techniques also side step the undesirable phenomenon of substrate inhibition, which almost invariably occurs when large amounts of substrate are added at a single time.

Multiphase Reactions. The use of organic solvents or aqueous–organic solvent mixtures as bulk media for biotransformations using enzymes or suspended cells is another powerful approach for feeding organic reactants, and incorporating biotransformations within conventional synthetic routes. From a practical, synthetic perspective, reactions in non-aqueous media provides three primary advantages: the ability to shift the thermodynamic equilibrium of hydrolytic reaction toward synthesis, the ability to solubilize a broad range of organic molecules at synthetically useful concentrations, and the ability to rapidly separate soluble reaction products from the insoluble biocatalyst. The inclusion of organic solvents may also minimize certain side reactions, and permit continuous extraction and recovery of reaction products. Much has been presented in the literature about other advantages of non-aqueous solvents, including improved thermostability, altered specificity, or decreased chance of contamination, which are of more limited applicability (144–146).

The primary difficulty with conducting biotransformations in the presence of organic solvents is lower catalytic activity and catalyst stability. Substantial literature is devoted to determining reasons for loss of catalytic activity, and methods for preventing it. A complete description is well beyond the scope of the present work, and is available from many excellent reviews (147–149). In many cases, the practice of non-aqueous biocatalysis, widely regarded as untenable $<$20 years ago, is today a successful reality. Practical guidelines for conducting biocatalytic reactions in the presence of organic solvents will be the focus here.

The behavior of whole-cell biocatalysts in the presence of organic solvents is somewhat distinct from that of isolated enzyme catalysts and will be treated separately. Several excellent reviews of activity preservation and solvent toxicity effects on whole-cell catalyzed reactions in organic solvents have been published (150-152). Much is still unknown about mechanisms for whole-cell solvent tolerance, due to the complex nature of the living cell, and some individual strains

exhibit large deviations from general trends. Empirically, however, several general recommendations can be made.

First, although no single solvent property has been definitively correlated to solvent tolerance, cell biocatalysts (both growing and resting) tend to maintain higher activity for a longer period with solvents of high hydrophobicity (normally expressed as solvent octanol–water partition coefficient, log P). Solvents with log $P > 4–5$ tend to make the most compatible media, while solvents of intermediate or low hydrophobicity (log $P = 0–4$) are often most toxic. Water-immiscible solvents are much better choices for the bulk organic phase than are water-miscible ones. In general, cell immobilization, usually by entrapment or encapsulation, significantly improves organic solvent tolerance, while permitting substrate access to the catalyst.

While hydrophobic solvents are biocompatible media for reactions with hydrophobic substrates, eg, steroids, many organic molecules of interest are of intermediate polarity and are not highly soluble in either nonpolar solvents or aqueous media. The addition of small quantities of a toxic solvent with good solvating power to a biocompatible, hydrophobic bulk solvent can provide the positive attributes of both; the biocompatible solvent extracts the toxic one away from the catalyst, yielding an organic phase capable of holding a suitable concentration of reactant.

Isolated microbial enzymes tend to exhibit much better retention of catalytic activity in the presence of organic solvents than whole cells. Like whole cells, enzymes tend generally to prefer more hydrophobic solvents with log $P > 2–4$, but tolerate a much broader range of solvents and solvent mixtures than do whole-cell catalysts. Polar solvents, such as acetonitrile, *tert*-butyl alcohol, monoglyme, and methyl *tert*-butyl ether preserve adequate catalytic activity of many enzyme catalysts.

As mentioned previously, however, general rules for non-aqueous biocatalysis are rare; indeed some highly tolerant whole-cell strains, eg, Pseudomonads, may serve as excellent recombinant hosts for nonaqueous biocatalysts, while important enzyme catalysts, eg, cytochrome P450s, exhibit a low tolerance even to minor levels of organic carrier solvents. Thus, the most prudent strategy presently is to screen an abbreviated list of good candidate organic solvents with each chosen biocatalyst.

Solid adsorbents can also be used when it is desired to deliver and recover reactants and/or products in a separate phase. Solid-phase resins can be more compatible with cell growth and enzyme activity, and can address problems due to compound instability, toxicity, or for ease of handling. As an example, 3,4-methylene-dioxyphenyl acetone was stereoselectively reduced to (*S*)-3,4-methylenedioxyphenyl isopropyl alcohal in 95% yield and 99.9% enantiomeric excess by *Zygosaccharomyces rouxii* (153). Both substrate and product were toxic to the biocatalyst, so polymeric hydrophobic resins, eg, XAD-7, were used to supply substrate to and remove product from the reaction mixture as it formed. Using the solid adsorbent increased fermentor productivity 15-fold.

In summary, to be considered general catalysts on a par with other, traditional chemical catalysts for organic synthesis, biocatalysts must be functional in a fair range of organic solvents. Practically, many organic molecules of interest for transformation have limited solubility in aqueous media, or in the highly

lipophilic solvents most often described in the literature for use with biocatalysts. Moreover, thermodynamic control of normally hydrolysis-favoring equilibria, ease of product recovery, and minimization of certain side reactions are also important motivations for conducting biocatalytic reactions in organic media. Over the last 10–15 years, significant strides have been made toward making practical, synthetic biocatalysis in nonaqueous media feasible.

Microbial Transformation Scale-Up. Fermentors are useful for larger scale biotransformations. With a fermentor it is possible to control culture parameters in ways and degrees not possible in flasks and tubes. Stirring and air-sparging devices allow the maximum possible aeration. Many parameters (eg, pH) can be measured and controlled continuously. Therefore, it is useful to have access to several bench-top fermentors (1–10 L) wonder for experiments that cannot be done conveniently in flasks and for scaling-up processes. Larger scale fermentor studies (20–1000 L) often involving sophisticated downstream processing will be more appropriate for pilot and larger scale processes. These will typically require the involvement of microbiology and fermentation experts with specially equipped laboratories.

5. Prospects for Further Advances

Enablement technologies are already starting to make an impact for future development of microbial transformations. Through advances in molecular biology, new biocatalysts are being produced solely from their DNA and RNA blueprints. Using RNA extracted from environmental samples, thousands of new enzymes have been made available by expressing the polymerase chain reaction (PCR) amplified sequences in suitable, generic microbial hosts. Shotgun cloning, and the effort devoted to genomic sequencing is providing many more opportunities to make additional genes, and the encoded biocatalysts, accessible to the synthetic chemist. Building on this, *in vitro* evolution approaches, eg, directed evolution and gene shuffling approaches permit the tailoring of enzymes for broader ranges of operation, higher efficiency, and new synthetic applications (111,154,155).

Additional processes are under development to take greater advantage of the synthetic potential of biocatalysis. Combinatorial biology attempts to engineer biosynthetic pathways within microorganisms to create modified versions of commercially important natural products (156,157). Combinatorial biocatalysis purports to make a general synthetic platform for compound derivatization by combining enzymatic, microbial, and chemical synthetic techniques (158,159). With the increasing importance of chiral synthesis and environmental safety, it is likely that biotransformation techniques will continue to increase in importance.

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