BIOCATALYSIS

1. Introduction

Bioorganic catalysis can be defined as the use of biological systems (whole cells or pure enzymes) to produce organic compounds. Bioorganic catalysis has been practiced from the ancient times mainly for the production of foods and beverages. The production of alcohol via fermentation, of vinegar via oxidation of ethanol by acetic acid bacteria, and the production of cheese via enzymatic breakdown of milk proteins are well-known examples. Biocatalysts are ever increasingly being exploited for the production of industrially important materials, in many cases competing with traditional chemical methods and in some instances performing reactions that traditional chemistry methods cannot. As a result, biocatalysis is now being considered as another implement in the chemist's arsenal for tackling chemical transformations.

One of the earliest examples of an industrial application of bioorganic catalysis is the chemoenzymatic synthesis of L-ephedrine and psuedoephedrine (1). In this process exogenous benzaldehyde was condensed with pyruvic acid produced within a yeast fermentation to yield L-phenylacetylcarbinol (L-PAC), which is a key intermediate in the production of L-ephedrine and psuedoephedrine. The synthesis of L-PAC is catalyzed by the pyruvate decarboxylase enzyme and can be produced at a price that competes with traditional chemical synthesis (2). In spite of the prevalent use of biocatalysts in organic synthesis some myths still exist, such as biocatalysts are sensitive, biocatalysts are expensive, biocatalysts have narrow substrate range, and biocatalysts cannot perform all possible chemical reactions.

Debunking the myth of biocatalysts is an ongoing task and there are many examples being presented to elucidate the many advantages of using biocatalysts

Table 1. **Enzymes Sales in Millions**[a]

Category	1980	1990	1999
detergent additives (protease, lipase, amylase, cellulase)	6.2	40	325
starch conversion (amylase, amyloglucosidase, fungal amylase, glucose isomerase)	69	135	181
food processing (lipase, pectinase, catalase, glucose oxidase, protease, papain, renin, pepsin)	47	90	146
textile processing (amylase, cellulase, catalase)	60	50	70
diagnostic	20	35	65
research	12	25	50
recombinant DNA	2	30	60
therapeutic (TPA, urokinase, others)	0	500	155
synthesis	0	0	10

[a] Dollar amounts do not incorporate inflation (Dr. Bernard Wolnak, data presented at "Enzymes for the Next Millenium, Chicago, USA, 2000").

for organic reactions. Even though most biocatalysts work in relatively moderate reaction conditions, advances in isolation and expression of extremophilic enzymes and genetic modifications of mesophilic enzymes have created biocatalysts that can tolerate substantially harsh conditions (3,4). Some enzymes isolated from hyperthermophilic organisms are active at temperature as high as 140°C and others isolated from psychrophilic organisms are active at temperatures as low as 4°C. Some enzymes from barophilic organisms isolated near deep-sea vents tolerate pressures as high as 100 bar. Even though enzymes are specific with respect to the type of reaction they catalyze, most of them have activity on a wide range of substrates. In addition, there are many instances where enzymes have been tailored using genetic engineering to suit the conditions in biotransformation processes. There are biologically catalyzed equivalents for almost all of the chemical reactions, even the Diels–Alder reaction and the Claisen rearrangement (5). Advances in genetic engineering and fermentation technology have enabled the production of many enzymes at high concentrations in high cell density fermentations, resulting in a substantial decrease in cost of manufacture of biocatalysts. This is evidenced by the increase in enzyme sales from $130 million in 1980 to $700 million at present (Dr. Bernard Wolnak, data presented at "Enzymes for the Next Millenium, Chicago, USA, 2000"). A breakdown in the enzyme sales during this time period in the many industries is shown in Table 1. However, not all enzymes are amenable to manipulations and cost contribution of the biocatalyst has to be evaluated on a case-by-case basis.

2. Whole Cells Versus Pure Enzymes

There are mainly two biological entities that can compete as bioorganic catalysts, and they are isolated enzymes and whole cells (microbial, plant, or animal) (6).

Several parameters that ultimately affect the cost of a process (product purity, throughput, stability) are important while selecting a particular form of biocatalyst in an industrial biotransformation process. An ideal process would be one where the culturing of the cells would also accomplish the biotransformation at a high product concentration. However, a lot of the substrates and products at any significant level are toxic to cell growth. In addition, separation of the product from the cell broth can be very difficult and there may be undesirable side reactions during cell growth. This necessitates the separation of cell growth and biotransformation. The only time whole cell biocatalysis is advantageous is when the biotransformation involves multiple enzymes, where cofactor regeneration is necessary, or when enzyme isolation is needed and is difficult.

The number and complexity of the steps in the preparation of the biocatalyst is proportional to the cost of the biocatalyst. Progress in genetic engineering has allowed the production of recombinant enzymes in microbial cells. However, production of all recombinant enzymes in microbial cells is not automatic and sometimes impossible. The cost of biocatalyst preparation has to be justified within the context of the biotransformation process involved. Purified enzymes have been used in many cases to demonstrate feasibility of a particular reaction. Separation of soluble enzymes from fermentation broth is cumbersome, and in addition there is the issue of enzyme stability during isolation. The overall cost of a biotransformation process can be reduced if the enzyme can be easily separated and reused. Immobilization of enzymes allows the recycle of enzymes, the ease of which is dependent on the method of immobilization. More than a hundred techniques for immobilization had been published by 1983 (6) and many more since (7). There are advantages and disadvantages to each method of immobilization and the decision of choosing one method over another is made on a case-by-case basis. The properties of an enzyme can change dramatically upon immobilization and this needs to be taken into account when immobilization is contemplated. Some of the problems encountered are enzyme inactivation, lowered enzyme activity, altered allosteric properties, and cost of immobilization. There are some advantages when enzymes are immobilized such as increased stability, high enzyme loading, altered pH, and temperature optima (6,7).

Immobilized cells could be used instead of immobilized enzymes to eliminate the costly isolation step while retaining the ability to recycle the biocatalyst. Typical cell immobilization methods are adsorption, gel entrapment, and compartmentalization in polymer matrices. The advantages for immobilized cells remain the same as for immobilized enzymes, except that improved enzyme stability is rarely observed. The disadvantages may be exacerbated because of the immobilization method and the fact that there is another barrier for the substrate to get to the enzyme, namely the cell membrane.

Crude enzyme probably represents the simplest form of prepared biocatalyst. The advantage is that there is little preparatory cost compared to immobilized enzymes, cells, or purified enzymes. The main disadvantage is that the biocatalyst cannot be recycled. Crude enzyme can be prepared by spray drying or freeze drying the cell culture. Freeze drying which is prevalent at the laboratory scale cannot be practiced economically at the large scale. The critical parameters in spray drying are the operating inlet and outlet temperatures and the residence time in the dryer. Aminotransferases synthesized in recombinant

Escherichia coli have been shown to retain >95% of their original activity after spray drying (Chiragene, Inc., unpublished data). Some of the advantages are that these spray-dried biocatalysts can be stored easily at room temperature, retain activity for an extended period of time, and can be used directly in the reaction without further processing.

3. Biocatalyst Performance

Biocatalyst performance parameters such as activity, selectivity, and stability can be altered by modifying the enzyme or the environment around it. The latter approach uses the principle of changing the solvent environment around the enzyme molecule (commonly referred to as solvent engineering) while the former approach uses the redesigning of enzyme to meet the desired goals. Solvent properties such as dipole, dielectric constant, hydrophobicity, and density have been shown to cause predictable effect on enzyme stability (8–10), activity, and enantioselectivity (11–16). The solvent engineering approach has been demonstrated mainly at small scale but has not been scaled up into commercial processes because enzyme catalytic efficiencies are 2–6 orders of magnitude lower in nonaqueous media than in aqueous solutions (17).

The other approach to improving enzyme performance parameters is by enzyme modification at the molecular level (commonly referred to as enzyme engineering). The enzyme can be altered to tackle the problem of production cost, poor activity, stereoselectivity, and stability. The earliest approach to engineering enzymes is through rational design on the basis of structure–function relationship (18). This requires knowledge regarding the protein structure either by x-ray crystallography or molecular modeling or a combination of both. This can be tedious in an industrial setting where the enzyme has to be improved within a matter of months if not weeks.

Error-prone polymerase chain reaction (PCR) is a powerful tool to introduce errors in the DNA sequence coding for the enzyme of interest (19–21). The advantage of this method is the speed at which mutations can be introduced and the fact that they can be targeted into the gene of interest. The result is a random mutation of the enzyme, resulting in many candidates in a short amount of time. A proper screening or selection method can then be used to pick the enzyme with the required property. With a little more knowledge on the effect of amino acid changes on changes in properties, this mutation strategy can be directed to improving a specific property by building on the initial mutations. Examples are the directed evolution of an esterase and subtilisin to perform in high concentrations of dimethylformamide (20,22,23). An alternative to single amino acid changes is the DNA shuffling method that recombines sequences from various versions of the enzyme to create a new enzyme with new properties (24). There are several published examples where significant improvements in enzyme activity, selectivity, stability, thermostability, or solvent tolerance have been achieved using both these methods (20,22,23,25–32).

In cases where there is no natural enzyme to catalyze the reaction catalytic antibodies have provided a starting point (33,34). The first report on the use of catalytic antibodies involved simple acyl transfer reactions (35,36). The list of

transformations has grown considerably since then to include ester and amide hydrolysis, lactonization, group eliminations, reductions, C–C bond formation and cleavage, Claisen rearrangement, and the Diels–Alder reaction (33,34). This technology has not matured to the stage where antibodies can be raised or produced in large quantities to carry out reactions at rates similar to existing enzymes. Another variation is the use of transferred active sites to inert protein scaffolds to create a synthetic enzyme (37,38). There is enough evidence in literature to indicate that any given enzyme can be modified significantly to cater to the needs of the biotransformation process.

4. Bioorganic Catalysis

The enzymes catalyzing oxidation/reduction reactions constitute an important class from an industrial perspective since industrial chemistry involves many oxidation/reduction reactions. The use of oxidoreductases for organic synthesis has been under intense investigation for the past several decades (39) and still continues.

Dehydrogenases, which constitute the largest class within the oxidoreductases, are enzymes that catalyze the reduction and oxidation of carbonyls and alcohols, respectively. Reduction of the carbonyl group can create a chiral center and therefore is very important from an industrial perspective. Oxidation reactions generally destroy the chiral center and can be useful where resolution of a racemic alcohol is needed. Detailed structural, mechanistic, and specificity data are available for this class of oxidoreductases (39). A broad range of substrate alcohols, from simple aliphatic to complex polycyclic, can be oxidized by three alcohol dehydrogenases (yeast, Horse liver, and *Pseudomonas testosteroni*) with overlapping specificities (40). Even though many oxidation/reduction reactions have been accomplished using dehydrogenases, there have been few that have been scaled for large-scale production. The main obstacles to the large-scale use of these enzymes are the cost of enzyme, cost of cofactor, and solubility of substrate.

Reduction of enzyme cost and increasing solubility of substrate are problems that apply to a lot of biotransformations. The solution to the problem of enzyme cost is to improve the performance significantly so as to utilize less of the enzyme and/or to produce the enzyme in recombinant organisms to improve productivity. The problem of solubility is often dealt with by using co-solvents to increase solubility of substrates or by running reactions in pure solvents. The more tenacious problem associated with biological oxidation/reduction reactions is the need for expensive cofactors such as NADH, NADPH, and FADH. These cofactors are usually required in stoichiometric amounts and that typically renders the biological route expensive. The only way the cost contribution from the cofactor can be reduced is via recycling of the cofactor. Several solutions for cofactor recycling have been presented and practiced with varied amount of success. Efficiency of cofactor recycling can be measured as the number of cycles the cofactor undergoes before it is destroyed (turnover number can be expressed as moles of product formed per mole of cofactor). Turnover numbers greater than tens of thousands need to be achieved in order for the recycling method to be

Table 2. **Turnover Numbers Achieved in Various Cofactor Recycling Methods**[a]

Method	Turnover number
chemical (sodium dithionite)	<100
electrochemical and photochemical	<1000
enzymatic (single or coupled)	10^3–10^5

[a] Ref. 50.

considered feasible at a large scale. Table 2 gives turnovers numbers for each of the methods used for cofactor recycling (5).

Chemical methods for cofactor regeneration using sodium dithionite, phenazine methosulfate, and flavin mononucleotide have been fairly successful. However the utility of chemical regeneration method depends on ease of separation of product and number of cofactor turnover in the reaction system (5).

Electrochemical methods have been studied as a means of regenerating cofactors (5,41). Electrochemical methods, although widely used in biosensors, need to demonstrate economic feasibility (high turnover number) before being accepted as method for regenerating cofactors. Another method is to use a second enzyme system to recycle the cofactor, and this has been successfully used in a small-scale process producing multi-kilogram quantities (42). Polyethylene glycol (PEG) derivatized NAD was used as cofactor in an ultrafiltration membrane reactor that allowed separation of cofactor/enzymes and substrates/products. Regeneration was provided by formate dehydrogenase (FDH) that catalyzed the oxidation of formate to carbon dioxide with the concomitant generation of PEG-NADH. FDH cost has been substantially reduced in order to make this recycling process economically feasible in some processes (39). Using the coupled enzyme process, cofactor turnover numbers of greater than 10^5 can be achieved, making it one of the best available methods (42). Unfortunately, the FDH cofactor regenerating system cannot be used with NADPH because the FDH does not accept NADP as a substrate.

A glucose dehydrogenase (GDH) cofactor regenerating system has been used to recycle NADPH and NADH via the oxidation of glucose to gluconolactone. Gluconolactone then spontaneously hydrolyses to form gluconic acid, making the reaction scheme favorable for both NADH and NADPH generation. Since glucose is a cheap substrate, this method can be very inexpensive, provided gluconic acid can be separated from the other products of the reaction. As with FDH the cost of GDH is a factor that has to be addressed. Other regenerating systems have been used such as glucose-6-phosphate dehydrogenase, alcohol dehydrogenases, and hydrogenase. The latter methods are less attractive than the FDH or GDH system for a variety of reasons that are not presented here (43).

The enzyme recycling principle can be applied using whole living cells instead of isolated enzymes (44–49). In this case, the main enzymatic reaction and the cofactor regeneration reaction are carried out during the growth of living cells. Growing cells produce cofactors using their intrinsic metabolic pathways from cheap carbohydrates and regenerate cofactor during their growth cycle. A mixed culture of *E. coli*, one expressing glucose dehydrogenase for cofactor recycling and the other expressing aldehyde reductase for asymmetric reduction of

ethyl 4-chloro-3-oxobutanoate, was used in a two-phase chiral alcohol production system successfully at laboratory scale (45). *Trichosporon capitatum, Geotrichum candidum*, Baker's yeast, and other whole cells have been used in a similar fashion to regenerate cofactor *in situ* during reductions of a variety of substrates (44,47–49). Even though some aspects of this method are attractive, there are drawbacks to whole-cell biocatalysis. Nonnatural substrates and products can be toxic to the cells at very low concentrations (0.1–0.3%) (5). Recovery of low concentration products from the reaction mixture may be troublesome with by-products from the growth of cells contaminating the product. Multiple enzymes can act on the substrate and impact the yield as well as stereoselectivity of the product. Side reactions on either substrate or product may reduce yield and purity. There are some solutions to these problems and they need to be evaluated on a case-by-case basis.

Enolate reductases which reduce C–C unsaturated bonds are another class of enzymes that require cofactor recycling. Since the products of these reactions can result in a chirally pure product, they constitute an important class of reactions. Typical biotransformations have utilized whole-cell biocatalysis rather than of isolated enzymes because of the ease of cofactor recycling on the laboratory scale. In addition to the problem of cofactor recycling, enolate reductases are inactivated by traces of oxygen (5).

Biological oxidation reactions achieve heteroatom oxygenation, aromatic hydroxylation, Bayer–Villiger oxidation, double bond epoxidation, and nonactivated carbon atom hydroxylation of substrates, which is difficult via conventional chemistry. The biological oxidation of primary and secondary alcohols to aldehydes is not of practical interest because these reactions are just as easily accomplished using conventional chemical methods, are theromodynamically unfavorable, have unfavorable reaction conditions, and in the case of secondary alcohols destroy an asymmetric center (5). The only context where it is meaningful is during the resolution of racemic alcohols. As discussed earlier, the regeneration of cofactor is a major stumbling block in this scheme.

The regioselective oxidation of polyols is of practical interest because biocatalysts can selectively oxidize one hydroxyl group without requiring any protection of the remaining hydroxyl groups. This is a feat that cannot be achieved by conventional chemical oxidants. Oxidation of glucose to gluconic acid using glucose oxidase is a prominent example (50). Another example is pyranose oxidase that is used in the synthesis of D-fructose from D-glucose and 5-keto-D-fructose from L-sorbopyranose. Selective oxidation of hydroxyl groups in steroids is an important reaction carried by cholesterol oxidase. Since substrate solubility is a problem in aqueous systems, steroid oxidations can be carried out in organic solvents using PEG modification of the enzymes as a method to make the enzymes soluble in organic solvents (5).

Oxygenases are enzymes that incorporate molecular oxygen directly into the substrate. Oxygenase-catalyzed oxidations are important since direct addition of molecular oxygen into unactivated organic substrates is very difficult to accomplish using conventional chemistry. Monooxygensases incorporate one atom of oxygen whereas dioxygensases incorporate two atoms of oxygen into a substrate. Because these enzymes are membrane bound and are difficult to isolate, most oxidations are carried out using whole cells. Main problems in this

reaction are further metabolism of products, low yield due to side reactions, and substrate and product toxicity. Many examples exist where these oxygenases were used to synthesize small quantities of material (5). The stereoselective hydroxylation of nonactivated carbons is very important because there is no equivalent traditional chemical method. This field burgeoned in the 1950s when steroid modifications were being investigated, and more recently some of the hydroxylations have been scaled up to produce commercial quantities of steroid (51). By screening for the appropriate organism it is now possible to selectively hydroxylate virtually any center in a steroid (5).

Another class of reactions that is very important is the Baeyer–Villiger reactions where ketones are oxidized into esters and lactones. The fact that there is chiral recognition by the enzymes sets them apart from conventional methods (5). Since flavin- and nicotinamide-dependent monooxygenases are usually involved in these reactions, whole-cell biocatalysis is utilized most of the time. One of the latest examples of a dioxygenase-based process is the production of indanediol, which is an intermediate in the synthesis of the protease inhibitor Crixivan (Merck & Co.) used in the treatment of AIDS (52,53). Growing recombinant *E. coli*, cells carrying the *Pseudomonas putida* toleuene dioxygenase and dihydrodiol dehydrogenase were used for the production of *cis*-(1*S*),(2*R*)-indandiol with an optical purity >99% ee.

Aldolase-catalyzed asymmetric C–C bond formations which are carried out in a neutral pH aqueous environment are a very important class of reactions. Stereocontrolled synthesis of D- or L-threo-phenylserine using L- or D-threonine aldolase has been accomplished on a preparative scale (54). This aldolase technology can generate other β-substituted serines and other derivatives starting from modified substrates. Other commercially important reactions catalyzed by aldolases are for the synthesis of unusual sugars, polyhydroxylated alkaloids, novel C–C polymers, and analogues of *N*-acetylneuraminic acid (5,55,56).

The formation of cyanohydrins is catalyzed by oxynitrilase. These enzymes catalyze the asymmetric additon of hydrogen cyanide to the carbonyl group of an aldehyde or ketone forming a chiral cyanohydrin (57). Chiral cyanohydrins are important intermediates in the synthesis of pharmaceuticals (58–60), agrochemicals (61), or liquid crystals (62,63). Since oxynitrilases are obtained mainly from plant sources, biocatalyst cost is a major issue. This limitation has been overcome in certain cases with the use of enzyme recycling either by immobilization or by using biphasic reaction systems (57).

Hydrolases catalyze the hydrolysis of various bonds such as amides and esters. Among the hydrolases, lipases, esterases, and proteases are most widely used enzymes. Hydrolases are routinely used in organic synthesis since they do not require cofactors and a large number of them possessing relaxed substrate specificities are available from different sources. Lipases are the most widely used hydrolases and they catalyze the hydrolysis of triglycerides into fatty acids and glycerol. On the basis of triglyceride hydrolysis, microbial lipases can be classified into two groups. Lipases of first group have no regiospecificity and release fatty acids from all three positions of glycerol. They completely hydrolyze the triglycerides to fatty acids and glycerol with diacylglycerol and monoacylglycerol as intermediates of the reaction. In contrast, lipases of second group release fatty acids regioselectively from the outer 1- and 3-positions of

triglycerides. Lipases have enormous potential in chemical synthesis because of several reasons: (*1*) lipases are stable in organic environment; (*2*) lipases possess broad substrate specificity; (*3*) lipases exhibit high regio- and enantioselectivity. A number of lipases have been isolated from fungi and bacteria and characterized. Lipases and esterases are used to prepare enantiomerically pure esters, acids, and alcohols (5). Two major procedures are used: hydrolysis of racemic ester in water and acylation (transesterification, esterification, transaminolysis) of alcohols in nonaqueous media such as organic solvents and supercritical fluids (64). Lipases and esterases from different sources were used to resolve the racemic mixture to produce S-enantiomers of Ibuprofen and Naproxen in excess of 95% ee (65–67). Another important application of lipases is the interesterification of triglycerides that are useful in the preparation of cocoa butter equivalents (68–70). The steadily growing interest in lipases for the organic synthesis is reflected by an increasing number of review articles covering application of lipases in biocatalysis (71,72).

What has been presented is a small sample of the variety of bioorganic transformations being carried out. In order to better describe the many hurdles faced in biotransformation processes, a case study is presented where an aminotransferase was used to convert a substituted tetralone to a chiral aminotetralin.

5. Case Study

Aminotrasferases are enzymes that transfer an amine group from a donor molecule to an acceptor molecule, resulting in a chiral amine. Chiral amines play an important role in pharmaceutical and fine chemicals (73,74). Chiral amines are also used as resolving agents for the preparation of chiral carboxylic acid. Chiragene, Inc., uses proprietary aminotransferase or transaminase technology to produce chiral amine or chiral amine derived molecules. Figure 1 shows the

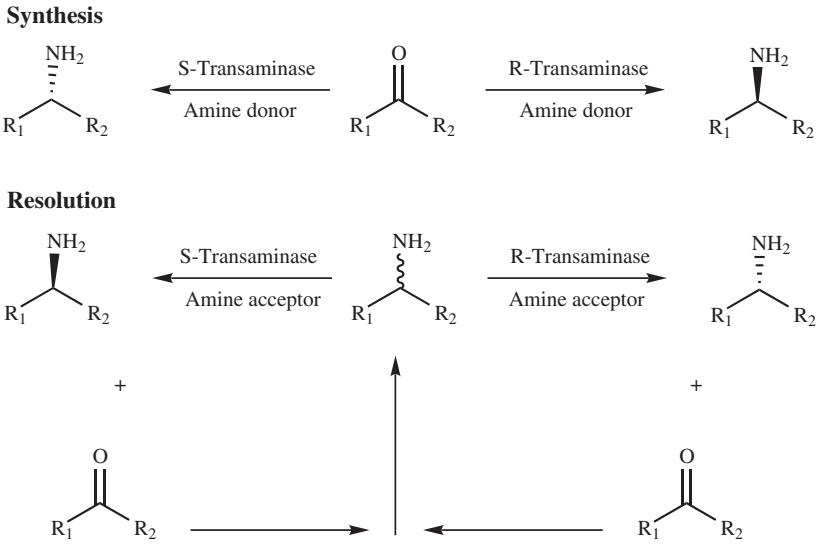

Fig. 1. Aminotransferase route to chiral amine.

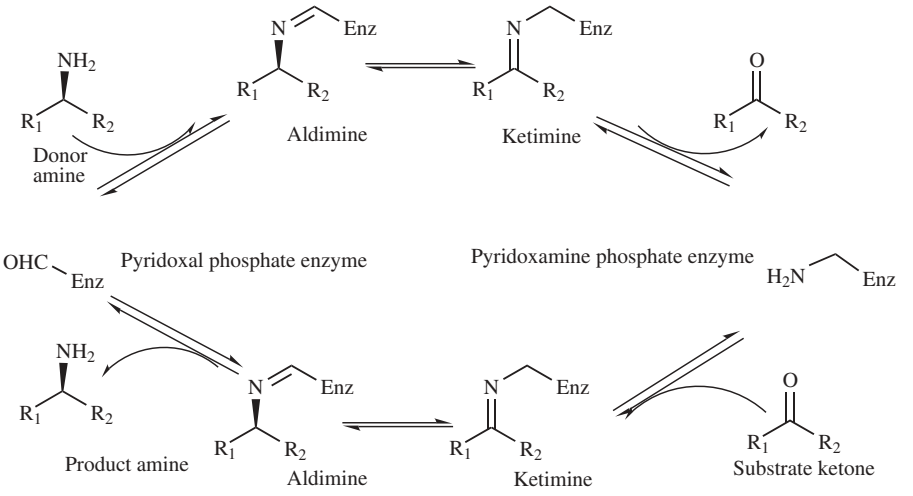

Fig. 2. Mechanism of transamination.

two possible reaction routes involved in a given transamination process. Synthesis involves the transfer of the amine group from a cheap amine donor (isopropylamine or aminobutane) to a prochiral ketone, whereas resolution involves selective transfer of an amine group from one isomer to an acceptor carbonyl group, leaving behind the corresponding ketone. Figure 2 describes the transamination reaction in detail. These enzymes require pyridoxal phosphate (PLP) as a cofactor to act as a shuttle to transfer the amine moiety. This cofactor is tightly bound to the enzyme and is not needed in stoichiometric amounts and hence does not pose the cofactor regeneration problems encountered in oxidation/reduction reactions.

Transamination is a cyclic process involving two steps to complete one cycle (75). As shown in Figure 2, during the first half of the cycle, the amine donor condenses with the aldehyde group of PLP to form a Schiff's base with the release of water. The imine complex thus formed tautomerizes to form a ketamine intermediate that undergoes hydrolysis to release the first product which is the ketone corresponding to the amine donor. At this point the amine group is transferred to the PLP attached to enzyme, converting it to pridoxamine phosphate (PMP). In the second half, the enzyme-PMP complex is attacked by acceptor ketone, followed by a reversal of steps observed in the first half of the reaction. At the end of the second half of the reaction, the acceptor ketone gets converted to the product amine and enzyme–PLP complex is generated. In the resolution mode racemic amine acts as an amine donor while any suitable carbonyl acts as an amine acceptor. Since aminotransferases are selective, only one of the isomers reacts, leaving the desired isomer untouched.

Figure 3 shows a block diagram of aminotranferase process. A typical reaction consists of buffer, PLP, amine donor, enzyme, and amine acceptor in aqueous solution. The pH is maintained using buffer at desired level. After the reaction is complete the biomass is removed by centrifugation. The product amine is isolated from the reaction mixture by solvent extraction. Both

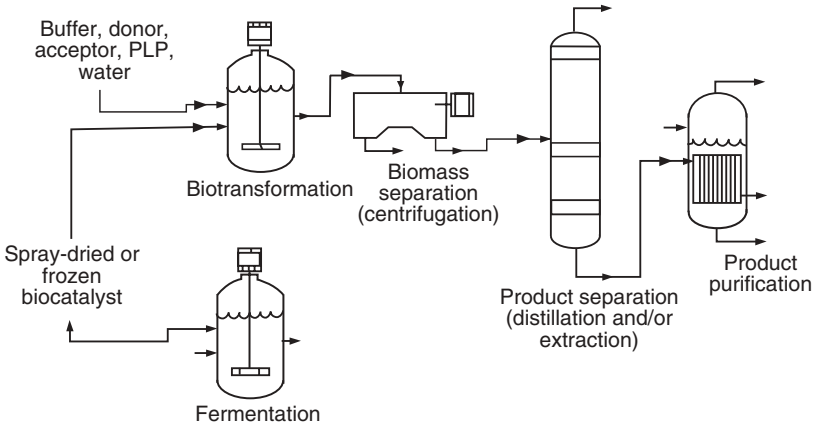

Fig. 3. Process diagram for chiral amine production using aminotransferase.

the synthesis and resolution approaches are tested in a laboratory scale to figure out the most economical route of producing the desired chiral amine molecule in hand. The route that is selected has performance criteria attached, such as the required yield and concentration. Some of the typical hurdles to attain the performance requirements are equilibrium limitation, low chiral purity, enzyme inactivation, and substrate solubility. The enzyme is tailored to meet the performance criteria using error-prone PCR and a colorimetric screen. Once a suitable biocatalyst is identified in the screen it is fermented at 16 L scale and spray dried for testing at small scale (50–100 mL reaction). If the desired performance is not reached, the best mutants are subjected to a second round of tailoring, screening, and testing. This is continued until the desired performance is reached, and this is illustrated in Figure 4. Once the desired improvement for a given process is

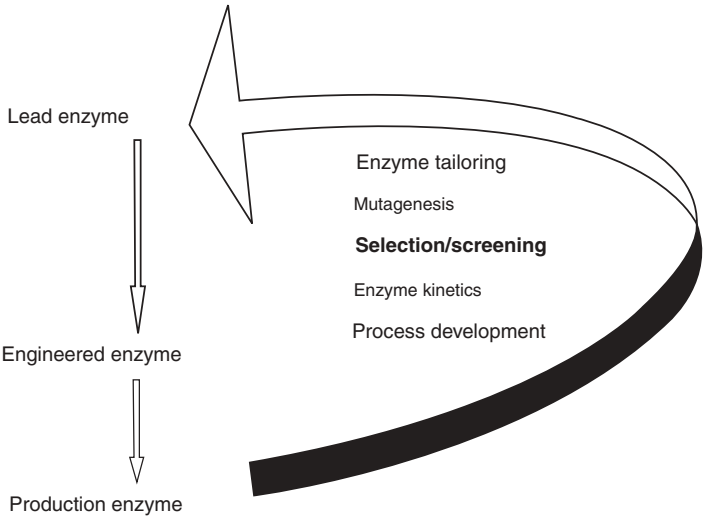

Fig. 4. Error-prone PCR approach to improve enzyme performance parameters.

reached the process is scaled up from 50 mL to 200 gal to 700 gal to 2000 gal reaction volume. Since the biotransformation reactions involve nearly homogenous reaction mixtures there is not much of a deviation from the laboratory-scale results. The decision to use synthesis or resolution depends on relative availability of substrate, cost of substrate, and enzyme activity. The direct synthesis of chirally pure amine is especially valuable where the ketone is expensive. One such example is the production of substituted 2-aminotetralins, starting from prochiral-substituted β-tetralones. Typical costs for the ketone range from \$100 to \$5000/kg, and in such a situation, an amine yield in excess of 90% is paramount.

After initial feasibility studies with one of the substituted β-tetralones, the concentration achieved in the synthesis mode was low (1–6 g amine in a liter). This throughput in the biotransformation process competes with the traditional chemical route. The performance criteria needed to make the biotransformation feasible required the throughput and the rate to be increased threefold while maintaining the stereoselectivity. Experiments showed that the reaction was equilibrium limited and the enzyme was experiencing inhibition/inactivation by substrate and product amine. Two possible ways of shifting equilibrium in the desired direction are (*1*) by increasing the concentration of one or both substrates and (*2*) by removing one or both of the products in the reaction. The latter method is more difficult because the product ketone or amine has to be removed selectively (ie, without affecting the concentration of substrate ketone or amine). The former method is easier because all it needs is additional substrate. Since the ketone is expensive it makes economic sense to increase the concentration of amine donor (isopropylamine or 2-aminobutane) that is relatively inexpensive. However, increasing the concentration of amine donor necessitates that the enzyme be active under these conditions. Since the other hurdle was enzyme inhibition/inactivation by amine engineering an enzyme tolerant to high donor amine concentration could conceivably result in overcoming both hurdles at the same time.

Using the error-prone PCR and a colorimetric screen, several thousand clones were screened for improved performance. The screen was designed to pick up clones that showed activity in the presence of high concentrations of donor amine. An additional indicator that was used to select the clones was the time it required to display activity. After three to four rounds of enzyme modification, an enzyme was identified that was tolerant to high concentrations of donor amine. This enzyme was tested in the biotransformations at small scale (20–50 mL) using the high donor amine concentration, and the product amine concentration was measured as a function of time. The results from this experiment are shown in Figure 5. The modification of the enzyme in conjunction with the appropriate screen resulted in an enzyme that achieved a fourfold increase in product amine concentration within 8–10 h without any change in stereoselectivity (>99% ee). This biotransformation process was scaled up to 2000 gal with reproducible results. Figures 6 and 7 show some of the improvements attained by the enzyme-modification technique in similar transamination reactions. Figure 6 illustrates a case where inactivation/inhibition by product amine was reduced and the enzyme stability was increased, resulting in a sixfold improvement in final product concentration. In addition to improving enzyme

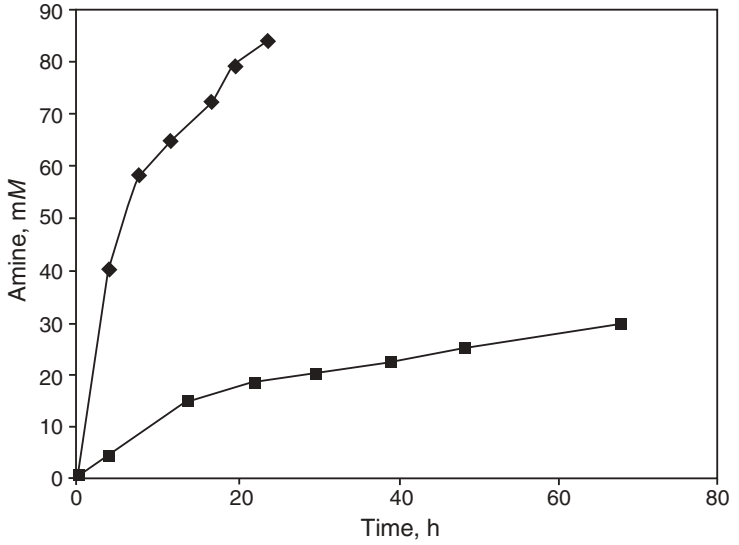

Fig. 5. Comparison of wild-type and modified enzyme. Improvements were made targeting amine tolerance and reaction rate. ◆ Modified; ■ wild type.

activity and stability it is possible to improve selectivity using the same technique. Figure 7 shows the improvement in enantioselectivity that was achieved starting from ~6% ee to >99% ee. These examples demonstrate the power of enzyme modification and its impact on achieving performance and economic targets for an industrial biotransformation process.

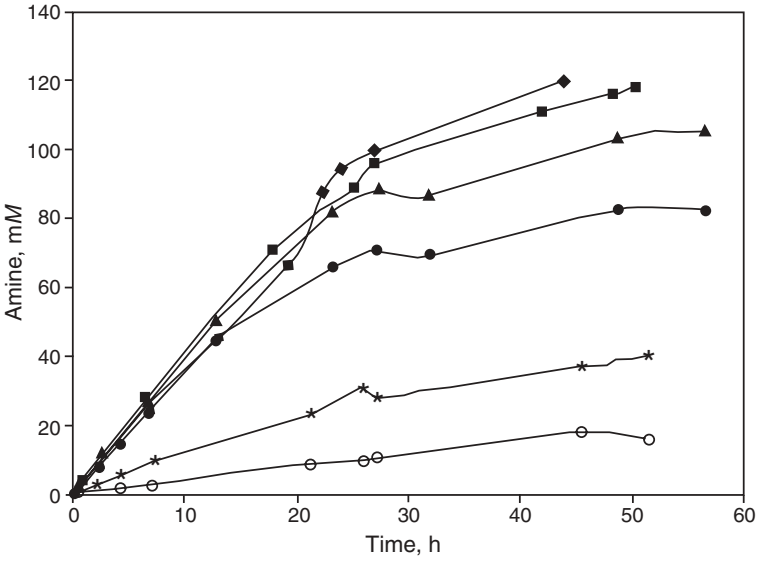

Fig. 6. Improvement of enzyme stability and acitivity using error-prone PCR. ◆ 4th generation; ■ 3rd generation; ▲ 2nd generation; ● 1st generation; ✱ 1st generation; ○ wild type.

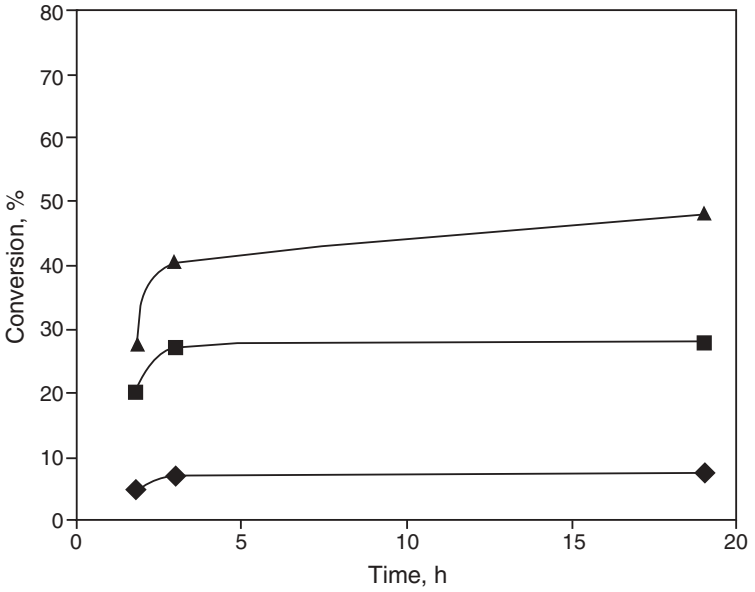

Fig. 7. Improvement in enzyme selectivity using error-prone PCR. ◆ Wild type, 6.5% ee; ■ 1st generation, 37.8% ee; ▲ 2nd generation, 99.4% ee.

6. Conclusions

Biocatalysis is growing very fast because of its unmatched stereoselectivity, regioselectivity, minimal waste, and relatively mild reaction conditions. In addition, recent development in solvent and protein engineering has made redesigning biocatalysts to suit the particular process easier. Major pharmaceutical companies are investing heavily in biocatalysis, indicating the utility and acceptance of biocatalysis as an additional tool in a scientist's hand. Enzymes from all different classes have been used to prepare chiral fine chemicals and pharmaceutical intermediates. Both whole cells and isolated enzymes have been used to catalyze the synthesis of various different chemical reactions. Although there is much to be done in this area, the future holds great promise in this burgeoning field.

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