Exploiting Enzymes: Technology and Applications

3

Much before enzymes were identified as discrete biochemical entities, they found favor through their useful properties. Early applications included use of enzyme preparations in meat tenderizing and starch hydrolysis. From the very beginning, commercial enzyme applications have largely belonged to a group of hydrolytic reactions. But a few oxidative enzymes were also exploited. While this trend holds even today, examples of designer enzymes and catalysts for more complex chemical processes are being developed. The first application of diastase (α-amylase) was by Jokichi Takamine. His 1894 patent (US Patent No. 525823) describes a process to make Taka-diastase from *Aspergillus oryzae*. This α-amylase was useful as a digestive aid, in eliminating starchy material from textiles and laundry. In a short but succinct paper, E.F. Leuchs (in 1931) described “the action of saliva on starch.” The possible practical utility of such activity was clearly anticipated by him. The last line of his report reads “it will be possible to use saliva and gastric juice of killed animals very successfully in cases of defective digestion.”

The quantity and quality of an enzyme are two critical parameters that define their application and extent of use. Industrial scale processes require enzymes (often in crude form) in tons, whereas precise clinical use mandates extreme purity and minimal or no contaminating factors. Accordingly, the enzyme production costs for different end objectives vary – they can be of high volume and low cost or low volume but of high cost. For instance, medically valuable products like streptokinase and asparaginase need to be very pure and are therefore expensive. Enzyme catalysts of practical import are sought by industries in many different ways. Significant among these are screening for useful activities from the naturally abundant diversity, modifying already available enzyme properties to suit our requirements, and genetically engineering desirable properties into these catalysts. We will briefly touch upon the applications of enzymes and industrial strategies with suitable examples in this chapter. Applications of enzymes and enzyme technology can occupy volumes, and many authoritative books are available for the interested reader.

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_3

3.1 Exploiting Natural Diversity

The rich biodiversity on earth goes hand in hand with naturally vast array of catalytic activities. A cleverly designed screen almost always leads to an enzyme with desired properties. Thermostable protease (from *Bacillus* strains) and DNA polymerase (from *Thermus aquaticus*) are two examples of enzymes chosen for high temperature stability. The range of natural diversity is obvious from the number of enzymes that have found niche applications in the processing of carbohydrate polymers, proteins, and lipids.

Enzymes for Bioprocessing Polysaccharides are the major biomolecules that comprise biomass on this planet. They serve two important functions – energy storage (such as starch) and structural rigidity (such as cellulose). It is therefore not surprising that enzyme technology took its roots through processes to hydrolyze these sugar polymers. Microbes (bacteria and fungi) constitute an abundant source of amylases and cellulases. Controlled hydrolysis of starch to sweeteners (and sugar substitutes) is a well-developed industry (Fig. 3.1). Various enzymes used in the starch saccharification process are α-amylases, β-amylases, glucoamylases, pullulanases, and glucose isomerase. Despite certain limitations, conversion of glucose to fructose through glucose isomerase is central to many sucrose substitutes – with distinct economic and manufacturing advantages.

Although there is an abundance of cellulose in nature, transforming cellulosic biomass into sugar has been a challenge. Concerted action of a bunch of enzymes (that constitute the "cellulase complex") is required for this (Payne et al. 2015). Significant advances in enzymatic processes to breakdown cellulose into fermentable sugars are being made. In the meanwhile, individual components (Table 3.1) of the cellulase complex have found application in textile and paper industry.

Proteases and lipases are next in order of significance in enzyme industry. Apart from the historical significance of papain and digestive enzymes (like trypsin and chymotrypsin), this class of enzymes has found wide-ranging applications in foods, detergents, and tanning of leather. Bacteria and fungi are ideal sources for the large-scale production of proteases (Li et al. 2013). Most important alkaline protease producers are *Bacillus* strains and fungi belonging to genus *Aspergillus*. Subtilisin is the best known bacterial protease additive of modern detergents. It has been extensively selected/modified for features like pH optimum and temperature stability. Chymosin (also known as rennin) is a milk coagulating enzyme from calf stomach, which is used for generations in cheese making. An equivalent enzyme from a microbial source was sought, and several *Mucor* strains are chosen to produce rennin substitutes.

Many other enzymes including lipases and pectinases are also available in industrial scale. A representative list of enzymes commonly used in industry is given in the table (Table 3.2).

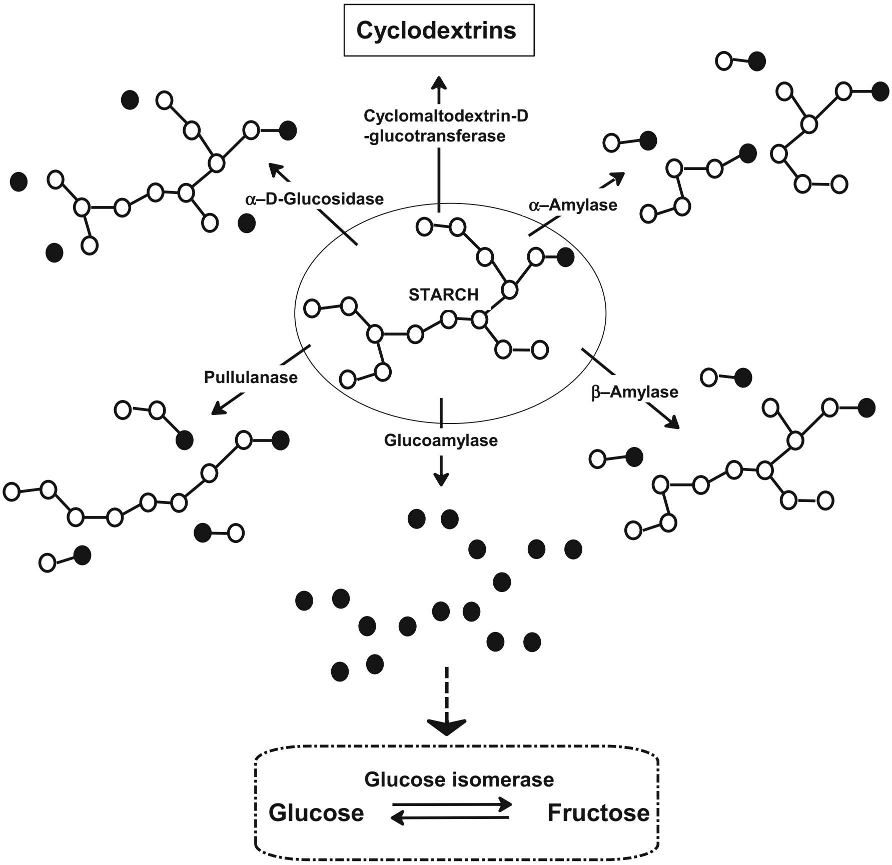

Fig. 3.1 **Significant steps and enzymes employed in starch processing**. Glucose residues of starch are schematically represented as circles. Filled circles indicate glucose residues whose C_1-OH has not entered into a glycosidic linkage (free reducing ends). Besides glucose isomerase, all the possible enzymatic modes of dismantling starch are shown. Some combinations of these enzymes are commercially available as industrial formulation

Enzymes in Pharma and Medical Applications Pharmaceutical industry is another big beneficiary of applied enzymology. Enzymes isolated from natural sources as well as those cloned and expressed (through genetic engineering) are in use. Enzymes and their critical study serve multiple purposes in drug discovery and development.

- Active principles of many effective drugs are enzyme inhibitors (Robertson 2005). An enzyme, critically located in the intermediary metabolism, may provide an excellent target to screen for such inhibitors. A few successful examples of drugs have panned out from such enzyme screens.

Table 3.1 Component activities of cellulase complex and their applications

Cellulase component	Substrate specificity	Application
β-Glucosidase (Cellobiase)	Cellobiose→Glucose	Saccharification
Cellobiohydrolase I (CBH1)	Cellulose→ Cellobiose (exo – Nonreducing end)	Biomass conversion
Cellobiohydrolase II (CBH2)	Cellulose→ Cellobiose(both exo and endo)	Biomass conversion
Endoglucanase I (EG1)	Cellulose (endo)	Textile/fabric softening,
		Biopolishing
Endoglucanase II (EG2)	Cellulose (endo)	Textile/fabric softening,
		Biopolishing
Xylanase	Xylan	Paper pulp deinking
All components	Cellulose and Xylan	Feed/fodder, biomass
		Conversion

Table 3.2 Large-scale use of enzymes in industry

Enzyme	Application
Acting on carbohydrates	
Amylases	Starch processing
Cellulase complex	Biomass conversion, textile industry
Pectinases, esterases	Food industry, fruit juice, brewing
Glucose isomerase, invertase	High fructose syrups, invert sugar
Acting on proteins	
Papain, pepsin	Meat and leather processing, treating dough
Rennin, chymosin	Cheese making
Subtilisin	Detergents, leather and wool processing
Acting on lipids and esters	
Lipases	Food and detergent industry, cocoa butter
Acting on antibiotics	
Penicillin acylase	Produce 6-aminopenicillanic acid (6-APA)

The concept of screening for enzyme inhibitors was first adopted by Hamao Umezawa's group in Japan (Umezawa 1982). Since then, many enzyme inhibitors have been discovered (few are listed in Table 3.3) and are in use. For instance, preventing absorption of dietary fat (triglycerides) can be a possible strategy to control obesity. An appropriate lipase from the digestive juices could serve as a target for this screening (Fig. 3.2).

Often the active chemical entity obtained from an enzyme screen may not find direct application. These lead compounds (inhibitors) are suitably altered/derivatized

Table 3.3 **Examples of enzyme-targeted screens for active principles**

Enzyme target	Screening outcome	End use
Pepsin	Pepstatin	Ulcers
Angiotensin converting enzyme	Captopril	Hypertension
HMG CoA reductase	Lovastatin	Hypercholesteremia
α-Amylase	Acarbose	Diabetes
Triacylglycerol lipase	Orlistat (lipostatin)	Obesity
Acetylcholine esterase	Rivastigmine	Alzheimer's disease
β-Lactamase	Clavulanic acid	Combination therapy

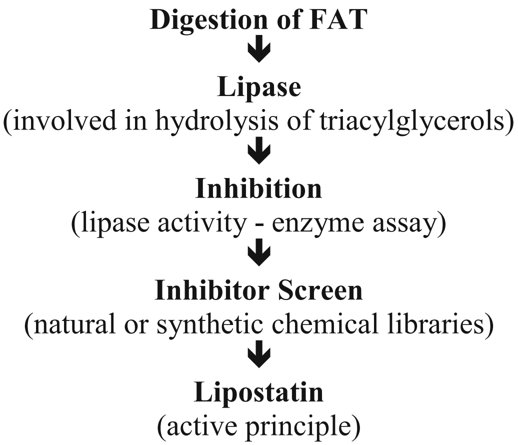

Fig. 3.2 **Flow chart outlining the design of a lipase inhibitor screen**

to achieve better bioavailability and reduce toxicity. An in-depth kinetic analysis of enzyme inhibition (concluded in Chap. 28) is at the heart of modern drug discovery programs.

- Besides being targets for inhibitor screens, many enzymes are employed as catalysts for synthesis. The whole range of β-lactam antibiotics available today includes a large number of semisynthetic penicillins and cephalosporins. While penicillin G is produced by fermentation, 6-aminopenicillanic acid – an important precursor for semisynthetic penicillins – is derived from it (Fig. 3.3). Penicillin acylase is a valuable commodity in the large-scale production of 6-aminopenicillanic acid.
- Because of their catalytic potential coupled with specificity, many enzymes are used as exquisite analytical tools. Alkaline phosphatase and peroxidase are two reporter enzymes of extensive history in ELISA (enzyme-linked immunosorbent assay). Here the desired specificity of interaction (through antibodies) is coupled to the signal amplification provided by enzyme catalysis. Enzymes as antibody-conjugates find routine use in detection of DNA/RNA/protein on blots. *Taq* DNA polymerase is extensively employed for DNA amplification through polymerase chain reaction (PCR). A number of metabolites are analyzed (in a clinical setting)

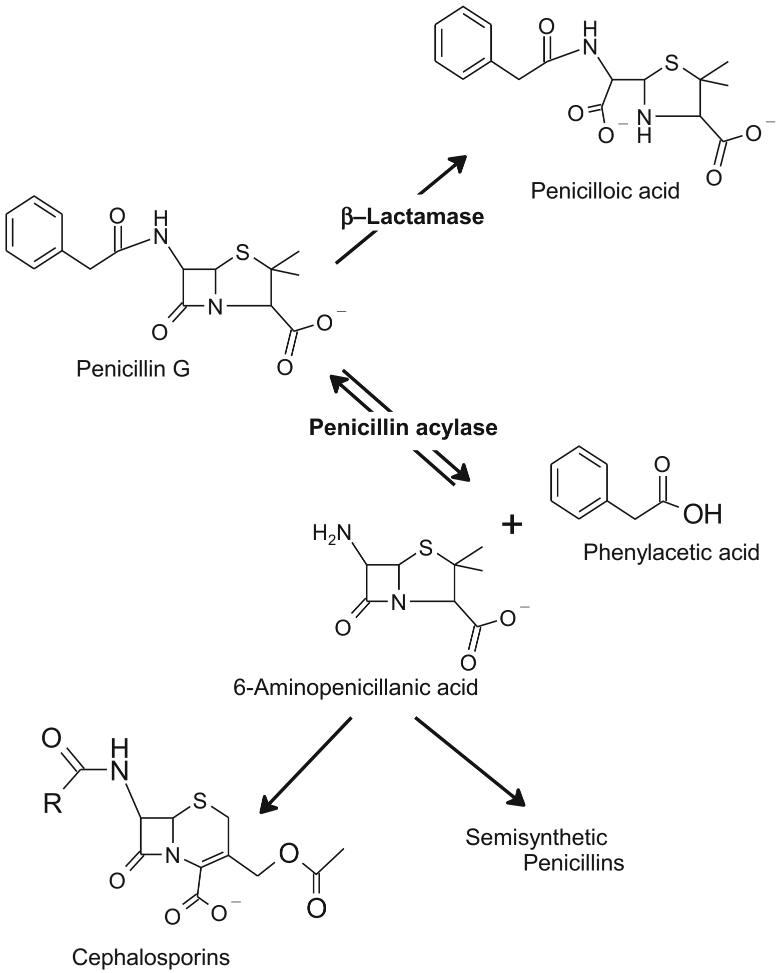

Fig. 3.3 **Enzymes and steps relevant to penicillin (β-lactams) industry**. Antibiotic resistance is often due to a β-lactamase; better antibiotics may be evolved by screening for novel structures that are not acted upon by the β-lactamase. Penicillin acylase is used to produce 6-aminopenicillanic acid (6-APA). 6-APA is an important precursor to make semisynthetic penicillins, both through enzymatic and chemical routes

through assays involving enzymes. A list of more commonly used enzymes and the corresponding analytes are given in Table 3.4. Some of these enzymes are also employed as a component of biosensors (see below).

- Enzymes have found medical applications in terms of diagnosis as well as therapy. A few enzymes find direct application as therapeutic agents in medicine. Best known examples from the market include diastase (α-amylase, digestive aid), asparaginase (leukemia, antitumor therapy), rhodanese (cyanide poisoning), and streptokinase (medication to dissolve blood clots). However, a large number

Table 3.4 Examples of enzymes for metabolite analysis

Enzyme	Analyte detected/estimated
Catalase	Hydrogen peroxide
Glucose oxidase	Glucose
Hexokinase	Glucose
Alcohol dehydrogenase	Ethanol
Lactate dehydrogenase	Lactate/pyruvate
Luciferase	ATP
Urease	Urea
Cholesterol oxidase	Cholesterol

Table 3.5 Examples of enzymes as clinical markers

Enzyme	Used as marker for
Lactate dehydrogenase (H4 isoform)	Heart diseases
Glutamate-oxoglutarate transaminase (SGOT), glutamate-pyruvate transaminase (SGPT)	Liver function
Creatine kinase	Myocardial infarction, skeletal muscle damage
Lactase	Lactose intolerance
Hexosaminidase A	Tay-Sachs disease
Acid phosphatase	Prostate cancer
Phenylalanine hydroxylase	Phenylketonuria (PKU)

of enzymes are routinely monitored as clinical markers (Table 3.5). Enzyme profiles from serum, amniotic fluid, urine, etc. are monitored, and their levels are often correlated with disease conditions and aid in the diagnosis of disease.

Enzymes and Issues of Safety Industrial preparation and use of enzymes come with its own safety and regulatory issues. Potential hazards from exposure to large quantities of a given enzyme include allergenicity, functional toxicity, chemical toxicity, and source-related contaminants. Large-scale manufacture of enzymes often ends with partially pure yet enriched preparations. Such material could contain potentially toxic chemicals (like mycotoxins) carried over from the source. Not all microorganisms are safe, and their trace contamination in the final enzyme preparations requires attention. For enzymes to be used in food ingredients, they must be GRAS (*generally recognized as safe*). Some enzymes like proteases are potentially dangerous – particularly upon exposure of sensitive tissues to concentrated preparations. Since enzymes are proteins, they can be potent allergens. Repeated exposure through inhalation or skin contact can trigger severe allergic response. Enzyme preparations especially handled in the form of dust, dry powder, or aerosol are harmful and must be avoided. Many issues of safety regarding free enzyme preparations may be overcome by using them in the immobilized, granulated, or encapsulated form.

3.2 Modifying Enzymes to Suit Requirements

Despite the vast natural diversity of biological catalysts, significant technology has developed to alter the properties of available enzymes. This tinkering has involved facets of their immobilization, chemical modification, genetic engineering, or their use in nonaqueous solvents.

Immobilization for Better Use Most natural enzymes isolated are in water soluble state. They cannot be stored in this form for long, often due to instability. Their immobilization is one way to enhance their shelf life (Mateo et al. 2007). In addition, immobilized enzymes are easy to recover and amenable to repeated use. This is an important consideration when the cost of enzyme is very high. The characteristics of the matrix are very critical in determining the performance of the immobilized enzyme system. These supports may be inorganic or organic according to the nature of their chemical composition. The physical characteristics like mean particle size, swelling behavior, mechanical strength, etc. decide the technical conditions in which the system is used. Enzymes may be quarantined on the matrix either irreversibly (by covalent bonding, entrapment, microencapsulation, cross-linking, etc.) or reversibly (by adsorption, ionic binding, affinity binding, disulfide bonds, or chelate/metal binding). The cost associated with the process of immobilization determines whether it is economically viable to do so.

Different means are adopted for enzyme immobilization in practice, and only a few are represented in Fig. 3.4. The field of enzyme immobilization technology and its applications has grown vastly over the years. Many books and volumes (*Methods in Enzymology* series) are available for detailed reference (Brena and Batista-Viera 2006). This section covers very briefly on this applied aspect of enzymes, and the reader is encouraged to refer the more specialized literature for the purpose.

The choice of immobilization method depends on the type of the enzyme and the nature of applications in question. Non-covalent confinement (like physical entrapment, microencapsulation, or electrostatic adsorption) methods at times may lead to enzyme leaching during operation. Covalent anchoring of enzymes on the other hand requires bifunctional cross-linking reagents and suitable functional groups on the enzyme surface. These functional groups must not be critical for enzyme activity however. A great deal of sophisticated chemistry has been developed to activate inert organic/inorganic polymers for subsequent enzyme immobilization. Carrier-bound (covalently linked to polymers; figure above) penicillin acylase is highly effective in the preparation of 6-APA and permits economic recycling of the catalyst. Penicillin acylase immobilized cassettes are available that function at >99% conversion efficiency even after 1500 cycles of use. Glutaraldehyde is used to cross-link glucose isomerase – the cross-linked material can be reused many times in the commercial process for production of fructose syrups from glucose. Detergent protease components act as allergens, and this problem was overcome through microencapsulation; the dustless protease preparations have reduced this risk. Some of the enzymes successfully immobilized for application are shown in Table 3.6.

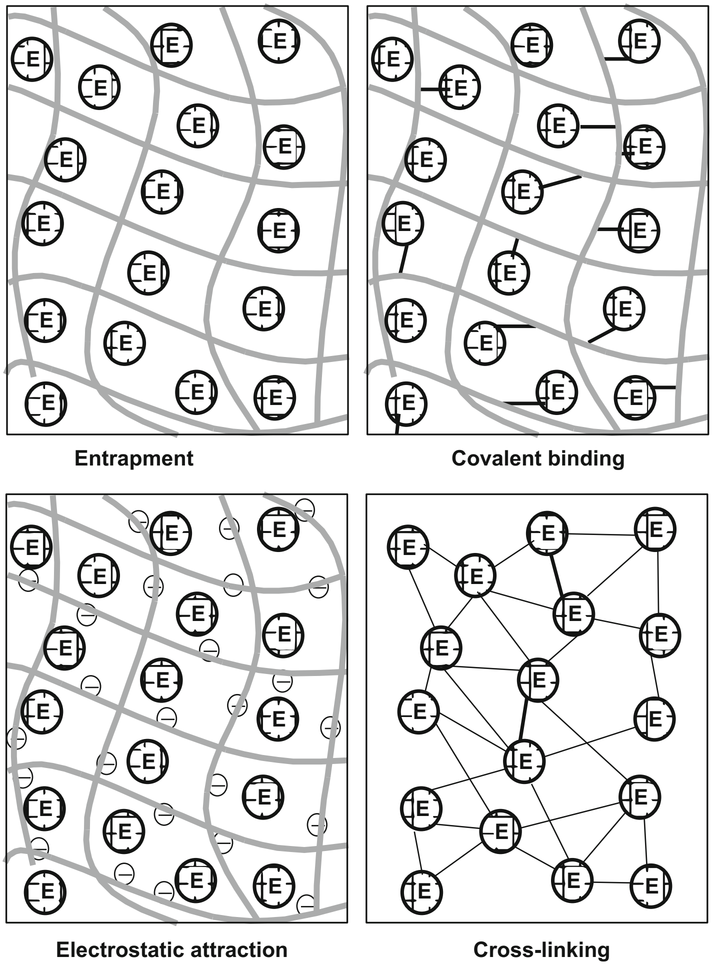

Fig. 3.4 Four different modes of enzyme immobilization

Table 3.6 Industrial uses of immobilized enzymes

Enzyme	Product
Aspartate ammonia lyase	L-Aspartic acid
Hydantoinase	L- and D-Amino acids
Thermolysin	Aspartame
Lactase	Lactose-free milk and whey
Invertase	Invert sugar
Glucose isomerase	High-fructose syrup
Lipase	Cocoa butter substitute
Penicillin acylase	6-APA and penicillins
Nitrile hydratase	Acrylamide

It is expected that the substrate specificity and the catalytic potential of the enzyme should not be unfavorably affected upon immobilization. The changed microenvironment could affect the enzyme stability to heat, pH, and proteolytic enzymes. A reduction in its activity may also occur due to associated conformational changes in the enzyme. The pH optimum of an immobilized enzyme may change by as much as 2.0 pH units due to microenvironment effects. It is observed that an anionic carrier (matrix) pushes the pH optimum to a more alkaline value, while a cationic carrier moves it to a more acidic value. These matrix (electrostatic field) effects are due to changes in the degree of ionization of amino acid residues on the enzyme; this effect is usually not observed in medium with high ionic strength – as salt ions counter the charges on the carrier.

The kinetic behavior of an immobilized enzyme can differ significantly from the free enzyme in solution. Both maximal velocity ($V_{\max}$) and Michaelis constant (K_M – apparent affinity for the substrate; see Chap. 15 for a detailed treatment) of the enzyme may be affected. The apparent K_M significantly decreases when the carrier used is of opposite charge to that of the substrate – largely due to electrostatic interactions causing the substrate to be at a higher concentration near the carrier. In general, partition effects (arising from ionic, electrostatic, or hydrophobic interactions) cause changes in the local concentration of the ligands/molecules which in turn affect the kinetic constants. The apparent K_M of an immobilized enzyme is also affected by diffusion factors. Both the diffusion of the substrate to and the diffusion of the product away from the enzyme influence enzyme activity. Restricted diffusion of bulk substrate to the immobilized enzyme leads to significantly higher K_M value. Poor product diffusion, away from the enzyme, obviously results in an inhibited enzyme. If diffusion constrains the highest substrate concentration achievable, then the immobilized enzyme may display an apparent $V_{\max}$ lower than the free soluble enzyme.

The objective of using enzyme-catalyzed reactions in industry is to convert given amount of the substrate maximally into the product. Integrated form of the rate equation (Michaelis-Menten equation) is better suited to analyze such systems. However, the integrated form of the rate equation is also valid only if (a) substrate concentrations far exceed that of the enzyme, (b) the overall reaction is irreversible, (c) enzyme is stable over the time period, (d) no product or substrate inhibition occurs, and (e) the system remains properly mixed (see Chap. 17 for a detailed treatment). Some of these factors can be tackled through choice of enzyme reactor configuration and process design. Continuous industrial processes could employ immobilized enzyme in stirred-tank, packed-bed, or fluidized-bed reactors or the enzyme immobilized on membranes, hollow fibers, or tubes. The flow of the reaction mixture may be exploited to input fresh substrate, remove the product formed, adjust pH, etc. Membrane cassettes of penicillin acylase have made the production of 6-APA very efficient – the reaction mixture pH can be maintained by dosing NaOH (pH stat) and continuously removing the product formed.

Enzyme Biosensors Biosensors are used to determine/monitor the concentration of substances (often metabolites) of biological interest. Enzymes play a major role in such analytical devices – to convert a biological response into an electrical signal. Specificity and signal amplification (catalysis) of an immobilized enzyme are ideally suited for this purpose. Besides the fact that they may be reused, the immobilized enzymes (with an elevated K_M value) give proportional change in reaction rate over a substantial linear range of the substrate concentrations. Also, often, these rates are independent of pH, temperature, ionic strength, and inhibitors – features advantageous in metabolite measurements in real analytical samples.

The critical component of the biosensor is the transducer which converts the outcome of an enzyme reaction into a measurable signal. Biosensors may have a transducer to exploit enzyme reactions with (1) heat generation (calorimetric biosensor, to measure H_2O_2 with catalase), (2) release or absorption of ions (potentiometric biosensor, glucose with glucose oxidase and urea with urease), (3) production of a current (amperometric biosensor – glucose with glucose oxidase, alcohol with alcohol oxidase, and cholesterol with cholesterol oxidase), and (4) absorption or emission of light (optical biosensor, peroxides with horseradish peroxidase and ATP with luciferase). Paper enzyme strips are also in use to measure/detect substances through colorimetry. Enzyme biosensors today occupy a substantial analytical market in health care, food industry, and environmental monitoring.

Function in Organic Solvents – Nonaqueous Enzymology Living systems are intimately linked with their aqueous environment. Being biological catalysts, enzymes are exquisite products of long biological evolution. Enzymes are easily denatured by organic solvents. However, some enzymes can tolerate high concentrations of water-miscible organic solvents in their aqueous surroundings. Even in a water-immiscible nonpolar solvent, enzymes do need a monolayer of water molecules covering their exposed surfaces and the active site (Halling 2004). In this arrangement – with an essential water layer – they can continue to function as catalysts even in an organic solvent. The early work of Bourquelot and others (since 1913) showed that few enzymes could act in the presence of >80% of organic solvents such as ethanol or acetone. This example may very well be the forerunner of nonaqueous enzymology and use of enzymes in organic solvents for synthetic applications. The synthesis of a glucoside (by maltase in 1898) highlighted very early the synthetic capabilities of an enzyme.

Enormous interest in biocatalysis in nonaqueous phase was triggered due to the merits of good enantioselectivity, reverse thermodynamic equilibrium, and no water-dependent side reactions. For most organic reactions, water is not an ideal solvent. But enzymes have evolved for the catalysis of reactions in water. It would be very useful to have enzymes perform catalysis in nonaqueous media. There are added advantages if this is made possible:

- Many substrates are more soluble in organic solvents than in water. Large initial concentrations can be achieved with hydrophobic substrates that are sparingly soluble in water. They continuously diffuse into the active site from the bulk solution (which is nonaqueous). Some products are labile in aqueous media, and thus water-dependent side reactions can be minimized.

- Enzymes naturally find themselves in an aqueous environment with 55.5 M of water. Introducing them into nonaqueous environment means a drastically reduced water concentration! There are important consequences of lower water activity – particularly with reactions in which water is added or removed. For instance, peptide bond synthesis involves elimination of water – a thermodynamically uphill task in water. However, this step is favored in a nonaqueous environment. As a consequence a hydrolytic enzyme could be endowed with synthetic potential. Most hydrolytic reactions – generally irreversible in water – can be made reversible. Esterases, glycosidases, and proteases can be used for synthesis.

All water-soluble enzymes possess a small but significant amount of strongly bound water – thereby resulting in a two-phase system within the nonaqueous bulk medium. The *water activity* – $\mathbf{a_w}$ – is defined as the partial vapor pressure of water in a substance divided by the standard state partial vapor pressure of water. It is indicative of the extent of water content around the enzyme molecules. Enzyme activity in nonaqueous media depends on the magnitude of a_w because it affects the extent of this bound water. Lower a_w may lead to a rigid enzyme with limited thermal motion and associated thermal stability. The miniscule water pool around the enzyme in an organic solvent (between 50 and 500 water molecules per enzyme molecule) retains the pH of the last aqueous solution from where it is derived. Therefore, it appears as if the enzyme remembers and functions in that pH (*pH memory*).

The enzyme is inactivated if the tightly bound water layer is stripped off or diluted by the organic solvent phase. The stability or inactivation of an enzyme is thus dictated by the polarity of the solvent used. A useful measure of this polarity is **log *P*** – the logarithm of the partition coefficient of the organic solvent (X) between n-octanol and water. Log *P*, the partition coefficient, is a measure of hydrophobicity of organic solvent. The lower the *P* value, the more polar (hydrophilic) is the solvent.

$$\log P = \log\left(\frac{[\mathrm{X_{Octanol}}]}{[\mathrm{X_{Water}}]}\right)$$

As an empirical rule, enzymes are generally inactivated by solvents with log $P < 2$ but are little affected by more hydrophobic solvents with log $P > 4$.

Industrial focus on the use of enzymes in nonaqueous environments and reverse micelles systems is on the rise, with applications in foods, medicine, and industry. Being chiral catalysts, they are often valuable in resolving racemic mixtures of important drugs and intermediates in pharmaceutical industry. In practice it is common to find applications of nonaqueous enzymology in transglycosylation, transesterification, and transpeptidation reactions. An important example of the reversal of peptide bond hydrolysis is aspartame (α-L-aspartyl-L-phenylalanyl-*O*-methyl ester) synthesis. The protease thermolysin is used to condense L-aspartic acid with the methyl ester of L-phenylalanine and produce aspartame (sweetener and a sugar substitute). Similarly, suitable glycosidases are used to synthesize cyclodextrins. Interesterification reactions with lipases in nonaqueous solvents have found many applications. Consider an esterase catalyzing the hydrolytic reaction:

$$R-COOX+H-OH\rightarrow R-COOH+X-OH$$

The same enzyme, in the presence of another alcohol (Y-OH), but in the absence of water (low a_w), can bring about efficient catalysis of an ester exchange shown below:

$$R-COOX+Y-OH\rightleftarrows R-COOY+X-OH$$

For example, isoamyl acetate (a banana fruit flavor) is produced from ethyl acetate and isoamyl alcohol. Yet another interesting patented example of transesterification with a lipase is the preparation of cocoa butter substitute from palm oil.

3.3 Genetic Engineering and Enzymes

Large-scale production of enzymes is often a prerequisite for most applications. Obtaining them from animal and plant material – though of historical importance – has become progressively difficult for economic and ethical reasons. Therefore, the extant microbial biodiversity is routinely screened for enzymes with similar/desirable properties. Microbial rennin (a substitute for chymosin) produced from *Mucor* spp. is a case in point. Another exciting option is to produce the required enzyme through recombinant DNA technology, preferably in a microbial host.

The formidable tools of genetic engineering have allowed the expression and management of enzyme structures almost at will. Detailed recipes of recombinant DNA techniques are available in many texts and protocol books, while we sketch a brief outline of these steps here. Systematic manipulation of the DNA sequence at the molecular level is the essence of genetic engineering. This means we can cut and patch DNA fragments/ gene(s) for any enzyme protein from diverse living organisms. These recombinant DNA molecules – capable of expressing a natural or a mutant enzyme protein – can then be moved into a suitable host for protein expression. When this genetic information is expressed (by transcription and translation) in the new host, it can produce an enzyme protein that is even foreign to it! A general strategy for genetic engineering is outlined in Fig. 3.5. There are essentially four stages to this powerful technique.

Isolation of the Gene/ORF/cDNA for the Enzyme Protein Restriction endonucleases of different sequence specificities are employed to cut out the desired gene or the ORF (open reading frame) encoding that enzyme from a given source of DNA. The required DNA fragment may be amplified by PCR (polymerase chain reaction) or the corresponding cDNA may be obtained through reverse transcriptase-PCR of the

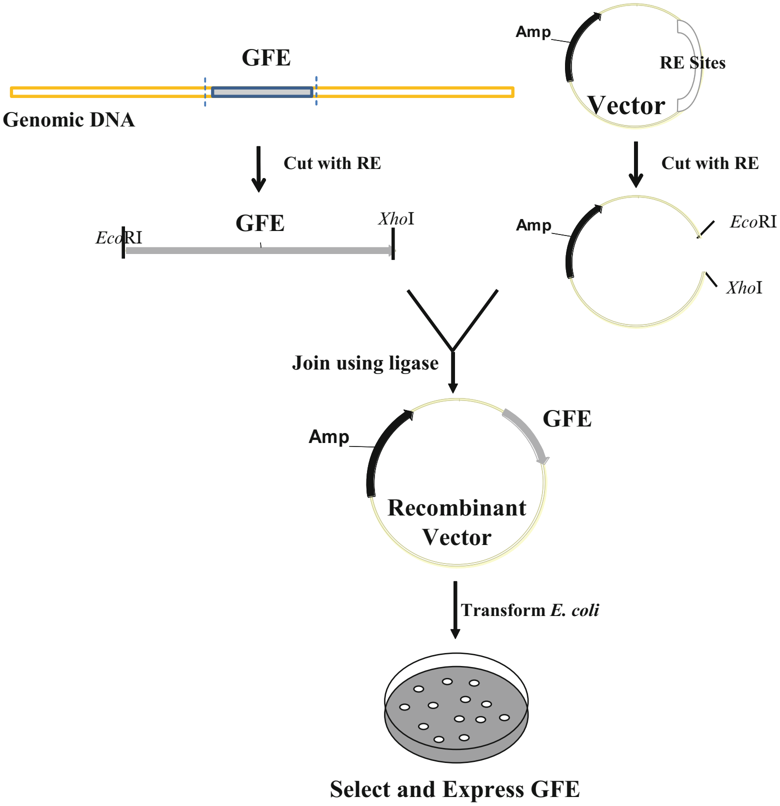

Fig. 3.5 A general genetic engineering strategy for enzyme expression

relevant mRNA. Alternatively, the required DNA may also be synthesized chemically – if the desired nucleotide or amino acid sequence is known.

Insertion of the Gene Into an Expression Vector The gene/ORF/cDNA/ is integrated (linked) into another piece of DNA, the vector DNA, in order to promote its uptake and replication in a suitable host organism. In general, the DNA vectors are designed (engineered) to have a unique site for restriction endonuclease and carry a marker to facilitate selection of genetically modified host cells (transformants). The chimerical recombinant DNA is introduced in appropriate host for expressing the enzyme protein. Typically, a bacterial plasmid vector is used to transform bacteria (Fig. 3.5). For animal and plant cells, their respective viral DNAs are often used as vectors.

Transformation of the Host Cell The recombinant DNA vector is introduced into a host cell either directly (by the process of transformation) or by infecting it using a viral vector.

Detection of the Inserted Gene The presence of the foreign DNA insert in the host is detected by molecular tools like Southern blotting (DNA-DNA hybridization), PCR, etc. The functional expression of the recombinant DNA may be directly monitored through enzyme activity and the presence of the protein.

At its simplest level, genetic engineering tools allow us to produce any enzyme protein in the common bacterium *Escherichia coli*. One could choose from bacteria, yeast, plant, or mammalian cells as hosts for optimal expression and/or appropriate posttranslational processing (like glycosylation, etc.) of the protein. Expression in a homologous host is generally successful. One of the principal reasons that an enzymologist manipulates DNA blueprint of an enzyme is to modulate the existing feature or to create new ones. Approaches like site-directed mutagenesis (SDM) and directed evolution of enzymes are routine nowadays. We will have more to say on these genetic engineering strategies for enzyme design/redesign later in this book (Chap. 39 "Future of Enzymology – An Appraisal"). In addition, genetic engineering has made optimal enzyme production possible in many different ways. We will simply illustrate the field with a few examples:

1. Enzyme overproducing strains have been constructed by suitably overcoming regulation at the level of feedback inhibition, transcription, translation, or secretion of the enzyme protein. Expression of amylases is often under the control of carbon catabolite repression (glucose repression). Yet another bottleneck with respect to protease production is nitrogen metabolite regulation. Both bacterial and fungal strains, with mutations for deregulation, constitutive expression, and hypersecretion, find utility in enzyme production. Suitable genetically stable mutant strains combining many such features have found their place in enzyme industry.

   Individual component activities of the cellulase complex and their specific combinations find industrial applications (Table 3.1). Producer strains (like *Trichoderma reesei*) overexpressing individual activities as well as deleted in each one of them are available. Well-defined cocktail of cellulase components are suitable in textile industry and in biomass conversion.
2. Heterologous expression, even of mammalian or plant enzymes, in a convenient microbial host. The recombinant chymosin (bovine) was produced in a fungus by introducing a stable expression construct. The difficult challenges however include ability to obtain economically viable levels of secretion, stability of the expressed protein, and attaining proper posttranslational modifications like glycosylation, if any.
3. Once the structural gene for the enzyme is cloned and expressed, it is feasible to generate mutant forms of this enzyme. Tinkering with enzyme properties like stability, pH optimum, specificity, and regulatory features is possible. Protein and enzyme engineering through site-directed mutagenesis has found direct applications in the enzyme industry. Subtilisin was engineered for better stability.

Glucose isomerase was improved for its metal preference, substrate specificity, and pH optimum. DNA polymerase with high fidelity for PCR applications is another fruitful example.

The impact of genetic engineering on the field of modern enzymology may be further gauged by examples presented in Chap. 39 ("Future of Enzymology: An Appraisal").

3.4 Summing Up

Being superbly crafted catalysts of nature, enzymes found their use very early in the game. The first patent for an enzyme application was in 1894, even before their chemical nature was known! Subsequently, bioprospecting for enzymes with unique properties has continued unabated. The entire industry to process starch has evolved by exquisite use of enzymes from across the three domains of life. In the clinical and pharmaceutical setting, enzymes have served as disease markers, analytical tools for metabolite measurements, and biosensors and targets for drug discovery. In an industrial process, the given amount of substrate has to be maximally converted to product. Immobilized enzymes have reduced the cost by permitting their reuse and reaction scale up. Although evolved essentially for catalysis in an aqueous environment few enzymes are able to function in organic solvents. This has expanded the scope of their utility in terms of the types of reactions that can be carried out; many hydrolases may now be used to drive the reactions in reverse for synthetic purposes.

Having tasted their potential in industry, enzymes are being genetically engineered for desired features and also for efficient large-scale production. With a better understanding of how these catalysts work, the range of their applications in various industries has expanded. While it is beyond the scope for an elaborate coverage, the present chapter has attempted to provide a focused overview. For more detailed treatments, the reader may refer to the cited literature.

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