

Applied Biocatalysis: An Overview

Munishwar N Gupta* and Ipsita Roy

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110 016, India.

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*"Rather I prize the doubt,
Low kinds exist without,
Finished and finite clods,
Untroubled by a spark"*

Robert Browning

Introduction

The title of this overview/mini-symposium may raise a few eyebrows. Why applied biocatalysis? Why not simply biocatalysis? Is there a difference? The following anecdote may give a clue. Michael Faraday presented his discovery of electricity to the British government. The Lord of Exchequer asked him the possible use of such a discovery. Faraday's answer showed his brilliance. He replied: "My Lord, one day you will be able to tax it!" He was given the green signal for further work. It is a thin line which divides fundamental and applied sciences. If you put a biocatalyst to use for a specific purpose and the application is economically viable (or at least shows potential), we can call it applied biocatalysis. The adjective keeps out conventional enzymology, e.g. purifying an enzyme with 5-10 steps.

A few years back, Whitesides and associates wrote a very good article on perceived advantages and disadvantages of using enzymes¹. The latter included "expensive, unstable, restricted to predominantly aqueous environments and difficult to manipulate". This overview will show that these disadvantages have been more or less overcome. (In fact, the various papers in this mini-symposium illustrate this with specific examples). Much of the biotechnology today deals with either producing enzymes/proteins or using them! Applied Biocatalysis is an area which is truly multi-disciplinary in nature: microbiologists, tissue

culture experts, chemical engineers, biomedical engineers and immunologists merely constitute an illustrative list.

The history of applied biocatalysis is nearly as old as human civilization. Earliest applications were empirical and were more of recipe than science. Moreover, these applications were there even before the concept of biocatalyst or enzyme was known to us. Table 1 summarizes these early applications and discoveries and some corresponding current perspectives.

Production of Enzymes

The production of therapeutic proteins, which has been made possible by new discoveries in biotechnology, has in 2001, generated sales exceeding \$25 billion¹⁰. This, combined with the commercial need of vaccines, industrial biocatalysts, enzymes for synthesis of fine chemicals by 'green' routes and diagnostic kits, have necessitated a 'second look' at the cost factor in downstream processing of proteins. This stage also faces challenges from the fact that the target product forms only a minor part of a highly complex broth.

Apart from conventional methods of extracting enzymes from animal, plant and microbial sources, today one can produce enzymes by cloning, tissue culture or transgenic technique¹¹. All stages from the point of production to obtaining the enzyme with "adequate purity level" constitute downstream processing¹². In the case of intracellular enzymes, downstream processing starts with cell disruption¹³ by chemical, mechanical or biochemical means. For all enzymes, the next step is solid/liquid separation for removing the suspended impurities and cell debris. Beyond this, there are no set rules but the trend is to use methods of increasingly higher resolution. Thus, precipitation → chromatography → affinity chromatography have, over the years, formed the basic outline for the purification protocol.

* Author to whom correspondence may be addressed.
Tel.: 91-11-659 1503
Fax: 91-11-658 1073
Email: mn_gupta@hotmail.com

Table 1—Historical Perspective and Some Current Trends in Applied Biocatalysis

- Production of soy derived foods in China and Japan. Microbial amylases and proteases were involved².
- Homer described “the production of cheese by stirring milk with a twig of fig tree”. Ficin, obviously, was involved².
Milk kept in a bag made from the stomach of a recently slaughtered calf gave a drier substance which gained a flavour after some time. Thus was born the art of cheesemaking with the help of rennin². Our more recent knowledge tells us that calf rennin catalyzes the proteolytic cleavage of a single peptide bond (Phe¹⁰⁵-Met¹⁰⁶) in the κ -casein molecule, initiating protein precipitation³.
We now also know that changes in texture and flavour of cheese are associated with the further hydrolysis of milk proteins⁴.
Proteins → Peptides → Amino acids → Volatile flavours and aromas
Occasional shortages of calf rennin (chymosin) have prompted the use of fungal proteases from *Endothia parasitica*, *Mucor meihei* and *M. pucillus* as substitutes. Global demand for chymosin is estimated to be worth about \$100 million a year. On March 23, 1990, chymosin became the first genetically engineered protein to be used in food processing⁵.
Rennet (a mixture of rennin and pepsin) incidentally was also the first enzyme preparation to be marketed, by a company called Chr. Hansen (Copenhagen, Denmark) in 1875².
- Otto Rohm obtained trypsin-in-detergent patent in 1913. This led to the introduction of the first enzymatic detergent ‘Burnus’ consisting of pancreatin and sodium carbonate⁶. However, it was quite some time before enzyme-based detergents became commonly acceptable. Like many other applications of enzymes, this story is linked with the issues of stability and stabilization of enzymes. The enzymes incorporated in the detergents should be stable at alkaline pH, at fairly high temperatures and in the presence of chelating agents and surfactants. Around the early sixties, “everybody wanted Biotex, the protease containing detergent”². However, there was the scare of allergic responses in the late sixties. Thus, it is interesting to note that by the early seventies, enzymes were withdrawn from detergents³. Granulation techniques allowed their reintroduction in the detergents as it was found that it was the inhalation of dust formed in the enzyme powder which was responsible for the allergic responses³.
Protein engineered subtilisin addressed the aspect of operational stability³. Today, enzyme-based detergents constitute a major application of biotechnology. The detergent industry accounts for ~ 30 % of total sales of enzymes. Apart from proteases and lipases, amylases and cellulases are also used.
Apart from detergents for textiles, enzymes are also used in cleaning agents for other purposes, e.g. lipases in contact lens cleaners and windshield washing fluids, etc.².
- **Starch processing:**
Today, polysaccharide-degrading enzymes constitute a major component of the market for industrial enzymes. Within this, starch-degrading enzymes represent the biggest portion. α -Amylase and glucoamylase yield sugars. The latter can either be fermented to alcohol (an energy source) or converted into products like high fructose corn syrup (HFCS) (industrial sweetener) by glucose isomerase. The enzymology of starch degradation is depicted in Fig. 1.
A major constraint has been that while industrially useful (thermostable enzymes for starch liquefaction) α -amylases are unstable below pH 6.0, glucoamylase requires pH of around 4.2-4.5 for further hydrolysis to give glucose. pH adjusting means additional cost, more so if glucoamylase action has to be followed by glucose isomerase (with pH optimum around 7-8). Few years back, Shaw *et al.* (1999) have reviewed protein engineering of α -amylase for functioning at low pH⁷. This perhaps gives a good idea of what applied biocatalysis is all about!
- **Pectin hydrolysis:**
It was in 1930 that Kertesz filed a patent for “enzymatic clarification of unfermented apple juice”⁸. Today, enzymes are used in a variety of ways: in extraction, clarification and modification of fruit juices⁹. While pectinase is a key enzyme (see Fig. 2), many other enzymes are also employed in specific cases. For example, addition of cellulases helps in releasing desired coloured compounds in the case of black currants and red grapes. Some fruits like pear are rich in arabans and arabanase is required for clarification of pear juice. Naringinase is required for debittering, especially in cases like grapefruit juice. Another interesting requirement is that of glucose oxidase for removal of O₂ from “headspace” in the case of wine, beer and fruit juice. This cuts down non-enzymatic browning of these beverages. This is one area where knowledge of biocatalysis has become an acceptable practice at industrial level. For example, while technology for production of French cider has essentially remained unchanged since 1909, in about 30 % cases, the industry has switched over to the use of Rapidase Pomaliq (a mixture of pectinases, hemicellulases and cellulases by Gist-Brocades, Netherlands) for cutting down the process time by enzymatically hydrolyzing pectic material in apples⁸.
- Today, there is hardly any industry where enzymes are not used. Animal feed, production of low lactose milk and whey hydrolysis, edible oil extraction, textiles, leather and paper industries are some other notable examples.

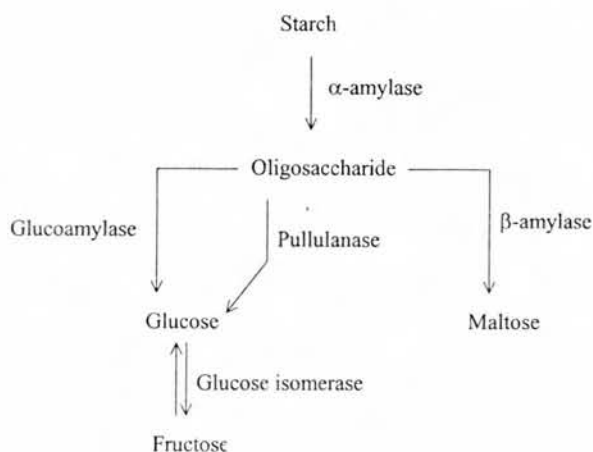


Fig. 1—Schematic diagram of enzymatic degradation of starch. [α -Amylase hydrolyzes internal α -1,4 linkages (endohydrolase). β -Amylase removes maltose units from the non-reducing end (exohydrolase). Glucoamylase hydrolyzes α -1,4 glycosidic bonds and removes glucose units from the nonreducing end of the oligosaccharide. Pullulanase, on the other hand, hydrolyzes α -1,6 glycosidic bonds. Glucose isomerase converts glucose to fructose and is used for producing industrial sweeteners as fructose is much more sweet than glucose].

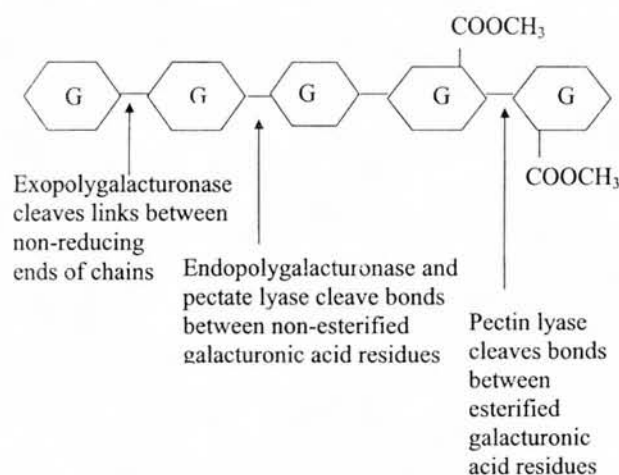


Fig. 2—Schematic diagram of enzymatic degradation of pectin

Modern downstream processing is based upon the realization that a limited number of unit processes is an essential feature of an economical protocol. This has resulted in two broad approaches:

- To deal with crude suspensions directly and skip the solid/liquid separation step. This has been possible by using two-phase extractions, expanded bed chromatography and in recent years, three phase partitioning.
- To bring up affinity-based processes right in the beginning and not at the polishing stage. This has been made possible by interfacing affinity

interactions with precipitation, membrane separations, fluidized beds, two-phase and three-phase partitioning methods.

Both broad approaches coincide in shaping many modern bioseparation techniques. This is one area of biocatalysis where much has been happening in the last decade or so. Hence this will be dealt with in somewhat greater detail. A review of some of the important developments follows.

Expanded Bed Chromatography

In this approach, the use of tailor-made media allows one to operate chromatography in a fluidized bed mode. Particulate adsorbents, if packed in a column, can not deal with crude suspensions as clogging of adsorbent beds will take place. Batch adsorption in stirred tank is an option in such cases, but shows poor resolution and capacity. A fluidized bed in which inter-particle distance in adsorbent is increased, creating voids through which suspended impurities pass through, is best suited to capture the protein from the crude extract. The best way of fluidizing the adsorbent particles in practice is by a liquid flow directed upwards. Such fluidized beds can combine clarification, concentration and (varying extent of) fractionation in a simple unit process¹⁴. More sophisticated designs and more detailed discussion can be found in some of the reviews^{15,16}. Operationally, an expanded bed chromatography protocol follows the standard sequence (of packed beds): equilibration, sample application, wash, elution and cleaning-in-place (CIP). Expanded bed chromatography has already been successfully applied in many cases. Some illustrative examples from our laboratory are given in Table 2.

Affinity Precipitation

Affinity precipitation involves precipitation of the target protein in free solution, mostly using a reversibly soluble-insoluble macroaffinity ligand^{17,18}. This macroaffinity ligand consists of a polymer which is so designed that its solubility is controlled by altering some solvent parameter such as pH, temperature, addition of metal ions, etc. In some fortuitous cases, the polymer itself has an affinity for the protein. In other cases, a ligand is coupled to the polymer. Since the target protein has affinity for the macroaffinity ligand, it is selectively precipitated along with the macroaffinity ligand upon the necessary change in the controlling parameter. It can be recovered using specific or non-specific eluents

Table 2—Some examples of the use of the technique of expanded bed chromatography (Adapted from Ref. 12)

Enzyme/ Protein	Source	Matrix	Yield (%)
α -Amylase	<i>Bacillus amyloliquefaciens</i>	Alginate	92.5
	Porcine pancreas		99.7
	Wheat germ		89.8
	<i>Scytalidium thermophilum</i>		80.2
Cellulase	<i>Aspergillus niger</i>	Cellulose beads	91
		Chitosan	80
Amylase inhibitor	Wheat germ	Cu ²⁺ -linked agarose	83
Phospholipase D	Peanut	Crosslinked alginate	78
Alkaline phosphatase	Chicken intestine	Dye-linked cellulose beads	70
Pullulanase	<i>Bacillus acidopullulyticus</i>	Alginate	97
Polyphenol oxidase	<i>Duranta plumieri</i>	Streamline DEAE	77

and the macroaffinity ligand is available for reuse. The process thus results in concentration and purification of the target protein and is amenable to scale-up. It can also be used directly with crude culture broth, unlike chromatographic techniques where fouling of the column (especially in the packed bed mode) is a recurring problem when using extracts containing particulate matter. Some illustrative examples are tabulated in Table 3.

Three Phase Partitioning

Precipitation with salts and organic solvents are well-established techniques for concentration of crude protein extracts. Three phase partitioning (TPP) was first developed as a bridge between upstream and downstream processes³². The basic protocol involved is outlined in Fig. 3. The mechanism of how partitioning operates is not very clearly understood. It is believed to be the result of a collective operation of principles involved in numerous techniques such as conventional salting out, Morton's *n*-butanol extraction method, isoionic precipitation, cold cosolvent precipitation and osmolyte and kosmotropic precipitation of proteins³³. In an effort to understand the mechanism involved, proteinase K was subjected to three phase partitioning. The X-ray diffraction patterns of crystals of pure native proteinase K and TPP-treated proteinase K (TPK) were compared³⁴. As a result of this treatment, the specific activity of TPK had gone up by 2.1 times. The attention was thus focussed on the binding region of the enzyme. The

Table 3—Some examples of proteins purified by affinity precipitation

Polymer	Enzyme/Protein purified	Reference
Eudragit S-100	Xylanase	19
	IgG-type monoclonal antibody	20
Alginate	Pectinase	21
	α -Amylase	22
	Phospholipase D	23
	Lipase	24
Chitosan	Wheat germ lectin	25
	Lysozyme	26
Copolymer of 1-vinylimidazole and N-vinylcaprolactum	Soybean trypsin inhibitor	27
Poly (N-isopropylacrylamide)	Alkaline protease	28
Copolymer of 1-vinylimidazole and N-isopropylacrylamide	Amylase inhibitor	29
Galactomannan	Human IgG	30
Poly (N-isopropylacrylamide)	Avidin	31

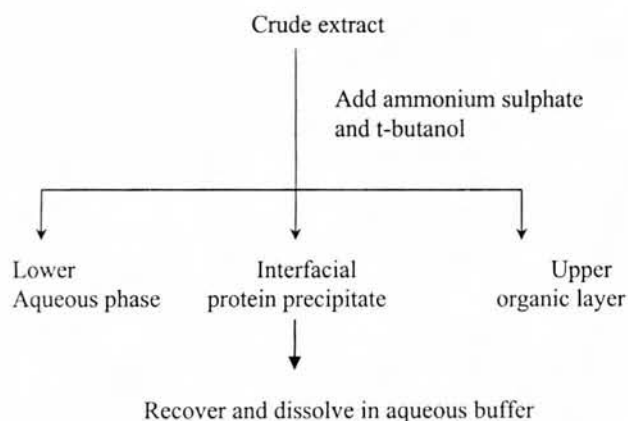


Fig. 3—Flowsheet for three phase partitioning

most striking change in TPK was found in the conformation of the side chains of a number of residues. The residues indicated more than one conformational state for their side chains. This structure (TPK) corresponds to the highest observed B-factor for proteinase K. Thus, the increased flexibility of the molecule is responsible for higher activity of the enzyme.

Some examples from our laboratory, listing the success of this technique, are shown in Table 4. It has been shown that interfacing a metal affinity based step with TPP protocol makes the latter a highly selective technique. Metal affinity based separations (of which immobilized metal ion affinity chromatography (IMAC) is the most frequently used version) exploit the affinity of surface histidine, cysteine and tryptophan residues in the protein molecule for metal ions like Cu^{2+} , Zn^{2+} and Ni^{2+} . Soybean trypsin inhibitor (STI) has surface histidine residues and has been purified via metal affinity. The selectivity and usefulness of metal ion-assisted TPP has been evaluated by purifying STI from soybean meal³⁹.

A modification in this process has been introduced by macroaffinity ligand facilitated three phase partitioning (MLFTPP). As discussed above, there are many polymers which show affinity for the enzymes. Using these polymers to capture the target enzymes in the TPP mode, enhances the selectivity of this simple process. This has been used to purify xylanase using Eudragit S-100 and the polymer is available for reuse after the end of one purification cycle⁴⁰.

Other Emerging Techniques

Simulated Moving Bed Technology

Continuous chromatographic separation processes based on the simulated moving bed (SMB) techno-

Table 4—List of enzymes/proteins purified by three phase partitioning

Source	Enzyme/Protein	Reference
Chicken intestine	Alkaline phosphatase	35
<i>Dacus carota</i>	Phospholipase D	36
Wheat germ	α -Amylase/Proteinase K inhibitor	37
Tomato	Pectinase	38
Soybean	Trypsin inhibitor	39
<i>Aspergillus niger</i>	Xylanase	40

logy have been gaining increasing importance since the beginning of the last decade for applications in the fine chemical and pharmaceutical industries, in particular for the resolution of enantiomers⁴¹. The focus has shifted from sugar and petrochemical industries for very large scale fractionation when the technology was introduced in the late 1950s. The technology has evolved out of true moving bed (TMB) concept. The solid adsorbent particles flow along the column countercurrent to the fluid stream at a velocity that pushes mixture components (which are to be separated) in opposite directions. The mixture is fed in the middle and components A and B can be collected from the top and bottom portions. Apart from the most critical choice of flow rates, design of a TMB (or SMB) also has to consider feed concentrations, number of columns per zone, column length, column diameter and particle size.

Nicoud has described the use of moving bed for removal of ammonium sulphate from a protein⁴². Purification of trypsin from porcine pancreatic extracts, purification of human serum albumin, separation of myoglobin and lysozyme have shown the potential of this approach⁴². Gottschlich and Kasche have purified a monoclonal antibody from a cell culture supernatant with yield exceeding 90%⁴³. SDS-PAGE of the feed and the pooled stream confirmed the removal of more than 99% of the contaminating proteins. SMB technology offers several advantages over conventional preparative chromatographic techniques: a continuous process, it can be allowed to run unattended and ensures stable quality of the product. Productivity per unit mass of the stationary phase is high (the column works in the non-linear region of the adsorption equilibria of the

components to be separated, i.e. under overload conditions, thus ensuring optimal use of the stationary phase) and the recycling of the fluid phase implies that the solvent requirement is low. Also, the technique is more robust since high purity of the product can be attained with a smaller number of theoretical plates. At present, however, this technology is limited to efficient and complete separation of a mixture of two proteins.

Perfusion Chromatography

In contrast to conventional chromatography media, perfusion chromatography media particles have two discreet classes of pores. Large "throughpores" allow convection flow to occur through the particles themselves, quickly carrying sample molecules to short "diffusive" pores inside. By reducing the distance over which diffusion needs to occur, the time required for sample molecules to interact with interior binding sites is reduced. Diffusion is no longer limiting and flow rates can be dramatically increased—without any loss of resolution or capacity. Separations can be achieved at 1,000 to 5,000 cm hr⁻¹ compared to 50 to 360 cm hr⁻¹ for conventional media. The technique is associated with high throughput and high dynamic capacity. Fahrner and Blank have shown that an on-line chromatographic assay can reliably control antibody loading in real-time protein A affinity chromatographic purification of a recombinant antibody from clarified Chinese hamster ovary cell culture fluid⁴⁴. Hizel *et al.* have described a new procedure for the isolation, purification and quantification of the product of the oncosuppressor gene *brca1* in tumour cells⁴⁵. It involves [³⁵S] methionine labelling of intracellular proteins followed by two perfusion chromatographies. DNA-binding proteins were isolated by heparin affinity chromatography on Poros 20 HE. BRCA1, which includes a zinc finger, could be eluted along with all the nucleic acid-binding proteins using a salt gradient.

Monoliths

Monoliths are continuous stationary phases that are cast as a homogeneous column in a single piece and prepared in various dimensions with agglomeration-type of fibrous microstructures. They exhibit high efficiencies even at high flow rates due to their fast convective mass transfer and can therefore be used at very high mobile phase velocities (up to 400 ml min⁻¹), leading to high productivities^{46,47}.

Monoliths have been applied for the fast separation and purification of proteins, DNA, smaller molecules like organic acids⁴⁸, hydroxybenzoates, oligonucleotides and peptides^{49,50}. Quantitative analysis of affinity interactions between antibodies and immobilized group specific ligands (protein A, G and L) has been attempted using high performance monolith affinity chromatography (HPMAC)⁵¹. Monoliths have been used for in situ solid phase peptide synthesis⁵² and immobilization of polynucleotide phosphorylase⁵³. The benefits of using monolithic columns include miniaturization leading to extremely fast separation (within seconds) and easy scale-up. Further applications of this upcoming technology include the areas of capillary electrochromatography, chip technology and scale-up in purification⁵⁴.

Tailoring Enzyme Properties or Biocatalyst Engineering

Early efforts in tailoring enzymes to suit specific needs utilized chemical modifications and chemical crosslinking⁵⁵. The key issue has been of enhancing stability (especially thermal stability)⁵⁶. Immobilization techniques brought in reusability and possible stabilization⁵⁷. Both noncovalent and covalent methods of immobilization have proved useful. Two relatively recent developments in this area have been:

- (i) Introduction of crosslinked enzyme crystals, CLECTM, by Altus Biologicals. These microcrystalline preparations are reported to be stable in both aqueous and nonaqueous media⁵⁸.
- (ii) Design of smart biocatalysts. This has mostly consisted of creating bioconjugates of enzymes with smart polymers⁵⁹. This enables the biocatalyst to be in free solution and one can transform macromolecular and insoluble substrates. After use, the biocatalyst can be recovered by applying suitable stimulus which is chosen depending upon the smart polymer. Temperature, pH and addition of chemicals are some of the stimuli which have been used. An attractive design is to build in a smart domain within the enzyme structure⁶⁰.

Protein engineering has been a more powerful tool for altering specific properties of enzymes⁶¹. While early applications again focussed on enhancing stability, more innovative approaches like changing pH optima (for efficient biodegradation of starch) are beginning to emerge⁷. Directed evolution is the

latest tool which attempts at creating enzymes with suitable regio- and stereo-selectivity and suitable kinetic properties⁶². This continues to use recombinant methods but takes advantage of high throughput screening methods to reach a pre-selected property.

Bioconversions, Organic Synthesis and Non-Aqueous Enzymology

Converting biomass to value-added products has been a major class of bioconversions. Some of these have already been mentioned in Table 1. Hydrolysis of cellulose and lignin to sugars and finally to bioethanol has been an active area which has recently been reviewed by Himmel *et al.*⁶³. Starch degradation has already been mentioned. Starting with corn starch, production of high fructose corn syrup (HFCS) is one of the top few applications of enzymes in the industry¹. Enzymes are increasingly being employed to develop 'green technology' for replacing processes based upon aggressive chemicals⁶⁴. A less-known example in this category is the extraction of edible oils by aqueous enzymatic oil extraction process^{65,66}. The process essentially degrades macromolecular structures entrapping oil bodies and thus facilitates the oil recovery.

Development of methodology for use of enzymes under low water conditions constitutes a milestone for applied biocatalysis. Both "nearly anhydrous systems" pioneered by the groups of Klibanov⁶⁷, Mattiasson⁶⁸ and Halling⁶⁹ and "reverse micellar systems" developed by the group led by Levashov and Martinek⁷⁰, Luisi⁷¹ and more recently by Cabral⁷², have made it possible to use hydrolases for synthesis. Lipases, undoubtedly, have been used more often, followed by proteases and carbohydrases. Several interesting phenomenon like pH memory, unusually high thermal stability and molecular imprinting have been observed with the use of enzymes in "neat organic solvents"⁷³. Again the major issue here has been that of stabilizing the enzymes against inactivation by organic solvents: Polar solvents are known to be more damaging as compared to non-polar solvents⁷⁴. Again, a variety of techniques like chemical modification, immobilization and protein engineering (used for enzyme stabilization in aqueous media against denaturing conditions) have been used with varying success⁷⁵. Recent work with directed evolution is especially relevant to the use of enzymes for obtaining drug intermediates⁷⁶. This is an area where much is going on and it is likely to prove

immensely useful for the overall purpose of synthesis of fine chemicals and pharmaceuticals.

Exposure to organic solvents as such can have some unusual and interesting consequences. It has been shown that in the presence of about 5-10% organic solvent, various enzymes show higher activity⁷⁷. It has also been observed that heating enzymes in the presence of organic solvents at moderately high temperatures results in obtaining enzymes with higher turnover numbers⁷⁸.

Biosensors and Diagnostics

Biosensors are basically created by interfacing a biocatalyst with a transducer. The former may be an enzyme, tissue slice, or whole organism. The purpose of the biocatalyst component is to detect the presence of a chemical substance and amplify the 'signal' via its turnover number. The transducer converts it into an observable parameter. Depending upon the latter, we have enzyme electrodes⁷⁹, fibre optic sensors (optodes)⁸⁰, field effect transistors (FETs)⁸¹ and thermistors⁸². The applications of biosensors extend to diverse areas like clinical chemistry and health care, veterinary, agriculture and food sciences, fermentation and pharmaceutical production and environmental control and pollution monitoring. With the advent of smart materials, the last few years have seen design of 'molecular gates' and 'valves' which sense and respond to the presence of certain metabolites⁸³.

ELISA, based upon antigen-enzyme or antibody-enzyme bioconjugates is the best example of use of biocatalyst diagnostics⁸⁴. On-line analyzers using flow injection (FIA) in combination with expanded beds and perfusion chromatography are relatively recent examples^{85,86}.

Proteomics and Beyond

Proteomics represents the most recent and still fashionable face of applied biocatalysis. Genomics has given us the information. Proteomics has to tell us the "action part". Which enzyme/protein occurs where, how much, along with what other proteins and does what, are the questions which define the contours of this emerging science. It may be interesting for the reader to refer to a recent debate on what proteomics should mean⁸⁷! Right now, for most of the people, it means mass spectrometry (electro spray and occasionally MALDI-TOF) or 2-D electrophoresis. There are some more sensible views expressed, which foresee that more sophisticated

bioseparation strategies at the semi-preparative level will have to be developed⁸⁸. Thus, once again, we may come back full circle. The old protein chemistry suddenly became fashionable and was reborn as protein folding. May be, we will see renaissance of downstream processing as a part of proteomics.

Thus, the area of applied biocatalysis, which perhaps started by stirring milk with the twig of a fig tree, has come a long way. Today it involves more sophisticated tools and strategies.

“Where is the knowledge we have lost in information?”

T. S. Eliot

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