

Immobilized enzymes in bioprocess

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Immobilization of biocatalysts helps in their economic reuse and in the development of continuous bioprocesses. Biocatalysts can be immobilized either using the isolated enzymes or the whole cells. Immobilization often stabilizes structure of the enzymes, thereby allowing their applications even under harsh environmental conditions of pH, temperature and organic solvents, and thus enable their uses at high temperatures in nonaqueous enzymology, and in the fabrication of biosensor probes. In the future, development of techniques for the immobilization of multienzymes along with cofactor regeneration and retention system can be gainfully exploited in developing biochemical processes involving complex chemical conversions. The present review outlines some of the above aspects, and delineates the present status and future potentials of immobilized enzymes and nonviable cells in the emerging biotech industries.

BIOTECHNOLOGY is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. This is mainly because, unlike the chemical catalysts, the biological systems have the advantages of accomplishing complex chemical conversions under mild environmental conditions with high specificity and efficiency. Biological systems help in ingredient substitution, processing aid substitution, more efficient processing, less undesirable products, increased plant capacity, increased product yields, and improved or unique products. The variety of chemical transformations catalysed by enzymes has made these catalysts a prime target of exploitation by the emerging biotech industries.

Despite these advantages, the use of enzymes in industrial applications has been limited by several factors, mainly the high cost of the enzymes, their instability, and availability in small amounts. Also the enzymes are soluble in aqueous media and it is difficult and expensive to recover them from reactor effluents at the end of the catalytic process. This restricts the use of soluble enzymes to batch operations, followed by disposal of the spent enzyme-containing solvent. Over the last few decades, intense research in the area of enzyme technology has provided many approaches that facilitate their practical applications. Among them, the newer technological developments in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploi-

tation of biocatalysts in industry, waste treatment, medicine, and in the development of bioprocess monitoring devices like the biosensor.

Immobilization means associating the biocatalysts with an insoluble matrix, so that it can be retained in a proper reactor geometry for its economic reuse under stabilized conditions. Immobilization thus allows, by essence, to decouple the enzyme location from the flow of the liquid carrying the reagents and products. Immobilization helps in the development of continuous processes allowing more economic organization of the operations, automation, decrease of labour, and investment/capacity ratio. Immobilized biocatalysts offer several other advantages, notable among them is the availability of the product in greater purity. Purity of the product is very crucial in food processing and pharmaceutical industry since contamination could cause serious toxicological, sensory, or immunological problems. The other major advantages include greater control over enzymatic reaction as well as high volumetric productivity with lower residence time, which are of great significance in the food industry, specially in the treatment of perishable commodities as well as in other applications involving labile substrates, intermediates or products¹.

Immobilized nonviable cells as an economical source of enzymes

Biocatalysts can be immobilized using either the isolated enzymes or the whole cells or cellular organelles. Immobilization of whole cells has been shown to be a better alternative to immobilization of isolated enzymes²⁻⁴. Doing so avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment thus protecting it from inactivation either during immobilization or its subsequent use in continuous system. It may also provide a multipurpose catalyst, specially when the process requires the participation of number of enzymes in sequence. The major limitations which may need to be addressed while using such cells are the diffusion of substrate and products through the cell wall, and unwanted side reactions due to the presence of other enzymes. The cells can be immobilized either in a viable or a nonviable form. Immobilized nonviable cell preparations, which are normally obtained by permeabilizing the intact cells, for the expression of intracellular activity are useful for simple processes that

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require single-enzyme with no requirement for cofactor regeneration, like hydrolysis of sucrose or lactose²⁻⁴. On the other hand immobilized viable cells, which serve as 'controlled catalytic biomass', have opened new avenues for continuous fermentation on heterogeneous catalysis basis by serving as self-proliferating biocatalysts^{4,5}. The present review will focus mainly on immobilized isolated enzyme systems, and the use of immobilized nonviable cells as a source of enzymes. For the aspects on the immobilized viable cell systems, the readers can refer to another article in this issue. Some of the aspects on the applications of immobilized viable cells in biochemical process development and monitoring have also been reviewed recently⁶.

Most of the enzymes used at industrial scale are normally the extracellular enzymes produced by the microbes. This has been mainly due to their ease of isolation as crude enzymes from the fermentation broth. Moreover, the extracellular enzymes are more stable to external environmental perturbations compared to the intracellular enzymes. However, over 90% of the enzymes produced by a cell are intracellular. The economic exploitation of these, having a variety of biochemical potentials, has been limited in view of the high cost involved in their isolation. Also, compared to extracellular enzymes, the intracellular enzymes are more labile. Delicate and expensive separation methods are required to release the enzymes undamaged from the cell, and to isolate them. This increases the labour and the cost of the enzyme. These problems could now be obviated by the use of permeabilized cells as a source of enzyme. Permeabilization of the cells removes the barrier for the free diffusion of the substrate/product across the cell membrane, and also empties the cell of most of the small molecular weight cofactors, etc., thus minimizing the unwanted side reactions. Side reactions, which can occur due to the presence of other enzymes in a cell, can also be minimized by inactivating such enzymes prior to or after immobilization^{7,8}. Such permeabilized cells, which are often referred to as nonviable or nongrowing cells, can be exploited in an immobilized form as a very economical source of intracellular enzyme for simple bioconversions like hydrolysis, isomerization and oxidation reactions that do not need a cofactor-regeneration system⁴. The decision to immobilize cells either in a viable or nonviable form is very important and depends on their ultimate application. Thus the permeabilized *K. fragilis* cells, which are nonviable, convert lactose in milk to glucose and galactose⁹, whereas the viable (nonpermeabilized) cells convert the lactose to ethanol and are useful in the complete desugaring of milk¹⁰.

A variety of physical, chemical, and enzymatic techniques have been developed for the permeabilization of cells^{9,11-16}. Some techniques like entrapment in polyacrylamide itself have been shown to result in permeabilization of the cells¹¹. A few typical examples of immobilized

enzyme preparation obtained using permeabilized cells are listed in Table 1 (refs 17-30). It is evident that most of the major industrially important intracellular enzymes can now be immobilized using whole cells.

Techniques and supports for immobilization

A large number of techniques and supports are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Therefore, it will not be possible to suggest any universal means of immobilization. It can only be said that the search must continue for matrices which provide facile, secure immobilization with good interaction with substrates, and which conform in shape, size, density and so on to the use for which they are intended. Care has to be taken to select the support materials as well as the reagents used for immobilization, which have GRAS status, particularly when their ultimate applications are in the food processing and pharmaceutical industries. Macromolecular, colloidal, viscous, sticky, dense or particulate food constituents or waste streams also limit the choice of reactor and support geometries. Commercial success has been achieved when support materials have been chosen for their flow properties, low cost, non-toxicity, maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability, and ease of immobilization³¹. The variety of techniques and supports investigated for the immobilization have been reviewed in a number of articles and books^{1-4,32,33}. The objective of the preceding part of the paper is to review briefly, in the light of current developments, some approaches that have been used for the immobilization of biocatalysts with reference to the studies carried out in the author's laboratory as well as few others.

Techniques for immobilization have been broadly classified into four categories, namely entrapment, covalent binding, cross-linking and adsorption. A combination of one or more of these techniques has also been investigated. It must be emphasized that in terms of economy of a process, both the activity and the operational stability of the biocatalysts are important. They determine its productivity, which is the activity integrated over the operational time.

Entrapment

Entrapment has been extensively used for the immobilization of cells, but not for enzymes. The major limitation of this technique for the immobilization of enzymes is the possible slow leakage during continuous use in view of the small molecular size compared to

Table 1. Some examples of immobilized enzyme preparations obtained using nonviable cells and cellular organelles

Enzyme	Source	Technique/support	References
Invertase	<i>Saccharomyces cerevisiae</i>	Entrapment in polyacrylamide, alginate	17, 18
Invertase	<i>S. cerevisiae</i>	Adhesion to cotton threads, wool	19, 20
Inulinase	<i>S. marxianus</i>	Entrapment in open pore gelatine	21
Catalase	<i>S. cerevisiae</i>	Entrapment in polyacrylamide	11
Catalase	<i>Trigonopsis variabilis</i>	Entrapment in polyacrylamide	22
D-amino acid oxidase	<i>T. variabilis</i>	Entrapment in alginate, polyacrylamide, gelatine	23, 24, 25
Alcohol dehydrogenase	<i>S. cerevisiae</i>	Entrapment in polyacrylamide	26
Penicillin acylase	<i>Escherichia coli</i>	Entrapment in polyacrylamide	14
Urease	Ureolytic cells	Adhesion to cotton threads	27
L-Histidine ammonia lyase	<i>Achromobacter liquidum</i>	Entrapment in polyacrylamide	28
Fumarase	Liver mitochondria	Entrapment in polyacrylamide	7
Fumarase	<i>Brevibacterium flavus</i>	Entrapment in carrageenan	29
Aspartase	<i>E. coli</i>	Entrapment in carrageenan	30
Lactase	<i>Kluyveromyces fragilis</i>	Different supports	–
Glucose isomerase	cf Table 2	cf Table 2	

the cells. Biocatalysts have been entrapped in natural polymers like agar, agarose and gelatine through thermoreversible polymerization, but in alginate and carrageenan by ionotropic gelation^{3,4,33}. A number of synthetic polymers have also been investigated. Notable among them are the photo-crosslinkable resins, polyurethane prepolymers³⁴, and acrylic polymers like polyacrylamide^{17,24}. Among these, the most widespread matrix made from monomeric precursors is the polyacrylamide gel. Polyacrylamide may not be a useful support for use in food industry in view of its toxicity, but can have potentials in the treatment of waste and in the fabrication of analytical devices containing biocatalysts. One of the major limitations of entrapment technique is the diffusional limitation as well as the steric hindrance, especially when the macromolecular substrates like starch and proteins are used. Diffusional problems can be minimized by entrapment in fine fibres of cellulose acetate or other synthetic materials³² or by using an open pore matrix³⁵. Recently, the development of so-called hydrogels and thermoreactive water-soluble polymers, like the albumin-poly (ethylene glycol) hydrogel, have attracted attention in the field of biotechnology³⁶. In the area of health care, they offer new avenues for enzyme immobilization. Such gels with a water content of about 96% provide a microenvironment

for the immobilized enzyme close to that of the soluble enzyme with minimal diffusional restrictions³⁷.

Covalent binding

Covalent binding is an extensively used technique for the immobilization of enzymes, though it is not a good technique for the immobilization of cells. The functional groups extensively investigated are the amino, carboxyl, and the phenolic group of tyrosine^{1,32}. Enzymes are covalently linked to the support through the functional groups in the enzymes, which are not essential for the catalytic activity. It is often advisable to carry out the immobilization in the presence of its substrate or a competitive inhibitor so as to protect the active site. The covalent binding should also be optimized so as not to alter its conformational flexibility. Some of these problems however, can be obviated by covalent bonding through the carbohydrate moiety when a glycoprotein is concerned. A number of industrially useful enzymes are glycoproteins wherein the carbohydrate moiety may not be essential for its activity. In general, functional aldehyde group can be introduced in a glycoprotein by oxidizing the carbohydrate moiety by periodate oxidation without significantly affecting the enzyme activity. The

enzyme could then be covalently linked to a support containing an alky amine group through Schiff's base reaction. Enzymes like glucose oxidase, peroxidase, invertase, etc. have been immobilized using this technique^{32,38,39}. Covalent binding has been extensively investigated using inorganic supports. Enzymes covalently bound to inorganic supports have been used in the industry⁴⁰. Enzymes have also been bound to synthetic membranes, thus integrating biconversion and downstream processing^{41,42}. Large-scale processes using such an approach have been demonstrated for the preparation of invert sugar using invertase⁴³.

Cross-linking

Biocatalysts can also be immobilized through chemical cross-linking using homo- as well as heterobifunctional cross-linking agents¹. Among these, glutaraldehyde which interacts with the amino groups through a base reaction has been extensively used in view of its GRAS status, low cost, high efficiency, and stability⁴⁴. The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen^{4,32}. Studies from our laboratory have shown the use of raw hen egg white as an economic, easily available novel proteinic support rich in lysozyme for the immobilization of enzyme or nonviable cells either in a powder⁴⁵⁻⁴⁷, bead⁴⁸ or highly porous foam^{49,50} form, using glutaraldehyde as the cross-linker. The unique feature of this support is the large concentration of lysozyme naturally present in hen egg white which gets co-immobilized, thus imparting the bacteriolytic property to the support^{49,51}. Adsorption followed by cross-linking has also been used for the immobilization of enzymes. The technique of cross-linking in the presence of an inert protein can be applied to either enzymes or cells. The technique can also be used for the immobilization of enzymes by cross-linking the cell homogenates⁵². Osmotic stabilization of cellular organelles⁵³ or halophilic cells¹⁵ prior to immobilization using cross-linkers has also shown promise.

Adsorption

This is perhaps the simplest of all the techniques and one which does not grossly alter the activity of the bound enzyme. In case of enzymes immobilized through ionic interactions, adsorption and desorption of the enzyme depends on the basicity of the ion exchanger. Moreover, a dynamic equilibrium is normally observed between the adsorbed enzyme and the support which is often affected by pH as well as the ionic strength of the surrounding medium. This property of reversibility of binding has often been used for the economic recovery of the support. This has been successfully adapted in industry for the resolution of racemic mixtures of amino acids, using amino acid acylase^{1,3}. A variety of commercially available

ion exchangers have been investigated for this purpose³². One of the techniques, which has gained importance more recently, is the use of polyethylenimine for imparting polycationic characteristics to many of the neutral supports based on cellulose or inorganic materials⁵⁴. Enzymes with low pI, like invertase⁵⁵, urease⁵⁶, glucose oxidase⁵⁷, catalase⁵⁷, and other enzymes⁵⁴ have been bound through adsorption followed by cross-linking on polyethylenimine-coated supports.

Immobilization of enzymes through hydrophobic interaction has also shown promise^{58,59}. One of the important features of this technique, which is of great significance, is that, unlike ionic binding, hydrophobic interactions are usually stabilized by high ionic concentrations, thus enabling the use of high concentrations of substrates as desired in an industrial process without the fear of desorption. Other types of strong interactive binding techniques have also been reported for the reversible immobilization of enzymes. A typical example is the immobilization of soybean β -amylase on phenylboronate agarose, which can be reversed for the recovery of the support using sorbitol⁶⁰. Varieties of biospecific interactions have also been investigated for the reversible immobilization of enzymes by adsorption. Enzymes like acetyl choline esterase, ascorbic acid oxidase, invertase, peroxidase, glucose oxidase, etc. have been immobilized by biospecific-reversible immobilization on lectin-bound supports^{61,62} and invertase, using polyclonal antiinvertase antibodies⁶³.

Traditional enzyme immobilization procedures involve isolation of the enzyme, followed by use of several steps for the immobilization. Costs of enzyme purification, the immobilization procedure, bioreactor operational stability, and bioreactor regeneration are the major factors that determine the cost of a bioreactor process. Development of techniques for the simultaneous isolation and immobilization of enzymes from crude extracts has obviated these problems. Some typical examples include the immobilization of a streptavidin- β -galactosidase fusion protein expressed in *Escherichia coli* and bioselectively adsorbed from a crude cell lysate to biotin which is covalently immobilized on controlled pore glass⁶⁴, and the simultaneous purification and immobilization of D-amino acid oxidase from *Trigonopsis variabilis* cell lysate adsorbed on phenyl sepharose⁵⁹. The technique can have future potentials especially in the downstream processing and immobilization of enzyme/proteins obtained by recombinant DNA technology.

Techniques for the adhesion of whole cells on polymeric surfaces are also currently gaining considerable importance. The major advantage for the cells immobilized through adhesion is reduction or elimination of the mass transfer problems associated with the commonly used gel entrapment method. The technique of immobilization usually being followed is the microbial colonization by recycling of the cell suspension along

with nutrients, such that a biofilm is gradually formed. This often results in the immobilization of cells in a viable form for use in heterogeneous fermentations⁶⁵. Useful techniques have been developed also for the immobilization of nonviable cells to be used as an enzyme source for simple chemical conversions. Notable among them include treating the cells or the support with trivalent metal ions like Al^{3+} or Fe^{3+} or charged colloidal particles⁶⁶, and use of polycationic polymers like chitosan⁶⁷. Novel techniques have been developed to adhere cells strongly on a variety of polymeric surfaces including glass⁶⁸, cotton cloth⁶⁹, cotton threads¹⁹, and other synthetic and inorganic surfaces using polyethylenimine⁷⁰. This technique has also been recently used for the simultaneous filtration and immobilization of cells from a flowing suspension, thus integrating downstream processing with bioprocessing⁷¹. This technique may have future potentials for the immobilization of cells for food applications. Polyethylenimine is nontoxic and the United States of America Food and Drug Administration has permitted its use as a direct food additive under the Food Drug and Cosmetic Act⁵⁴.

Immobilized enzymes in organic solvents

In recent years, much research has centered on the conduct of enzyme reactions in organic solvents. It is now well established that hydrolytic enzymes can catalyse esterification and transesterification reactions in monophasic organic solvents and in water-organic biphasic systems. Conventional immobilized enzymes are principally used to facilitate catalysts recovery. Even though enzymes by themselves are insoluble in organic solvents, in addition to others, the prime importance to the use of enzymes in organic media is the necessity to avoid enzyme deactivation or denaturation. A number of enzymes which are used in organic solvents have been immobilized using a variety of techniques with a view to stabilizing them⁷². In systems containing enzymes immobilized on solid supports and working in organic media, the support has a significant influence on the total enzyme activity, and can also displace the reaction equilibrium (hydrolysis towards synthesis) due to the interaction of the support with the water molecules. Thus the choice of a suitable support material, proper water content, and the selection of the organic solvents are crucial for the use of immobilized enzymes in organic media^{73,74}. Another approach that has been explored is by chemical cross-linking of enzyme crystals, thereby stabilizing the crystalline lattice and its constituent enzyme molecules, which result in forming highly concentrated immobilized enzyme particles that can be lyophilized and stored indefinitely at room temperature. Cross-linked enzyme crystals retain catalytic activity in harsh conditions, including temperature and pH extremes, exogenous proteases, and

exposure to organic or aqueous solvents^{75,76}. Lyophilized cross-linked enzyme crystals can be reconstituted easily in these solvents as active monodisperse suspensions. Cross-linked enzyme crystals have shown promise in a variety of applications like the synthesis of aspartame, using thermolysin⁷⁶, and for the resolution of chiral esters⁷⁷. The techniques of immobilization can also be extended to obtain organic solvent-soluble enzymes by treating them with hydrophobic molecules like the lipids⁷⁸.

Immobilization of enzymes for the fabrication of biosensors

Most of the techniques described above have been used for the immobilization of biocatalyst for biosensor applications⁷⁹. The choice of the support and the technique for the preparation of membranes has been dictated by the low diffusional resistance of the membrane coupled with its ability to incorporate optimal amount of enzyme per unit area. In this respect, stable membranes have been prepared by binding glucose oxidase to cheese cloth in the fabrication of a glucose biosensor^{80,81}. Enzymes entrapped inside the reversed micelle have also shown promise in the fabrication of biosensors⁸². Cross-linked enzyme crystals (CLCs) described above provide their own supports and so achieve enzyme concentration close to the theoretical packing limit in excess of even highly concentrated enzyme solutions. In view of this, CLCs are particularly attractive in biosensor applications where the largest possible signal per unit volume is often critical⁷⁵.

Sensors based on small transducer or thinner enzyme immobilized membranes (miniature biosensors) are also emerging. The development of molecular devices incorporating a sophisticated and highly organized biological information processing function, is a long-term goal of bioelectronics. For this purpose, it is necessary in the future to develop suitable methods for microimmobilizing the proteins/enzymes into an organized array/pattern, as well as designing molecular structures capable of performing the required function. A typical example is the microimmobilization of proteins into organized patterns on a silicone wafer based on a specific binding reaction between streptavidin and biotin combined with photolithography techniques⁸³. Immobilized enzymes have also been used for various other analytical purposes. A recent development has been in obtaining a stable dry immobilized enzyme, like acetylcholinesterase, on polystyrene microtitration plates for mass screening of its inhibitors in water and biological fluids⁸⁴.

Immobilized multienzymes and enzyme-cell co-immobilizates

A common feature of metabolic pathways in intermediary metabolism is that the product of one enzyme in sequence

is the substrate for the next and so forth. One of the advantages of such an arrangement is that a favourable high local concentration of intermediates in the micro-environment of an enzyme system can be created. Such an approach perhaps will be useful in the development of enzymatic processes requiring multiple enzymes. The enzymes in question can be simultaneously bound to the same support or could be simultaneously immobilized through entrapment^{1,34}. Kinetic advantages of this system, such as reduction in the lag and significant enhancement in the final product, over mixed soluble enzymes or immobilized and then mixed systems have been demonstrated^{1,34,85}.

Often a cell may not contain all the necessary enzyme compliments for carrying out a required process. Thus *Saccharomyces cerevisiae*, a potential ethanol fermentor, cannot utilize lactose or starch since it lacks the enzyme β -galactosidase and amylase, respectively. There is considerable interest in genetically modifying such cells for the expression of the deficient enzymes. The success will depend on the plasmid stability, and quite often on the ability of these cells to secrete these enzymes for their action on the raw materials present in the growth medium. Recently, genetically engineered cells have been obtained which can not only produce the deficient enzyme but can also transport it to the periplasmic place and naturally immobilize it⁸⁶. Another more practical approach is to immobilize the deficient enzyme directly on the cell wall. This approach which is termed as enzyme-cell co-immobilization permits the tailoring of a whole cell for specific complex chemical conversion by combining the biochemical potential of both the cell and the additional enzyme from another source⁸⁷. A few techniques developed in our laboratory include the binding of the glycoprotein glucose oxidase onto the microbial cell walls, using Con A^{85,88}. Enzymes can also be immobilized on the microbial cell surfaces coated with poly-ethylenimine through adsorption followed by cross-linking⁸⁹. The different techniques for the preparation of enzyme-cell co-immobilization, and their applications have been reviewed⁸⁷.

Immobilized enzymes for the regeneration of cofactors

Enzyme technology has dealt mainly with simple reactions which do not require coenzymes. One of the major challenges for enzyme technologists is in the application of cofactor-dependent enzymes. Over 25% of the known enzymes require cofactors like NAD/NADH, ATP/ADP, etc. which, unlike the enzymes, undergo stoichiometric changes during a biochemical reaction. The living cells have circumvented this problem either by introducing certain cofactor-recycling enzyme systems in their metabolic pathways or through their recycling via

the electron transport system in the case of aerobic metabolism. *In vitro* applications of such enzymes will largely depend on developing efficient *in vitro* cofactor recycling systems. These can be obtained by co-immobilizing another enzyme so that it can recycle the cofactors, e.g. alcohol dehydrogenase in the presence of ethanol can be used for the recycling of NAD to NADH in a bioprocess catalysed by a NADH-dependent enzyme. Choice of the coupling enzyme for such application has been made based on its ability to use an economical substrate like ethanol or formate (formate dehydrogenase) as well as one which results preferably in a volatile byproduct like CO₂ or acetaldehyde so as to minimize the downstream processing problems^{1,3,32,33}. In addition to the recycling of the cofactor, other important criteria for development of cofactor-dependent biochemical process is also the retention of cofactors. Currently methods are available for the covalent binding of the cofactors along with the enzymes on polymeric supports or directly on enzymes or on soluble high molecular weight polymers like dextran or polyethylene glycol^{32,33}. Membrane reactors containing continuous cofactor regenerating systems have been used in the large-scale production of sugar derivatives, and optically active α -hydroxy acids and α -amino acids⁹⁰. Studies from our laboratory have shown that such cofactor regenerating system can be obtained by immobilization of permeabilized yeast cells as a source of alcohol dehydrogenase^{26,91}. Multienzymes and cofactors immobilized within microcapsules have been used for the multi-step conversion of lipophilic and lipophobic substrates⁹². A number of approaches have also been investigated for the regeneration of ATP. One of these viable processes makes use of acetate kinase which can phosphorylate ADP using acetyl phosphate as the phosphate donor^{32,33}.

Applications of immobilized enzymes

The first industrial use of an immobilized enzyme is amino acid acylase by Tanabe Seiyaku Company, Japan, for the resolution of racemic mixtures of chemically synthesized amino acids. Amino acid acylase catalyses the deacetylation of the L form of the *N*-acetyl amino acids leaving unaltered the *N*-acetyl-D amino acid, that can be easily separated, racemized and recycled. Some of the immobilized preparations used for this purpose include enzyme immobilized by ionic binding to DEAE-sephadex and the enzyme entrapped as microdroplets of its aqueous solution into fibres of cellulose triacetate by means of fibre wet spinning developed by Snam Progetti. Rohm GmbH have immobilized this enzyme on macroporous beads made of flexiglass-like material^{93,94}.

By far, the most important application of immobilized enzymes in industry is for the conversion of glucose syrups to high fructose syrups by the enzyme glucose

isomerase⁹⁵. Some of the commercial preparations have been listed in Table 2. It is evident that most of the commercial preparations use either the adsorption or the cross-linking technique. Application of glucose isomerase technology has gained considerable importance, especially in nontropical countries that have abundant starch raw material. Unlike these countries, in tropical countries like India, where sugarcane cultivation is abundant, the high fructose syrups can be obtained by a simpler process of hydrolysis of sucrose using invertase. Compared to sucrose, invert sugar has a higher humectancy, higher solubility and osmotic pressure. Historically, invertase is perhaps the first reported enzyme in an immobilized form⁹⁶. A large number of immobilized invertase systems have been patented⁹⁷. The possible use of whole cells of yeast as a source of invertase was demonstrated by D'Souza and Nadkarni as early as 1978 (ref. 98). A systematic study has been carried out in our laboratory for the preparation of invert sugar using immobilized invertase or the whole cells of yeast^{17-19,38,45,55,68,69,71,98}. These comprehensive studies carried out on various aspects in our laboratory of utilizing immobilized whole-yeast have resulted in an industrial process for the production of invert sugar.

L-aspartic acid is widely used in medicines and as a food additive. The enzyme aspartase catalyses a one-step stereospecific addition of ammonia to the double bond of fumaric acid. The enzymes have been immobilized using the whole cells of *Escherichia coli*. This is considered as the first industrial application of an immobilized micro-

bial cell. The initial process made use of polyacrylamide entrapment which was later substituted with the carragenan treated with glutaraldehyde and hexamethylenediamine. Kyowa Hakko Kogyo Co. uses Duolite A7, a phenolformaldehyde resin, for adsorbing aspartase used in their continuous process⁹⁹. Other firms include Mitsubishi Petrochemical Co.¹⁰⁰ and Purification Engineering Inc¹⁰¹. Some of the firms, specially in Japan like Tanabe Seiyaku and Kyowa Hakko, have used the immobilized fumarase for the production of malic acid (for pharmaceutical use)⁹⁴. These processes make use of immobilized nonviable cells of *Brevibacterium ammoniagenes* or *B. flavus* as a source of fumarase. Malic acid is becoming of greater market interest as food acidulant in competition with citric acid. Studies from our laboratory have shown the possibility of using immobilized mitochondria as a source of fumarase⁶.

One of the major applications of immobilized biocatalysts in dairy industry is in the preparation of lactose-hydrolysed milk and whey, using β -galactosidase. A large population of lactose intolerants can consume lactose-hydrolysed milk. This is of great significance in a country like India where lactose intolerance is quite prevalent¹⁰². Lactose hydrolysis also enhances the sweetness and solubility of the sugars, and can find future potentials in preparation of a variety of dairy products. Lactose-hydrolysed whey may be used as a component of whey-based beverages, leavening agents, feed stuffs, or may be fermented to produce ethanol and yeast, thus converting an inexpensive byproduct into a highly

Table 2. Commercial immobilized glucose isomerase preparations

Company	Enzyme source	Immobilization procedure
Clinton Corn Processing	<i>Streptomyces ribigenosus</i> , <i>S. wedmorensis</i>	Enzyme adsorbed on ion-exchanger
Novo Industry	<i>Bacillus coagulans</i>	Enzyme mixed with inorganic diluent and formed into solid spheres or cell lysate cross-linked with glutaraldehyde
Miles Laboratory	<i>Streptomyces olivaceus</i>	Glutaraldehyde cross-linked whole cells
Miles Kali Chemie	<i>Streptomyces</i> sp.	Heat-fixed cells cross-linked with glutaraldehyde
Snamprogetti	<i>Streptomyces</i> sp.	Enzyme entrapped in cellulose triacetate fibres
Gist Brocades	<i>Actinoplanes missouriensis</i>	Cells entrapped in gelatine, and cross-linked by glutaraldehyde
Mi-Car Int.	<i>Streptomyces olivaceus</i>	Glutaraldehyde cross-linked whole cell granules
ICI Americas Inc.	<i>Arthrobacter</i> sp.	Flocculated cells extruded and dried as cylindrical pellets
CPC Int. Inc.	<i>Streptomyces olivochromogenes</i>	Adsorption on alumina/other ceramics/ ion-exchange resin
Corning Glass Works	<i>Streptomyces olivochromogenes</i>	Enzyme adsorbed on controlled pore alumina
Sanmatsu	<i>Streptomyces</i> sp.	Enzyme adsorbed on anion exchange resin
Denki Kagaku-Nagase	<i>Streptomyces phaeochromogenes</i>	Cells entrapped in polymer and granulated

nutritious, good quality food ingredient⁹⁹. The first company to commercially hydrolyse lactose in milk by immobilized lactase was Centrale del Latte of Milan, Italy, utilizing the Snamprogetti technology. The process makes use of a neutral lactase from yeast entrapped in synthetic fibres¹⁰³. Specialist Dairy Ingredients, a joint venture between the Milk Marketing Board of England and Wales and Corning, had set up an immobilized β -galactosidase plant in North Wales for the production of lactose-hydrolysed whey. Unlike the milk, the acidic β -galactosidase of fungal origin has been used for this purpose³¹. Some of the commercial β -galactosidase systems have been summarized in Table 3. An immobilized preparation obtained by cross-linking β -galactosidase in hen egg white (lyophilized dry powder) has been used in our laboratory for the hydrolysis of lactose⁴⁷. A major problem in the large-scale continuous processing of milk using immobilized enzyme is the microbial contamination which has necessitated the introduction of intermittent sanitation steps. A co-immobilizate obtained by binding of glucose oxidase on the microbial cell wall using Con A has been used to minimize the bacterial contamination during the continuous hydrolysis of lactose by the initiation of the natural lacto-peroxidase system in milk⁸⁸. A novel technique for the removal of lactose by heterogeneous fermentation of the milk using immobilized viable cells of *K. fragilis* has also been developed¹⁰.

One of the major applications of immobilized enzymes in pharmaceutical industry is the production of 6-aminopenicillanic acid (6-APA) by the deacylation of the side chain in either penicillin G or V, using penicillin acylase (penicillin amidase)¹⁰⁴. More than 50% of 6-APA produced today is enzymatically using the immobilized route. One of the major reasons for its success is in obtaining a purer product, thereby minimizing the purification costs. The first setting up of industrial process for the production of 6-APA was in 1970s

simultaneously by Squibb (USA), Astra (Sweden) and Riga Biochemical Plant (USSR). Currently, most of the pharmaceutical giants make use of this technology. A number of immobilized systems have been patented or commercially produced for penicillin acylase which make use of a variety of techniques either using the isolated enzyme or the whole cells^{100,105,106}. This is also one of the major applications of the immobilized enzyme technology in India. Similar approach has also been used for the production of 7-aminodeacetoxy-cephalosporanic acid, an intermediate in the production of semisynthetic cephalosporins.

Immobilized oxidoreductases are gaining considerable importance in biotechnology to carry out synthetic transformations. Of particular significance in this regard are oxidoreductase-mediated asymmetric synthesis of amino acids, steroids and other pharmaceuticals and a host of speciality chemicals. They play a major role in clinical diagnosis and other analytical applications like the biosensors. Future applications for oxidoreductases can be in areas as diverse as polymer synthesis, pollution control, and oxygenation of hydrocarbons¹⁰⁷. Immobilized glucose oxidase can find application in the production of gluconic acid, removal of oxygen from beverages, and in the removal of glucose from eggs prior to dehydration in order to prevent Maillard reaction. Studies carried out in this direction in our laboratory have shown that glucose can be removed from egg, using glucose oxidase and catalase which are co-immobilized either on polycationic cotton cloth⁵⁷ or in hen egg white foam matrix⁵⁰. Alternatively, glucose can also be removed by rapid heterogeneous fermentation of egg melange, using immobilized yeast¹⁰⁸. Immobilized *D*-amino acid oxidase has been investigated for the production of keto acid analogues of the amino acids, which find application in the management of chronic uremia. Keto acids can be obtained using either L- or D-amino acid oxidases. The use

Table 3. Commercial immobilized lactase preparations

Company	Enzyme source	Immobilization procedure
Gist Brocades	<i>Saccharomyces lactis</i>	Enzyme entrapped in cellulose triacetate fibres
Snamprogetti	<i>Kluyveromyces lactis</i>	Enzyme entrapped in cellulose triacetate fibres
Corning Glass Works	<i>Aspergillus niger</i>	Enzyme covalently bound to silica beads
Valio Laboratory	<i>A. niger</i>	Enzyme adsorbed/cross-linked to phenol formaldehyde resin
Sturge	<i>A. niger</i>	Enzyme covalently bound to silanized Mn-Zn ferrite
Rohm GmbH	<i>A. oryzae</i>	Enzyme covalently bound to macroporous plexiglass beads
Sumitomo	<i>A. oryzae</i>	Enzyme covalently bound to macroporous amphoteric ion exchange resin
Amerace Corp.	<i>A. oryzae</i>	Enzyme covalently bound to microporous PVC-silica sheet

of D-amino acid oxidase has the advantage of simultaneous separation of natural L-isomer from DL-recemates along with the conversion of D-isomer to the corresponding keto acid which can then be transaminated in the body to give the L-amino acid. Of the several microorganisms screened, the triangular yeast *T. variabilis* was found to be the most potent source of D-amino acid oxidase with the ability to deaminate most of the D-amino acids¹⁰⁹. The permeabilized cells entrapped either in radiation polymerized acrylamide²⁴ Ca-alginate²³ or gelatin²⁵ have shown promise in the preparation of α -keto acids. Another interesting enzyme that can be used profitably in immobilized form is catalase for the destruction of hydrogen peroxide employed in the cold sterilization of milk. A few reports are available on its immobilization using yeast cells^{11,22}.

Lipase catalyses a series of different reactions. Although they were designed by nature to cleave the ester bonds of triacylglycerols (hydrolysis), lipase are also able to catalyse the reverse reaction under microaqueous conditions, viz. formation of ester bonds between alcohol and carboxylic acid moieties. These two basic processes can be combined in a sequential fashion to give rise to a set of reactions generally termed as interesterification. Immobilized lipases have been investigated for both these processes. Lipases possess a variety of industrial potentials starting from use in detergents; leather treatment controlled hydrolysis of milk fat for acceleration of cheese ripening; hydrolysis, glycerolysis and alcoholysis of bulk fats and oils; production of optically pure compounds, flavours, etc. Lipases are spontaneously soluble in aqueous phase but their natural substrates (lipids) are not. Although use of proper organic solvents as an emulsifier helps in overcoming the problem of intimate contact between the substrate and enzyme, the practical use of lipases in such pseudohomogeneous reactions poses technological difficulties. Varieties of approaches to solve these, using immobilized lipases, have recently been reviewed¹¹⁰.

Significant research has also been carried out on the immobilization and use of glucoamylase. This is an example of an immobilized enzyme that probably is not competitive with the free enzyme and hence has not found large-scale industrial application¹¹¹. This is mainly because soluble enzyme is cheap and has been used for over two decades in a very optimized process without technical problems. Immobilization has also not found to significantly enhance the thermostability of amylase¹¹¹. Immobilized renin or other proteases might allow for the continuous coagulation of milk for cheese manufacture¹¹². One of the major limitations in the use of enzymes which act on macromolecular substrates or particulate or colloidal substrates like starch or cellulose pectin or proteins has been the low retention of their realistic activities with natural substrates due to the steric hindrance. Efforts have been made to minimize these

problems by attaching enzymes through spacer arms¹¹³. In this direction, application of tris (hydroxymethyl) phosphine as a coupling agent¹¹⁴ may have future potentials for the immobilization of enzymes which act on macromolecular substrates. Other problem, when particulate materials are used as the substrates for an enzyme, is difficulty in the separation of the immobilized enzyme from the final mixture. Efforts have been made in this direction to magnetize the biocatalyst either by directly binding the enzyme on magnetic materials (magnetite or stainless steel powder) or by co-entrapping magnetic material so that they can be recovered using an external magnet^{98,115}. Magnetized biocatalysts also help in the fabrication of magnetofluidized bed reactor¹¹⁶.

A variety of biologically active peptides are gaining importance in various fields including in pharmaceutical industries and in food industries as sweeteners, flavourings, antioxidants and nutritional supplements. Proteases have emerged over the last two decades as powerful catalysts for the synthesis and modification of peptides. The field of immobilized proteases may have a future role in this area^{117,118}. One of the important large scale applications will be in the synthesis of peptide sweetener using immobilized enzymes like the thermolysin¹¹⁹. Proteolytic enzymes, such as subtilisin, α -chymotrypsin, papain, ficin or bromelain, which have been immobilized by covalent binding, adsorption or cross-linking to polymeric supports are used (Bayer AG) to resolve A N-acyl-DL-phenylglycine ester racemate, yielding N-acyl-D-esters or N-acyl-D-amides and N-acyl-L-acids¹⁰⁰. Immobilized aminopeptidases have been used to separate DL-phenylglycinamide racemates¹⁰⁰. SNAM-Progetti SpA-UK have used the immobilized hydroxypyrimidine hydrolase to prepare D-carmamyl amino acids and the corresponding D-amino acids from various substituted hydantoins¹⁰⁰.

Conclusion

Achieving technical success is seldom sufficient, a lot of other factors have to be fulfilled before industrial success is assured. The immobilized glucose isomerase was adopted only when the market was ready and the technical problems were solved. The same could be said about penicillin acylase. Immobilized enzymes have to be very heat stable, work in a reliable optimized process system, and the products have to be cheap. If there is a cheap source of soluble enzyme in the market or if other processes are well established, it is doubtful whether the newly developed immobilized enzyme process can survive as in the case of glucoamylase. The cost considerations must take into account such factors as cell production, matrix type and form, immobilization conditions, reactor design, and product purification. Economic and advantageous processes can be devised but

their general industrial adoption will need time and encouragement. Past experience clearly suggests that we must learn to work with unpurified enzymes like the whole cells or the cell homogenates. A significant future is expected for development of processes using multi-enzyme systems, specially those involving cofactor regeneration for the production of high value compounds. The initial industrial success in this direction is very encouraging⁹⁰ and may hold a vast potential for the future. The immobilized enzyme technology may also help in the future for integrating bioprocess with downstream processing with an effort to increase the productivity while minimizing product recovery cost. Immobilized enzyme technology may also be useful in nonaqueous enzymology, not only in terms of stabilization of the biocatalysts but also in the development of continuous bioreactors. One of the rapidly emerging areas in the field of sensors^{120,121} is the development of biosensors for industrial process control wherein immobilized enzyme technology will play a pivotal role. Stabilization of enzymes (heat) will be crucial for some of the applications. On the other hand, cold active enzymes also may gain potentials in certain biocoversions. Immobilized viable cells, which are discussed elsewhere, will also have future potentials, specially in revolutionizing the fermentation processes⁶. It is most likely that the future developments will come from within the industry itself and the role of academic researchers will probably remain to some extent as the laying of the theoretical foundations for this work and development of new approaches. Thus, there are interesting possibilities within the field of immobilized enzymes and it is imminent that in the future many applications will be replaced by immobilized systems and many more new systems will become technically as well as commercially feasible.

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