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Industrial Applications of Free and Immobilised Enzymes

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The methods for the preparation and purification of immobilised enzymes are briefly reviewed and examples are given of the utilisation in industry (mainly not in the food industry) of enzymes such as amylases, proteinases, cellulases, glucose oxidase, glucose isomerase, lipase, and invertase. Various procedures for the immobilisation of enzymes are described. The principal aspects of catalysis by immobilised enzymes are examined, particularly from the standpoint of the stability of the enzymes, reaction kinetics, and the influence of diffusion. Certain examples of the employment of enzymes incorporated in fibres are presented (hydrolysis of penicillin, cleavage of milk lactose, and isomerisation of glucose). The bibliography includes 65 references.

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In recent years, enzymatic reactions have found extensive applications in chemical and pharmaceutical industries. The present review is concerned with examples of the industrial (in the main not in the manufacture of food products) employment of enzymatic reactions, achieved both with the aid of enzymes in solution and with the aid of immobilised enzymes, which is particularly important in connection with their increasing value as industrial catalysts†.

I. ENZYMES IN SOLUTION

For technical and economic reasons, microbiological systems are the most important source of enzymes used in industry. The relevant micro-organisms are grown in surface or bulk-phase cultures. Aqueous extracts containing enzymes are then subjected to further treatment by various methods for the purpose of concentration, partial purification, stabilisation, and conversion of the enzyme material into the finished commercial product.

Extracellular enzymes are normally used, which makes it possible to isolate them relatively simply, and does not require the disruption of cells, although nowadays this can be easily done with the aid of modern homogenisers [1]. The scheme for the isolation of enzymes illustrated in Fig. 1 is usually employed [2].

When necessary, preservatives are added, for example, toluene, organic acids, phenolic compounds, quaternary ammonium salts, or sodium fluoride. Enzymes are precipitated by adding to the purified solutions or liquid concentrates, under strictly controlled conditions, precipitants such as organic solvents (acetone, ethanol), inorganic salts (sodium or ammonium sulphate, disodium hydrogen phosphate), or macromolecular compounds [poly(ethyleneglycol)]. The precipitated enzyme, isolated by filtration or centrifugation, is dried at atmospheric pressure or *in vacuo* at a low temperature, and the solid concentrate is ground to the required particle size. The concentrate obtained contains, apart from the required enzyme, other proteins and also moisture, carbohydrates, and inorganic salts [3]. Since the cost of the preparation is usually more important for commercial purposes than high purity, it is

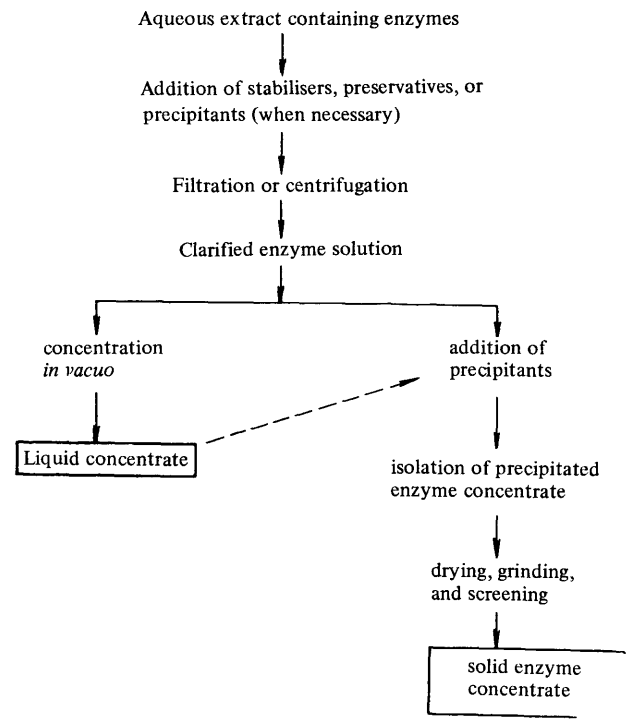


Figure 1. Scheme for the isolation of enzymes.

† At the present time, this problem is dealt with most completely in the collected volume edited by I.V.Berezin, "Immobilizovannye Fermenty" (Immobilised Enzymes), Izd. Moskov. Gos. Univ., Moscow, 1975 (a). The kinetic and thermodynamic aspects of catalysis by immobilised enzymes are described by I.V.Berezin, A.M.Klibanov, and K.Marinek, Uspekhi Khim., 44, 17 (1975) [Russian Chemical Reviews, No.1 (1975)].

not subjected to further purification. However, in certain cases the presence of contaminating enzymes or other substances in the preparation is undesirable if they can have an unfavourable influence on products or processes. Thus the enzymes used for special analytical purposes, certain pharmaceutical processes, and research must be carefully purified[2]. In the manufacture of high-purity enzymes, it is necessary to employ special methods, for example fractional precipitation, differential adsorption and elution[4], chromatography[5], electrophoresis[6], dialysis[7], crystallisation, and lyophilisation.

Table 1. Some industrial enzymes and their microbiological sources[8].

Source	Enzyme	Microorganism
Mould fungi	amylases	*Aspergillus oryzae*
	glucosidases	*Aspergillus flavus*
	proteinases	*Aspergillus niger*
	pectinases	*Aspergillus niger*
	glucoseoxidase	*Penicillium notatum*
	catalase	*Aspergillus niger*
Bacteria	amylases	
	proteinases	*Bacillus subtilis*
	penicillinase	
Yeast	invertase	*Saccharomyces cerevisiae*
	lactase	*Saccharomyces fragilis*

Table 1 lists the commercial enzymes manufactured mainly from various strains of mould fungi, bacteria, and yeast[8]. The most important examples of such enzymes are amylase, certain other carbohydrases, proteinases, glucose oxidase, and pectinase. Methods involving bulk-phase (immersed) cultures are used for their manufacture.

In writing the review of the applications of enzymatic processes in solution in "non-food" industry, the author found that, apart from the manufacture of starch and detergent powders (which will be considered in greater detail in view of their commercial importance), enzymes are used in textile, paper, leather, pharmaceutical, and other branches of industry.

In the textile industry, bacterial amylases are used both for the preparation of modified starch adhesives and for the removal of adhesives from fabrics. Preparations of bacterial amylases, which retain their activity at much higher temperatures than amylases from moulds, have to a larger extent replaced malt and pancreatin. In the paper industry, certain bacterial amylases are used to modify starch for coating paper. Proteinases from *Bacillus subtilis*[9] are employed in the manufacture of tanned leather to remove hair and largely for the softening of leather after tanning by the proteolytic decomposition of gelatin formed from collagen. Such treatment enhances the protective properties and improves the structure of leather[10]. Proteinase preparations should also find future applications as depilatory agents (preparations for the removal of hair) in cosmetics[11].

Mould or pancreatic amylases, proteinases, and lipases and mould cellulases are used as digestion-promoting agents. Steptokinase, trypsin, and other proteinases have found applications in the treatment of wounds and as anti-inflammatory or fibrinolytic agents. Among hydrolytic enzymes, invertase (catalysing the hydrolysis of sucrose to glucose and fructose), used in the preparation of highly concentrated invert sugar syrups and for special purposes, for example for the preparation of soft kinds of chocolate, is of considerable industrial interest. In contrast to the sucrose obtained by acid hydrolysis, enzymatically inverted sucrose does not have an undesirable taste and colour caused by decomposition products (for example, furfural). Of the two possible enzymes hydrolysing sucrose, only β-fructofuranosidase is used. In this case, the centre of attack is the fructose end of the sucrose molecule. This intracellular enzyme, obtained from yeast (usually from *Saccharomyces cerevisiae*), retains its catalytic properties at temperatures of 65–70°C and has the maximum activity at pH 4.5. The purification of invertase by the removal of the majority of impurities increases the sensitivity of the enzyme to physical damage[12]. Unpurified preparations containing small amounts of other enzymes (catalase, hexokinase) are therefore usually employed.

Amylases are extracellular enzymes hydrolysing starch molecules to dextrins and a set of compounds with lower molecular weights consisting of glucose units. They are divided into two classes: endoamylases and exoamylases. Endoamylases act at random on the 1 → 4 linkages in starch, liberating certain reducing groups and forming dextrins with various chain lengths. The 1 → 6 linkages,, which are the branching points in amylopectin and glycogen, are not attacked under these conditions. Exoamylases attack polysaccharides only from the non-reducible terminal groups. They can either cleave each linkage with formation of glucose only (amyloglucosidases) or they can rupture alternate bonds with formation of maltose (β-amylases, which are given this name because the maltose obtained has the β-configuration).

Amyloglucosidases from *Aspergillus niger* are capable of hydrolysing both 1 → 4 and 1 → 6 glucoside linkages in oligosaccharides, but in the latter case the rate of reaction is much lower[13].

Table 2. Microbial amylases*.

Properties	Bacteria		Mould fungi		Other sources	
	solubilisation	saccharification	*Rhizopus*	*A. oryzae* or *A. niger*	*Endomycopsis*	*Oospora*
Thermal stability, °C	65—90	55—70	50—65	55—70	35	50—70
pH stability	4.8—10.6	4.0—7.8	5.4—7.0	4.7—9.5	6.0—7.5	6.0—10.5
Optimum pH	5.4—6.0	4.8—5.2	3.6	4.9—5.2	5.4	5.6
Activity/mg	1800	1190	475	980	760	970
Adsorption on starch	++	—	+	+	+	+
Stabilisation by Ca^{2+} ions	+	—	—	+	?	+
Effect on starch: maximum degree of hydrolysis, % principal product	35 dextrin, maltose	70 glucose, maltose, maltotriose	48 maltose	48 maltose	90 glucose	37 dextrin, maltose
Effect on maltose	—	—	—	—	+	—
Effect on phenylmaltose	—	+	+	+	+	—

*According to data of Dr. J. Fukumoto.

Microbiological α-amylases are obtained either from moulds (mainly *Aspergillus orizae*) or from bacteria (mainly from *Bacillus subtilis*). All the known sources of amylases are listed in Table 2.[13] Commercial α-amylases, obtained from various bacteria and moulds, are characterised by different temperature and pH optima, different stabilities, and different extents to which they decompose starch. In view of its high thermal stability, bacterial α-amylase from thermophilic strains of

Bacillus subtilis can be used to solubilise starch. For this purpose, it is added to the doughy mass of the non-gelatinised starch grains (preferably from potatoes or sweet potatoes), which proceed to the gelatinisation stage and are mixed in the reactor[14]. Industrial processes using glucoamylase from surface *Rhizopus* cultures have been devloped in Japan[15]. Japanese investigators have also suggested that bulk-phase cultures of *Endomyces* strains be used for the preparation of glucoamylase[16]. Processes for the preparation of glucoamylase from *Aspergillus* in immersed cultures have been developed in the USA. This method gives higher yields. The enzyme from *Aspergillus* has excellent properties—a high temperature optimum, a higher stability, and a wider pH range in which it retains its activity.

All commercial glucoamylase preparations must be completely freed from transglucosidase, which is responsible for the formation of small amounts of oligosaccharide impurities, particular isomaltose, which hinder the manufacture of glucose. Several methods have been proposed whereby it is also possible to reduce the content of transglucosidase in glucoamylase preparations: (a) special cultures have been developed, which do not produce transglucosidase; (b) the unpurified enzyme is treated with agents which remove transglucosidase [these include clay minerals[17], sulphonated alcohols[18], $Mg(OH)_2$,[19] and acids[20]]; ion-exchange resins[21] etc. are also employed.

Amylolytic enzymes are used mainly in the manufacture of starch syrups (concentrated aqueous solutions of starch hydrolysates) and crystalline glucose (dextrose). The employment of suitable combinations of α- and β-amylases, amyloglucosidases, isoamylases, or maltases makes it possible to obtain an infinite variety of starch hydrolysis products and hence syrups with any desired properties, for example with a maltose content exceeding 80%.[22]

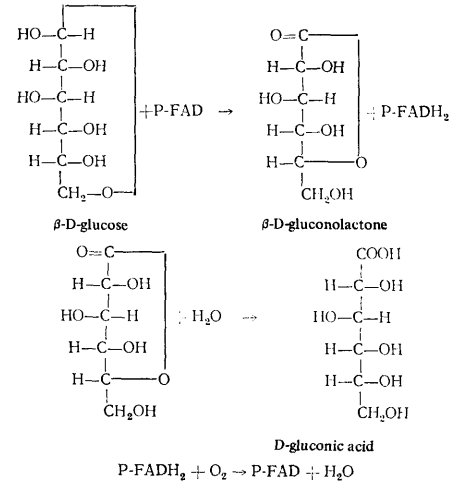
Another interesting enzyme catalysing the conversion of glucose into fructose, is glucose isomerase obtained from certain *Streptomyces* strains and bacteria. Depending on the temperature, the degree of conversion can reach approximately 50%. Since fructose is sweeter than glucose, sweeter syrups can be obtained in this way[14].

Another class of hydrolytic enzymes which are of industrial importance consists of proteinases. They can be of animal origin, for example trypsin, which is obtained from bovine pancreas as a side product after the extraction of insulin and has extensive pharmaceutical applications. Other proteinases are of plant origin, for example ficin, papain, or bromelain, and are mainly used as agents for tenderising meat. Proteinases can be obtained by microbiological methods, with the aid of both mould fungi and bacteria; several species of bacteria are normally employed—such as *Bacillus subtilis*, *Aspergillus niger*, and *Aspergillus oryzae*.

Microbiological proteinases are divided into three groups: acid, neutral, and alkaline[9]. Acid proteinases, which originate mainly from moulds (*Aspergillus*, *Rhizopus*) and are most active in pH range 2–5, are insensitive to the action of compounds with thiol groups, substances forming chelates with metals, and heavy metals, and catalyse the hydrolysis of many peptide linkages. Neutral proteinases are zinc-containing metalloenzymes which are therefore deactivated by chelating agents. The optimum conditions for these enzymes are pH 7–8 and a temperature in the range 45–50°. They do not exhibit esterase activity and mainly cleave peptide linkages of the following type: His-Leu, Ala-Leu, Tyr-Leu, Gly-Phe, and Phe-Phe.

Alkaline proteinases constitute the most important group, since they are widely used in combination with detergents for the removal of stains of protein origin, for example, blood, casein, etc. The most familiar enzymes of this group are subtilisins[9], for example subtilisin *Novo* or subtilisin *Carlsberg* from various *Bacilli* or strains of *Bacillus subtilis*. Alkaline proteinases are active over a wide pH range, from 6 to 11, the maximum activity being shown at pH 9.5–10.5. They exhibit a high esterase activity and cleave peptide linkages of several types.

Proteinases are also used in small amounts in dry cleaning, for the isolation of silver from used photographic film, and for the preparation of protein hydrolysates. Yet another enzyme, namely glucose oxidase, an FAD-dependent aerobic dehydrogenase, has found extensive industrial applications. It converts glucose into gluconic acid with simultaneous formation of equimolar amounts of hydrogen peroxide via the following reactions:



$$P\text{-}FADH_2 + O_2 \rightarrow P\text{-}FAD + H_2O$$

(Here P-FAD = glucose oxidase).

The microbiological source of this enzyme is *Penicillium notatum* and particularly *Aspergillus niger*. The intracellular enzyme from *A.niger* has an optimum in the pH range 4.0–6.5. The commercial enzyme contains catalase, which facilitates its industrial application. Glucose oxidase is used to remove oxygen from preserved non-alcoholic beverages, mayonnaise, and fruit juices, and also for the removal of glucose from eggs before drying.

Catalase, which decomposes hydrogen peroxide to water and oxygen, is used in the manufacture of cheese in order to decompose hydrogen peroxide residues after cold sterilisation, in the manufacture of foam rubber (foam plastics) and porous cement, and to accelerate the bleaching of fur and bird feathers.

Lipase, one of the hydrolytic enzymes, is contained in pancreatin and is produced by many micro-organisms. It is used commercially as an agent promoting digestion, in enzymatic dry cleaning apparatus, for the elimination of waste, and for the decomposition of fats to the fatty acids constituting the basis of ordinary soap[11].

Cellulases are new industrial carbohydrases. The cellulase complex is a mixture of extracellular enzymes (mainly from *Aspergillus niger* or *Trichoderma viridae*)[23] containing, firstly, an enzyme which acts non-specifically, hydrolysing highly oriented solid celluloses (C_1) and, secondly, a multicomponent fraction of $\beta(1 \rightarrow 4)$glucanases

(called C_X) both of the exo-type (these remove single glucosidic units from the non-reducible ends of the cellulose chain) and of the endo-type, which yield cellulodextrins, and, thirdly, β-glucodimerases, which act on glucose β-dimers, for example cellobiose. The employment of the cellulase complex from *Trichoderma viridae* made it possible to hydrolyse pretreated cellulose free from lignin to give unpurified extracts containing 8% of glucose [23,24]. Later investigations employing cedar sawdust yielded solutions containing more than 20% of glucose [25]. These extracts can be used to manufacture fermentation products or individual cell proteins. Other promising applications of cellulase may be its use for the removal of plant fibres from wool, or as agents promoting digestion, or for enzymatic dry cleaning.

II. IMMOBILISED ENZYMES

The advantages of immobilised enzymes as catalysts have been described in numerous reviews. Their use in chemical catalysis and automatic analysis is based on the possibility of their repeated use in combination with a high selectivity of the catalysis and a low cost of their preparation and purification; in many cases, the reaction volume can also be reduced. For research purposes, immobilised enzymes can be regarded as specific reagents readily removed from the system, which do not contaminate the products obtained. Furthermore, immobilisation makes it possible to increase the stability of enzymes. Artificially immobilised enzymes can be used, like model systems, to investigate the mechanism of the action of enzymes in biological membranes.

For biomedical purposes, enzymes can be used only in the immobilised form, in order to prevent the allergic response by the organism or undesirable interactions between the enzyme and individual components of the organic solutions being treated. Covalently bound enzymes have assumed increasing importance in the isolation of coenzymes and natural enzyme inhibitors by affinity chromatography from unpurified extracts or partly purified solutions.

These varied and attractive possibilities drew the attention of many investigators during the last few years to the investigation of immobilised enzymes. Various methods for the preparation of immobilised systems have been discovered and the activities of the enzymes obtained have been investigated. The theoretical aspects of the kinetics of the action of these enzyme systems are being vigorously studied together with the engineering problems of the construction of reactors using immobilised enzymes.

Immobilisation Methods

In the discussion of the problem of the utilisation of immobilised enzymes as industrial catalysts, we shall consider only relatively cheap carriers and simple methods for the attachment of enzymes.

Four main methods are used to prepare water-insoluble enzymes: (a) physical incorporation (i.e. incorporation into porous materials, whose cavities are bounded by walls impermeable to proteins but allowing the passage into the cavities of substances with low molecular weights). As an example of such carriers, one may quote polyacrylamide or starch gel, microcapsules, and fibrous matrices. (b) Covalent binding of enzymes to organic or inorganic carriers via the reactions of the functional groups of the proteins which are not responsible for catalytic activity. (c) The formation of covalent intermolecular bonds in the interaction of enzymes with the corresponding bifunctional reactants. (d) Adsorption on insoluble matrices (on ion-exchange resins or an inert support, for example as a result of hydrophobic interactions).

A combination of (c) and (d), i.e. adsorption of the protein on an appropriate carrier followed by the formation of intermolecular bonds, is being successfully used. Table 3 presents some of the most important examples of covalent binding.

We shall now consider the advantages and disadvantages of some of the immobilisation methods quoted above.

Table 3. Methods for the covalent binding of enzymes.

Activator	Carrier
Isothiocyanate	Sephadex
	Enzacryl AA
Azide	CM-cellulose
	Enzacryl AH
Carbodi-imide	CM-cellulose
	acrylamide - acrylic acid
Maleic anhydride	ethylene-maleic anhydride copolymer
Diazo-compounds	Enzacryl AA
	Polystyrene
	p-aminophenylalanine-leucine copolymer
Cyanogen halide	cellulose
	Sephadex
	polysaccharide
N-Ethyl-5-phenyl-isoxazolium-3-sulphonate	polyacrylic acid
	polyglutamic acid
	CM-cellulose
Trichloro-*s*-triazine	cellulose
	DEAE-cellulose
	DE-cellulose
Glutaraldehyde	AE-cellulose
	nylon
Silane	glass
Bisdiazobenzidine-2,2'-disulphonic acid	collodion

Physical incorporation in the gel. The inclusion of enzymes in gels such as three-dimensionally "cross-linked" polyacrylamide or starch has the advantage that it can be carried out under fairly mild conditions. Here the carrier has a minimal influence on the immobilised enzyme. The absence of covalent bonds between the enzyme and the matrix makes this method applicable, in principle, to any enzyme.

The relative inaccessibility of enzymes to substrates with large molecular weights, the appreciable influence of the diffusion of substrates and products, and the possible losses of the enzyme as a result of its elution from loose network gels must be regarded as disadvantages of the method [26]. An interesting variety of this immobilisation procedure involves enclosure in microcapsules [26] made of thin spherical semipermeable nylon or collodion membranes, which are used mainly for biomedical purposes.

Incorporation in fibrous structures. The method of incorporating enzymes in a fibrous structure developed in our laboratory belongs to the category of physical incorporation method. It involves the preparation of an emulsion by mixing an aqueous solution of the enzyme with a solution of the polymer in a solvent immiscible with water. The emulsion is then forced through thin draw plates into an immiscible liquid, which results in the coagulation of the polymer [28]; the protein is then included in the pores of the moist, coiled synthetic fibre. The pore size can be varied within certain limits

by altering the experimental conditions. Fig. 2 presents an electron microscope photograph of a transverse section through the fibre. This "trapping" technique is very simple, can be applied to any proteins, and makes it possible to achieve both the simultaneous incorporation of several different proteins in the matrix and the incorporation of large amounts of a single protein. The purity of the protein does not affect the "trapping" and the method can be used to incorporate individual organelles, cells, or spores. The enzymes incorporated into the matrix by this procedure are protected from the action of microbes and macromolecular substances produced by microbes, for example proteinases.

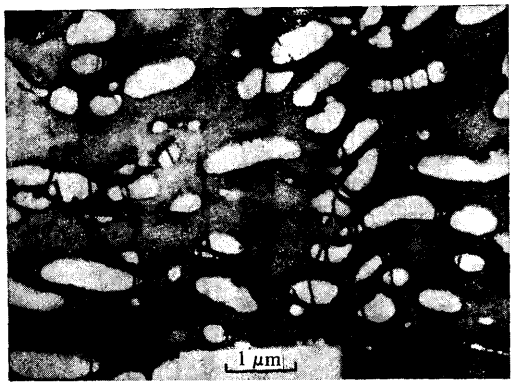


Figure 2. An electron microscope photograph of the transverse section through polymeric fibres.

Evidently in the future it will be possible to increase the stability of the incorporated enzyme by selecting as carriers polymers capable of interacting with the protein globule of the enzyme, stabilising the latter.

Covalent binding. The covalent binding of enzymes to a solid matrix is one of the most widely used methods which have been investigated in detail. Covalent binding has the advantage that the systems obtained are not broken down by altering the pH or the ionic strength and following the addition of the substrate. However, by altering the chemical structure of the enzyme, this method of binding can also alter its reactivity (either as a result of the blocking of the active enzyme centre by the binding reaction or as a result of the involvement in the reaction of the aminoacid residues which participate in the mechanism of enzyme action [26]). Mild conditions, moderate ionic strengths, pH 4–10, and a temperature in the range 0–35 °C should obtain during the fixation process. In the course of immobilisation, the active site of the enzyme must be protected by employing covalent blocking agents or by carrying out the reaction in the presence of a substrate or inhibitors. The carrier must be hydrophilic, its porosity must be high, and it is usually necessary to convert it into an active form for reaction with the enzyme. The particle size determines to a large extent the amount of bound enzyme per gram of carrier [29]. An increase of the surface area of the carrier increases the amount of bound enzyme per unit weight and an increase of the number of hydrophilic regions in the polymer also usually increases its binding capacity.

The most suitable centres for the binding of enzyme molecules to the matrix are the terminal amino-groups as well as the lateral amino groups in the lysine residues. The imidazole rings of histidine and the guanidine groups of arginine can participate in the binding process. The acid carboxy-groups of the glutamic and aspartic residues and the C-terminal groups of the proteins can also be effective. Another binding site may be the hydroxyphenyl group of the tyrosine residue, which readily interacts with diazonium reagents.

The immobilisation of enzymes by the formation of intermolecular bonds has been achieved successfully using bifunctional reagents containing two identical functional groups [30] as well as different groups or groups having different reactivities.

It is noteworthy that, although the method of covalent binding of enzymes to suitable carriers does prevent the elution of the enzyme and makes it possible to control the physical properties and particle size of the final product, nevertheless it suffers from certain limitations owing to the sensitivity of many enzymes to chemical modification [30].

Adsorption and methods involving the formation of cross links. Various adsorbents are used to adsorb enzymes—both charged resins and neutral surfaces. The adsorption method does not guarantee complete immobilisation. High concentrations of the salt or substrate increase the rate of desorption of the enzyme. This can be avoided by the formation of intermolecular bonds in proteins after their adsorption. Thus highly active synthetic membranes have been obtained by the formation of a "network" of enzyme molecules adsorbed on collodion [30] or on cellophane membranes [31], or by the formation of a network of enzyme and inactive protein molecules [31].

III. PRINCIPAL ASPECTS OF CATALYSIS BY IMMOBILISED ENZYMES‡

Stability of Immobilised Enzymes

The activity of immobilised enzymes, referred to unit weight, depends on the amount of bound enzyme, which can vary from several milligrams to several grams per gram of carrier, and on the activity of the enzyme itself, which varies from $<10\%$ to $>100\%$ of the initial value and can actually exceed the activity which the enzyme would have had in the same amount in solution. This depends largely on the various microenvironments of the enzyme on the surface and in the pores of the polymer matrix.

Immobilisation as a rule increases the effective lifetime and thermal stability of the enzyme, but in some cases the opposite effect is also possible: a carrier containing hydrophobic groups to a large extent denatures the protein, while a hydrophilic carrier with a positive or negative charge can influence the stability of the immobilised enzyme as a result of electrostatic interactions between the bound enzyme and the carrier [30]. Proteolytic enzymes are stabilised on immobilisation also because their autolysis is prevented.

The form of the pH dependence on the activity of the immobilised enzyme can differ appreciably from the

‡ These processes are described in greater detail in Berezin's collective volume (a) and particularly in the review of Berezin et al. (b) (see the footnote to p. 1067).

analogous relation for the same enzyme in solution owing to the changes induced by the polyelectrolyte carrier molecule on the effective acid dissociation constants of the ionisable groups in the active centre of the enzyme. However, more important interactions of the charge–charge type take place between the ionic charges of the substrate or other components in solution and the residual charges on the matrix. If the solution components have a charge opposite to that of the matrix, their concentration near the surface and in the pores of the matrix becomes higher than in the bulk of the solvent; the opposite behaviour is observed for charges of the same sign [33]. For example, the alkaline branch of the pH dependence of the activity of ficin bound to CM-cellulose is displaced by 0.5 of a unit towards higher pH values, since the concentration of protons within the matrix increases owing to their interaction with the field of the negatively charged carboxy-groups [33]. These anomalies disappear at high ionic strengths.

Reaction Kinetics

Enzyme molecules are bound to the carrier as a rule at several points, which leads to the formation of a certain number of cross links and this may hinder the access of the substrate to the bound or shielded enzyme molecule [34]. Another factor influencing the concentration of the substrate in the microenvironment of the active centre of the bound enzyme is diffusion. The particles of the immobilised enzyme in an aqueous suspension are surrounded by an unstirred layer of solution in which a substrate concentration gradient is established [35]. As a result, the saturation of the immobilised enzyme by the substrate takes place at substrate concentrations higher than are required for the saturation of the active centre in solution. This leads to an increase of the effective Michaelis constant. Owing to diffusion, insoluble enzymes exhibit an activity maximum only at fairly high rates of stirring of the solution [36–38]. An example of the favourable influence of diffusion is provided by insoluble polyenzyme systems which are more effective than the individual enzymes in solution, since the product formed in the initial stages does not diffuse instantaneously into the bulk phase but remains on the support at high concentrations, which favours the subsequent stages of the reaction [39].

The effective Michaelis constant for the immobilised enzyme depends on the charge of the matrix if the substrate is also charged [26]: it increases when the signs of the charges of the matrix and the substrate are the same, since the substrate is repelled by the matrix, and decreases for the opposite signs of the charges, since the substrate is then attracted to the matrix and its concentration in the microenvironment of the enzyme is higher than in the bulk of the solution. The Michaelis constant can vary by an order of magnitude and more as a result of these factors.

IV. EXAMPLES OF THE POSSIBLE INDUSTRIAL APPLICATIONS OF IMMOBILISED ENZYMES

Separation of Racemic Mixtures

Numerous studies have been made on the resolution of DL-aminoacids using acylases obtained from microorganisms and pig kidneys.

Chibata and coworkers [40–46] were successful in separating DL-methionine on acylases. There have been reports about the construction of an industrial apparatus with an output of 20 tonnes per month. The author used various methods for the immobilisation of enzymes: (a) ionic adsorption on DEAE-cellulose and DEAE-Sephadex; (b) covalent binding to iodoacetylcellulose; (c) incorporation in a polyacrylamide gel.

Pig kidney acylase, incorporated in fibrous cellulose triacetate, was investigated in the author's laboratory. A highly reactive catalyst was obtained (17 700 μmole h^{-1} per gram of dry fibre at 37°C). Under the conditions of their utilisation at 25°C, the activity of these fibres fell by 25% in 650 h, during which 550 g of L-methionine had been obtained per gram of the dry fibre.

Saccharification of Starch

Lilly and coworkers [47] carried out the covalent binding of amyloglucosidase to DEAE-cellulose with the aid of 2-amino-4,6-dichloro-*s*-triazine. The activity of the resulting enzyme is not specific. However, one should note that it remains constant during 28 days of employment of the enzyme. In a subsequent study [48], it was reported that the protein can be bound in amounts up to 120 mg per gram of carrier and that the activity is approximately 20–30% of that of the initial enzyme. Under the experimental conditions at 50°C, the activity decreases by approximately 30% in 21 days, but at 25°C the enzyme is completely stable.

Smiley and coworkers [49] investigated the ionic binding of amyloglucosidase to DEAE-cellulose. It was found that the activity is 55% of the initial value and remains constant for three weeks at 40°C. Amyloglucosidase from *Aspergillus niger*, incorporated in a polyacrylamide gel [50], has an activity equal to 20% of that of the enzyme in solution, a high thermal stability, and a pH dependence analogous to that for the initial enzyme. The activity persists for approximately 15 h at 60°C and then decreases at a rate of 1.5% per hour. At 50°C the activity is constant for 150 h, after which inactivation begins.

Glucoamylases incorporated in cellulose triacetate fibres were investigated in the author's laboratory [51]. The activity of the insoluble residues in relation to solubilised starch is one tenth of its activity in relation to maltose (possibly because the diffusion of starch molecules, having a high molecular weight, is hindered). The activity of fibres with attached enzyme remains constant during employment for six months in the reaction with maltose at 25°C; in 30% solutions of solubilised starch at 45°C, the activity of the fibres persists for 300 h.

A method has been proposed for the separation of the reaction products from the enzyme and the substrate by means of ultrafiltration membranes [52]. In this case, the enzyme is retained by the membrane and can be used again.

The covalent binding of amyloglucosidase to porous glass was achieved recently [53]. The yield of the binding reaction amounts to 40–60% and may even reach 80%. In the usual method of preparation at 45°C, the average lifetime is 650 days. It has been calculated that 500 columns (with a volume of one cubic foot, i.e. 0.028 m^3) with immobilised glucoamylase would produce sufficient glucose to supply the requirements of the USA.

Numerous studies have been made on the immobilisation of α- and β-amylases. However, the activity of the resulting insoluble derivatives is very low (6–15%) and their stability is unsatisfactory. The immobilisation of

pullulanase[54] by covalent binding to the acrylamide–acrylic acid copolymer with the aid of carbodi-imide was reported recently. The degree of binding is 34% and the activity 43%, but the system obtained is unstable.

Isomerisation of Glucose

Glucose isomerase has been immobilised by incorporation in polyacrylamide gel and also as a result of its covalent binding to porous glass. The enzyme proved to be unstable in both cases.

Experiments have been carried out on the incorporation of glucose isomerase in cellulose triacetate fibres[55]. Highly active fibres were obtained and the activity remained virtually constant for two months at 45°C.

The Reduction of Lactose in Milk Products

Immobilised β-galactosidase has been obtained by various investigators. For example, Sharp et al.[56] achieved the covalent binding of the enzyme from *E.coli* to triazinylcellulose derivatives. The stability of the specimens obtained depended markedly on temperature and the presence of possible poisons.

The treatment of milk with immobilised fungal lactase, obtained in the polymerisation of acrylic monomers in the form of spherical pellets, has been reported[57]. The amount of enzyme incorporated is 5.6 mg per gram of carrier. The activity of the specimens is 36%.

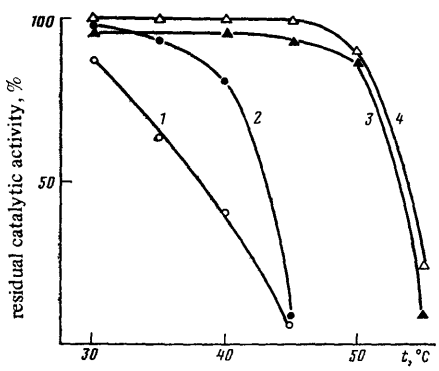


Figure 3. Thermal stability of free yeast β-galactosidase: 1) after incubation for 22 h; 2) after incubation for 6 h; thermal stability of free β-galactosidase from *E.coli*: 3) after incubation for 22 h; 4) after incubation for 6 h.

The properties of yeast β-galactosidase incorporated in cellulose triacetate fibres have been investigated in the author's laboratory[58,65]. The activity of the immobilised β-galactosidase is about 40% of the activity of the free enzyme. Both the free and the immobilised enzymes have an activity maximum at pH 6.0–7.0 (Fig. 3) and 37°C. Milk and pure lactose are hydrolysed at approximately the same rates. The immobilised galactosidase retains the initial activity during employment for 80 days. When very active fibres containing 1500 units of the enzyme per gram of fibre are used, it is possible to hydrolyse milk lactose at low temperatures (5°C) without the risk of altering the organoleptic properties of the treated milk/or of contamination by bacteria (Table 4). The experimental apparatus operating under these conditions demonstrates the possibility of the industrial hydrolysis of milk lactose, which would solve the problem of the elimination of lactose from food products.

Table 4. The influence of the density of filling of columns with cellulose triacetate fibre incorporating yeast β-galactosidase on the hydrolysis of milk lactose.

Amount of fibre, g per ml of column volume	Degree of hydrolysis of milk lactose, %*			
	30 min	60 min	120 min	180 min
0.115	7.75	12.4	23.7	33.7
0.250	7.75	15.0	27.0	38.5
0.400	11.0	14.4	29.8	40.7

*500 ml of milk at 25°C circulated through the column at a rate of 10 litre h^{-1}.

Hydrolysis of Penicillin

Penicillin acylase has been immobilised by covalent binding to derivatives of chloro-*s*-triazinylcellulose[59,60]. It has been shown[59] that the immobilised enzyme has an activity of 25% of the value in solution and retains it during employment for eleven weeks at 37°C. In another investigation[60], the best specimens had an activity between 70 and 120 μmole min^{-1} per gram of carrier, which is 45–80% of the activity of the enzyme in solution.

Penicillin acylase from *E.coli* has been immobilised in the author's laboratory[61] on cellulose triacetate fibres. The activity reaches 40 μmole min^{-1} per gram of carrier and remains constant for 150 days at 37°C. The degree of hydrolysis of penicillin on the enzyme amounts to 98%.

Insoluble specimens with an activity of 110 μmole min^{-1} per gram of carrier have now been obtained in the author's laboratory; with their aid, it is possible to hydrolyse a 12% solution of penicillin in a short time. The synthesis of ampicillin from 6-aminopenicillanic acid and phenylglycine esters has been achieved on the same fibres in a yield of about 70%. Furthermore, the hydrolysis of cephalosporins, which are penicillin derivatives, also takes place in a high yield.

Rearrangements in Steroids

The cells of *Curvularia lunata* trapped in a polyacrylamide gel are used for the 11-β-hydroxylation of Reichstein compounds[62]. The same paper reports the preparation of immobilised *Corynebacterium simplex* cells, which retain the Δ-1-dehydrogenase activity and can be used in columns. However, in both cases the activity of the systems is too low for industrial application.

Consecutive Reactions

The possibility of catalysing a number of consecutive reactions without the isolation of intermediates is of great

theoretical and practical interest. Brown et al.[63] investigated the cleavage of glucose by four glycolytic enzymes: hexokinase, phosphoglucoisomerase, phosphofructokinase, and aldolase. The enzymes are incorporated in a polyacrylamide gel and placed in a column in the required sequence.

Mosbach and Mattiasson[39,64] investigated enzyme complexes bound to the same matrix. They obtained[39] β-galactosidase, hexokinase, and glucose-6-phosphate dehydrogenase bound to Sephadex. It was found that a bound complex of this kind has a higher activity than in solution; the authors explained this by the influence of diffusion.

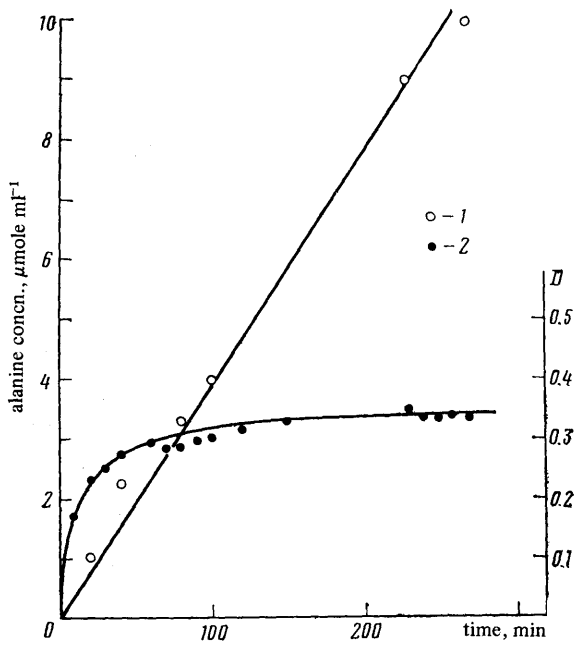


Figure 4. Kinetics of the formation of alanine catalysed by lactate dehydrogenase and alanine dehydrogenase immobilised on fibres: 1) increase of alanine concentration; 2) variation of NADH concentration (D = optical density at 340 nm).

Lactate dehydrogenase and alanine dehydrogenase have been incorporated in fibres in the author's laboratory in order to obtain alanine from lactate and ammonia:

$$(1)\ CH_3CHOHCOO^{(-)} + NAD^+ \rightleftarrows CH_3COCOO^{(-)} + NADH + H^+ ;$$
$$(2)\ CH_3COCOO^{(-)} + NH_3 + NADH + H^+ \rightleftarrows CH_3{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COO^{(-)} + NAD + H_2O .$$

Fig. 4 presents typical kinetic curves showing that the concentration of alanine increases linearly, while the concentration of NADH reaches a steady-state value in approximately 100 min. The time required for the attainment of a steady-state concentration is determined by the diffusion of NADH from the microcavities in the fibres to the bulk of the solution.

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