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Current Applications of Immobilized Enzymes for Manufacturing Purposes

POUL BØRGE POULSEN

Novo Industri A/S, Novo Allé, DK-2880 Bagsværd, Denmark

Introduction

Ever since the industrial use of enzymes began almost 100 years ago, people have discussed whether and how it would be possible to reuse them. In fact, what was being discussed was whether and how immobilized enzymes could be made industrially important.

According to the Working Party on Immobilized Biocatalysts within the European Federation of Biotechnology (1983), immobilized biocatalysts are defined as enzymes, cells or organelles (or combinations of these) which are in a state that permits their reuse. That means that immobilized enzymes can be, for example, insoluble enzymes used in a fixed-bed reactor, or soluble enzymes used in a semipermeable membrane reactor. The scope of this chapter is to describe the enzymes (and *not* organelles or whole cells) which today are used for manufacturing purposes in industry. That means that this chapter does not intend to cover, for example, immobilized enzymes for analytical purposes, or those for medical use. The term 'whole cells' is not well defined. Some of the applications which will be dealt with in this article involve immobilized whole cells; however, these cells contain only one enzyme which is utilized in the process. Furthermore, such cells are often partly broken down before or during the immobilization step.

The author also interprets the term 'manufacturing purposes' in such a way that immobilized enzymes being studied under pilot plant conditions will not be covered.

The final limitation is that this chapter is based on published data, which are extremely sparse, even for the longest-established example of an immobilized enzyme—immobilized penicillin G acylase. This is because such products mainly have been developed and manufactured by the self-same company which uses each particular immobilized enzyme, or by an industrial consortium. Most data for these processes are still regarded as confidential 'know-how' by the companies involved.

This leaves those enzymes which are reused today in industrial plants for manufacturing purposes, and with the additional limitation that most data can

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be given only for those enzymes which are marketed (i.e. produced by one company and used by another).

Immobilized enzymes in general

THE SCOPE OF ENZYME TECHNOLOGY

This is indicated by the list of the types of reactions catalysed by enzymes (Dixon and Webb, 1979; *see also* Chapter 1):

—Oxidation
—Reduction
—Inter- and intramolecular transfer of groups
—Hydrolysis
—Cleavage of covalent bonds by elimination
—Addition of groups to double bonds
—Isomerization

Hence virtually all organic and many inorganic reactions can be catalysed by an enzyme or several enzymes acting in sequence. Of course most of these reactions can also be catalysed by non-biological, chemical catalysts. One may therefore ask: When should enzymes be considered as catalysts for a certain reaction and when should they be avoided and non-biological, chemical catalysts chosen?

Enzymes have the advantage of being able to differentiate between chemicals of closely related structures, e.g. between stereoisomers, and of being effective at low temperatures (0–110°C) and at 'neutral' pH values (pH 2–12). This usually gives fewer by-products, a simpler purification process, and improved quality of the final product. Furthermore, enzymes are non-toxic and readily degradable (non-polluting), and they can be produced in unlimited quantities.

Enzymes can often replace strong acids and bases. This means that the use of specially resistant materials in reaction vessels is not necessary, thus saving money. It also means that the presence of large amounts of salts (which otherwise would have to be removed after the reaction) can be avoided. This can contribute both to financial savings and to a reduction in environmental pollution.

Enzymes have their limitations in the relatively fragile nature of the amino acid building blocks and the even more fragile tertiary structure of the protein molecule. These factors make enzymes sensitive to high temperature, extreme pH values, aggressive chemicals, and in some cases, to organic solvents.

It will not be possible to apply enzymes in reactions involving chemicals that denature proteins, i.e. destroy their tertiary structure. In addition, the presence of chemicals that inhibit the catalytic properties of the enzymes must be avoided. Furthermore, there are some processes, particularly in the petrochemical industry, which are so highly developed that one cannot expect further improvement by use of enzymes. Finally, the cost of enzymes will preclude their application in process stages that function well with inexpensive chemicals.

The important factor, costs, encouraged trials for reusing enzymes at a very

early stage. Enzymes adsorbed to charcoal were described by Nelson at the beginning of this century but the system was very unstable. In the 1950s, Georg Manecke was the first really to succeed in making relatively stable systems: however, he could not convince industry of the importance of further development of his systems. It was the group of chemists working with Ephraim Katchalski-Katzir in Israel in the 1960s who really opened the eyes of industry to the world of immobilized enzymes. The first immobilized enzyme products to be scaled up to pilot plant level and industrial manufacture (in 1969) were immobilized amino acid acylases (i.e. Chibata and colleagues at Tanabe Seiyaku Company in Japan), penicillin G acylase (M. D. Lilly, University College, London, and Beecham Pharmaceuticals, England) and glucose isomerase (Clinton Division of Standard Brands, now Nabisco Brands, USA, from the 'know-how' of Takasaki and his colleagues at the Fermentation Research Institute, Chiba City, Japan) (Klibanov, 1983).

IMMOBILIZATION TECHNIQUES

During the past ten years, more than a hundred immobilization techniques have been worked out (Zaborsky, 1973; Mosbach, 1976; Trevan, 1980). They can be divided into the following five groups (Klibanov, 1983):

1. Covalent attachment of enzymes to solid supports

In the laboratory a variety of supports have been used, e.g. porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers, and metallic oxides. Enzymes are usually immobilized through their amino or carboxyl groups. In most instances, the immobilization procedure consists of at least two steps: activation of the support and enzyme attachment *per se*.

2. Adsorption of enzymes on to solid supports

Ion exchangers readily adsorb most proteins and have therefore been used for enzyme immobilization. This immobilization procedure is simple: an enzyme solution is added to the support, mixed, and surplus enzyme is then removed by washing.

3. Entrapment of enzymes in polymeric gels

In this approach an enzyme is added to a solution of monomers before the gel is formed. Gel formation is then initiated either by altering the temperature or by adding a gel-inducing chemical. As a result, the enzyme becomes trapped in the gel.

4. Cross-linking of enzymes with bifunctional reagents

Among the most popular cross-linkers are glutaraldehyde, dimethyl adipimidate, and aliphatic diamines. The first two directly cross-link enzymes through their

amino groups. Diamines cross-link enzymes through carboxyl groups following activation of these groups with carbodiimides. Cross-linking may be both intermolecular and intramolecular.

5. Encapsulation of enzymes/soluble enzymes in semipermeable membrane reactors

In this approach, enzymes are enveloped within various forms of membranes (e.g. between sheets or within hollow capsules or fibres consisting of semipermeable membranes) that are impermeable to enzymes and other macromolecules but permeable to substrates and products of low molecular weight.

Which of these immobilization techniques has become most popular in industry? Unfortunately there is no clear answer, for the immobilized enzyme products which will be discussed later cover all five groups.

From the above, it can be deduced that there are several optimal immobilization methods. Every enzyme product and every enzyme process each needs its own optimization. The following are some examples of critical optimization factors:

1. Necessary degree of purification of the enzyme;
2. Apparent immobilization yield;
3. Pressure/shear stability of the insoluble enzyme;
4. Degree of leakage (enzyme, monomers, etc.);
5. (Apparently) changed kinetics (e.g. increased by-product formation);
6. System stability during application;
7. Variable costs, necessary investments;
8. Acceptable storage stability;
9. Can the immobilized enzyme be transported to the user?
10. Does the user like the immobilized enzyme product/ process (easy to use, inexpensive, etc.)?

Why are enzymes sometimes immobilized? The primary reason concerns running costs: in some cases it is much more economical to reuse the enzymes instead of using them once only. The second reason is that sometimes this leads to easier purification of the product. The third reason is that sometimes this entails lower investment costs.

On the negative side, immobilized enzymes cost more than those enzymes used once only. Usually, more sophisticated process equipment is needed. In the production process other problems arise, such as increased risk of contamination, need for extra control of temperature and pH, and substrate purity. Diffusion resistance may cause increased levels of by-products and may also reduce the apparent activity.

The conclusion is that the result of a cost–benefit analysis must be the factor to decide whether immobilization of an enzyme is needed.

Immobilized enzymes currently in industrial use

The immobilized enzymes used today in industry are listed in *Table 1*, together with names of producers. This appears to be an impressive list—eight different

Table 1. Immobilized enzymes currently used in industry.

Industrially used immobilized enzymes	Producers (listed alphabetically)
Aminoacylase (EC 3.5.1.14)	Amano, Japan; Tanabe, Japan
Amyloglucosidase/glucoamylase (exo-1,4-α-D-glucosidase, EC 3.2.1.3)	Tate & Lyle, UK
Glucose isomerase (xylose isomerase, EC 5.3.1.5)	CPC, US; Finn Sugar, SF; Gist Brocades, NL; Godo Shusei, Japan; Miles, US; MKC, West Germany; Nagase, Japan; Novo Industri, DK; UOP, US
Hydantoinase (dihydropyrimidinase, EC 3.5.2.2)	Amano, Japan (?); Snam Progetti, It. (?)
Lactase (β-D-galactosidase, EC 3.2.1.23)	Corning Glass, US, Snam Progetti, Italy; Valio, SF
Nitrilase (EC 3.5.5.1)	Nitto, Japan
Penicillin G acylase (EC 3.5.1.11)	Bayer, West Germany; Beecham, UK; Gist Brocades, NL; Snam Progetti, Italy; Pfizer, US; Toyo Jozo, Japan
Penicillin V acylase (EC 3.5.1.11)	Biochemie, Austria; Novo Industri, DK

enzymes and possibly 20 different suppliers. However, a more precise description of the present situation is that only one immobilized enzyme is used in large tonnage, and several in considerably smaller amounts. The same picture holds good for the producers, where less than five companies cover more than 90–95% of the market (*Table 2*).

Immobilized glucose isomerase

Glucose isomerase is an intracellular enzyme which is found in several micro-organisms and used in the production of fructose from glucose. Fructose syrup is competing with sucrose on the industrial market. Ideally, glucose isomerase will produce an equilibrium mixture of glucose and fructose, although in practice (for economic reasons) the mixture will contain about 42% fructose, 52% glucose and 6% dextrins. This mixture is sweeter, weight for weight, than glucose and about as sweet as sucrose or invert sugar (a 50/50 mix of fructose and glucose).

From the outset, in the 1950s and 1960s, it was obvious that the enzyme would have to be reused in order to prove economical. The price of raw sugar in the sixties was US $0.05/lb (0·45 kg) and fructose syrups could not be produced more cheaply. The enzymatic productivity was only 50–100 kg syrup d.s. (dry solids) per kg of the expensive cells containing the enzyme. Furthermore, cobalt salts had to be added to the reaction mixture and quantitatively removed after the isomerization. The cells autolysed during the reaction, thus making it difficult to produce a pure syrup.

Table 2. Immobilized enzymes—annual product consumption (1982—worldwide).

Immobilized enzyme product (listed alphabetically)	Size
Aminoacylase	Presumably less than 250 t L-amino acid produced per year Estimated enzyme amount: less than 5 t/year Largest producer: Amano, Japan (?)
Amyloglucosidase/glucoamylase	Presumably less than 5000 t syrup d.s. produced per year Estimated enzyme amount: less than 1 t/year Largest producer: Tate & Lyle, UK
Glucose isomerase	Approx. 2150000 t 42% HFCS and 1450000 t 55% HFCS produced per year (after Poulsen 1981/82) Estimated enzyme amount: 1500–1750 t/year Largest producers: Novo Industri, DK and Gist Brocades, NL
Hydantoinase	Presumably less than 50 t D-phenylglycine produced per year Estimated enzyme amount: less than 1 t/year Largest producer: ?
Lactase	Presumably less than 10000 tons d.s. lactose hydrolysates produced per year Estimated enzyme amount: less than 5 t/year Largest producer: Valio, SF
Nitrilase	Presumably less than 5 t acrylamide produced per year Estimated enzyme amount: less than 0·1 t/year Largest producer: Nitto, Japan
Penicillin G acylase	Approx. 4000 t 6-APA produced per year Estimated enzyme amount: 3–4 t/year (after Godfrey & Reichelt) Largest producers: Gist Brocades, NL, Beecham, UK, Toyo Jozo, Japan
Penicillin V acylase	Approx. 500 t 6-APA produced per year Estimated enzyme amount: approx. 1 t/year Largest producers: Biochemie, Austria and Novo Industri, DK

For comparison, the largest enzyme for once-only use is amyloglucosidase, of which 15000 tons are produced annually (substrate approx. 20×10^6 t dry matter) and used for syrup, ethanol, and beer production

The Clinton Division of the American Standard Brands Co. (now Nabisco Brands Co.) was the first to produce an immobilized glucose isomerase. Their product was based on technical expertise developed by Takasaki and his co-workers at the Fermentation Research Institute in Chiba City, Japan. However, 1969 was not the best time for marketing this product: the disadvantages were obvious; it was a new process for the industry with a complicated system of filter presses, and it necessitated equipment to remove the cobalt which was necessary for the reaction.

In November 1974, the spot prices of raw sugar reached US $0.6/lb (0·45 kg) and this made immobilized glucose isomerase commercially viable. At the same time, a group at Novo Industri A/S succeeded in producing an immobilized glucose isomerase product (Zittan, Poulsen and Hemmingsen, 1975; Hemmingsen, 1979) which could compete economically, could be used without the addition of cobalt, and could withstand the pressure in the large industrial fixed-bed reactors where heights of 7 m are not unusual.

Because nearly all the disadvantages had been eliminated the sales boomed. In 1976, 750 tonnes (t) of immobilized glucose isomerase were used to produce around 800 000 t of 42% fructose in the US alone. Thus, by the time that the sugar price fell again to US $0·15 in the last months of 1976, the new process and product were established. The 42% high fructose corn syrup (HFCS) was sold at a lower price than sucrose, but in 1978 the next development took place: a 55% HFCS became available at a price only 15–25% higher than that of the 42% syrup. This product was the result of a new fractionation technology, involving chromatographic separation of fructose from glucose by means of ion-exchange resins. In test panels, this 55% fructose syrup was found to be equivalent to invert syrup; in comparison to 42% syrup, the glucose and polysaccharide contents were lowered.

Table 3. Actual sales of HFCS and sucrose—USA†

Product	1977	1978	1979	1980	1981	1982
Industrial sucrose, 1000 t	6110	5990	5820	5500	5120	4790
HFCS						
42%	965	1100	1225	1320	1450	1450
55%	0	75	365	590	950	1450
Total, 1000 t	7075	7165	7410	7410	7520	7690
HFCS penetration, %	14	16	21	26	32	35

† Poulsen and Christensen (1982)

It is estimated that, in 1982, 1200–1500 t of immobilized glucose isomerase were used to produce 1450 000 t (dry weight) of 42% HFCS and 1450 000 t of 55% HFCS (*Table 3*).

Table 4 lists the major markets for HFCS in the USA. Japan is the second largest producer of high fructose syrups with an annual production of about $0{\cdot}5 \times 10^6$ t HFS. Other countries with high fructose production are Belgium, France, Italy, Spain, Yugoslavia, Hungary, Greece, Pakistan, S. Korea, Canada and Argentina, but in all these countries the scale of production is much less than that in the US and in Japan.

Table 4. Major markets for HFCS, USA†

Use	Sugar (1000 t)	1981 HFCS (DS) (1000 t)	HFCS penetration (%)	Estimated long-term penetration (%)
Baking	1160	385	25	25
Confections	873	11	1	5
Dairy products	408	181	31	35
Beverage	1659	1483	47	90
Processed foods	204	102	33	40
Canning	226	308	58	75
Other food and non-food uses	644	0	—	—
Total	5174	2470	32	52

† Poulsen and Christensen (1982).
Source: McKeany-Flavell Co. Inc., Sweeteners' Brokers San Francisco, June 7, 1982 and United States Department of Agriculture.

Table 5. Glucose isomerase products commercially available on a tonne scale (listed alphabetically)

Company	Immobilization method
Finn Sugar, SF	—
Gist Brocades, NL	*Actinoplanes missouriensis* cells mixed with gelatin and cross-linked with glutaraldehyde
Godo Shusei, Japan	Enzyme source: *Streptomyces phaechromogenes* (?)
Miles Labs, US Miles Kali Chemie, D	*Streptomyces olivaceus* cells cross-linked with glutaraldehyde or purified enzyme from *Streptomyces rubiginosus* immobilized on SiO_2 beads
Nagase, Japan	*Streptomyces* sp. (?)
Novo Industri, DK	*Bacillus coagulans* cell homogenate cross-linked with glutaraldehyde
UOP, US	*Streptomyces olivochromogenes* purified and immobilized with polyethyleneimine and glutaraldehyde on ceramic carrier Al_2O_3

Table 6. Glucose isomerase—typical application data.

Temperature	55–60°C
pH	7·5–8·0
Dextrose concentration	40–50% (w/w)
Additive	0–1 g $MgSO_4$, $7H_2O$/ℓ
Inhibitor	Ca^{2+}
Application time†	2–4 months
Productivity	2000–22000 kg/kg

†Time for which the enzyme product may be employed—typically down to 25% residual activity.

We learn from this that the use of immobilized glucose isomerase seems to be a success only in those nations which are net sugar importers. There must be substantial consumption of liquid sugar through a well-developed processed food and beverage industry. An abundant starch supply must be available. A labour force educated in the operation of sophisticated processing equipment must be available. Finally, a very good market incentive must exist during the start-up of the new process. The general layout of the HFCS production process is shown in *Figure 1*. All commercially available immobilized glucose isomerase products (*Table 5*) are used under more or less the same application conditions (*Table 6*). The development of an immobilized glucose isomerase process has entailed considerable effort by the companies involved (Hemmingsen, 1979; van Tilburg, 1983). The first step was to find a suitable micro-organism which produced an acceptable enzyme cheaply and in large quantities. The discovery by R. O. Marshall and E. R. Kooi in 1957 that xylose isomerase from *Pseudomonas hydrophila* could isomerize D-glucose to D-fructose was quite a novelty. For the organisms investigated, the conversion of glucose to fructose required the previous isomerization of the phosphorylated sugar by the enzyme glucosephosphate isomerase (EC 5.3.1.9). Marshall and Kooi further pointed out, however, that the presence of arsenate in the reaction mixture was essential in order to obtain a proper conversion. This fact, and the fact that the formation of xylose isomerase was absolutely dependent on the presence of xylose in the

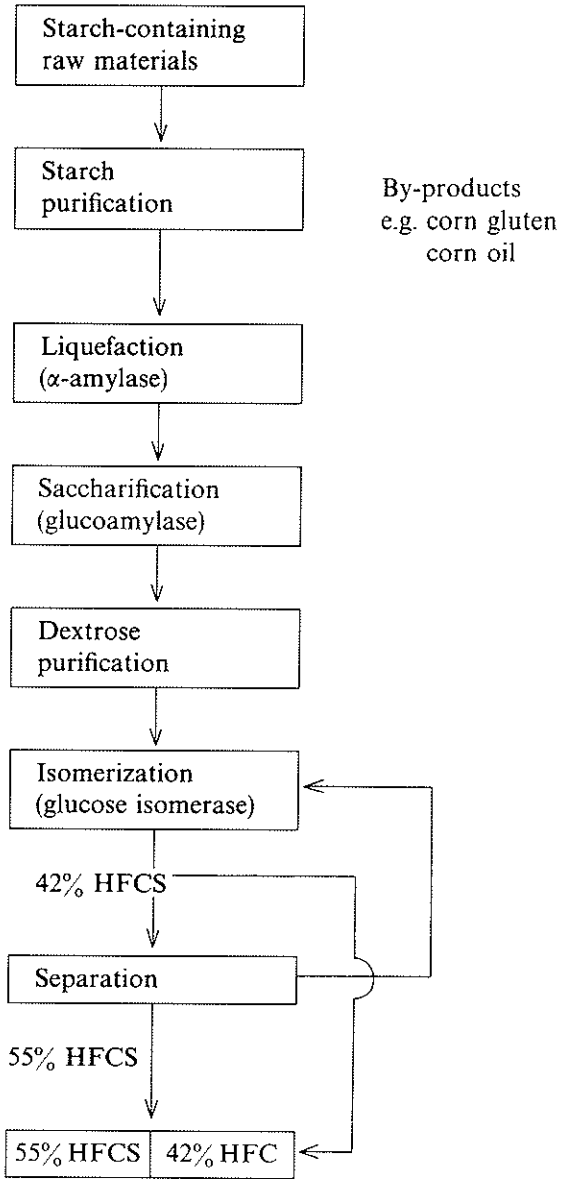

Figure 1. General process layout for HFCS (high fructose corn syrup).

growth medium, meant that Marshall and Kooi's enzyme could not be exploited commercially.

The important step forward came in the mid-1960s when enzyme-producing micro-organisms of the genera *Streptomyces*, *Actinomyces* and *Bacillus* began to be investigated. The glucose isomerases obtained here were fairly thermostable (i.e. they could withstand 10 minutes at 80–90°C). The only cofactors necessary were Mg^{2+} and Co^{2+}. However, as Co^{2+} is undesirable in industrial processes, it was important that the enzyme also worked reasonably well without this. A problem which initially caused considerable concern was the fact that the pH optimum was undesirably high (pH 9·3–9·5): under alkaline conditions, apart from the formation of coloured by-products, a non-metabolizable sugar (D-psicose) is produced in hot glucose and fructose solution. New mutants were soon found, with pH optima about 7–8 and a concomitant satisfactory reduction in psicose formation. This development has been described in more detail by van Tilburg (1983).

The next step was to find an acceptable immobilization procedure. Why immobilize glucose isomerase? The main reason is that this is necessary in order to keep the holding time within satisfactory limits. A holding time longer than 3 hours will easily cause by-product formation. The same activity per volume with soluble once-only enzyme would be considerably more expensive. As the holding time is minimized in a packed-bed or fluidized-bed reactor, the research soon concentrated on finding an immobilization procedure which would yield a product that could withstand such conditions. The immobilization procedure also had to be cheap (i.e. involve food-grade chemicals), to result in high yield, and to avoid the involvement of too many unit operations. The first product marketed by Clinton Corn in the US was simply glucose isomerase fixed in whole cells by heat treatment: however, as this material is very soft, the product had to be used in a very expensive filterpress system. The technology soon improved to granulated flocculated cross-linked whole cells. The final winners in the mid-1970s were products such as granulated glutaraldehyde cross-linked cell homogenate, and cells entrapped in gelatine cross-linked with glutaraldehyde. These particles of the immobilized enzyme were so strong that they could withstand the pressure in columns 7 m high with a holding time of less than 1 hour.

The final step comprised process optimization, i.e. finding the temperature, pH, concentration and necessary degree of substrate purification, which resulted in the highest productivity and therefore the most economical process.

The development of immobilized glucose isomerase has been documented fully by Zittan, Poulsen and Hemmingsen (1975), Hupkes (1978), Hemmingsen (1979), Norsker, Gibson and Zittan (1979), Roels and van Tilburg (1979a), van Tilburg and Roels (1982) and van Tilburg (1983); *see also* Chapter 2.

Immobilized penicillin G/V acylases

The only penicillins which can be produced in high yields by fermentation are penicillin G (phenyl acetyl-6-APA) and penicillin V (phenoxy acetyl-6-APA). However, micro-organisms resistant to these two penicillins have always existed

Chemical process	pen $\xrightarrow{SiMe_2Cl_2}$ (pen-COO)$_2$SiMe$_2$ $\xrightarrow[\text{base}]{PCl_5}$ imidochloride $\xrightarrow[-40°C]{\text{n-BuOH}}$ iminoether $\xrightarrow{\text{hydrolysis}}$ 6-APA
Enzymatic process	pen $\xrightarrow[\sim \text{pH } 7,\ 35°C,\ 4\text{–}15\%\ \text{w/v pen}]{\text{enzyme}}$ 6-APA + side chain

Figure 2. Conversion of penicillin G or V to 6-APA.

and more and more resistant mutants have appeared. In order to enlarge the antibiotic spectrum and improve the pharmacokinetic properties of these antibiotics towards the resistant strains, the G and V side chains have been removed and new side chains have been coupled to 6-APA (6-aminopenicillanic acid). Several semisynthetic penicillins, in particular ampicillin (D-phenyl glycyl-6-APA) and amoxycillin (*p*-OH-D-phenyl glycyl-6-APA) have proved to be effective against strains resistant to the parent penicillins V and G.

Two types of process are possible for the deacylation step—a chemical process and an enzymatic process—(*Figure 2*). The chemical route has been used for longer than the enzymatic route: it proceeds at −40°C, involves organic solvents and must be absolutely free from water—factors which all make the process difficult. The enzymatic process was developed in the 1960s and soon became an accepted alternative to the chemical route as the process conditions were very undemanding; however, the weak point was the enzyme costs.

The penicillin acylases (both the V and the G acylases) are intracellular enzymes produced by several micro-organisms. Typically, G acylases are found in bacteria such as *E. coli* and V acylases in fungi such as *Bovista plumbea*. The first major problem to overcome was elimination of β-lactamases from the acylase preparations: β-lactamase (EC 3.5.2.6) is unwanted because it opens the lactame ring in 6-APA, thereby destroying it. This problem was solved by mutation and chemical treatments.

Initially, the cell suspensions containing acylase were used only once, as in the case of glucose isomerase. Productivity was very low, only about 0·5–1 kg 6-APA per kg *E. coli* suspension. In 1969, Beechams and Malcolm Lilly discovered a method of immobilizing the *E. coli* cells and were able to increase productivity thereby. Few technical data have been published but the immobilization method was probably the DEAE cellulose method. In the 1970s this method was abandoned: Bayer switched to a polymethacryl glutaraldehyde method and Beechams to a cyanogen-bromide-activated dextran/sephadex method; productivity increased to approximately 50 kg/kg (early 1970s).

In principle, these methods are still used today. The productivities obtained are presumably now in the range 100–2000 kg/kg. The current annual 6-APA production is also thought to be about $8–10 \times 10^3$ t, more than half of which is produced by the enzymatic route. However, these figures are tentative as little is published by the companies involved.

Beechams is probably the largest producer of 6-APA from penicillin G by the enzymatic route. Their process data are presumably those given by Godfrey and Reichelt (1983) and shown in *Table 7*. Toyo Jozo, Japan has also developed an immobilized penicillin G acylase; their product information is shown in *Table 8*. Pfizer also claims to have had a penicillin G acylase system operating for several years.

Table 7. Typical process data for immobilized penicillin G acylase†

Enzyme product	Rigid granules or dextran/sephadex
Reactor	Column
Substrate	Penicillin G or certain semisynthetic cephalosporins (4–15% w/v)
Temperature	35–40°C
Inlet pH	7–8
Operating life	2000–4000 hours
Productivity	1000–2000 kg/kg enzyme

‡ According to Godfrey and Reichelt (1983)

Table 8. Process data for immobilized penicillin G acylase†

Enzyme product	Pen G acylase (*B. megatherium*) covalent bound to glutaraldehyde-activated porous polyacrylonitrile fibres
Reactor	Parallel column system
Substrate	Penicillin G (10% w/v)
Temperature	30–36°C
Inlet pH	$8·4 \pm 0·1$
Operating life	Approx. 1200 hours
Productivity	500–700 kg/kg enzyme

† According to Toyo Jozo, Japan.

Table 9. Process data from immobilized penicillin V acylase†

Enzyme product	Penicillin V acylase cell homogenates covalently bound with glutaraldehyde
Reactor	Stirred batch or column
Substrate	Penicillin V (4–12% w/v)
Temperature	35°C
Inlet pH	7·5–8·0
Operating life	1000–2000 hours
Productivity	200–600 kg/kg enzyme

† Data from Novo Industri A/S, Denmark.

Penicillin V acylases are manufactured by Biochemie and Novo. However, the only published data are of the Novo process (*Table 9*).

Immobilized penicillin G and V acylases have become a success because the current very low enzyme consumption has made this process the most economical one. The process involves an aqueous system (without organic solvents) and proceeds at room temperature (not at energy-demanding temperatures of -20°C and lower). Interesting future developments are expected as the use of semisynthetic cephalosporins rapidly becomes more and more widespread.

Immobilized lactase

To produce 1 kg of cheese, about 10 ℓ of milk are needed, and 9 ℓ of whey are produced as a by-product (Harju and Kreula, 1980). Whey is a yellowish liquid containing 6% dry matter (4·7% lactose, 0·7% protein and 0·5% salts). In a few countries whey is now being used either as pig food or, in a converted form, for human consumption; however, in many countries whey is still discarded as waste. Lactose, the main component of whey, is causing some problems because of properties such as low solubility and low sweetness. If hydrolysis of lactose is combined with ultrafiltration, demineralization, etc., several potentially useful lactose hydrolysates can be produced (*see* Chapter 12).

Lactose in whey can be hydrolysed to glucose and galactose either enzymatically or with acid. Hydrolysis of lactose in milk is a more difficult problem: in this case the enzymatic routes are the only feasible ones. The lactases available can be divided into two groups: neutral lactases (yeasts and bacteria, pH optimum 6–8) and acid lactases (moulds, pH optimum 3–5). Whey is an excellent substrate for microbes and usually the microbial growth is the decisive factor when a process is to be set up.

As previously mentioned, lactose hydrolysis can be accomplished with acid. Because of its high temperature and very low pH, acid-catalysed hydrolysis is suitable only for fairly pure lactose solutions, because otherwise the dark colour and unpleasant taste arising from this process will necessitate costly purification processes. When mineral acids (1–3 M HCl, 0·5 M H_2SO_4) are used, the temperature must be fairly low (50–60°C) to limit side reactions, and up to 48 h may be needed to reach a high degree of hydrolysis. With strong cationic resins the process with deproteinized whey can be improved because no acid is added and a high temperature (90–100°C) can be used. The rate of reaction is greatly accelerated and, in addition, a continuous process may be developed. In a column operation the residence time needed is 0·5–2·0 h (Harju and Kreula, 1980).

An alternative to this acid process is the enzyme process. First, a simple batch process utilizing soluble (once-only) lactase was proposed for both milk and whey. However, in order to limit microbial contamination, a low temperature (10°C) was chosen. This again demanded a high level of lactase in order to keep the hydrolysis time down to 24 h. With such an enzyme level, the cost of the hydrolysis was relatively high.

Because of the drawbacks in batch hydrolysis, several methods have been developed for immobilization of lactase. When lactase is immobilized, the cost of hydrolysis may be reduced if the operational life of the system is long enough. The microbial growth in the reactor, however, needs special attention. The use of sanitizers is difficult because they may easily inactivate the enzyme. If an acid-stable mould lactase is immobilized, the pH of whey can be lowered to 3·5 where the growth of most of the microbes is inhibited.

Many factors are to be considered in optimization of an immobilized lactase system. Besides the temperature, pH and substrate concentration, the activity and operational life of the preparation, the microbial growth and the reactor

Table 10. Large-scale production of immobilized lactase

Manufacturer	Enzyme	Carrier
Corning Glass Works	*A. niger* lactase	Silica beads
Snam Progetti	Yeast lactase	Cellulose acetate fibres
Valio Finnish Co-op Dairies Association	*A. niger* lactase	Adsorption resin

design may all affect the economics of the operation. At least three immobilized lactase systems have been developed on a large scale (*Table 10*).

The first industrial concern commercially to hydrolyse lactose in milk by means of immobilized lactase was Centrale del Latte of Milan, Italy, utilizing Snam Progetti hollow-fibre technology (Marconi and Morisi, 1979). A plant with a capacity of 10000 ℓ/day was opened in 1975, using a low-temperature batch process. In 1978, Valio started their first industrial operation in Koria, Finland (Harju, 1982). Two 300 ℓ columns were able to treat 20000 ℓ of whey per day. Today, the capacity has been increased to two 600 ℓ columns with a whey capacity of 80000 ℓ/day. The Valio process can operate with ordinary whey as well as demineralized whey and the ultrafiltration permeate.

As described, the immobilized lactase can now be designated as used on an industrial scale. However, it is remarkable that, after nearly 10 years, fewer than 10 plants are utilizing this technology. Reasons for this slow development could be:

1. It is cheaper to produce sweeteners from starch than from whey;
2. The need for milk improvement is apparently not great enough when all factors are considered;
3. If the whey causes effluent disposal problems, there are several other ways of using it (e.g. production of fodder or of ethanol);
4. The immobilized enzyme process still has too many weak or undesirable points, such as the risk of contamination, slow rate, and unacceptable cost.

Other immobilized enzymes

IMMOBILIZED AMINOACYLASE

When amino acids are produced by chemical synthetic methods, optically inactive racemic mixtures of L- and D-isomers are obtained. To obtain the L-amino acid (which is the form existing in nature) from the chemically synthesized D,L forms, optical resolution is necessary. The enzymatic method for optical resolution using aminoacylase is one of the most advantageous procedures for industrial production of optically pure L-amino acids (*Figure 3*). *Aspergillus oryzae* aminoacylase has versatile substrate specificity and can be used for the production of many L-amino acids. Since 1954, the enzymatic resolution method has been used by Tanabe Seiyaku Co., Osaka, Japan for the industrial production of several L-amino acids (Chibata *et al.*, 1976).

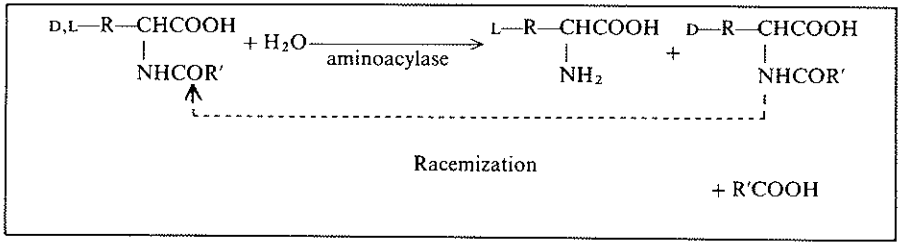

Figure 3. Optical resolution using aminoacylase.

Until 1969, the enzyme reaction was carried out in a batch process by incubating a mixture containing substrate and soluble enzyme, a procedure which had some disadvantages for industrial use. To overcome these disadvantages, the immobilization of aminoacylase and the continuous optical resolution of D,L-amino acids using a column packed with immobilized enzyme was started in 1969. The immobilization method selected was adsorption of aminoacylase to DEAE-Sephadex. When this product was used at 50°C, it lost 50% of its activity after 65 days, but could be reactivated, thus increasing its useful life. Chibata *et al.* (1976) claim that, when using the immobilized enzyme, purification becomes simpler and the yield higher than when native enzyme is used; labour costs are also significantly reduced. The overall operating cost of the immobilized enzyme process has been described as 60% of the conventional batch process using the native enzyme.

Immobilized aminoacylase has been used for production of L-alanine (214 kg after 24 h in 1000 ℓ reactor), L-methionine (715 kg), L-phenylalanine (594 kg), L-tryptophan (441 kg) and L-valine (505 kg). In 1982, Degussa in West Germany reported that they had started a plant with annual output 60 t. They use the soluble (native) aminoacylase from Amano, Nagoya, Japan in a membrane reactor, which has resulted in lower enzyme activity losses, reduced contamination, and easier sterilization than if an insolubilization technique was used. Degussa are currently producing 240 t/year.

IMMOBILIZED AMYLOGLUCOSIDASE/GLUCOAMYLASE

Soluble glucoamylase has been used industrially for more than 20 years. It is an extracellular enzyme produced by fungi such as *Aspergillus niger* and *Rhizopus* spp. The enzyme is used in the saccharification of dextrins to glucose, primarily in the starch industry, but also in such industries as distilling and brewing.

The object of immobilizing glucoamylase was to improve the economics of the process, to reduce the size of the saccharification tanks, and at the same time to maintain the quality of the product.

Perhaps the most crucial factor is the percentage of glucose obtainable. The

residence time with soluble glucoamylase is normally in the range of 48–110 h in the dextrin saccharification process. This means that the tank volume required becomes very large, about 7–15 m^3/t d.s. (dry solids) daily production capacity. In this process, glucose percentages of about 95–96 (at a total solids concentration of approximately 30 w/w%) are routinely obtained in industry. However, the highest figures reported in the literature with immobilized glucoamylase products have been 94% which is considered to be significantly lower than 95–96% because this depression has a marked effect on the yield of crystalline dextrose or on the yield of HFCS. The reason for the lower figure is thought to be diffusion resistance. The product glucose is resynthesized, for example to isomaltose (Roels and van Tilburg, 1979b).

This situation might be acceptable if the economics of the process were significantly improved, but this is not yet the case. The important factor as far as the economics are concerned is productivity at an acceptable temperature. A low reaction temperature requires larger tank volumes and special equipment to reduce (or eliminate) the risk of contamination. On the other hand, a high reaction temperature gives a faster enzyme decay. Rugh, Nielsen and Poulsen (1979) report that 55°C is the optimal temperature: however, the immobilized glucoamylase had to be used for 700 h (and there is only 25% residual activity after 700 h at 55°C) before the process became economically superior to that using soluble glucoamylase. Taking the lower product quality into consideration, the whole immobilized glucoamylase concept becomes non-viable (for the dextrin-to-glucose process under discussion).

The concept of immobilized glucoamylase seems to be viable for one process only, i.e. the saccharification of the so-called raffinate. Raffinate (which has a typical composition of 7% fructose, 84% glucose, 4% maltose and maltulose, 2% isomaltose, and 1% each of other disaccharides, DP3 and higher saccharides) is the side stream (or mother liquors) which appears when 42% fructose syrups are upgraded to 55%. The reason why this stream is easier to saccharify is primarily that the dry substance level is 20% w/w or lower. Tate and Lyle are marketing an immobilized glucoamylase for that purpose (*A. niger* adsorbed to granulated charcoal).

IMMOBILIZED HYDANTOINASE

Immobilized hydantoinase is used in the production of optically active amino acids, starting from the racemic amino acid hydantoins which are hydrolysed stereospecifically by the hydantoinase to the corresponding carbamoyl derivatives and finally transformed into the optically active amino acids.

The principle of the preparation of D-phenylglycine, an important intermediate for the synthesis of semisynthetic penicillin and cephalosporins, is illustrated in *Figure 4*. Several hydantoinases apparently exist—some are strictly specific for D-hydantoins, others hydrolyse only the L-form.

According to newspaper reports, the Kanegafuchi Company of Singapore use immobilized hydantoinase(s) for the production of the semisynthetic penicillin and cephalosporin side chains, D-phenylglycine and *p*-OH-phenylglycine.

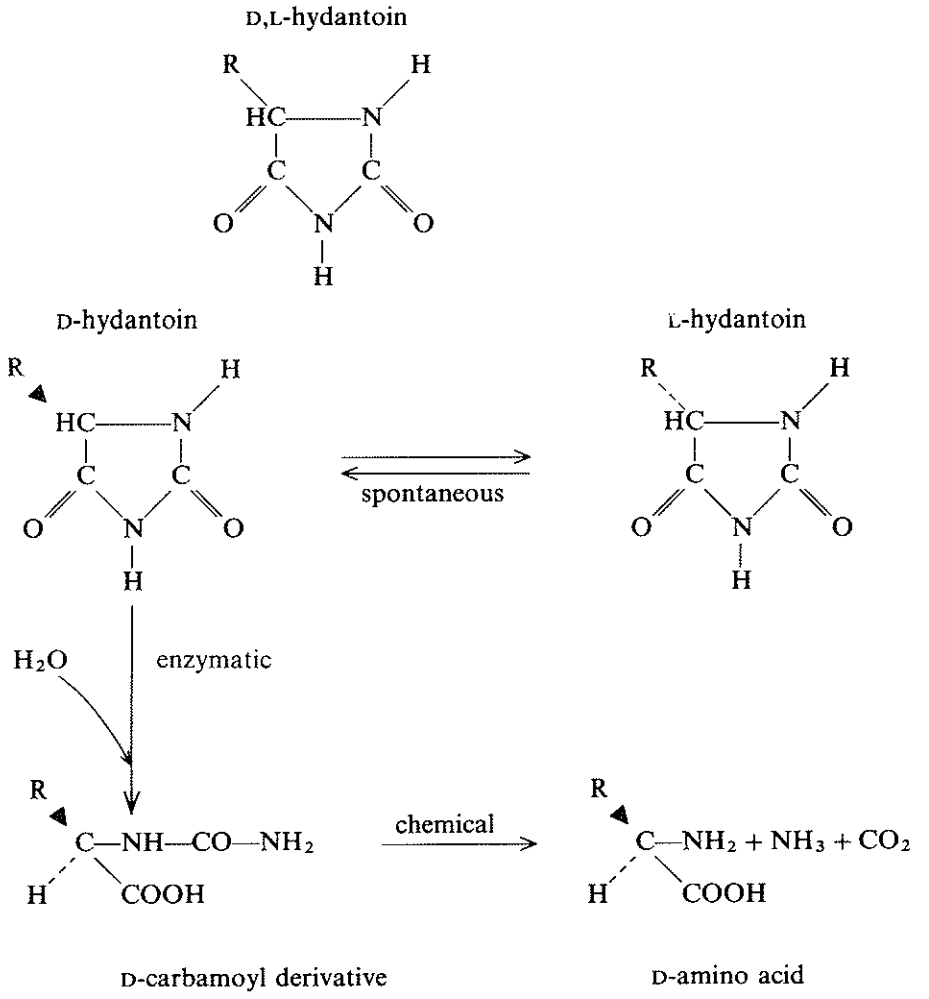

Figure 4. Preparation of an optically active amino acid (D-phenylglycine) using immobilized hydantoinase.

IMMOBILIZED NITRILASE

A new and interesting use of immobilized enzymes is just about to be introduced on a commercial scale, namely the use of immobilized nitrilase for the production of acrylamide from acrylonitrile. Acrylamide has been widely used as a starting material for the production of various polymers for such uses as flocculants, stock additives and polymers of petroleum recovery.

The 'chemical process' for acrylamide involves a copper catalyst. This catalyst is difficult to regenerate and the separation and purification of acrylamide is complex. Furthermore, it would be preferable to produce acrylamide under moderate reaction conditions because compounds like acrylamide contain double bonds and therefore are easily polymerized. Nitto Chemical Industry is about to introduce an enzymatic process on a commercial scale, but only their

patents have been published so far. According to these patents (Watanabe, 1982; Watanabe, Sakashita and Ogawa, 1981; Watanabe, Satoh and Takano, 1981), nitrilase from *Corynebacterium* is immobilized in a cationic acrylamide-based polymer gel. One hundred per cent conversion is claimed when a concentration of 5% (w/w) acrylonitrile or less is used in aqueous solution at 0–10°C and pH 7–8·5. Further news of this promising development is awaited with interest.

Conclusions—the future

The first conclusion is that, among the numerous projects which have been described in the literature, there have been very few immobilized enzyme systems which can be described as an industrial success. The only two (or perhaps three) are immobilized glucose isomerase and the immobilized penicillin G and V acylases, and even these have been produced for a long time. All the established processes pertain to carbohydrate processing, to penicillin/cephalosporin processing, and to separation of amino acids. It seems that many projects have been started before the real need and the characteristics of the market have been determined. To obtain a technical success is very seldom sufficient and many other criteria must be fulfilled before industrial success is assured. The immobilized glucose isomerase did not become a success until the market was ready and the technical problems were solved. The same could be said for the immobilized penicillin G and V acylases. It should be borne in mind that the immobilized enzyme systems will have to be very cheap, very heat-stable, and will have to work in reliable optimized process systems. In addition, if there is a cheap soluble enzyme on the market or if other processes are well established, then it is very doubtful whether a newly developed immobilized enzyme system can survive.

No single immobilization method seems to be generally applicable if an optimized immobilized enzyme system is required. One has to be flexible and let the process conditions and the nature of the enzyme determine the optimal method of immobilization. The choice of immobilization method therefore appears to be only a question of engineering.

What will the future bring with regard to new immobilized enzyme systems? That is, of course, a very difficult question. However, the newest immobilized enzyme system, the immobilized nitrilase, could become an opening to a new area. In the past, immobilized enzymes opened routes to new products or alternative routes to chemical routes. Besides continuation of this development, the future could also show more examples of substitution of inorganic catalysts within organic synthesis. Organic synthesis could mean synthesis of lipids, fats and petrochemicals. This would further mean that, whereas all existing immobilized enzyme systems work in an aqueous phase, more research and development will be needed on topics such as enzyme behaviour in two-phase systems, organic solvents, etc. If organic chemists could be convinced that the use of immobilized enzyme systems would also offer them a choice in their process optimization, then the immobilized enzyme field could gain a new, strong impetus.

To sum up, there are interesting perspectives within the field of immobilized

enzyme systems, but we will have to develop better process techniques with complicated systems, we will have to be very selective with respect to the processes we try to scale up, and we will have to improve our analysis of the target market.

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