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Immobilization of Polysaccharide-degrading Enzymes

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Introduction

The development of biomass conversion processes for the commercial use of complex plant polysaccharides is a major focus of biotechnology research. Lignocellulosics, which comprise the cell wall materials of all woody plants, grasses and cereals, constitute the largest single biomass resource on Earth (Tan, Wong and Saddler, 1985; Tan *et al.*, 1985). Similarly, starch, the principle food reserve polysaccharide in the plant kingdom, is the next most abundant renewable biopolymer, and forms the primary source of carbohydrates in the human diet (Galliard, 1985). Polysaccharides derived from these sources continuously accumulate in large quantities as by-products of the forestry, pulp and paper, agricultural, food processing, sweetener, brewing and textile industries (Biely, 1985; Wood, 1985). Technologies that could make use of these biopolymers have several advantages including a reduction of waste biomass, an enhancement of the economic competitiveness of bioconversion technologies through the use of abundant and inexpensive starting materials, and the potential development of new products from renewable resources (Saddler and Brownell, 1982). The application of enzymatic degradations is a highly efficient means of conducting biomass conversion processes to create specific, high-quality and valuable products from waste biomass.

At present, the most commercially important enzymes are those that hydrolyse starch (amyloglucosidase, α-amylase, β-amylase, pullulanase), whereas those that degrade plant fibres, including cellulose (cellulases), xylan (xylanases) and pectin

Abbreviations: BSTR, batch stirred tank reactor; CMC, carboxymethylcellulose; CNBr, cyanogen bromide; ConA, concanavalin A; CSTR, continuous stirred tank reactor; DEAA, diethylaminoacetyl; DEAHP, *N*, *N*-diethylamino-2-hydroxypropyl cellulose; HFCS, high-fructose corn syrup; HFS, high-fructose syrup; HPLC, high-pressure liquid chromatography; PBR, packed bed reactor; PE, pectin esterase; PEG, polyethylene glycol; PEI, polyethyleneimine; PG, polygalacturonase; PMGL, endopolymethylgalacturonate lyase; PNPG, *p*-nitrophenyl glucosides; PVA, polyvinylalcohol; STR, stirred tank reactor.

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(pectinases), or other plant polysaccharides, such as dextran (dextranase) and inulin (inulinase), continue to gain in industrial importance for the production of specific products. Glucose is the most commercially successful product of the enzymatic hydrolysis of both cellulose and starch and has a vast number of uses, including as a feedstock additive for single-cell protein processes, as a fermentable compound in the brewing industry, and in the production of chemical reagents and fuels, such as ethanol (Woodward and Zachry, 1982; Cheetham, 1985; Woodward, 1989). Furthermore, when cellulose or starch degradations are combined with the use of glucose isomerase the ultimate product is fructose syrup, which has wide uses in the baking, soft drink and confectionery industries (Fogarty, 1983). Glucose/fructose syrups can also be produced in other ways. For example, the hydrolysis of inulin-rich plants, such as the Jerusalem artichoke, by inulinases offers a direct production of fructose syrups, whereas the hydrolysis of sucrose or raffinose by invertase is another commercial method for the production of glucose/fructose syrups (Goodwin and Mercer, 1983).

Pectin degradation by pectic enzymes constitutes a common industrial practice for improving the quality and yield of fruit and vegetable juices during processing (Rexová-Benková and Mracková-Dobrotová, 1981; Borrego *et al.*, 1989). Xylanase enzymes have been applied to the pulp and paper industry (Dekker, 1983) and to fruit and vegetable juice production (Biely, 1985), and the hydrolysed products of xylan can be used for the production of fermented chemical reagents (Roy, Roy and Duke, 1984; Yu and Saddler, 1985). Dextranases have direct application in the production of molasses and sucrose syrups from sugar cane and sugar beet (Margaritis and Pace, 1985).

The enzymatic degradation of biopolymers offers several advantages to a commercial process:

1. enzymatic methods are highly efficient and environmentally safe procedures for biomass conversion;
2. enzymes exhibit excellent specificity with respect to substrates used and products created; and
3. enzymatic conversions can be carried out under conditions of moderate pH and temperature (Chakrabarti and Storey, 1988).

By contrast, acid-catalysed breakdown methods frequently result in the formation of undesirable by-products (Coker and Venkatasubramanian, 1985).

However, enzymatic degradation still has some disadvantages, chiefly problems of cost and reusability of catalysts. The production of purified enzymes for bioconversion processes is a major expenditure. Furthermore, reuse of enzyme catalysts is impractical in many systems because they are difficult and uneconomical to recover from a reaction mixture (Hartmeier, 1985). The economics of commercial biomass conversion processes demand that optimum conditions be determined for enzyme production and use (Schafhauser and Storey, 1992a). Two lines of research are aimed at lowering the costs of enzymatic procedures through:

1. the development of new strains of microbial mutants with higher specific activities of enzymes than those currently in use; and
2. the development of immobilization methods for the attachment of enzymes to solid supports to allow for their repeated use (Takeuchi and Makino, 1987; Woodward, 1987).

This latter topic is the subject of the present chapter.

IMMOBILIZED ENZYMES

The application of immobilized enzyme technology has great potential for improving the industrial uses of enzymes. The overall cost of an enzyme-catalysed industrial process can be greatly reduced if the enzymes can be easily recovered and reused, usually by simple filtration or centrifugation (Ohba *et al.*, 1978; Cheetham, 1985). Immobilization can also reduce the conversion time because very high enzyme concentrations can be used and recovered (Pitcher, 1980). Kinetic properties and pH or temperature optima of immobilized enzymes can also frequently be manipulated to favour specific applications, and enzyme stability is often enhanced upon immobilization (Klibanov, 1979). Enzymes within the same family, as well as those than can operate under similar conditions of pH and temperature, can also be co-immobilized to create multienzyme systems or pathways. The advantages of such systems are that faster reaction rates and more efficient substrate hydrolysis can often be achieved by a co-immobilized enzyme system compared with the same enzymes free in solution (Cheetham, 1985).

An estimation of the commerical potential of a particular immobilized enzyme system requires a thorough knowledge of certain parameters affecting its behaviour under operational conditions (Ohba *et al.*, 1978). The activities and properties of an enzyme can change quite dramatically upon immobilization, and can be attributed to conformational effects, microenvironmental effects and diffusional or mass-transfer effects (Kobayashi and Laidler, 1972). During immobilization enzymes generally experience some conformational changes which can result in changes in substrate affinity or allosteric properties, subunit dissociation or even enzyme inactivation (Klibanov, 1979; Cheetham, 1985). However, the immobilization step, which frequently involves multipoint binding of the catalyst molecule to a support, typically results in a more stable enzyme. Thus, immobilized enzymes are generally more resistant to unfolding and deactivation in response to shifts in pH or temperature, or exposure to chemicals and impurities in the biopolymer substrate mixture, and are less accessible to microbial attack relative to the free enzymes (Klibanov, 1979).

Once immobilized, enzymes come under the influence of the microenvironment of the support which may be drastically different from that which the enzyme experiences free in solution (Klibanov, 1979). Substrates and supports of opposite charge can result in attractive forces which may enhance mass transfer and increase the local concentrations of substrates (Kobayashi and Laidler, 1972). Electrostatic effects and shifts in pH optima also occur when enzymes are immobilized on to charged surfaces (Klibanov, 1979). A negatively charged matrix usually results in an alkaline shift in the pH optimum, due to the creation of a microenvironment of H^+ ions surrounding the matrix, which lowers the pH of the microenvironment surrounding the enzyme compared to that of the bulk solution (where the pH is measured). The opposite effect is created by the use of a positively charged matrix (Zaborsky, 1973). The same is true for non-polyelectrolytic supports if the partition coefficient for H^+ ions in the microenvironment and the bulk solution is not unity (Klibanov, 1979). The microenvironment of the immobilized enzyme can also possess buffering properties that can stabilize the enzyme against pH changes (Klibanov, 1979).

Since enzymes are frequently immobilized within porous bodies or retained by semipermeable membranes, mass-transfer limitation problems, particularly with substrates of large molecular weight, usually occur. Mass-transfer effects can limit the efficiency of the catalytic action of the enzyme as well as change its apparent kinetic behaviour (D.D. Lee *et al.*, 1976). Strongly diffusion-limited systems can also give the impression of stability (Caldwell *et al.*, 1976a,b). Diffusion limitation results in the creation of an unstirred layer of solvent called the Nernst layer, which surrounds water-insoluble particles, creating a concentration gradient (Zaborsky, 1973). As a result, the immobilized enzymes will usually become saturated with substrates at a higher concentration than that normally required for saturation of the free enzyme, giving rise to higher K_m values (Zaborsky, 1973). Diffusion limitation affects initial rate assays and, thus, distorts the standard plots used to estimate kinetic constants (Pitcher, 1980). Diffusion limitation effects are more apparent at high enzyme activities, and their presence in a system can be most easily detected with certain types of assays, such as via analysis of activity versus assay temperature (Arrhenius plots) (Pitcher, 1980).

External mass-transfer resistance depends primarily on particle size and fluid velocity around the particle, which is influenced by mixing conditions in stirred tanks and fluid velocity in mixed beds. Increases in stirrer speed or flow rate have been shown to minimize the problem (Bucholz and Klein, 1987). Internal mass transfer (or pore diffusion effects) is another phenomenon affecting the efficiency of an enzyme when immobilized on to porous supports. However, the apparent activity of such immobilized enzymes can be increased by either using smaller support particles, thus decreasing the diffusion path length, or by reducing the activity of the enzyme through lower enzyme loadings or lower reaction temperatures (Pitcher, 1980).

ENZYME IMMOBILIZATION METHODS AND BIOREACTOR TYPES

The economics of immobilized enzyme technology are dependent on several factors that should be considered when choosing an immobilization technique, including the cost of the support and any necessary chemicals, and whether the method is simple, reproducible, mild, cheap and safe. The resulting immobilized enzyme is worthwhile provided that the preparation remains firmly bound and stable over a wide range of conditions and with repeated uses (Kennedy, 1985; Sórensen and Emborg, 1989). Immobilization represents an added expense and operation for industries. Therefore, it must be shown that the extra costs involved can be justified by the potential savings of such a system after it is successfully implemented.

Several methods of enzyme immobilization have been considered:

1. adsorption on to solid supports;
2. covalent attachment to water-soluble or water-insoluble supports;
3. covalent cross-linking to bifunctional reagents;
4. entrapment in polymeric gels; and
5. encapsulation in microcapsules or hollow fibres (Klibanov, 1979).

Each enzyme immobilization method has its own advantages and disadvantages (*Table 1*).

Table 1. Summary of immobilization methods for polysaccharide-degrading enzymes

Method	Advantages	Disadvantages	Types of supports
Adsorption	Fast, easy, inexpensive No chemical reactants Easily regenerated and reusable Supports are often natural products	Enzymes easily desorbed due to changes in pH, ionic strength and substrate concentration	Collagen, chitin, bone, cotton, cellulose, dextran, ion-exchange resins, hydrophilic membranes, polyethylene glycol
Covalent attachment	Firm and permanent binding	Frequently causes enzyme inactivation and/or alters catalytic activity Chemical reagents often unsuitable for food industry	Nylon, silica, alumina, celite, bentonite, clay, titania, magnetite, hydrophilic membranes, polyvinyl alcohols
Covalent cross-linking	Firm and permanent binding Enhanced binding and stability compared with above methods	Immobilization procedures are long Added expense of chemical reagents Can alter catalytic activity	Glutaraldehyde, concanavalin A, polyethyleneimine, formaldehyde, aminopropyltriethoxysilane, epichlorohydrin
Entrapment	Gentle, simple No chemical modification of enzyme	Harsh polymerizing agents Unsuitable for enzymes that hydrolyse high molecular weight polymers	Gelatin, agarose, polystyrene, polyurethane, acrylamide, calcium alginate
Encapsulation in microcapsules, hollow fibres	No chemical modification of enzyme Easily scaled-up	Viscous solutions can clog reactors Substrate concentration gradients at support surface	Gelatin, nylon, polysulphone, calcium alginate, methyacrylate, polyvinyl alcohol

Immobilization by physical adsorption and ionic binding are the easiest and fastest methods (Pitcher, 1980). Since no reactive species are involved there is little or no conformational change to the enzyme following immobilization (Kennedy, 1985). However, because the binding forces involved are not permanent, changes in ionic strength, pH or substrate concentration can cause enzyme desorption (Solomon, 1978; Kennedy, 1985). In spite of this, such supports are popular, for they are usually easily regenerated and reused (Stanley *et al.*, 1976; Antrim and Auterinen, 1985).

Covalently bound enzymes give enhanced stability due to the strength and permanence of the bonds created (Solomon, 1978; Storey, Duncan and Chakrabarti, 1990). However, the conditions for achieving immobilization are more complicated and harsh, making enzymes more prone to inactivation (Kennedy, 1985). Furthermore, covalent binding methods are not used extensively in some fields, such as food processing, because the chemical reagents used for binding are not allowed in the final product (Pitcher, 1980).

Entrapment methods are usually gentle and simple, resulting in high concentrations of contained enzyme without chemical modifications or bindings that may lead to activity loss (Mazid, 1993). However, due to diffusion limitations, such methods are often unsuitable for the immobilization of enzymes that hydrolyse large molecular weight substrates (Solomon, 1978; Storey, Duncan and Chakrabarti, 1990).

A variety of bioreactor types are available for use in immobilized enzyme processes, depending on the requirements of a particular system. These include the stirred tank reactor (STR), continuous stirred tank reactor (CSTR), batch stirred tank reactor (BSTR), packed bed reactor (PBR), and membrane reactor (Gemeiner, Stefuca and Báles, 1993). The high recirculation rates of the STR minimize both the destabilization of the enzyme caused by any localized changes in reaction parameters (e.g. pH) and film diffusion resistance (Roy *et al.*, 1989). BSTR operations are broadly applicable, flexible and relatively inexpensive, whereas CSTR processes, by comparison, demand greater initial capital output for reactor design and control automation, but are more catalytically efficient processes, producing a more reliable product in a shorter reaction time (Celebi, Tsai and Tsao, 1991; Mazid, 1993). The effective use of a PBR depends on the fluidity of the reactor liquid. High molecular weight, viscous solutions can clog the column, resulting in a pressure drop across the bed (Ohba and Ueda, 1980; Pieter, Bardeletti and Coulet, 1992; Gemiener, Stefuca and Báles, 1993). Alternatively, substrate concentration gradients can develop due to internal and external diffusion limitations, which can in turn influence the catalytic efficiency (Chen and Wu, 1987). Similarly, enzymes immobilized on to small particles can also lead to pressure build-up when used in a PBR (Y.Y. Lee *et al.*, 1976).

Membrane reactors, such as the hollow fibre bioreactor and the membrane recycle bioreactor, exploit the differences in size between the enzyme and substrate trapped on one side of the membrane and the smaller molecular weight products which are freely diffusible and can be removed continuously from the reaction mixture (Darnoko, Cheryan and Artz, 1988). Although membrane bioreactors generally show higher productivities than batch processes, the catalytic efficiency can be limited due to the formation of a 'gel layer' on a membrane surface and a resultant decrease in overall permeation (Darnoko, Cheryan and Artz, 1988).

TYPES OF IMMOBILIZATION SUPPORTS

In order to be considered for use as enzyme immobilization supports, compounds must possess an adequately sized porous surface for binding, be strong, inert, and chemically and physically stable (Caldwell *et al.*, 1976a,b). A variety of diffusion supports are currently known (*Table 1*), and new ones are continually being developed and applied.

Organic compounds, including natural polymers (e.g. collagen, chitin, cellulose, bone and gelatin) and synthetic polymers (e.g. nylon, ion-exchange resins and polyurethanes) have been used in immobilized enzyme reactors. Granular chicken bone (Biobone™) and chitin are ideal supports, easily obtained from meat and seafood processing wastes, and are abundant, inexpensive, non-toxic and food grade (Findlay, Parkin and Yada, 1986; Emneus, 1993). Cellulosic materials are also inexpensive and available in many different physical forms (e.g. fibres, microcrystals, beads and membranes) (Gemeiner, Stefuca and Báles, 1993), but exhibit high hydrodynamic resistance due to compaction and clogging when used in a column (Sharma and Yamazaki, 1984). Cotton flannel exhibits high protein adsorption and shows good flow-through properties (Yamazaki, Cheok and Fraser, 1984). Gelatin is a good support matrix for entrapping enzymes, having a marked swelling power and low resistance to mass transfer (De Alteriis *et al.*, 1985).

Polyurethane foams offer a stable and homogeneous distribution of enzymes throughout the matrix and are flexible and non-reactive once polymerized (Chakrabarti and Storey, 1988). Nylon is an inexpensive support that is also available in different physical (e.g. films, membranes and powder) and chemically modified forms (Iborra *et al.*, 1992). Hydrophilic membranes, traditionally used for nucleic acid blotting and hybridization techniques, have a high protein binding capacity and good flow-through properties and represent another potential enzyme support matrix (D.Y. Schafhauser-Smith and K.B. Storey, unpublished results).

Enzymes covalently bound to polymeric carriers (e.g. styrene, acrylamide) are the most commonly used supports in continuous column reactions (Woodward, 1987). However, such polymers are relatively soft and thus prone to column plugging when operating at high flow rates and high substrate concentrations (Martensson, 1974a; Nithiandam *et al.*, 1981).

Inorganic supports (e.g. silica, alumina, celite, bentonite) have been used as carriers for the immobilization of numerous enzymes and are often preferred over organic supports due to their availability in a wide range of controlled particle sizes and porosities, their reduced susceptibility to microbial attack, and their overall mechanical and chemical stability (Kobarzewski and Paszczynski, 1983; Bajpai and Margaritis, 1987; Shimizu and Ishihara, 1987). Inorganic magnetic supports have additional advantages in that they allow for easy separation of immobilized enzymes from STR hydrolyslates through the application of a magnetic field (Dekker, 1990).

Derivatization, synthetic and chemical transformation methods are often used in an effort to make carrier surfaces more suitable for enzyme binding. Such modifications typically result in the incorporation of reactive amino and carbonyl groups on to the support surface hydroxyl groups (Weetall, 1993). Such measures may also serve to coat and thus strengthen the walls of the support, resulting in greater physical, chemical and hydrodynamic properties (Lopez-Santin *et al.*, 1983). Derivatization

can also introduce spacer groups, which increase the local surface area of the support and consequently reduce the steric hindrance in the immediate vicinity of the enzyme (Abdel-Naby, 1993). However, multiple fixation of an enzyme to the more reactive matrix surface can cause a decrease in catalytic activity owing to a decrease in the flexibility of the enzyme.

One of the most common means of introducing free amino groups on to a support is by a silanization reaction involving aminopropyltriethoxsilane (Wojick *et al.*, 1987; Weetall, 1993). Cross-linking with glutaraldehyde will generate reactive surface aldehyde groups (Wojcik *et al.*, 1987). Polyethyleneimine (PEI), a polybasic aliphatic amine, is routinely used either as a cross-linking agent or as a carrier in a number of immobilized biosystems (Bahulekar, Ayyangar and Ponrathnam, 1991). Concanavalin A (ConA) is a useful ligand for the immobilization of such industrially important glycoproteins as glucose oxidase, invertase, cellulase and amyloglucosidase (Saleemuddin and Husain, 1991). ConA-linked matrices usually exhibit high activity retention and enhanced stability against various types of inactivation (Saleemuddin and Husain, 1991).

The parameters used for the coupling of an enzyme to a particular matrix are usually optimized to allow for the best binding conditions for each particular enzyme (Martensson, 1974a). However, in order to be cost-effective, the ideal immobilization procedure should be a fast and simple one. Enzyme adsorption onto granular chicken bone is a firm and immediate reaction, with activity being retained after only a 20 min binding period (Schafhauser and Storey, 1992a,b). Enzyme immobilization onto hydrophilic polyurethane foam is also fast, requires no special catalysts, and results in irreversible binding (Chakrabarti and Storey, 1988, 1989; Storey, Duncan and Chakrabarti, 1990). However, enzyme immobilization onto alkylamine derivatives of metal-activated glass beads crosslinked with glutaraldehyde is a very long, multistep process (Cabral, Norais and Cardoso, 1981). The preparation of PEI cloth, together with the enzyme immobilization step, is also a long process (approximately 19 h) (Yamazaki, Cheok and Fraser, 1984). The use of methacrylate co-polymers as supports for enzyme immobilization required intensive carrier preparation (Nithiandam *et al.*, 1981; Svec and Kalal, 1978) as well as very long immobilization times (Svec and Kalal, 1978).

IMMOBILIZED BIOCATALYSTS

A number of processes utilizing immobilized biocatalysts are now well established on a commercial level. Immobilized enzymes have been used in affinity chromatography and synthetic chemistry as well as for the production of enzyme electrodes, thermistors and biosensors (Cheetham, 1985). Biosensors, which use O_2 or H_2O_2 electrodes in conjunction with an immobilized oxidase have found use in the food and drink industry. The 'Model 2700 Industrial Analyzer', marketed by Yellow Springs Instruments is one such analyser, which provides a method for determining levels of glucose and sucrose in the sugar, molasses and confectionery industries (Luong, Groom and Male, 1991). Units are also available for the detection of total starch, fructose and dextrose (Mazid, 1993). However, other than glucose isomerase, few of the enzymes with applications for plant biomass conversion processes are presently used in immobilized forms on a commercial scale (Jensen and Rugh, 1987).

Although immobilized enzymes, in competing with free enzymes, may have the advantage of increased stability and catalyst reuse, there are several reasons why immobilized enzymes are still not used in a number of traditional enzyme applications. Where the free enzyme is cheap and the process is already developed, a change to an immobilized system may not be financially beneficial (Hartmeier, 1985). Furthermore, immobilized enzyme preparations have been plagued by high capital costs arising from the use of expensive support materials and from enzyme activity losses caused by harsh immobilization conditions, enzyme instability or reduction in catalytic efficiency once bound (Klibanov, 1979; Kennedy, 1985).

Presented in this review is a compilation of the status of immobilized enzyme technology involving many of the industrially important polysaccharide-degrading enzymes. The review highlights the different immobilization methods that have been used for each enzyme and evaluates the usefulness of immobilized preparations compared with the corresponding free enzyme, and with respect to such production characteristics as pH and temperature optima, operational and storage stability, catalytic function, and the effect of diffusion limitation on productivity. Discussion will centre on practical ideas, with emphasis on the newest literature, and general considerations behind choosing a matrix and coupling a ligand, along with some of the more common problems often encountered with their implementation. It is beyond the scope of this chapter to review extensively all the published work on this subject. Several recent and excellent reviews are available in the areas of immobilized enzyme theory (Zaborsky, 1973; Cheetham, 1985; Kennedy, 1985; Buchholz and Klein, 1987), immobilized enzyme supports and coupling methods (Hartmeier, 1985; Bahulekar, Ayyangar and Ponrathnam, 1991; Saleemuddin and Husain, 1991; Weetall, 1993), and immobilized enzyme uses (Coker and Venkatasubramanian, 1985; Heath and Belfor, 1990; Chang and Furusaki, 1991; Luong, Groom and Male, 1991; Gemeiner, Stefuca and Báles, 1993; Mazid, 1993). Several excellent reviews also focus on specific polysaccharide-degrading enzymes: cellulases (Kyosov and Consultant, 1986; Knowles, Lehtovaara and Teeri, 1987; Woodward, 1987, 1989; Klyosov, 1990; Pitson, Seviour and McDougall, 1993), hemicellulases (Biely, 1985), amylases (Fogarty, 1983; Ward, 1985; Wiseman, 1985), glucose isomerases (Chen, 1980; Verhoff *et al.*, 1985; Jensen and Rugh, 1987), or their substrates: starch (Solomon, 1978; Banks and Muir, 1980; Kandler and Hopf, 1980; Galliard, 1985; Finn, 1987; Kennedy *et al.*, 1987), and lignocelluloses (Wood, 1985; Lewis and Paice, 1989; Coughlan and Hazlewood, 1993a).

The immobilization of starch-degrading enzymes

The main commercial sources of starch are corn in the US, wheat in Canada and Australia, and grain (wheat and barley) or root (potato) crops in Europe (Kennedy *et al.*, 1987). Cassava is one of the most efficient producers of carbohydrates, and is the fourth most important source of food energy in tropical countries after rice, cane sugar and corn (Pitcher, 1980). Sweet potatoes, taro and tannia are also good sources of starch (Klyosov and Consultant, 1986).

Starch is the same regardless of source, but starch granules are of a size and shape that is characteristic of individual species. The site of the main storage area of starch varies from plant to plant, for example, being the seed in corn, the tubers in potato and

the pith in sago. Starch granules are typically 25% amylose and 75% amylopectin (Banks and Muir, 1980). The characteristic features of amylose and amylopectin are their linear chains of (1-4)-linked α-*D*-glucopyranosyl residues; in addition, amylopectin has 1,4,6-tri-O-substituted residues which act as branch points (Kennedy *et al.*, 1987). Significant portions of *D*-glucose-6-phosphate and lipid material have been found in starches. Up to 1 residue in 6 in amylopectin is phosphorylated whereas the lipid material is believed to be complexed with the amylose structure (Kennedy *et al.*, 1987). Amylose is a linear molecule in the molecular weight range of 1.6×10^5 to 7.1×10^5 and varies with the source and maturity of the starch (Kennedy *et al.*, 1987). In neutral solutions amylose normally exists as a random coil but in the presence of complexing agents (e.g. iodine, fats, polar organic solvents) it forms a regular helical structure fixed via hydrogen bonding. Amylopectin has a highly branched structure with a molecular weight of about 10^8. Branches are believed to be arranged in either a comb-like or a tree-like fashion (Kennedy *et al.*, 1987). Natural starches suffer from poor solubility, inconsistent viscosity and susceptibility to acid degradation. For industrial use this can cause problems; for example, the properties of a food product containing natural starch can change on storage. For this reason, most industries make extensive use of modified starches. Most processes rely on heat and/or acid treatment which results in starches with lower viscosity and an increased number of branched chains (Finn, 1987). As a result, modified starches are more soluble and have a lower tendency towards 'set back' or gelling on standing.

AMYLOGLUCOSIDASE

Amyloglucosidase (or glucoamylase, EC 3.2.1.3.) is an exo-splitting enzyme that catalyses, by a multichain mechanism, the stepwise hydrolysis of α-1,4 linkages and, to a lesser extent, α-1,6 linkages from the non-reducing ends of starch (and glycogen) (Fogarty, 1983). Preparations may be obtained easily from culture filtrates of several fungal species of *Aspergillus* and *Rhizopus*, and from certain yeasts and bacteria (Fogarty, 1983). Amyloglucosidase is one of the most industrially important enzymes, and is used in the large-scale production of high-glucose syrups (96–98% *D*-glucose) from starch (Fogarty, 1983). High-glucose syrups are used either for the production of crystalline *D*-glucose or as a starting material for the production of high-fructose syrups which are extensively used in the baking, soft drink and confectionery industries (Fogarty, 1983).

Compared with the large-scale degradation of starch to glucose by the soluble enzyme (typical conditions are pH 4.5, 55–65°C for 48–72 h), immobilization of amyloglucosidase reduces the conversion time because very high enzyme concentrations can be used and recovered (Pitcher, 1980). The immobilization of amyloglucosidase on to different inert supports by entrapment, adsorption or covalent binding systems has been widely studied in the hopes of attaining a more controlled reusable system that can be successfully used commercially. However, immobilized amyloglucosidase has not yet found commercial success because of the difficulty in obtaining similar or higher glucose yields than with the free enzyme.

To be useful, an immobilized enzyme must remain stable and active. Schafhauser and Storey (1992b) demonstrated that 14% of added amyloglucosidase remained stable and firmly bound to granular chicken bone following repeated washes,

maintained maximum activity following 5 cycles of operation both at 23°C and 55°C, and showed a storage half-life of 144 days at 4°C. Unlike the free enzyme, immobilized amyloglucosidase was protected from the inhibitory effects of Triton X-100, ethanol, KCl and pH extremes (Schafhauser and Storey, 1992a). The high hydrophilicity of the collagen present in bone helps to prevent enzyme denaturation (Coulet, 1974). Storey, Duncan and Chakrabarti (1990) cross-linked 25% of added amyloglucosidase on to the polyurethane prepolymer Hypol^{R}FHP2002 and showed improved enzyme stability under several denaturing conditions (NaCl, urea, ethanol, 95°C), and an improved storage stability of the bound (70%) over the free (50%) enzyme after 2 months at 4°C. Amyloglucosidase immobilized on to acid-hydrolysed starch-g-polyacrylonitrile resulted in a stable and active enzyme, with glucose productivities reaching 2.7 g g^{-1} carrier h^{-1} and half-lives up to 1800 h in a continuous stirred tank reactor maintained at 50°C, pH 4, and using maltose as the substrate (Slinginger, Fanta and Abbott, 1988). Amyloglucosidase bound to chitin particles packed into a 180 ml two-phase bench scale reactor (liquid expanded bed) fed with hydrolysed manoic starch (15% w/v), operated continuously for 20 days, obtaining 96% conversions of starch to glucose with a hydraulic residence time of 4 h (Freire and Sánt'Anna, 1990). In another study, 19% of added amyloglucosidase bound ionically on to co-polymers of methacrylate and maintained good storage stability with no loss of activity during 47 days of storage (Nithiandam *et al.*, 1981). Glutaraldehyde-cross-linked silanized glass has been shown repeatedly to be a good enzyme support matrix. Cabral, Novais and Cardoso (1981) bound 12% of added activity on to glutaraldehyde-cross-linked metal-activated glass beads and demonstrated a long reaction half-life (1500 h) in a draw-and-fill basket reactor using 1% w/v soluble starch with no leakage. Other authors obtained a glucose productivity of 2.6 g g^{-1} carrier h^{-1} and a half-life of 2550 h (at 50°C and pH 4.5) with amyloglucosidase bound to glutaraldehyde-cross-linked glass beads (D.D. Lee *et al.*, 1976). Amyloglucosidase immobilized on to magnetic microparticles made of PEI-coated magnetite cross-linked with glutaraldehyde continuously hydrolysed a 30% w/v maltodextrin solution at 50°C and pH 4.5 in a fluidized bed reactor; continuous two week hydrolysis resulted in 70% conversions with only 5% of initial immobilized amyloglucosidase activity lost (Pieter, Bardeletti and Coulet, 1992). This system had the added advantage that the particles were easily settled because of their high density (5 g ml^{-1}) and the loss of ultrafines due to washout was prevented by surrounding the top of the bed with an electromagnet (Peiter, Bardeletti and Coulet, 1992). Amyloglucosidase immobilized on to silanized alkylamine glass beads in a packed bed reactor maintained 80% of maximal operational activity after 30 days of continuous use with 1% soluble starch, while showing only a 20% drop in storage stability (Kennedy, 1985). Amyloglucosidase coupled to anion exchange resin resulted in a system that showed no activity loss when stored at 4°C for 6–8 months, was reusable for at least 10 cycles, showed an increased stability towards urea and metal ions, and could convert high concentrations of starch hydrolysate (32% w/w) into 96–98% w/w glucose in 24 h at 50°C and pH 4.5 (Nehete *et al.*, 1987). Amyloglucosidase immobilized in an ultrafiltration reactor (YM-5 flat sheet membrane) for the continuous hydrolysis of cassava starch, showed conversions to glucose of 64% at 22°C and 97% at 55°C in 2 h (Darnoko, Cheryan and Artz, 1988).

Many researchers, in an effort to enhance and retain enzyme binding to their chosen

support material, have taken the extra step of using chemical cross-linking reagents such as carbodiimide and glutaraldehyde in an effort to enhance enzyme binding (Caldwell *et al.*, 1976b; D.D. Lee *et al.*, 1976; Svec and Kalal, 1978; Kobarzewski and Paszczynski, 1983; Nehete *et al.*, 1987; Freire and Sánt'Anna, 1990; Pieter, Bardeletti and Coulet, 1992; Emneus, 1993). Amyloglucosidase immobilized on to granular chicken bone cross-linked with silanized glutaraldehyde, silanized carbodiimide, and DEAE showed a 10–40% increase in the amount of activity bound over non-derivatized bone (Schafhauser and Storey, 1992a). Enhanced binding of amyloglucosidase to chitin was also obtained following pretreatments of the support with 25% glutaraldehyde (D.Y. Schafhauser-Smith and K.B. Storey, unpublished results).

Some enzyme systems are very unstable once immobilized or are easily inactivated or desorbed from the support, due to unfavourable conformational changes of the bound enzyme or due to the formation of weak bonds that are easily disrupted by environmental changes (pH, ionic strength of buffer) (Kennedy, 1985). Svec and Kalal (1978), using amyloglucosidase immobilized on to poly[(glycidyl methacrylate) co(ethylene dimethacrylate)] carriers, found a drop in temperature stability and a 70% loss of bound activity after 20 h, partly due to inactivation, partly to further coupling reactions of the enzyme. Amyloglucosidase bound to DEAE-cellulose-activated cyanuric chloride retained 62% of added activity and showed starch conversions of 56%; however, there was constant enzyme leakage with successive buffer washings at pH 3–6.6, a drop in activity in successive batch type processes, and a loss of temperature stability (Tomar and Prabhu, 1985). Amyloglucosidase immobilization on to the gel-forming polymer, Hypol^{R}8190H, resulted in only 1–4% of active bound activity, and a high loss (>68%) of total enzyme activity added (Storey, Duncan and Chakrabarti, 1990). The immobilization of amyloglucosidase on to hydrophilic membranes retained 46 to 51% of added activity, but resulted in higher rates of temperature deactivation, as well as a 30–40% loss of bound activity after nine cycles of use (D.Y. Schafhauser-Smith and K.B. Storey, unpublished results).

A suitable immobilized enzyme system should show functionality within the optimal industrial working range for amyloglucosidase (pH 4.5, 60°C), or show an enhanced range of productivities. The immobilization of amyloglucosidase usually results in pH stability equal to that of the free enzyme (pH 4.5) (Stanley *et al.*, 1978; Slinginger, Fanta and Abbott, 1988; Storey, Duncan and Chakrabarti, 1990; Schafhauser and Storey, 1992a), and maintains an optimal temperature of 50–60°C or better (Svec and Kalal, 1978; Nithiandam *et al.*, 1981; Storey, Duncan and Chakrabarti, 1990; Schafhauser and Storey, 1992a). However, Slinginger, Fanta and Abbott (1988) found catalyst decay at 60°C with enzyme immobilized on to starch graft co-polymers but showed that by operating at 40°C instead of 60°C the half-life of the enzyme could be increased sixteen-fold while decreasing the productivity by a factor of only 4. Thus, the major considerations that influence the choice of the most profitable operating temperature include the required production rate based on demand, capital availability for investment in reactor volume, the length of downtime and the expense of recharging the reactor (Slinginger, Fanta and Abbott, 1988).

The net charge on a carrier can have a profound influence on the pH activity behaviour of an immobilized enzyme due to the influence of the microenvironment surrounding it (Nithiandam *et al.*, 1981; Slinginger, Fanta and Abbott, 1988). An

acidic shift in pH optimum from 5.5 to 4.6 occurred upon amyloglucosidase immobilization on to 2-dimethylaminoethyl methacrylate (Nithiandam *et al.*, 1981). As this carrier has a net positive charge, the shift to the lower pH optimum upon immobilization can be explained by a depletion of H^+ ions in the vicinity of the enzyme compared with H^+ concentration in the bulk solution (Nithiandam *et al.*, 1981). Amyloglucosidase immobilized to DEAE-cellulose showed a narrowing of the pH optimum from 3.8–5.2 to 3.6 (Tomar and Prabhu, 1985). The optimum pH range also became narrower with immobilization on to poly[(glycidyl methacrylate) co(ethylene dimethacrylate)] carriers (Svec and Kalal, 1978). However, Nehete *et al.* (1987) found that immobilization on to polystyrene anion-exchange resin did not alter the pH optimum of the enzyme, indicating an absence of partitioning effects of the substrate in the microenvironment of the immobilized enzyme.

Researchers frequently report changes in enzyme kinetic parameters upon immobilization, usually as the result of harsh immobilization procedures, retention by semipermeable membranes, or immobilization within porous bodies. Active site modification due to multiple point attachment of enzymes on to matrix surfaces may cause a decrease in substrate affinity (Zaborsky, 1973). Such problems are usually compounded by the use of high molecular weight substrates which cause mass transfer limitation problems. Starch, being a macromolecule, frequently shows diffusion limitation not only with respect to enzyme bound within the porous interior of a carrier, but also with surface-bound enzyme. As a result, the catalytic action and apparent kinetic behaviour of enzymes can be compromised (D.D. Lee *et al.*, 1976).

Some immobilized enzyme systems suffer from a high diffusion resistance as well as an inefficient coupling method (Nithiandam *et al.*, 1981). Darnoko, Cheryan and Artz (1988) determined that the viscosity of even a 1% w/w cassava slurry was too high, causing stirring and fouling of the membrane and excessive pressure build-up. Amyloglucosidase immobilized on to co-polymers of methacrylate showed a ten-fold increase in K_m, as well as a two-fold increase in specific activity (Nithiandam *et al.*, 1981). Amyloglucosidase immobilized on to poly[(glycidyl methacrylate) co(ethylene dimethacrylate)] carriers showed 1.8- to 2.3-fold increases in K_m values, using 1% starch (Svec and Kalal, 1978). The immobilized amyloglucosidase preparation of Tomar and Prabhu (1985) showed a 1.3-fold increase in K_m, and Lineweaver–Burk plots which indicated substrate inhibition. Amyloglucosidase immobilized on to polystyrene anion-exchange resin showed a 1.7-fold increase in K_m using a 32% w/w starch solution (Nehete *et al.*, 1987). Amyloglucosidase bound to hexyl-Sepharose, using soluble starch substrate, also showed a marginal increase in the apparent K_m (Caldwell *et al.*, 1976b).

Amyloglucosidase bound to granular chicken bone showed no significant change in its K_m values, but a 1.7-fold decrease in specific activity occurred with either Lintner or potato starches as substrates (Schafhauser and Storey, 1992a). Other kinetic data from Lineweaver–Burk plots and Arrhenius plots indicated that there were no diffusional limitations imposed by the binding to bone. However, tests using solutions of whole starch indicated that the free enzyme was 23% more effective at hydrolysing the insoluble portions of starch. Vigorous mixing helped to alleviate the problems of external mass transfer resistance, since a 3.3-fold increase in starch hydrolysis was apparent in mixed, compared with unmixed, preparations (Schafhauser and Storey, 1992a).

D.D. Lee *et al.* (1976) immobilized amyloglucosidase on to porous silica (400 Å pore diameter) packed into a recirculating batch reactor operated at high flow rates to eliminate diffusion resistance. Nehete *et al.* (1987) also found that increasing the stirring speed and decreasing the matrix particle size did not change the activity of the immobilized enzyme, thus indicating no diffusion limitation problems. The size of the glass beads (125 μm) used by Toldra, Jansen and Tsao (1992) for the immobilization of amyloglucosidase was small enough to avoid diffusion resistance.

Amyloglucosidase immobilized on Celite R649 (mean pore size 0.25 μm) was as efficient as the soluble enzyme in catalysing the hydrolysis of maltose, but less efficient in hydrolysing 30% w/v maltodextrin. The immobilization of amyloglucosidase on porous glass fibres showed 98.8% conversion of a 28% maltose solution and 93.5% conversion of a 39% dextrin solution (Toldra, Jansen and Tsao, 1992). Not surprisingly, Slinginger, Fanta and Abbott (1988) found K_m values for maltose largely unchanged when amyloglucosidase was immobilized on to starch graft polyacrylonitrile. Thus, lower product yields can occur when high molecular weight substrates are used, for they are either excluded from small carrier pores or diffuse into these pores at a much slower rate than lower molecular weight substrates. Also, glucose can form reversion products if it is slow in diffusing out of the carrier pores (Slinginger, Fanta and Abbott, 1988; Celebi, Tsai and Tsao, 1991; Emneus, 1993). This is not the case with free amyloglucosidase, where no decrease in yield occurred when high molecular weight substrates were used (D.D. Lee *et al.*, 1976; Storey, Duncan and Chakrabarti, 1990; Schafhauser and Storey, 1992a; Toldra, Jansen and Tsao, 1992). Solutions to the problem of maintaining high yields with real feeds can include increasing enzyme reactor residence times or pre-hydrolysing feeds with α-amylase to hasten the process.

Diffusion limitation can also affect the binding of the enzyme itself on to a support. The yield and activity of bound amyloglucosidase was higher when the enzyme was immobilized on to supports with larger pores (Wojcik *et al.*, 1987; Emneus, 1993). Gels with pore diameters of 50, 100, 130 Å showed 3.2, 12.4 and 16.9 units of bound amyloglucosidase per gram of gel, respectively.

Reduced catalyst activity of immobilized enzymes is not always the result of diffusion limitation. Inefficient coupling methods can also have the same result. For example, when amyloglucosidase was immobilized on to silanized alkylamine glass beads linked by carbodiimide coupling, by a modified enzyme carbohydrate, or by glutaraldehyde cross-linking, the percentage of added protein that remained bound was 29, 56.8 and 64.8%, respectively, whereas activity retention was only 11.5, 10.4 and 11.5% (Kobarzewski and Paszczynski, 1983). Amyloglucosidase immobilized on to the DEAE-, carbodiimide-or glutaraldehyde-derivatized granular chicken bone showed two-to sixfold increases in K_m compared with enzyme bound to underivatized bone (D.Y. Schafhauser-Smith and K.B. Storey, unpublished results). Immobilization of amyloglucosidase on to polyurethane foam resulted in a small, 1.1-fold, increase in K_m (Storey, Duncan and Chakrabarti, 1990). However, since high molecular weight compounds such as blue dextran could readily penetrate the foam, the large substrates (>200 kDa) were hydrolysed as effectively as smaller ones, it appeared that the K_m change was not caused by diffusion resistance in this case.

Those immobilization supports that can load high activities of enzyme per gram of support are ideal for scale-up purposes (Svec and Kalal, 1978; Kobarzewski and

Paszczynski, 1983; Storey, Duncan and Chakrabarti, 1990; Pieter, Bardeletti and Coulet, 1992; Schafhauser and Storey, 1992a). The shorter residence times that would result would decrease the number of side reactions and thus lower refining costs (Fogarty, 1983). However, Kobarzewski and Paszczynski (1983) found that the immobilization of amyloglucosidase on to glass beads in amounts greater than 10 mg protein g^{-1} beads showed only a slight increase in substrate hydrolysis, consequently decreasing the practical and economical value of the product. Enzyme loading must be optimized by considering the tradeoff between less efficient enzyme use at high loadings, and the need for greater carrier and reactor size and consequently greater immobilization cost at low enzyme loadings (Pitcher, 1980). Inexpensive supports can help to offset such extra costs (D.D. Lee *et al.*, 1976; Stanley *et al.*, 1978; Cabral, Novais and Cardoso, 1981; Freire and Sánt'Anna, 1990; Storey, Duncan and Chakrabarti, 1990; Schafhauser and Storey, 1992a; Toldra, Jansen and Tsao, 1992).

α-AMYLASE

α-Amylase ([1-4]-α-*D*-glucan amyloglucosidase, EC 3.2.1.1) is a calcium metalloprotease that is widely distributed in nature and found in animal, plant and microbial species (Solomon, 1978; Kennedy *et al.*, 1987). α-Amylase hydrolyses the α-*D*-(1,4) links between the α-*D*-glucopyranosyl residues of starch in a random endo-fashion, but is unable to hydrolyse the α-*D*-(1,6) bonds of amylopectin (Kennedy *et al.*, 1987). The end products are glucose, maltose and α-limit dextrins containing α-(1,6)-glycosidic bonds (Solomon, 1978). α-Amylases have application in the food processing industry for the production of dextrins and glucose syrups for the brewing and baking industries (Solomon, 1978; Kennedy *et al.*, 1987). The microbial α-amylases, in particular, are widely used commercially in the production of specific oligosaccharides. The enzyme from each species produces primarily a single specific oligosaccharide; for example, the major hydrolysis products of the enzymes from *Bacillus lichenformis, B. amyloliquefaciens* and *B. subtilis* are maltopentaose, maltoheptaose and maltohexaose, respectively (Kennedy *et al.*, 1987). The pH and temperature optima for maximum enzyme activities are also species-dependent, but generally occur within a pH range of 4.8–6.5 and a temperature range of 70–80°C (Kennedy *et al.*, 1987).

Sadhukhan *et al.* (1993) immobilized α-amylase from *Myceliophthora thermophila* by covalent binding to cyanogen bromide (CNBr)-activated Sepharose and by entrapment within polyacrylamide gels or calcium alginate beads in order to determine the best carrier for use in the saccharification of starch. Immobilization to calcium alginate beads gave the best improvement in α-amylase stability; compared with the free enzyme, immobilized α-amylase showed a substantial increase in thermal stability (optimal temperature rose from 60 to 65°C), a broadening of the optimal pH range (pH 4–8 versus pH 5.6) and an increase in storage stability (a half-life of 110 days versus 5 days at 28°C). However, 60% of activity was lost after six cycles of reuse (with 1% starch substrate, pH 5.5, 60°C); this was due to enzyme leakage as the result of changes in the gel structure caused by the continuous agitation.

De Cordt *et al.* (1992) analysed the reaction kinetics of the thermostable α-amylase from *B. lichenformis* in free form and when covalently bound to glutaraldehyde-activated glass beads, for application as a possible time–temperature integrator for

evaluation of heat processes. The rate of thermal inactivation (D-value) was shown to be faster for the free enzyme (6–157 min) than the immobilized α-amylase (17–620 min) at 95°C, with the range of values reflecting the variety of different environmental reaction conditions tested (pH, ionic strength, Ca^{2+} concentration). Further studies analysed the performances of three different linking agents (glutaric dialdehyde, benzoquinone, s-trichlorotriazine) for α-amylase immobilization on to porous glass beads (de Cordt, 1994); all three linkers gave a similar improvement in immobilization yield and improved enzyme resistance to heat inactivation to a similar extent.

α-Amylase immobilized by attachment to soluble amino-S-triazinyl derivatives of dextran 2000, DEAE-dextran 2000 and CM-cellulose retained up to 65%, 90% and 19% of added activity, but specific activities decreased by 33%, 67% and 40%, respectively, compared to the free enzyme (Wykes, Dunnill and Lilly, 1971). All bound enzymes were stabilized in the presence of Ca^{2+} and starch, with a marked thermal stability of CM-cellulose-bound α-amylase. Indeed, the CM-cellulose-bound enzyme had a 2.6-fold greater operational half-life than the free enzyme in continuous hydrolysis reactions of soluble corn starch in an ultrafiltration reactor at 70°C (Wykes, Dunnill and Lilly, 1971).

β-AMYLASE

β-Amylase (α-1,4-glucan maltohyrolase, EC 3.2.1.2) hydrolyses α-1,4-glycosidic linkages from the non-reducing ends of starch in an exo-fashion, releasing β-maltose units (Fogarty, 1983). The enzyme occurs widely in higher plants and microorganisms (Kennedy *et al.*, 1987). Maltose and high-maltose syrups have a variety of commercial uses in the confectionery, baking and brewing industries, due to their low viscosity in solution, low hygroscopicity, resistance to crystallization, low sweetness, heat stability and lack of colour formation (Fogarty, 1983); the syrups are also used in the manufacture of vaccines and antibiotics (Ohba, Shibata and Ueda, 1979).

The high production costs associated with the continuous use of fresh enzyme in industrial saccharification processes limits the commercial potential of the process and such has been the case to date with β-amylase. Furthermore, the β-amylases derived from plants are expensive and tend to be unstable, whereas β-amylases from microbial sources are even less active and thermostable (Saha and Shen, 1987). An immobilized β-amylase preparation that could retain high activity and functional stability would, therefore, have considerable industrial potential.

Many attempts have been made at producing an active and stable immobilized β-amylase, and a number of immobilization methods have resulted in improved functional characteristics over that of the native enzyme. The covalent coupling of β-amylase to soluble conjugated polysaccharides (dextran, amylose) made the bound enzyme as accessible as the native enzyme to macromolecular starch (Lenders and Crichton, 1984). The immobilized β-amylase also showed greater temperature stability, and could hydrolyse starch at a temperature 20°C higher than the native enzyme. CNBr-activated epichlorohydrin-cross-linked Sepharose covalently bound 35% of added barley β-amylase activity, and resulted in an enhanced temperature stability as well as increased storage and operational stabilities compared with the native enzyme (Vretblad and Axen, 1973).

β-Amylase immobilized on to polystyrene cation-exchange resin (IR-120 A13+)

would not dissociate following treatments with high concentrations of buffers, salts or EDTA solutions and retained up to 60% of activity after 3 months; furthermore, the bound enzyme showed a broader temperature optimum compared with the free enzyme (Roy and Hegde, 1987). Maeda, Tsao and Chen (1978) immobilized soybean β-amylase on to porous cellulose beads, retaining 59–69% of added activity; the immobilized enzyme showed characteristics similar to that of the native enzyme with respect to starch conversion rates, and temperature and pH optima and stabilities. Operational and storage half-lives were 24 days at 50°C and 40 days at 40°C, respectively, in packed bed reactors (Maeda, Tsao and Chen, 1978). Barley β-amylase bound firmly to granular chicken bone (21% of added activity) and, compared with the free enzyme, was less affected by treatments with detergents, ethanol, high salt and pH extremes (Schafhauser and Storey, 1994). Although pH stability did not improve, bone-immobilized β-amylase showed an increase in high-temperature stability. Sweet potato β-amylase remained bound to *p*-aminobenzylcellulose in the presence of high salt concentrations, showed improved temperature stability, and maintained full activity when stored in a dry state for 6 months (Ohba, Shibata and Ueda, 1979). Ramesh and Singh (1981) immobilized 36% of added sweet potato β-amylase activity on to alkylamine glass and showed a 1.4-fold drop in activation energy and an increased thermal stability over that of the native enzyme. β-Amylase immobilized on to arylamine glass was less stable, showing enhanced pH and thermal stability with 48% of added activity remaining bound, but a 1.5-fold drop in activation energy (Ramesh and Singh, 1981).

Although many immobilized enzyme systems have been tested with β-amylase, the enzyme has, in most cases, proven to be too unstable and immobilization has not been enough of a stabilizing factor to maintain acceptable enzyme activities over an extended period of time. The low binding ability of some supports (3–7% of added β-amylase) could be as much the result of the unstable enzyme as of other factors, such as the blocking of active sites upon immobilization or a destabilizing effect of the support on the enzyme (Roy and Hegde, 1987; Germain and Crichton, 1988). Long-term stability of β-amylase bound to Biobone was poor, with 50% of activity lost after overnight storage at 23°C (Schafhauser and Storey, 1994). However, this instability was not due to enzyme leaching from bone itself, for assays conducted in the presence versus absence of bone washings showed similar rates of substrate hydrolysis. Caldwell *et al.* (1976a) showed that the continuous use of sweet potato β-amylase immobilized on to epichlorohydrin-cross-linked hexyl-Sepharose resulted in activity leakage at a rate of 0.5% h^{-1}. The enzyme also showed poor storage and thermal stability once bound, losing 50% of bound activity after 9 days' storage at 25°C, with even greater losses at storage temperatures of 35°C and 45°C. Caldwell *et al.* (1976a) concluded that β-amylase, once immobilized, can undergo unfavourable conformational changes that can ultimately lead to activity loss, a situation that is enhanced with increasing thermal motion at higher temperatures.

The parameters affecting the coupling of enzyme to matrix can be varied to give the best possible conditions for binding and operational activities. Takeda and Hizukuri (1975) showed a 160% increase in β-amylase activity in presence of Triton X-100. Martensson (1974a), who immobilized barley β-amylase on to a carbodiimide-activated co-polymer of acrylamide–acrylic acid, demonstrated the protective effects of reduced glutathione and serum albumin for stabilizing β-amylase activity and

thereby enhancing conversion rates. As β-amylase is very sensitive to the oxidation of some thiol groups necessary for enzymatic activity, reduced glutathione would impart stability through an antioxidant effect (Martensson, 1974a). the presence of serum albumin could offer stability through increased protein–protein interactions due to higher protein concentrations (Martensson, 1974a). This study also demonstrated increased operational (5.5 and 4 days versus 2 days) and storage (110 and 90 versus 18 hours) half-lives for glutathione- and albumin-protected immobilized β-amylase, compared with the unprotected bound enzyme, respectively. By contrast, the stability of β-amylase immobilized on to granular chicken bone was not improved when both immobilization and storage were conducted in the presence of bovine serum albumin, Triton X-100, dithiothreitol or mercaptoethanol (Schafhauser and Storey, 1994). Overnight stability was achieved only when the immobilized enzyme was stored in a substrate solution or when the enzyme was immobilized to derivatized bone in the presence of 1 M sorbitol or sucrose. Sugars and polyols are known protein stabilizers, which function by preferentially hydrating protein surfaces and, thereby, enhancing protein associations (Arakawa and Timasheff, 1982). Under these conditions, a storage half-life of 65 days was achieved, but only 30% of activity was maintained after five cycles of use at 45°C (Schafhauser and Storey, 1994).

Reticulating agents which serve to rigidify protein structure are valuable for increasing enzyme stability, and Germain and Crichton (1988) increased the stability of sweet potato β-amylase immobilized to porous silica by prior treatments involving enzyme attachment to soluble dextran or cross-linking with glutaraldehyde. Porous silica retained 45% of added dextran- and glutaraldehyde-modified β-amylase, but only 4% of added native enzyme. The modified-immobilized enzyme also showed greater operational and thermal stabilities compared with those of the native immobilized enzyme, for the temperatures at which 50% of activity remained bound after 10 minutes were 65°C for native β-amylase, 66°C for glutaraldehyde-cross-linked β-amylase, and 74°C for dextran-modified β-amylase (Germain and Crichton, 1988).

The immobilization of β-amylase also leads frequently to reduced catalytic activity. This may be caused by conformational changes that alter enzyme substrate affinity, or by slow intraparticle substrate diffusion or film diffusion, brought on by the large molecular weight and highly viscous starch substrates that are not conducive to rapid transport (Caldwell *et al.*, 1976a; Hon and Reilly, 1979). β-Amylase showed a six-fold increase in K_m upon immobilization on to cation-exchange resin (Roy and Hegde, 1987) and an eight-fold increase following its immobilization on to hexyl-Sepharose (Caldwell *et al.*, 1976a). The enzyme showed 1.7-and two-fold increases in K_m as well as 1.5- and two-fold decreases in specific activity upon immobilization on to alkylamine and arylamine glass (Ramesh and Singh, 1981). A 67-fold increase in K_m following the immobilization of β-amylase on to porous cellulose beads was substantially reduced after the immobilized preparation was ground into powder form to greatly increase the surface area (Maeda, Tsao and Chen, 1978). Film diffusion at flow rates below a linear velocity of 3 cm min^{-1} was also apparent (Maeda, Tsao and Chen, 1978).

The K_m values for native, glutaraldehyde-cross-linked and dextran-conjugated β-amylase, using soluble starch as the substrate, increased 33-, 3.5- and 1.4-fold, respectively, following their immobilization on to porous silica (Germain and Crichton, 1988). While the modified enzymes showed significant improvements to enzyme

catalytic activity following immobilization, the systems still suffered from steric hindrance and/or diffusion limitation that could not be resolved by an increased stirring rate of the mixture. The authors also suggested that the modified β-amylase may be too big to penetrate into the porous support. By contrast, β-amylase bound to granular chicken bone showed a 1.2-fold decrease in the K_m for soluble starch, and both Lineweaver–Burk and Arrhenius plots were similar for the bound and free enzymes, all these results indicating that immobilization did not hinder the approach of high molecular weight substrates to this support (Schafhauser and Storey, 1994). However, the increase in surface area achieved by powdering the bone resulted in a 1.5-fold increase in enzyme activity.

PULLULANASE

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41), also known as the 'debranching enzyme', catalyses the hydrolysis of α-*D*(1,6) and to a lesser extent the α-*D*(1,3) and α-*D*(1,4) glycosidic bonds of pullulan, amylopectin and glycogen in a random endo-fashion (Solomon, 1978; Ram and Venkatasubramanian, 1982; Yang and Coleman, 1987). The end products are maltotriose and maltoriosyl oligosaccharide side-chains of varying length (Fogarty, 1983). Useful preparations of pullulanases are obtained from a variety of micro-organisms, including *Aerobacter aerogenes*, *Escherichia intermedia* and *Streptomyces flavochromogenes* (Fogarty, 1983). Pullulan, a linear polymer of maltotriose linked by α-(1,6) glucosidic bonds, and waxy maize, a starch containing 80% α-(1,6) glycosidic links, are the common substrates used for pullulanase analysis (Solomon, 1978; Ram and Venkatasubramanian, 1982).

Pullulanase is widely used as an analytical tool for elucidating the structures of polysaccharides and in the determination of chain length of glycogen and amylopectin, which is important in the diagnosis of two types of glycogen storage disease (Martensson and Mosbach, 1972). For industrial use in bioconversion, the addition of pullulanase in combination with α-amylase, β-amylase or amyloglucosidase can greatly increase the purity of high-maltose and high-glucose syrups produced from starch (Yang and Coleman, 1987). Because the enzyme is of limited use on its own, only a few studies have analysed pullulanase immobilization alone, but other studies, considered later in this review, have assessed the improvements in product output brought about by the co-immobilization of pullulanase along with starch- or cellulose-degrading enzymes.

A key effort of investigators developing immobilized pullulanase systems has been the improvement of functional and thermal stability. Pullulanase from *Klebsiella aerogenes* covalently bound to azide-linked bovine-hide collagen membranes, showed improved thermal and operational stability compared with that of the native enzyme (from 2 to 7 days at 50°C) (Ram and Venkatasubramanian, 1982). *Streptomyces flavochromogenes* pullulanase pretreated with tannic acid to facilitate binding on to TEAE-cellulose, showed an operational half-life of 15 days at 50°C, and remained stable following 6 months of storage (Ohba *et al.*, 1978). The bound enzyme was also greatly affected by the presence of calcium salts, showing a substantial increase in temperature stability at low levels of Ca^{2+} (2–5 mM), whereas amounts greater than 10 mM resulted in enzyme desorption.

Lenders and Crichton (1984), who conjugated pullulanase to soluble poly-

saccharides (dextrans, amylose), showed a direct correlation between enzyme coupling to polymers of increasing molecular weight and greater thermal stability, resistance to urea denaturation and lower K_m values; such enhanced stabilities were attributed to the formation of multiple covalent linkages between the enzyme and the polysaccharides. Pullulanase from *Aerobacter aerogenes*, cross-linked to a co-polymer of acrylamide–acrylic acid (Biogel CM-100), retained 43% of added activity, but showed no improvement in stability over that of the free enzyme, with a half-life less than 30 min at 55°C (Martensson and Mosbach, 1972). However, coupling in the presence of substrates resulted in a fivefold increase in activity. Aware of the enzyme's sensitivity to heat denaturation, Martensson (1974b) studied the operational stability of pullulanase immobilized to a co-polymer of acrylamide–acrylic acid, at three different substrate concentrations and at three different temperatures. A stabilizing effect of high substrate concentrations was apparent, but half-lives were still low, ranging from 3 to 9 days at 40°C and 2 days at 50°C.

The immobilization of fibre-degrading enzymes

CELLULASE

Cellulases are complex multicomponent enzyme systems produced by micro-organisms, plants and perhaps some animals, that can function synergistically to hydrolyse cellulosic materials into glucose (Klyosov and Consultant, 1986; Woodward, 1989). Wood-decaying fungi far outnumber bacteria with respect to the number of cellulolytic organisms identified so far, primarily because they produce and secrete more cellulases (Saddler, 1986). Fungi such as *Trichoderma reesei* and *Aspergillus niger* are the most common commercial sources of extracellular cellulases, having specific activities greater than cellulases from other sources (Mandels *et al.*, 1981). The cellulase system can be divided into three classes, including exoglucanases such as cellobiohydrolase (1,4-β-*D*-glucan cellobiohydrolase, EC 3.2.1.91), endoglucanases (1,4-β-*D*-glucan glucanhydrolase, EC 3.2.1.4) and β-glucosidase (β-*D*-glucoside glucohydrolase, EC 3.2.1.21) (Cheetham, 1985; Woodward, 1989; Klyosov, 1990). The exo- and endoglucanases adsorb on to and act synergistically on crystalline cellulose to produce cellobiose and *D*-glucose, whereas β-glucosidase catalyses the hydrolysis of cellobiose to *D*-glucose (Woodward and Zachry, 1982). The ability of cellobiohydrolases and endoglucanases to degrade cellulose is directly correlated with the degree to which they are adsorbed to its surface, with a greater degree of adsorption resulting in a higher reaction rate and greater glucose yield (Klyosov and Consultant, 1986). Enzymes isolated from different sources can occur as isozymes and can differ in their molecular characteristics, mode of action, absorptive ability, catalytic activity and substrate specificity (Klyosov, 1990). As only one of each of the constituent enzymes is required for effective hydrolysis, the variety and/or effectiveness of cellulase complexes from different cellulase sources is quite vast (Woodward, 1989; Klyosov, 1990).

Cellulose is a major component of plant biomass and is a renewable resource, abundantly available in the form of hardwood and softwood trees, agricultural and citrus crop residues, and municipal wastes (Jain and Wilkins, 1987; Woodward, 1987; Grohmann and Baldwin, 1992). Natural cellulose is a large and complex glucose

polymer. It can consist of up to 10 000 glucose units and can contain both highly ordered (crystalline) regions and more randomized (amorphous) regions, as well as forming associations with lignin and hemicellulose (Knowles, Lehtovaara and Teeri, 1987). In deference to these inconsistencies, a wide variety of cellulose-related substrates have been used for cellulase characterization studies, including soluble substrates, such as carboxymethylcellulose (CMC), cellobiose and *p*-nitrophenyl glucosides (PNPG), and insoluble substrates, such as cotton, filter paper, solka floc, amorphous cellulose and microcrystalline cellulose (Avicel) (Knowles, Lehtovaara and Teeri, 1987).

Potentially, the glucose derived from the breakdown of cellulose can be used for a vast number of processes, including as a feedstock additive for single-cell protein processes, a fermentable sugar for the production of chemical precursors and fuel, and a precursor for fructose syrups for the sweetener industry (Woodward and Zachry, 1982; Cheetham, 1985). However, the enzymatic production of glucose from cellulose has not attained commercial success. Cellulase enzymes have low specific activities for insoluble cellulosics and, thus, a high ratio of enzyme to substrate is necessary to achieve productive hydrolysis in a reasonable period of time (Takeuchi and Makino, 1987). Optimizing the conditions to maximize the activities of a multienzyme catalyst, which can involve potentially inhibitory substrate and product levels, as well as a variety of soluble and insoluble substrates and products, is a complex process (Saddler, 1986). Enzyme costs are also an important feature in the economy of enzymatic saccharification of cellulosic materials, comprising, for example, nearly 50% of the outlay required to produce sugar from corn stover (Woodward, 1987). Thus, the use of soluble cellulases for the production of inexpensive products is an expensive undertaking.

Efforts to retrieve and recycle free cellulases from fermentor reactors have not been successful (Mes-Hartree *et al.*, 1987). However, free cellulase activity was successfully recovered and reused from a spent reactor hydrosylate of steam-exploded aspen wood by a few minutes' contact with fresh substrate (Lamed *et al.*, 1991). The development of reusable cellulase systems has been an area of active study as it is seen as a practical solution for overcoming waste and high costs.

The catalytic ability of an immobilized cellulase (particularly one immobilized by entrapment or on to an insoluble support) hydrolysing an insoluble substrate should be greatly impaired, for the hydrolysis of insoluble cellulose requires both cellobiohydrolases and endoglucanases to adsorb to the surface of the substrate to initiate depolymerization (Woodward, 1987, 1989). For example, it has been shown that up to 50% of initial soluble cellulase activity can remain bound to lignocellulosic substrates following a hydrolysis reaction (Mes-Hartree *et al.*, 1987). In spite of this, immobilized cellulases can successfully hydrolyse cellulose substrates, albeit not to the same degree as the native enzyme with respect to insoluble substrates. However, as a compensation, many immobilized cellulase preparations have resulted in firmly bound, reusable enzymes, showing stability properties better than the free enzyme systems. *Trichoderma reesei* cellulase adsorbed to concanavalin A cyanogen bromide (ConA-CNBr)-activated Sepharose showed no enzyme leakage during normal operations or following treatment with urea (Woodward and Zachry, 1982). *Aspergillus niger* cellulase bound to poly-*L*-glutamic acid showed improved temperature and pH stability compared with the native enzyme, and could maintain 90% of its activity

after 20 days storage (Takeuchi and Makino, 1987). Cellulases covalently bound to polyurethane particles maintained 100% of activity following more than 15 repeated uses (Kumakura, 1985). CMC cellulases immobilized on to polyvinylalcohol (PVA) and polyethylene glycol (PEG) showed no activity loss or leakage after 3 and 12 cycles of use, respectively (Mishra, Deshpande and Rao, 1983; Kumakura and Kaetsu, 1985). Cellulase from *T. reesei* covalently bound to polyurethane foam retained 33% of added activity, and showed a half-life greater than 6 weeks (assessed with 1% CMC at 23°C, pH 4.5) as well as improved pH and thermal stabilities (Chakrabarti and Storey, 1988). Cellulases immobilized on to a collagen fibril matrix saccharified a 0.33% solution of Avicel to better than 80% completion in a fluidized bed reactor at 30°C (Karube *et al.*, 1977). Cellulase enzymes from *Macrophomina phasedina* entrapped in 5% acrylamide could hydrolyse filter paper and cotton to 41% and 6.75%, respectively, over 120 h, and remained stable after 25 repeated uses (Roy, Roy and Duke, 1984).

In an effort to improve the limited degree of hydrolysis seen with immobilized enzymes against insoluble celluloses, some investigators have introduced spacer molecules between the immobilized enzyme and the support matrix, ideally allowing for increased mobility of the enzyme and, thus, a more effective interaction between the insoluble cellulose and insoluble enzyme. Cellulase immobilized on to CNBr-Sepharose and CH-Sepharose gels containing hydrocarbon spacers showed higher activity towards cellobiose, acid-swollen cellulose and cellulose powder, compared with cellulase immobilized directly on to gels (Chim-anage *et al.*, 1986). Similarly, cellulases immobilized on to CNBr-activated Sepharose, ConA-Sepharose and CNBr-activated glass beads hydrolysed alfalfa, wheat straw and pine needles to a greater extent than did the free enzyme (Fadda *et al.*, 1984). In particular, ConA-Sepharose showed 1.5- to 1.7-fold greater activity than the native enzyme against those substrates (Fadda *et al.*, 1984). The cellulase complex from *T. reesei* immobilized on to CNBr-activated ConA-Sepharose retained full activity against CMC, but only 9% and 29% activities against Avicel and cellobiose, respectively (Woodward and Zachry, 1982).

However, cellulase enzymes that are required to adsorb to the cellulose surface for proper function may not be bound strongly enough to their supports to resist desorption tendencies. At the very least, enzymes immobilized in such a way as to restrict the movement required for adsorption and desorption on to the substrate could suffer activity loss and loss of catalytic properties.

Trichoderma reesei cellulase bound to glutaraldehyde-activated nylon retained 60% of initial activity and became more thermally stable (Jain and Wilkins, 1987). However, bound activity was lost after five cycles of use, and the bound enzyme hydrolysed only 33% as much Avicel as the native enzyme over 24 h at 50°C (Jain and Wilkins, 1987). ConA-Sepharose cellulase lost between 30 and 50% of activity after only five repeated uses (Fadda *et al.*, 1984) and *T. reesei* cellulase entrapped in acrylate polymers suffered from leakage over long periods of time (Higa, Del Mastro and Castagnet, 1986). This desorption, however, could be prevented by the addition of silica gel or PEG 600 to the preparation.

Cellulases have also been immobilized on to water-soluble supports with the assumption that, as long as recovery was possible by filtration or centrifugation, such immobilized enzymes would offer greater interaction between the constituent enzyme and insoluble cellulosic substrates (Woodward, 1989), and would be more

efficient catalysts compared with the free enzyme. However, no significant improvement of cellulase function was found for immobilization on to soluble versus insoluble supports. *Aspergillus niger* cellulase immobilized on to poly-*L*-glutamic acid could be recovered easily from the product solution and reused, being soluble in neutral and alkaline conditions, and insoluble at acidic pHs (Takeuchi and Makino, 1987). The immobilization of the cellulase complex to polyethylene glycols (PEG) successfully hydrolysed cellulose powder at 40°C (Kumakura and Kaetsu, 1985). Enzyme activity was also shown to be affected by an increase in the molecular weight of PEG (and the corresponding viscosity increase of the carrier); thus, cellulase bound to PEG 200 retained 60% of its original activity but only 40% of activity was retained when PEG 600 was the carrier. Cellulase from *Penicillium funiculosum* bound to carbodiimide-linked PVA showed enhanced stability, converting two-fold more alkali-treated bagasse than the native enzyme after 48 h (Mishra, Deshpande and Rao, 1983).

An understanding of the complex process of cellulase hydrolysis of cellulosics is further complicated when one considers the number of catalytic changes that could occur to the component enzymes upon immobilization. Furthermore, one would expect that any steric hindrances between the immobilized enzymes and the substrate, soluble or not, would prevent the usual binding of cellulase to substrate and thus affect enzyme catalytic properties. This subject has been addressed by surprisingly few investigators. The cellulase complex entrapped within 5% acrylamide showed K_m values only slightly higher than the native enzyme with respect to the hydrolysis of CMC and cellobiose, with enzyme activities increasing with decreasing particle size (Roy, Roy and Duke, 1984). Cellulase immobilized on to polyurethane particles also experienced an increase in activity as a function of decreasing particle diameter (Kumakura, 1985).

Not only do some immobilized cellulases utilize some insoluble forms of cellulose, but some make more efficient use of them when compared to free cellulases, thus resulting in more efficient catalysis (Fadda *et al.*, 1984; Chakrabarti and Storey, 1988). Polyurethane foam apparently presented little or no diffusional barrier to penetration by high molecular weight cellulose substrates, for compared with their respective activities against soluble CMC, the relative activities against microcrystalline cellulose were 20% for the immobilized enzyme and only 16% for the native enzyme (Chakrabarti and Storey, 1988). In addition, a substantial 7.5-fold decrease in K_m CMC occurred following cellulase immobilization on to foam. Furthermore, ConA-Sepharose-, CNBr-Sepharose- and CNBr-glass bead-immobilized cellulase showed only slight increases in K_m values (1.1-, 1.3- and 1.3-fold, respectively) compared with the free enzyme. The immobilized enzymes were also more resistant to inhibition by glucose and cellobiose, suggesting higher β-glucosidase and cellobiase activities (Fadda *et al.*, 1984). Cellulase immobilized to ConA-Sepharose could carry out long-term high rates of hydrolysis of microcrystalline cellulose to a greater extent than equal amounts of free enzyme (17 and 4 μmol ml^{-1} *D*-glucose, respectively) (Woodward and Zachry, 1982). Immobilization may have altered the conformation of the active site or regulatory and effector sites of one or more of the enzymes in the multienzyme complex, resulting in one or more effects such as increased affinity of endoglucanase for cellulose, improved enzyme efficiency, reduced feedback inhibition of cellobiohydrolase by cellobiose, or improved function of β-glucosidase that

reduces cellobiose accumulation (Klyosov and Consultant, 1986; Chakrabarti and Storey, 1988).

β-GLUCOSIDASE

β-Glucosidase, which hydrolyses cellobiose to produce glucose, is essential to the complete enzymatic conversion of cellulose to glucose and is, thus, a subject of research interest in its own right. β-Glucosidase is subject to thermal inactivation and can be limited by glucose inhibition; it is considered to be the rate-limiting factor in the multienzyme process that converts cellulosics into glucose (Sundstrom *et al.*, 1981; Matteau and Saddler, 1982; Roy *et al.*, 1989). Furthermore, cellulase complexes often contain insufficient concentrations of β-glucosidase to prevent the accumulation of cellobiose, and high cellobiose levels inhibit the endoglucanase components of most cellulase systems (Sundstrom *et al.*, 1981; Woodward, 1989). Thus, supplementation of the cellulase mixture with additional β-glucosidase can lower or prevent the accumulation of inhibitory cellobiose, and enhance the rate and extent of saccharification (Sundstrom *et al.*, 1981; Roy *et al.*, 1989). β-Glucosidases are also useful in the preparation of aromatic oils as additives in the food industry (Iborra *et al.*, 1992).

The addition of purified β-glucosidase to a soluble cellulase system (Cellulosome) can result in the hydrolysis of accumulated cellobiose with a resulting ten-fold increase in cellulose degradation (Lamed *et al.*, 1991). The use of an immobilized β-glucosidase would be a further improvement, imparting all of the associated benefits of an immobilized enzyme system, including enhanced stability, reusability and reduced enzyme cost. Thus, a commercial process could become economically viable if an immobilized β-glucosidase was used. Numerous methods, of varying utility, have been developed for immobilizing β-glucosidase.

Mycelial-associated β-glucosidase encapsulated in calcium alginate beads hydrolysed cellobiose and salicin (a cellobiose analogue) to 47% and 30% conversions, respectively, in a packed bed reactor at 50°C (Matteau and Saddler, 1982). Although rapid thermal inactivation occurred at temperatures higher than 60°C, the reactor showed half-lives greater than 1000 h when operating continuously at 50°C (Matteau and Saddler, 1982). Nylon powder retained 67% of added *Penicillium funiculosum* β-glucosidase (Aguado, Romero and Rodriguez, 1993) whereas approximately 50% of added β-glucosidase remained active when immobilized to a series of (hydrophobic) alkyl and aryl derivatives of cotton cloth (Sharma and Yamazaki, 1984). Sweet-almond β-glucosidase maintained stability for 1 h at 80°C immobilized to an ultrafiltration membrane, unlike the free enzyme, which showed a rapid loss of activity under similar conditions (Sarkar and Burns, 1983). β-Glucosidase incorporated into thin films by gelation with α-casein, retained all bound activity following 10 cycles of hydrolysis of PNPG, and could be stored at 5°C for at least 1 year without activity loss or leakage (Motoki *et al.*, 1987). Woodward and Capps (1992) treated *A. niger* β-glucosidase with glutaraldehyde prior to entrapment within calcium alginate gel spheres to prevent a previously encountered enzyme leakage problem. This treatment resulted in bound enzyme activities similar to the native enzyme, as well as enhanced thermal stability (Woodward and Capps, 1992). The enzyme from *Alcaligenes faecalis* retained 25% of added activity on PNPG-CNBr-activated cellulose and

showed a slight increase in temperature stability (Srinivasan and Bumm, 1974). β-Glucosidase linked to controlled pore alumina retained 50–60% of added activity, losing only 10% of activity after 500 h continuous use at 50°C against cellobiose (Sundstrom *et al.*, 1981). Polyurethane foam retained 73% of added β-glucosidase activity, and maintained essentially 100% activity after 100 h of continuous use (Chakrabarti and Storey, 1988).

Barley-meal β-glucosidase immobilized on to Sepharose, glutaraldehyde-cross-linked glass beads or phenolic resin (Duolite) retained 35%, 21% and 42% of added activity, respectively (Simos and Georgatsos, 1990). However, only Sepharose-linked β-glucosidase showed a significant increase in thermostability, whereas the cross-linked supports showed poor stability in general (Srinivasan and Bumm, 1974). β-Glucosidase bound to CNBr-activated Sepharose and entrapped within polyacrylamide gels retained 33% and 51% of added soluble enzyme activity, and showed improved temperature and storage stabilities (half-lives of 70 and 90 days, respectively) compared to the free enzyme (half-life of 4 days) but both lost approximately 30% of initial activity after four cycles of use (Roy *et al.*, 1989). *Aspergillus niger* β-glucosidase bound to a polyethyleneimine glutaraldehyde-activated magnetic support retained only 15–27% of added activity, with the continual loss of bound activity attributed to physical loss during both washing and recovery steps (Dekker, 1990). However, the immobilized enzyme could be recovered easily from lignocellulosic solids suspension in a stirred batch reactor by applying a magnetic field (Dekker, 1990). Cassava-leaf β-glucosidase bound to photo-cross-linkable prepolymer resins could be reused repeatedly and stored at 4°C without any significant loss of activity for up to 2 years (Yeoh, 1992). β-Glucosidase immobilized on to hydrazine-derivatized nylon required a long and arduous support preparation and immobilization procedure (over 16 h) (Iborra *et al.*, 1992). Although retaining 30% of added activity, the bound enzyme showed storage and operational half-lives of only 30.7 and 20.1 days, and no improvement in pH or thermostability. However, the bound enzyme showed improved catalysis over the native enzyme, with a 20% decrease in K_m for picrocrocin (the glycoside precursor of safranal oil) and an 80% decrease in K_m for glucose.

Although immobilization usually imposes steric and diffusional limitations on enzyme activity, an immobilized β-glucosidase, since it is hydrolysing a soluble substrate, should experience relatively few alterations in catalytic properties. However, β-glucosidase immobilized on to controlled pore alumina was apparently subject to diffusion limitation, showing increasing activity (from 50% to 90% of initial bound activity) with increasing liquid velocity through the packed bed (Sundstrom *et al.*, 1981). A 3.6-fold increase in K_m following immobilization was also observed, but activities of both native and bound enzyme followed a linear Arrhenius relationship with respect to temperature (Sundstrom *et al.*, 1981). Beta-glucosidase immobilized on to Sepharose, duolite or glass also showed slight increases in K_m values with respect to the free enzyme (by 10%, 40% and 80%, respectively) (Simos and Georgatsos, 1990). The 10% and 20% increases in K_m values seen following immobilization of β-glucosidase on to polyacrylamide gel and CNBr-activated Sepharose were also attributed to mass transfer limitation (Roy *et al.*, 1989). In addition, cellulose bound β-glucosidase showed an Arrhenius plot unlike that of the native enzyme, with a higher activation energy at temperatures greater than 45°C. A fivefold increase in K_m also occurred, but this was attributed to enzyme hydrolysis

occurring on both the added substrate and the β-1,4 glucosidic linkages of the cellulose matrix (Srinivasan and Bumm, 1974).

XYLANASES

Lignocellulosics, which comprise the cell wall materials of all woody plants, grasses and cereals, constitute the largest single biomass resource on Earth (Tan, Wong and Saddler, 1985). They are a complex matrix principally composed of cellulose (40–60%), hemicellulose (20–30%) and lignin (15–30%) that is highly resistant to breakdown and hydrolysis (Dekker, 1983; Kluepfel *et al.*, 1990). Lignin, an aromatic polymer composed of phenylpropanoid subunits (Woodward, 1984), is not saccharide-based and, therefore, will not be discussed further in this review. Cellulose, essentially composed of non-branching polymers of *D*-glucose, retains the same structure in all plants (Biely, 1985). However, xylans, the major component of hemicelluloses, have, by comparison, much more complex structures that can vary from one plant species to another (Biely, 1985). Xylans are composed of a backbone of β-1,4-linked xylopyranose residues with side-chains of acetate, *L*-arabinofuranose, *D*-glucuronic and 4-*O*-methyl-*D*-glucuronic acid at key branch points (Biely, 1985; Wong, Tan and Saddler, 1986; Christov and Prior, 1993). Hetero-1,4-β-*D*-xylans constitute the major hemicellulosic component of the Gramineae (grasses and cereals) and angiosperms (hardwoods), whereas the hetero-1,4-β-*D*-mannans are more abundant in gymnosperms (softwoods) (Dekker, 1983).

Celluloses and xylans, the two most abundant renewable polysaccharides in nature, continuously accumulate in large quantities as waste products from the forestry, pulp and paper, agricultural, and food processing industries (Biely, 1985; Wood, 1985). Most of the biotechnological research concerned with the utilization of these waste materials has dealt with the cellulose portion for the production of liquid fuel and other cehmical products (Saddler and Brownell, 1982; Biely, 1985). The utilization of the xylan content of lignocellulosics has equal biological potential, and the development of new technologies that utilize xylans are important in several ways, including: the reduction of waste biomass, the enhancement of economic competitiveness of bioconversion technologies through the use of these abundant, inexpensive and presently under-utilized starting materials, and the development of new products from these renewable resources (Saddler and Brownell, 1982; Dekker, 1983; Yu *et al.*, 1987).

In the pulp and paper industry, xylanases can be used for the removal of contaminating hemicellulose components from high-grade cellulose pulps, for the manufacturing of dissolving pulp (Dekker, 1983; Senior *et al.*, 1988), for the processing of plant fibre sources such as flax and hemp (Biely, 1985), for the production of construction materials and as potential bleaching agents (Kluepfel *et al.*, 1990). Xylanases can also be used in the manufacture of liquid coffee, for the adjustment of wine characteristics, for the clarification of juices and for liquifying fruits and vegetables (Biely, 1985; Lewis and Paice, 1989). The hydrolysed products of xylan, namely xylose and xylo-oligosaccharides, can be used for the production of such fermentation products as ethanol, butanol and acetone (Roy, Roy and Duke, 1984; Yu and Saddler, 1985), as a supplement for microbial feedstocks or for the production of xylitol sweeteners (Abdel-Naby, 1993).

The structural rigidity of lignocelluloses necessitates that they be pretreated before being used in specific processes. Pretreatment methods include steam explosion, ball milling, swelling (with concentrated acidic or basic solutions) or enzymatic treatment (using brown- or white-rot fungi) to enhance their susceptibility to hydrolysis (Saddler and Brownell, 1982). Such preprocessing helps to increase the accessibility of cellulose and xylan by loosening these components from the binding constraints of lignin, disrupting crystalline and cell wall structures, and enhancing substrate solubility (Saddler and Brownell, 1982).

Xylans can be converted to monosaccharides by acid or enzymatic hydrolysis. However, like most acid hydrolysis reactions, the formation of toxic compounds is problematic and can hinder further microbial fermentation processes (Biely, 1985). Xylan hydrolysis by enzymatic means would allow for more efficient and controlled substraate use and product formation. Due to the level of complexity and variability that exists among xylans from different sources, their complete hydrolysis requires the synergistic action of a multitude of enzymes (Asther and Meunier, 1993; Coughlan and Hazlewood, 1993b). The enzymes involved in the hydrolysis of xylan are classified as either esterases, which rupture the ester linkages found throughout the xylan polymer, or hydrolases, which hydrolyse the glycosidic bonds of xylan (Lewis and Paice, 1989; Coughlan and Hazlewood, 1993a). The hydrolase class of enzymes includes: endoxylanases (EC 3.2.1.8), which randomly dismember the xylan backbone into shorter xylo-oligosaccharides; β-xylosidases (EC 3.2.1.37), which cleave the xylo-oligosaccharides in an exo-manner, producing xylose; and α-arabinofuranosidases (EC 3.2.1.55) and α-glucuronidases (EC 3.2.1.1), which remove the arabinose and 4-O-methylglucuronic acid substituents, respectively, from the xylan backbone (Biely, 1985; Dekker, 1983). As the result of the multifarious nature of xylans and the enzymes involved in its degradation, the analysis and characterization of xylan-degrading enzymes has proceeded slowly, compared with the cellulases, both with respect to obtaining suitable substrates for the analysis of enzyme activity, and with respect to the purification and characterization of the individual enzyme components. However, an excellent review of the subject is newly available (Coughlan and Hazlewood, 1993a).

Most biotechnological approaches to xylanolytic enzyme use have focused on the xylanases and β-xylosidases, the primary enzymes involved in xylan hydrolysis, with relatively little information available on the xylan-debranching enzymes (Christov and Prior, 1993). The role of β-xylosidase is analogous to that of β-glucosidase in the breakdown of cellulose by cellulases (Dekker, 1983). Both xylanase and β-xylosidase production are inducible when micro-organisms are grown on substrates such as xylans, or methyl β-*D*-xylopyranosides, respectively (Dekker, 1983). While xylan-degrading enzymes are produced in many organisms, those of technological interest arise from fungal sources, such as *Aspergillus* (Shimizu and Ishihara, 1987; Stålbrand *et al.*, 1992; Abdel-Naby, 1993), *Trichoderma* (Saddler and Brownell, 1982; Shimizu and Ishihara, 1987; Royer and Nakas, 1989), *Zymononas* (Saddler and Brownell, 1982), *Streptomyces* (Kleupfel *et al.*, 1990), *Thermoascus* (Senior, Mayers and Saddler, 1989) and *Aureobasidium* (Leathers, 1989) or bacterial sources such as *Cryptococcus* (Stålbrand *et al.*, 1992), *Streptomyces* (Kleupfel *et al.*, 1990) and *Bacillus* (Senior, Mayers and Saddler, 1989). Their high yields and relative ease of production make these systems ideal for commercial exploitation.

In spite of this, the costs associated with enzyme production for bioconversion processes are a major expenditure. Economics demand that optimum conditions be determined for the production and use of enzymes (Senior, Mayers and Saddler, 1989). The use of immobilized xylanolytic enzymes could improve enzyme productivity, due to the ease of recovery and reuse. To date, however, there have been relatively few attempts to immobilize xylanolytic enzymes and, of these, most have been limited to xylanases and β-xylosidases.

Shimizu and Ishihara (1987) coupled the hemicellulases of *T. reesei* and *A. niger* to a variety of supports, including porous silica, alumina and titania, with $TiCl_4$ (Titanium tetrachloride) and glutaraldehyde as the binding agents, showing enzyme loadings in the range 10–50 mg enzyme g^{-1} carrier. Using the hemicellulose-derived substrates xylan, aryl-B-xyloside, 4-O-methylglucuronoxylan and *p*-nitrophenyl-β-*D*-xylopyranoside, the immobilized activities were found to be diffusion limited, showing only 3–53% of the activity of the free native enzymes against the same substrates (Shimizu and Ishihara, 1987). However, the enzyme preparations from both sources remained stable upon immobilization, maintaining their temperature optima and showing stable rates of xylan conversions of 36–64% over 48 h.

Roy, Roy and Duke (1984) successfully immobilized both the xylanase and β-xylosidase enzymes from *Macrophomina phaseolina* by entrapment within acrylamide polymers, maintaining full activity against xylan and *o*-nitrophenol-β-*D*-xylopyranoside, respectively, after 25–27 reuses at 38°C. Xylanase showed greater temperature stability following immobilization, maintaining activity at rates as high as that of the free enzyme after 25 days at both 38° and 50°C, whereas the gel-bound β-xylosidase showed substantial reductions in activity after 4 days at 50°C. K_m values increased following immobilization by 1.8- and 1.6-fold for β-xylosidase and xylanase activities, respectively; these changes were apparently influenced by diffusion limitations, for when gel-bound β-xylosidase was ground into smaller segments using a mortar and pestle, the K_m increase was only 1.3-fold (Roy, Roy and Duke, 1984).

Oguntimein and Reilly (1980) immobilized *A. niger* β-xylosidase on to 10 different support carriers in the hope of obtaining a stable enzyme preparation; $TiCl_4$-linked alumina proved to be the best support, binding 70% of added enzyme activity. Abdel-Naby (1993) conducted a thorough study involving the immobilization of *A. niger* xylanase and β-xylosidase on to an assortment of carriers, including tannin-chitosan, Dowex-50W, glutaraldehyde-cross-linked chitosan and polyacrylamide gel beads. Both enzymes maintained the greatest stability and activity when immobilized by cross-linking or entrapment. The absorbed and ionically bound enzymes showed a decrease in thermal stability, whereas the opposite was true for the covalently bound and entrapped enzymes. Polyacrylamide retained the highest amount of added xylanase (15–25%) and β-xylosidase (16–33%) activities, and maintained continuous (albeit low) rates of larchwood xylan and *p*-nitrophenyl-β-*D*-xylopyranoside hydrolysis for over 40 days in packed bed reactors. Similarly, xylanase covalently bound to chitosan lost only 19% of its initial rate after continuous operation for 40 days, whereas the enzymes immobilized by adsorption and ionic forces lost 82% and 67%, respectively. In general, when immobilized by these methods, both xylanase and β-xylosidase showed decreases in specific activities to only 15–24% and 13–32% of the values for the corresponding soluble enzymes, respectively, as well as corresponding 23–42% and 7.5–36% increases in K_m values for substrates, respectively.

Such deviations from the free enzyme values were attributed to structural changes resulting from immobilization, as well as problems with diffusion resistance and steric hindrance (Abdel-Naby, 1993).

Stålbrand *et al.* (1992) exploited the occurrence of spontaneous mycelial pellet formation in three strains of *Aspergillus* as a possible source of immobilized β-xylosidase. Such self-immobilized enzyme systems can occur in shake flask cultures after inoculation with conidia. In general, the mycelial pellets of *A. phoenicis* had the highest pellet-bound β-xylosidase activities (280 U g^{-1} dry weight of mycelia) and maintained stability after storage at 50°C for 333 h. However, the mycelial pellets of *A. phoenicis* QM 329 and ATCC 13157 suffered from enzyme leakage, and the mycelial pellets of *A. terreus* QM 1991 were limited by both leakage and instability.

Clearly, much more research still needs to be done in the area of immobilized xylanolytic enzymes in order to improve the competitive edge of potential commercial bioconversion processes. Several areas need to be explored. For example, the co-immobilization of xylanase and cellulase enzymes could allow the efficient use of both the xylan and cellulose components of substrates (Yu and Saddler, 1985). The co-immobilization of several xylanase enzymes would also allow for a more complete degradation of the xylan. Wong, Tan and Saddler (1986) found that by using a mixture of the three soluble xylanases from *T. harzianum*, the overall hydrolysis of aspenwood xylan was increased by 30%. Furthermore, the co-immobilization of enzymes which liberate the side-chain sugars from the xylan backbone, such as α-*L*-arabino-furanoside and α-glucuronidase, or the enzymes involved in the degradation of acetyl xylans, would create new substrate sites for further xylanase activity (Biely, 1985). The results of such work would allow for the direct conversion of xylan to useful products by simple and inexpensive methods. Such steps are necessary, for a critical limitation in the commercialization of xylanolytic systems is the incomplete utilization of xylans (Biely, 1985).

PECTINASES

Pectins are a class of complex acidic polysaccharides of high molecular weight (25–360 kDa) that are present in varying amounts in higher plants. Their presence affects the consistency, turbidity and appearance of fruit juices (Fogarty and Kelly, 1983; Ward, 1985; Borrego *et al.*, 1989). Pectins consist of α-1,4-*D*-galacturonic acid polymers of varying methyl ester content, which are often complexed with *L*-araban and β-1,4-*D*-galactan polymers (Dorfman, 1955; Ward, 1985). The methoxy content of pectin varies among plant sources and changes during the development and ripening stages of fruits and vegetables. The degree of esterification of the pectins of fruits ranges from 90–95% for apples to 60–70% for oranges and only 30–35% for grapes (Ward, 1985). The two major groups of pectolytic enzymes include the pectin esterases (PE) (pectinhydrolase, EC 3.1.1.11), which hydrolyse the methyl groups that are esterified to galacturonic acid residues, and the polygalacturonases (PG) (poly[1,4-α-*D*-galacuronide]glycanohydrolase, EC 3.2.1.15), which are depolymerizing enzymes capable of hydrolysing α-1,4-galacturonosyl linkages (Dorfman, 1955; Ward, 1985; Romero *et al.*, 1989). Although PE is not a polysaccharide-degrading enzyme, PG function is dependent on the action of PE, since PG can only hydrolyse pectic substrates with a 60% or lower degree of esterification (Borrego *et*

al., 1989). Another important group is the pectic lyases (EC 4.2.2.2) such as endopolymethylgalacturonate lyase (PMGL), which cleaves the α-1,4-galacturonosyl linkages by elimination of (Δ)-4,5-*D*-galacturonate residues at the non-reducing side of the bond being split (Ward, 1985). Enzymes of this class are not hydrolytic, but act in concert with the other pectinases for the complete breakdown of pectins. Pectolytic enzymes originate from a range of plant and microbial species. Pectin esterases are commonly found in tomato (Borrego *et al.*, 1989; Romero *et al.*, 1989), *Fusarium oxysporum* and *Clostridium multifermentans*, whereas the polygalacturonases have been isolated from a variety of fungi (*Aspergillus, Fusarium, Rhizopus*) (Romero *et al.*, 1989) and bacteria (*Erwinia aroideae, Bacillus*) (Ward, 1985). Pectic lyases also originate from fungal (*Fusarium, Aspergillus, Penicillium*) and bacterial (*Bacillus, Erwinia, Clostridium, Streptomyces*) sources (Ward, 1985).

Pectin, which can be present in either soluble or insoluble (protopectin) forms, causes the cloudy and highly viscous properties of fruit and vegetable extracts, and these undesirable properties can be reduced or dissipated by treatments with pectic enzymes (Ward, 1985). Indeed, pectin degradation by pectic enzymes is a common industrial practice for improving the quality and yield of fruit and vegetable juices during processing (Ward, 1985; Borrego *et al.*, 1989). Pectinases have similar uses in the treatment of coarse fruit pulp and peel in the preparation of natural cloudifiers for use in the beverage industry, as well as in the preparation of citrus oils, the production of baby foods from macerated fruits and vegetables, and in wine production for increased yields and juice clarification (Ward, 1985).

The use of immobilized pectinolytic enzymes for continuous use in such applications would be beneficial were it not for the highly viscous substrates, which could effectively reduce the catalytic ability of an immobilized enzyme (Romero *et al.*, 1989). Thus, a successful and productive immobilized pectic enzyme reactor must be able to combat the hydrodynamic limitations of the substrate and remain stably bound to allow for high rates of conversion over long periods of operation (Lozano *et al.*, 1990).

Lozano *et al.* (1990) immobilized a commercial pectolytic enzyme preparation (Pectinol D) on to nylon membrane for use in a cross-flow packed bed reactor for the hydrolysis of 0.5% citrus pectin at pH 4 and 40°C. The cross-flow reactor design was superior to a packed bed reactor model, showing higher rates of pectin hydrolysis and higher recycling flow rates. This, in turn, effectively reduced the external film diffusion limitations on the system due to the viscous substrate. However, the system was compromised because the high flow rates resulted in enzyme deactivation due to shearing stress.

Tomato pectin esterase (PE) covalently bound to porous glass, retained only 0.32% of added activity and suffered from diffusion limitations when using citrus pectin as the substrate (Weibel *et al.*, 1975). Severe internal mass-transfer restrictions were highlighted by the twelve-fold increase in PE activity that occurred after the glass-bound enzyme was ground into smaller fragments. Citrus pectin was separated into low and high molecular weight fractions and used for kinetic studies; the kinetic behaviour of soluble PE was unaffected by the molecular weight of the substrate but the immobilized enzyme showed a five-fold increase in K_m for the high molecular weight fraction, as compared with the low molecular weight fraction (Weibel *et al.*, 1975). Immobilized PE also showed a reduction in thermal and storage stability,

compared with the native enzyme, with a half-life of only 2 weeks at 25°C (Weibel *et al.*, 1975).

Borrego *et al.* (1989) immobilized tomato pectin esterase on to glyceryl-coated controlled-pore glass by four different coupling methods, in order to determine the most efficient enzyme system for the hydrolysis of citrus pectin. Although pectin esterase coupled to the thiol-derivatized glass showed the highest activity yield of 3.2%, this low value, together with the very low specific activity obtained, suggested that interactions between the enzyme and the high molecular weight substrates were severely limited by steric restrictions. Pectinesterase bound to 20 nm pore glass showed the highest rate of demethylating activity, whereas the use of 46 nm pore sized glass resulted in a decrease in activity. This was attributed to internal diffusion limitations of the pectin (MW 290 kDa) to pectin esterase immobilized within the 46 nm glass pores, whereas the 20 nm pored sized glass was inaccessible to both enzyme and substrate. A slightly higher catalytic efficiency, together with a 1.9-fold drop in K_m for citrus pectin following enzyme immobilization on to the 20 nm pore sized glass, implied that a positive conformational change had occurred. Indeed, fluorescence spectra revealed that the immobilized enzyme had undergone a partial unfolding of its tertiary structure. The immobilized enzyme remained fully stable in a continuous stirred tank reactor (0.5% pectin, pH 7, 50 nM NaCl) for 10 h, after which a steady deterioration of the support and enzyme activity occurred as the result of continuous stirring (Borrego *et al.*, 1989).

In an extension of these studies, Romero *et al.* (1989) immobilized endo-*D*-polygalacturonase from *Rhizopus* spp. on to the same porous glass support modified by the four different coupling methods. Enzyme activity, which was measured as a decrease in the viscosity of a commercial methyoxylated pectin solution at 40°C, pH 5 and 50 mM NaCl, was highest when immobilization was on to aminoaryl-derivatized glass. That greater activity was achieved against low MW pectin (MW 34 kDa) over high MW citrus pectin (MW 280 kDa) also implied limitation by steric hindrances. As seen with immobilized pectin esterase, the highest specific activity was achieved at a 20 nm pore size, and the decrease in activity seen at 46 nm pore size was the result of a higher degree of diffusion limitation (Romero *et al.*, 1989).

Van Houdenhoven, de Wit and Visser (1974) demonstrated that the continuous dydrolysis of 1% sodium pectate by a Sepharose 4B column containing immobilized *Saccharomyces fragilis* polygalacturonase was suitable for the preparation of pectic oligomers. The production of a series of such oligomers, which could be useful for the analysis of the kinetic properties of pectin-degrading enzymes, was found to vary with reaction pH. In addition, the degree of substrate degradation, as determined by thin layer chromatography, was dependent on the methyl ester and foreign sugar content and the largest number of oligomers was produced at the slowest elution flow rate (9.5 ml h^{-1}).

Aspergillus niger endo-*D*-galacturonase, covalently bound to continuous flow columns of methacrylate gel by a series of different spacer groups, showed a decrease in enzyme activity against sodium pectate in all instances, compared with the free enzyme (Rexová-Benková and Mracková-Dobrotová, 1981). Although the K_m values for sodium pectate were not affected by either immobilization or by the length of the spacer groups, the relative enzyme activity increased with increasing spacer group length, due to a progressively decreased effect of steric hindrance and diffusion

limitation. Analysis of the products of immobilized and soluble enzyme hydrolysis by gel permeation chromatography showed that the randomness of the degradation by the bound enzyme was reduced because of the restriction of enzyme action to the peripheral areas of the substrate. Furthermore, analysis of immobilized enzyme activity against oligo-*D*-galactosiduronic acids of various sizes indicated that, at a common spacer length, activity was inversely proportional to substrate size, due to the limited access of bulkier molecules to the immobilized enzyme.

Endopolymethylgalacturonate lyase (PMGL), immobilized on to titanium-linked DEAE-cellulose and titanium-linked porous glass, retained only 0.46% and 9.2% of added activity, respectively (Hanisch, Rickad and Nyo, 1978). Most notable was the change in pH optimum from 5.3 for free PMGL to pH 3.7–4.7 when bound to DEAE-cellulose, and to pH 6.2–6.9 when bound to porous glass; these are typical effects for binding to polycationic versus polyanionic supports, respectively. The charged supports also influenced the catalytic activity of the bound enzyme. The attractive forces between pectin, which is negatively charged, and the polycationic DEAE-cellulose resulted in a 43% decrease in K_m whereas the repulsive forces between the pectin and the polyanionic glass support were manifest in a doubling of K_m.

The immobilization of other polysaccharide-degrading enzymes

DEXTRANASE

Dextran is a class of α-*D*-glucose branched polymers, in which the majority (95%) of the residues are linked by α-1,6-glucosidic bonds, but also some (5%) α-1,3-glucosidic linkages (Ward, 1985). Produced in abundant quantities by infection with *Leuconostoc mesenteroides* or related species in mechanically or weather-damaged sugar cane and sugar beet, dextrans can increase the viscosity of juices and thus cause problems during the clarification and evaporation of juices and in the concentration of molasses (Margaritis and Pace, 1985; Ward, 1985). However, these problems can be overcome by treating contaminated juices with dextranases for 15 min at 50–55°C, between the extraction and clarification stages of sugar production (Ward, 1985). Apart from their use in the sugar industry, other successful applications of dextranases include the production of controlled pore molecular sieves composed of dextran, and use as a toothpaste additive to help remove dental plaque (Margaritis and Pace, 1985; Wiseman, 1985).

Dextranses (1,6-α-glucan-6-glucanohydrolyases, EC 3.2.1.11) are most active in the pH range 5–7 and at temperatures up to 60°C (Ward, 1985; Wiseman, 1985). The major end products of dextran hydrolysis by dextranases are isomaltose and isomaltotriose (Wiseman, 1985). Dextranases that function in an endo-fashion are produced from strains of *Penicillium* and *Flavobacterium*, as well as *Klebsiella aerogenes, Lipomyces starkeyi* and *Fusarium monoliformi* (Smiley, Boundy and Hensley, 1982; Prabhu and Prabhu, 1985; Ward, 1985; Koenig and Day, 1989), whereas exo-dextranases are produced by *Bacillus coagulans* and *Arthrobacter globiformis* (Wiseman, 1985). Immobilization methods for dextranases have not been well documented but a few recent papers on the subject exist.

Prabhu and Prabhu (1985) covalently bound 50% of added dextranase activity from *P. aculeatum* on to glutaraldehyde-treated bentonite for the production of a stable clarifying agent for sugar cane juices. When immobilized, the enzyme opti-

mum pH range for activity narrowed, the K_m for dextran increased by 1.3-fold, and heat stability was reduced. However, in the presence of 20% sucrose, immobilized dextranase showed enhanced thermal stability, maintained high operational activity following six cycles of repeated use, and showed a 60% hydrolysis of dextran at 50°C in 1 h (Prabhu and Prabhu, 1985).

Smiley, Boundy and Hensley (1982) showed that the action pattersn of endo-dextranases from *P. funiculosam* and *P. lilacinum* are altered when covalently bound to silanized and glutaraldehyde-treated silica beads. The active immobilized enzyme produced a substantial amount of reducing sugars but its action had little effect on the viscosity of a high molecular weight dextran (2000 kDa) at pH 5.1 and 40°C. By contrast, soluble dextranase only slightly increased the level of reducing sugars but produced a rapid decrease in substrate viscosity. However, the hydrolysis of low molecular weight dextrans (150 kDa) resulted in similar hydrolysis products for bound and free enzyme. The authors suggested that immobilized dextranase can hydrolyse relatively few internal bonds of the high molecular weight dextrans, and, as a result, the bound dextranase converts from an endo- to an exo-type of action and produces high levels of reducing sugar (Smiley, Boundy and Hensley, 1982).

INULINASE

The use of fructose as an alternative sweetener to sucrose for the food and beverage industry has gained in popularity in recent years due to its more beneficial medicinal and nutritive properties, as well as its high sweetness level (Gupta *et al.*, 1990). Apart from sucrose and starch, there are several other potential commercial sources of fructose, namely, the β-*D*-fructofuranose polymers. These fructose polymers occur in two forms, both containing a terminal sucrose residue (Goodwin and Mercer, 1983). Fructose units linked by β-β-(2,1) glycosidic bonds belong to the inulin class, whereas those linked via β-(2,6) bonds are classified as phlean (Goodwin and Mercer, 1983). Inulin is the most common fructose storage polysaccharide in nature. It is a sizeable compound consisting of 30–35 straight-chain fructose units with a molecular weight of approximately 5000 (Goodwin and Mercer, 1983; Bajpai and Margaritis, 1985). Inulins are found primarily in the Compositae family (e.g. dahlia, Jerusalem artichoke, chickory) (Goodwin and Mercer, 1983; Uhm *et al.*, 1987) and are inexpensive, high-yielding sources of fructose that are generating considerable commercial interest as a carbohydrate source for the production of foodstuffs for humans and livestock, as well as for many other industrial processes (Kosaric *et al.*, 1989). In particular, the Jerusalem artichoke tuber is approximately 80% inulin by weight (Kim and Rhee, 1989) and this inulin source has stimulated considerable experimental interest.

Inulinases (2,1-β-*D*-fructan fructanohydrolase, EC 3.2.1.7) occur in a variety of plants, particularly species with inulin-containing roots and tubers, as well as in various yeasts (*Kluyveromyces, Debaryomyces, Candida*) (Bagsvaerd, 1981; Guiraud, Demeulle and Galzy, 1981; Kim, Byun and Uhm, 1982; Bajpai and Margaritis, 1985, 1987) and fungi (*Aspergillus, Penicillium, Fusarium*) (Bagsvaerd, 1981; Kim and Rhee, 1989; Gupta *et al.*, 1990). Three functional types are known: exo-inulinases, the most common, hydrolyse the terminal β-2,1- and β-2,6-fructofuranoside linkages of inulin; endo-fructan hydrolases hydrolyse inulin to fructo-oligomers, and fructo-

transferases split difructose anydride off the inulin chain to produce a difructose dianhydride (Beck and Praznik, 1986).

Conventional methods of industrial high-fructose syrup production involve a multistep process employing several amylolytic enzymes as well as glucose isomerase, for the net conversion of starch to syrups typically containing 42–55% fructose (Kosaric *et al.*, 1989). By contrast, the direct production of fructose from inulin by inulinase is a single-step reaction that can result in yields of up to 95% fructose (Kim and Rhee, 1989). The potential commercial benefit of an inulin–inulinase system for fructose production is obvious. The inulin-based substrates, simple straight-chain polymers of fructose, are ideally suited for hydrolysis. To date, however, inulinases have been considered too thermally unstable for industrial use, where high operational temperatures are required to prevent microbial contamination (Bagsvaerd, 1981). Inulinases typically have temperature optima of 45–55°C and they show a rapid loss of activity at 60°C. In an effort to overcome this problem, Bagsvaerd (1981) discovered strains of *Aspergillus* that produce more thermally stable inulinases; these maintained 95% of activity after 20 min reactions at 60–65°C, and showed activity optima at 60°C (Kosaric *et al.*, 1989). Another alternative for overcoming the problem of low thermostability is enzyme immobilization, since immobilization is cost-effective and typically results in improved operational parameters. However, despite the potential advantages of immobilized inulinases, there are only a few in-depth studies on this subject.

DEAE-cellulose retained as much as 75% of added *Debaryomyces phaffii* inulinase activity, and the immobilized enzyme showed an increase in temperature optimum and thermal stability, and an operational half-life that was five times longer than that of the free enzyme at 50°C (Guiraud, Demeulle and Galzy, 1981). However, the immobilized enzyme could not be used to its fullest potential, for the optimum pH for the immobilization step (pH 5) and the optimum operational pH (pH 2.5) did not coincide; this resulted in either low activity yields or enzyme desorption. Kim and Rhee (1989) immobilized 23% of added *A. ficuum* inulinase on to glutaraldehyde-cross-linked chitin, and produced fructose from Jerusalem artichoke tubers by continuous and batch reactor processes. The immobilized enzyme demonstrated high operational stability and a storage half-life of 560 h in 10% inulin, as well as greater thermostability than the free enzyme. Kim, Byun and Uhm (1982) found that 53% of added *K. fragilis* inulinase activity was retained on aminoethylcellulose-linked glutaraldehyde, and showed that inulin had protective effects in enhancing thermal and operational stabilities. The immobilized enzyme activity was maintained for 4 h at 55°C in the presence of inulin, whereas a rapid loss of activity occurred at temperatures greater than 50°C in the absence of substrate. Also, the bound enzyme showed operational half-lives of 17 and 14 days in the presence of a Jerusalem artichoke tuber extract or a 7% (w/v) inulin solution, respectively. Using metal-link chelation, Gupta (1994) immobilized 40% of purified *K. fragilis* inulinase on cellulose; the half-life of the immobilized enzyme was 5 days at 25°C and the optimal temperature for enzyme function (55°C) was not changed by immobilization.

Fusarium oxysporum inulinase immobilized by entrapment in polyacrylamide gel retained 45% of added activity, showed an enhanced temperature optimum (45°C) compared to the free enzyme (37°C), and a storage half life of approximately 5 days at 25°C (Gupta *et al.*, 1990). Ninety per cent of immobilized enzyme was maintained

after storage for 24 h at 45°C in the presence of 1% chickory root inulin, but without substrate all activity was lost. The immobilization of *Kluyveromyces marxianus* yeast cells by entrapment in gelatin also gave a very stable inulinase preparation which showed only a 2% loss of activity after continuous operation for 240 h and maintained full activity after 10 repeated uses in 7 h batch cycles at 50°C (Bajpai and Margaritis, 1985). Bajpai and Margaritis (1987) also immobilized 72% of added inulinase activity on to titanium chloride activated molecular sieves, resulting in an enzyme preparation that could function optimally at a higher temperature, was more thermostable than the free enzyme, and showed storage and operational half-lives of 83 and 30 days, respectively.

Inulinase immobilized on to glutaraldehyde-cross-linked chitin showed high conversion yields of 90 and 55% after 10 h in batch reactors and continuous packed bed column reactors, respectively, with productivities as high as 61 g l^{-1} h^{-1} (Kim and Rhee, 1989). The continuous reactor operation of gelatin-entrapped yeast cells demonstrated maximum productivities of 100 g l^{-1} h^{-1} and inulin conversions of 80% (Bajpai and Margaritis, 1985). High-fructose syrups were also produced in batch reactors with the gelatin-entrapped cells, resulting in 93% and 90% hydrolysis of Jerusalem artichoke tuber extracts or commercial inulin substrates, respectively (Bajpai and Margaritis, 1985). Inulinase adsorbed on to DEAE-cellulose maintained a constant K_m value, and the amount of inulin hydrolysis could be increased by decreasing substrate concentration and flow rate (Guiraud, Demeulle and Galzy, 1981). Total hydrolysis of the substrate was obtained using an inulin concentration of 5 g l^{-1} and a flow rate of 48 ml h^{-1}.

Immobilization of *K. marxianus* inulinase on to molecular sieves via titanium chloride activation, resulted in a two-fold increase in the K_m value for chickory root inulin (Bajpai and Margaritis, 1987). Aminoethylcellulose-immobilized inulinase showed both batch and column reactor conversions of 90% for Jerusalem artichoke extract, and high productivities of 102 mmol l^{-1} h^{-1} at 40°C and pH 5 (Kim, Byun and Uhm, 1982). An 8% increase in K_m for inulin upon immobilization was attributed to modest diffusion limitation due to an external film layer, which could be removed by increasing the reactor flow rate.

The immobilization of mono- and disaccharide-utilizing enzymes

GLUCOSE ISOMERASE

Glucose isomerase or xylose isomerase (EC 5.3.1.5) catalyses the reversible isomerization of xylose to xylulose and, of greater commercial importance, the isomerization of glucose to fructose for the production of high-fructose syrups (HFS or HFCS when corn is the substrate) (Bucke, 1981; Antrim and Auterinen, 1985). Glucose isomerase is an intracellular enzyme produced by microbial organisms from a range of genera, principally *Streptomyces, Bacillus, Pseudomonas, Lactobacillus* and *Anthrobacter* (Bucke, 1981; Al-Tai, Ali and Abdul-Razzak, 1987).

Before 1970, the principle source of food sweeteners was sugar cane and sugar beets (Verhoff *et al.*, 1985). However, with a 1.7-fold greater sweetening ability, HFCS was found to be a good alternative to sucrose for use as a food sweetener in the soft drink and candy industries (Chen, 1980; Bucke, 1981). Industrial processes treat

corn starch first with amylases to produce glucose syrups and then use glucose isomerase to produce syrup with a significant fructose content (42% at equilibrium) (Verhoff *et al.*, 1985). Soluble glucose isomerases have been used by corn-syrup producers for decades (Jensen and Rugh, 1987). Unfortunately, the cost of the isomerization step was huge due to the expense of using fresh glucose isomerase each time (Verhoff *et al.*, 1985). Alkaline isomerization is an alternative, but produces excessive colour and undesirable side-products (Cheetham, 1985). Thus, to make this process economically feasible, glucose isomerase is now used in an immobilized form (Jensen and Rugh, 1987).

The commercial processes for glucose isomerase immobilization are of two types, cell-free enzyme immobilization and whole-cell immobilization (Jensen and Rugh, 1987). In 1969, Takasaki patented the first whole-cell immobilized glucose isomerase. This batch isomerization process, which used heat-fixed *Streptomyces wedmorensis* cells containing glucose isomerase, produced a 48% fructose product (70°C, pH 7.5, 10 mM Mg^{2+}) in 24 h with an operational half-life of approxiamtely 170 h (Jensen and Rugh, 1987). Other methods of micro-organism immobilization include flocculation, entrapment, adsorption and cross-linking (Chen, 1980; Jensen and Rugh, 1987). Although immobilized cell systems do not require enzyme isolation or purification steps, they usually produce less stable glucose isomerase preparations and can suffer from product contamination by cell debris, as compared with methods that immobilize pure enzyme (Chen, 1980; Jensen and Rugh, 1987).

Critical to the expansion of the sweetener industry has been the considerable effort devoted to the development of reusable, pure enzyme-immobilized glucose isomerase systems. Indeed, the enzymatic conversion of glucose to fructose by immobilized glucose isomerase on an industrial scale was the first successful application of immobilized enzyme biotechnology (Chen, 1980). The first reusable, pure enzyme-immobilized glucose isomerase, patented in 1970 by the Clinton Corn Company, used adsorption on to DEAE-cellulose and gave 50% isomerization (pH 6.5, 60°C) and an activity half-life of approximately 200 h. However, these early systems were very susceptible to changes in process conditions (Jensen and Rugh, 1987). The recent literature on the immobilization of glucose isomerase is vast and cannot be mentioned fully here. However, good comprehensive reviews of the subject are provided by Jensen and Rugh (1987) and Chen (1980).

Immobilized glucose isomerase is available commercially. Glucose isomerase immobilized on to SiO_2 beads can be purchased under the trade name Optisweet 22 from Kali-Chemie and the enzyme bound to derivatized alumina particles can be obtained from UOP Inc. Gist Brocades and Miles Laboratories Inc. immobilize whole cells by entrapment in gelatin treated with glutaraldehyde (Maxazyme) or by flocculation of whole cells treated with glutaraldehyde (Taka sweet) (Gaikwad and Deshpande, 1992). The following discussion highlights only a fraction of the papers published on the subject of glucose isomerase immobilization. We have chosen to focus on some of the different methods of glucose isomerase immobilization as well as the various factors involved in the successful function of an immobilized glucose isomerase bioreactor, including the maintenance of high enzyme activity, stability and reusability.

Glucose isomerase has a characteristically high reaction temperature and thermal stability (Bucke, 1981) and immobilized glucose isomerase usually maintains the pH

(operational range pH 7–9) and temperature stabilities (70–90°C) similar to the free enzyme (Y.Y. Lee *et al.*, 1976; Huitron and Limon-Lason, 1978; Gaikwad and Deshpande, 1992; Schafhauser and Storey, 1992b; Deshmukh, Choudhury and Shankar, 1993). Antrim and Auterinen (1985) demonstrated the successful immobilization of glucose isomerase on to DEAE-cellulose, with industrial-sized columns showing a high immobilized activity of 1500 U g^{-1}, a productivity greater than 9 metric tons of 42% fructose syrup solids per kilogram of immobilized enzyme, and a half-life of 70 days. Glutaraldehyde-activated Indion 48-R retained 30–40% of added glucose isomerase activity and could convert 36% glucose syrups into 18.5% fructose in 5 h (Deshmukh, Choudhury and Shankar, 1993). Glutaraldehyde-cross-linked porous glass particles bound 56% of added glucose isomerase activity, with an enzyme loading of 48 mg g^{-1} glass and a half-life reaching 240 days at 50°C (Y.Y. Lee *et al.*, 1976). A silanized and glutaraldehyde-treated silicon oxide based carrier bound as much as 30 units g^{-1} of support material without a decrease in activity (Sórensen and Emborg, 1989). Granular chicken bone bound up to 32% of added enzyme and binding was stable even after exposure to a wide variety of chemical and physical conditions, including treatments with detergents, organic solvents and high salt solutions (Schafhauser and Storey, 1992b). Enzyme activity was also stable after five cycles of operation but the operational half-life was only 168 h at 55–60°C. Glucose isomerase immobilized on to polyurethane foam retained 55% of added activity but the storage half-life was only 160 h at 55°C (Storey and Chakrabarti, 1990).

Increases in K_m values for glucose of 1.6- to 3-fold following immobilization are not unusual for glucose isomerase (Huitron and Limon-Lason, 1978; Volkin and Klibanov, 1989; Storey and Chakrabarti, 1990; Schafhauser and Storey, 1992b). Such changes could be due to partial inactivation due to immobilization in an unfavourable configuration, or to steric hindrance from overcrowding of the immobilized enzyme (Kennedy, 1985). However, effects of immobilization on glucose isomerase kinetic properties are fairly inconsequential compared with the effects of immobilization on many other enzyme systems.

Immobilized glucose isomerase is rarely affected by pore diffusion limitation or external mass-transfer resistance problems, as both the substrate and product molecules are small (Bucke, 1981). Experiments that typically identify diffusion-limitation problems, such as comparisons of the Arrhenius and Lineweaver–Burk plots for free versus immobilized enzymes, or measurement of reaction rates for the enzyme bound to supports of varying pore or particle sizes, have concluded that no appreciable diffusion limitation exists (Sórensen and Emborg, 1989; Storey and Chakrabarti, 1990; Schafhauser and Storey, 1992b). Similarly, experiments conducted to assess the presence of external mass-transfer limitations, such as increasing the agitation speed of the bioreactor, had minimal effect on the apparent kinetic properties of the immobilized glucose isomerase and, therefore, showed no restrictions on the catalytic efficiency of the immobilized enzyme (Huitron and Limon-Lason, 1978; Schafhauser and Storey, 1992b). However, Y.Y. Lee *et al.* (1987), in comparing the reaction rates of immobilized glucose isomerase at changing flow rates in a recirculated packed bed reactor, observed significant resistance to substrate diffusion through the external liquid film when the flow rate was below a certain velocity. Upon comparing the reaction rates of two support sizes (310 v. 20 μm diameter), the authors showed that the reaction rate differences above 60°C were the result of a suppressed reaction rate

caused by diffusion resistance in the micropores of the enzyme support.

Enzyme stability is one of the principal factors affecting reactor productivity for free or immobilized glucose isomerase (Chen and Wu, 1987). The primary causes for activity loss are poor binding stability, thermal inactivation, impurities, and the generation of chemical inactivators at high temperature and at pH extremes (Y.Y. Lee *et al.*, 1976; Huitron and Limon-Lason, 1978; Klibanov, 1979; Chen, 1980; Bucke, 1981; Antrim and Auterinen, 1985; Chen and Wu, 1987; Jensen and Rugh, 1987; Volkin and Klibanov, 1989; Schafhauser and Storey, 1992b). Thus, few immobilized enzyme systems have the properties necessary for economically feasible, commercial production of HFCS (Verhoff *et al.*, 1985; Gaikwad and Deshpande, 1992). But due to its commercial importance, extensive research has focused on new methods of immobilization that could result in a more stable glucose isomerase, for even a modest increase in the half-life of the enzyme would substantially reduce the production cost of HFCS (Klibanov, 1979; Volkin and Klibanov, 1989).

Poor binding stability is a common problem in immobilized enzyme technology. Immobilization by absorption tends to be weaker, although this is not always the case. Glucose isomerase immobilization on to an anion-exchange resin showed a 35% loss of enzyme activity after seven cycles of reuse (Gaikwad and Deshpande, 1992) whereas glucose isomerase bound to glutaraldehyde-activated Indion 48-R lost approximately 40% of initial bound activity after five cycles of use (Deshmukh, Choudhury and Shankar, 1993). Immobilization by entrapment in polyacrylamide (Stranberg and Smiley, 1971) also resulted in very unstable preparations. Chitin cross-linked with glutaraldehyde retained 40% of added glucose isomerase activity, but showed a decreasing half-life with increasing flow rates at 60°C (7 days at 7.8 ml h^{-1} and 16.5 days at 5 ml h^{-1}) (Stanley *et al.*, 1976). These supports, however, can be regenerated and reused (Antrim and Auterinen, 1985; Sórensen and Emborg, 1989).

Glucose isomerase is a metalloenzyme, containing distinct metal-binding sites which require a combination of cobalt, magnesium and manganese for maximum activity (Verhoff *et al.*, 1985; Marg and Clark, 1990). Glucose isomerase immobilized on to porous glass particles showed 39% and 40% increases in activity in the presence of 10 mM Mg^{2+} and 0.1 mM Co^{2+}, respectively (Y.Y. Lee *et al.*, 1976). Co^{2+} also stabilized glucose isomerase against heat denaturation and increased the enzyme half-life ten-fold. However, Co^{2+} is very toxic and must be removed when the reaction is complete in order to meet government standards for food products (Verhoff *et al.*, 1985; Jensen and Rugh, 1987). This problem can be alleviated by adding 1.5–50 mM Mg^{2+}, which allows full expression of free and immobilized glucose isomerase activity in the absence of cobalt (Huitron and Limon-Lason, 1978; Antrim and Auterinen, 1985; Chen and Wu, 1987; Sórensen and Emborg, 1989; Volkin and Klibanov, 1989; Storey and Chakrabarti, 1990; Schafhauser and Storey, 1992b). The addition of bisulphate in the range of 0.1–2 mM also helps to maintain enzyme stability and prevent oxidation (Antrim and Auterinen, 1985; Sórensen and Emborg, 1989; Storey and Chakrabarti, 1990; Schafhauser and Storey, 1992b).

The degree of stability given to glucose isomerase by the presence of ligands (glucose, fructose, HFCS) has also been extensively studied. Substrate protection of an enzyme may be due to the accompanying stability of the tertiary protein structure when the enzyme combines with the substrate to yield a complex form (Chen and Wu, 1987). A knowledge of enzyme protection by a substrate may be used in selecting the

operating conditions of enzyme reactors, optimizing reactor performance and enzyme replacement policy (Y.Y. Lee *et al.*, 1976; Chen and Wu, 1987). Indeed Y.Y. Lee *et al.* (1976) observed an eight-fold increase in the half-life for glucose isomerase immobilized on to porous glass beads in the presence of 1.5 M fructose. Substrates or products can also stabilize enzymes against heat-induced unfolding by forming intermediate complexes which have a slower thermal decay rate compared with that of the uncomplexed enzyme (Klibanov, 1979; Ahern and Koibanov, 1987; Chen and Wu, 1987; Volkin and Klibanov, 1989). Glucose isomerase immobilized on to glutaraldehyde-cross-linked glass beads showed a half-life of 37 min at 90°C in the presence of HFCS, but less than 1 min its absence (Volkin and Klibanov, 1989). Immobilized glucose isomerase also showed an increasing half-life from 120 to 190 hours with increasing glucose concentrations of up to 3 M at 70°C (Chen and Wu, 1987).

Although most glucose isomerases exhibit high temperature optima, industrial bioreactors carry out the reaction at 55–57°C, as the enzyme is subject to thermal inactivation with long-term use at high temperatures. For example, glucose isomerase covalently bound to glutaraldehyde-cross-linked porous glass beads showed a functional half-life of only 120 min at 80°C (Volkin and Klibanov, 1989). At any given temperature, irreversible thermoinactivation of an enzyme is caused by a combination of covalent and conformational processes. Volkin and Klibanov (1989) provided a mechanistic description of the irreversible thermoinactivation of glucose isomerase under normal operating conditions. Thermoinactivation of free or immobilized glucose isomerase at 90°C in the presence of HFCS was shown to be the result of deamidation of the enzyme's asparagine/glutamine residues. The authors also showed that the time-dependent decay of enzyme activity in industrial bioreactors is brought about by oxidation of the enzyme's cysteine residues (a significant problem since total exclusion of O_2 in a bioreactor is not feasible) and/or heat-induced deleterious reactions with HFCS or its impurities.

The purity of the substrates and products are also critical factors for glucose isomerase activity and stability. Saccharified corn syrups usually contain particulate matter and soluble impurities such as amino acids, peptides, lipids and metals which can cause enzyme inactivation or inhibition if not removed; decreases in productivity of as much as 50% have been recorded (Jensen and Rugh, 1987).

Jensen and Rugh (1987) described various types of bioreactors used for HFCS production and their optimization. Batch processes typically function in the presence of Co^{2+} and at low pH values, in order to stabilize the enzyme and to limit by-product formation during the long residence times of the reaction. Plug flow (fixed bed) processes are more advantageous, as much higher rates of productivity and shorter residence times can be achieved; these both eliminate the need for Co^{2+} and considerably reduce by-product formation. Production capacity can also be adapted according to demand by adjusting isomerization temperature and age of the enzyme. When a high syrup production capacity is needed, the isomerization temperature is increased and old enzyme is continuously replaced by new, high-activity enzyme. The lowest cost of conversion is obtained by running at a low temperature (e.g. 56–57°C) and by utilizing the enzyme until only 10% residual activity remains. Syrups containing 55, 60 and 90% fructose can now be manufactured from corn syrup, but at much lower quantities than the 42% fructose syrups (MacAllister, 1980).

INVERTASE

Invertase (β-*D*-fructofuranosidase, EC 3.2.1.26) is an enzyme, typically found in baker's yeast (*Saccharomyces cerevisiae*), that can hydrolyse disaccharide compounds with unsubstituted β-*D*-fructofuranosyl residues, such as sucrose and raffinose, into their constituent monosaccharides (Goldstein and Lampen, 1975; Lopez-Santin *et al.*, 1983). The invert sugar syrups that result find use in the confectionery, beverage and bakery industries (Mansfeld, Schellenberger and Römbach, 1992). Invertase also exhibits transferase activity at high substrate concentrations (e.g. 1 M sucrose), and is capable of transferring the β-*D*-fructofuranosyl residue to primary alcohols (Goldstein and Lampen, 1975). Notably, the very first study of an immobilized enzyme occurred in 1916 and involved the stable immobilization of invertase on to activated charcoal (Nelson and Griffin, 1916). Immobilized invertase preparations are now commercially available from several companies, including Gist-Brocades Inc. (Maxinvert), Rohm GmbH (Plexazym IN) and Tate and Lyle Inc. (charcoal-bound invertase). However, although yeast invertase is used commercially for the production of glucose/fructose syrups, immobilized invertase has not found widespread use (Yamazaki, Cheok and Fraser, 1984), possibly because most invertase preparations suffer from substrate (sucrose) inhibition (Dickensheets, Chen and Tsao, 1977).

Yeast invertase is a glycoprotein; 50% of its molecular weight is carbohydrate, mainly glucan and mannan (Lampen, 1971). The immobilization of glycoprotein enzymes via their carbohydrate moieties is advantageous, since the bonds always occur away from the enzyme active site, thus allowing the enzyme to maintain all of its original activity (Marek, Valentová and Kás, 1984). The covalent coupling of yeast invertase on to *Ocimum basilcum* seeds through its carbohydrate moieties resulted in an alkaline shift in pH optimum (from pH 3.8 to 4.4) and an increase in thermostability with no appreciable change in temperature optimum (Melo and D'Souza, 1992). The immobilized enzyme could be reused more than 10 times in batch process without appreciable loss of activity.

Marek, Valentová and Kás (1984) compared the pH optima and kinetic constants of invertase immobilized on to various solid supports, in an effort to determine the ideal conditions for attraction and binding of invertase via its carbohydrate moiety. All of the supports tested contained groups that reacted with the invertase aldehyde groups activated by periodate oxidation. Enzyme conjugates prepared by direct reaction to glycidyl methacrylate, styrene-divinyl benzene or bead cellulose had higher pH optima and higher K_m values for sucrose (2.8-, 3.8- and 2.0-fold higher, respectively), compared with the native enzyme. By comparison, invertase immobilized on to the same supports by the modified Ugi method (addition of 20 ml cyclohexyl isocyanide and 40 ml 0.5 M acetic acid to the enzyme/support mixture followed by incubation with shaking at 4°C for 6 h), showed overall higher enzyme activities, higher pH optima and lower K_m values than the corresponding immobilized enzymes prepared by direct action.

Yeast invertase immobilized on to ConA-Sepharose through its carbohydrate moiety also retained high enzyme activity (70%) and showed enhanced stability at 60°C and in the presence of 3 M urea, compared to the native enzyme (Iqbal and Saleemuddin, 1983, 1985). Stability was further improved when the immobilized

enzyme was cross-linked with glutaraldehyde. The continuous column hydrolysis of 1 M sucrose by ConA-Sepharose-bound invertase resulted in over 80% sucrose conversions at 30°C and at a flow rate of 100 ml h^{-1}, with the column retaining over 80% of bound activity after 60 days (Iqbal and Saleemuddin, 1983, 1985).

Baker's yeast invertase immobilized on to microcrystalline cellulose, DEAE-or CM-Sephadex, and insolubilized ConA retained 46, 41, 70 and 73% of added activity, respectively (Woodward and Wiseman, 1978). ConA- and microcrystalline-cellulose-bound invertase were more thermally stable, particularly in the presence of sucrose, and showed broader pH optima (pH 2.5–6) and unaltered K_m values, compared with the native enzyme. By contrast, DEAE- and CM-Sephadex bound invertase both showed a 3.6-fold increase in the K_m value for sucrose and had poor thermal stability, losing all activity after 1 min of heating at 65°C. However, stability was restored in the presence of 20 mM succinic acid and 20 mM ethylenediamine, respectively, suggesting that new stabilizing salt-linkages had formed between the enzymes and these compounds to replace those that were broken in the enzyme during the immobilization step. These results, together with additional chemical modification experiments, led the authors to conclude that the alteration of either amino or carboxyl groups would result in the loss of conformational stability, unless these stabilizing salt-linkages were preserved.

Invertase immobilized on to *N,N*-diethylamino-2-hydroxypropyl (DEAHP)-cellulose beads remained firmly bound, with a similar temperature optimum, a slightly higher thermal stability and a more acidic pH optimum, compared with the native enzyme (Hradil and Svec, 1981). Furthermore, the enzyme retained the same activation energy but showed a slight increase (20%) in the K_m value upon immobilization. The amount of enzyme adsorbed on to DEAHP-cellulose was proportional to the porosity and the exchange capacity of the beads, with activities in a stirred tank reactor exceeding 8000 mmol min^{-1} g^{-1} in 10% sucrose at 30°C and pH 5.

Candida utilis invertase immobilized on to porous cellulose beads by ionic-guanidino attachment showed a broader pH optimum (from pH 4 to 5.4) compared with the native enzyme (pH 4.1), a 1.8-fold lower activation energy and, unlike native invertase, remained active at temperatures above 70°C (Dickensheets, Chen and Tsao, 1977). However, the 2.8-fold higher K_m value, together with a 1.7-fold higher K_i value for sucrose, suggested that immobilized invertase activity was limited by diffusion effects. This was verified by the fact that a minimum column flow rate of 18 ml min^{-1} was required to eliminate the effect of external film diffusion on the reaction rate of bound invertase. Furthermore, a comparison of the reaction rates of invertase bound to cellulose beads of three different mesh sizes revealed pore diffusion effects with the smallest mesh bead size (Dickensheets, Chen and Tsao, 1977). Diethylaminoacetyl (DEAA)-cellulose retained 30–45% of added *C. utilis* invertase activity by ionic interactions, and the immobilized enzyme showed similar K_m values, storage stability, heat stability and temperature optimum to native invertase (Maeda and Suzuki, 1973). An immobilized column preparation catalysed continuous inversion of 50% sucrose for 9 days at 30°C.

Yeast invertase covalently bound to glutaraldehyde-cross-linked polystyrene anion-exchange resin showed continuous sucrose hydrolysis in both 3 ml packed bed reactors and 50 litre pilot-scale reactors, without loss of efficiency or operational stability (Mansfeld, Schellenberger and Römbach, 1992). Studies of the dependence

of productivity on substrate concentration, temperature and catalyst stability also found that the loss of reactor productivity due to product inhibition at sucrose concentrations higher than 2.1 M could be overcome by increasing the reactor temperature from 40 to 50°C (Mansfeld, Schellenberger and Römbach, 1992). The 60% decrease in half-life of the catalyst that resulted from the higher temperature could be overcome by using a higher sucrose concentration (2.4 M). The half-life of the catalyst increased from 15 to 131 days at higher substrate concentrations, due to substrate protection. As a result, the productivity of the 50 litre immobilized invertase reactor was 0.76 kg sucrose hydrolysed h^{-1} l^{-1} at 40°C and a substrate concentration of 2.4–2.6 M. Sucrose conversions of 92% could be maintained for 1 year under these conditions. This demonstrates the value of searching for the optimal compromise between different operational parameters in order to achieve optimal results with respect to economy and catalyst exploitation (Mansfeld, Schellenberger and Römbach, 1992).

Stefuca, Gemeiner and Báles (1988) compared the reaction kinetics and stability of chlorotriazinyl cellulose-bound invertase in a stirred tank reactor (STR) and a packed bed reactor (PBR). Invertase in the STR, when maintained at an agitation speed of 300 rev min^{-1}, showed no change in K_m values and, thus, was not limited by external diffusion. In the PBR, the reaction rate and K_m value for invertase was affected by external mass transfer but only when the flow rate dropped from 4 to 0.5 ml min^{-1}. However, the beads in both reactors were deformed or partially destroyed under the reactor conditions. Under optimal storage conditions (in the presence of formaldehyde) the immobilized invertase maintained 100% activity after 40 days at 4°C. Monsan and Combes (1992) compared the activities of invertase immobilized on to corn grits and operating in either a continuous stirred tank reactor (CSTR) or a packed bed reactor (PBR) of 100 ml volumes. The productivity of the PBR was 30% and 40% higher than that of the CSTR when using either low (0.4 M) or high (2.44 M) sucrose concentrations, respectively. The PBR was successfully scaled up to 17.6 litres, achieving productivities of 0.45 kg sucrose hydrolysed h^{-1} l^{-1} or 93% sucrose conversions at 2.5 M substrate and 50°C.

Invertase entrapped in gelatin discs hardened with formaldehyde was limited by sucrose resistance to diffusion into the insoluble matrix (de Alteriis *et al.*, 1985). The 39% enzyme activity yield that was obtained under conditions of pH 5 and 30°C was nearly doubled after immobilized invertase was crushed into smaller segments (de Alteriis *et al.*, 1985). Invertase covalently bound to glutaraldehyde-cross-linked polyethyleneimine-derivatized cotton cloth showed in a decreased optimal pH range with significant activity loss above pH 5 (Yamazaki, Cheok and Fraser, 1984). However, the enzyme preparation maintained full activity after 3 months' storage at 30°C in 1 M sucrose. López-Santin *et al.* (1983) immobilized 20–30% of added invertase on to cyanuric chloride-activated clay; the immobilized enzyme showed a broader pH optimum than its free counterpart but kinetic parameters were similar in assays at 25°C and 0.3 M sucrose. However, immobilized enzyme batch reactors were still limited by extensive substrate and production inhibition as seen with the free enzyme at 5% sucrose, and also showed significant levels of thermal deactivation above 30°C.

Co-immobilization studies

A new generation of enzyme technology has explored the concept of co-immobilizing enzymes on to a single support, for the creation of a conversion system that can degrade complex substrates into monomer sugars in a single process. Reactions catalysed by a co-immobilized enzyme system could offer a variety of advantages over a comparable series of individual soluble enzyme reactions, including:

1. reduction of the diffusion distance between enzymes for substrates and products;
2. prevention of the accumulation of inhibitory concentrations of intermediates and products in reactors;
3. a potential reduction in the overall amount of enzyme required;
4. an improved sugar yield and/or shorter residence times;
5. a potential increase in the half-lives of constituent enzymes due to the stability imparted by the close proximity of multiple enzymes on a single matrix; and
6. a potentially greater cost-effectiveness with respect to energy and equipment use (Mårtensson, 1974a,b; Hägerdal, 1980; Chakrabarti and Storey, 1990; Xiao *et al.*, 1990).

Such advantages are in addition to those basic properties already associated with the use of immobilized enzymes.

The greatest potential for success occurs when the enzymes involved have similar characteristics (optimum pH and temperature, heat stabilities) and when they are co-immobilized in a ratio that has been optimized to give the highest enzyme conversion rates possible without overcrowding the support (Chakrabarti and Storey, 1989; Schafhauser and Storey, 1993). Although relatively few immobilized multienzyme systems have been developed, such processes show great potential as a more advanced and efficient method of polymer degradation (especially starch and cellulose) for the production of high-sugar syrups.

CO-IMMOBILIZED ENZYMES FOR STARCH DEGRADATION

β-Amylase can hydrolyse the α-1,4-glycosidic links, but is unable to bypass the α-1,6-glycosidic linkages in the amylopectin portions of starch and does not attack maltotriose; hence, the use of β-amylase alone results in incomplete degradation of branched polymers. The end products typically resulting from β-amylase hydrolysis of liquified starch are 55% maltose, 15% maltotriose and 30% β-limit dextrins (Finnsugar Co.Ltd. production information). Realizing the limitations imposed by the specificity of β-amylase, researchers have focused on developing multienzyme systems that combine pullulanase and β-amylase to produce a high-maltose product from starch.

Ohba and Ueda (1980) combined a pullulanase complex immobilized to TEAE-cellulose with sweet potato β-amylase bound to *p*-aminobenzyl cellulose in the same reactor in order to produce a functioning multienzyme complex. Unlike the immobilized β-amylase alone, the reactor containing the mixture of both enzymes could completely convert starch to maltose at 50°C and showed a long operational half-life (20 days) (Ohba and Ueda, 1980). Måartensson (1974b) co-immobilized β-amylase and pullulanase on to acrylamide–acrylic acid co-polymers but the

system showed intraparticle/film diffusion resistance and steric hindrance despite vigorous shaking of the mixtures. Although activation energies and Arrhenius plots for the free and co-immobilized enzyme systems were similar, product yields were strongly influenced by flow rate, and the K_m value for starch increased by 5.8-fold with immobilization. Furthermore, the stability of β-amylase and pullulanase, even when co-immobilized, was still dependent on substrate concentration and remained sensitive to temperature. Operational stability improved with increasing substrate concentration for the co-immobilized enzymes, but half-lives decreased from 20 days at 40°C to 1–5.6 days at 50°C (Mårtensson, 1974b).

Amyloglucosidase can attack both α-(1,4) and α-(1,6) bonds in starch to produce glucose. However, the efficiency of conversion is increased if the debranching enzyme, pullulanase, is added (Galliard, 1985). The enzymes function interdependently. Pullulanase, by debranching starch, makes it more accessible to amyloglucosidase which has higher reaction rates on linear glucose polymers (Ram and Venkatasubramanian, 1982). The amyloglucosidase–pullulanase complex covalently coupled to polyurethane foam retained 32% of combined added activity (compared with a soluble mixture of the two enzymes) and showed no activity loss after 4 months storage at 4°C (Chakrabarti and Storey, 1990). The similar pH and temperature optima of the individual enzymes allowed for enhanced activities when co-immobilized and doubled the glucose yield over immobilized amyloglucosidase alone (Chakrabarti and Storey, 1990).

The co-immobilization of amyloglucosidase and pullulanase on to azide-linked bovine hide collagen membranes resulted in enhanced levels of substrate hydrolysis (4–5% dextrose equivalent) over that of immobilized amyloglucosidase alone; pullulanase was especially effective in enhancing activity in the final stages of long-term hydrolysis (e.g. 160 h) (Ram and Venkatasubramanian, 1982). Co-immobilization also greatly improvd the stability of pullulanase, which remained active for at least 6 days compared with only 1 day for the free enzyme. At 50°C, pH 5.0 was considered to be the optimal operational pH. Although immobilized pullulanase would show greater activity and stability at pH 6 or 7, these higher pHs had an adverse effect on the activity of amyloglucosidase (Ram and Venkatasubramanian, 1982).

The addition of pullulanase to amyloglucosidase immobilized on to granular chicken bone imparted a certain degree of instability not seen when amyloglucosidase was immobilized alone; storage half-life decreased (70 days versus 144 days, respectively), activity was lost with continuous reuse (24% lost versus 100% remaining after five cycles) and a significant activity loss occurred in the presence of 0.2 and 0.5 M KCl (Schafhauser and Storey, 1993). However, the co-immobilization of pullulanase with amyloglucosidase maintained high temperature optima and resulted in higher product yields than those produced by immobilized amyloglucosidase alone, particularly in the later stages of a hydrolysis reaction (4–24 h). Furthermore, the co-immobilized enzymes maintained a broader pH range of activity (from pH 3 to 6.5) which was particularly different from the free-enzyme mixture on the alkaline side of the range, the free-enzyme mixture retaining only about 60% of maximal activity at pH 6.5.

With near-optimal activities of the bone-immobilized amyloglucosidase/pullulanase still retained at pH 6.5, the possibility of adding glucose isomerase into the immobilized enzyme system became feasible. Such an enzyme complex would allow the

complete conversion of starch to fructose. A one-step conversion process would be ideal for, at present, the processes of starch hydrolysis and glucose isomerization using soluble enzymes must, of necessity, be conducted in two separate stages using very different reaction conditions (particularly of pH) (Verhoff *et al.*, 1985). Furthermore, the enriched glucose product afforded by co-immobilized amyloglucosidase and pullulanase activity would lead to a higher conversion efficiency and reduced reaction time for the isomerization step, for high-fructose syrups are more easily produced by starting with as high a glucose concentration as economy allows (Antrim and Auterinen, 1985).

The co-immobilization of amyloglucosidase, pullulanase and glucose isomerase on to granular chicken bone resulted in a sufficient broadening of the operational parameters that the three enzymes could successfully catalyse a one-step conversion process that converted glycogen to fructose (Schafhauser and Storey, 1992c). Since the immobilized complex could not hydrolyse insoluble starch in any appreciable amount, glycogen was used as the substrate for experimental optimization of the system. The reaction pH used with the three-enzyme co-immobilized system strongly influenced the yield and ratio of the hexose sugars produced. Lowering the reaction pH increased the glucose yield by increasing the activity of amyloglucosidase and pullulanase, but fructose production dropped due to the inhibition of glucose isomerase. This is a positive result, however, for it demonstrates that the product ratios can be altered readily by a simple manipulation of pH in order to suit different goals of HFCS producers. The greatest fructose yield achieved was 20% at the optimal pH of 6.5–7 of the co-immobilized system, versus 30% at pH 7.5–8 for a mixture of the free enzymes. Reactor pH could also be favourably manipulated during the hydrolysis reaction to increase fructose output; a neutral pH in the early stages of the reaction favoured glucose production, whereas a more alkaline pH in the later stages favoured fructose production, resulting in a net 25% fructose product solution.

Xiao *et al.* (1990) separately immobilized amyloglucosidase on to anilinosulphonic-linked polystyrene beads and glucose isomerase on to triethanolamine-linked polystyrene beads and then combined the two in the same reactor. The mixture carried out simultaneous saccharification and isomerization reactions successfully at pH 6.6 and 60°C. The charged supports altered the pH profiles of the two enzymes such that immobilized amyloglucosidase and glucose isomerase retained high activities (90% and 55%, respectively) at neutral pH. By contrast, soluble amyloglucosidase and glucose isomerase showed much lower activities (40–50%) due to their very different pH optima (pH 4.5 and 9.5, respectively).

Wheat β-amylase and *A. niger* amyloglucosidase were successfully co-immobilized on to glutaraldehyde-cross-linked silica beads due to the similarities in their pH optima (Bohnenkamp and Reilly, 1980). By co-immobilizing different ratios of β-amylase and amyloglucosidase in batch reactors with increasing process times, syrup products of a variety of compositions were produced (Bohnenkamp and Reilly, 1980). Hägerdal (1980) successfully co-immobilized sweet almond β-amylase and *Saccharomyces cerevisiae* cells for the production of ethanol from cellobiose. At 22°C the complex, which was entrapped into calcium alginate gel, showed approximately 50% conversion of cellobiose to glucose, with 40% of the resulting glucose fermented to ethanol.

CO-IMMOBILIZED ENZYMES FOR CELLULOSE DEGRADATION

Natural cellulases from most fungal sources are limited in their overall activity by low amounts of β-glucosidase (Klyosov and Consultant, 1986) and by cellobiose inhibition of cellobiohydrolase (Sundstrom *et al.*, 1981). Although advantageous to the growth of the fungus, this is a limiting factor with respect to their industrial application for cellulose processing. Improved rates of cellulose hydrolysis and improved glucose yield can be achieved under lab or industrial conditions by combining additional β-glucosidase with cellulases (Sundstrom *et al.*, 1981).

The co-immobilization of *Trichoderma reesei* cellulase and *A. niger* β-glucosidase on to polyurethane foam resulted in a 2.5- and 4-fold greater rate of glucose production for soluble cellulose (CMC) and insoluble cellulose (microcrystalline), respectively, compared with immobilized cellulase alone (Chakrabarti and Storey, 1989). A ratio of 30:2 units of immobilized cellulase:β-glucosidase was optimal for maximum cellulose hydrolysis, which represents a five-fold increase in β-glucosidase content compared with the natural content of the fungal cellulase complex (Chakrabarti and Storey, 1989). Furthermore, the long operational half-life (approximately 500 h) and the capacity for long-term linear rates of hydrolysis of microcrystalline cellulose, implied that the co-immobilization of β-glucosidase with cellulase resulted in a stable complex which eliminates the problem of cellobiose feedback inhibition of the cellulase complex.

Co-immobilization of cellulase and β-glucosidase on to polyurethane foam also resulted in an alkaline shift in the pH optimum from 5 to 6 with a significant amount of activity still maintained at pH 7 and 8 (Chakrabarti and Storey, 1989). This, plus the fact that foam-immobilized glucose isomerase also showed significant activity at a pH as low as 6.8, justified experiments on the co-immobilization of all three enzymes in an attempt to create a single-process conversion of cellulose to fructose. Such a one-step conversion process is not possible with the soluble enzymes under similar reaction conditions. The formation of an operational immobilized multienzyme complex would allow for greater rates of cellulose conversion, for the ultimate product fructose, unlike glucose, is a poor feedback inhibitor of β-glucosidase (Woodward and Arnold, 1981).

Like the multienzyme system designed for the one-step conversion of starch to fructose (Schafhauser and Storey, 1992c), the specific reaction pH used with the co-immobilized complex of cellulase, β-glucosidase and glucose isomerase on polyurethane foam strongly influcenced the yield and ratio of the hexose sugars produced (Storey and Chakrabarti, 1990). Lowering the pH increased the total yield of hexose sugars by increasing the activity of cellulase and β-glucosidase, but reduced the fructose yield by inhibiting glucose isomerase activity. Increasing the pH had the opposite effect. However, unlike the complex of amyloglucosidase, pullulanase and glucose isomerase, the immobilized complex of cellulase, β-glucosidase and glucose isomerase became a better catalyst than a mixture of the free enzymes. The co-immobilized system showed a fructose yield as high as 30% from insoluble as well as soluble cellulose substrates at its pH optimum of 7 (Storey and Chakrabarti, 1990).

CO-IMMOBILIZED ENZYMES FOR PECTIN DEGRADATION

Compared with the individually immobilized enzymes, a co-immobilized system including both pectin esterase (PE) and polygalacturonase (PG) should represent a logical and beneficial advance for industrial processes such as decreasing the viscosity of pectin-containing solutions and enhancing the rate of juice clarification. The depolymerizing activity of PG is dependent on the demethoxylating activity of PE, such that PG cannot hydrolyse the glycosidic linkages of *D*-galacturonic acid until they have been sufficiently demethoxylated (i.e. by about 45%).

Romero *et al.* (1989) co-immobilized tomato PE with a *Rhizopus* spp. PG on to an aminoaryl-derivatized porous glass support, and showed that the co-immobilized enzymes were much more effective than the individual immobilized enzymes in reducing the viscosity of both high molecular weight citrus pectin and low molecular weight methoxylated pectin. The pH optima for stability and activity of the two enzymes overlapped sufficiently (for PE pH 5–8 and for PG pH 3.5–6.5) to result in significant activities of both enzymes when the co-immobilized system was run at pH 5.9. Co-immobilized PE and PG showed better activity when immobilized on to 46 nm pore size glass as compared with 20 nm pore size glass. The 46 nm pore size glass had a larger and more accessible surface area (both internal and external) for enzyme binding. The pores of 20 nm pore sized glass, however, were too small to allow for enzyme entrance and binding and, as a result, the enzymes were limited to the external surface of the support for binding.

Manjón *et al.* (1992) also co-immobilized the two enzymes on to porous glass and showed that although PG acts as an activity enhancer for PE activity, both soluble and immobilized PE was competitively inhibited by polygalacturonic acid, the substrate of PG. Whereas PE showed a decrease in temperature optimum with co-immobilization (from 60 to 45°C), the temperature optimum (rising from 40 to 60°C) and the temperature stability of PG increased substantially in the co-immobilized system. However, the co-immobilized enzymes suffered from diffusional limitations, with K_m values increasing by 1.3-fold for PE and by 1.7-fold for PG. Both enzymes showed greater operational stability after co-immobilization, with an increase in half-life during continuous operation of 1.2-fold for PE and 1.6-fold for PG.

INVERTASE CO-IMMOBILIZATION WITH OTHER ENZYMES

Various studies have also attempted to immobilize invertase along with other enzymes to achieve the production of specific sugar products. Inman and Hornby (1974) co-immobilized yeast invertase and glucose oxidase on to the inner surface of nylon tubes in an attempt to create a one-step sucrose analyser; however, the system showed no functional improvements as compared with the combined activities of the individually immobilized enzymes. It was suggested that α-*D*-glucose, the hydrolysed product of sucrose, must first undergo mutarotation to β-*D*-glucose before it can be oxidized by glucose oxidase. Thus, the co-immobilization of the two enzymes offered no improvements in the system, as mutarotation was the rate-limiting step for the reaction.

Yeast invertase and *Penicillium chrysogenum* glucose oxidase were co-immobilized on to urea- and formaldehyde-activated cheesecloth for the detection of

saccharose in solution (Krysteva and Yotova, 1992). The immobilized enzyme preparation was used in conjunction with a 'liquid' dextran catalase, and saccharose concentration was measured indirectly by means of an amperometric oxygen electrode. The co-immobilized invertase and glucose oxidase maintained constant pH optima (pH 5.0 and 5.8, respectively) and showed slight increases in their temperature optima (60° and 35°C, respectively). The immobilized preparation remained highly stable and active, maintaining reproducible readings with short response times (2–3 min) over a 30 day period, and showing no detectable change in enzyme activity when stored at 4°C for 1 year. Kiba (1993) immobilized invertase and pyranose oxidase on to porous glass beads for use as column reactors in a HPLC system for the detection of sucrose and glucose; the hydrogen peroxide produced was monitored chemiluminometrically. The system was highly sensitive, with detection limits of 2–3 $\times 10^{-7}$ M and a linear response up to 5×10^{-4} M.

Ro and Kim (1991) co-immobilized invertase bound to glutaraldehyde-cross-linked chitin with *Zymomonas mobilis* cells entrapped in calcium alginate beads for the continuous production of gluconic acid and sorbitol from sucrose. The optimum pH for the oxidoreductase enzyme of immobilized *Z. mobilis* (pH 6.2) and for immobilized invertase (pH 4.5) were close enough that the co-immobilized system could function at a compromise pH of 6.0. Immobilized invertase was less sensitive to substrate inhibition than the free enzyme (i.e. inhibition was first seen at 20% sucrose v. 5% sucrose, respectively), possibly due to the formation of a concentration gradient between the bulk solution and the pores of the alginate beads. The co-immobilized system operating in a continuous stirred tank reactor showed maximum productivities of 4.6 g l^{-1} h^{-1} with 20% sucrose as substrate, but the operational half-life was only 170 h due to abrasion of the beads. A packed bed reactor, which was subsequently designed to overcome this problem, showed maximum productivities of 5.2 g l^{-1} h^{-1} for 250 h with 93% substrate conversion after 22 h (Ro and Kim, 1991).

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