

Industrial use of immobilized enzymes

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Although many methods for enzyme immobilization have been described in patents and publications, relatively few processes employing immobilized enzymes have been successfully commercialized. The cost of most industrial enzymes is often only a minor component in overall process economics, and in these instances, the additional costs associated with enzyme immobilization are often not justified. More commonly the benefit realized from enzyme immobilization relates to the process advantages that an immobilized catalyst offers, for example, enabling continuous production, improved stability and the absence of the biocatalyst in the product stream. The development and attributes of several established and emerging industrial applications for immobilized enzymes, including high-fructose corn syrup production, pectin hydrolysis, debittering of fruit juices, interesterification of food fats and oils, biodiesel production, and carbon dioxide capture are reviewed herein, highlighting factors that define the advantages of enzyme immobilization.

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1. Introduction

Many methods for enzyme immobilization have been described in the patent and academic literature, although relatively few

have been successfully commercialized on a large scale.^{1–11} The number of cited applications for immobilized enzymes is also substantial, but again only a limited number are deployed in the marketplace, many of which were developed decades ago.^{4–8} To understand this apparent discrepancy, an understanding of the fundamental drivers for commercial acceptance of immobilized enzyme products is required. In this critical review we highlight the development and attributes of several established and emerging industrial applications for

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Table 1 Attributes of immobilized biocatalysts

Advantages	Disadvantages
Amenable to continuous and batch formats	Loss of enzyme activity upon immobilization
Reuse over multiple cycles possible	Unfavorable alterations in kinetic properties
Improved stability over soluble enzyme forms	Cost of carrier and fixing agents
Favorable alterations in pH and temperature optima	Cost of immobilization process
Sequester enzyme from product stream	Mass transfer limitations
Co-immobilization with other enzymes possible	Subject to fouling

immobilized enzymes, highlighting factors that enable commercial success.

The use of enzymes in industry has attracted attention for well over a century and continues to expand.^{1–4} Enzymes demonstrate high turnover numbers and enormous reaction rate accelerations, in some cases exceeding 10⁸-fold over background.^{2,12} Enzymes in insoluble form are essentially a specialized form of heterogeneous catalyst in that they can be recovered and reused, often retain activity for long periods and are amenable to a wide variety of process formats.^{4–11} In contrast to many chemical heterogeneous catalysts, immobilized enzymes produce highly-selective reaction outcomes, in both structural and stereochemical terms, under relatively mild conditions.¹² It is also important to recognize the limitations of immobilized enzymes (Table 1). For example, industrial enzymes such as proteases, amylases and cellulases are often used to modify macromolecules, surfaces, and complex mixtures. In these contexts, the effectiveness of immobilized enzymes is often considerably less than that of their soluble equivalents. This is primarily due to diffusional constraints imposed by attachment to solid supports which affects the ability of substrates to access enzyme active sites and for products to exit to bulk solution.^{3–8}

Immobilization of an enzyme can be considered to be a specialized type of enzyme formulation. The role of any formulation is to maximize the stability, both enzymatic and physical, of an enzyme in a form that best suits its application. Given the significant degree to which the physical and chemical properties of the support influence catalytic performance, an appreciation of materials science is required for the successful development of an immobilized enzyme catalyst.^{8–11,13} Beyond the technical issues concerning enzyme production and immobilization are those associated with process engineering and the need to understand where value can best be generated. Overall, the industrial relevance of immobilized biocatalysts is primarily application driven, in that there has to be a differentiating advantage offered by an immobilized enzyme product over either soluble enzymes or chemical catalysts.

2. Economics of immobilized enzymes

The world market for industrial enzymes exceeded \$US3.3 billion in 2010 and is projected to grow to around \$4.5 to \$5 billion by 2015 at a compounded annual growth rate of 7–9%.^{14,15} This market is dominated by products containing non-immobilized enzymes, predominantly hydrolases (*e.g.* amylases, proteases, cellulases and lipases). These products are either liquid concentrates, or enzyme granules that release the soluble enzyme upon dissolution. Enzymes for use in non-industrial markets, primarily for pharmaceutical, diagnostic and research applications, accounted for around \$2.4 billion in 2010.^{15,16} Sales of enzymes for biocatalysis, many of which are used in immobilized form, were valued at \$160 million in 2010 and projected to increase to \$230 million by 2015 (not including captive use by companies producing their own immobilized enzymes).¹⁵ The market segments for enzymes is depicted in Fig. 1.

Current sales of immobilized enzymes themselves amount to only a small fraction of the total enzyme market, down significantly from 1990, when they accounted for nearly 20%



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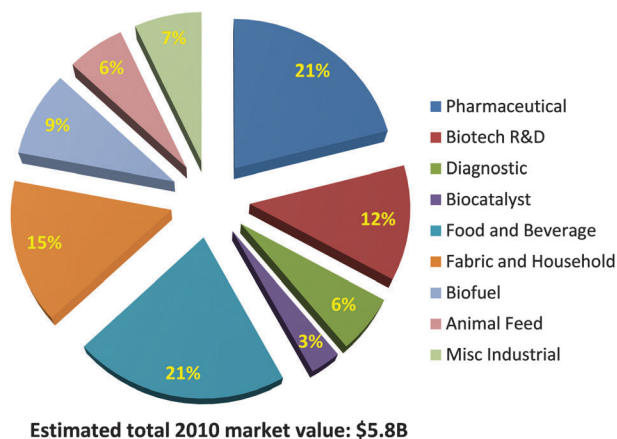


Fig. 1 A breakdown of the World enzyme market in 2010 by sector (reprinted with permission from The Freedonia Group Inc.).¹⁵

of all industrial enzyme sales.¹⁷ Why the apparent disconnect between the thriving scientific field of enzyme immobilization and the very modest market share of immobilized enzymes relative to the enzyme market at large? From the enzyme producer's perspective, the cost of producing an immobilized form of an enzyme must enable a new application, or offer some other benefit relative to the soluble form of the enzyme. The fact that immobilized enzymes can often be reused does not directly benefit the enzyme producer, but often provides incentive for customers to purchase an immobilized enzyme product. In some cases, an enzyme producer may attempt to gain some value beyond enzyme sales alone, for example by securing intellectual property on the processes and products derived from the use of immobilized enzyme. In other instances a customer in need of an immobilized enzyme will purchase a soluble form of the enzyme, and prepare an immobilized form of the enzyme themselves, or outsource this task to companies that specialize in immobilization.¹⁸ Established chemical companies increasingly produce and immobilize their own enzymes in parallel with process development.¹⁸ Overall, product opportunities need to be chosen carefully to ensure that an immobilized enzyme is really needed and offers reasonable return. The established large-scale applications for immobilized enzymes are shown in Table 2.

It is often incorrectly assumed that industrial enzymes are very expensive and that these costs drive the need for an

immobilized, recyclable form of biocatalyst.² In reality the cost of most industrial enzymes is in the \$50 to \$500 per kg enzyme protein range, and they are often only a minor component in overall process economics.^{2,5,6} For example, the total cost of enzymes for starch-derived ethanol is around 1 cent per liter. In these instances, the additional expenses associated with enzyme immobilization are not worth the return that might be gained from enzyme recycle. The cost contribution from an immobilized enzyme is dependent on the number of times the enzyme is reused, an indirect measure of total productivity on a kg product per kg biocatalyst basis. This amount varies between a few hundred \$ per kg for specialty chemicals, down to a few cents per kg for bulk chemicals, and is often in the range of \$0.1 to \$10 per kg.^{2,6}

The early success of immobilized glucose isomerase (IGI) holds several lessons for the design of future immobilized biocatalysts, both isolated enzyme and whole cell products. Many factors came together to drive the success of IGI, summarized in a review by Pedersen.¹⁹ This suggests that the development and application of immobilized enzyme products requires a good understanding of both technical and economic factors, as well as a good sense of the larger market forces at play.^{4,7}

3. Immobilized glucose isomerase

3.1 Background

Glucose isomerase (GI), also known as xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), is one of the most important industrial enzymes in commerce today, driven primarily by the rise of D-fructose as a sweetener for beverages and foodstuffs.²⁰ Although D-xylose is the native substrate, the enzyme has broad substrate specificity and efficiently converts D-glucose to D-fructose (Scheme 1). A number of reviews have been published on both glucose isomerase and its industrial use.^{21–23} The immobilized form of glucose isomerase (IGI) is used in all commercial applications and is considered to be the paradigm for an immobilized enzyme product.^{7,19,21–23} Given this success, it is worth reviewing the properties of glucose isomerase, the processes used for immobilization and practical application of this enzyme.

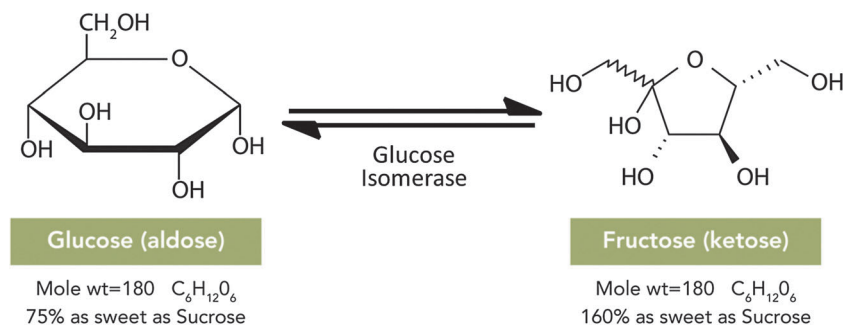
3.2 Properties of glucose isomerase

3.2.1 Enzyme sources and industrial production. D-Xylose isomerases (EC 5.3.1.5), commonly known as glucose isomerases, are widely distributed in nature and are produced

Table 2 Large scale industrial processes utilizing immobilized biocatalysts

Enzyme	Form ^a	Process	Product scale (ton per year)	Ref.
Glucose isomerase	CWC, IME, CIE	High fructose corn syrup from corn syrup	10 ⁷	7,19,21–23
Nitrile hydratase	CWC	Acrylamide from acrylonitrile	10 ⁵	334–336
Lipase	IME	Transesterification of food oils	10 ⁵	205–209
Lactase	IME	Lactose hydrolysis, GOS synthesis	10 ⁵	337–339
Lipase	IME	Biodiesel from triglycerides	10 ⁴	269,271–277
Penicillin G acylase	CIE	Antibiotic modification	10 ⁴	340–342
Aspartase	CWC, IME	L-Aspartic acid from Fumaric acid	10 ⁴	343–345
Thermolysin	IME	Aspartame synthesis	10 ⁴	346–348
Lipase	IME, CIE	Chiral resolution of alcohols and amines	10 ³	349–351

^a CWC = cross-linked whole cell; IME = immobilized enzyme; CIE = covalently immobilized enzyme.



Scheme 1 Isomerization of D-glucose to D-fructose.

by many microorganisms.²² They can be placed into four different categories depending on substrate preference and cofactor requirements.²² Marshall and Kooi first characterized a D-glucose isomerizing enzyme from *Pseudomonas hydrophila* in 1957.²⁴ The enzyme had a preference for D-xylose, but also accepted D-glucose as an alternative substrate, albeit with a K_m 160-fold higher. Production of the enzyme required fermentation media containing both xylose and the isomerization reaction itself was enhanced by arsenate. Tsumura and Sato filed a patent in 1966 on a xylose isomerase (EC 5.3.1.5) that was able to isomerize D-glucose to D-fructose without requiring either arsenate or NAD⁺.²⁵ Additional work led to the identification of a *Streptomyces* strain (YT-5) that expressed a xylose isomerase without the need for xylose induction, instead being able to utilize xylan from corn cobs and other sources.^{26,27} All subsequent commercial processes for HFCS production were based upon D-xylose isomerases that did not require either xylose, or high levels of Co²⁺, an environmental hazard, for production and enzyme stability. Glucose isomerase is typically produced in a submerged, aerobic fermentation process with batch times of 2–3 days.^{22,28} In most cases the enzyme is not secreted, being either expressed intracellularly or associated with cellular mycelia. There are ongoing efforts directed toward the discovery and engineering of glucose isomerases with improved thermostability, reduced dependence on metal ions for activity, lower pH optima and greater resistance to Ca²⁺ ions and other inhibitors.^{29–32}

3.2.2 Biochemical properties of glucose isomerase. Xylose (glucose) isomerases isomerize a range of monosaccharides other than D-xylose.²² The K_m for D-glucose is typically higher than that for D-xylose and ranges from 0.086 to 0.920 M.²¹ The conversion of D-glucose to D-fructose is a slightly endothermic keto–enol isomerization reaction with a ΔH of 5 kJ mol^{−1} and an equilibrium constant (K_{eq}) of approximately 1 at 298 K.³³ The α -anomer of D-glucose is the preferred substrate for most glucose isomerases, although the β -anomer has also been shown to be a substrate in some cases, for example the enzyme from *Streptomyces murinus*.³⁴ The position of isomerization equilibrium is a function of temperature, tending to favor fructose formation at higher temperatures as shown in Fig. 2. At 60 °C, the temperature at which most glucose isomerases are used commercially, the conversion of dextrose produces around 50% fructose, whereas at 90 °C the equilibrium mixture contains 56% fructose.³³

Both type I and type II glucose isomerases have been described, differing in the N-terminal sequence.³⁵ Almost all glucose isomerases exist as homotetramers. The *Streptomyces rubiginosus* enzyme, the structure of which was first determined in 1984, has a molecular weight of 173 kDa, composed of four 43 kDa monomers (Fig. 3).³⁶ Glucose isomerases require divalent metal ions for both maximal catalytic activity and enzyme stability. The preferred ion for enzyme activity is Mg²⁺, followed by Mn²⁺ and Co²⁺. Two metal ions are bound near the active site and play a direct role in the catalytic mechanism.^{37,38} Additional metal binding sites provides conformational stability and contribute to maintenance of the overall quaternary tetrameric structure of the active complex. In this case Co²⁺ is the preferred ion. Glucose isomerases are inhibited by a number of other metal ions, including Ca²⁺, Zn²⁺, Ni²⁺ and Hg²⁺. Glucose isomerase is also inhibited by sugar alcohols including xylitol, sorbitol, arabitol and mannitol. The thermal inactivation mechanism of glucose isomerase has been studied by several groups and seems to be driven by a combination of cysteine oxidation and Maillard adducts between lysine residues and sugars.^{29,39,40}

The activity of glucose isomerase is defined in terms of glucose isomerase units (GIU), being defined as the amount of enzyme required to isomerase 1 μ mol of glucose to fructose per minute under defined assay conditions. The mechanism of glucose isomerase has been debated over the years as being one of three possibilities; (i) a base-catalyzed proton transfer *cis*-ene diol mechanism, (ii) a hydride-shift mechanism, or (iii) a hydride shift mediated by metal ions.^{22,31} X-ray crystallography alone is insufficient to discriminate between these possibilities, largely due to ambiguities as to the position of hydrogen atoms which scatter X-rays poorly.⁴¹ Katz and coworkers sought to define the ionization state of active site residues of the *S. rubiginosus* enzyme using time-of-flight neutron diffraction and determined the presence of an unionized water molecule, interacting with both the substrate and one of the metal ions present at the active site.⁴² A combination of neutron and X-ray diffraction studies have refined understanding of the catalytic mechanism, but key aspects, such as the exact route by which a hydrogen atom moves from C2 to C1 of the substrate, remain to be elucidated.⁴³

3.3 Methods for GI immobilization

Both the application requirements and the biochemical properties of glucose isomerase have driven the development of immobilized

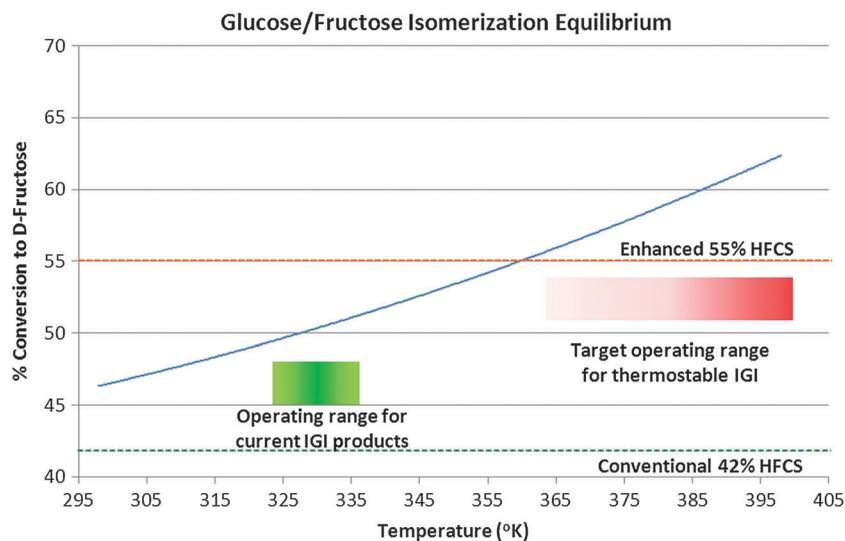


Fig. 2 The glucose/fructose equilibrium is shown as a function of temperature (blue line). The operating temperature of current IGI products, used to produce 42% HFCS, is shown by the green bar. Thermostable GI would need to operate at temperatures over 365 K (red bar) in order to produce 55% HFCS.

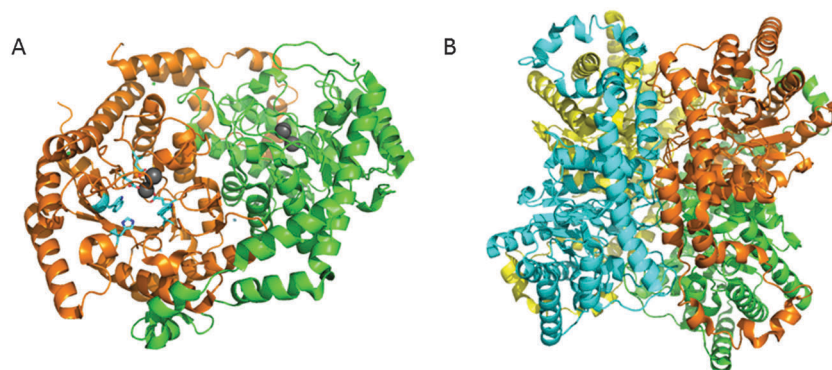


Fig. 3 Structure of Glucose Isomerase from *Streptomyces rubiginosus* in (A) dimeric form with metal ions depicted as grey spheres and active site residues in cyan, and (B) as a tetramer.

forms of the enzyme.^{7,19} The high K_m for D-glucose (>0.1 M) meant that elevated enzyme concentrations were required to push the reaction to near equilibrium within a practical timeframe. Additional factors included the cost of production and recovery of GI, an intracellular enzyme, as well as the process advantages inherent to using an immobilized form of the enzyme. Many methods for the immobilization of glucose isomerase have been developed and commercialized over the years, beginning in 1967.^{21–23,44} Immobilization of GI was considered a mature technology by the late 1970's when over 6 distinct glucose isomerase products were on the market (Table 3).^{3,23,44} In recent years there has been a consolidation whereby two products currently dominate the market, driven by the overall process economics of IGI production and use by HFCS producers.¹⁵

3.3.1 Crosslinking of whole cell preparations. Whole cell immobilization for IGI production was initially demonstrated by Takasaki and Kamibayashi using a heat-fixation technique.⁴⁵ Glucose isomerase-expressing cells of *Streptomyces* sp. were held at elevated temperatures (60 to 80 °C) for short periods,

leading to a crosslinked matrix composed of denatured cellular protein and other components. The advantage of the process were two-fold in that the treatment fixed the GI in a form that could be used in plug flow reactors, in addition to denaturing cellular proteases that might otherwise degrade the GI enzyme. Clinton Corn Processing Company, a division of Standard Brands Co., introduced a discontinuous process for glucose isomerization based on Takasaki's method into the US in 1967.⁴⁶ The process converted corn-derived glucose syrup into a mixture containing 42% fructose.

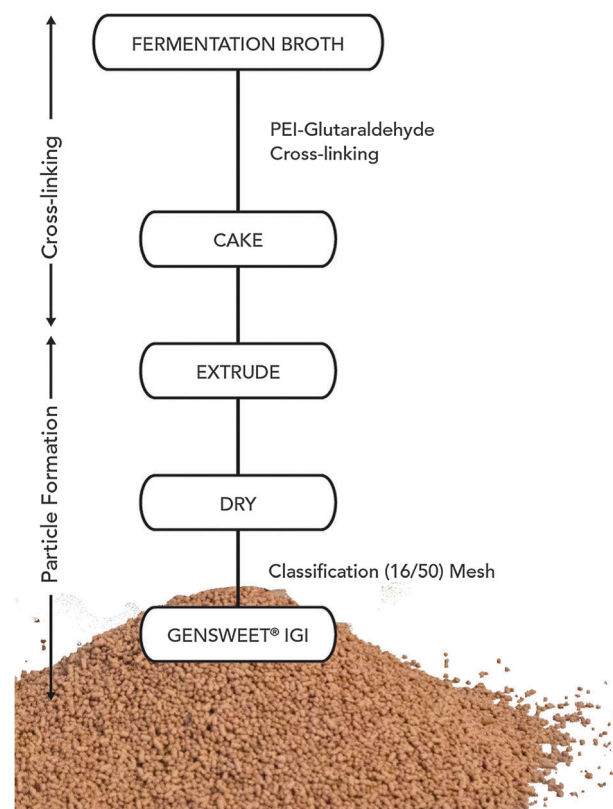
Chemical fixation techniques were developed at around the same time, based on both inorganic and organic cross-linking agents, for example, one process utilized salt solutions such as CoCl_2 , FeCl_3 , CaCl_2 etc. to fix glucose isomerase within the *Streptomyces* cells.⁴⁷ Organic acids including citrate were also used as fixing agents.⁴⁸ Glutaraldehyde (50 wt% solution) was determined to be a very effective fixing reagent, first described in 1973 using *Streptomyces olivaceus* cells.⁴⁹ This method was further refined whereby homogenized *Bacillus coagulans* cells

Table 3 Examples of commercial immobilized glucose isomerase products^{22,23,44}

Product	Producer	GI source	Description	Currently sold?
Optisweet [®] 22	Miles-Kali/Solvay	<i>S. rubiginosus</i>	Adsorption of GI on to SiO ₂ followed by crosslinking with glutaraldehyde	N
TakaSweet [®]	Miles Labs/Solvay	<i>Flavobacterium arborescens</i>	Polyamine/glutaraldehyde crosslinked cells extruded and spheronized	N
Maxazyme [®] GI	Gist-Brocades	<i>A. missouriensis</i>	Crosslinked cells entrapped within gelatin beads	N
Ketomax GI-100	UOP	<i>S. olivochromogenes</i>	Glutaraldehyde crosslinked GI adsorbed to PEI-treated alumina	N
Spezyme [®]	Genencor	<i>S. rubiginosis</i>	Crystallized crosslinked GI adsorbed to granular DEAE-cellulose	N
Sweetase [®]	Denki Kagaku-Nagase	<i>S. phaeochromogenes</i>	Heat-treated cells entrapped within polymer beads	N
Sweetzyme [®] T	Novozymes A/S	<i>B. coagulans</i>	Glutaraldehyde crosslinked whole cell homogenate containing inorganic carrier	Y
GENSWEET [®] SGI	Genencor/DuPont	<i>S. murinus</i>	Soluble GI product for adsorption to DEAE-cellulose anionic resin	Y
GENSWEET [®] IGI	Genencor/DuPont	<i>S. rubiginosis</i>	PEI/glutaraldehyde crosslinked cells, mixed with inorganics (clay, DE)	Y

were crosslinked with glutaraldehyde, followed by mechanical formation into granules suitable for deep bed reactors.⁵⁰ Flocculation agents were also used in some variations of this method. Glutaraldehyde remains an important component of current commercial glucose isomerase products including Sweetzyme[™] and GenSweet[®] IGI, marketed by Novozymes A/S and Genencor (now DuPont Industrial Biosciences), respectively.^{51–53} Incorporation of polyethylenimine and inorganic carriers such as bentonite clay and diatomaceous earth led to further improvements in product quality and performance, and this process is still in use today.⁵⁴ Following the crosslinking procedure, the composite is dewatered and mechanically fashioned into particles by extrusion/marumerization before drying in a fluidized bed dryer. The resulting immobilized GI is extremely stable, with a half-life of over 1 year when used in a packed bed reactor at 60 °C. Fig. 4 depicts the steps in the large scale immobilization process.

3.3.2 Adsorption-based methods. Immobilization methods based on ionic adsorption of isolated GI to resins, both organic and inorganic, were developed in parallel to the whole cell methods described above. While procedures to release and purify the free enzyme from host cells incurred additional expense, there were considerable operational advantages associated with the physical forms of these products. Adsorption of the GI isolated from *Streptomyces phaeochromogenes* to DEAE-Sephadex[®] was first described by Tsmura and coworkers in 1967.⁵⁵ The method was subsequently improved to the extent that it became commercially feasible.⁵⁶ In this instance, GI derived from *Streptomyces* sp. was bound to DEAE-cellulose either in the presence or absence of associated cellular material such that the activity of the resulting material was at least 3 IGIU cc^{−3}. Under optimal conditions this method produced material with activities of over 2500 IGIU g^{−1}. The inventors also described adsorption of GI to polymeric anion-exchange resins such as Amberlite[™] IRA-938. Despite the high catalytic density of these products, they could only be used in shallow bed reactors due to their inherent compressibility. Development of granular products with higher mechanical stability allowed for operation in deep-bed reactors. Antrim and coworkers developed

**Fig. 4** Process for large scale immobilization of glucose isomerase.⁵³

a regenerable product based upon a granular DEAE-cellulose-polystyrene-TiO₂ resin with particle sized in the 400 to 800 micron range.⁵⁷ The process involved the electrostatic binding of highly purified GI from *S. rubiginosus* to the resin and resulted in IGI preparations with catalytic densities of up to 1500 IGIU g^{−1}. The loading process could be performed in either batch or column mode.⁵⁸ The immobilized enzyme was found to be very stable, with a half-life of over 1800 hours under plant operating conditions. A key innovation in this method was the ability to regenerate the resin by addition of soluble GI to compensate for the loss of enzyme activity over time. This allowed the process

to be run under constant flow conditions, in contrast to alternative processes where flow rates through a given bed needed to be reduced over time in order to maintain a constant degree of isomerization to fructose. The business model for this product, currently sold under the trade name GENSWEET[®] SGI by DuPont Industrial Biosciences (formerly Genencor), was also novel, with customers leasing the support and purchasing soluble GI as needed to maintain the desired level of enzyme activity. This product has mostly been replaced by GENSWEET[®] IGI, described above.⁵³

3.3.3 Covalent attachment to inorganic supports. Methods for the covalent attachment of glucose isomerase and other enzymes to inorganic carriers, such as alumina, titania and controlled-pore size glass were developed at Corning Glass Works in the late 1960's and early 1970's.^{59,60} Surface modification of the inorganic carrier using an aminosilane was followed by covalent attachment of enzymes, mediated by a coupling reagent such as a carbodiimide. The resulting materials had catalytic activities of several hundred IGIU g⁻¹ and were found to have improved microbial stability, relative to other forms of IGI. Many patents were filed on variations on this process. For example, Eaton and Messing invented a method for the immobilization of glucose isomerase on MgO/Al₂O₃-based porous supports.⁶¹ The advantage of using magnesium oxide in the composite related to providing the Mg²⁺ required by glucose isomerase, in addition to providing a favorable microenvironment with regard to pH.

3.3.4 Matrix entrapment methods. Entrapment of free enzyme or cells in a cross-linked matrix represents another general approach for enzyme immobilization.⁵⁻¹¹ Early efforts included entrapment of soluble glucose isomerase within cellulose triacetate fibers⁶² and collagen films.⁶³ Gist-Brocades commercialized a process in 1976 whereby cells of *Actinoplanes missouriensis* expressing GI were entrapped within a glutaraldehyde-crosslinked gelatin matrix.⁶⁴ The process began by mixing cellular mycelia containing GI with a gelatin solution at above 40 °C, followed by addition to a water immiscible solvent with stirring, resulting in coagulated particles with a diameter of around 1 mm. The particles were washed with a water miscible solvent such as ethanol and then treated with a 2.5% solution of glutaraldehyde in cold water. Following washing with water and either ethanol or acetone, the particles were dried, or alternatively stored in a NaCl solution containing 0.3% formaldehyde. The activity of the wet particles was reported to be as high as 6000 IGIU g⁻¹. The product was sold under the tradename Maxazyme[®] Immob-GI and could be used in a variety of formats including batch, fixed bed and expanded bed reactors. Optimal performance required cobalt ions for enzyme stability and magnesium ions for maximal enzyme activity. Calcium ions were detrimental to enzyme activity. Half-lives of around 500 hours were observed when the product was used in column format using typical corn-derived glucose syrup.

3.3.5 Recent developments in GI immobilization. Glucose isomerase is one of the most extensively-studied enzymes and serves as an excellent benchmark for new immobilization methods. Immobilization of glucose isomerase within interpenetrating polymer networks (IPNs) was reported by Demirel

and coworkers.⁶⁵ Interpenetrating polymer networks are a type of hydrogel comprising a mixture of two or more crosslinked polymers. They have been used in biomedical applications due to their interesting mechanical properties and high biocompatibility. Glucose isomerase was entrapped within poly(acrylamide), poly(acrylamide)-κ-carrageenan and poly(acrylamide)-alginate matrices by *in situ* radical polymerization in the presence of enzyme. Stabilities of 82%, 33% and 32% relative to initial activity were noted after 42 days storage at 4 °C, respectively. Optimal pHs and temperatures were similar to the free enzyme, but diffusional resistances were thought to be responsible for the altered kinetic properties noted in the immobilized forms. The authors of the study concluded that the polyacrylamide-only network had a pore structure more amenable to enzyme stability and mass transport.

Plasma modification of surfaces has been used to introduce reactive functionalities onto otherwise inert polymers to enable subsequent covalent enzyme immobilization.⁶⁶ Glucose isomerase was immobilized onto polyethersulfone membranes that had been modified with amino-functionalities using a plasma deposition technique.⁶⁷ A range of amines was examined, with allylamine/Ar plasma proving to be the most effective in maximizing the activity of the immobilized enzyme.

Entrapment of enzymes using silica sol-gel techniques has been widely applied for enzyme immobilization. An early report by Braun described the entrapment of enzymes in silicate sol-gel glasses.⁶⁸ Dunn and coworkers were granted a patent on improved methods for sol gel entrapment of a range of enzymes that included glucose isomerase in the claims.⁶⁹ Sol gel techniques were used to entrap non-growing bacterial cells expressing GI within silica xerogels.⁷⁰ A wild type *Arthrobacter nicotiana* strain that naturally expressed GI was compared to a recombinant *E. coli* BL21 (DE3) strain expressing the *A. nicotiana* xylA gene. Cobalt salts and chemical crosslinkers, either glutaraldehyde or a carbodiimide, were also incorporated into the formulations, which were dried, crushed and fashioned into granules 1–4 mm in diameter. The materials derived from the recombinant *E. coli* strain exhibited both higher activity and stability, relative to the *A. nicotiana* strain. Under continuous isomerization conditions at 62–65 °C with a 3 M fructose feed, the stability of the two optimized formulations was 60 h and 25 h, respectively. The authors noted that the optimal loading of cellular biomass was 40–60% (w/w) for *E. coli*, but only 15% for *A. nicotiana*, based on the maximal loading that would still produce biocatalyst granules with acceptable mechanical properties.

Immobilization of GI and other enzymes within nanoporous supports such as mesoporous silica (MPS) has received much attention in recent years.^{9,10,13,71} The advantage of MPS over traditional supports relates to the very uniform pore size, very high total surface areas and the hierarchical organization of the material, allowing for improved mass transport between the pores and bulk solution. Ackerman and coworkers have published several reports on the properties of enzymes immobilized within functionalized mesoporous silica (FMS) where the pore surfaces are modified with amino, carboxy or other functional groups *via* a silane linker.^{72–74} By tailoring pore size and functional

group selection, Ackerman and coworkers claim it is possible to induce favorable conformational changes in immobilized enzymes such that significant activity enhancements over the free, unimmobilized enzyme are seen. For example, glucose isomerase was immobilized within both unfunctionalized SBA-15 mesoporous silica (UMS), as well as amino-functionalized SBA-15 (FMS). Both simple adsorption and covalent attachment techniques were used in the case of the latter material. Non-porous silica (NPS) was chosen as a control support. In contrast to earlier work with organophosphate hydrolases, there was no enhancement of specific activity of immobilized GI over that of the unimmobilized enzyme, although it is still noteworthy that the two were comparable given that immobilization typically results in reductions in specific activity. The study also found that urea was capable of significantly enhancing the specific activity of a previously frozen sample of GI at concentrations between 0.4 and 6 M, above which losses in specific activity were seen. The degree of specific activity enhancement was most pronounced for GI spontaneously adsorbed into FMS, where a 128% increase in activity was observed *versus* untreated soluble GI. The covalently immobilized material showed a lower degree of enhancement, at 90% relative to the control. Interestingly, urea at concentrations of 0.4 to 5.2 M enhanced the specific activity of the soluble enzyme by 43%, over the untreated control. The authors concluded that both urea and the confining effect of immobilization within FMS promoted rearrangement into a more productive form and presented protein fluorescence data as evidence of conformational changes.

A rapid method for enzyme immobilization in polyamine-precipitated silica was developed by Chang and coworkers.⁷⁵ The inspiration for this method was driven by insights into the template assisted deposition of biosilicates by diatoms and other marine organisms. The method is scalable (>100 g) and uses relatively inexpensive materials (phosphoric acid, sodium silicate, cationic polymers and enzymes).⁷⁶ The extent of enzyme capture is influenced by a number of factors including the concentration of the enzyme, polyamine and silicate components, as well as the temperature, pH and order of addition during the process. Immobilization of *Streptomyces* glucose isomerase using this technique resulted in the capture of 75% of the protein and retention of 35% of the specific activity relative to the unimmobilized enzyme.⁷⁵

Substrate pretreatment. The effect of substrate pretreatment on the activity of GI prior to immobilization has been studied by a number of research groups.^{77,78} These studies were motivated by the hypothesis that binding of substrate to the active site protects the native conformation of the enzyme during the immobilization process. For example, Song and coworkers immobilized *S. rubiginosus* GI on aminopropylsilane-activated silica gel using glutaraldehyde as a cross-linking agent, resulting in multipoint covalent attachment of the enzyme to the support.⁷⁹ The enzyme was immobilized with and without substrate pretreatment, using both D-xylose and D-glucose. After optimizing the pretreatment conditions with regard to time, temperature and

agitation, the authors reported that pretreated immobilized GI (PIGI) displayed 2.5 fold higher activity relative to non-pretreated IGI. The effect of D-xylose was greater than D-glucose, possibly related to the fact that the enzyme has a lower K_m for the former substrate.

Thermostable immobilized glucose isomerases. Much effort has been directed toward the development of glucose isomerases with enhanced thermostability, both through protein engineering, as well as the discovery of novel new enzymes.^{22,29,31,32,80–83} The quest for such enzymes is driven by the fact that at 90 °C it is possible to produce HFCS containing 55% D-fructose directly from glucose syrup, without the need for chromatographic enrichment.³³ Despite these efforts, thermostable glucose isomerases have not been applied commercially, in large part due to the additional requirement for operation at pH values below 6 given the instability of fructose at elevated temperatures. A comparison of two hyperthermophilic class II glucose isomerases from *Thermotoga* sp. and the class I enzyme from *Streptomyces murinus* was performed with the intent of establishing isomerization performance at elevated temperatures.⁸⁴ The enzymes were immobilized by covalent attachment to carboxylic acid-functionalized porous glass beads using carbodiimide-mediated coupling in the presence of 600 mM xylose and 5 mM Mg^{2+} . A commercial IGI preparation (Sweetzyme® IT) was included in the study for comparison under realistic processing conditions. It was found that the hyperthermophilic *Thermotoga* enzymes were capable of commercially-relevant productivities (~2000 kg product per kg biocatalyst) at 80 °C, although this required the presence of cobalt ions to maximize enzyme stability. The study concluded that thermostable GI's had some commercial promise, but that further development with regard to thermostability/thermoactivity and reduced pH optima was required. Harris and coworkers constructed a fusion protein by combining a chitin-binding domain from the hyper-thermophile *Pyrococcus furiosus*, with the N-terminus of a thermostable type II xylose isomerase (TNXI) isolated from *Thermotoga neapolitana*.⁸⁵ The intent was to create a thermostable enzyme that could be readily immobilized by interaction of the carbohydrate binding domain (CBD) with chitin beads. The resulting fusion protein (TNXI-CBD) was capable of binding to chitin and was found to be more thermostable than the soluble wild-type enzyme, with half-lives at 100 °C of approximately 20 minutes and 7 minutes, respectively. Interestingly, the unimmobilized fusion protein (TNXI) displayed the highest thermostability of all, with a $T_{1/2}$ at 100 °C of nearly 1 hour. The authors inferred that immobilization of the fusion compromised the overall improvement in thermostability, relative to the soluble fusion.

Coimmobilization with other enzymes. Coimmobilization of glucose isomerase with other enzyme activities offers the possibility of direct production of fructose syrups directly from glucose precursors (starch, cellulose, etc.) in combined unit operations. In practice, the savings derived from reduced capital intensity need to compensate for the fact that in such systems, glucose production can often be rate-limiting, especially

where macromolecular substrates are involved. Coimmobilization of glucose isomerase and glucoamylase was reported by Ge and coworkers using a layer-by-layer technique whereby the two enzymes were adsorbed sequentially to trimethylamine-modified polystyrene.⁸⁶ The K_m of the immobilized GI alone was found to increase by 28%, from 0.6 M to 0.77 M, while the V_{max} decreased by 11.5%. When combined with glucoamylase, the dual-activity biocatalyst was able to convert soluble starch and two dextrin grades (DE-5 and DE-27) into fructose at a rate exceeding that of the equivalent amounts of the soluble enzymes. The authors cite the broader pH-activity profile of the immobilized GI *versus* the soluble enzyme as one of the reasons for this improvement, as well as the possibility of favorable proximity effects in the co-immobilized system. A similar technique was also used for the coimmobilization of glucose isomerase with cellulase, enabling the production of glucose–fructose mixtures directly from cellulose.⁸⁷

3.4 Immobilized glucose isomerase processes

The use of immobilized glucose isomerase (IGI) for the production of HFCS represents the largest commercial process involving an immobilized enzyme, both in terms of the amount of enzyme sold, as well as the volume of product produced.^{3–5,7,19,21–23} Over 500 tons of IGI are manufactured annually, enabling the production of approximately 10 million tons of HFCS per annum.^{4,22} The first process for enzymatic production of HFCS using glucose isomerase was a batch process developed by Takasaki and Tanabe in the mid-1960's.^{26,45,46} Clinton Corn Brands, a US-based corn processing company obtained a license for this technology in 1966 and was the first company to commercialize the enzymatic production of HFCS in 1967. An enzyme-based process had a number of advantages over the alternative alkaline catalysis method, including improved product appearance and quality. Development of an immobilized form of glucose isomerase made continuous processing possible and delivered greatly improved economics. Within the space of 10 years several immobilized forms of GI had been developed and commercialized.^{3,23,44} Several different reactor formats were used, including stirred tank, fluidized bed, shallow and deep fixed bed reactors, the choice of which was often dictated by the form of enzyme available.⁴⁴

Contemporary processes for HFCS production are carried out in fixed bed reactors arranged in parallel and operated in a continuous manner.⁵³ Corn-derived D-glucose syrup is converted into a mixture containing approximately 42% D-fructose, 50% D-glucose, 6% maltose, 2% maltotriose and traces of other sugars. Higher concentrations of fructose, such as the 55% HFCS grade used in most soft drinks, are derived from chromatographic enrichment of the 42% grade to 90% D-fructose (HFCS-90), which is typically blended with HFCS-42 to produce HFCS-55. Crystalline D-fructose with a purity of over 99% (w/w) is also produced from HFCS-42. In contrast to ethanol production, HFCS is derived from corn-wet milling operations. Fig. 5 shows the different operations used to convert corn to HFCS.⁵³

Corn-derived starch is first converted into D-glucose by the steps of liquefaction and saccharification through the action of

thermostable α -amylases and glucoamylase, respectively.⁸⁸ The saccharified liquor is then clarified by filtration and refined by passage through carbon and ion-exchange filters. Evaporation results in concentration to glucose syrup with a dry solids content of 45 to 55% (w/w). Magnesium is added to a level of 30 to 50 ppm in order to maintain the activity of the immobilized glucose isomerase, in addition to sodium bisulfite (100 mM) which acts as a preservative. The pH is adjusted to between 7.8 and 8.2 prior to a final filtration step. The glucose liquor feed is fed in a down-flow manner into a series of fixed bed reactors arranged in parallel and held at around 60 °C. The flow rate through a given column is controlled so as to achieve the desired degree of isomerization, which is a function of the catalytic activity of the IGI bed and the flow rate. Suppliers of immobilized GI typically provide recommended operating parameters (Table 4) and kinetic rate equations that allow their customers to calculate the required process conditions for optimal use of their products.⁵³ Effluent with a fructose content of 42% w/w is typical output.

This stream is directed through carbon and ion exchange columns and concentrated in order to produce final product. A portion of the 42% fructose stream is enriched by chromatography to syrups with a fructose content of over 90% (HFCS-90). Blending of this enriched material with 42% HFCS gives the 55% HFCS grade used in beverages. A number of considerations impact the process economics and operating conditions for glucose isomerization, including the capital associated with both enzyme reactors and chromatography columns, the operating lifetime of both biocatalyst and ion exchange resins and the need to minimize side reactions and metal ion levels so as to produce a food quality product. While current IGI products have been well optimized over the years, there are still opportunities to further improve the process, given the factors mentioned above.

A number of alternate reactor formats for glucose isomerization have also been studied on a small scale, examples of which are discussed below. Dehkordi and coworkers describe the kinetic advantages of a two impinging jets reactor (TIJR) process over conventional stirred batch and packed bed reactors for the isomerization of glucose to fructose by IGI.⁸⁹ The principle of an impinging jet reactor involves creating a turbulent mixed flow region (*i.e.* the impingement region) by bringing two streams (gas, liquid, solid and mixtures thereof) together within a reactor that enable continuous recycling of the streams.^{90,91} Significant improvements in both heat and mass transfer are seen relative to stirred batch and fixed bed reactors, in particular for mixed phase systems such as those involving immobilized enzymes.⁹¹ A commercial immobilized glucose isomerase (Sweetzyme® T) was studied using a TIJR that included a rotating inner cylinder that could be controlled independently of flow through the system.⁸⁹ The advantage of this modification is that it allows for suspension of solid particles, even at low mass flow rates, in addition to increasing both the degree of turbulence and particle residence time within the reactor. Experimental results were obtained from stirred batch, packed bed and TIJR processes. A comparison to

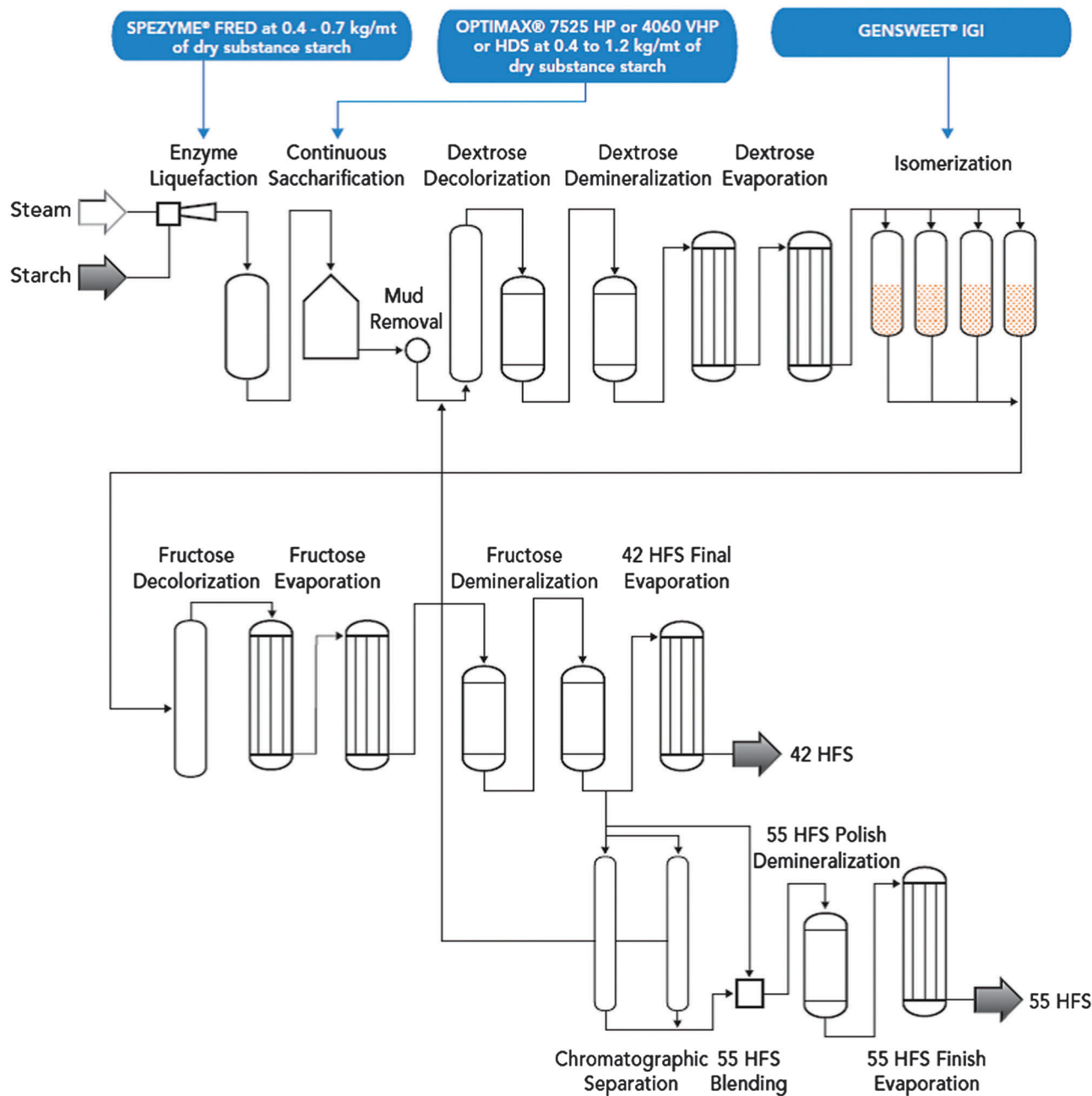


Fig. 5 Layout of a corn wet milling plant.⁵³

a downflow jet loop (DJR) reactor was also included.⁹² The degree of glucose conversion (fractional conversion %) for each reactor type was compared as a function of residence time. The TIJR setup achieved dramatically higher fractional conversions for both short (60 s) and longer (120 s) mean residence times, relative to the other reactor formats (Table 5). The authors modeled the experimental data using a two parameter Markov chains model to account for the flow characteristics within the TIJR system.⁹¹ The enhanced performance of this TIJR format was attributed to elimination of the external mass-transfer resistance around the IGI particles, as well as the high intensity mixing attained within the impingement zone and the reactor

itself. In a subsequent study, the same group investigated another variation on the TIJR theme whereby the reactor included a perforated plate designed to retain the IGI particles within the high-intensity mixing zone region of the reactor.⁹³ The authors concluded that the jet Reynolds number was one of the more important parameters for predicting TIJLR performance.

Glucose isomerization has also been studied using a simulated moving bed (SMB) reactor format.^{94–97} Also known as reactive chromatography, a SMB reactor combines reaction and substrate-product separation.⁹⁸ This permits reactions limited by thermodynamic equilibrium, such as glucose isomerization, to be driven to completion by continuous removal of fructose from the system.

Table 4 Recommended operating parameters for industrial immobilized glucose isomerase (GENSWEEET[®] IGI)⁵³

Parameter	Target range
Dry substance	45–52%
Temperature	53–60 °C
Monosaccharide	>95%
pH	7.6–7.8 (at room temp.)
Magnesium	30–50 ppm
Bisulfite (as SO ₂)	80–150 ppm
Calcium	<2 ppm
Refining	Carbon and ion exchange
Final check filtration	<10 microns

Table 5 Fractional conversion (%) of D-glucose to D-fructose in various reactor formats⁹²

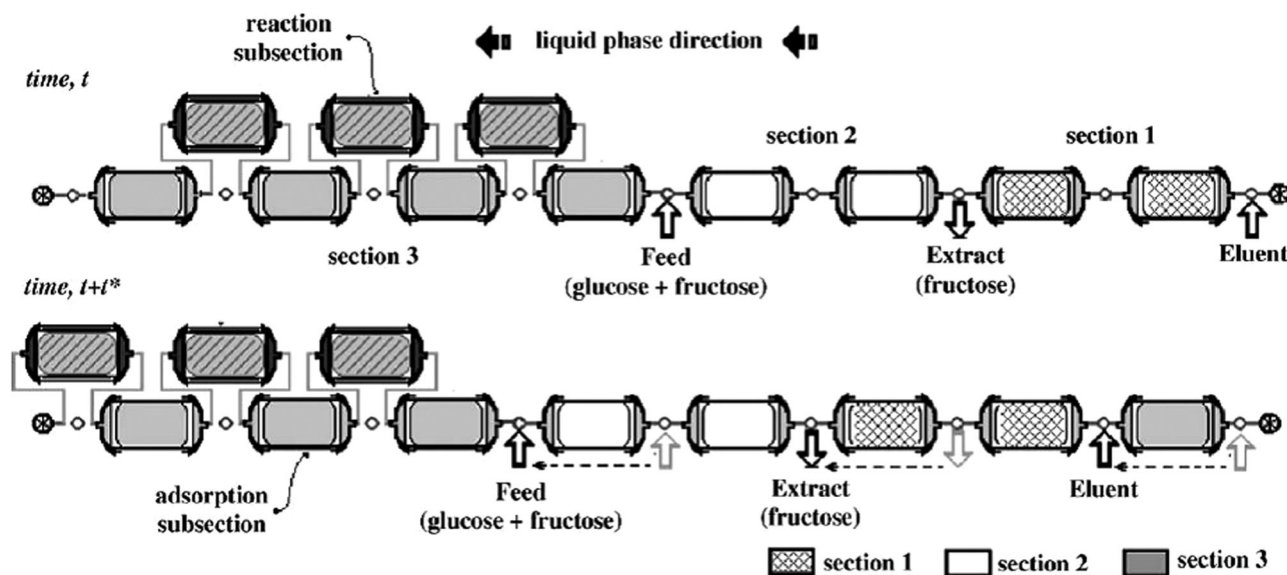
Mean residence time (s)	Reactor types			
	BSTR	PBR	DJR	TJJR
60	2	10	2.5	37
120	3	15	12	50

Operation of the SMBR format requires careful control over both flow rates and the switching of external feed streams (feed, eluant and extract). A related technique, simulated moving bed chromatography, is used on an industrial scale to separate crude sugar streams into component monosaccharides, having been originally developed in the 1980's.⁹⁹ Borges da Silva and coworkers published two studies in 2006 that describe a SMBR system for glucose isomerization using a combination of three separation stages containing ion-exchange resins, and a series of reaction stages containing immobilized glucose isomerase in fixed bed format (Fig. 6).^{97,100} The principle of operation involves directing a feed stream containing glucose into a reaction column where it is partially isomerized to fructose in the first of several isomerization reactors. The mixture is then

directed to the separation stages under conditions that retard fructose movement relative to glucose. Fructose-rich extract is removed from the system at a point (with regard to time and position) where the fructose/glucose ratio is greatest. The glucose-enriched mixture remaining in the system is directed back to a subsequent reaction column where further glucose isomerization occurs and the cycle is repeated. In this manner, Borges da Silva and coworkers used a combination of mathematical modeling and experimental data derived from isolated reaction and separation processes to predict that a conversion of glucose to fructose of over 90% could be achieved with a modification of the SMBR process originally proposed by Hashimoto.⁹⁴

Alternative means for *in situ* product removal (ISPR) of fructose from HFCS streams have also been proposed.¹⁰¹ The use of 3-aminophenylboronic acid (3-APBA) resin to selectively complex fructose from a glucose–fructose mixture was studied and shown to be capable of enriching a 55/45 glucose–fructose mixture to 27% glucose and 73% fructose.¹⁰¹ Similarly, a 77/23 xylose–xylulose mixture was enriched to 48% xylose and 52% xylulose. Optimal results were obtained when 3-APBA was attached to an epoxy-functional resin (Eupergit[®] C), *versus* attachment *via* an amide bond to a carboxy-functional resin.

3.4.1 Kinetic studies of IGI. Industrial IGI products are considered to be robust biocatalysts from both a productivity and stability perspective. Current products have operational lives of well over 1 year and produce up to 23 000 kg of HFCS per kg of biocatalyst.⁵³ Nonetheless, catalytic activity decreases over time as a function of the operating parameters, as well as the intrinsic properties of the biocatalyst, both biochemical and physical.¹⁰² The ability to model biocatalyst performance over time allows the determination of optimal operating conditions and the refinement of cost models for the overall process.^{102,103} The kinetic properties of immobilized glucose isomerase have been studied by numerous research groups who have sought to

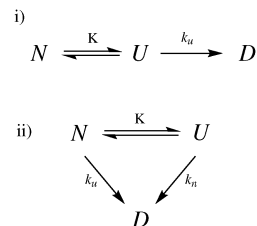
**Fig. 6** SMBR unit combining adsorbers and bioreactors for glucose isomerization (reprinted from ref. 97 with permission from Elsevier).

characterize a range of kinetic properties including process productivity and stability,^{102–106} thermal inactivation rates,^{40,107} the nature and effect of diffusional constraints,^{108–110} substrate preferences,³⁴ and the effects of substrate protection on inactivation rates.^{77,78}

One of the key issues for immobilized enzymes in general relates to limitations in mass transfer relative to soluble forms of the enzyme, the effect of which is to lower the effective specific activity of the biocatalyst.^{3–8} Both external and internal mass transfer limitations need to be considered given that most immobilized glucose isomerase products consist of granular, porous solids. Dadvar and Sahimi constructed a multiscale 3D pore network model in order to determine the degree to which blockage of micropores within enzyme granules contributes to the loss of activity over time.¹¹¹ The model suggested that pore blockage is a significant factor in activity loss, most likely acting in tandem with other enzyme denaturation mechanisms.

Experimental determination of the half-life of an immobilized biocatalyst is particularly challenging in cases where the operational lifetime is months or years and generally requires accelerated stability studies under conditions considerably harsher than normal. This problem was addressed in a study by Gibbs and Bommarius using a combination of both modeling and experimental observations.¹¹² The authors derived an equation for predicting the total turnover number (ttn) of a commercial glucose isomerase product (Gensweet[®] IGI) by determining enzyme kinetic performance over a range of temperatures. Two variations of a Lumry–Eyring kinetic model were applied to describe the pathways leading to irreversible enzyme inactivation (Scheme 2).¹¹³ The first version assumes that the native form of the enzyme (N) is first reversibly converted to an inactive form (U), governed by an equilibrium constant K . The inactive form U can be irreversibly converted to a completely deactivated form (D) at a rate given by k_u . An extended form of this model allows for direct irreversible conversion of the native form to the deactivated form at a rate of k_n .

The time- and temperature-dependant expression derived from these models allowed the prediction of several important kinetic parameters including the Gibbs free enthalpies of both activation ($\Delta G_{\text{cat}}^\ddagger = 88 \pm 0.9 \text{ kJ g}^{-1} \text{ mol}^{-1}$) and deactivation ($\Delta G_{\text{u}}^\ddagger = 125 \pm 0.7 \text{ kJ g}^{-1} \text{ mol}^{-1}$), in addition to the unfolding enthalpy ($\Delta H_{\text{eq}} = 90 \pm 0.3 \text{ kJ g}^{-1} \text{ mol}^{-1}$) and temperature ($T_{\text{m}} = 354 \pm 3.4 \text{ K}$). The authors noted that these values were consistent with prior literature values, as well as those predicted using isothermal batch experiments. There was significant discrepancy between the predicted ttn values obtained using these parameters, which ranged from 10^5 to 10^7 , and those reported for IGI under commercial operating conditions ($\text{ttn} = 10^4$). A possible explanation lies in the fact that the enzyme is unlikely to be operating at the maximal possible rate under commercial processing conditions where the fructose content approaches equilibrium (42 to 45% w/w), in contrast to this study, where conversions of 18 to 21% fructose were achieved. The authors also noted that prediction of the temperature for achieving optimal yield requires the definition of additional parameters such as the minimal acceptable space-time yield.



Scheme 2 (i) The Lumry–Eyring model for enzyme denaturation and (ii) an extended Lumry–Eyring model. The equilibrium constant between the native (N) and unfolded (U) species is represented by K , whereby $K = [U]/[N]$. The rate of conversion of U to the denatured species (D) is given by k_u . In the extended model the direct conversion of N to D is given by k_n .

3.5 Applications of immobilized glucose isomerase

3.5.1 High fructose corn syrup production. The dominant application for glucose isomerase is for the isomerization of corn-derived D-glucose to D-fructose in the form of high fructose corn syrup (HFCS). HFCS is entirely produced using immobilized glucose isomerase biocatalysts on an immense scale, exceeding 10^7 tons HFCS per year. The major current producers of IGI are DuPont Industrial Biosciences (formerly Genencor) and Novozymes A/S. Initial development of an enzymatic process for fructose production began in Japan in the 1950's and was soon extended to the United States, in part due to a shortfall in sucrose supply due to the Cuban revolution in 1958. Another key driver was the fact that the alternative chemical isomerization processes produced a low-quality product.²² Steady growth in the technology for production and immobilization of glucose isomerase in the subsequent two decades was aided by spikes in sucrose prices in the 1970's. D-Fructose, in the form of 42% high fructose corn syrup (HFCS), overtook sucrose as the dominant sweetener in the United States by 1978. The technology for HFCS production had improved to the point that manufacture of 55% HFCS syrup was economical and led to the adoption of HFCS by most beverage companies, including Coca-Cola and PepsiCo by 1984. On a weight basis D-fructose is 160% as sweet as sucrose and over twice as sweet as D-glucose (Fig. 7). The 55% grade is equivalent or slightly greater in sweetness as compared to sucrose. The major producers of HFCS are Archer Daniels Midland Co., Cargill, Ingredion Inc. and Tate & Lyle Ingredients America Co. HFCS in its various forms, is used in many food and beverage products, listed in Table 6.

In recent years, fructose-based products, termed collectively as high fructose corn syrup (HFCS), have relinquished some market share, in part due to concerns of the levels of consumption and a perceived association with the ongoing obesity epidemic.¹¹⁴ Others point out the advantages of HFCS sweeteners, for example D-fructose has a greater sweetening effect relative to sucrose on a caloric basis and has the lowest glycemic index of the simple sugars.^{22,115}

Glucose isomerase has also been used in tandem with lactase to sweeten D-lactose containing beverages and to reduce lactose content.¹⁹ A mixture of a commercial immobilized GI (SweetzymeTM) and an immobilized L-arabinose isomerase

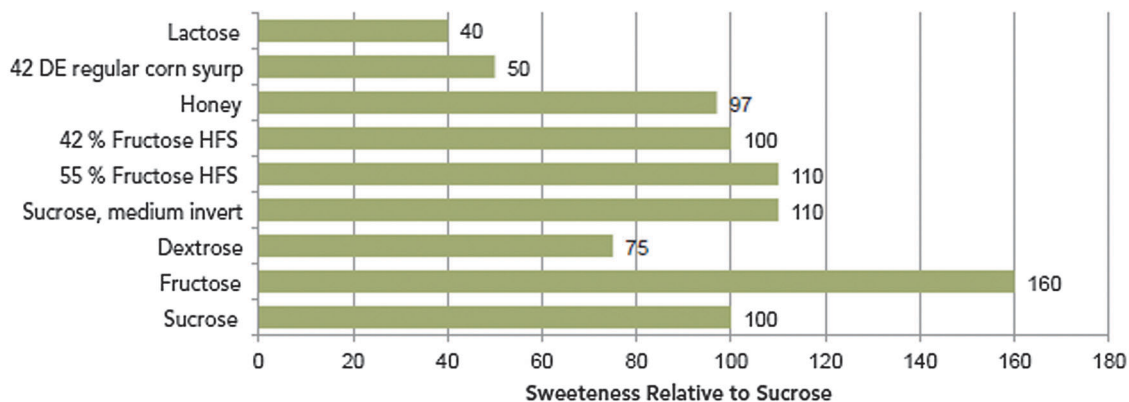


Fig. 7 Relative sweetness of common sugars.

(araA) was used to convert D-lactose into a mixture of D-glucose, D-galactose, D-fructose and D-tagatose.¹¹⁶ D-Tagatose is of interest as a sweetener as it has an even lower caloric value than D-fructose when benchmarked against sucrose on an equivalent sweetness basis.^{117,118}

3.5.2 Other applications of immobilized glucose isomerase.

Glucose isomerase has been used to enhance the conversion of sugars derived from cellulosic biomass to ethanol.^{119–121} The depolymerization of hemicelluloses generates pentose sugars including D-xylose, which is not readily fermented to ethanol by commonly used yeast ethanologens.¹²² In 1980, Wang and coworkers utilized different forms of GI to convert D-xylose to D-xylulose, which was converted to ethanol by *Schizosaccharomyces pombe* and *Kluyveromyces lactis*.¹¹⁹ The study found that Maxzyme GI, a soluble GI, was most effective in terms of the amount of ethanol produced from a 5% xylose solution, however only if Bovine serum albumin (BSA) was present in the fermentation media, presumably to minimize proteolytic degradation of the GI. An immobilized GI product (Taka Sweet from Miles Laboratories) was also evaluated and while this preparation was less effective in terms of final ethanol titers, a lower susceptibility to protease degradation was also observed. While this work clearly demonstrated the concept, the overall extent of conversion of D-xylose to ethanol was very low. Around the same time, Gong, Flickinger and Tsao filed a European patent (EP 38723) on a process for the conversion of D-xylulose to ethanol by fermentation utilizing

yeasts selected from *Saccharomyces*, *Candida*, *Torula*, *Schizosaccharomyces*, *Pichia* and *Hansenula* species.¹²³ The inventors cited processes where the xylose isomerase activity was expressed by the ethanologen, in addition to processes where soluble or immobilized xylose isomerase was used to convert D-xylose to D-xylulose prior or concurrent with fermentation to ethanol. One of the main hurdles for this technology was that the optimal conditions for fermentation to ethanol, typically pH values below 5 and temperatures in the 30 to 35 °C range, do not correlate to the optima for the GI enzymes utilized (typically pH 7 to 8 and 60 °C). This issue was addressed by Chandrakant and Bisaria who utilized a GI from *Candida boidinii* with an optimal pH of 4.5 to 5 and a temperature optimum of around 35 °C.¹²¹ An immobilized form of the enzyme was used to convert a mixture of D-xylose and D-glucose to ethanol in a simultaneous isomerization fermentation (SIF) experiment utilizing *S. cerevisiae* as the ethanologen. The authors immobilized the GI on eggshells, using glutaraldehyde as a crosslinker and reported 47% retention of enzymatic activity relative to an equivalent amount of the soluble form of the enzyme. The study examined the conversion of D-xylose to ethanol alone, and in the presence of D-glucose. In both cases, D-xylose conversion to ethanol was in the 42 to 45% range at yields of 0.36 to 0.40 g ethanol per g of xylose.

Another impediment to the conversion of D-xylose to ethanol is the unfavorable equilibrium at fermentation temperatures that favors D-xylose over D-xylulose by a factor of 4 : 1. Rao and coworkers developed a two-fold approach that addressed this issue, as well as the pH mismatch between conditions optimal for GI activity and fermentation mentioned above.¹²⁴ The authors added tetrahydroxyborate (0.05 M) to the reaction mixture to drive the equilibrium toward D-xylulose, a result of the preferential complexation of borate to D-xylulose over D-xylose. In addition, the authors co-immobilized urease and glucose isomerase by adding urease to a commercial IGI preparation (Sweetzyme™). The immobilized urease converted urea present in the reaction medium into ammonia, raising the pH within the immobilized enzyme particles and enhancing the activity of the proximal GI, despite the fact that the bulk medium was held at a pH of 4.5. No conversion to D-xylulose was seen in the absence of added urea. Overall, the authors

Table 6 Uses of high-fructose corn syrup (source: USDA: (<http://www.ers.usda.gov/topics/crops/sugar-sweeteners/background.aspx>))

Product	Application	% of total market (%)
HFCS-90	Production of HFCS-42 and 55	>95
	Natural and light foods	<5
HFCS-55	Beverage industry	>90
HFCS-42	Beverage industry	41
	Processed foods	22
	Cereal and bakery products	14
	Multiple use food manufacture	12
	Dairy industry	9
	Confectionary	1

claimed an 86% conversion of D-xylose to D-xylulose under these conditions.

More recently, several groups have focused on improvements in the yield of ethanol from D-xylose utilizing immobilized forms of GI. In both cases, the authors cite practical issues with the alternative approach of expressing xylose isomerase activity in wild yeast ethanologens, which include lower ethanol yields and tolerance, as well as concerns about GMO organisms in general.¹²⁵ Silva and coworkers coimmobilized glucose isomerase with *S. cerevisiae* cells and demonstrated ethanol production from glucose-xylose mixtures during a simultaneous isomerization fermentation (SIF) process.¹²⁶ The authors first immobilized a commercially available soluble glucose isomerase (GENSWEET® SGI) on chitosan beads, mediated by glutaraldehyde cross-linking, attaining a preparation with high specific activity (1700 IU g⁻¹). The immobilized biocatalyst was then co-immobilized with *S. cerevisiae* cells in a calcium alginate gel. The resulting enzyme/yeast biocatalyst was able to convert a glucose-xylose mixture to ethanol with productivity of 0.25 g ethanol per L h⁻¹ and a total xylose consumption of 75.4%. Additional byproducts included glycerol, acetate and xylitol. Miller *et al.* compared the effect of immobilized glucose isomerase on ethanol production from different carbon sources using two *Saccharomyces* strains (*S. pastorianus* and *S. cerevisiae*).¹²⁷ They found ethanol yields were enhanced on feedstocks such as D-fructose, as well as D-xylose containing substrates. They concluded that the effect was due to an overall increase in D-glucose uptake rate. Overall, the authors found that *S. pastorianus* was more efficient at converting cellulosic biomass derived sugars into ethanol, attaining a yield of 0.35 g ethanol per g of supplied nutrients. It was noted that this yield is comparable to the best results obtained with recombinant strains of *S. cerevisiae*.¹²⁸

3.5.3 Fructooligosaccharide production. Fructooligosaccharides (FOS) are a class of non-digestible oligosaccharides (NDO) used as prebiotics in foodstuffs. Structurally they are defined as containing one D-glucose residue and 2 or more β-D-fructosyl residues, essentially β-1,2-fructofuranosyl derivatives of sucrose. Kestose (GF2), nystose (GF3) and 1-fructofuranosylnystose (GF4) comprise the FOS molecules of greatest interest. The enzymes that catalyze the formation of FOS from sucrose are called β-fructosyltransferases (FTase; EC 2.4.1.9) and are found in a number of microorganisms.¹²⁹ The products of the reaction include FOS oligosaccharides in addition to D-glucose, which acts as a competitive inhibitor and can limit the extent of conversion. Accordingly, several groups have used enzymes such as glucose isomerase and glucose oxidase to reduce the amount of glucose in the reaction mixture. Hendersen and

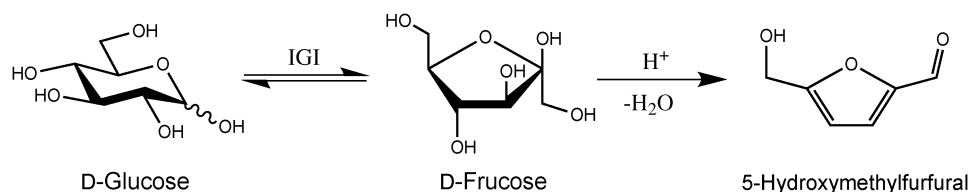
coworkers filed a patent application on a system whereby sucrose is converted *in situ* to FOS with a fructosyltransferase.¹³⁰ Additional enzymes (glucose oxidase, glucose isomerase) are used to convert the D-glucose byproduct to other sugars. The invention is intended for use in fruit juice and other beverages, as well as sweet syrups. Fructooligosaccharide production by *Aspergillus oryzae* N74 was studied both experimentally and through kinetic modeling.¹³¹ Immobilized GI was found to have a slight positive effect on FOS yield, with an increase of 4.7% over a control without IGI, giving a total yield of 58.3% g g⁻¹ on sucrose. The ratio of glucose to fructose decreased by a factor of 6.2, which the authors pointed out might confer advantages in downstream purification given the lower levels of residual sucrose and glucose in the FOS product.

3.5.4 5-Hydroxymethylfurfural synthesis. Acid-catalyzed dehydration of D-fructose gives 5-hydroxymethylfurfural (HMF), a platform chemical that can be converted to fuels and chemicals.^{132–135} A combined chemoenzymatic route for the conversion of glucose to HMF utilizing both glucose isomerase and an acid catalyst was proposed recently (Scheme 3).¹³⁴ Inclusion of borate ions in the medium drove the isomerization reaction toward fructose. The hydrolytic instability of HMF has led to a search for non-aqueous solvents compatible with both isomerization and dehydration steps. Ståhlberg and coworkers screened a range of ionic liquids and found that glucose isomerase retained activity in several cases, with the best results obtained with a mixture of *N,N*-dibutylethanolammonium octanoate (DBAO) and water.¹³³

The conversion of D-glucose to HMF using IGI and seawater has been demonstrated.¹³⁵ Isomerization of glucose to fructose was followed by oxalic acid catalyzed dehydration of fructose to 5-HMF, a reaction enhanced by high salt concentrations. The HMF product was continuously extracted into a 2-methyltetrahydrofuran (2-MeTHF) organic phase and sent to recovery. The remaining aqueous stream, comprising around 50% glucose, 5% 2-MeTHF and oxalic acid was first subjected to a crystallization step to recover oxalic acid, and then returned to the isomerization reactor. The study showed that the IGI used (Sweetzyme™ IT) was able to isomerize a 50% glucose stream in seawater containing 2-MeTHF without loss of activity over several recycle steps.

3.6 Outlook for immobilized glucose isomerase

Glucose isomerase remains as the most successful immobilized enzyme product ever developed in terms of both enzyme sales and the total volume of product produced. Current commercial products are the result of decades of iterative improvements and display very high productivities and overall robustness. Further improvements are still actively sought, for example



Scheme 3 Conversion of D-glucose to HMF.

enzymes capable of operating at higher temperatures and lower pH.²¹ The goal remains to enable a process that can produce 55% D-fructose directly, without the need to apply chromatographic enrichment.

A number of alternate chemical processes for glucose isomerization have been described, beginning with an alkaline process originally developed in 1895, known as the Lobry de Bruyn-Alberda van Ekenstein transformation.¹³⁶ These methods had several drawbacks including unwanted generation of mannose, psicose and other contaminants, in addition bitter flavors and a dark appearance. The lack of commercially viable alternatives for D-fructose production was a major factor in the rapid adoption of the enzymatic process. Research into chemical catalysts for glucose isomerization did not cease however, and continues to be pursued. For example, Moliner and coworkers recently described how Ti- and Sn-zeolites effectively catalyze the isomerization of glucose to fructose over a range of temperatures.¹³⁷ A subsequent study investigated the mechanism by which hydrophobic Sn- and Ti-zeolites catalyze the isomerization reaction, concluding that a metal-assisted hydride shift process that mimicked the enzymatic process was responsible.¹³⁸ This highlights another important consideration in the development of immobilized biocatalysts – industry will favor the best process from an economic perspective, regardless of whether the catalyst is synthetic or biological in nature. As such, it is important that the relative advantages of chemical and biological catalysis are carefully assessed on an application by application basis. Life-cycle analysis (LCA) is one way to quantify the relative merits of different catalysts on the overall economic and environmental impact of a given process and is discussed toward the end of this review.¹³⁹

4. Emerging immobilized enzyme applications

There are very few industrial applications of immobilized enzymes at large scale; as mentioned in Section 2, above, there often is not a significant economic benefit for utilization of an immobilized enzyme when compared to the cost of using an unimmobilized form of the same biocatalyst. A review of current and past publications in the area of enzyme immobilization indicates that there has been significant activity for the development of process that employ immobilized enzymes for food applications, including pectin hydrolysis and the reduction of bitter components in the production of fruit juices, and for the interesterification of food fats and oils, the latter being practiced commercially.

4.1 Pectin hydrolysis

Pectin consists of a complex mixture of polysaccharides that is the major component of the primary cell wall of most plants, and is also present in the middle lamella between plant cells.¹⁴⁰ Chemically, pectin is composed of branched heteropolysaccharides containing from a few hundred to *ca.* one thousand building blocks per molecule, with a backbone consisting of galacturonic acid residues part of which are methylesterified. The pectin molecule (Fig. 8) is generally agreed to consist of a chain structure of axial-axial α -(1,4)-linked D-galacturonic acid units, containing blocks of L-rhamnose rich regions, with mainly arabinose, galactose and xylose as side chains. The carboxylate groups of the galacturonic acid are partially esterified by methyl groups, and partly or completely neutralized by sodium, potassium or ammonium ions. Some of the hydroxyl groups on C2 and C3, may be acetylated.¹⁴¹ Various enzymes are responsible for pectin depolymerization, acting on the main galacturonate backbone;^{140,142} in particular endopectinlyase (PL; EC 4.2.2.2, EC 4.2.2.9 and EC 4.2.2.10) and endopolygalacturonase (PG; EC 3.2.1.15 and 3.2.1.67) act on pectin and polygalacturonic acid, respectively. Pectinesterase (PE; EC 3.1.1.11) is responsible for pectin de-esterification, producing a polysaccharide that can be hydrolyzed by polygalacturonase.

Pectin produces turbidity and undesired solid suspensions in fruit juices, and commercial production of fruit juices often utilizes pectin lyase to degrade and remove pectin, resulting in increased yields, improved liquefaction, clarification and filterability of juices, and reduction in viscosity. Pectin lyase is active towards hydrolysis of highly esterified pectins, such as apple pectin, through a β -eliminating cleavage of glycosidic linkages.¹⁴³ Although the use of an immobilized enzyme for hydrolysis of a macromolecular substrate can be problematic due to potential diffusional or structural limitations of contacting the immobilized enzyme with the substrate, it has been demonstrated that pectin lyase can be immobilized on a variety of supports and still remain active for pectin hydrolysis. Immobilization on solid supports enables catalyst recycle, and the implementation of a continuous process for pectin degradation during fruit juice production. Pectin lyase has been immobilized on several supports, including alginate gel,¹⁴⁴ EUDRAGIT® L100-55,¹⁴⁵ bentonite,¹⁴³ γ -alumina,¹⁴⁶ nylon,^{147–150} chitin,¹⁴⁹ porous glass and DEAE cellulose activated with titanium salt,¹⁵¹ and Eupergit® C.¹⁵²

A comparison was made of the catalytic capability of alginate-immobilized pectin lyase present in several commercial enzymic mixtures (Rapidase® C80 (Gist Brocades), Biopectinase® CCM (Quest International), Grindamyl™ 3PA (Danisco) and Pectinex®

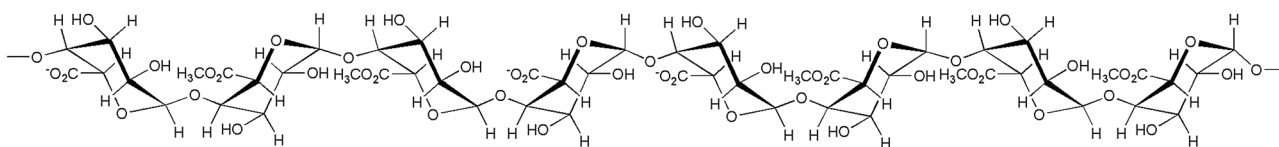


Fig. 8 Structure of the pectin molecule. Only one chain of the major component of pectins, partially methyl-esterified galacturonic acid, is represented. Side chains of galactose, arabinose and xylose residues are not included (adapted from ref. 140).

3XL (Novozymes)).¹⁴⁴ Maximum percentage of immobilization (10.6%) was obtained with Rapidase[®] C80. Immobilization parameters were determined and the effects of immobilization on the pH and temperature profile, kinetic parameters, thermal stability and operational stability of the immobilized enzyme preparation were also studied. The pH and temperature at which activity of soluble and immobilized enzyme was maximal were 7.2 and 55 °C. Immobilized Rapidase C80 was recycled in four consecutive batch reactions without measurable loss of activity, where a reduction of viscosity of apple pectin solution at 40 °C was about 35% after 30 min of treatment.

A commercial pectolytic enzyme preparation (Pectinex[®] Ultra SP-L from *A. aculeatus*, Novozyme) was immobilized onto porous anion exchange resins (Styrene-DVB macroporous based resins (Dowex Marathon WBA)) *via* electrostatic adsorption, which makes possible regeneration of the support.¹⁵³ As both pH and ionic strength are important parameters associated with immobilization by electrostatic adsorption, pH was kept constant at 4.5 during the trials and immobilization processes. The activity of immobilized enzyme was determined by measuring pH, dry matter content and viscosity of carrot puree. Enzyme immobilized particles were added to the carrot puree at 1.5 g particle/100 g puree at pH 4.5 and 35 °C to degrade soluble and insoluble pectin and haze-provoking polysaccharides. The immobilized enzyme reduced the viscosity of the carrot puree from 90 to 6.5 Poise after 60 min of incubation. While the viscosity and pH of the puree were decreased, dry matter content and total yield were found to be increased because of the polysaccharide degradation. An average juice yield increase of 30.23% was obtained compared to the yield obtained from non-enzymic processed carrot juice. Immobilized enzyme activity loss was found to be only 6.5% when the immobilized enzyme was used in five consecutive batch reactions.

Pectin lyase from *Penicillium italicum* was immobilized on Nylon 6, and the resulting immobilized enzyme demonstrated increased thermal stability, a pH activity optimum shifted towards lower pH values, increased stability at low pH values, and improved operational stability.¹⁴⁸ The enzyme was immobilized by covalent binding to Nylon 6; after an acid cleavage of the amide groups of nylon, the enzyme was coupled to the resulting primary amino groups of the support *via* glutaraldehyde crosslinking. The effect of the immobilized enzyme on the viscosity of three different fruit juices (melon, apricot, and peach) was evaluated, and a viscosity reduction of at least 25% was observed after 60 min of incubation for each fruit juice. No loss of activity was observed when the immobilized enzyme was recycled in twelve consecutive batch reactions. Michaelis–Menten constants for the immobilized enzyme activity for hydrolysis of citrus pectin (DE 70%) at pH 6.0 were lower than that for the soluble enzyme.

Multipoint attachment has been used to immobilize pectinase on an agar-gel support.¹⁵⁴ The activated glyoxyl agar-gel support was prepared by etherification of 6% agar-gel with glycidol and further oxidation of the resulting glyceryl agar-gel by NaIO₄. After immobilization, the optimal pH and temperature curves for

enzymatic activity were slightly broadened. The immobilized enzyme exhibited higher thermal stability compared to the free form and also compared to pectinase immobilized on alginate support using glutaraldehyde as a coupling agent.¹⁵⁵ The maximal activity of immobilized pectinase was obtained at 5 °C, pH 3.6, with a 24 h reaction time at an enzyme dose of 0.52 mg protein per g gel; these conditions increased the thermal stability of the immobilized pectinase 19-fold compared to the free enzyme at 65 °C. The immobilized enzyme also exhibited great operational stability, and an 81% residual activity of the immobilized enzyme was observed after 10 batch reactions with catalyst recycle.

The co-immobilization of the pectolytic enzymes endopolygalacturonase (PG), endopolygalacturonase (PG) and pectinesterase (PE) present in a commercial formulation (Pectolyase Y23 from *Aspergillus japonicus* (Sheishin Corporation, Japan) has been reported using γ -alumina spheres as support and *o*-phosphoryl-ethanolamine as organophosphate bidentate linker.¹⁴⁶ The fragility of γ -alumina and its low resistance to mechanical friction stress made the use of the γ -alumina–enzyme complex in a stirred reactor problematic, so the immobilized biocatalyst was contained in a thermostated column and the substrate solution (cloudy apple juice) recycled through the packed bed. Despite low amounts of PG and PL and only traces of PE co-immobilized on the functionalized γ -alumina spheres, efficient depolymerization of pectin and polygalacturonic acid in raw apple juice was demonstrated; only a small decrease in catalytic activity was detected after five biocatalyst uses.

The use of a pectin-degrading enzyme has been evaluated as a method for increasing the flux in the microfiltration, ultrafiltration and reverse osmosis of fruit juices and other pectin-containing liquid food products, where pectin is considered to be a major contributor to flux decline and to difficulties in membrane cleaning.¹⁵⁶ By immobilizing pectinase on the membrane surface, the potential benefits of the pectin degradation at the membrane–solution interface were achieved with a minimal effect on the properties of the clarified concentrate. A titania microfiltration membrane having an average pore diameter of 0.05–0.1 μm was washed with aqueous HNO₃ at pH 2.5 for 30 min and rinsed with water, then the pectinase (polygalacturonase from *Aspergillus niger* (EC 3.2.1.15)) was adsorbed on the membrane by circulating a dilute solution of the crude enzyme through the membrane; approximately 5.0 g m^{−2} of pectinase was adsorbed. Solutions of citrus and apple pectin (1.0 g dm^{−3}) were used to test the immobilized-enzyme membrane, and a flux increase in microfiltration of the pectin solutions was obtained, indicating that the presence of immobilized pectinase on the titania microfiltration membrane produced a higher fouling resistance than that observed for the control membranes without immobilized pectinase. The resistance associated with concentration polarization, or resistance reversible after a water rinse, was reduced enough under these filtration conditions to significantly increase the flux over that obtained with the control membranes.

PL immobilization on organic (cellulose and its derivatives, or Amberlite[™] XAD) and inorganic (sulphides, γ -alumina, and

bentonite) matrices through simple adsorption or after activation with glutaraldehyde has been examined.¹⁴³ The acrylic resins XAD-7 or XAD-8) were also activated, after basic hydrolysis, with carbodiimide, thionyl chloride, and trichlorotriazine. After purification, the commercial formulation Cytolase PCL5 (Genencor) was employed as PL source, and after esterification, apple pectin was used as the enzyme substrate. Immobilization on cellulose and its derivatives gave poor results for both catalytic activity and stability. Among XAD-amberlites, XAD 7 activated with trichlorotriazine produced an immobilized PL with acceptable adsorption and immobilization yields, as well as good specific activity (335 U g^{-1}); although poor stability was improved by treatment of the immobilized enzyme with glutaraldehyde, the catalyst activity half-life (213 h) remained too low for commercial use. Immobilization of PL on glutaraldehyde-activated bentonite in the presence of albumin, although exhibiting lower specific activity (65 U g^{-1}) than on XAD 7, was considerably more stable, with a half-life of 650 h. Further advantages of this bentonite-immobilized enzyme included a slight shift in the optimum pH toward acidic values and its greater relative activity at lower temperatures. Bentonite was found to be particularly suitable as a support for enzyme immobilization in this application due to its nontoxicity (food grade), ready availability, low cost, microbiologic stability, good thermal and mechanical resistance, and the possibility of being activated by simple and inexpensive methods.

Two commercial ion exchangers were evaluated for the immobilization of a commercial enzyme formulation containing pectinase, pectin esterase and pectate lyase.¹⁵⁷ Carboxylic acid cation exchanger Ostion KM (a methacrylate polymer) was used without further chemical modification for covalent binding by carboxylic group derivatization. Ostion AT (a weakly basic anion exchanger based on polystyrene crosslinked with 4% divinylbenzene substituted with diethylamine) had no functional groups for immobilization of proteins, and was chemically derivatized to produce a support with aromatic primary amino groups that were crosslinked to protein using glutaraldehyde. The operational stability of the immobilized enzymes was measured in a packed bed reactor using 0.5% pectin solution in 0.1 M acetate buffer, pH 4.9 at 30 °C; the KM-aminopolystyrene and glutaraldehyde supported enzyme had a $t_{1/2}$ of 456 days, and the hexamethylenediamide supported enzyme (Ostion KM) had a $t_{1/2}$ of 403 days. The AT-poly(aminostyrene)-glutaraldehyde-pectolytic enzyme was chosen for further characterization using 1% pectin solution or apple juice at 30 °C and pH 4.5. The immobilized enzyme produced a reduction in viscosity that was 80% of that produced using soluble enzyme; this lower degree of viscosity reduction was ascribed to the negative effect of insoluble colloidal particles present in the natural juice on the activity of the immobilized enzyme.

4.1.1 Impact of reactor design on immobilized pectinase efficiency. One approach to ameliorating the negative impact of colloidal particles in fruit juice on the activity of immobilized pectin-hydrolyzing enzymes was to evaluate a particular reactor. A cross-flow reactor with enzymes immobilized on a membrane surface has been shown to offer several advantages for viscous

substrate solution processing, since the external diffusional limitations are reduced by the high recycling flow rate.¹⁵⁸ This type of bioreactor was used for the hydrolysis of whey and vegetable proteins and for the development of a mathematical model for diffusion and reaction at only one recycling flow rate.^{159,160} Synthetic membranes containing active covalently-bound pectolytic enzymes have been previously developed¹¹ and applied in a cross-flow reactor configuration for the continuous clarification of apricot juice with excellent results.¹⁶¹ The kinetics and operational behavior of immobilized pectolytic enzyme in a membrane cross-flow reactor as a function of the recycling flow rate was determined, and the effect of recycling flow rate, filtrate flow rate, reaction volume, and amount of immobilized enzyme on the conversion profiles was reported.¹⁶² A pectolytic enzyme preparation (Pectinol D from Rohm GmbH) containing five polygalacturonases, four pectin lyases and one pectinesterase was immobilized on a nylon 6 membrane using polyethyleneimine (PEI) and glutaraldehyde. Citrus pectin having a 40–60% degree of esterification was used as substrate. A Minitan S[®] system (Millipore) was used as the crossflow biocatalytic reactor, and this mode of operation was compared to using the immobilized Pectinol D on nylon pellets in a packed bed reactor. The operational stability of immobilized Pectinol D in the crossflow reactor was tested by recycling the pectin solution through the module at 40 °C and at different flow rates. The system was operated continuously at a filtrate flow rate of 0.6 mL min^{-1} .

The continuous processing of citrus pectin by the immobilized Pectinol D in either a cross-flow or packed bed reactor produced a viscosity drop greater than 50%, sufficiently high for the product to be considered as clarified. In the cross-flow reactor, the immobilized Pectinol D demonstrated the highest pectic substrate conversion (Fig. 9); this was due to the higher mass-transfer rate produced by the recycling flow rate that approximated the observed kinetic parameters to their reported intrinsic values, which were previously worked out in a batch reactor (and where the kinetic parameters were severely affected by external diffusional limitations). Under these conditions, the catalytic efficiency of the immobilized biocatalysts was the highest they could exhibit. Although the immobilized enzyme on nylon pellets in a packed bed reactor had a higher operational stability than the crossflow reactor (due to the pellets experiencing lower shear stress), the cross-flow reactor had a higher catalytic efficiency than the packed bed reactor, possibly due to the lower pressure drop when using the membrane configuration; the high viscosity of the substrate solution caused a pressure drop in the packed bed reactor that reduces the substrate conversion capability of the immobilized enzyme. The use of the cross-flow system provided an efficient method to process high-viscosity pectin solutions, where enzyme deactivation due to shear stress produced by tangential flow was largely compensated by a high conversion capability.

4.2 Reduction of bitter components in fruit juices

Naringin (4',5,7-trihydroxyflavanone-7- β -L-rhamnoglucoside-(1,2)- α -D-glucopyranoside), is the principal bitter flavonone glycoside

and primary bitter component in fruit juices such as grapefruit juice,^{163–165} where the typical concentration is 400 mg L⁻¹.¹⁶⁶ Commercial methods to debitter juices typically employ neutral or ion exchange resin technology, where naringin is absorbed from clarified juice by passing through a divinylbenzene polymer approved for food use.¹⁶⁷ Use of adsorption for debittering, is non-specific, and can alter the chemical composition of juices, either through chemical reactions or by removal of nutrients, flavor or color. The resin must also be regenerated using chemicals that cannot be recycled, and regeneration produces a waste stream that adds a disposal cost to the process.

As an alternative to physical adsorption techniques, debittering can be achieved by treatment of juice with naringinase, an enzyme complex obtained from fungi such as *Penicillium decumbens*, *Aspergillus sp.*, *Aspergillus niger*, *Aspergillus oryzae* or *Rhizopus nigricans*. It is a heterodimer of 168 kDa composed of two subunits, α -L-rhamnosidase (EC 3.2.1.40) and β -D-glucosidase

(EC 3.2.1.21).^{164,168–171} Naringin is first hydrolysed by α -L-rhamnosidase into rhamnose and prunin (trihydroxyflavone-7-glucoside); prunin, which is one-third as bitter as naringin, can be further hydrolysed by the β -D-glucosidase into glucose and tasteless naringenin (4',5,7-trihydroxyflavanone) (Scheme 4). Naringinase has been immobilized by entrapment or by adsorption on or covalent attachment to insoluble carriers, as well as in hollow fiber cartridges (Table 7).

4.2.1 Debittering of fruit juice. Naringinase has been immobilized by entrapment in poly(vinylalcohol) cryogels.¹⁷² The highest activity yield (91.6%) was observed when using a PVA concentration of 8% (w/v); at lower PVA concentrations, the porosity of the gel increased and retention of the enzyme decreased. The optimum pH (4.5) for the entrapped enzyme was the same as for free enzyme; high activity at this pH was optimal for fruit juice processing where the pH of fruit juices is often less than 5. A low operational pH would also limit microbial contamination of the reaction mixture. The operational stability of the entrapped naringinase was determined by measuring naringin hydrolysis in simulated fruit juice in consecutive 24 h batch reactions at pH 3.2 and 20 °C using 10% (w/v) PVA-naringinase beads, where the immobilized enzyme was washed with 0.2 M acetate buffer (pH 5.0) after each batch reaction; a 64% reduction in the immobilized enzyme activity was observed after only three batch reactions with biocatalyst recycle.

Nunes *et al.* reported the use of polyvinyl alcohol-alginate beads for immobilization of naringinase from *Penicillium decumbens*.¹⁷³ The highest naringinase activity yield in 2 mm-dia. 10% PVA, 1% alginate beads was 80%, and the pH and temperature optimum were 4.0 and 70 °C, respectively. Naringin bioconversion was measured using 0.5 g L⁻¹ naringin in acetate buffer (0.02 M, pH 4.0), at 30 °C. The Michaelis constant ($K_{m,app}$) and the maximum reaction velocity ($V_{max,app}$) were evaluated for both free ($K_{m,app}$ = 0.233 mM; $V_{max,app}$ = 0.13 mM min⁻¹) and immobilized naringinase ($K_{m,app}$ = 0.349 mM; $V_{max,app}$ = 0.08 mM min⁻¹). The residual activity of the immobilized enzyme was followed in eight consecutive batch runs with a retention activity of 70%; the observed loss

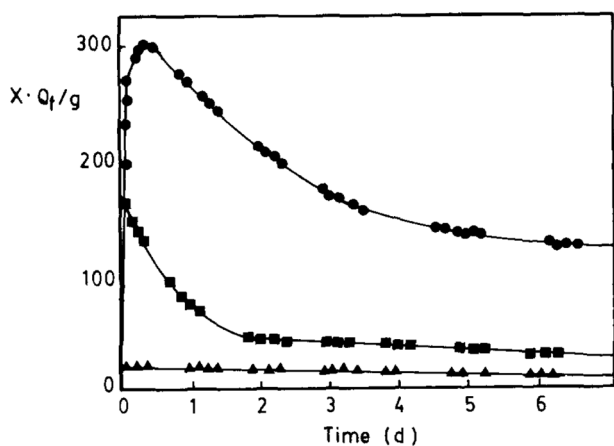
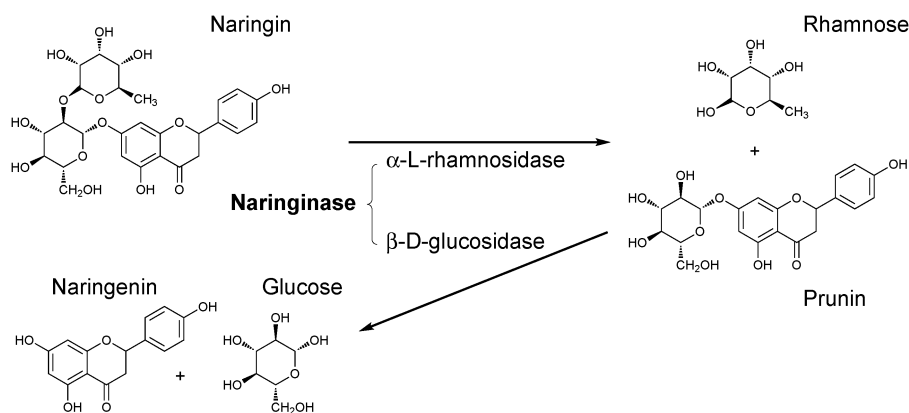


Fig. 9 Substrate conversion capability of immobilized Pectinol D with time in: (●) cross-flow reactor, (Q_f = 100 mL min⁻¹); (■) packed-bed reactor with membrane circles; (▲) packed-bed reactor with pellets. Conversion capability (XQ_f/g) was calculated as substrate conversion times the outlet flow per amount of immobilized enzyme expressed in grams (reprinted from ref. 162 with permission from Elsevier).



Scheme 4 Hydrolysis of naringin into prunin, rhamnose, naringenin and glucose by naringinase containing α -L-rhamnosidase and β -D-glucosidase activities (adapted from ref. 171).

Table 7 Immobilization methods for naringinase

Support	Method	Ref.
Ferromagnetic supports	Covalent attachment	187 (Soria <i>et al.</i> , 2012)
Poly(vinylalcohol) cryogels	Entrapment	172 (Busto <i>et al.</i> , 2007)
Celite	Adsorption	186 (Sekeroglu <i>et al.</i> , 2006)
Styrene/maleic anhydride copolymer	Adsorption	188 (Goldstein <i>et al.</i> , 1971)
Tannin-aminohexyl cellulose	Adsorption	189 (Ono <i>et al.</i> , 1978)
Chitin/glutaraldehyde/borohydride	Entrapment/crosslinking	191 (Tsen, 1988), 192 (Tsen, 1984)
Hollow fiber reactor	Physical separation	199 (Tsen, 1991), 197 (Olson <i>et al.</i> , 1979), 198 (Gray and Olson, 1981)
Controlled pore glass	Covalent attachment	193 (Roitner <i>et al.</i> , 1984)
Silicate/glutaraldehyde	Covalent attachment	196 (Birkner <i>et al.</i> , 1989)
Glycophase-coated porous glass	Covalent attachment	194 (Manjón <i>et al.</i> , 1985)
Bagasse	Covalent attachment	190 (Afaq <i>et al.</i> , 1997)
Alginate	Entrapment	174 (Puri <i>et al.</i> , 1996), 175 (Pedro <i>et al.</i> , 2007)
κ -Carrageenan	Entrapment	195 (Ribeiro <i>et al.</i> , 2008)

of activity was ascribed to leaching of the enzyme from the support when the immobilized enzyme was washed with buffer prior to recycle in a subsequent batch reaction. After 6 weeks, upon storage in acetate buffer pH 4 at 4 °C, the immobilized biocatalyst retained 90% of the initial activity.

Naringinase derived from *Penicillium* sp. (containing both α -rhamnosidase and β -glucosidase activities) was entrapped in 2% calcium-crosslinked alginate beads.¹⁷⁴ The operational stability of the immobilized naringinase was satisfactory for a naringin solution, but when used for debittering of kinnow mandarin juice in consecutive batch reactions, the catalyst activity slowly decreased to 50% of initial activity after twelve recycles, likely due to particulate matter from the kinnow juice clogging the pores of the alginate beads; leaching of the enzyme from the alginate beads was not detected. The use of high pressure to improve the catalytic activity of naringinase immobilized in calcium alginate beads has been reported.¹⁷⁵ When evaluated for hydrolysis of naringin (0.86 mM) in 20 mM acetate buffer (pH 4.0) at 30 °C, naringinase entrapped in Ca-alginate beads displayed higher activity and a 65% higher maximum initial rate ($V_{\text{max,app}} = 0.069 \text{ mM min}^{-1}$), and a 70% lower $K_{\text{m,app}}$ (0.097 mM) at 160 MPa when compared to kinetic parameters measured at atmospheric pressure. The use of this high-pressure method was evaluated for hydrolysis of naringin in fruit juices;¹⁷⁶ grapefruit juice was centrifuged at 8000 rpm prior to use in bioconversions run using four parts (by volume) juice to 1 part immobilized enzyme. A debittering of 75% occurred at 37 °C for 20 minutes when using particulate-free grapefruit juice at a pressure of 160 MPa.

The use of wood chips for covalently immobilizing naringinase for debittering of kinnow mandarin juice was also evaluated.¹⁷⁷ Cedar wood chips (30–40 mesh) were first washed with sodium acetate buffer (10 mM, pH 7.0) and then repeatedly treated with HCl (1 M) and NaOH (1 M) prior to reaction with glutaraldehyde (GA) and subsequent immobilization of naringinase at 37 °C. Centrifuged kinnow mandarin juice was reacted with 0.2 U L⁻¹ of immobilized naringinase at 45 °C for 1 h. Only 76% naringin was hydrolysed in 1 h, with no increase in conversion at longer reaction times. The enzyme did not leach from the support, and no loss of activity was observed when the immobilized enzyme was used in seven consecutive batch reactions with catalyst recycle.

The immobilization of enzymes in packaging materials has been proposed as a form of “active packaging”,¹⁷⁸ and the immobilization of naringinase in films for packaging materials has been reported by Soares and Hotchkiss.¹⁷⁹ Naringinase from *Penicillium* sp. was immobilized in cellulose acetate films with up to 23% efficiency. The enzyme activation energy decreased upon immobilization (from 14.2 to 11.0 Kcal mol⁻¹), and the K_{m} for immobilized naringinase (2.1 mM) was lower than for free enzyme ($K_{\text{m}} = 3.6 \text{ mM}$). Cellulose acetate films containing immobilized naringinase reduced the naringin level in grapefruit juice at common refrigeration temperature.

Enzyme activity in cellulose acetate films was less affected by lower temperature than free enzyme and did not lose activity during dry storage, demonstrating the potential for use as an inner film layer in citrus juice packages to reduce naringin. When the film-to-product ratio (cm² film per mL juice) was 7.2, 10° Brix grapefruit juice stored with immobilized naringinase showed a 60% naringin hydrolysis in 15 days at 7 °C. The surface-area to product-volume ratios were noted to be higher than those used in many packaging applications (typically 0.6 to 1.0), therefore the authors concluded that enzyme activity per unit area would have to be increased, or the packaging would need to be redesigned to increase the ratio.

Naringinase immobilized on mesoporous silica MCM-41 *via* adsorption with glutaraldehyde was used to debitter white grapefruit juice at 60 °C for 30 min.¹⁸⁰ The immobilized catalyst demonstrated excellent thermal stability and storage stability, and retained about 45% of its initial activity after six batch reactions with catalyst recycle. The immobilization and evaluation of naringinase as crosslinked enzyme aggregates (CLEAs) has also been reported.^{181–183} In a study by Ribeiro and Rabaça,¹⁸³ ammonium sulphate, polyethylene glycol 6000 and *tert*-butyl alcohol were screened as precipitating agents and glutaraldehyde as cross-linking agent at different pH, time, and temperature conditions, but only *tert*-butyl alcohol and crosslinking with glutaraldehyde (3%) at pH 4.0 and at temperature between 7 and 10°C produced CLEAs. The operational stability of NGase-CLEAs was evaluated at 30 °C, pH 4.0, 100 rpm with naringin in a concentration of 0.5 mg mL⁻¹. After each run of 60 minutes, the biocatalyst was separated and washed with acetate buffer 20 mM, pH 4.0. The naringinase residual activity was above 90% until the third catalyst recycle, where the catalyst activity decreased to 30% of initial activity.

Although not directly evaluated for the debittering of fruit juices, naringinase was immobilized in a sol-gel matrix of tetramethoxysilane (TMOS) and glycerol, and the effect of increasing concentration of co-solvents on the stability of both soluble and immobilized naringinase expressing α -L-rhamnosidase and β -D-glucosidase activities was evaluated.¹⁸⁴ Sol-gel immobilization stabilized naringinase in co-solvent systems employing dimethyl sulfoxide, *N,N*-dimethylmethanamide, methanol, ethanol, acetone, tetrahydrofuran, 1,2-dimethoxyethane and 1,4-dioxane, where an increase of at least 4 half-lives was observed for the immobilized enzyme. The half-life of α -L-rhamnosidase and β -D-glucosidase increased 21-fold and 59-fold, respectively, in aqueous solution containing 10% tetrahydrofuran.

Naringinase has also been encapsulated in ionic liquid (IL)-based silica gel matrices, to further improve naringinase activity and stability for glycoside hydrolysis.¹⁸⁵ The ILs [EMIM][DMP], [C₂OHMIM][BF₄], [BMIM][MeOEtOEtOSO₃] and [E2-MPy][ESO₄] were tested for production of the sol-gel matrices as the unique additive with TMOS, and separately in combination with glycerol. Immobilized enzyme activity and stability was evaluated in nineteen consecutive batch reactions with catalyst recycle, and for both α -L-rhamnosidase and β -D-glucosidase, enzymatic efficiency was higher in sol-gel matrices of TMOS/glycerol/ILs than for matrices of TMOS/ILs; the presence of glycerol resulted in higher efficiencies for the two enzyme activities of about 60% and 75%, respectively.

Naringinase from *Penicillium decumbens* was immobilized by adsorption onto diatomaceous earth (Celite[®], Hyflo supercel) and the activities of free and immobilized naringinase (optimum pH, optimum temperature and kinetic parameters) for naringin hydrolysis were compared.¹⁸⁶ The retained activity of celite-adsorbed naringinase was found to be 83% under optimum immobilization conditions. Reactions were run using 0.52 mM naringin solution containing 0.1 mg mL⁻¹ soluble or immobilized enzyme. The values of $K_{m,app}$ and $V_{max,app}$ obtained were 1.22 mM and 0.45 μ mol min⁻¹ mg⁻¹ enzyme for free enzyme and 2.16 mM and 0.3 μ mol min⁻¹ mg⁻¹ enzyme for immobilized enzyme, respectively. The activity of immobilized enzyme was evaluated in consecutive batch reactions (pH 3.5, 60 °C); a rapid drop in retained activity to 60% initial activity after 3 recycles was observed due to desorption of the enzyme from the support.

α -L-Rhamnosidase from *Aspergillus terreus* was covalently immobilized on ferromagnetic supports prepared from polyethylene terephthalate (Dacron-hydrazide), polysiloxane-polyvinyl alcohol (POS-PVA), and chitosan.¹⁸⁷ Use of a magnetic support has the potential to facilitate separation of the catalyst from other insoluble components present during debittering of grapefruit and other citrus juices. The supports were magnetized by thermal coprecipitation using ferric and ferrous chlorides, and immobilization was carried out using glutaraldehyde. *p*-Nitrophenyl α -L-rhamnoside or naringin were employed as enzyme substrate. The activity of the Dacron-hydrazide and POS-PVA immobilized enzyme was significantly higher than that of the chitosan derivative. The activity-pH and activity-temperature profiles for all immobilized enzymes were not significantly different from

that of the free enzyme. The Dacron-hydrazide immobilized enzyme was stable in the temperature range of 40–70 °C, and the POS-PVA and chitosan derivatives were stable up to 60 °C. The Dacron-hydrazide immobilized enzyme was preferred to the other two supports, demonstrating the best performance with catalyst recycle for naringin hydrolysis.

Additional supports for immobilization of naringinase include: copolymers of styrene and maleic anhydride,¹⁸⁸ tannin-aminoethyl cellulose,¹⁸⁹ bagasse,¹⁹⁰ chitin (covalent attachment using glutaraldehyde and sodium borohydride),^{191,192} controlled pore glass,¹⁹³ glycophasic-coated controlled pore glass,¹⁹⁴ κ -carrageenan¹⁹⁵ and silicate,¹⁹⁶ for fruit and vegetable juices debittering. The use of hollow fiber reactors for hydrolysis of naringin in unclarified grapefruit juice has also been evaluated.^{197–199}

4.2.2 Enhancing aroma in wine. Volatile components responsible for the aroma of wines, such as linalool, geraniol, nerol, citronellol and α -terpeniol, are present in the grape skin as odorless diglycosides of terpenes, for example, α -L-arabinofuranosyl- β -D-glucopyranosides and α -L-rhamnopyranosyl- β -D-glucopyranosides, which upon hydrolysis release volatile terpeniol. Spagna *et al.* have shown that purified α -L-rhamnosidase from *A. niger* increases the aroma of a model wine solution containing aromatic precursors extracted from Muscato grapes.²⁰⁰ Caldini *et al.* have conducted kinetic and immobilization studies of fungal glucosidases for aroma enhancement in wine.²⁰¹ The enzyme preparation of *A. niger* contained β -glucosidase, α -arabinosidase, and α -rhamnosidase activities in a ratio considered suitable for aroma enhancement in wine making. The three activities were immobilized to a silanized bentonite solid carrier with glutaraldehyde with the aim of developing a continuous process for wine aroma enhancement.

4.3 Interesterification of food fats and oils

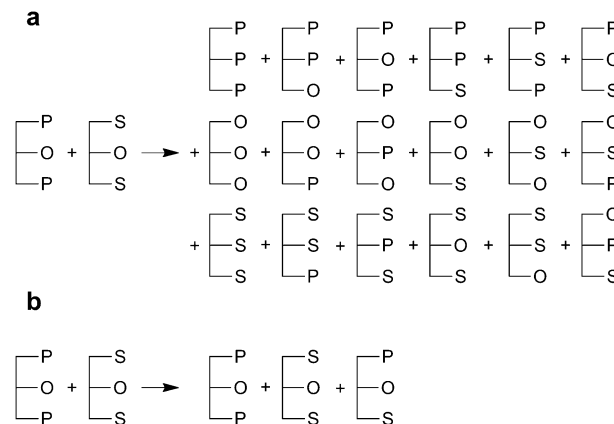
The physical properties of fats and oils used by food manufacturers is generally specific to their source, where fats and oils from each source may have a different distribution of fatty acids in their triacylglycerols, and where the acyl groups of the triacylglycerols are distributed in a non-random pattern.²⁰² Fractionation, hydrogenation and chemical interesterification (CIE) have each been employed for modification of the melting and crystallization properties of food oils and fats, particularly for the production of margarine and baking fat,²⁰³ where mixing of two or more fats is often insufficient to produce the right melting and/or crystallization properties. The use of partial hydrogenation of fats can produce oils and fats with the desired melting properties, but it can also result in the production of high *trans* fat levels, which substantially increases the risk of coronary heart disease.²⁰⁴ Enzymatic interesterification (EIE) of food oils and fats allows for improved control of final product composition when compared to CIE,^{205–209} and EIE is now practiced commercially as a means to produce oils and fats with desired physical properties but with elimination or reduction in production of *trans* fats.²¹⁰ There is also an opportunity to utilize EIE to markedly increase overall production of fats and oils intended for food use by upgrading alternative sources of fats and oils that are currently

not used in food production due to their chemical and/or physical properties.

EIE of long chain triacylglycerols (triglycerides) is usually performed using a lipase as catalyst. In water, lipases catalyze the hydrolysis of carboxylic acid ester bonds present in triglycerides, but under reaction conditions where water activity is low, lipase can catalyze esterification of carboxylic acids by alcohols, or the transesterification of carboxylic acid esters. For the interesterification of triglyceride mixtures, the type of lipase utilized will determine the degree of randomization of acyl groups among the triglycerides present.²¹¹ When using a non-specific lipase (e.g., *Candida cylindracea*, *Corynebacterium acnes*, *Candida antarctica* or *Staphylococcus aureus*) acyl groups at the *sn*-1, *sn*-2 and *sn*-3 positions of the triglyceride are exchanged, and the product distribution is similar to what is produced by CIE. Lipases that are *sn*-1,3 specific (*Mucor miehei*, *Rhizopus arrhizus*, *Aspergillus niger*, and *Thermomyces lanuginosus* (formerly *Humicola lanuginosa*)) typically do not exchange acyl groups at the *sn*-2 position due to steric hindrance, although intramolecular transesterification of diacylglycerol intermediates can occur over extended reaction times, leading to exchange at the *sn*-2 position as well; the product distribution of interesterified fats is therefore more limited than that produced by CIE (Scheme 5).

4.3.1 Initial commercial development of EIE. EIE was first evaluated for the production of a cocoa butter equivalent (CBE) that employed the *sn*-1,3 specificity of a variety of fungal lipases. Unilever immobilized a variety of lipases by adsorption onto diatomaceous earth and used the resulting biocatalyst to convert a mixture of palm mid fraction and stearic acid into a CBE-like product containing an increased level of the desired triglycerides, 1(3)-palmitoyl-3(1)-stearoyl-2-monooleine and 1,3-distearoyl-2-monooleine.^{211–214} A mixture of palm mid fraction (1 part) and myristic acid (0.4 parts) dissolved in 100–120 °C petroleum ether (3.2 parts) was saturated with water and then continuously pumped at a flow of 22 mL h^{−1} through a bed of hydrated catalyst (5.0 g) prepared from *Rhizopus niveus* lipase and diatomaceous earth (kieselguhr) at 40 °C.²¹¹ Complete interesterification was obtained over 400 h of continuous reactor operation; during steady-state operation, water present in the feed to the reactor led to the generation of a small quantity of diacylglycerol and free fatty acids. Water is required for enzyme-catalyzed interesterification because interesterification is initiated by a partial hydrolysis of enzyme-bound triglyceride to free diglyceride and an acyl-enzyme intermediate;²¹⁵ subsequent to this first step, another free diglyceride reacts with the acyl-enzyme intermediate, leading to ester exchange.²¹⁶ Although some water is necessary for the enzyme to be active and effective for interesterification, and excess of water can result in triglyceride hydrolysis rather than interesterification, and also accelerate the isomerization of diacyl glycerides, where the acyl group originally present at the *sn*-2 position can migrate to the *sn*-1 or *sn*-3 position.

The Fuji Oil Company developed a EIE process using a 1,3-specific lipase from *Rhizopus niveus* adsorbed on diatomaceous earth to catalyze the transesterification between 1,3-dipalmitoyl-2-olein (from palm oil mid fraction) and ethyl stearate to give a cocoa



Scheme 5 (a) Triacylglycerol products from the transesterification of two triacylglycerols, 1,3-dipalmitoyl-2-oleoyl glycerol and 1,3-distearoyl-2-oleoyl glycerol, using either a non-specific lipase or chemical esterification. (b) Transesterification products of 1,3-dipalmitoyl-2-oleoyl glycerol and 1,3-distearoyl-2-oleoyl glycerol, using a 1,3-specific lipase (adapted from ref. 205).

butter substitute that was suitable for chocolate manufacture; the transesterification activity of the biocatalyst and the diglyceride content of the product were each proportional to the water content of the reaction mixture.^{217–219} This immobilized enzyme catalyst also required the addition of water to the immobilized enzyme or to the reaction mixture to obtain high enzyme activity, and this water was gradually lost as the triglyceride was converted into diacyl and monoacyl glycerol *via* hydrolysis. The continuous addition of water was required to maintain enzyme activity.

Subsequent to this early work, continuous enzymatic interesterification processes were developed for the production of margarine and shortening hardstocks, where EIE produces fully-randomized products with physicochemical properties that are almost identical to the products obtained by CIE,²²⁰ and where EIE-produced fats generally contain less partial glycerides and have a higher natural tocopherol content and less color than chemically-interesterified fats. Christensen *et al.* described the use of a combination of silicate support and an organic binder to produce a granulated enzyme particle.²²¹ The lipase used was derived from *Thermomyces lanuginosus* (TLL), where the lipase was expressed and secreted by an *Aspergillus* sp. production host. The lipase was distributed throughout the porous particles having a typical particle size of 300–1000 μm, with a mean of 500–600 μm. The particle size distribution and porosity enabled the use of this biocatalyst in a plug flow, packed bed continuous reactor. The enzyme particle also binds the required amount of water needed to maintain enzyme activity in a continuous process, obviating the need to add water to the reaction mixture, and reducing or eliminating the production of mono- and diacyl glycerols. The commercialization of this process was enabled by the production of a robust enzyme particle (Novozymes Lipozyme[®] TL IM) containing a cost-effective lipase.^{207,209,222}

Archer Daniels Midland commercialized EIE-based production of *trans*-free margarines and shortenings using Lipozyme[®] TL IM in July 2002.^{207,209} To address the gradual loss of enzyme

activity in a single fixed bed reactor when operated continuously for commercial production, several reactors were operated in series. This mode of operation did not require complete conversion using a single reactor; as the first reactor in a series was operated down to effectively zero activity, the immobilized enzyme in the first reactor was replaced without disrupting production, and the recharged reactor was then operated as the last reactor in the series (Fig. 10). The U.S. Environmental Protection Agency awarded ADM a Presidential Green Chemistry Award In 2005 for its use of EIE for manufacture of NovaLipid™ interesterified oils.

In 2009, Alimentos Polar Comercial C.A., in Venezuela, reported the start-up of its new De Smet Ballestra Interzym Interesterification plant, with a capacity of 80 tpd, and in 2011, Industrias ALES in Manta, Ecuador announced its plans for construction of a 40 tpd Enzymatic Interesterification plant, also to be built by De Smet Ballestra. The De Smet Interzym process utilizes Lipozyme® TL IM as an alternative to both chemical interesterification and hydrogenation. A pre-treated blend of fats is pumped through a number of packed bed reactors (usually three or four) placed in series and kept at a temperature of around 70 °C; typical flow rate is 1–2 kg oil per kg enzyme per hour.²²⁰ Using freshly-deodorized oil blends as feed, 2.5–4.0 tonnes of oil were interesterified with 1 kg of immobilized enzyme, where the enzyme remains active for 2500–4000 h. The enzymatically interesterified oil does not need bleaching, only mild deodorization to remove some free fatty acid (FFA) and off-flavors. Based on the reported enzyme productivity and 2008 enzyme pricing, the enzyme cost was approximately 25 US dollars per ton of oil, therefore, if an

amount of sodium methylate catalyst equal to 0.1% was used in a chemical interesterification process, the total costs of both interesterification processes were about equal.²²⁰

IOI-Loders Croklaan utilizes EIE for commercial production of Betapol®, a vegetable fat blend that has been specially developed for infant formulas (Akoh 2002);²²³ it also produces a variety of fats for margarine and baked goods under the Crokvitol™ product name, using enzymatic interesterification at facilities in Rotterdam, Netherlands, with production capacity of 100 000 tons per year.^{224,225} In addition to requiring less process steps than CIE, production using enzymatic interesterification was reported to save 23 kg CO₂ per ton of end product when compared to the chemically-catalyzed process.

4.3.2 Further development of EIE. Palm stearin (PS) and flaxseed oil (FSO) were used to modify the physicochemical and nutritional properties of anhydrous butterfat (ABF), where either an immobilised non-specific lipase (Novozym SP435, *Candida antarctica* lipase B) or an immobilised 1,3-specific lipase (Lipozyme® RM IM, *Rhizomucor miehei* lipase) was employed as catalyst.²²⁶ The reaction was carried out for 24 h at 60 °C in shake flasks, and various proportions of ABF, PS and FSO were enzymatically interesterified to produce functional spreads that included α -linolenic acid as an omega-3 fatty acid. After 24 h interesterification, decreased saturated fatty acid (SFA) contents in low-*trans* spreadable fats (LTSFs) were observed, with ranges from 67% to 41%. The *sn*-2 positional fatty acid composition of selected LTSFs produced using non-specific and *sn*-1,3 specific lipases was compared, and no difference associated with positional substrate specificity was

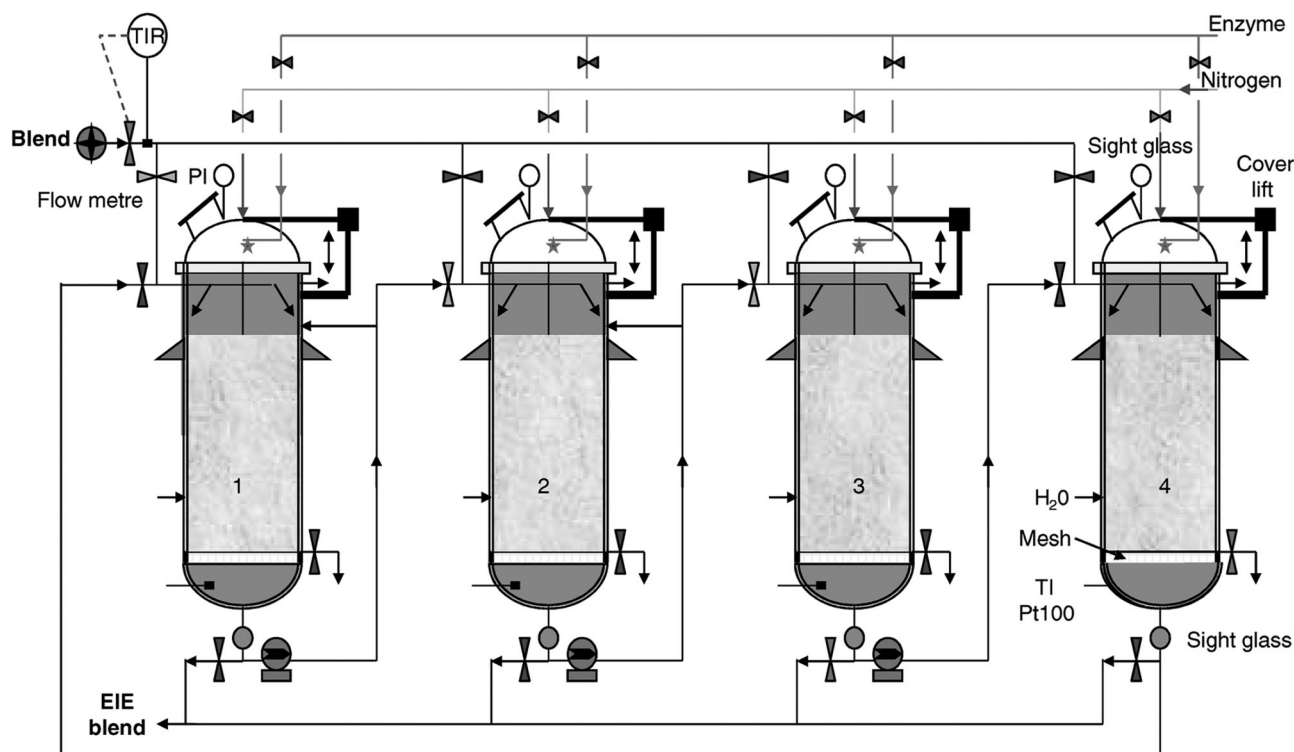


Fig. 10 Layout of reactors for EIE (reprinted from ref. 222 with permission from John Wiley & Sons).

observed. The *trans* fatty acids in the LTSFs was not more than 2%. EIE of these same substrates was also evaluated at 60 °C in a continuous packed bed reactor containing Lipozyme[®] RM IM, where the effect of substrate flow rate (contact time) on the degree of interesterification was monitored by analyzing the melting and crystallization behavior of the interesterified product, as well as its TAG composition.²²⁷

Enzymatic interesterification of palm stearin with coconut oil was conducted in batch reactions using a combination of two lipases (Ibrahim 2008).²²⁸ Palm stearin (PS), which has a relatively simple fatty acid composition (combined oleic and palmitic >85%), and coconut oil (CO), which enriches the composition with medium-chain fatty acids, was employed as the typical substrate system for margarine fat studies. 1,3-Specific lipases from different sources, existing in either free (*Pseudomonas fluorescens* lipase) or immobilized form (lipases from *Thermomyces lanuginosa*, *Rhizomucor miehei* and *Candida antarctica* B), were employed as biocatalysts for enzymatic interesterification. The combination of Lipozyme[®] TL IM and RM IM was found to generate a positive synergistic action at all test mixing ratios when compared to use of either lipase alone. Only equivalent amounts of Lipozyme[®] TL IM with Novozym[®] 435, or Lipozyme[®] RM IM with Novozym[®] 435, produced a significant synergistic effect as well as an enhanced degree of interesterification. A dual enzyme system, consisting of immobilized lipases and a non-immobilized one (Lipase AK), in most cases enhanced the activity of the free lipase. In contrast to the synergistic effects observed for combinations of lipases in batch reactions, no apparently synergistic or antergic effects were observed when performing these same reactions in a packed bed reactor, reportedly due to an ameliorative mass transfer by convection in the batch reaction that is lacking in a continuous operation.

Rønne *et al.* evaluated the lipase-catalyzed interesterification of butterfat blended with rapeseed oil (70/30, wt/wt) both in batch and in continuous reactions, with the objective of developing a process to modify butterfat through enzymatic interesterification with rapeseed oil.²²⁹ Rapeseed oil has been widely used for the manufacture of butter as a blend to increase the nutritional values of butter, as well as to improve spreadability. The oil contains a large amount of oleic acid as well as a mixture of linoleic (ω6) and linolenic (ω3) acids. Compared to simple blending, the interesterified product was expected to have an extended shelf life. Six commercial immobilized lipases (Table 8) were screened in batch reactions, and Lipozyme[®] TL

IM and Lipozyme[®] RM IM were chosen for further studies in a continuous packed bed reactor. TL IM produced the fastest rates of reaction, where equilibrium with a residence time of *ca.* 30 min, whereas RM IM required 60 min (Fig. 11). The effect of reaction temperature was more pronounced for RM IM; there was no significant effect on degree of interesterification when the temperature was increased from 60 °C to 90 °C when using TL IM, whereas RM IM was positively affected when the temperature was increased from 40 °C to 80 °C (Fig. 12). During prolonged continuous use, the activity of TL IM remained constant for the first five days, after which activity decreased over the next ten days to 40% of initial activity. The release of short-chain fatty acids from butterfat during EIE resulted in an unacceptable sensory quality of the initial product, but short-chain FFAs would normally be removed by deodorization in a subsequent process step.

The structure of human milk triacylglycerol is unique, where 60–70% of palmitic acid (16:0) is located at the *sn*-2 position, and stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) are preferentially esterified in the *sn*-1/3 positions.²³⁰ Pancreatic lipase selectively cleaves the fatty acids in the *sn*-1 and *sn*-3 positions, therefore palmitin is mainly present as efficiently-absorbed 2-monopalmitin. Free palmitic acid forms poorly-absorbed calcium soaps in the intestine, resulting in reduced absorption of both calcium and fat, therefore the position of C16:0 on the triglyceride is of considerable significance for the absorption of fat and minerals in infants. Using a combination of EIE (employing Lipozyme[®] RM IM), fractionation and batch deodorization, a mixture of butterfat, soybean oil and rapeseed oil was used to produce human milk fat substitute (HMFS) with a molecular structure and fatty acid composition that was very similar to that of human milk fat.²³¹ The oxidative stability of the HMFS oil was lower than that of the reference oil with the same fatty acid composition, but oxidation did not lead to a severe increase in rancidity during storage; off-flavors such as burnt and bitter were detected, and further optimization of the deodorization process was required to remove these off-flavors.

Teichert and Akoh have reported the enzymatic interesterification of stearidonic acid (SDA, C18:4*n* – 3)-enriched soybean oil and tripalmitin to produce structured lipids (SLs) enriched with palmitic acid (PA) at the *sn*-2 position of the triacylglycerol,^{232,233} where the resulting SLs may also be useful as human milk fat analogues for infant formula. SDA soybean oil contains approximately 20% stearidonic, 24% linoleic, and

Table 8 Immobilized commercial lipases and their characteristics (reprinted from ref. 229 with permission from the American Chemical Society)

Brand	Lipase species	Carrier	Specificity	Water content (wt%)	Porosity
Lipozyme TL IM	<i>Thermomyces lanuginosus</i>	Silica granules	<i>sn</i> -1,3-Specific	6.0	0.77
TL-lab-immobilized	<i>Thermomyces lanuginosus</i>	Accurel EP 100	<i>sn</i> -1,3-Specific	5.8	
Lipozyme RM IM	<i>Rhizomucor miehei</i>	Macroporous resin	<i>sn</i> -1,3-Specific	3.2	0.45
Novozym 435	<i>Candida antarctica</i> lipase B	Macroporous polymer based on methyl and butyl methacrylic esters	Non-specific	4.1	0.65
Lipase PS-C-I	<i>Burkholderia cepacia</i>	Ceramic particles	Non-specific ^a	4.3	
Lipase PS-D-I	<i>Burkholderia cepacia</i>	Diatomaceous earth	Non-specific ^a	3.7	

^a No clear claim.

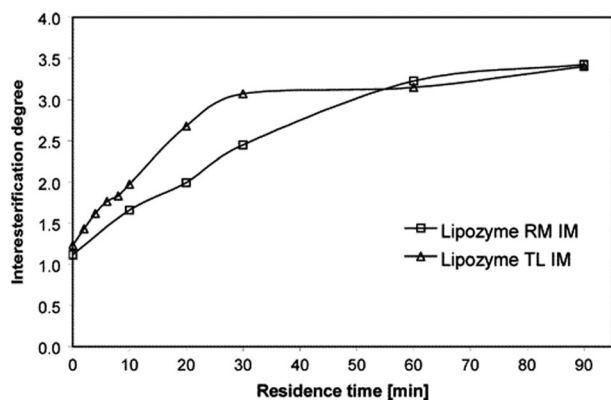


Fig. 11 Effect of residence time on the interesterification degree in a continuous reaction. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzymes, Lipozyme TL IM and Lipozyme RM IM; enzyme amount, approximately 15 g in both cases; reaction temperature, 60 °C; room temperature, 60 °C (reprinted from ref. 229 with permission from the American Chemical Society).

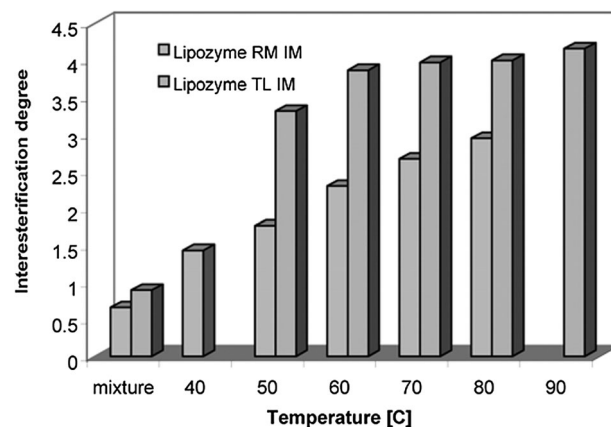


Fig. 12 Effect of temperature on the interesterification degree in a continuous reaction. Experimental conditions: packed bed reactor (200 mm × 1.5 mm); substrate, butterfat and rapeseed oil (70/30, w/w); enzymes, Lipozyme TL IM and Lipozyme RM IM; enzyme amount, approximately 15 g in both cases; flow rate, 0.9 mL min⁻¹; residence time, 30 min; room temperature, 60 °C (reprinted from ref. 229 with permission from the American Chemical Society).

12% palmitic acids, whereas PA is the second major fatty acid found in breast milk at approximately 18–26%.^{234–237} Using interesterification with either Novozym[®] 435 or Lipozyme[®] TL IM, a SL was produced consisting of over 60% PA at the *sn*-2 position, and containing over 6% of total SDA. Either enzyme could be used to produce a SL suitable for a human milk fat analogue that had the potential to deliver absorption characteristics and FA content similar to human milk fat, with health benefits associated with omega-3 fatty acids. Human milk fat substitute has also been produced by enzymatic interesterification of a vegetable oil blend comprising palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil, formulated in the mass ratio of 4.0:3.5:1.0:1.5:0.2, catalyzed by lipase from *Thermomyces lanuginosa* (Lipozyme[®] TL IM), where the reaction product contained a similar triacylglycerol (TAG) structure to that of human milk fat (HMF).²³⁸ The predominant TAGs of the reaction product contained mainly a 1,3-diunsaturated-2-saturated structure, like HMF.

Lipozyme[®] TL IM was used for enzymatic interesterification of anhydrous milkfat (AMF) with linseed oil (LO) in binary blends and with rapeseed oil (RO) in one ternary blend.²³⁹ The decrease in solid fat content and in dropping point temperature obtained with increasing content of LO and interesterification in binary blends resulted in good plastic properties for the products originating from the blends 70/30 and 60/40. A ternary blend composed of AMF/RO/LO 70/20/10 gave satisfactory rheological and oxidative properties, fulfilling the requirements for a marketable spread. The oxidative stabilities of the interesterified blends and blends with 50 ppm of α -tocopherol added as antioxidant were evaluated.²⁴⁰ Peroxides appeared to be the only significant oxidation products after 12 weeks of storage at 4 °C. Interesterification led to variable effects on the oxidation of fat mixtures, depending on composition and temperature. The enrichment of AMF by enzymatic interesterification with selected vegetable oils comprising polyunsaturated fatty acid for production of structured lipids was demonstrated, although the oxidative state of raw oils and the storage conditions

both impact storage stability; the addition of *R*-tocopherol did not enhance storage stability at low temperature.

Milk fat and canola oil blends were enzymatically interesterified using the *Rhizopus oryzae* lipase (L036P, Biocatalysts (Cardiff, UK))²⁴¹ or *Aspergillus niger* (Lipase A, Amano)²⁴² immobilized in polysiloxane–polyvinyl alcohol (SiO₂–PVA), to assess the effect of different proportions of milk fat mass and different temperatures on the composition as well as texture properties of the obtained product. Milk fat was obtained from commercial butter after melting at 50–60 °C followed by centrifugation to separate the aqueous phase. Milk fat was also intramolecularly interesterified using the *sn*-1,3 specific lipase *Rhizomucor miehei* (Lipozyme[®] RM IM), resulting in a decrease in the content of triacylglycerol (TAG) groups from TAG C34 to TAG C42 in modified fat, with a simultaneous increase of long-chain TAG C44 to TAG C52 triacylglycerols content.²⁴³

Caprine (goat) milk has been recommended as a potential alternative to human milk for infant nutrition, as it is less allergenic and more digestible when compared to bovine milk; a significant quantity of caprine milk is reported to be consumed by infants and patient suffering from allergies to cow's milk.^{244,245} Enzymatically-modified vegetable oil blends have been incorporated into skim caprine milk to produce goat milk-based infant formula analogs, homologous to human milk.²⁴⁶ A modified lipid containing palmitic, oleic, and linoleic acids, resembling the composition of human milk fat, was synthesized by enzymic interesterification of tripalmitin and a vegetable oil blend containing a 2.5:1.1:0.8 ratio of coconut, safflower, and soybean oils. *Rhizomucor miehei* lipase (Lipozyme[®] RM IM) was used as the biocatalyst. The highest incorporation of palmitic acid was obtained at 12 h of incubation at 55 °C with a substrate molar ratio of 1:0.4 tripalmitin to vegetable oil blend, whereas interesterification for 12 h at a 1:1 molar ratio produced a product that had a greater resemblance to the fatty acid composition of human milk.

Because of its high melting point and low level of unsaturated fatty acids (C18:2, C18:3), beef tallow is not used directly as a food ingredient, but is used as frying fats or shortenings. For food applications, it is typically fractionated and/or modified by interesterification with edible oils. Beef tallow was interesterified with sunflower oil using immobilized lipase from *Rhizomucor miehei*,²⁴⁷ where the emulsions prepared on the basis of fats which were formed during interesterification in the presence of the catalyst Lipozyme[®] IM (where the amount of water was 10 and 15%) showed high stability. In related work, beef tallow stearin was blended at various proportions with rapeseed oil and the blends interesterified using sodium methoxide or immobilized lipases from *Rhizomucor miehei* (Lipozyme[®] IM) and *Candida antarctica* (Novozym[®] 435). The total fatty acid composition of fats before and after interesterifications remained unchanged, but their distributions were random after chemical interesterification and close to random when Novozym[®] 435 was used. When Lipozyme[®] IM was used the fatty acid composition at the *sn*-2 position remained practically unchanged compared with the starting blend.^{248–250}

Kokum fat is extracted from Kokum nuts (*Garcinia indica*), and is rich in stearic acid and low in palmitic acid,²⁵¹ making it less atherogenic than fats with higher palmitic or other saturated fatty acid contents. Mahua oil is non-edible and is isolated from the seeds of the Mahua tree (*Madhuca indica*).²⁵² Enzymatic interesterification using Lipozyme[®] TL IM was found to be effective in modifying the thermal behavior and solid fat content (SFC) of a mahua oil and kokum fat (1:1) blend.²⁵³ The melting profile of the blend subjected to EIE for 1 h resembled that of commercial milk fat and one interesterified for 6 h showed a wider melting range, similar to that of hydrogenated fats used for culinary and bakery purposes.

A blend of palm stearin and soybean oil, (70/30, wt%) targeted for the production of hard stock used in “low *trans*” stick margarine or puff pastry margarine formulations was employed in a comparison of EIE and CIE.²⁵⁴ Solid fat content profile, TAG distribution and corresponding calculated degree of interesterification were used to monitor the conversion during the batch-wise enzymatic reaction with Lipozyme[®] TL IM. EIE was evaluated in both batch and continuous enzymatic. A chemically interesterified blend was chosen as reference for comparison of melting properties (solid fat content profile, melting point) and product quality (partial acylglycerol content, residual acidity, color and tocopherol content) of the interesterified products, before and after final deodorization. Better oil quality, mainly in terms of acidity, free tocopherol and partial acylglycerol content, was obtained after EIE.

The lipase-catalyzed intramolecular interesterification of refined, bleached, deodorized palm olein was studied in a continuous packed-bed reactor operating at 65 °C with Lipozyme[®] TL IM (Huey 2009).²⁵⁵ Initially, the palm olein consisted of 53.7% of diunsaturated TAG (mainly POO; P = palmitic acid and O = oleic acid) and 30.9% of di-saturated TAG (mainly POP) with a minor content of tri-unsaturated TAG (8.9%) and tri-saturated TAG (0.1%). After interesterification, the total tri-saturated and tri-unsaturated TAG groups were increased while the total di-unsaturated and di-saturated TAG groups were decreased.

EIE for the production of margarine fats has utilized a variety of different oils and fats. The enzymatic interesterification of palm stearin with *Cinnamomum camphora* seed oil to produce zero-*trans* medium-chain triacylglycerols-enriched fat used Lipozyme[®] TL IM.²⁵⁶ Production of a cocoa butter substitute by dry fractionation, partial hydrogenation, chemical and enzymatic interesterification of tea seed oil was evaluated using Lipozyme[®] TL IM.²⁵⁷ The immobilized *sn*-1,3-specific lipase from *Rhizomucor miehei* (Lipozyme[®] RM IM) was used to catalyze the interesterification of palm mid fraction (PMF), palm kernel stearin (PKS) and medium chain triacylglycerols (MCT) under controlled reaction parameters such as enzyme load, time course and temperature to produce a low calorie cocoa butter substitute.²⁵⁸ Lard is the only animal fat that has a similar structure to human milk fat. Structured lipids produced *via* EIE of lard and soybean oil blends catalyzed by Lipozyme[®] TL IM produced interesterified blends of lard and soybean oil having properties and chemical compositions similar to human milk fat.^{259,260}

trans-free interesterified fat has been produced by interesterification of a mixture of rice bran oil, palm stearin and coconut oil in a Lipozyme[®] TL IM-catalyzed reaction.²⁶¹ Coconut oil has relatively high medium-chain triacylglycerol (MCT) content, mainly saturated fatty acids with chain lengths from 6 to 12 carbon atoms. MCTs are hydrolyzed faster and more completely than long-chain triacylglycerols (LCTs), the hydrolysis products of MCTs are absorbed faster than those of LCTs, and MCTs are very stable to oxidation because of their saturation. The interesterified fats exhibited desirable physical properties and suitable crystal form (β' polymorph) for possible use as a spreadable margarine stock.

Two isomers of conjugated linoleic acid (CLA), *cis*9, *trans*11, and *trans*10, *cis*12, have been reported to inhibit tumor growth, reduce atherosclerotic risk and reduce body fat.^{262–264} The major natural sources of CLAs are fat tissues of ruminants (meat and dairy products); vegetable oils contain only small amounts of CLA, and consumption of dietary CLA has decreased over time as milk and animal fats have been replaced by vegetable oils. EIE (with immobilized non-specific *Candida antarctica* lipase) was employed using CLA structured lipids (CLA SL, as soft stock) and palm stearin (PS, as hard stock) in different proportions, to determine the best blend as the fat phase for possible production of a margarine enriched in CLA;²⁶⁵ only the interesterified blend of PS/SL 30:70 was suitable for the formulation of margarines.

High-oleic sunflower oil (HOSO) is high in monounsaturated fatty acids, and consumption of oleic acid has been shown to have beneficial effects on human health. Fully hydrogenated canola oil (FHCO) is a hardstock that is free of *trans* fatty acids and is high in stearic acid: it can be used to impart a solid consistency in blends with vegetable oils. The effects of chemical and enzymatic interesterification on the composition and physical properties of FHCO and HOSO blends was evaluated, using immobilized non-specific *Candida antarctica* lipase in EIE.^{266,267} Both enzymatic and chemical interesterification of FHCO and HOSO blends changed the physical properties of the blends to more closely

resemble those of puff pastry, stick, and general-purpose margarines and shortenings, in particular those containing 20–40% hardstock.

Semi-solid fats were prepared *via* enzymatic interesterification of extra-virgin olive oil (EVOO) and fully hydrogenated palm oil (FHPO) in batch reactors using Novozym[®] 435, Lipozyme[®] TL IM or Lipozyme[®] RM IM as biocatalyst, varying the initial weight ratio of EVOO to FHPO from 80:20 to 20:80.^{268,269} Fats prepared using large proportions of FHPO contained high levels of TAG groups containing only saturated fatty acid residues, and high levels of TAG groups containing both saturated and unsaturated fatty acid residues were found in fat products obtained with the lowest proportions of FHPO. Independently of the initial wt ratio of EVOO to FHPO, the interesterified products were characterized by a higher molar ratio of unsaturated to saturated fatty acid residues at the *sn*-2 position, were softer over a wide temperature range, exhibited lower oxidative stabilities and were completely melted at lower temperatures than the corresponding physical blends. The interesterified products may be useful for formulating margarines, frying fats, *etc.*

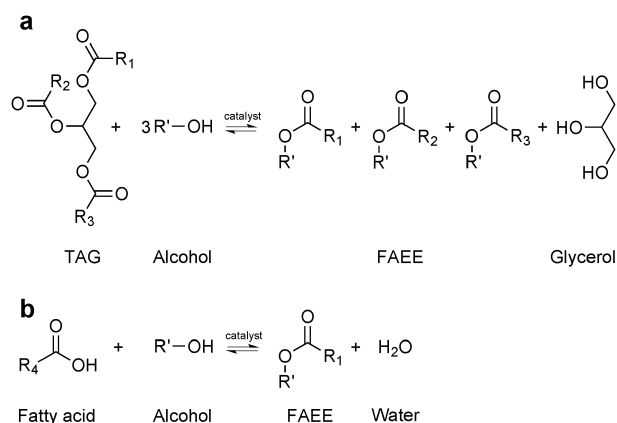
4.4 Biodiesel

Biodiesel is produced by the chemical or lipase-catalyzed esterification of fatty acids or transesterification of oils and fats with short-chain alcohols to produce fatty acid alkyl esters (FAAEs) (Scheme 6); methanol is most commonly used to produce fatty acid methyl esters (FAMES). The oils and fats, and their corresponding fatty acids, that are used to produce biodiesel are obtained from a variety of animals, plants and microorganisms: plant oils such as soybean oil, jatropha oil, palm oil, cottonseed oil and sunflower oil; microbial sources of oils from micro-algae or oleaginous yeasts; animal fats such as tallow, lard and grease; waste cooking oil and industrial waste oil.²⁷⁰ The chemical process for biodiesel production typically uses sodium methoxide to convert plant oil-based triglycerides to FAME, where the resulting glycerol is contaminated with high concentrations of alkali salts and is difficult and costly to purify. Lipases can be used to catalyze the production of FAAEs under milder conditions and with production of less alkali salts than the chemical process. A major economic hurdle for use of the enzymatic process is the need to recover and recycle the enzyme catalyst; immobilization of the enzyme on a solid support often stabilizes the biocatalyst activity and allows for facile catalyst recovery and recycle. Several recent reviews have summarized the latest methods employed for stabilization of enzyme activity *via* immobilization, where the immobilized enzyme is employed for production of biodiesel.^{269,271–277}

When producing FAME using immobilized *Candida antarctica* lipase B, a significant decrease in enzyme activity has been observed when stoichiometric amount of methanol was added at the start of the reaction.²⁷⁸ One solution to this problem was to add methanol at a 1:1 molar ratio to triglyceride in the first stage of transesterification, then two additional equivalents of methanol sequentially when the previous equivalent of methanol was converted to ester; using sequential methanol addition and

immobilized *Candida antarctica* lipase as catalyst, a 98.4% conversion of vegetable oil to FAME was obtained.²⁷⁹ In a recent study by Maceiras *et al.*,²⁸⁰ transesterification reactions of waste frying oil were carried out in presence of two different acyl acceptors, methanol and 2-propanol. A decrease in the deactivation rate of immobilized lipase (Novozym[®] 435) was observed when 2-propanol was substituted for methanol as acyl-acceptor. The difference between methanol and 2-propanol on lipase deactivation was greater when using immobilized enzyme than when using unimmobilized *C. antarctica* lipase B, where no difference was observed when using the two acyl acceptors. The immobilization support used to prepare Novozym[®] 435 was reported to adsorb primary alcohols such as methanol more readily than secondary alcohols such as propanol, and when the alcohol is adsorbed to the immobilized enzyme, the entry of triglycerides is blocked and the enzyme deactivation is enhanced, causing the reaction to stop. One of the main causes of deactivation of the enzyme was due to the immiscibility between triglycerides and methanol.

Microbial lipases from *Thermomyces lanuginosus* (TLL) and *Pseudomonas fluorescens* (PFL) have been immobilized on a commercial matrix Toyopearl[®] AF-amino-650M (Tosoh Bioscience) by multipoint covalent attachment.²⁸¹ The immobilization support was functionalized with glycidol, epichlorohydrin and glutaraldehyde prior to covalently attaching lipase to the carrier surface, and the resulting biocatalysts evaluated using both aqueous (hydrolysis of olive oil emulsion) and non-aqueous (butyl butyrate synthesis) reaction conditions. Biodiesel synthesis was subsequently evaluated using TLL and PFL immobilized on glyoxyl-resin activated with glycidol and epichlorohydrin. Babassu and palm oils were employed as feedstocks using an ethanol to babassu oil molar ratio of 9:1, and ethanol to palm molar ratio of 18:1, and reactions utilized a catalyst loading of 2 mg of immobilized protein per gram of oil. TLL immobilized on glyoxyl-resin had the highest hydrolytic activity and thermal stability, between 27 and 31 times more stable than the corresponding soluble lipase, and showed the highest activity towards transesterification of vegetable oils. Immobilized



Scheme 6 Synthesis of FAAE: (a) Transesterification of TAG and short-chain alcohols leading to FAAE and glycerol; (b) esterification of fatty acid and short-chain alcohol leading to FAAE and water. R_{1–4} = acyl residues, R' = alcohol moiety (R' = CH₃ for methanol, R' = CH₂CH₃ for ethanol) (reproduced from ref. 272 with permission from Springer).

PFL catalysts were less active and thermally stable than the immobilized TLL catalysts.

Thermomyces lanuginosus lipase (TLL) has also been immobilized on an aldehyde-Lewatit[®] (Lew-TLL) carrier and evaluated for biodiesel production *via* the enzymatic transesterification of soybean oil with ethanol.²⁸² Lewatit[®] VP OC1600 (Bayer) consists of poly(methyl methacrylate-*co*-divinylbenzene) and has an average particle size of 315–1000 μm , surface area of 130 $\text{m}^2 \text{g}^{-1}$, and a pore diameter of 150 \AA ;²⁸³ it is used for the commercial production of Novozym[®] 435 (immobilized CALB).²⁸⁴ In this instance, the support was modified to obtain aldehyde groups on its surface, allowing for covalent attachment of TLL with a 90% recovery of lipase activity (200 U g^{-1}). Lew-TLL was 10-fold more thermally stable (60 $^{\circ}\text{C}$, pH 7) than the commercial TLL preparation, Lipozyme[®] TL-IM. Transesterification of ethanol and soybean oil was carried out with a 7.5:1 molar ratio of ethanol:soybean oil, 15% immobilized enzyme and 4% water at 30 $^{\circ}\text{C}$. In the presence of *n*-hexane, the transesterification reached 100% conversion, while in solvent-free system the yield was 75%. Transesterification was subsequently carried out using three stepwise additions of ethanol, producing 80% conversion while a two-step ethanolysis (2 molar equivalents added initially at 30 $^{\circ}\text{C}$ and 1 molar equivalent at 6 h) produced 100% conversion after 10 h of reaction in both solvent and solvent-free systems.

The influence of the support surface on the loading and enzymatic activity of immobilized *Pseudomonas fluorescens* lipase was compared for sunflower oil ethanolysis using different porous supports: polypropylene (Accurel[®]), polymethacrylate (Sepabeads EC-EP), silica (SBA-15 and surface modified SBA-15), and an organosilicate (MSE).²⁸⁵ The different functional groups occurring on the support surface allowed either physical (Accurel[®], MSE, and SBA-15) or chemical adsorption (Sepabeads EC-EP and SBA-15-R-CHO). A low enzyme surface coverage was used to produce catalysts where the effect of the surface functional groups only would be compared; effects due to pore size and surface area were negligible. The surface-modified SBA-15 (SBA-15-R-CHO) allowed the highest loading, whereas the lipase immobilized on the MSE was the most active biocatalyst.

Biodiesel has been produced by the enzymatic transesterification of palm oil with methanol, ethanol, 1-propanol, isopropanol, 1-butanol and isobutanol.²⁸⁶ *Candida antarctica* lipase B was immobilized on granular activated carbon (ACG-E) and activated carbon cloth (ACC-E) and used as a biocatalyst. In the conversion of palm oil to alkyl esters using granular activated carbon as a support, isobutanol gave the highest conversion of 100%, isopropanol 86%, 1-butanol 77%, 1-propanol 68% and ethanol 57%, while only 48% methyl ester was observed with methanol. With activated carbon cloth used to support the enzyme, isobutanol gave the highest conversion of 82%, isobutanol 72%, isopropanol 59%, 1-butanol 45% and propanol 40%, while only 28% methyl ester was observed with methanol. As has been observed with other immobilized CALB catalysts, the degree of deactivation was found to be inversely proportional to the number of carbon atoms in the linear lower alcohols.

Acyl transfer, as an alternative to enzyme-catalyzed methanolysis of triglycerides, has been reported to improve immobilized lipase stability.²⁸⁷ When using methyl acetate in place of methanol as the source of alkanol for production of biodiesel, triacetyl glycerol was produced as reaction byproduct in place of glycerol, and the triacetyl glycerol had no negative effect on the activity of the lipase. The feasibility of producing biodiesel from sunflower oil using immobilized *Candida antarctica* lipase (Novozym[®] 435) and methanol in a solvent-free system was evaluated using a response surface methodology and a 5-level/4-factor central composite design for a comprehensive study of parameters affecting biodiesel synthesis.²⁸⁸ Optimal reaction conditions (45 $^{\circ}\text{C}$, 3% enzyme based on oil weight, 3:1 methanol:oil molar ratio, no added water) resulted in >99% oil conversion to FAME over 50 h of reaction, but catalyst activity decreased markedly over the course of repeated runs. Alternative acyl acceptors (2-propanol, *n*-butanol and methyl acetate) were subsequently tested for biodiesel production with respect to their effects on the enzyme activity and stability. Methyl acetate produced a FAME yield of 96% and increased the half-life of the immobilized lipase by about 20-fold relative to methanol. The reaction was evaluated in both a batch stirred tank reactor and a packed bed reactor, where the kinetics in a packed bed reactor system were preferred; no loss in productivity was observed for up to 72 h of operation.

When water is present during enzymatic biodiesel production, the production of FAMES is accompanied by hydrolysis and esterification reactions that result in co-production of free fatty acids that result in a highly acidic product mixture. The use of a reaction medium with low water activity was evaluated, where Novozym[®] 435 was employed for conversion of waste frying oil to FAME using methanol as acyl acceptor, and 3 \AA molecular sieves to reduce water activity.²⁸⁹ The anhydrous conditions enabled the esterification of FFAs present in the feedstock during the initial phase of the reaction. Using this anhydrous medium, a decrease in both the acid value and the diglycerides content in the product was obtained, improving FAME yield. The enzyme activity could not be recovered after a single batch reaction, either by washing the immobilized enzyme with acetone or by washing with fresh waste frying oil. The use of the moderately-polar solvent *tert*-butanol as a co-solvent led to a stable catalysis using Novozym[®] 435 even after 17 successive cycles of FAME production under anhydrous conditions.

Phospholipids present in plant-derived oils can be problematic for phase separation of biodiesel from reaction mixtures containing significant concentrations of water, and removal of these phospholipids prior to transesterification by a degumming process can increase yield of recovered biodiesel.²⁹⁰ A two-step enzymatic processes (degumming and transesterification) was carried out for the production of biodiesel from crude canola oil (typically containing 100–300 ppm phospholipids), where degumming was performed using phospholipase A2. The initial phospholipid content was reduced to less than 5 ppm by enzymatic degumming. A combination of *Rhizopus oryzae* and *Candida rugosa* lipases immobilized on silica gel was utilized

for transesterification optimization experiments, and optimal reaction conditions utilized 24.4% (w/w) immobilized catalyst, 13.5% (w/w) buffer solution, and 15.8% (w/w) methanol based on oil mass. Conversion rate of degummed crude canola oil to FAME was determined to be 88.9% under optimal conditions.

The production of byproduct glycerol during plant oil transesterification can negatively impact reaction efficiency and productivity, where glycerol can adsorb onto enzyme-immobilization carriers and form a hydrophilic layer which makes the lipases inaccessible to hydrophobic substrates. Unreacted alcohol may preferentially diffuse into the glycerol layer covering the immobilized lipase, resulting in lipase inactivation by a locally high alcohol concentration.²⁹¹ Facile removal of glycerol can be achieved by dialysis,²⁹² extraction,²⁹³ and adding organic solvents,²⁹⁴ but these methods require capital investment for biodiesel purification. The use of an enzymatic packed-bed reactor (PBR) integrated with a glycerol-separating system has been evaluated for the solvent-free production of biodiesel fuel.²⁹⁵ Novozym[®] 435 was used as catalyst, and a mixture of rapeseed and soybean oils was used as substrate. When using a glycerol-separating tank, the outflowing liquid from the bottom of the PBR (consisting of fatty acid methyl ester, residual glycerides, and glycerol) were retained for a sufficient period of time to allow glycerol to accumulate at the bottom of the separation tank due to its higher density and hydrophilicity. For long-term operation of the PBR without significant loss of lipase activity, methanol concentration in the effluent was maintained at less than 2%; under this condition, glycerol was also removed successfully by the separation tank (99.7% of theoretical yield).

Biodiesel production by a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases immobilized on silica gel was evaluated using supercritical carbon dioxide as solvent;²⁹⁶ when the batch process was performed under optimal conditions, the biodiesel conversion yield was 99.13%. *Pseudomonas cepacia* lipase was immobilized onto electrospun polyacrylonitrile (PAN) nanofibrous membranes for biodiesel production from soybean oil;²⁹⁷ under optimal reaction conditions, the biodiesel conversion of soybean oil was 90%, and the immobilized *P. cepacia* lipase retained 91% of its initial activity after 10 recycles in batch reactions.

4.4.1 Biodiesel commercialization using immobilized enzymes. Tan *et al.*²⁷⁰ and Zhang *et al.*²⁷² have reported on two separate commercial processes for biodiesel production in China. Lvming Environmental Technology Co. Ltd. (Shanghai, China) started commercial production of biodiesel in 2007 using an enzymatic production line with a capacity of 10 000 tons, employing a process developed at the Beijing University of Chemical Technology that utilized a commercial immobilized lipase (LS-10A, *Candida* sp. 99–125) as catalyst. High acid value (AVN160 mg KOH g⁻¹) waste cooking oil was used as substrate, where the enzyme dosage was 0.4% relative to the weight of oil. The process was conducted in a stirred tank reactor, and a centrifuge was used to separate glycerol and water. Yields of FAMEs achieved 90% under optimal conditions. A second process for biodiesel using enzymatic catalysis was commercialized in 2006 by Hainabaichuan Co. Ltd., Hunan Province, with a capacity of 20 000 tons per year (enlarged to 40 000 tons per year in 2008); this process employed technology developed by

Tsinghua University, where Novozym[®] 435 was used as the catalyst, and waste palm oil, waste edible oil, or oil with high acid value as feedstock.

Piedmont Biofuels announced in February 2012 that they scaled up an enzymatic transesterification process in a commercial facility located in Pittsboro, North Carolina.²⁹⁸ The process used *Candida antarctica* Lipase B (CALB) from Novozymes to continuously esterify FFA in oil or biodiesel. The enzyme was immobilized on a support with a particle size of 0.3–0.7 mm. The enzyme and support remain in the reactor and enzyme did not leach into the feed stream. The process used continuous moisture removal to both increase the reaction rate and to achieve the lowest possible amount of FFAs. Biodiesel must contain less than 0.25% FFA (acid number less than 0.5), and using palm fatty acid distillate, conversion to less than 0.25% FFA was achieved in a batch process without caustic stripping. There was no limit to the amount of free fatty acids or moisture in the incoming feed, however, higher moisture resulted in a slower flow rate, therefore less than 1500 ppm moisture was recommended for the process feedstock.

Purolite (Bala Cynwyd, PA) and Transbiodiesel (Shfar-Am, Israel) announced in 2010 their intent to manufacture and market enzyme-loaded ion exchange resins for the simultaneous esterification of free fatty acids and transesterification of fats and oils. Transbiodiesel has previously implemented its technology at Zohar Dahlia, a detergent producer, which will make biodiesel and use the glycerin coproduct as a component in its liquid soaps.^{299,300} Sunho Biodiesel Corporation (Taipei, Taiwan) has also developed a commercial process for biodiesel using immobilized enzyme technology.³⁰¹

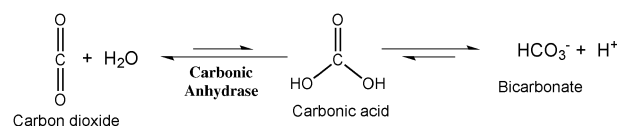
Whereas biodiesel is currently being produced commercially, although in volumes that are small relative to global annual demand for diesel fuel, and government mandates for biodiesel production are helping to drive commercialization, there are numerous issues that will ultimately determine the commercial viability of biodiesel production. The use of biomass-derived fatty acids as a feedstock replacement for plant-derived triglycerides can disconnect biodiesel production from the “food vs. fuel” debate, and leveraging what has been learned from the commercialization of enzymatic food oil interesterification may lower the cost for use of enzymes (soluble or immobilized) to a point where enzyme cost alone is not a significant contributor to the overall cost of manufacture of a diesel fuel. The additional issues of moderating oil prices, the sustainability of biofuel production, and the impact on water resources required for feedstock production will also need to be addressed to enable the commercial success of enzymatic biodiesel production.

4.5 Carbonic anhydrase

It is well documented that CO₂ levels in the atmosphere are increasing at a rapid rate due mostly to emissions caused by human activities. CO₂ is a major greenhouse gas that contributes to global warming. Of all the CO₂ emissions, flue gas from power plants constitutes a large share of the total CO₂ emitted. There are several ongoing studies to develop effective methods for capturing CO₂ from flue gas and either injecting it

Yu *et al.* reported the immobilization of bovine carbonic anhydrase II to mesoporous silica (surface area 533 m² g⁻¹) that was functionalized with carboxyl groups.³⁰⁷ In this system, the

Bovine carbonic anhydrase immobilized using alginate³¹⁴ was as efficient as free enzyme in CO₂ capture (calcite formation) and retained 67% activity through 6 cycles of operation. The immobilized enzyme also had better thermostability and storage stability compared to free enzyme.



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Surfactant modified silylated chitosan beads were shown to be very effective in adsorbing carbonic anhydrase from *Bacillus pumilus*.³¹⁵ In an optimized immobilized enzyme system, the activity and stability of immobilized enzyme at higher temperatures were better than free enzyme, whereas the catalytic efficiency of the immobilized enzyme, as measured by *p*-nitrophenyl acetate hydrolysis and the conversion of CO₂ to bicarbonate, was similar to that of free enzyme at low temperatures.

Another approach used to immobilize carbonic anhydrase is by displaying the enzyme on the surface of *E. coli*.³¹⁶ Carbonic anhydrase from *Helicobacter pylori* was expressed on the outer membrane of *E. coli* using a surface-anchoring system derived from ice nucleation protein (INP) from *Pseudomonas syringae*. This system was used to demonstrate better stability of the cell displayed enzyme *versus* free enzyme and showed the feasibility of CO₂ capture in a contained liquid membrane system.

Carbonic anhydrase in a liquid membrane is another way of immobilizing and reusing the enzyme. Such liquid membrane reactors have been designed by Cowan *et al.*³¹⁷ and Bao and Trachtenberg.³¹⁸ In this contained liquid membrane reactor, mutually orthogonal sets of feed and sweep fibers are immersed in a buffer containing carbonic anhydrase. Feed gas consisting of high concentrations of CO₂ enters the reactor in a set of feed fibers and CO₂ diffuses outwards through the fibers into the liquid membrane where it is converted by the enzyme into bicarbonate. Bicarbonate then diffuses into nearby sweep fibers where it reacts with carbonic anhydrase and releases CO₂ which diffuses inwards through the fibers and is swept away by vacuum or a carrier gas to keep a concentration gradient between the two fiber sets.

Carbonic anhydrase can be immobilized by a variety of methods including covalent coupling through chemical means or through the display on a cell surface and by adsorption/entrapment in a solid or gel matrix or in a liquid membrane. The results from these various immobilization techniques are very similar in that the immobilized enzyme generally has equivalent or lower activity compared to the free enzyme, but in all cases the enzymes have significantly better stability than the free enzyme. This is true regardless of the enzyme source (bovine, human, microorganisms). The selection of the immobilization matrix and method has a significant impact on the microenvironment of the enzyme. This microenvironment and the inherent stability of the enzyme contribute to the stability of the immobilized enzyme system. Therefore it is important to select a naturally occurring stable enzyme^{319,320} or a protein engineered enzyme with enhanced stability³²¹ for this process.

Other factors to consider during immobilization of carbonic anhydrase for CO₂ capture are mass transfer issue at the gas liquid interface that limits the substrate (CO₂) transport to the active site of the enzyme, and inhibition of the overall reaction by product due to the reverse reaction. Therefore, selection of the immobilization matrix, reactor design, and product removal are critical for successful development of this application. Studies are underway to address these issues.^{322,323} Even though there are no reported enzyme systems that can meet the stability, performance and cost requirements for the process of

CO₂ capture from flue gas, successful development of an enzyme system is expected in the near future.

Although power generation is the biggest market for CO₂ capture systems, financial incentives to deploy these systems are lacking unless government regulations restricting CO₂ emissions from power plants are mandated. However there are other potential markets for cost effective CO₂ capture system. Natural gas processing, industrial hydrogen production and fertilizer (urea) manufacturing are areas where CO₂ sequestration is needed. Coupling CO₂ capture with CO₂ enhanced oil recovery (EOR) where CO₂ can be injected into depleted oil wells to recover untapped oil could be a major near-term opportunity. Given these opportunities, the development of immobilized carbonic anhydrase biocatalysts will continue, ultimately leading to commercial deployment.

5. Life-cycle assessment of immobilized enzyme products

There is broad agreement in the scientific community that Life-Cycle Assessment (LCA) is one of the best methodologies for evaluation of environmental burdens associated with industrial processes.¹³⁹ In addition to identifying energy and materials used as well as waste and emissions released to the environment, LCA also allows identification of opportunities for environmental improvement.^{139,324} In a more general sense, sustainable bio-processes are processes that minimize environmental impacts, are economically attractive and socially responsible.³²⁵ The use of enzymes in industrial processes is often linked to reduced consumption of energy, chemical inputs and waste streams.^{326,327} Oxenbøll and Ernst examined several enzymes used in the food industry and determined their impact on greenhouse gas emissions relative to non-enzymatic processes.³²⁸ In one example it was shown that the use of a phospholipase for degumming of vegetable oil resulted in a 44 ton reduction in greenhouse gases (as CO₂ equivalents) per 1000 ton of oil produced. The majority of these savings were realized through improvements in the yield of oil, which resulted in reduced agricultural intensity, a major greenhouse gas contributor.

Although a minor fraction of the overall enzyme market, immobilized enzyme systems have improved and enabled many processes and minimized their undesired impacts.³²⁶ For example, immobilized enzymes allow for continuous processing, which in turn can lead to lower production costs and energy consumption. Chemical consumption and waste stream generation can also be substantially reduced through the use of immobilized enzymes. It should also be recognized that while important, the potential environmental benefit obtained from using immobilized enzymes is not typically the primary driver in process development, with economic considerations often taking precedence. Several recent reports have applied LCA methodology to study the advantages of immobilized enzymes for lipase-mediated interesterification of triglycerides,²⁰⁹ biodiesel production,^{329–331} and the production of pharmaceutical intermediates.³³²

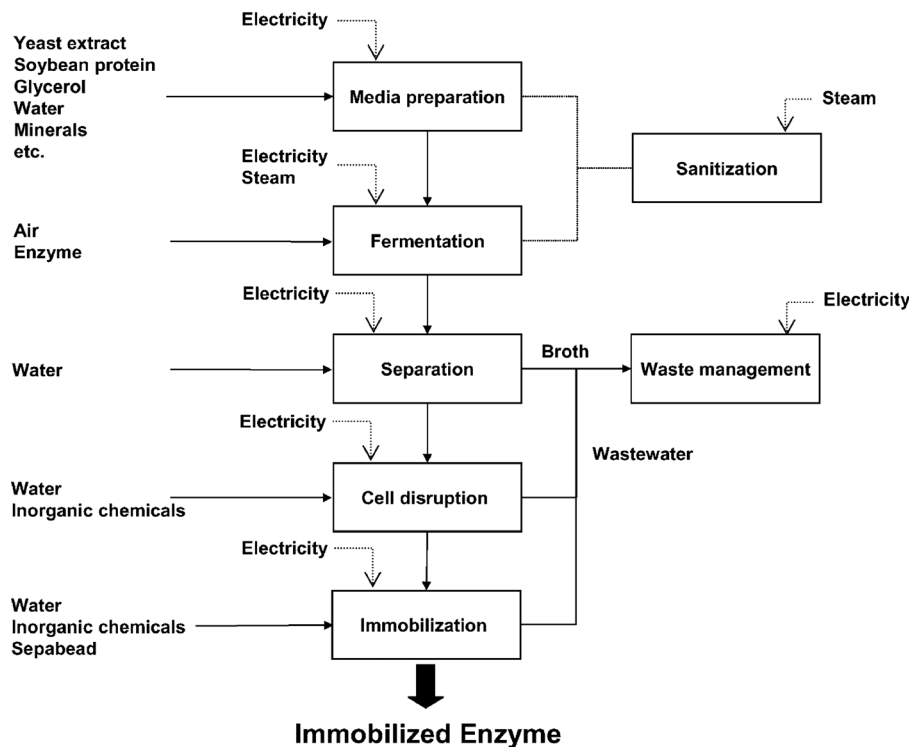


Fig. 13 LCA analysis of inputs required to produce an immobilized enzyme (reproduced from ref. 332 with permission from Springer).

Holm and Cowan applied LCA to compare chemical and immobilized enzymatic routes for interesterification in the oil and fats industry.²⁰⁹ EIE was found to have a lower environmental impact relative to CIE due to generation of fewer by-products, lower operating temperatures and reduced consumption of water. As a result, EIE was judged to have a significant net environmental benefit relative to CIE with regard to energy use, global warming, acidification, and smog formation.

In recent years, biodiesel production using biocatalyst technology has drawn increased attention relative to chemical catalytic approach because the conventional production of biodiesel by chemical catalysts is energy consuming, leads to undesirable side products, requires expensive wastewater treatment, and makes it difficult to recover the glycerol produced as a byproduct.^{271–277,329} Enzymatic production of biodiesel mitigates many of these difficulties and is workable at milder conditions (see Section 4.4). A review of enzymatic biodiesel production concluded that low cost immobilization technology is a key requirement if the biological route is to match chemical process economics.³²⁹ A biocatalyst cost of \$25 per ton of biodiesel, comparable to that of chemical catalysts, was cited as a future target. In a detailed report, Raman *et al.* applied LCA methodology to compare chemical and enzymatic methods for biodiesel production.³³⁰ The overall conclusion was that an immobilized enzyme catalyst had less environmental impact as compared to processes using either an alkali or a soluble biocatalyst, although this advantage was a function of the extent of enzyme reuse.

Both economic and environmental impacts of immobilized enzymes are a function of overall biocatalyst productivity. A

threshold of 5–10 tons of product per kg of immobilized biocatalyst has been cited as necessary for industrial relevance.^{2,6,329} In addition to the production costs of the enzyme, the added impact of both the immobilization matrix manufacture and the immobilization process itself must be taken into account (Fig. 13). For example, in an LCA study undertaken by Kim *et al.*, the production of immobilized aldolase was found to have a larger environmental impact than other enzymes evaluated because of a larger energy intensity and lower enzyme production yield.³³² The immobilization process, particularly Sepabead[®] production, was identified as the primary greenhouse gas emission source for the immobilized enzyme (51–83%). Much of this impact was due to Sepabead[®] raw materials, namely glycidyl methacrylate and ethylene dimethacrylate. The immobilization process was also found to be the primary contributor to acidification and in two out of three cases, to eutrophication. This study highlights the impact of carrier choice on the environmental profile of an immobilized enzyme product. Thus the use of an immobilization matrix with environmentally benign character and milder immobilization process conditions is desirable.

6. Conclusions

Despite ongoing challenges, the commercial development and large-scale application of immobilized enzymes will continue to expand, for chemical production as well as in consumer applications. A more holistic understanding of the factors that justify implementation of immobilized enzyme processes will drive the next generation of biocatalysts and build upon the early successes within the industry. Traditionally, the

differentiating advantage of an immobilized biocatalyst was often realized in cases where a defined product stream was required. Examples include the production of HFCS, amino acids, pharmaceutical intermediates and chemical monomers. Food applications will continue to dominate the application of immobilized enzymes on a volume basis, however emerging applications for biodiesel production and carbon capture might ultimately be applied on an immense scale.

The advent of nanotechnology and integrated systems engineering promises to move immobilized enzymes products into other areas of application, such as those involving insoluble or macromolecular substrates, biosensors and intelligent materials. One exciting possibility is the combination of enzymes with inorganic catalysts in multi-catalyst systems capable of 'one-pot' synthesis of chemicals and materials.³³³ The need to develop more sustainable processes and reduce greenhouse gas emissions will also favor the development of this field. While it might be true that the implementation of immobilized enzyme systems to date has not lived up to initial, optimistic expectations, the outlook is bright given current industry trends combined with the rapid evolution of immobilized enzyme technology.

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