Molecular Catalysis 479 (2019) 110607

Contents lists available at ScienceDirect

Molecular Catalysis

journal homepage: www.elsevier.com/locate/mcat

Industrial applications of immobilized enzymes—A review

Alessandra Basso*, Simona Serban

Purolite, Unit D, Llantrisant Business Park, Llantrisant, CF72 8LF, United Kingdom

ARTICLE INFO

Keywords:
Industrial biocatalysis
Immobilized enzymes
Immobilized lipases
Immobilized glycosidases
Stereochemistry

ABSTRACT

The use of immobilized enzymes is now a routine process for the manufacture of many industrial products in the pharmaceutical, chemical and food industry. Some enzymes, such as lipases, are naturally robust and efficient, can be used for the production of many different molecules and have a wide range of industrial applications thanks to their broad selectivity. As an example, lipase from *Candida antarctica* (CalB) has been used by BASF to produce chiral compounds, such as the herbicide Dimethenamide-P, which was previously made chemically. The use of the immobilized enzyme has provided significant advantages over a chemical process, such as the possibility to use equimolar concentration of substrates, obtain an enantiomeric excess > 99%, use relatively low temperatures (< 60 °C) in organic solvent, obtain a single enantiomer instead of the racemate as in the chemical process and use a column configuration that allows dramatic increases in productivity. This process would not have been possible without the use of an immobilized enzyme, since it runs in organic solvent [1].

Some more specific enzymes, like transaminases, have required protein engineering to become suitable for applications in production of APIs (Active Pharmaceutical Ingredients) in conditions which are extreme for a wild type enzyme. The process developed by Merck for sitagliptin manufacture is a good example of challenging enzyme engineering applied to API manufacture. The previous process of sitagliptin involved hydrogenation of enamine at high pressure using a rhodium-based chiral catalyst. By developing an engineered transaminase, the enzymatic process was able to convert 200 g/l of prositagliptin in the final product, with e.e. > 99.5% and using an immobilized enzyme in the presence of DMSO as a cosolvent [2].

For all enzymes, the possibility to be immobilized and used in a heterogeneous form brings important industrial and environmental advantages, such as simplified downstream processing or continuous process operations. Here, we present a series of large-scale applications of immobilized enzymes with benefits for the food, chemical, pharmaceutical, cosmetics and medical device industries, some of which have been scarcely reported on previously.

In general, all enzymatic reactions can benefit from the immobilization, however, the final choice to use them in immobilized form depends on the economic evaluation of costs associated with their use versus benefits obtained in the process. It can be concluded that the benefits are rather significant, since the use of immobilized enzymes in industry is increasing.

1. Introduction

Operating at the crossroads between biochemistry, organic chemistry, microbiology and molecular biology, biocatalysis is an example of a viable and sustainable interdisciplinary technology. The first wave of biocatalysis started over a century ago, when scientists discovered that whole living cells could perform valuable chemical transformations such as production of a precursor of L-ephedrine [3].

During the second wave of biocatalysis, in the last quarter of the 20th century, enzymes, substrate, and media screening and initial protein engineering technologies allowed the substrate range of enzymes to be extended to unnatural compounds.

The third wave of biocatalysis started in the late 1990's with the so-called "direct evolution" [4], when advanced molecular biology and high-throughput screening were introduced to achieve rapid and extensive modification of biocatalysts. We are now rapidly approaching the fourth wave, where protein engineering efforts are enabling biocatalytic access to novel and often non-natural reactions [5].

Thanks to major achievements made during the years in enzyme development and protein engineering, and to the green chemistry advantages characterizing biocatalytic processes, more and more molecules have been and are being synthesized using enzymes.

* Corresponding author.
E-mail addresses: alessandra.basso@purolite.com (A. Basso), simona.serban@purolite.com (S. Serban).

https://doi.org/10.1016/j.mcat.2019.110607
Received 18 March 2019; Received in revised form 26 August 2019; Accepted 30 August 2019
Available online 18 September 2019

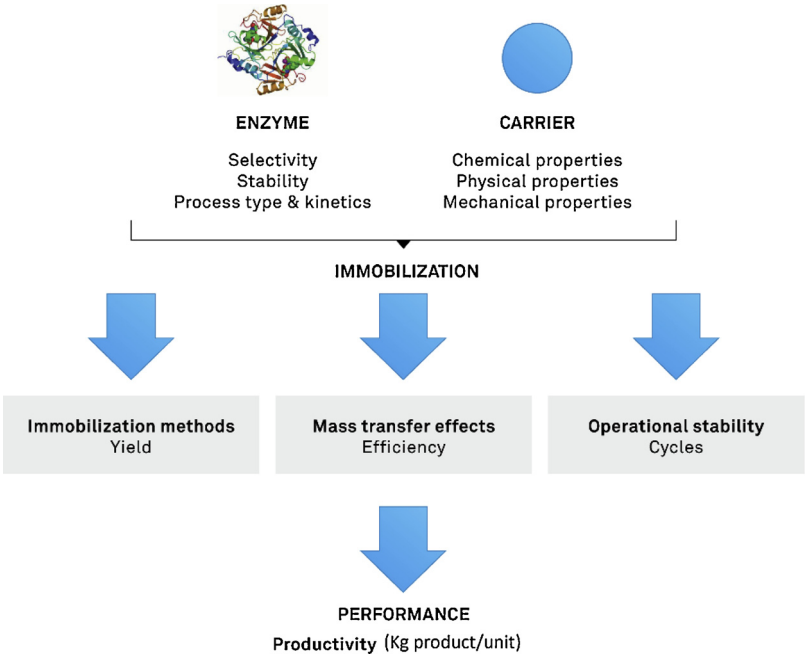

Fig. 1. Factors affecting enzyme immobilization.

A good example of protein engineering coupled to a successful enzyme immobilization which enabled the transition from a chemical process to a green process is the use of engineered transaminase for sitagliptin manufacture as reported in paragraph 5.4 [2]. Lipases have also been modified to broaden their selectivity and accept different substrates, as in the case of acrylate synthesis (paragraph 4.2) [6] and in the case of cocoa butter analogues (paragraph 3.6) for the synthesis of 1,3-triglycerides [7]; immobilization on solid supports is, in both cases, the key for successful industrial application.

In industry the choice of using free soluble enzyme or enzyme in insoluble form is largely dependent on the cost of the enzyme and the application. Enzymes in an insoluble form are essentially a specialized form of heterogeneous catalysis in that they can be recovered and reused, often maintaining activity for large periods of time and being applicable to a variety of process formats [8].

Immobilization of an enzyme means combining the selectivity, stability and kinetic of that enzyme with the physical and chemical properties of the carrier in a specialized formulation which has as primary role maximizing the stability both physical and enzymatic of the biocatalyst (Fig. 1).

There are many different methods used for enzyme immobilization, but industry always prefers simple and cost-effective methods. The most used methods are based on physical immobilization (adsorption or physical entrapment) and chemical immobilization (covalent binding and cross linking). We here refer to interesting reviews for further reading [9–15].

Alongside the advantages, it is important to recognize the limitations of immobilized enzymes (Table 1). For example, the fast kinetic of native enzymes such as amylases or proteases is dramatically reduced when immobilized due to diffusion restrictions, making the immobilized enzyme less economical.

Overall, the industrial relevance of immobilized enzymes is mainly application driven, in that there must be a differentiating advantage offered by such biocatalyst over soluble enzymes, whole cells or chemical catalysts.

In industrial processes, the cost contribution from an immobilized enzyme is dependent on the reaction kinetic and specificity but also on the number of cycles the enzyme is reused as an indirect measure of total productivity on a kg product per kg biocatalyst basis [16]. In this sense, valuable guidelines were provided to evaluate catalyst production cost in biocatalytic processes, and a range of productivity was recommended: 2000–10000 kg product / kg immobilized enzyme for bulk commodity products, going all the way to 50–100 kg product / kg immobilized enzyme for high-value pharma products [17].

The challenge in scaling up processes using immobilized enzymes is mainly cost driven and before using an immobilized enzyme, the economic evaluation of the overall process is performed, considering all associated costs:

- native enzyme
- resin for enzyme immobilization
- downstream processing
- reactors
- disposal of immobilized enzyme after use
- regeneration of carriers.

This analysis is providing an estimation of the minimum number of recycles of the immobilized enzyme to make the process economically

Table 1
Advantages and disadvantages of immobilized enzymes in industrial processes.

Advantages	Disadvantages
• Easy separation of biocatalyst • Reduced costs of downstream processing • Multiple use of biocatalyst (recycling) • Better stability, especially towards organic solvents and higher temperatures • Use of fixed bed or batch reactors without need of membrane to isolate enzyme from product • Co-immobilization with other enzymes is possible	• Lower enzyme activity compared to native enzyme • Additional costs for carriers and immobilization • Lower reaction rates compared to native enzymes • Subject to fouling • Disposal of exhausted immobilized enzyme (incineration)

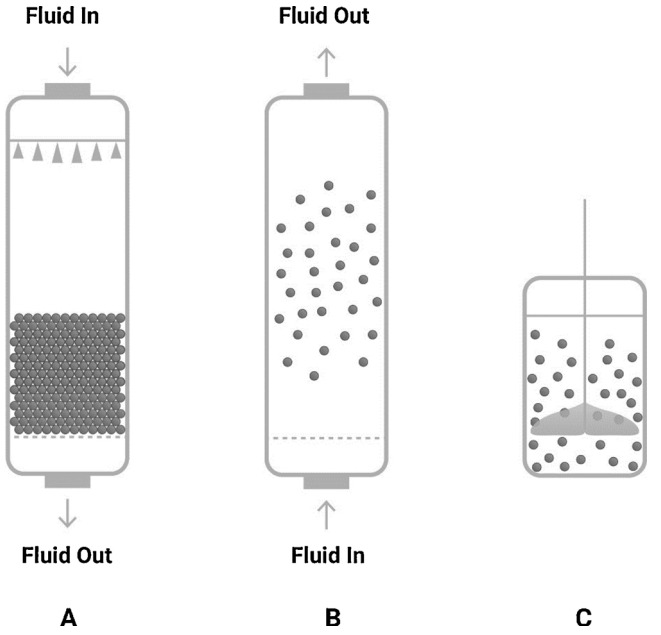

Fig. 2. Typical reactor configurations used in industry with immobilized enzymes. (A) Fixed bed reactor, (B) Fluidized bed reactor, (C) Stirred reactor.

advantageous compared to the use of a native enzyme.

This suggests that the use of immobilized enzyme biocatalysts requires a good understanding of both technical and economic factors. The review will report on examples of applicability, use and advantages of immobilized enzymes as biocatalysts in large-scale industrial process.

2. Typical reactors used in industry for biocatalysis reactions

Use of immobilized enzyme allows to simplify process set up compared to the use of native enzymes [18].

When it comes to the choice of reactor to use with immobilized enzymes, different options are available, as shown in Fig. 2.

Stirred batch reactors (Fig. 2C) are typically used by the pharmaceutical industry, since volume of the reactor is rather small (maximum of a few m^3) and product manufactured per batch can be several hundred kilograms [19]. The reactor is equipped with a retaining stainless-steel filter at the bottom for easy liquid removal and immobilized enzyme re-cycling, and a stirrer that allows controlled and gentle mixing without affecting the mechanical stability of the immobilized enzyme. The retaining multilayer stainless-steel filter is adjusted to the particle size of the immobilized enzyme, with the target to enable fast filtration, retain all immobilized enzyme in the reactor, avoid breakage of the immobilized enzyme, and easy cleaning. A polypropylene multilayer filter can also be used but has the disadvantage of being more fragile to pressure. Jacketed reactors allow for accurate control of the temperature reaction parameter. In general, the costs of stirred batch reactors are quite low compared to other configurations, such as packed bed reactors.

The packed bed column reactors (Fig. 2A) are preferred by food and chemical industry, where volumes of manufactured products are very high, and productivity over time is a key factor. A typical packed column reactor for manufacture of sweeteners by immobilized enzyme can have internal volume of 10 m^3 and some food processes run, as an example, at multiple ton per hour in packed bed columns. Process productivity and economics are very important considerations when using packed bed reactors for large volumes of price-sensitive products. Reactors are quite a big investment in this case, considering the valve set up required to run in a continuous way, the necessary inline controls, and resistance to high pressures.

An alternative to packed bed reactors is the fluidized bed reactor (Fig. 2B), a good option when highly viscous substrates and products, such as oils, are handled, and immobilized enzyme particles are maintained in suspension by the up-flow stream of substrate.

When designing a biocatalysis process using immobilized enzymes for industrial production of key molecules, some variables need to be identified and carefully optimized [20].

1) Reaction temperature: this parameter has a great impact on kinetics. Typically, immobilized enzymes are more stable and can operate at higher temperature (approximately 10 °C higher) than non-immobilized enzymes.
2) Amount of immobilized enzyme used: this is a key factor impacting on costs. Typical industrial operations in batch mode or fluidized mode run at 3–10 % w/v of immobilized enzyme / reaction total volume.
3) Substrates excess: when more than one substrate is involved in a biocatalyzed reaction, the target becomes to work at equimolar concentrations, since this significantly affects costs during downstream processing.
4) Immobilized enzyme replacement: the biocatalysts' replacement, after prolonged reactions times (cycles in batch reactors or operation time in columns) is a key decision that impacts costs. Typically, industry replaces the immobilized enzyme when the residual activity is between 50 and 10% of the initial activity. When using packed column operations, a solution is also to partially replace the front end of the column with fresh enzyme, thus increasing operational life of the column.

3. Immobilized enzymes in the food industry

As opposed to the pharmaceutical industry and some parts of the chemical industry, the requirements in the food industry are to produce very large quantities of cost-sensitive products. In this case, the cost of the biocatalyst (immobilized enzyme) must be low, therefore, it has to show good operational stability allowing for a large number of cycles to be performed. Very often in the food industry, the continuous flow operational configuration is preferred over batch processes, especially when large quantities of products are to be made. Examples of immobilized enzymes used at large scale in continuous process for production of foodstuffs such as high fructose corn syrup (HFCS), cocoa butter analogues, allulose, galacto-oligosaccharides, etc. are presented below (Table 2) and in detail in the following paragraphs.

3.1. D-glucose/xylose isomerase for production of HFSC

The use of immobilized D-Glucose/xylose isomerase (EC 5.3.1.5) in the production of so-called "high fructose corn syrup" (HFCS) represents the largest commercial process involving an immobilized enzyme, both in terms of the amount of enzyme used, as well as the volume of product produced [21]. In fact, over 500 tons of immobilized D-Glucose/xylose isomerase are consumed annually, enabling the production of approximately 10 million tons of HFCS per annum [8]. HFCS is further used in production of fructose as a sweetener for beverages and foodstuffs, or for direct use as a food component.

Although D-xylose is the native substrate of D-Glucose/xylose isomerase, the enzyme has broad substrate specificity and efficiently converts D-glucose to D-fructose, in this industrial application (Fig. 3) [16,22–24].

The position of isomerization equilibrium is a function of temperature, tending to favor fructose formation at higher temperatures. At 60 °C, the temperature at which most immobilized D-Glucose/xylose isomerase are used commercially, the conversion of dextrose produces around 50% fructose in the HFCS, whereas at 90 °C the equilibrium mixture contains 56% fructose [22].

Nowadays, processes for HFCS production are carried out in fixed bed reactors arranged in parallel and operated in a continuous manner

Table 2
Use of immobilized enzymes for the industrial manufacture of food ingredients.

Product	Application	Immobilized enzyme	Reaction type	Reaction media[a]	Type of immobilization
HFCS	Sweetener	D-Glucose/xylose isomerase	Isomerization	A	Adsorption on silica
Allulose	Sweetener	3-epimerase	Epimerization	A	Adsorption/ionic
Tagatose	Sweetener	1. Beta-galactosidase 2. Epimerization by baker yeast	Hydrolysis	A	Adsorption/ionic on ion exchange styrene/DVB polymer
GOS	Synthesis of oligosaccharides	Beta-galactosidase	Synthesis	A	Covalent immobilization on synthetic polymers
Vitamin C ester	Antioxidant	Lipase B from *Candida antarctica*	Transesterification	B	Adsorption on methacrylate/divinylbenzene copolymer
Triglycerides	Cocoa butter equivalent	Lipase from *Thermomyces lanuginosus* (TL)	Transesterification	B	Adsorption on silica
Triglycerides	Cocoa butter equivalent	Lipase from *Rhizomucor miehei* (RM)	Transesterification	B	Adsorption/ionic on ion exchange styrene/DVB polymer
Omega-3 ethyl esters	Omega'3 from fish oil	Lipase B from *Candida antarctica*	Esterification	B	Adsorption on methacrylate/divinylbenzene copolymer
Hydrolysed lactose	Lactose-free dairy products	Beta-galactosidase	Hydrolysis	A	Adsorption/ionic on ion exchange modified cellulose fiber

[a] A: liquid phase is formed by water or aqueous buffers. B: liquid phase is formed by neat liquid reagents only, without the additions of organic solvents or aqueous buffers.

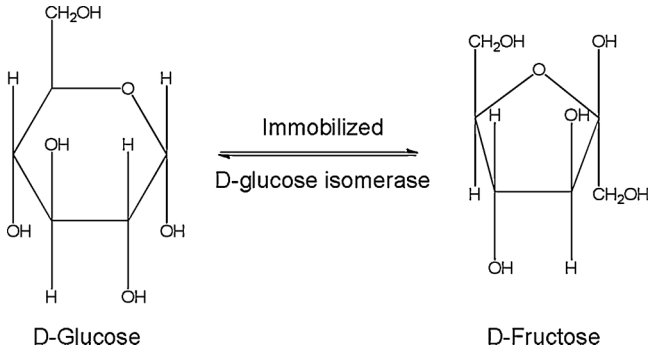

Fig. 3. Manufacture of fructose by immobilized D-Glucose/xylose isomerase in aqueous media.

[25]. Corn-derived D-glucose syrup is converted into a mixture usually named HFCS-42 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 a final 90% D-fructose (HFCS-90). HFCS-90 is then blended with HFCS-42 to produce HFCS-55 (55% fructose).

Commercial crystalline D-fructose with a purity of over 99% (w/w) can also be obtained starting from HFCS-42.

The two commercial preparations of immobilized D-glucose / xylose isomerase, mostly used for these productions, are based on inexpensive inorganic carriers such as bentonite clay and diatomaceous earth with enzyme cross-linked by glutaraldehyde, an approved reagent for enzyme immobilization by FDA under 21 CFR 173.357) [26]. The obtained composite is dewatered and mechanically extruded before drying in a fluidized bed dryer. The resulting immobilized D-glucose / xylose isomerase (Sweetzyme and GenSweet, marketed by Novozymes and Genencor now DuPont Industrial Biosciences, respectively) [27] are extremely stable, with a half-life of over one year when used in a packed bed reactor at 60 °C [28,29].

3.2. Epimerase for allulose

Allulose is a "zero-calorie" sweetener and has a sweetness suggested to be similar to dextrose (Fig. 4).

D-allulose is the C-3 epimer of D-fructose (Fig. 4), and the structural difference between allulose and fructose results in allulose not being metabolized by the human body and thus having zero calories. Therefore, allulose is a good candidate as a sweet bulking agent, having similar properties to typical monosaccharides.

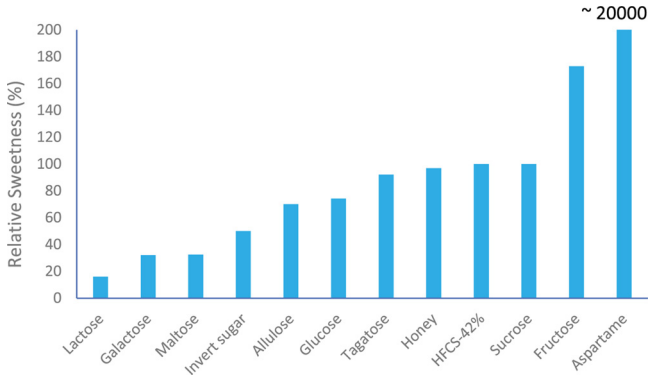

Fig. 4. Relative sweetness (%) of natural sugars and sweeteners. Sucrose is reference and is set at 100. Aspartame is out of scale with a value of 20,000. All sweeteners with relative sweetness (%) lower that sucrose are less sweet that sucrose when comparing same amount of sweetener. On the contrary, fructose is almost twice sweeter than sucrose.

The primary target market for allulose is food and beverage manufacturers; replacing dextrose, fructose or HFCS by allulose in their products significantly reduces calories without altering properties imparted by the sugar component, such as bulking, browning, texture and sweetness.

Although it is not metabolized by the human body, allulose is naturally present in processed cane and beet molasses, steam-treated coffee, wheat plant products and high fructose corn syrup with a typical total daily intake greater than 0.2 g per day.

In recent years, allulose was recognized as GRAS (Generally Regarded As Safe) in the United States and it is being commercialized and used as a foodstuff in other countries such as Mexico, Columbia, Chile and Costa Rica. Allulose is not an approved foodstuff in Europe but several allulose producers are currently applying for its approval as a novel food ingredient in the European Union.

In 2011 CJ CheilJedang Corp reported that ketose-3-epimerases (EC 5.1.3.31) expressed in different microorganisms can interconvert fructose and allulose [30–32].

The technical challenge was to create an enzyme able to convert fructose to D-psicose, by epimerasization of hydroxy group in position 3, using a D-psicose epimerase derived from *Agrobacterium tumefaciens*. The new enzyme was characterized by thermostability and high activity (Fig. 5).

However, the major step towards industrialization came from Tate & Lyle in 2014 with the development of a full industrial process using improved enzymes immobilized on an ion exchange resin [33] and the development of a sweetener composition including fructose, sucralose and allulose [34]. The use of immobilized enzyme opened the door to the production of allulose using continuous packed bed configurations, with significant cost reductions.

In fact, the process for manufacture of allulose is a continuous fixed bed reactor with fructose solution at pH 8.0 pumped in and recirculated continuously at a rate of 8 bed volumes per hour, with the jacketed columns heated to a temperature of 57 °C.

The process is now industrialized and in March 2017, Tate & Lyle launched DolciaPrima® crystalline allulose using the above manufacturing process [35].

3.3. Beta-galactosidase for tagatose

Tagatose is another emerging sweetener, derived from animal sources. Tagatose is a sweetener with 92% sweetness of sucrose (Fig. 4), but only 38% the calories of sugar, with a much lower glycemic index. Tagatose can be obtained from lactose which has only 16% of the sweetening power of sucrose. Lactose is a natural sugar contained in milk, typically comprising 2–8 % of its weight.

In 2014, Inalco has reported a process for manufacture of tagatose using immobilized beta-galactosidase [36]. This process proposed the recovery and valorization of lactose contained in whey permeates at a concentration of 18%. Tagatose can be obtained by a first hydrolysis of lactose using immobilized beta-galactosidase (EC 3.2.1.23) to obtain glucose and galactose. Glucose is removed by subsequent deglycosylation with baker's yeast; tagatose is obtained by the subsequent epimerization of galactose with aerated $Ca(OH)_2$ (Fig. 6).

Inalco has developed a cost-effective ionic/covalent immobilization of beta-galactosidase on ion exchange resin using primary or tertiary amine functionalized styrene-divinyl benzene pre-activated with glutaraldehyde.

The great economic advantage of using immobilization on ion exchange resins in food applications is the possibility to regenerate resins using cost-effective reagents: when enzyme is exhausted and activity is too low, the carrier can be regenerated in situ using NaOH and HCl, which remove the enzyme and clean the resin from fouling matter. A new batch of enzyme is typically added directly in the column containing the ion exchange resin and immobilization takes place. In food industry this is usually done for a number of cycles varying from 5 to 10.

Starting from a solution of lactose at about 13% (w/w), immobilized beta-galactosidase is used to produce galactose and a final concentration of tagatose of 4% w/w.

Reports were also published on the use of immobilized l-arabinose isomerase (EC 5.3.1.4) to produce tagatose in stirred tank or continuous flow operations [37,38]. In 2000 a Korean company Tongyang Confectionary Co. published a patent presenting the use of l-arabinose isomerase from recombinant *Escherichia coli* for the production of tagatose. However, the immobilized enzyme on Ca alginate beads showed lower production yield compared to the use of free enzyme [39]. The difficulty to regenerate resins as Ca alginate is also a limiting step in the industrialization so, the economic advantage of using immobilized enzymes in this case is lower compared to the use of native enzymes.

3.4. β-Galactosidase for galacto-oligosaccharides

In general, galacto-oligosaccharides (GOS) and oligosaccharides in general have received a lot of attention lately, mainly due to their many beneficial health effects and wide applications as prebiotic food [40–42]. GOS comprise a chain of galactose units that arise through consecutive transgalactosylation reactions, with a terminal glucose unit (Fig. 7). The enzyme β-galactosidase (EC 3.2.1.23) can be used to produce GOS [41,43]. The degree of polymerization of GOS can vary quite significantly, ranging from 2 to 9 monomeric units, depending mainly on the source of enzyme used and the conversion degree of lactose. Moreover, the enzyme can also act in a synthetic way and be used to synthetize new molecules starting from galactose to other chemicals. Consequently, they have potential application in the production of food ingredients, pharmaceuticals and other biological active compounds [44].

Covalently immobilized β-galactosidase can be used successfully in packed bed or plug-flow reactors for continuous production of GOS from lactose [45,46]. It was found that high concentrations of lactose in the reaction or lactose slurry would favor the stability of the immobilized enzyme used in this process. Operating pH 7, 65% w/w lactose slurry and 60 °C of temperature allow the formation of up to 57% GOS at 24 h reaction time. Such process was considered suitable for GOS synthesis in an industrial process by Friesland Brands B.V. and allows for the preparation of a 57% GOS content which is close to the required final product of 75% GOS syrup [47].

3.5. Lipase CalB for vitamin C esters

L-Ascorbic acid (vitamin C) is the major water-soluble natural antioxidant. Acting as a free radical scavenger, L-ascorbic acid and its derivatives react with oxygen, thus removing it in a closed system. Also, esters of L-ascorbic acid with long-chain fatty acids (E-304) are

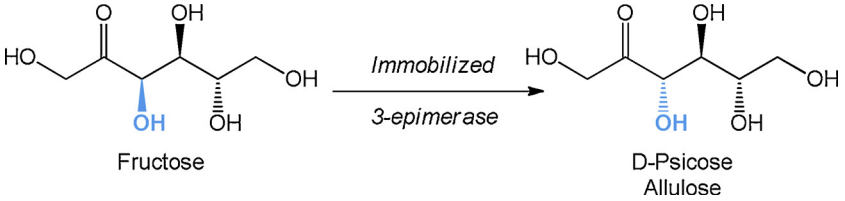

Fig. 5. Manufacture of allulose by immobilized epimerase in aqueous media.

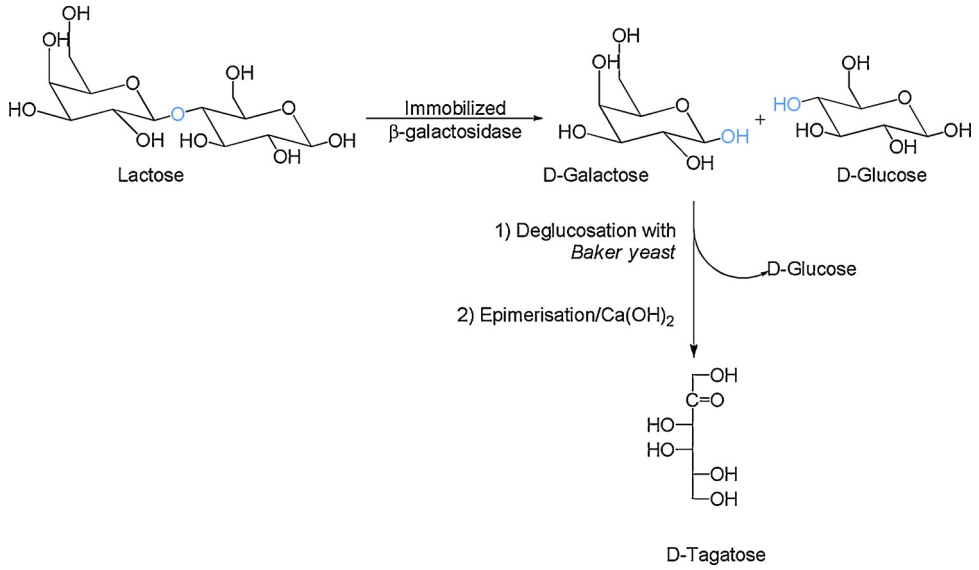

Fig. 6. Manufacture of tagatose by first hydrolysis of lactose by immobilized β-galactosidase and subsequent epimerization.

employed as antioxidants in foods rich in lipids due to their solubility in fats compared to Vitamin C, which is insoluble in oils [48].

Ascorbyl palmitate and stearate are currently produced by reacting ascorbic acid with sulphuric acid followed by re-esterification with the corresponding fatty acid, and subsequently purified by re-crystallization. This chemical process has some disadvantages such as the use of strong acids, the low yields due to non-regioselective reactions and the need for tedious product isolation [49]. The biocatalytic methods described below employ the immobilized lipase B from *Candida antarctica* (CalB) as biocatalyst and free fatty acids or activated esters such as acyl donors (Fig. 8) [50].

The biocatalytic conversion can achieve levels of approx. 95% conversion depending on the operating temperature, the efficiency of the side product (water) removal, and the length of the fatty acid.

Although enzymatic synthesis offers some advantages compared with the current chemical processes, such as lower reaction temperatures, purer product and reduced downstream processing, most of the production of ascorbyl esters is still performed by chemical synthesis, so this biocatalyzed process is still in its initial development stage. This is due to the long reaction time required by the enzymatic process and the high costs of the immobilized enzymes compared to the chemical catalysts.

3.6. Lipase RM and lipase TL for cocoa butter analogues

The origin of vegetable oils (i.e. palm, rapeseed, canola, sunflower, etc.) determines the physical properties of fats and oils used by food manufacturers, since every oil has a different distribution and type of fatty acids in the 1, 2, 3 positions of triacylglycerides [51].

Fractionation, hydrogenation and chemical interesterification have each been largely employed for obtaining the desired melting properties of food oils and fats, particularly in the production of margarine and baking fat [52,53].

Compared to chemical interesterification, the enzymatic interesterification of food oils and fats offers the great advantage of better control of final product composition [54–56].

Due to this selectivity, enzymatic transesterification by 1, 3 selective lipases (EC 3.1.1.3) is now used for commercial production of oils and fats with desired physical properties and with elimination of hydrogenated trans fats that cause serious health concerns [57].

Lipases that are *sn*-1,3 specific (*Mucor miehei*, *Rhizopus arhizus*, *Aspergillus niger*, and *Thermomyces lanuginosus,* formerly *Humicola lanuginosa*) typically do not exchange acyl groups at the 2 position due to steric hindrance (Fig. 9), although some intramolecular transesterification of diacylglycerol intermediates can occur over extended reaction times.

Enzymatic transesterification was initially evaluated for the production of a cocoa butter equivalent that employed the sn-1,3 specificity of a variety of fungal lipases. Cocoa butter equivalents are semi-solid oils that typically melt at 37 °C; they are obtained from cheaper sources than cocoa, such as palm, sunflower or rapeseed oil.

In the 1980's Unilever developed a lipase from *Rhizopus niveus* immobilized by adsorption onto diatomaceous earth. The aim was to convert a mixture of palm mid fraction and stearic acid into a cocoa butter equivalent containing an increased level of the desired triglycerides, 1(3)-palmitoyl-3(1)-stearoyl-2-monooleine and 1,3-distearoyl-2-monooleine [58–62].

At a similar period of time, the Fuji Oil Company also developed a 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. The aim was to produce a cocoa butter substitute suitable for chocolate manufacture [63,64].

Following this early work, continuous enzymatic interesterification processes were developed and applied in the production of margarine and shortening feedstock. This is due to the fact that the enzymatic

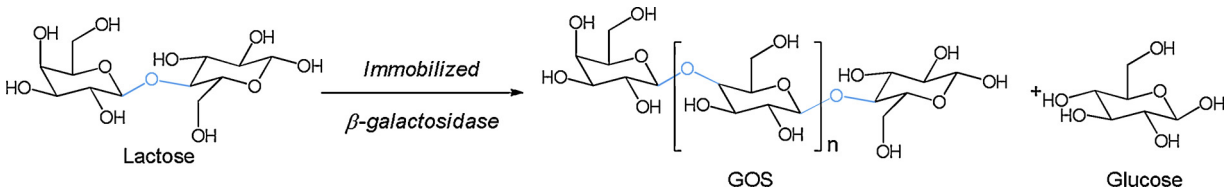

Fig. 7. Simplified enzymatic reaction to produce galacto-oligosaccharides (GOS) from lactose by β-galactosidase catalyzed elongation of lactose by glycosidic linkages; n = . 2–9.

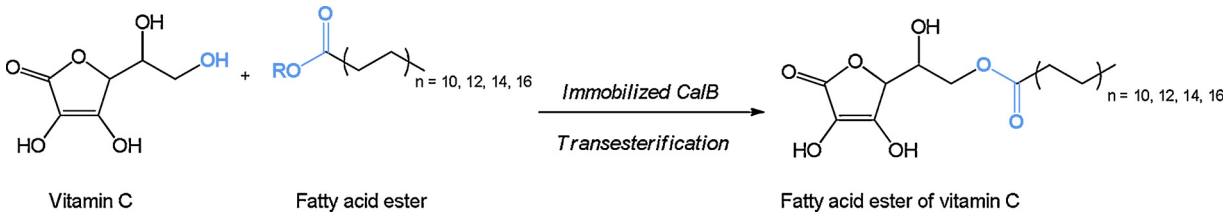

Fig. 8. Manufacture of vitamin C fatty acid ester by transesterification catalyzed by immobilized CalB.

transesterification leads to fully-randomized products with almost identical properties to the products obtained by chemical transesterification, but with the advantage of fewer partial glycerides, higher natural tocopherol content and less undesired color [57].

In 2002, Novozymes started the commercialization of Lipozyme® TL IM, a robust cost-effective lipase from *Thermomyces lanuginosus* (TL) [65,66] immobilized on a silicate support and an organic binder in form of a granulate (300–1000 µm size). The large particle size distribution enabled the use of this biocatalyst in a plug flow, packed bed continuous reactor and the hydrophilic carrier retains the required water needed to maintain enzyme activity in a continuous process. Recently, Novozymes has launched a new preparation of immobilized Lipase TL IM, named Novozym® 40086, which benefits from a better polymeric resin in form of spherical beads instead of silica granulates for enzyme immobilization without the need to further engineer the enzyme. This offers benefits over silica when packed into columns.

In July 2002 Archer Daniels Midland (ADM) implemented the transesterification-based production of trans-free margarines and shortenings using Lipozyme TL IM in sequential reactors configuration [67]. The U.S. Environmental Protection Agency awarded ADM a Presidential Green Chemistry Award in 2005 for its use of enzymatic interesterification 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 ton per day (tpd), and in 2011, Industrias ALES in Manta, Ecuador, announced its plans for construction of a 40 tpd enzymatic transesterification 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 in a packed bed reactor [68].

IOI-Loders Croklaan in Rotterdam, Netherlands utilizes enzymatic transesterification for commercial production of Betapol, a vegetable fat blend that has been specially developed for infant formulas and a variety of fats for margarine and baked goods under the SansTrans™ product name, with production capacity of 100 000 tons per year [69,70].

3.7. Lipase CalB for omega'3 ethyl esters

Polyunsaturated fatty acids (PUFA), such as omega-3 fatty acids, are vital to everyday life and function. For example, the beneficial effects of omega-3 fatty acids such as m-5,8,11,14,17-eicosapentaenoic acid (EPA) and c/s-4,7,10,13,16,19-docosahexaenoic acid (DHA) on lowering serum triglycerides are now well established. These compounds are also known for other cardioprotective benefits [71–73].

Omega-3 fatty acids are often derived from marine fish, microbial, and/or algal oils. Such sources typically provide the PUFA in a triglyceride form where other undesired fatty acids (e.g. saturated fatty acids) are present alongside a desired PUFA in the triglyceride molecule. The food industry is focusing efforts on modifying the composition of omega-3 fish oils to improve organoleptic and bioavailability properties.

Pronova BioPharma Norge AS reported on a process for separating ethyl ester EE fractions enriched in EPA (eicosapentaenoic acid, C20:5) from free fatty acid fraction (FFA) enriched in DHA (docosahexaenoic acid, C22:6) by direct esterification of fish oil free fatty acids with ethanol using immobilized CalB followed by distillation [74].

The process is described as follows: starting solution containing 14% EPA and 15% DHA, 3 equivalents of ethanol and mixed at 40 °C in the presence of 5% w/w immobilized CalB on styrene/divinyl benzene (Novozym 435) as biocatalyst. Over 78% conversion of ethyl ester (EE) and 22% remaining free fatty acid (FFA) was reached after 4 h reaction and the residual FFA was increased to contain 49% DHA and 6% EPA (Fig. 10).

Ocean Nutrition Canada Ltd. reported a method of modification of omega-3 fish oils using immobilized lipase from *Thermomyces lanuginosus* (EC 3.1.1.3.) for hydrolysis of the glyceride, followed by a separation of the free saturated fatty acids FFA from the glycerides and a final enzymatic esterification of the hydrolyzed glyceride by immobilized CalB with a polyunsaturated fatty acid in water free medium [75]. The whole process is intended to increase the concentration of polyunsaturated fatty acid in an oil composition.

DSM has reported the use of immobilized CalB for the esterification and consequent reduction of PUFA in fish oils [76]. In the DSM process, FFA fish oil and glycerol were added to a fixed-bed enzyme reactor able

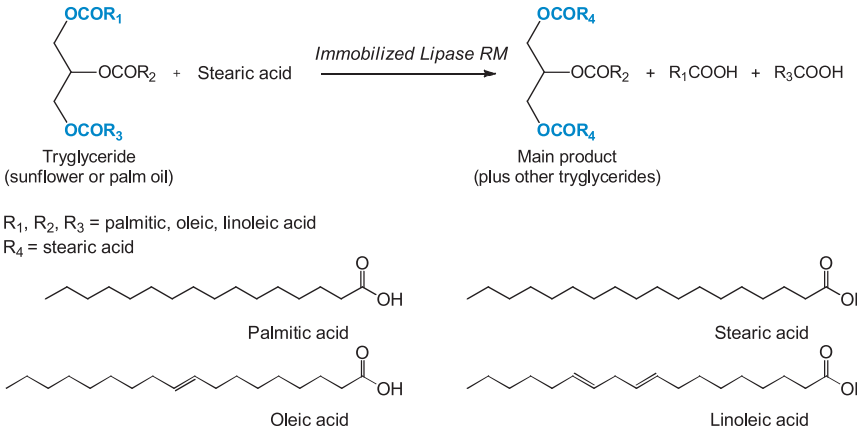

Fig. 9. Manufacture of cocoa butter analogues by transesterification catalyzed by 1,3 selective immobilized lipase from *Rhizomucor miehei* (RM) in water free system. In the example a vegetable oil (from sunflower or palm oil) is reacted with stearic acid using immobilized lipase RM or TL and the final product is a mixture of triglycerides containing predominantly stearic acid in the positions 1 and 3.

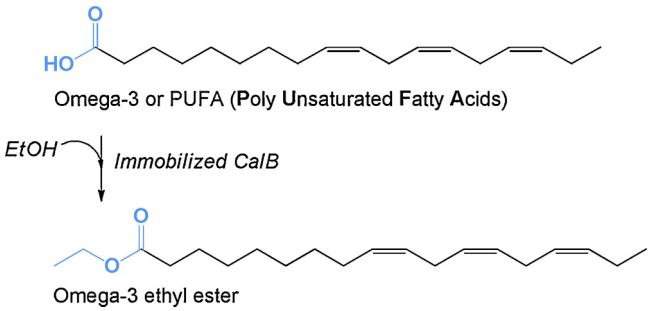

Fig. 10. Manufacture of omega-3 ethyl ester by esterification catalyzed by immobilized CalB in water free media.

to process 4000 and 8000 Kg per run, using immobilized CalB on polymeric beads based on styrene/divinyl benzene (XAD-1180). The system was heated at 70 °C for a reaction cycle of 24 h and final composition was 15% FFA and 85% triglyceride.

3.8. Beta-galactosidase for lactose hydrolysis

The concentration of lactose, a disaccharide composed of glucose and galactose, is 4.3–4.5 % in cow's milk and represents 38–40 % of the total milk solids. Lactose in milk and milk products is not hydrolyzed in the stomach or in the upper section of the small intestine and passes in the next section of the intestine where beta-galactosidase (lactase) produced by intestinal flora hydrolyses it into galactose and glucose [77]. It is estimated that an average of 65% of the population (up to 90% in some Asian communities) is not able to secrete enough beta-galactosidase, creating many health problems by making these people lactose intolerant.

Removal of lactose from milk and milk products makes them acceptable to lactose-intolerant people, so the dairy industry has devoted high resources to develop the hydrolysis of lactose by beta-galactosidase.

Since the sweetening power of lactose, glucose and galactose is 20, 70 and 58% respectively to that of sucrose, the hydrolyzed milk is much sweeter than ordinary milk (Fig. 4).

The industry has developed two hydrolytic methods using beta-galactosidase. The enzyme β-galactosidase (EC 3.2.1.23), most commonly known as lactase, hydrolyses lactose into its monomers: glucose and galactose and it is used to remove lactose from milk (Fig. 11) [78,79]. The simplest but more expensive is the addition of beta-galactosidase to whole milk and after the enzymatic hydrolysis is complete the enzyme is deactivated by heat treatment (typically combined with pasteurization).

The other process involves the use of immobilized beta-galactosidase on the skimmed milk and when hydrolysis is complete, the fat is added again to the hydrolyzed milk to adjust its content. This process has of course, compared to the addition of free enzyme, the advantage of recycling the immobilized enzyme and a final product free from additional components such as enzymes, which are potential allergens.

In the 1970's, Snam Progetti, Italy, developed an immobilized galactosidase from Saccharomyces lactis entrapped in cellulose triacetate fibers that was used successfully in Centrale del Latte, Italy and Snow Brand Milk Products, Japan. The reactor developed was a rotary horizontal column reactor and in 1977 an industrial plant was operating 10 tons milk per day; the immobilized enzyme was used for 50 cycles with a loss of enzyme activity of less than 9% [80].

Corning Glass Works developed a pilot process based on a column reactor with 6800 liters of liquid whey treated per day. The immobilized enzyme has a life of at least 4000 h with the galactosidase being immobilized on alumina particles [81,82].

In another similar application Sumitomo Chemical, Japan, developed an immobilized beta-galactosidase from *Aspergillus oryzae* where the enzyme was covalently bound to an ion-exchange resin based on polyphenolic formaldehyde (Duolite type resins) [83]. Such process was successfully used by Drouin Cooperative Butter Factory, Australia. The immobilization was achieved by exploiting the isoelectric point (pI) of the enzyme and the charges on the resin.

The main issue of using immobilized beta-galactosidase is the microbial contamination of the biocatalyst and the need to design specific process to control this. Therefore, the use of native enzyme is still predominant in industry.

4. Immobilized enzymes in chemical industry

In the last 20–30 years, the focus of the chemical industry has turned increasingly towards the implementation of green and sustainable processes to produce bulk chemicals, but also chiral molecules for a variety of applications. Similarly to the food industry, in the chemical industry the biocatalysts must generate products at ton scale making recyclability a key factor alongside selectivity and high reaction turnover. This is somewhat different to the pharmaceutical industry, where the biocatalysts are being used to produce hundred kg scale of final product. The examples in Table 3 below will give an overview on several large-scale industrial processes employing immobilized enzymes for bulk chemicals manufacturing.

4.1. Lipase CalB for chiral amines

Chiral amines play an important role in stereoselective organic synthesis. They are used directly as resolving agents, building blocks or chiral auxiliaries. While classically available through racemic resolution with optically active acids, biotechnological approaches also open a way to chiral amines. The enantioselective hydrolysis of amides was previously reported using either dinitrogen tetroxide or by chiral separation [84] which is however industrially not feasible.

In 1997 BASF reported a novel method for manufacture and purification of chiral amines, using an enzymatic process instead of previously reported chemical methods [85].

The process allows to obtain both enantiomers with enantiopurity > 99% by resolution of a racemate using 3 steps (Fig. 12).

The initial step involves chiral acylation of rac-phenylethylamine with *i*-propyl methyloxyacetate in methyl tert-butyl ether (MTBE), using immobilized CalB to give a mixture containing S-phenylethylamine and the corresponding amide of R-phenylethylamine. The

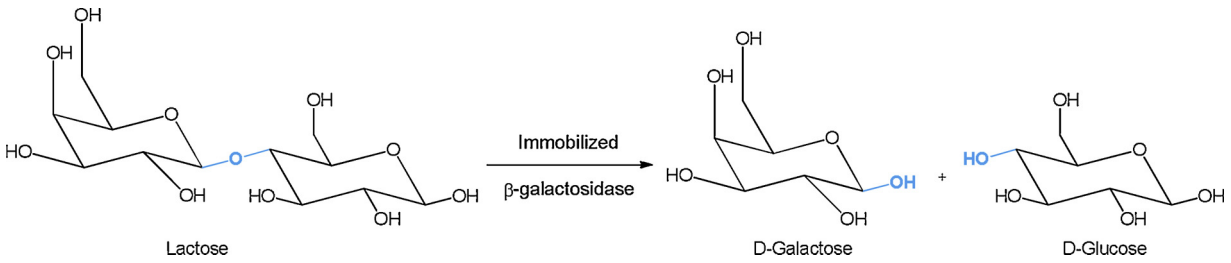

Fig. 11. Hydrolysis of lactose by immobilized β-galactosidase in aqueous media.

Table 3
Main industrial applications using immobilized enzymes for the manufacture of bulk chemicals such as chiral compounds, polymers and surfactants.

Product	Application	Immobilized enzyme	Type of immobilization	Reaction media[a]	Reaction type
(S)-phenylethylamine	Chiral amines	Lipase B from *Candida antarctica*	Adsorption on methacrylate/divinylbenzene copolymer	A	Transamidation
Methylacrylate derivatives	Polymer monomers	Lipase B from *Candida antarctica*	Adsorption on polyethylene	B	Transesterification
Organosilicone polyamide	Polymers	Lipase B from *Candida antarctica*	Adsorption on methacrylate/divinylbenzene copolymer	B	Amidation
Outlook (Dimethenamid-P)	Herbicide	Lipase B from *Candida antarctica*	Adsorption on methacrylate/divinylbenzene copolymer	B	Transamidation
Myristyl myristate	Surfactant	Lipase B from *Candida antarctica*	Adsorption on methacrylate/divinylbenzene copolymer	B	Esterification
L/amino acid	L-amino acids	Aminoacylase	Adsorption on ion exchange resins	C	Hydrolysis

[a] A: liquid phase is formed by organic solvents. B: liquid phase is formed by neat liquid reagents only, without the additions of organic solvents or aqueous buffers. C: liquid phase is formed by water or aqueous buffers.

biocatalytic transformation is performed in very high substrate concentrations (300 g/l of 1-phenylethylamine and *i*-propyl methyloxyacetate) with a 50% conversion in a continuous flow reactor.

The second step involves separation of unreacted amine. S-Phenylethylamine can be recovered by fractionation distillation and then separated from the amide with enantiopurity of 99% [1].

The final step involves the cleavage of amide by hydrolysis using ethylene glycol at 170 °C in presence of NaOH, giving *R*-phenylethylamine with e.e. of 99%.

The process has been applied to a variety of aromatic amines such as substituted phenylethylamines, bicyclic amines, aliphatic amines, cycloaliphatic amines, aryl alkyl amines and has been scaled up by BASF. These chiral amines are commercialized under the brand ChiPros® [86].

As an additional note, BASF has explored the potential of other enzymes rather than lipases to introduce chirality in building blocks. Some examples are enoate reductases (EC 1.3.1.31) for the stereoselective reduction of C=C bonds or alcohol dehydrogenase (EC 1.1.1.1) for the reduction of ketones [87].

4.2. Lipase CalB for acrylates and organosilicone esters or amides

Acrylates are key building blocks used in the production of polymeric-based materials, including biopolymers, as demonstrated by the intense research activities in this field from companies such as Sumitomo [88]. Traditional chemical processes for producing acrylate esters employ high temperatures frequently leading to polymerization of acrylic double bonds. To avoid these unwanted reactions, polymerization inhibitors and acid catalysts have been introduced allowing for reactions at 80 to 100 °C. Enzymatic processes conducted at moderate temperature might be beneficial due to their selectivity, so additives like inhibitors can be omitted with additional benefits of energy saving and better final quality and purity of the product. Selectivity of enzymes is a further advantage if there are several reactive functionalities (hydroxyls, amines, thiols) in the molecule of interest.

The key for transforming a biocatalyzed process in a robust and scalable industrial process was the engineering of CalB to produce a mutant able to accept unnatural substrates as acrylates, since the native CalB does not catalyze acrylation reactions with a yield high enough for industrial applications. The enzyme engineering performed by BASF was able to transform the process in a green and energy efficient technology [89].

The mutant CalB was immobilized by adsorption on polymeric resins such as polypropylene (Accurel® MP1000) before being used in a biocatalytic process run in solvent free neat reagents (methacrylate and hydroxypropylcarbamate) at 40 °C obtaining 97% conversion in 6 h. In this case it is interesting to note that free enzyme, i.e. non-immobilized, was not active due to instability and inaccessibility to substrates.

The biocatalytic process can produce mixtures or enantiomeric pure acrylates with good yields, as presented in Fig. 13.

Immobilized CalB has also found an interesting application in the industrial production of polymers as oleamides, used in plastics processing as slip and antiblocking agents [90]. Synthesis of oleamide is normally performed by enzymatic amminolysis of triolein with ammonia catalyzed by CalB immobilized on polymeric beads (Novozym 435) [91]. Dow Corning, in collaboration with Prof. Gross, reported, for the first time, the production of polymers consisting of organosilicone esters or amides prepared by enzyme catalysis [92]. Fig. 14 shows as an example of this application, the synthesis of an ester of diacid siloxane with hexanol in equimolar concentrations and with immobilized enzyme < 10% w/v. Other examples also report the synthesis of polymers analogous to Nylon starting by diamine disiloxane and dimethyl adipate using immobilized CalB and achieving a molecular weight of approx. 2100 [92].

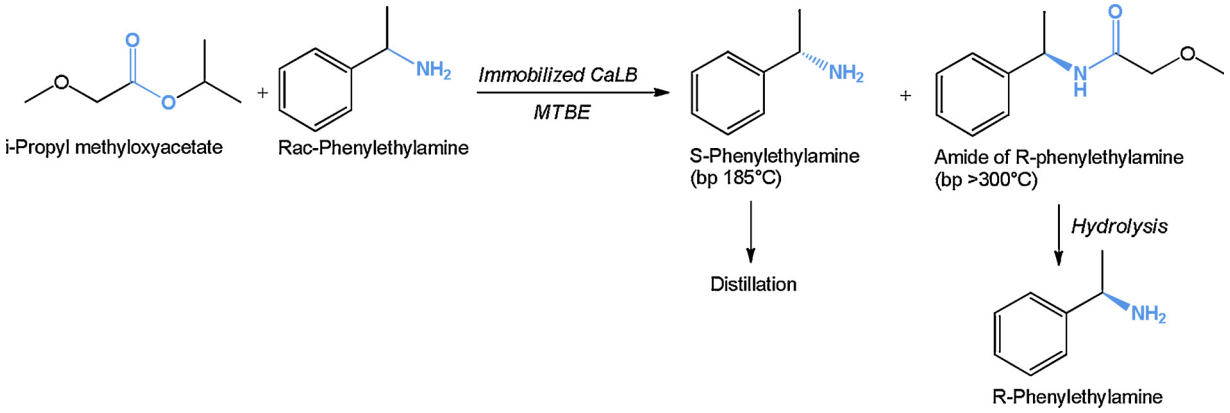

Fig. 12. Manufacture and separation of both chiral amines by immobilized CalB in organic solvent.

4.3. Lipase CalB for Dimethenamid-P

In the vast range of herbicides, there is a group of selective herbicides that are active against the target plant species by enzyme inhibition of fatty acid biosynthesis. Within this group are a number of active compounds that possesses a common chemical structure, the aryloxy-substituted phenoxypropionate with a chiral center (Fig. 15).

In fact, similar to pharmaceutical molecules, it was identified that a major part of the herbicidal activity resides in only one of the enantiomers. In this case, the *R*-configuration at position 2 is the most active so there has been considerable effort from companies to develop cost-effective routes to manufacture single enantiomers. Using a single enantiomer instead of a racemate does not mean only reduced environmental impact but also reduced cost for producers and for the farmer. Biocatalysis proved to be an excellent tool to achieve such enantioselectivity [93].

Metolachlor (discovered in 1970 and commercialized by Ciba-Geigy in 1976) and dimethenamid (discovered by Sandoz in 1986) were originally developed as a racemic mixtures [94]. Following their discovery, it was identified that a major part of the herbicidal activity resides in only one of the enantiomers.

In the case of (*S*)-Metolachlor (Fig. 16), the active ingredient in Dual Magnum® (one of the most important grass herbicides for use in maize), the chemical manufacturing process utilized hydrogenation of an imine with the enantiomerically pure ligand "xyliphos", catalyzed by an iridium catalyst system [95–97].

Dimethenamid was initially developed by Sandoz and the process was sold to BASF in 1996. BASF then successfully developed the route to the single enantiomer Dimethenamid-P (Fig. 17), that involves a selective acylation of (*S*)-1-methoxy-2-aminopropane with ethyl 2-methoxyacetate [1] or a longer chain ester [98] catalyzed by immobilized lipase CalB. In the case of dimethenamid, the chirality of the molecule lies in the 1-methoxy-2-aminopropane part of the structure.

The reaction takes place using neat reagents without addition of organic solvent at 20–60 °C and achieves conversions of > 60% with (*S*)-amine ee > 99% using immobilized CalB (Novozym 435 or L2 from Chirazyme), whereas the unwanted (*R*)- amide is further recycled. The desired (*S*)- amine can be recovered from the unwanted (*R*)-amide and alcohol by-product by either extraction or distillation. The recovered (*S*) amine is further reacted to obtain the Dimethenamid-P. This process was further simplified by using immobilized lipase which can be easily separated in the process and used for further racemic resolution cycles.

The process was also refined allowing the reuse the unwanted enantiomer, in form of (*R*)-amide by BASF and others [99,100].

Dimethenamid-P is part of the commercial product sold under the brand name of Outlook™ by BASF. The product is specifically used for the control of grasses and small-seeded broadleaf weeds such as water hemp, pigweed and nightshade.

4.4. Lipase CalB for myristyl myristate (cosmetic)

The growing trend for natural ingredients and environmentally-responsible processes in the cosmetics market has encouraged manufacturers to find new greener and cleaner manufacturing methods.

Specialty esters such as ceramides and emollient esters for cosmetic formulations were manufactured using strong acid catalysts at high temperatures, with undesired by-products removable by energy-intensive purifications [101]. Other methods of producing cosmetic esters require organic solvents that are potentially hazardous to workers and the environment.

In early 2000, several companies, such as Evonik [102] and Eastman Chemical Company [103], began investigating the use of immobilized CalB as catalysts to produce cosmetic esters at mild temperature. The biocatalytic process is driven to high conversion by continuous removal of the water produced during the esterification (Fig. 18) or a low molecular weight alcohol from transesterification of an ester [104]. The biocatalytic reaction to produce myristyl myristate was performed using equimolar amounts of myristyl alcohol and myristic acid, at 60 °C in presence of 1% w/w of immobilized CalB (Novozym 435).

In order to improve the stability of the immobilized CalB, a silicone coating was performed on the biocatalysts. This enabled an increase in the half-life time of the biocatalyst from 1 day to 23 days and subsequently significantly increased the productivity from 100 $g_{product}/g_{imm\ CalB}$ to 2300 $g_{product}/g_{imm\ CalB}$.

The low temperature (60 °C) used during processing conditions and the CalB specificity do not lead to the formation of undesirable by-products that usually contribute to color or odor in final emollient

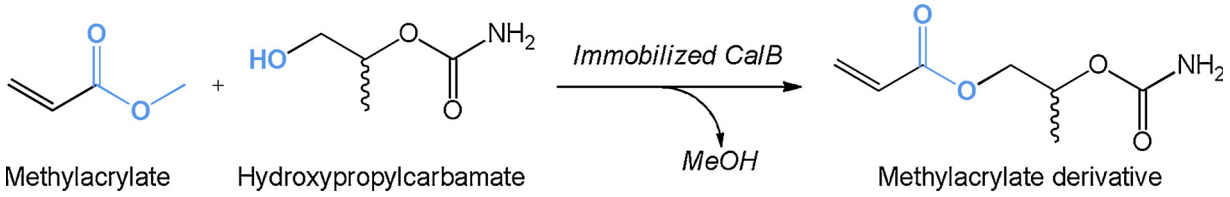

Fig. 13. Methacrylate derivative production by transesterification reaction in water free media using immobilized CalB as biocatalyst.

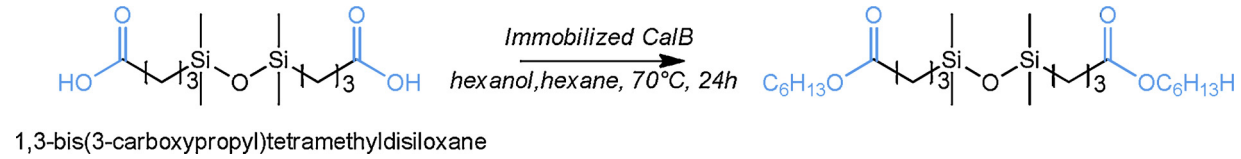

Fig. 14. Synthesis of esters of 1,3-bis(3-carboxypropyl) tetramethyldisiloxane.

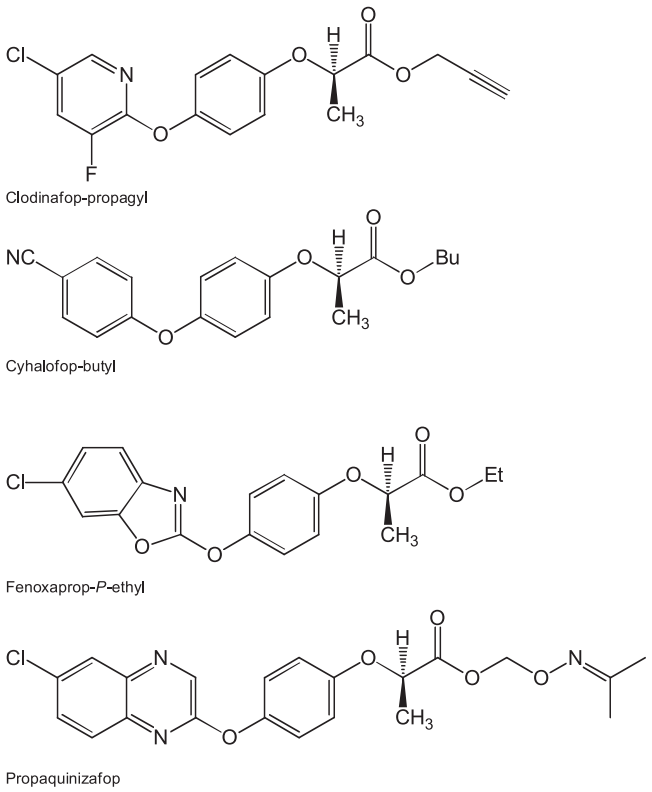

Fig. 15. Examples of phenoxypropionate herbicides with chiral center (R-configuration).

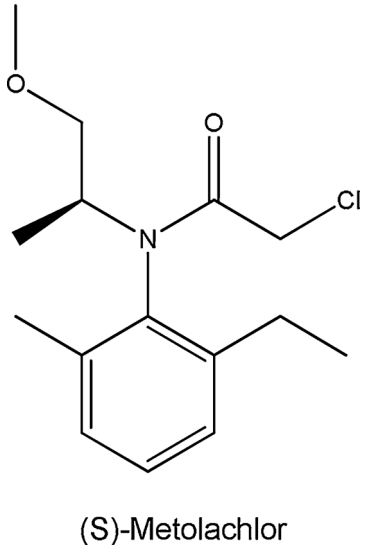

Fig. 16. Chemical structure of (S)-Metolachlor.

esters. The immobilized enzyme is integrated into a fixed-bed reactor with a circulation loop, through which the reaction charge is pumped long enough to reach the complete conversion, thus ensuring long life to the biocatalyst.

During the process life cycle assessment performed by Evonik, it was demonstrated that energy consumption was reduced by more than 60%, and emissions of different pollutants was reduced by 60% - 90%. Emissions of greenhouse gases were reduced from 1518 to 582 kg CO_2-equivalents when making 5 tons of esters - a reduction of 62%. Significant reductions were also measured in sulphur dioxide (acidification potential), phosphates (nutrient enrichment potential) and volatile organic compounds (smog formation potential), all due to the very mild biocatalyzed reaction conditions.

About 50 different emollient esters are now available on the market and Evonik manufactures 4 of them by utilizing immobilized enzymes, as shown in Table 4 [105].

As a recognition of the great impact of this green route, in 2009 Eastman Chemical Company was recognized during the Presidential Green Chemistry Challenge of the Greener Synthetic Pathways Awards for the manufacture of emollient esters such as 2-ethylhexylpalmitate by using immobilized CalB [106].

4.5. Aminoacylase for L-aminoacids

An early industrial application of an immobilized enzyme was the use of the aminoacylase from *Aspergillus oryzae* (EC 3.5.1.14) to resolve racemic mixtures of amino acids.

Chemically synthesized racemic N-acyl-DL-amino acids are hydrolyzed at pH 8.5 to produce free L-amino acids plus the non-hydrolyzed N-acyl-D-amino acids (Fig. 19) These products are easily separated by differential crystallization and the N-acyl-D-amino acids is further racemized chemically (or enzymically) and reprocessed.

The enzyme is immobilized by adsorption onto anion exchange resins (e.g. DEAE-Sephadex) [107] and has an operational half-life of about 65 days at 50 °C in aqueous media. The immobilized enzyme may be re-activated in situ by simply adding more enzyme. The immobilized enzyme has proved to be more efficient than the use of free enzyme mainly due to the more efficient use of the substrate also due to reductions in the cost of enzyme and labor. This aminoacylase from *Aspergillus oryzae* has versatile substrate specificity and several L-amino acids can be produced using this method, such as: L-methionine, L-phenylalanine, L-tryptophan, L-valine, etc. [108]. This biocatalytic process was implemented at industrial scale in early 1970's at Tanabe Seiyaku Co. Ltd. for the production of many L-amino acids and it is considered perhaps the first example of industrial biocatalytic process using immobilized enzyme. The L-amino acids produced via this process were utilized as building blocks in the chemical industry but also in the food industry.

5. Immobilized enzymes in the pharmaceutical industry

Biocatalysis and its advantages, such as selectivity, safety and sustainability in industrial processes, recently became a very appealing technology for the pharmaceutical industry. Through protein engineering, it was shown that unnatural molecules could be used as substrates for enzymes operating in organic solvents and producing high yielding chemo-, regio-, and enantioselective compounds [109,110]. The production of such high-value molecules by traditional chemical synthesis would present real challenges, hence the important investments made by the pharmaceutical industry into high speed

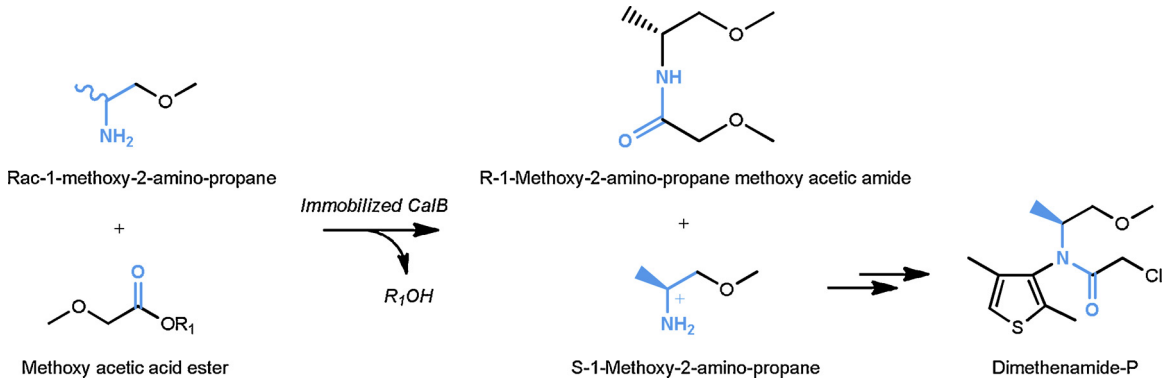

Fig. 17. Manufacture of Dimethenamide-P by enantioselective transamidation by immobilized CalB.

throughput protein engineering in order to produce enzymes capable of meeting industry requirements [111]. In Table 5 and the following paragraphs we present important achievements in the use of immobilized enzymes to produce chiral molecules of pharmaceutical interest.

5.1. Lipase CalB for Odanacatib

Lipases, in general, and lipase B from *Candida antarctica* (CalB) (EC 3.1.1.3) in particular, are commonly used catalysts for the production of cosmetic additives for personal care, active pharmaceutical ingredients and food ingredients due to their high regio-, chemo- and enantioselectivity.

In its immobilized form, CalB finds numerous applications in industrial processes due to its broad selectivity and high tolerance to organic solvents and temperature. Many of these industrial applications are presented in this paper.

One noteworthy API produced by biocatalysis, is the manufacture of Odanacatib using immobilized CalB, introduced by Merck in 2011 [112]. Odanacatib is a potent cathepsin K inhibitor that was discovered in 2008 [113] and subsequently evaluated for treatment of osteoporosis in post-menopausal women, now discontinued.

In the process reported by Merck, the key step is the complete ring opening by ethanolysis of azlactone. The reaction catalyzed by immobilized CalB gives 95% conversion of the desired (*S*)-γ-fluoroleucine ethyl ester (Fig. 20) in methyl *tert*-butyl ether (MTBE), using high substrate concentration (200 g/L of azlactone) in a continuous plug flow reactor at 60 °C [114]. The process was scaled up at 100 Kg scale with yields > 90% and ee of 88%.

The key to success for the process was the use of a different carrier than the one that is normally employed for CalB immobilization, which enabled a 99.9% reduction in cost when compared to using the commercially available Novozym 435 (CalB immobilized on a divinylbenzene/methacrylate carrier). In fact, when immobilizing CalB on an octadecyl functionalized methacrylate resin, the stability and activity was significantly increased compared to the commercially available preparation Novozym 435 and this was attributed to the optimal interaction of the hydrophobic octadecyl groups with lipases, as reported by Purolite [115]. Interesting to note is that the octadecyl functionalized carrier also shows good performance in the immobilization of transaminase, as reported by Merck [116,117].

5.2. Lipase CalB for Sofosbuvir

Hepatitis C is an infectious disease caused by Hepatitis C virus (HCV), a member of the hepacivirus genera within the *Flaviviridae* family. Hepatitis C is the leading cause of chronic liver disease worldwide [118]. The infection is often asymptomatic, but its chronic infection can lead to the scarring of the liver and finally, to cirrhosis, which is generally apparent after many years. In some cases, liver cirrhosis can develop into liver failure, cancer, oesophageal and gastric varices. HCV is transmitted primarily by direct contact with infected blood.

Hence, the interest in developing improved methods of treating hepatitis C that can overcome the high mutagenicity of HCV and the presence of different genotypes and subtypes [119–122].

In 2014, Gilead patented a new compound that inhibits the hepatitis C virus NS3 protease, which has the great advantage of inhibiting multiple genotypes of the hepatitis C virus [123–125]. This compound, commercialized as Sofosbuvir, reported also by Sandoz with a new crystalline formulation [126], is used for the treatment of HCV infection and the related symptoms (Fig. 21).

Sofosbuvir is a large molecule with a synthesis that requires many chemical steps, and immobilized CalB is used in the patent from Gilead in the process for the enantioselective hydrolysis of an acetate ester into chiral alcohol. The CalB immobilized on a divinyl benzene / methacrylate polymer (Novozym 435) was used in MTBE saturated with aqueous 0.1 M phosphate buffer pH 7 at a temperature of 10 °C; the conversion of the racemate to the desired enantiomer is almost 40% (Fig. 22).

In 2014, Chemelectiva-HC-Pharma reported another biocatalytic process to manufacture an intermediate of sofosbuvir [127]. The process involves also the use of immobilized lipase CalB but the catalyzed reaction interestingly is on a different position of the molecule. In fact, immobilized CalB is used for a regioselective mono-deacetylation of sofosbuvir intermediate in polar protic organic solvent to give the corresponding alcohol at 60 °C (Fig. 23).

5.3. Penicillin G amidase for β-lactam antibiotics

Over time, many discoveries in science have been fortuitous and penicillin is a very good example [128]. In 1928, Alexander Fleming discovered that some fungi, such as *Penicillium notatum,* were able to produce a beriolytic substance that was able to inhibit the growth of staphylococci [129]. However, more than a decade passed

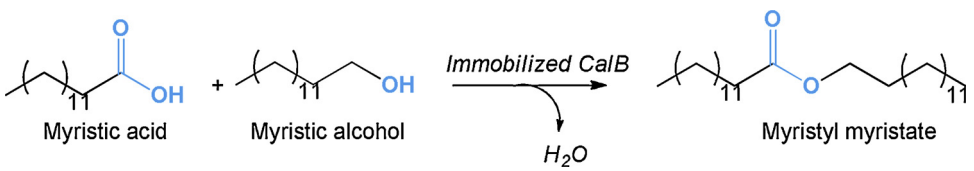

Fig. 18. Esterification by immobilized CalB in water free media of myristic acid with myristic alcohol to produce the surfactant myristyl myristate.

Table 4
Emollient esters manufactured by Evonik and Eastman using immobilized CalB.

Emollient	Reaction	Aspect and properties	Application	Manufactured by
Myristyl myristate	Myristic acid (C14) + myristyl alcohol (C14)	White, wax-like substance, melting point 35-40 °C, CAS no 3234-85-3	Easily spreadable oil component especially in lotions, and to improve the consistency of water/organic (W/O) emulsions	Evonik
Decyl cocoate	Coconut fatty acid (C8-C18) + decyl alcohol (C10)	Liquid at room temperature, CAS no 92044-87-6	Primarily used in face care products and in O/W-type sunscreen formulations	Evonik
Cetyl ricinoleate	Ricinoleic acid (C18) + cetyl alcohol (C16)	Off white solid at 25 °C, CAS no 10401-55-5	Skin care products, decorative cosmetics and lipsticks	Evonik
Isocetyl palmitate	Palmitic acid (C16) + isocetyl alcohol (C16)	Colorless liquid, CAS no 127770-27-8	Substitute for mineral oil in skin care products, especially for dry skin	Evonik
2-Ethylhexylpalmitate	2-Ethylhexanol (C8) + palmitic acid (C16)	Colorless liquid, CAS no 29806-73-3	Skin care, sunscreen formulations	Eastman

afterFleming's discovery before Florey and Chain, in 1940, finally isolated penicillin G (6-aminopenicillanic acid, 6-APA) Fig. 24 [130].

After 20 years of clinical use, the penicillin G was phased out in the late 1960s due to increasing microbial resistance, but the acylation of 6-APA with various side-chains, afforded new active semi-synthetic penicillins. The introduction of semi-synthetic β-lactam antibiotics transformed the 6-aminopenicillanic acid (6-APA) into a major pharmaceutical intermediate [131,132]. The most important examples are ampicillin and amoxicillin (Fig. 24), in which the side-chain of 6-APA is D-phenylglycine and 4-hydroxyphenylglycine, respectively. Many years later, semi-synthetic β-lactams antibiotics still find a wide range of applications due to their broad and great antibacterial activity, representing more than 50% of the antibiotic prescriptions (including beta-lactams, tetracycline, macrolides, quinolones and sulphonamides) [133].

In 1960, the discovery of penicillin G acylase (penicillin amidohydrolase EC 3.5.1.11) was reported independently by four industrial research groups [134–137]. The newly-discovered serine hydrolase penicillin G acylase was initially employed as an industrial catalyst for the manufacture of 6-APA from penicillin G and the traditional chemical procedures were gradually being replaced by bio-transformations.

Over the years, penicillin G acylase variants with improved stability were obtained by screening and by employing recombinant DNA technology. These new enzymes, combined with efficient immobilization methods [138], made the recycling of the biocatalyst possible, generating a more efficient production of β-lactams and dramatic reductions in enzyme costs [139–142].

Nowadays, the industrial production of β-lactam antibiotics occurs using two different penicillin G acylases, one designed for the hydrolysis of benzylpenicillin (HydPGA) to give the β-lactam nucleus 6-APA and one designed for the synthesis (SynPGA) of semi-synthetic β-lactams as ampicillin or amoxicillin (Fig. 24) [143].

The enzymatic cleavage of penicillin G to 6-APA is run efficiently in batch mode reactors in water with robust immobilized penicillin G acylase mainly on hydrophilic resins such as epoxy methacrylate resins; the biocatalyst can be recycled up to 1000 cycles.

Even if the hydrolysis of benzylpenicillin is a well-established process, the synthesis of β-lactam antibiotics using immobilized SynPGA can still be a challenge.

The enzymatic synthesis of β-lactam antibiotics can, in principle, be accomplished either via thermodynamically controlled reversal of the hydrolytic reaction or via kinetically controlled transacylation in water [144].

The main obstacle of the kinetically controlled synthesis of beta-lactams using immobilized SynPGA (the currently used industrial process), is the possible hydrolysis of the sidechain donor (phenylglycine or 4-hydroxyphenylglycine) as well as of the product. To avoid such unwanted reactions, the process is carried out in an excess of the side-chain donor making the downstream treatment more laborious.

The synthesis/hydrolysis ratio (S/H, mol product per mol hydrolyzed side-chain donor formed) is often used as an indicator of the viability of the process [143]. The intrinsic acyl transfer efficiency (S/H) of the biocatalytic process using immobilized SynPGA has been overcome thanks to the improvement of SynPGA and now ampicillin and amoxicillin are being manufactured in batch mode in aqueous solution with approximately 500 cycles biocatalyst reuse.

5.4. Transaminase for sitagliptin

The immobilization of transaminases (EC 2.6.1) for the industrial production of APIs represents the first successful industrial example of using other enzyme than the hydrolases series. The initial enzyme development using a combination of modelling and directed evolution led to an active and stable R-selective amine transaminase for the synthesis of sitagliptin, a key blockbuster from Merck for treatment of diabetes

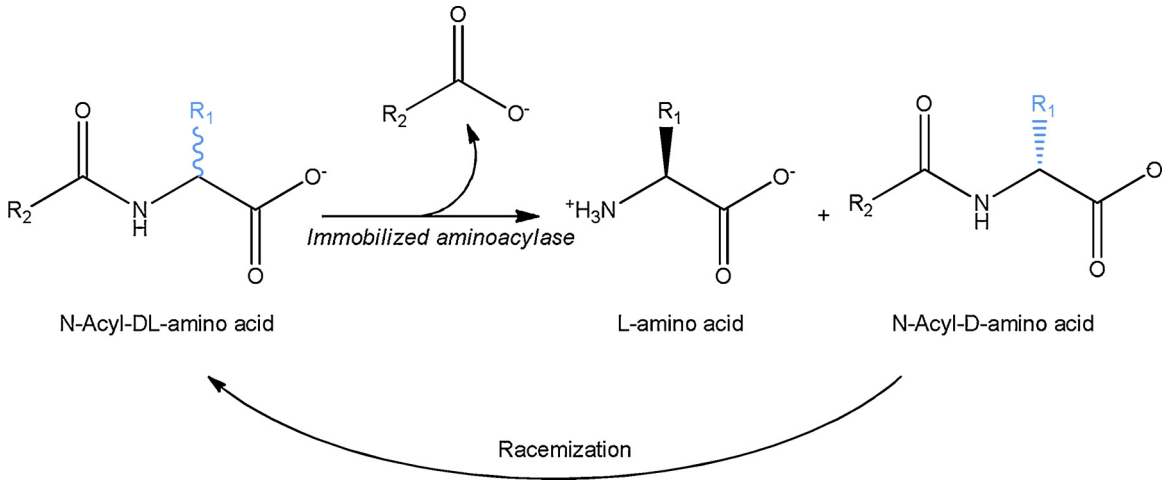

Fig. 19. Biocatalytic route for L-amino acid preparation using immobilized aminoacylase.

Table 5
Main industrial applications using immobilized enzymes for the manufacture of different APIs using immobilized hydrolases and transferases.

API	Target application	Immobilized enzyme	Type of immobilization	Reaction media[a]	Reaction type
Odanacatib	Osteoporosis treatment	Lipase B from *Candida antarctica*	Adsorption on octadecyl polymethacrylate resin	A	Esterification
Sofosbuvir	Treatment of hepatitis C	Lipase B from *Candida antarctica*	Adsorption on methacrylate/divinylbenzene copolymer	A	Hydrolysis
6-APA	Beta-lactams	Penicillin G Amidase (hyd)	Covalent on epoxy or amino methacrylate polymer	B	Hydrolysis
Amoxicillin/ ampicillin	Beta-lactams	Penicillin G Amidase (syn)	Covalent on epoxy or amino methacrylate polymer	B	Amidation
Sitagliptin	Antidiabetic	Transaminase	Adsorption	A	Transamination

[a] A: liquid phase is formed by organic solvents. B: liquid phase is formed by water or aqueous buffers.

[2]. The use of organic solvents is necessary to increase the poor solubility of sitagliptin and its derivatives therefore the use of a non-immobilized transaminase limits the choice to water and miscible solvents such as dimethyl sulfoxide (DMSO), making the downstream processing difficult [116,145].

On the contrary, using immobilized transaminase (immobilized by adsorption on a functionalized octadecyl methacrylate) [117] opens the possibility to use neat organic solvents and recycle the immobilized transaminase without loss of enzyme activity. Merck reported conversions > 80% in isopropylacetate with very high substrate concentration (200 g/L of ketoamide and 80 mL/L isopropylamine) at 60 °C.

This is an excellent example of the success of a combined approach enzyme engineering and process optimisation using immobilized enzymes: this approach was able to replace a chemical process based on hydrogenation of enamine at high pressure with a biocatalyzed process able to give 99.5% enantiomeric excess and able to convert very high concentrations (up to 200 g/l) of prositagliptin in the final product, using an immobilized enzyme in the presence of DMSO as a cosolvent.

The previous existing asymmetric hydrogenation of an enamine at high pressure (250 psi, 17 bar) using a rhodium -based chiral catalyst was in fact giving ee of only 97% (Fig. 25) [146].

The great novelty of the process lies in the engineering of a transaminase that was lacking activity towards prositagliptin. Thanks to a rational engineering of the enzyme performed by the team Codexis-

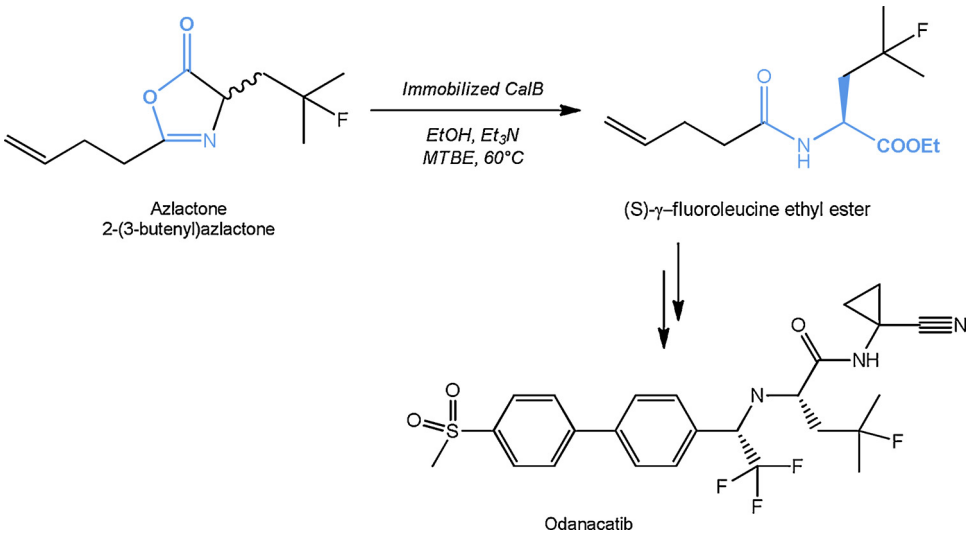

Fig. 20. Lactone opening and esterification by immobilized CalB in organic solvent to produce chiral intermediate of drug odanacatib.

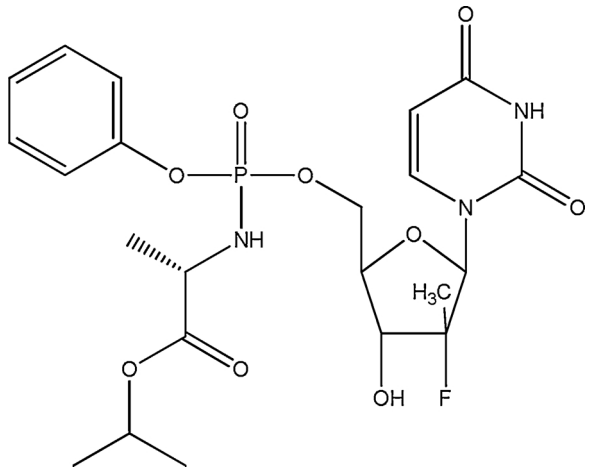

Fig. 21. Chemical structure of Sofosbuvir molecule, inhibitor of the hepatitis C virus NS3 protease.

Merck, the final mutant enzyme was able to not only perform the desired reaction with high yields, but also to tolerate high denaturing solvent concentration, to work in extremely high concentrations and to be immobilized for recycling purposes [2]. The great achievement is also in the use of a different enzyme than hydrolases that are normally used in industrial applications.

6. Immobilized enzymes in medical devices and biosensors

6.1. Lipases in medical devices

In patients with cystic fibrosis and exocrine pancreatic insufficiency, fat malabsorption can lead to decreased caloric intake and deficiencies of fatty acids (FA), such as docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), which are important for growth and development [147,148]. Patients with cystic fibrosis use pancreatic enzyme replacement therapy in conjunction with meals to increase the absorption of fat and other nutrients [149]. Thousands of cystic fibrosis patients in USA use enteral nutrition that allows them to meet growth and weight goals. However, formulas available contain triglycerides rather than fatty acids due to the poor stability of hydrolyzed fats.

Alcresta Therapeutics, in collaboration with ChiralVision, has developed a single-use cylindrical cartridge with a chamber closed by frits containing digestive immobilized enzyme that connects in-line with the enteral feeding set [150,151]. The cartridge contains covalently immobilized lipase on a methacrylic polymer [152] (EC 3.1.1.3, from *Chromobacterium viscosum*, *Pseudomonas fluorescens*, *Burkholderia cepacia*, or *Rhizopus oryzae*) on polymeric beads that hydrolyses up to 90% of the fats in the enteral formula as it is passed through the cartridge [153]. The device increases life expectancy of the patients since it helps to improve fat absorption, consequently improving cognitive ability, and improving chronic lung disease, with a reduced length of time required for parenteral nutrition [154].

6.2. Urease in medical devices

Urease (EC 3.5.1.5) is a highly specific enzyme that catalyzes the hydrolysis of urea to ammonium and carbon dioxide [155].

Applications of the urease are various, ranging from the hydrolysis and removal of urea in waste water effluents, beverages and foods, to more specialized and sophisticated usage, such as the removal of urea from blood for extracorporeal detoxification or in the dialysate regeneration system for artificial kidneys.

Urea is a major metabolic end product and its removal has been a major problem for patients suffering from renal failure [156–158]. Immobilized urease can be used in the dialysis system of an artificial kidney machine to remove the urea. The introduction of immobilized urease allowed for the small, portable dialysis machines based on sorbent regenerative dialysis systems to be manufactured [159,160]. Several varying approaches have been tried in the past for urea removal, and there is currently renewed interest in a number of new alternative approaches.

Looking at the urease-cation exchanger approach, there are several technologies that can be mentioned: the sorb system which is a conventional divalent-selective cation exchanger employed by Sorb, Renal Solutions Inc., Fresenius Medical Care [160,161], the zeolite (aluminosilicate) monovalent-selective cation exchanger [162], the Zirconium silicate: crystalline monovalent- selective cation exchanger [163,164], a resin-based system is used by Baxter [165]. Other approaches, such as at Exxon, are using urease and liquid membrane acid-filled microcapsules [166].

Of all the historical efforts, the only practical device using urease that has emerged for clinical use has been based on the sorbent column. Portable dialysis systems, such as the REDY, Allient or AWAK sorbent systems, employed a urease to convert urea, which (unlike other metabolic wastes such as creatinine and uric acid) is not readily adsorbed by activated carbon, into ammonium ions and bicarbonate ions [167,168].

Commercially, such dialysis devices using immobilized urease were available in 1980 but their production stopped in 1990. Since 2000, although mainly developmental, such systems emerged again as viable technological alternative and much interest is taken by companies, AWAK system [169,170] being an example of such commercially available technology.

6.3. Enzymes in biosensors

Enzyme-based biosensors represent a major application of immobilized enzymes, not only in medicine and clinical diagnostics [171,172] but also in food [173], food safety [174], agricultural industries [175] as well as environmental monitoring [176].

Such applications of immobilized enzymes deserve a book of their own, so we shall highlight, very briefly, some important points about enzymatic biosensors based on immobilized enzymes.

Depending on the assay type, two fundamental classes of enzymatic biosensors can be distinguished.

In the first group, the enzyme detects the presence of a substrate, or co-substrate / co-factor. A typical example is a glucose biosensor used for blood glucose monitoring in people affected by diabetes and produced at large scale by companies such as: LifeScan, Abbott, Roche Diagnostics, etc. The glucose strip used in diabetes monitoring is estimated to reach in 2020 a global market of $18.5 billion [177].

The second group is based on the detection of enzyme inhibitors in the presence of a substrate. The most common example of this approach is the detection of organophosphate compounds used as pesticides [178,179] or warfare nerve agents [180].

The major advantage of these approaches is the high sensitivity and specificity of catalytically active enzymes towards their target molecules.

While the transducers used for enzymatic biosensors vary widely

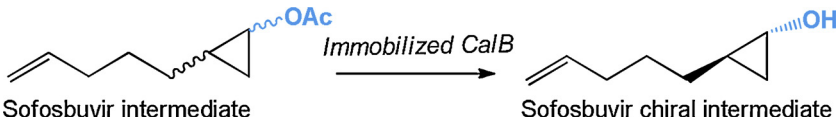

Fig. 22. Enantioselective hydrolysis of hepatitis C virus inhibitor sofosbuvir by immobilized CalB in organic solvent saturated with aqueous buffer as described by Gilead.

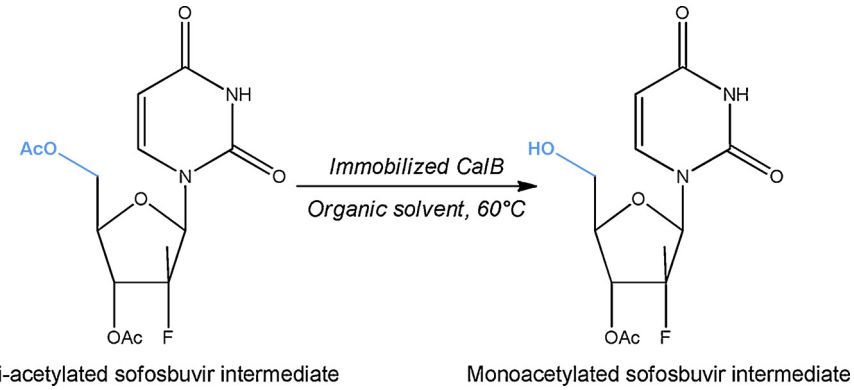

Fig. 23. Selective mono-deacetylation of a Sofosbuvir catalyzed by immobilized CalB in protic organic solvent to give the corresponding alcohol.

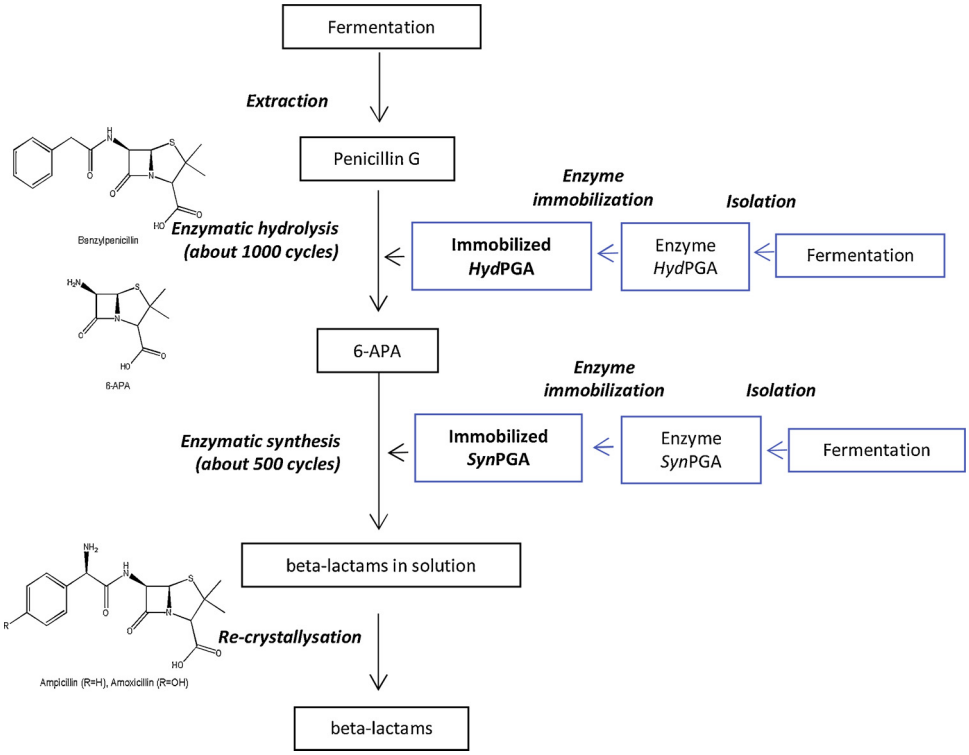

Fig. 24. Beta-lactam antibiotics (amoxicillin and ampicillin) synthesis using two different immobilized Penicillin G Amidase, in a sequential hydrolytic/synthetic process.

based on the detection technique, operating principle, construction, size etc., the electrochemical transducers are playing the most important role in medicine and clinical diagnostics [181].

The discovery and development of new enzymes, combined with the development of new performant materials for the transducer, as well as important advances in detection techniques, are continuously transforming the enzymatic biosensor landscape offering new opportunities for practical applications of such devices.

7. Conclusion

Immobilized enzymes provide substantial benefits to the industrial processes in which they are currently used. Some of the benefits are: process simplification, reduced environmental impact and a more sustainable process compared to chemical synthesis [106,182,183].

The food industry employs the highest amounts of immobilized enzymes in well-established industrial processes such as HFCS, amino acid, cocoa butter analogues production, which are used worldwide as major food ingredients. Close behind, the chemical industry has considerably expanded its biocatalytic processes in recent decades due to the ability to produce complex chiral molecules at low temperatures, such as chiral amines and herbicides. Often for these applications, immobilized native enzymes are used with little engineering necessary to modify their properties, since they are required on quite naturally similar substrates, usually in aqueous media.

Comparatively, the situation is rather different in the pharmaceutical industry. This industry is investing most in the development of biocatalytic processes and on improving performances of existing enzymes. Many of the enzymes used in the pharmaceutical industry are required to act towards unnatural molecules, very different from the natural enzyme substrates, and sometimes in extreme conditions, such as the presence of organic solvents or high substrate concentration. This has forced the industry into the search for, development of and improvement of new or existing enzymes for unnatural substrates in order to expand applications to a multitude of chemical molecules.

The need to develop more sustainable and economical processes, combined with the rapid progress in enzyme development and protein engineering, alongside the advantages of immobilized enzyme

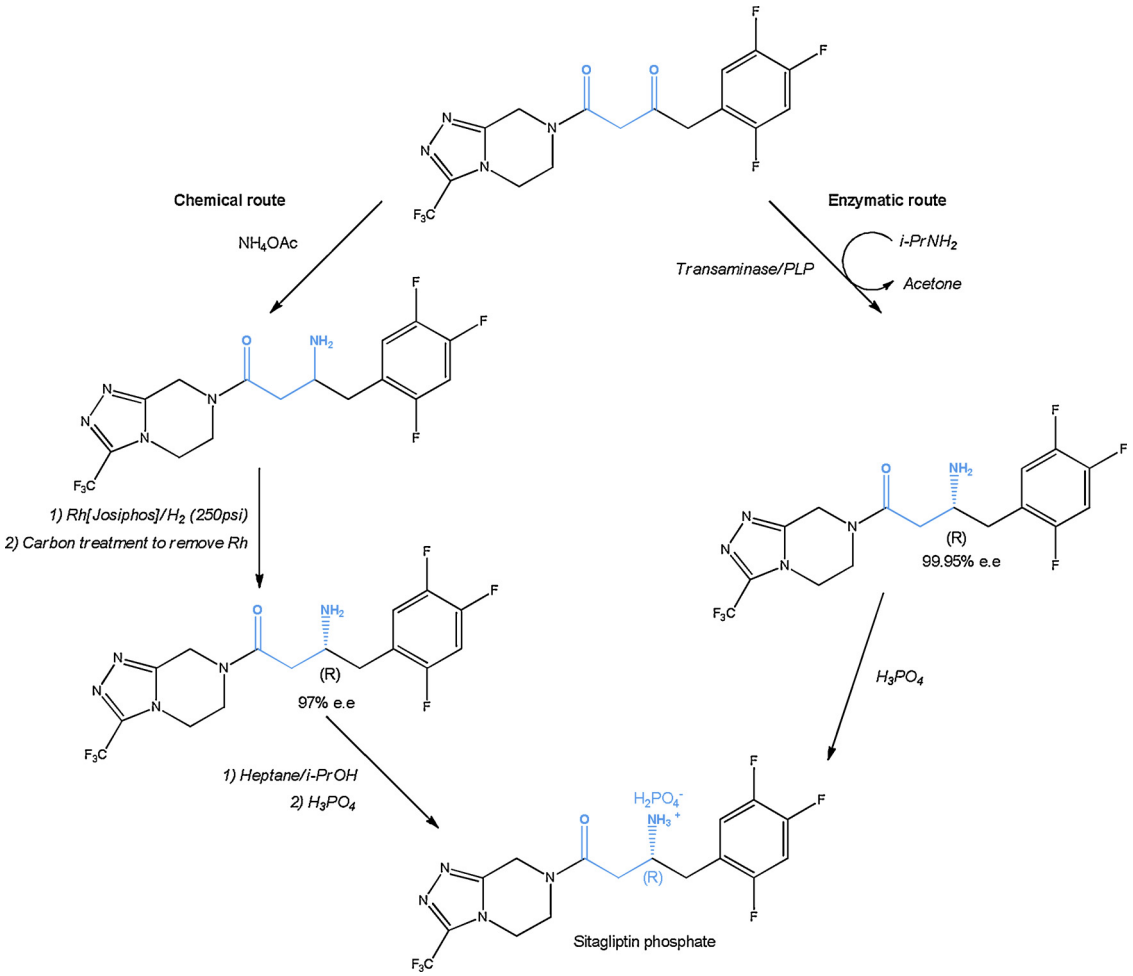

Fig. 25. Enzymatic *vs* chemical route to manufacture sitagliptin phosphate using immobilized transaminase or ruthenium catalysts.

technology shows a bright future for the use of biocatalysis in industrial processes.

References

[1] F. Balkenhohl, K. Ditrich, C. Nübling, Racemate Separation of Primary and Secondary Heteroatom-substituted Amine by Enzyme-Catalysed Acylation, WO1996023894A1, 1995.

[2] C.K. Savile, J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, G.J. Hughes, Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture, Science 329 (2010) 305–309, https://doi.org/10.1126/science.1188934.

[3] C. Neuberg, E. Welde, Phytochemical reactions III. Transformation of aromatic and fatty aromatic aldehydes into alcohols, Biochem. Z. 62 (1914) 477–481.

[4] U.T. Bornscheuer, G.W. Huisman, R.J. Kazlauskas, S. Lutz, J.C. Moore, K. Robins, Engineering the third wave of biocatalysis, Nature 485 (2012) 185–194, https://doi.org/10.1038/nature11117.

[5] U.T. Bornscheuer, The fourth wave of biocatalysis is approaching, Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 376 (2017) 1–7, https://doi.org/10.1098/rsta.2017.0063.

[6] B. Hauer, C. Kvarnstrom Branneby, K. Hult, A. Magnusson, A. Hamberg, An Enzymatically Catalyzed Method of Preparing Mono-Acylated Polyols, WO 2011/033039, (2011).

[7] S. Sellappan, C. Akoh, Application of lipases in modifications of food lipids, in: C.T. Hou (Ed.), Handb. Ind. Biocatal. CRC Press, 2005.

[8] P. Tufvesson, W. Fu, J. Skibsted Jensen, J.M. Woodley, Process considerations for the scale-up and implementation of biocatalysis, Food Bioprod. Process. 88 (2010) 3–11, https://doi.org/10.1016/j.fbp.2010.01.003.

[9] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C.S. dos Santos, Á. Berenguer-Murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, Biotechnol. Adv. 37 (2019) 746–770, https://doi.org/10.1016/J.BIOTECHADV.2019.04.003.

[10] C. Ortiz, M. Luján Ferreira, O. Barbosa, J.C. S dos Santos, R.C. Rodrigues, Á. Berenguer-Murcia, L.E. Briand, R. Fernandez-Lafuente, Novozym 435: the "perfect" lipase immobilized biocatalyst? Catal. Sci. Technol. 9 (2019) 2380–2420, https://doi.org/10.1039/c9cy00415g.

[11] A. De Simone, M. Naldi, M. Bartolini, L. Davani, V. Andrisano, Immobilized enzyme reactors: an overview of applications in drug discovery from 2008 to 2018, Chromatographia 82 (2019) 425–441, https://doi.org/10.1007/s10337-018-3663-5.

[12] M.P. Thompson, I. Peñ, S.C. Cosgrove, N.J. Turner, Biocatalysis using immobilized enzymes in continuous flow for the synthesis of fine chemicals, Org. Process Res. Dev. 23 (2019) 9–18, https://doi.org/10.1021/acs.oprd.8b00305.

[13] C. Bernal, K. Rodríguez, R. Martínez, Integrating enzyme immobilization and protein engineering: an alternative path for the development of novel and improved industrial biocatalysts, Biotechnol. Adv. 36 (2018) 1470–1480, https://doi.org/10.1016/J.BIOTECHADV.2018.06.002.

[14] A. Homaei, Enzyme immobilization and its application in the food industry, in: V. Ravishankar Rai (Ed.), Adv. Food Biotechnol. John Wiley & Sons Ltd, 2015, , https://doi.org/10.1002/9781118864463.ch09.

[15] B.R. Facin, M.S. Melchiors, A. Valériovalério, J.V. Oliveira, D. Débora De Oliveira, Driving immobilized lipases as biocatalysts: 10 years state of the art and future prospects, Ind. Eng. Chem. Res. 58 (2019) 5358–5378, https://doi.org/10.1021/acs.iecr.9b00448.

[16] R. DiCosimo, J. Mc Auliffe, A.J. Poulose, G. Bohlmann, Industrial use of immobilized enzymes, Chem. Soc. Rev. 42 (2013) 6437–6474, https://doi.org/10.1039/c3cs35506c.

[17] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J.M. Woodley, Guidelines and cost analysis for catalyst production in biocatalytic processes, Org. Process Res. Dev. 15 (2011) 266–274, https://doi.org/10.1021/op1002165.

[18] R.M. Lindeque, J.M. Woodley, Reactor selection for effective continuous biocatalytic production of pharmaceuticals, Catalysts 9 (2019) 262, https://doi.org/10.3390/catal9030262.

[19] A. Illanes, C. Altamirano, Enzyme reactors, Enzym. Biocatal. Springer Netherlands, Dordrecht, 2008, pp. 205–251, https://doi.org/10.1007/978-1-4020-8361-7_5.

[20] A. Illanes, L. Wilson, C. Vera (Eds.), Problem Solving in Enzyme Biocatalysis, John Wiley and Sons Ltd, 2013, , https://doi.org/10.1002/9781118341742.

[21] D. Crabb, J.K. Shettyt, Commodity scale production of sugars from starches, Curr. Opin. Microbiol. 2 (1999) 252–256.

[22] W.P. Chen, Glucose isomerase, Process Biochem. 15 (1980) 30–41.

[23] S.H. Bhosale, M.B. Rao, V.V. Deshpande, Molecular and industrial aspects of glucose isomerase, Microbiol. Rev. 60 (1996) 280–300.
[24] V.J. Jensen, S. Rugh, Industrial-scale production and application of immobilized glucose isomerase, in: K. Mosbach (Ed.), Methods Enzymol. - Immobil. Enzym. Cels, Part C, Elsevier Inc., 1987, pp. 356–370.
[25] G.E. Nedwin, V. Sharma, J.K. Shetty, Alpha-amylase blend for starch processing and method of use thereof, US 2014/0087429 A1, 2014.
[26] CFR - Code of Federal Regulations Title 21 CFR 173.357, (n.d.). https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=173.357 (Accessed 12 February 2019).
[27] Efficient conversion of dextrose to fructose | Novozymes, (n.d.). https://www.novozymes.com/en/advance-your-business/food-and-beverage/starch/sweetzyme (Accessed 1 August 2019).
[28] L. Zittan, P.B. Poulsen, S.H. Hemmingsen, Sweetzyme - A New Immobilized Glucose Isomerase, Starch - Stä¤rke 27 (1975) 236–241, https://doi.org/10.1002/star.19750270705.
[29] O.B. Jørgensen, L.G. Karlsen, N.B. Nielsen, S. Pedersen, S. Rugh, A new immobilized glucose isomerase with high productivity produced by a strain of Streptomyces murinus, Starch - Stärke 40 (1988) 307–313, https://doi.org/10.1002/star.19880400809.
[30] Deok-kun Oh, H.-J. Kim, Y.-J. Lee, S.-H. Song, S.-W. Park, J.-H. Kim, S.-B. Kim, D-psicose production method by D-psicose epimerase, US2011/8030035B2, 2011.
[31] K. Maruta, K. Yamamoto, T. Nishimoto, H. Chaen, T. Nakada, Ketose 3-epimerase, its preparation and uses, US2011/0275138A1, 2011.
[32] Y.H. Hong, J.H. Kim, S.B. Kim, J.H. Kim, Y.M. LEE, Immobilization of psicose-epimerase and a method of producing d-psicose using the same, WO 2011/040708 A3, 2011.
[33] R.D. Woodyer, R.W. Armentrout, 3-Epimerase, WO 2014/049373, 2014.
[34] R.D. Woodyer, J.C. Cohen, J.R. Bridges, Sweetener, US 2017/ 9635879 B2, 2017.
[35] Tate & Lyle Introduces DOLCIA PRIMA® Crystalline Allulose; Low-Calorie Solution Provides the Full Taste and Enjoyment of Sugar, But Without All the Calories, (n.d.). https://www.tateandlyle.com/news/tate-lyle-introduces-dolcia-prima-crystalline-allulose-low-calorie-solution-provides-full (Accessed 2 January 2019).
[36] J. Chini, B. Febbruari, M. Matulli, L. Vagnoli, Enzymes immobilized on styrene-divinyl benzene matrices and the use thereof in industrial productions., WO2014/006606 A1, 2014.
[37] B.-C. Lim, H.-J. Kim, D.-K. Oh, Tagatose Production with pH Control in a Stirred Tank Reactor Containing Immobilized L-Arabinose Isomerase from Thermotoga neapolitana, Appl. Biochem. Biotechnol. 149 (2008) 245–253, https://doi.org/10.1007/s12010-007-8095-x.
[38] D.-K.T. Oh, properties, applications, and biotechnological processes, Appl. Microbiol. Biotechnol. 76 (2007) 1–8, https://doi.org/10.1007/s00253-007-0981-1.
[39] P. Kim, H. Roh, S. Yoon, J. Choi, Biological tagatose production by recombinant escherichia coli, WO 00/68397, 1999.
[40] Z. Kovács, E. Benjamins, K. Grau, A. Ur Rehman, M. Ebrahimi, P. Czermak, Recent developments in manufacturing oligosaccharides with prebiotic functions, in: H. Zorn, P. Czermak (Eds.), Biotechnol. Food Feed Addit. Springer, Berlin, Heidelberg, 2014, pp. 257–295, , https://doi.org/10.1007/10_2013_237.
[41] A.-R.R. Park, D.-K.K. Oh, Galacto-oligosaccharide production using microbial β-galactosidase: current state and perspectives, Appl. Microbiol. Biotechnol. 85 (2010) 1279–1286, https://doi.org/10.1007/s00253-009-2356-2.
[42] M. Camara, V. Fernandez-Ruiz, P. Morales, M.C. Sanchez-Mata, Fiber compounds and human health, Curr. Pharm. Des. 23 (2017), https://doi.org/10.2174/1381612823666170216123219.
[43] J.R. Xavier, K.V. Ramana, R.K. Sharma, β-galactosidase: Biotechnological applications in food processing, J. Food Biochem. 42 (2018) e12564, , https://doi.org/10.1111/jfbc.12564.
[44] A. Basso, A. Ducret, L. Gardossi, R. Lortie, Synthesis of octyl glucopyranoside by almond b-glucosidase adsorbed onto Celite R-640 ®, Tetrahedron Lett. 43 (2002) 2005–2008, https://doi.org/10.1016/S0040-4039(02)00197-1.
[45] L.M. Huerta, C. Vera, C. Guerrero, L. Wilson, A. Illanes, Synthesis of galacto-oligosaccharides at very high lactose concentrations with immobilized beta-galactosidases from Aspergillus oryzae, Process Biochem. 46 (2010) 245–252, https://doi.org/10.1016/j.procbio.2010.08.018.
[46] R. Gaur, H. Pant, R. Jain, S.K. Khare, Galacto-oligosaccharide synthesis by immobilized Aspergillus oryzae b-galactosidase, Food Chem. 97 (2006) 426–430, https://doi.org/10.1016/j.foodchem.2005.05.020.
[47] F. Benjamins, L. Cao, A. Broekhuis, Production of galacto-oligosaccharides, WO 2015/034356A1, 2015.
[48] H. Burham, R.A.G.A. Rasheed, N.M. Noor, S. Badruddin, H. Sidek, Enzymatic synthesis of palm-based ascorbyl esters, J. Mol. Catal. B Enzym. 58 (2009) 153–157, https://doi.org/10.1016/J.MOLCATB.2008.12.012.
[49] S. Ferreira-Dias, G. Sandoval, F. Plou, F. Valero, The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries, Electron. J. Biotechnol. 16 (2013), https://doi.org/10.2225/vol16-issue3-fulltext-5.
[50] P. Villeneuve, Lipases in lipophilization reactions, Biotechnol. Adv. 25 (2007) 515–536, https://doi.org/10.1016/J.BIOTECHADV.2007.06.001.
[51] R.W. Stevenson, F.E. Luddy, H.L. Rothbart, Enzymatic acyl exchange to vary saturation in di- and triglycerides, J. Am. Oil Chem. Soc. 56 (1979) 676–680, https://doi.org/10.1007/BF02660072.
[52] L. Ahmadi, A.J. Wright, A.G. Marangoni, Structural and mechanical behavior of tristearin/triolein-rich mixtures and the modification achieved by interesterification, Food Biophys. 4 (2009) 64–76, https://doi.org/10.1007/s11483-009-9102-2.
[53] A.J. Dijkstra, Edible Oil Processing From a Patent Perspective, Springer US, Boston, MA, 2013, https://doi.org/10.1007/978-1-4614-3351-4.
[54] W.M. Willis, A.G. Marangoni, Enzymatic interesterification, in: C.C. Akoh (Ed.), Food Lipids, Marcel Dekker, 2002, pp. 839–875, , https://doi.org/10.1201/9780203908815.
[55] X. Xu, Z. Guo, H. Zhan, A.F. Vikbjerg, M.L. Damstrup, Chemical and enzymatic interesterification of lipids for use in food, in: F.D. Gunstone (Ed.), Modifying Lipids Use Food, Woodhead, 2006, pp. 234–272.
[56] C.T. Hou, Handbook of Industrial Biocatalysis, CRC Press, 2005, https://doi.org/10.1016/j.ijbiomac.2006.01.009.
[57] M. Asif, Process advantages and product benefits of interesterification in oils and fats, Int. J. Nutr. Pharmacol. Neurol. Dis. 1 (2011) 134–138, https://doi.org/10.4103/2231-0738.84203.
[58] M.H. Coleman, A.R. Macrae, Fat process and composition, US4275081, 1979.
[59] M. Coleman, A. Macrae, Fat process and composition, GB 1577933, 1976.
[60] P. Halling, A. Macrae, Fat processing, US4863860, 1989.
[61] R.A. Wisdom, P. Dunnill, M.D. Lilly, A. Macrae, Enzymic interesterification of fats: factors influencing the choice of support for immobilized lipase, Enzyme Microb. Technol. 6 (1984) 443–446, https://doi.org/10.1016/0141-0229(84)90093-0.
[62] A.R. Macrae, Microbial lipases as catalysts for the interesterification of oils and fats, in: C. Ratledge, P. Dawson, J. Rattray (Eds.), Biotechnol. Oils Fats Ind. Amer Oil Chemist Society, 1985, pp. 189–198.
[63] N. Sawamura, Transesterification of fats and oils, Ann. N. Y. Acad. Sci. 542 (1988) 266–269, https://doi.org/10.1111/j.1749-6632.1988.tb25840.x.
[64] T. Matsuo, N. Sawamura, Y. Hashimoto, W. Hashida, Method for enzymatic interesterification of lipid and enzyme used therein, EP0035883, 1981.
[65] A. Svendsen, M. Skjot, J. Brask, J. Vind, S.A. Patkar, Immobilised enzymes, WO 2007/080197 A2, 2007.
[66] R. Fernandez-Lafuente, Lipase from Thermomyces lanuginosus: uses and prospects as an industrial biocatalyst, J. Mol. Catal. B Enzym. 62 (2010) 197–212, https://doi.org/10.1016/J.MOLCATB.2009.11.010.
[67] H.C. Holm, D. Cowan, The evolution of enzymatic interesterification in the oils and fats industry, Eur. J. Lipid Sci. Technol. 110 (2008) 679–691, https://doi.org/10.1002/ejlt.200800100.
[68] W. De Greyt, A.J. Dijkstra, Fractionation and interesterification, in: A.J. Dijkstra, R.J. Hamilton, W. Hamm (Eds.), Trans Fat. Acids, Blackwell Pub, 2008, pp. 181–202, , https://doi.org/10.1002/9780470697658.
[69] L. Gambelli, R. Happe, S. Banni, Glyceride composition, WO 2015/014967 Al (2015).
[70] C. Akoh, X. Xu, Enzymatic production of Betapol and other specialty fats, in: Tsung Min Kuo, Harold Gardner (Eds.), Lipid Biotechnol. Marcel Dekker, 2002, pp. 461–478.
[71] K. Radack, C. Deck, G. Huster, The effects of low doses of n-3 fatty acid supplementation on blood pressure in hypertensive subjects, Arch. Intern. Med. 151 (1991) 1173–1180, https://doi.org/10.1001/archinte.1991.00400060097017.
[72] J.H. O'Keefe, W.S. Harris, Omega-3 fatty acids: time for clinical implementation? Am. J. Cardiol. 85 (2000) 1239–1241, https://doi.org/10.1016/S0002-9149(00)00735-9.
[73] J.A. Nettleton, Omega-3 Fatty Acids and Health, Springer US, Boston, MA, 1995, https://doi.org/10.1007/978-1-4615-2071-9.
[74] G. Gudmundur, A. Halldorsson, O. Thorstad, Lipase-catalysed esterification of marine oil, US 7491522B2, 2003.
[75] J. Kralovec, W. Wang, Immobilized enzymes and methods of using thereof, EP2439268B1, 2006.
[76] J. Kralovec, W. Wang, J.C. Barrow, Enzymatic modification of oil, WO 2009/040676A2, 2009.
[77] P. Brodelius, Industrial applications of immobilized biocatalysts, Adv. Biochem. Eng. Vol. 10 Springer-Verlag, Berlin/Heidelberg, 1978, pp. 75–129, https://doi.org/10.1007/BFb0004472.
[78] P.S. Panesar, S. Kumari, R. Panesar, Potential applications of immobilized β-Galactosidase in food processing industries, Enzyme Res. (2010) 473137, , https://doi.org/10.4061/2010/473137.
[79] M.W. Griffiths, D.D. Muir, J.D. Phillips, Thermal stable beta-galactosidase, US4332895, 1979.
[80] NIIR Board of Consultants & Engineers, Enzymes Bio-Technology Handbook, Asia Pacific Business Press, 2005.
[81] M.R. Kosseva, Use of immobilized biocatalyst for valorization of whey lactose, in: C. Webb, M.R. Kosseva (Eds.), Food Ind. Wastes, Academic Press, 2013, pp. 137–156, , https://doi.org/10.1016/B978-0-12-391921-2.00008-1.
[82] W.G. Flynne, Biotechnology and bioengineering, Nova Biomedical Books, (2008).
[83] H. Hirohara, H. Yamamoto, E. Kawano, S. Nabeshima, Immobilized lactase, its preparation and use, EP 0037667B1, 1981.
[84] E.H. White, The chemistry of the N-Alkyl-N-nitrosoamides. II. A new method for the deamination of aliphatic amines, J. Am. Chem. Soc. 77 (1955) 6011–6014, https://doi.org/10.1021/ja01627a064.
[85] K. Ditrich, F. Balkenhohl, W. Ladner, Separation of optically active amides, WO1997/10201A1, 1996.
[86] ChiPros® Chiral Amines for Your Innovation - BASF Intermediates, (n.d.). http://www.intermediates.basf.com/chemicals/chiral-intermediates/amines (Accessed 2 January 2019).
[87] A. Simon, U. Karl, Expanding the scope of industrial biocatalysis, Spec. Chem. Mag. (2010) 36–38.
[88] T. Ohrui, Y. Sakakibara, Y. Aono, M. Kato, H. Takao, T. Kawaguchi, Process for continuously synthesizing acrylic acid esters, US 3875212A, 1973.
[89] B. Hauer, C.K. Branneby, S. Maurer, P. Trodler, M. Miiller, CALB muteins and their use, US 8206969B2, 2012.

[90] F. van Rantwijk, M.A.P.J. Hacking, R.A. Sheldon, Lipase-catalyzed synthesis of carboxylic amides: nitrogen nucleophiles as acyl acceptor, Monatshefte Fuer Chemie/Chemical Mon. 131 (2000) 549–569, https://doi.org/10.1007/s007060070086.

[91] M.C. de Zoete, A.C. Kock-van Dalen, F. van Rantwijk, R.A. Sheldon, Lipase-catalysed ammoniolysis of lipids. A facile synthesis of fatty acid amides, J. Mol. Catal. B Enzym. 2 (1996) 141–145, https://doi.org/10.1016/S1381-1177(96)00025-2.

[92] K.F. Brandstadt, T.H. Lane, R.A. Gross, Enzyme catalyzed organosilicon esters and amides, US 2004/0082024A1, 2004.

[93] D. Jackson, Application of biocatalysis in the agrochemical industry, in: J. Tao, R.J. Kazlauskas (Eds.), Biocatal. Green Chem. Chem. Process Dev. John Wiley & Sons, 2011, pp. 255–276.

[94] H.-U. Blaser, H.-P. Buser, K. Coers, R. Hanreich, H.-P. Jalett, E. Jelsch, B. Pugin, H.-D. Schneider, F. Spindler, A. Wegmann, The chiral switch of metolachlor: the development of a large-scale enantioselective catalytic process, Chimia (Aarau) 53 (1999) 275–280.

[95] H.-U. Blaser, The chiral switch of (S)-metolachlor: a personal account of an industrial odyssey in asymmetric catalysis, Adv. Synth. Catal. 344 (2002) 17–31, https://doi.org/10.1002/1615-4169(200201)344:1<17::AID-ADSC17>3.0.CO;2-8.

[96] H.-U. Blaser, B. Pugin, F. Spindler, M. Thommen, From a chiral switch to a ligand portfolio for asymmetric catalysis, Acc. Chem. Res. 40 (2007) 1240–1250, https://doi.org/10.1021/ar7001057.

[97] J.R. Shroff, V.R. Shroff, B. Shanker, Hydrogenation of imines, US8461386B2, 2013.

[98] C. Nuebling, K. Ditrich, C. Dully, Optical resolution of primary amines by enantioselective acylation with a long-chain alkoxyalkanoate or phenoxyalkanoate ester in the presence of a lipase, DE19837745A1, 1998.

[99] H. Riechers, J. Simon, A. Hoehn, A. Kramer, F. Funke, W. Siegel, C. Nuebling, Racemization of optically active amines useful as pharmaceuticals or intermediates, by contacting in gaseous form with hydrogen and catalyst, giving high racemization degree and yield, DE19852282A1, 1998.

[100] K.S. Hayes, E.G. Lutz, M.G. Turcotte, Racemization of optically active alkoxyamines, US6060624A, 1999.

[101] M.B. Ansorge-Schumacher, O. Thum, Immobilised lipases in the cosmetics industry, Chem. Soc. Rev. 42 (2013) 6475–6490, https://doi.org/10.1039/c3cs35484a.

[102] R. Nieguth, M. Eckstein, L.O. Wiemann, O. Thum, M.B. Ansorge-Schumacher, Enabling industrial biocatalytic processes by application of silicone-coated enzyme preparations, Adv. Synth. Catal. 353 (2011) 2522–2528, https://doi.org/10.1002/adsc.201100421.

[103] S. Clendennen, J. Yuan, An Enzymatic Approach to Sustainable Manufacturing of Personal Care Ingredients: Reducing the Traditional Environmental Impact of a Consumer Product's Life Cycle, Euro Cosmet. 9 (2015) 334.

[104] T. Garcia, M. Martinez, J. Aracil, Enzymatic synthesis of Myristyl Myristate. Estimation of parameters and optimization of the process, Biocatal. Biotransformation 14 (1996) 67–85, https://doi.org/10.3109/10242429609106877.

[105] G. Hills, Industrial use of lipases to produce fatty acid esters, Eur. J. Lipid Sci. Technol. 105 (2003) 601–607, https://doi.org/10.1002/ejlt.200300853.

[106] E. US EPA, OCSPP, OPPT, Presidential Green Chemistry Challenge: 2009 Greener Synthetic Pathways Award, (n.d.). https://www.epa.gov/greenchemistry/presidential-green-chemistry-challenge-2009-greener-synthetic-pathways-award (Accessed 2 January 2019).

[107] I. CHIBATA, Application of immobilized enzymes for asymmetric reactions, in: V. 185 ACS Symposium Series (Ed.), Asymmetric React. Process. Chem. American Chemical Society, 1982, pp. 195–203, , https://doi.org/10.1021/bk-1982-0185.ch014.

[108] I. Chibata, T. Tosa, T. Sato, T. Mori, K. Yamamoto, Applications of immobilized enzymes and immobilized microbial cells for L-amino acid production, Immobil. Enzym. Technol. Springer US, Boston, MA, 1975, pp. 111–127, https://doi.org/10.1007/978-1-4615-8735-4_9.

[109] M. Goldsmith, D.S. Tawfik, Directed enzyme evolution: beyond the low-hanging fruit, Curr. Opin. Struct. Biol. 22 (2012) 406–412, https://doi.org/10.1016/J.SBI.2012.03.010.

[110] J.M. Palomo, J.M. Guisan, Different Strategies for Hyperactivation of Lipase Biocatalysts, Humana Press, 2012, pp. 329–341, https://doi.org/10.1007/978-1-61779-600-5_20.

[111] M.D. Truppo, Biocatalysis in the pharmaceutical industry: the need for speed, ACS Med. Chem. Lett. 8 (2017) 476–480, https://doi.org/10.1021/acsmedchemlett.7b00114.

[112] M.D. Truppo, G. Hughes, Development of an improved immobilized CAL-B for the enzymatic resolution of a key intermediate to odanacatib, Org. Process Res. Dev. 15 (2011) 1033–1035, https://doi.org/10.1021/op200157c.

[113] J.Y. Gauthier, N. Chauret, W. Cromlish, S. Desmarais, L.T. Duong, J.P. Falgueyret, D.B. Kimmel, S. Lamontagne, S. Léger, T. LeRiche, C.S. Li, F. Massé, D.J. McKay, D.A. Nicoll-Griffith, R.M. Oballa, J.T. Palmer, M.D. Percival, D. Riendeau, J. Robichaud, G.A. Rodan, S.B. Rodan, C. Seto, M. Thérien, V.L. Truong, M.C. Venuti, G. Wesolowski, R.N. Young, R. Zamboni, W.C. Black, The discovery of odanacatib (MK-0822), a selective inhibitor of cathepsin K, Bioorganic Med. Chem. Lett. 18 (2008) 923–928, https://doi.org/10.1016/j.bmcl.2007.12.047.

[114] J. Limanto, A. Shafiee, P.N. Devine, V. Upadhyay, R.A. Desmond, B.R. Foster, D.R. Gauthier, R.A. Reamer, R.P. Volante, An efficient chemoenzymatic approach to (S)-γ-fluoroleucine ethyl ester, J. Org. Chem. 70 (2005) 2372–2375, https://doi.org/10.1021/jo047918j.

[115] A. Basso, M. Hesseler, S. Serban, Hydrophobic microenvironment optimization for efficient immobilization of lipases on octadecyl functionalised resins, Tetrahedron 72 (2016) 7323–7328, https://doi.org/10.1016/j.tet.2016.02.021.

[116] M.D. Truppo, H. Strotman, G. Hughes, Development of an immobilized transaminase capable of operating in organic solvent, ChemCatChem 4 (2012) 1071–1074, https://doi.org/10.1002/cctc.201200228.

[117] A. Basso, W. Neto, S. Serban, B.D. Summers, How to optimise the immobilization of amino transaminases on synthetic enzyme carriers, to achieve up to a 13-fold increase in performances, Chem. Today 36 (2018) 40–42.

[118] N. Boyer, P. Marcellin, Pathogenesis, diagnosis and management of hepatitis C, J. Hepatol. 32 (2000) 98–112, https://doi.org/10.1016/S0168-8278(00)80419-5.

[119] A.U. Neumann, N.P. Lam, H. Dahari, D.R. Gretch, T.E. Wiley, T.J. Layden, A.S. Perelson, Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy, Science 282 (1998) 103–107, https://doi.org/10.1126/SCIENCE.282.5386.103.

[120] T. Fukumoto, T. Berg, Y. Ku, W.O. Bechstein, M. Knoop, H. Lemmens, H. Lobeck, U. Hopf, P. Neuhaus, Viral dynamics of hepatitis C early after orthotopic liver transplantation: evidence for rapid turnover of serum virions, Hepatology 24 (1996) 1351–1354, https://doi.org/10.1002/hep.510240606.

[121] E. Domingo, E. Martinez-Salas, F. Sobrino, J. Carlos De La Tortea, A. Portela, J. Ortin, C. L6pez-Galindezb, P. Pkez-Breaab, N. Villanuevab, R. Nhjera', S. Vandepol', D. Steinhauer', N. Depolo', J. Holland, The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance-a review, Gene 40 (1985) 1–8.

[122] M. Martell, J.I. Esteban, J. Quer, J. Genescà, A. Weiner, R. Esteban, J. Guardia, J. Gómez, Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution, J. Virol. 66 (1992) 3225–3229.

[123] A. Cagulada, J. Chan, L. Chan, D.A. Colby, Synthesis of an antiviral compound, US 2015/0175626A1, 2015.

[124] K. Bjornson, E. Canales, J.J. Cottell, K.K. Karki, Inhibitors of Hepatitis C Virus, US 2016/ 9296782 B2 (2016).

[125] C.Y. Yang, Hepatitis C treatments with Sofosbuvir, WO 2014/185995 Al, 2014.

[126] N. Martin, O. Schone, H.-P. Spitzenstatter, D. Benito-Garagorri, A process for preparing a crystalline form of sofosbuvir, WO 2016/156512 A, 2016.

[127] M. Gaboardi, G. Pallanza, G. Castaldi, M. Castaldi, Process for the preparation of Sofosbuvir, US 2017 / 0247404 A1, 2017.

[128] C.C. Muñiz, T.E.C. Zelaya, G.R. Esquivel, F.J. Fernández Perrino, Penicillin and cephalosporin production: a historical perspective, Rev. Latinoam. Microbiol. 49 (2007) 88–98.

[129] A. Fleming, On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ, Br. J. Exp. Pathol. 10 (1929) 226–236.

[130] H. Florey, E. Chain, N. Heatley, M. Jennings, A. Sanders, E. Abraham, M. Florey, Antibiotics. A Survey of Penicillin, Streptomycin, and Other Antimicrobial Substances from Fungi, Actinomycetes, Bacteria, and Plants, Oxford University Press, 1949.

[131] M.A. Wegman, M.H.A. Janssen, F. van Rantwijk, R.A. Sheldon, Towards biocatalytic synthesis of β-lactam antibiotics, Adv. Synth. Catal. 343 (2001) 559–576, https://doi.org/10.1002/1615-4169(200108)343:6/7<559::AID-ADSC559>3.3.CO;2-Q.

[132] M.M. Harold, J. Van Theodorus, M.D. Godfried, Process for the synthesis of cefaclor, WO 2006/069984 A2, 2005.

[133] European Centre For Disease Prevention And Control, Antibiotic Consumption in Europe, (2017) http://drive-ab.eu/wp-content/uploads/2014/09/WP1A_Final-QMs-QIs_final.pdf.

[134] F.R. Batchelor, F.P. Doyle, J.H.C. Nayler, G.N. Rolinson, Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations, Nature 183 (1959) 257–258, https://doi.org/10.1038/183257b0.

[135] G.N. Rolinson, F.R. Batchelor, D. Butterworth, J. Cameron-Wood, M. Cole, G.C. Eustace, M.V. Hart, M. Richards, E.B. Chain, Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis, Nature 187 (1960) 236–237, https://doi.org/10.1038/187236a0.

[136] C.A. Claridge, A. Gourevitch, J. Lein, Bacterial penicillin amidase, Nature 187 (1960) 237–238, https://doi.org/10.1038/187237a0.

[137] H.T. Huang, A.R. English, T.A. Seto, G.M. Shull, B.A. Sobin, Enzymatic hydrolysis of the side chain of penicillins, J. Am. Chem. Soc. 82 (1960) 3790–3791, https://doi.org/10.1021/ja01499a083.

[138] A.I. Kallenberg, F. van Rantwijk, R.A. Sheldon, Immobilization of Penicillin G acylase: the key to optimum performance, Adv. Synth. Catal. 347 (2005) 905–926, https://doi.org/10.1002/adsc.200505042.

[139] E.J. Vandamme, Biotechnology of Industrial Antibiotics, M. Dekker, 1984.

[140] E.J. Vandamme, Peptide antibiotic production through immobilized biocatalyst technology, Enzyme Microb. Technol. 5 (1983) 403–416, https://doi.org/10.1016/0141-0229(83)90021-2.

[141] J.G. Shewale, V.K. Sudhakaran, Penicillin V acylase: its potential in the production of 6-aminopenicillanic acid, Enzyme Microb. Technol. 20 (1997) 402–410, https://doi.org/10.1016/S0141-0229(96)00176-7.

[142] A. Parmar, H. Kumar, S. Marwaha, J. Kennedy, Advances in enzymatic transformation of penicillins to 6-aminopenicillanic acid (6-APA), Biotechnol. Adv. 18 (2000) 289–301, https://doi.org/10.1016/S0734-9750(00)00039-2.

[143] A. Bruggink, E.C. Roos, E. de Vroom, Penicillin acylase in the industrial production of β-Lactam antibiotics, Org. Process Res. Dev. 2 (1998) 128–133, https://doi.org/10.1021/op9700643.

[144] V. Kasche, Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, peptides and other condensation products, Enzyme Microb. Technol. 8 (1986) 4–16, https://doi.org/10.1016/

0141-0229(86)90003-7.
[145] M.D. Truppo, J.M. Janey, G. Hughes, Immobilized transaminases and processes for making and using immobilized transaminase, WO2012/177527A1 (2012).
[146] K.B. Hansen, Y. Hsiao, F. Xu, N. Rivera, A. Clausen, M. Kubryk, S. Krska, T. Rosner, B. Simmons, J. Balsells, N. Ikemoto, Y. Sun, F. Spindler, C. Malan, E.J.J. Grabowski, J.D. Armstrong, Highly efficient asymmetric synthesis of Sitagliptin, J. Am. Chem. Soc. 131 (2009) 8798–8804, https://doi.org/10.1021/ja902462q.
[147] P.M. Kidd, Omega-3 DHA and EPA for cognition, behavior, and mood: clinical findings and structural-functional synergies with cell membrane phospholipids, Altern. Med. Rev. 12 (2007) 207–227.
[148] N. Peretti, V. Marcil, E. Drouin, E. Levy, Mechanisms of lipid malabsorption in Cystic Fibrosis: the impact of essential fatty acids deficiency, Nutr. Metab. 2 (2005) 11–29, https://doi.org/10.1186/1743-7075-2-11.
[149] C. Fibrosis Foundation, Patient Registry Annual Data Report 2015, (2015) https://www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf.
[150] R. Gallotto, G.L. Loring, K. Gary, E.S. PARK, D.J. Brown, W.R.K. Schoevaart, M.C. A. Van Vliet, Enteral feeding device and related methods of use, US 20170105903, 2017.
[151] A.L. Margolin, Methods, compositions, and devices for supplying dietary fatty acid needs, WO 2013123139A8, 2013.
[152] Center for Food Safety and Applied Nutrition, Environmental Decisions - Environmental Decision Memo for Food Contact Notification No. 001190, (2012), pp. 2–5 (Accessed 28 January 2019), https://wayback.archive-it.org/7993/20171030193105/https://www.fda.gov/Food/IngredientsPackagingLabeling/EnvironmentalDecisions/ucm443618.htm.
[153] Relizorb (Immobilized lipase cartridge) - Formulas, (n.d.). https://www.relizorb.com/docs/pdfs/Compatible-Formulas-and-Pumps.pdf (Accessed 28 January 2019).
[154] S. Freedman, D. Orenstein, P. Black, P. Brown, K. McCoy, J. Stevens, D. Grujic, R. Clayton, Increased fat absorption from enteral formula through an in-line digestive cartridge in patients with cystic fibrosis, J. Pediatr. Gastroenterol. Nutr. 65 (2017) 97–101, https://doi.org/10.1097/MPG.0000000000001617.
[155] J.P. CHEN, S.H. CHIU, Preparation and characterisation of urease immobilized onto porous chitosan beads for urea hydrolysis, Bioprocess Eng. 21 (1999) 323–330.
[156] G. Bayramoğlu, H. Altınok, A. Bulut, A. Denizli, M.Y. Arıca, Preparation and application of spacer-arm-attached poly(hydroxyethyl methacrylate-co-glycidyl methacrylate) films for urease immobilisation, React. Funct. Polym. 56 (2003) 111–121, https://doi.org/10.1016/S1381-5148(03)00050-6.
[157] C.-C. Lin, M.-C. Yang, Urea permeation and hydrolysis through hollow fiber dialyzer immobilized with urease: storage and operation properties, Biomaterials 24 (2003) 1989–1994, https://doi.org/10.1016/s0142-9612(02)00611-7.
[158] A. Saxena, A. Bhattacharya, S. Kumar, I.R. Epstein, R. Sahney, Biopolymer matrix for nano-encapsulation of urease – a model protein and its application in urea detection, J. Colloid Interface Sci. 490 (2017) 452–461, https://doi.org/10.1016/J.JCIS.2016.11.030.
[159] J.W.M. Agar, Review: Understanding sorbent dialysis systems, Nephrology 15 (2010) 406–411, https://doi.org/10.1111/j.1440-1797.2010.01321.x.
[160] P.D. Updyke, H.F. Sandford, B.J.B.. Lipps, D.M.D.M. Zatezalo, M.J. Beiriger, M.J. Mullner, Dialysis systems and methods, EP 2219704B1, 2009.
[161] C. Curtin, B. Lipps, N. Ofsthun, H.F. Sandford, A. Stennett, U. Palmer David, Wearable kidney, WO 2007/103411A2, 2007.
[162] J.D. Sherman, Ion Exchange separations with molecular sieves zeolite, in: F.R. Ribeiro, A.E. Rodrigues, L.D. Rollmann, C. Naccache (Eds.), Zeolites Sci. Technol. NATO ASI Ser. Vol. 80 Springer Netherlands, Dordrecht, 1983, pp. 583–623, , https://doi.org/10.1007/978-94-009-6128-9_22.
[163] J.D. Sherman, D.S. Bem, G.J. Lewis, Process for removing toxins from blood using zirconium metallate or titanium metallate compositions, US6099737A, 1999.
[164] S.R. Ash, Extracorporeal blood detoxification by sorbents in treatment of hepatic encephalopathy, Adv. Ren. Replace. Ther. 9 (2002) 3–18, https://doi.org/10.1053/jarr.2002.30474.
[165] S. Karoor, B. Donovan, T.T. Hai, M. Katada, L. Lu, L. Martis, S. Morti, S. Mujais, P. J. Sanders, P.J. Soltys, R. Tandon, Method and composition for removing uremic toxins in dialysis processes, US7241272B2, 2007.
[166] T.C. Vogler, W.J. Asher, Liquid membrane capsule systems resistant to coalescence by means of an irreversible coating, US4244816A, 1981.
[167] M. Roberts, The regenerative dialysis (REDY) sorbent system, Nephrology 4 (1998) 275–278.
[168] S. Haroon, A. Davenport, Haemodialysis at home: review of current dialysis machines, Expert Rev. Med. Devices 15 (2018) 337–347, https://doi.org/10.1080/17434440.2018.1465817.
[169] http://awak.com/technology/ (Accessed 27 December 2018), (n.d.).
[170] C. Bluchel, P. Haywood, Sorbent for a dialysis device and dialysis system, WO2018/106185A2, 2018.
[171] O. Hosu, S. Mirel, R. Săndulescu, C. Cristea, Minireview: smart tattoo, microneedle, point-of-care, and phone-based biosensors for medical screening, diagnosis, and monitoring, Anal. Lett. 52 (2019) 78–92, https://doi.org/10.1080/00032719.2017.1391826.
[172] J.D. Habimana, J. Ji, X. Sun, Minireview: trends in optical-based biosensors for point-of-care bacterial pathogen detection for food safety and clinical diagnostics, Anal. Lett. 51 (18) (2018) 2933–2966, https://doi.org/10.1080/00032719.2018.1458104.
[173] S. Serban, A.F. Danet, N. El Murr, Rapid and sensitive automated method for glucose monitoring in wine processing, J. Agric. Food Chem. 52 (2004) 5588–5592, https://doi.org/10.1021/JF0494229.
[174] N. Gazel, H.B. Yildiz, Enzyme-based biosensors in food industry via surface modifications, Surf. Treat. Biol. Chem. Phys. Appl. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2016, pp. 227–252, https://doi.org/10.1002/9783527698813.ch7.
[175] M.L. Verma, Nanobiotechnology advances in enzymatic biosensors for the agri-food industry, Environ. Chem. Lett. 15 (2017) 555–560, https://doi.org/10.1007/s10311-017-0640-4.
[176] J.P. Hart, S. Serban, L.J. Jones, N. Biddle, R. Pittson, G.A. Drago, Selective and rapid biosensor integrated into a commercial hand-held instrument for the measurement of ammonium ion in sewage effluent, Anal. Lett. 39 (2006) 1657–1667, https://doi.org/10.1080/00032710600713545.
[177] Global Test Strip Market Research Report- 2021 | MRFR, (n.d.). https://www.marketresearchfuture.com/reports/test-strip-market-672 (Accessed 28 January 2019).
[178] B. Rajangam, D.K. Daniel, A.I. Krastanov, Progress in enzyme inhibition based detection of pesticides, Eng. Life Sci. 18 (2018) 4–19, https://doi.org/10.1002/elsc.201700028.
[179] F. Arduini, S. Cinti, V. Scognamiglio, D. Moscone, Nanomaterials in electrochemical biosensors for pesticide detection: advances and challenges in food analysis, Microchim. Acta 183 (2016) 2063–2083, https://doi.org/10.1007/s00604-016-1858-8.
[180] M. Pohanka, Cholinesterases in Biorecognition and biosensors construction: a review, Anal. Lett. 46 (2013) 1849–1868, https://doi.org/10.1080/00032719.2013.780240.
[181] P. Martinkova, A. Kostelnik, T. Valek, M. Pohanka, Main streams in the construction of biosensors and their applications, Int. J. Electrochem. Sci. 12 (2017) 7386–7403, https://doi.org/10.20964/2017.08.02.
[182] O. Thum, Sustainability that gets under the skin, Https://Personal-Care.evonik.com/Product/Personal-Care/Downloads/Public/elements25-Evonik-Newsletter-Sustainability.pdf (2008).
[183] R.A. Sheldon, D. Brady, Broadening the scope of biocatalysis in sustainable organic synthesis, ChemSusChem 12 (2019) 2859–2881, https://doi.org/10.1002/cssc.201900351.